SOME APPLICATIONS OF THE COLORIMETRIC
PHOSPHATE METHOD.

BY A. P. BRIGGS.

(From the Departments of Biological Chemistry and Internal Medicine,
Washington University School of Medicine, St. Louis.)

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Two years ago, a modification (1) of the Bell-Doisy phosphate
method was published and at that time technique was given
for its application to inorganic phosphorus in blood and urine.
Since then its use has been extended to a variety of analytical
procedures, some of which are given below.

In principle the method consists of the formation of phospho-
moiodybdic acid and its subsequent reduction by hydroquinone
and sulfur dioxide with the production of a stable blue color,
proportional to the inorganic phosphorus present. The excess
moiodybdic acid is not reduced.

Reagents.—The following reagents are used in all of the pro-
cedures to be given.

Molybdate Solution.—This contains 5 per cent ammonium
moiodybate in 5 n H₂SO₄ and is prepared as follows: Dissolve
25 gm. of ammonium moiodybate in 300 cc. of water and to this
add 75 cc. of concentrated H₂SO₄ diluted with 125 cc. of water.

Hydroquinone Solution.—This is a 1 per cent solution to which
a drop of concentrated H₂SO₄ is added to retard oxidation.

Sulfite Solution.—A 20 per cent solution of sodium sulfite
is used. The strength of sulfite solutions deteriorates by oxida-
tion, but the amount used in color development is sufficient pro-
vided an easily detectable odor of SO₂ is evolved.

Stock KH₂PO₄ Solution.—This is prepared from pure KH₂PO₄
which has been pulverized and dried for several days over con-
centrated H₂SO₄. 4.394 gm. are dissolved in a liter of distilled
water and then 5 cc. of CHCl₃ added as preservative. 1 cc. = 1
mg. of phosphorus.
Standard \(KH_2PO_4\) Solutions.—The following dilutions of the stock solution are prepared for standards in the various procedures. 100 cc. of the stock solution to a liter of water. 1 cc. = 0.1 mg. of phosphorus. From this solution three additional dilutions are made.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>50 cc. to 200 cc.</td>
<td>1 cc. = 0.025 mg. P.</td>
</tr>
<tr>
<td>50 &quot; &quot; 250 &quot;</td>
<td>1 &quot; = 0.02 &quot; &quot;</td>
</tr>
<tr>
<td>50 &quot; &quot; 500 &quot;</td>
<td>1 &quot; = 0.01 &quot; &quot;</td>
</tr>
</tbody>
</table>

Each of these solutions is preserved by \(CHCl_3\).

Phosphorus Compounds of the Blood.

I: Inorganic Phosphorus in Blood or Plasma.

The method previously published seems to have proved quite satisfactory in every respect except the intensity of the color produced. By a few slight changes the color has been considerably improved and the proportionality to inorganic phosphorus unimpaired. A much better color is obtained if the order of adding the sulfite and hydroquinone is reversed, that is by adding the molybdate and hydroquinone and then the sulfite after the green color appears. The development of color is retarded by large amounts of acid as was pointed out before. By using 1 cc. of the acid molybdate solution instead of 2 cc. an abundant excess of molybdate is provided and the diminished acidity allows the reduction to proceed to a much greater extent in the half hour allowed. The procedure, then, which seems best at present, is: Transfer a measured volume of blood or plasma to a small flask. Add 3 volumes of water and 1 volume of 20 per cent trichloroacetic acid, shake vigorously for a minute, and pour onto an ashless filter. Transfer 5 cc. of the filtrate to a 10 cc. graduated cylinder, to another cylinder transfer 3 cc. = 0.03 mg. of P of a standard \(KH_2PO_4\) solution. To each then add in the following order, 1 cc. of molybdate, 1 cc. of hydroquinone, and 1 cc. of sulfite. Dilute to 10 cc. and after a half hour compare. Set the standard at 30 mm.

By using a molybdate solution prepared with less \(H_2SO_4\), colors of still greater intensity may be obtained. If this is done trichloroacetic acid equivalent to that in the filtrate should be
added to the standard, so that reduction will proceed at the same rate.

In spite of the exposure to strong mineral acid, the results obtained by this method are not higher than those reported by others (2, 3). Apparently there is very little hydrolysis of organic phosphate. In order to get more definite information in regard to this question, comparison was made with the method of Bloor (2) which has been used by Buell (4) and others where it was desired to avoid hydrolysis of organic phosphates. A mixture was made from ten samples of blood brought into the routine laboratory. Duplicate determinations by the colorimetric technique above gave identical results, 4.50 mg. of P per 100 cc. Duplicate determinations by Bloor's method gave 4.46 and 4.44 mg. per 100 cc. These results confirm the notion that there is very little hydrolysis by the colorimetric reagents.

II. Inorganic Plus Hydrolysable Organic Phosphorus.

If the filtrate and inorganic reagents are heated in boiling water the results are a little higher for plasma and a great deal higher for whole blood, due to the presence of a labile organic phosphate reported by Zucker and Gutman (5). Inorganic plus hydrolysable organic phosphorus may be determined conveniently by the following technique. Transfer 5 cc. of the trichloroacetic filtrate from plasma (or 2 cc. of whole blood filtrate plus 3 cc. of water), to a test-tube graduated at 10 cc. To a similar tube transfer 3 cc. of standard KH₂PO₄ solution equivalent to 0.03 mg. of P and add to this tube 2 cc. of water. Then to each tube add 1 cc. of molybdate, 1 cc. of hydroquinone, and 1 cc. of 10 N H₂SO₄; mix these solutions well with a stirring rod, as the acid molybdate tends to layer in the bottom. Rinse off the stirring rod, insert rubber stoppers loosely into the tubes, and immerse them in boiling water for 15 minutes (an hour for whole blood filtrates). Cool to room temperature under the tap and then add to each tube 1 cc. of the sulfite solution and dilute to 10 cc. Mix and after 10 minutes compare in the colorimeter.

The 10 N H₂SO₄ may be prepared with sufficient accuracy by diluting 30 cc. of concentrated H₂SO₄ with 70 cc. of water. If too much acid is added the reduction by hydroquinone is pre-
vented and if too little acid is present molybdic acid as well as phosphomolybdic acid will be reduced. Sulfite should not be added until after the heating, as different amounts of \( \text{SO}_2 \) are driven off from the separate tubes with the result that different degrees of reduction are obtained and the colors produced do not match.

Since the amount of hydrolysable phosphate in plasma is small, it has been recommended by Myers (6) that a similar technique be used for the clinical determination of inorganic phosphorus in serum. Analysis of three human plasmas for inorganic phosphorus and inorganic plus hydrolysable phosphorus gave results shown in Table I.

<table>
<thead>
<tr>
<th>No.</th>
<th>Phosphorus per 100 cc. plasma.</th>
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<tbody>
<tr>
<td></td>
<td>Inorganic P.</td>
</tr>
<tr>
<td></td>
<td>mg.</td>
</tr>
<tr>
<td>I</td>
<td>2.88</td>
</tr>
<tr>
<td>II</td>
<td>2.91</td>
</tr>
<tr>
<td>III</td>
<td>3.32</td>
</tr>
</tbody>
</table>

These results indicate that values about 10 per cent higher may be expected where reduction is accelerated by heat.

III. Total Acid-Soluble Phosphorus.

This may be determined colorimetrically on a small amount of the trichloroacetic filtrate digested with nitric and sulfuric acids. For accurate results it is necessary to drive off all the nitric acid and very little of the sulfuric acid. By substituting 30 per cent \( \text{H}_2\text{O}_2 \) for nitric acid the digestion is very much facilitated; Merck's White Label Superoxal has been used.

Procedure.—Transfer 2 cc. of the trichloroacetic filtrate to a large Pyrex tube graduated at 25 cc. and add 1 cc. of 10 \( \times \) \( \text{H}_2\text{SO}_4 \). Drop in a glass bead and heat over a micro burner until the fumes of \( \text{SO}_3 \) appear. Turn off the flame and allow the tube to cool for about a minute. Add a drop of 30 per cent \( \text{H}_2\text{O}_2 \), cover the tube with a watch-glass and heat over the micro burner for 10 minutes, adjusting the flame rather low so as to avoid loss of \( \text{SO}_2 \). Cool the tube a few seconds in running water and dilute with 18 cc. of distilled water. To a similar tube transfer 10 cc.
of a standard KH₂PO₄ solution = 0.1 mg. of P, 1 cc. of 10 N 
H₂SO₄, and 7 cc. of distilled water. To each tube add 2 cc. of 
the molybdate solution and 2 cc. of the hydroquinone solution. 
Cover the tubes with small beakers and heat a half hour in boil-
ing water. Cool, add 1 cc. of the sulfite, dilute with water to 
25 cc., mix, and after 10 minutes compare.

The oxidation of charred carbonaceous material is very prompt 
after the addition of a drop of the 30 per cent H₂O₂: The heating 
for 10 minutes is directed to decompose excess peroxide, which 
if present would interfere with color production.

Lipoid Phosphorus.

Randles and Knudson (7) have given a procedure for the 
determination of lipoid phosphorus using the original Bell-
Doisy method of color development. By application of the 
technique given above for total acid-soluble phosphorus to the 
evaporated residue from an aliquot of the alcohol-ether filtrate 
one gains the advantage of the stable acid color for comparison.

Determination of Calcium as Phosphate.

Calcium may be determined on 5 cc. of serum by the oxalate-
permanganate method with an error of ±5 per cent. When the 
analysis is carried out on smaller amounts of material the error 
is correspondingly greater, due chiefly to limitations of the per-
manganate titration.

Upon considering the possibility of determining small amounts 
of calcium as phosphate the following points were observed.

1. Very small quantities of calcium may be completely pre-
 precipitated with a moderate excess of oxalate.

2. The precipitate could be separated by centrifugation and 
decomposed with a drop of concentrated HCl and a few drops of 
30 per cent H₂O₂.

3. The calcium could be precipitated from the resulting small 
volume of solution by the addition of a few drops of a phosphate 
solution and a few drops of strong ammonia.

4. The calcium phosphate could be washed with ammoniacal 
20 per cent alcohol and the phosphate determined colorimetrically.
5. Calcium forms a variety of phosphates, their composition depending upon the alkalinity and the relative amounts of CaO and H₃PO₄ present (8). However, when a dilute acid solution of calcium containing excess phosphate is made alkaline with ammonia, the precipitate formed contains an amount of phosphorus corresponding very closely to the formula Ca₃(PO₄)₂.

These principles are utilized in the following procedure, an application to blood or plasma. Transfer 10 cc. of the trichloroacetic filtrate (= 2 cc. of plasma) to a Pyrex test-tube (16 mm. × 150 mm.), graduated at 15 cc. Add a drop of methyl red as indicator and while agitating with a rubber tipped stirring rod add dilute ammonia (concentrated ammonia diluted with 3 volumes of water) until the color changes to yellow, then add a few drops of 5 per cent acetic acid to produce the neutral brown. Add 1 cc. of 4 per cent ammonium oxalate and rub the sides of the tube with rubber tipped rod until the precipitate forms. Rinse off the stirring rod and allow 2 hours standing for complete precipitation. Centrifugate 10 minutes at 1,500 r.p.m. and pour off the fluids into a similar tube for the determination of magnesium. To the residue of calcium oxalate add 1 drop of concentrated HCl and 0.5 cc. of 30 per cent H₂O₂ and heat 30 minutes in boiling water. Then add 0.5 cc. of 2 per cent KH₂PO₄ and 3 drops of concentrated ammonia (or 1 drop after the precipitate forms). Allow 30 minutes standing for complete precipitation, then add 20 cc. of the wash solution, centrifugate 10 minutes at 1,500 r.p.m., and pour off the fluids. Add 20 cc. of the wash solution, then with the rubber policeman scrub the sides of the tube and mix up the precipitate. Centrifugate again and pour off the fluids. To the residue of Ca₃(PO₄)₂ add 5 cc. of water and to a similar tube add 5 cc. of a standard KH₂PO₄ solution equivalent to 0.1 mg. of P. To each add 1 cc. of the molybdate, 1 cc. of the hydroquinone, and 1 cc. of the sulfite. Dilute with water to 15 cc. and after a half hour compare. 1 mg. of P is equivalent to 1.935 mg. of Ca.

Reagents.—30 per cent H₂O₂, Merck’s White Label Superoxal was found satisfactory. The wash solution contains 200 cc. of 95 per cent alcohol and 50 cc. of concentrated ammonia per liter.

Analysis of known calcium solutions by this procedure gave theoretical results. In Table II are given comparative results by the permanganate and colorimetric methods.
Remarks.—Where precipitates are to be separated by centrifugation and “pouring off” of fluids, it is desirable to have the insoluble material in a very finely divided state in order that the forces of cohesion may be more effective. When calcium oxalate or magnesium ammonium phosphate are “rubbed down” a very finely divided precipitate is formed, in contrast to the relatively coarse grained precipitates obtained when they are allowed to form on standing. Calcium phosphate is precipitated at once on adding excess ammonia, but in a finely divided form, well suited to the technique of separation described. Calcium phosphate is of course not so insoluble in water as calcium oxalate, but this is offset by the use of alcohol as a wash fluid. After pouring off twice as described there remains less than 0.001 mg. of soluble phosphate.

**TABLE II.**

*Analysis of Trichloroacetic Beef Serum Filtrate.*

<table>
<thead>
<tr>
<th>By permanganate.</th>
<th>By colorimetric.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.3 mg. per 100 cc.</td>
<td>10.20 mg. per 100 cc.</td>
</tr>
<tr>
<td>10.3 &quot; &quot; 100 &quot;</td>
<td>10.30 &quot; &quot; 100 &quot;</td>
</tr>
<tr>
<td>10.0 &quot; &quot; 100 &quot;</td>
<td>9.92 &quot; &quot; 100 &quot;</td>
</tr>
<tr>
<td>10.13 &quot; &quot; 100 &quot;</td>
<td>9.92 &quot; &quot; 100 &quot;</td>
</tr>
<tr>
<td>9.80 &quot; &quot; 100 &quot;</td>
<td>9.92 &quot; &quot; 100 &quot;</td>
</tr>
<tr>
<td>9.99 &quot; &quot; 100 &quot;</td>
<td>9.99 &quot; &quot; 100 &quot;</td>
</tr>
</tbody>
</table>

Determinations by the permanganate method were made on 25 cc. of the filtrate, equivalent to 5 cc. of serum. For the determinations by the colorimetric method 10 cc. portions were used.

**Determination of Magnesium.**

The technique in use at present for blood is as follows: To the fluid poured off from the calcium oxalate precipitate, and received in a Pyrex test-tube (16 × 150 mm.), graduated at 15 cc. add 1 cc. of 2 per cent KH₂PO₄ and 1 cc. of concentrated ammonia. Rub down the precipitate with a rubber policeman as described for calcium oxalate, rinse off the policeman, and allow 4 hours standing for complete precipitation. Centrifugate 10 minutes at 1,500 R.P.M. and pour off the fluids. Wash with 20 cc. of the alcoholic wash fluid as described for calcium phosphate.
Centrifugate again and pour off. Dissolve the precipitate and develop the color as described for calcium. For a standard use 3 cc. of a KH$_2$PO$_4$ solution equivalent to 0.075 mg. of P or 0.0588 mg. of Mg.

Magnesium in Urine.

A rapid and accurate technique for determining magnesium without separation of calcium is as follows: Transfer 1 or 2 cc., depending on the amount of magnesium expected, of clear acid urine to a Pyrex test-tube similar to that described above. Add a drop of methyl red and then dilute ammonia drop by drop until the color changes to brown. Adjust with a few drops of 5 per cent acetic acid if too much ammonia is added. Add 1 cc. of 4 per cent ammonium oxalate and rub down the calcium oxalate; after 2 hours standing add 1 cc. of 2 per cent KH$_2$PO$_4$, 1 cc. of concentrated ammonia, and rub down the MgNH$_4$PO$_4$. Allow 2 hours standing for complete precipitation. Wash twice with the alcohol wash solution by centrifugation and pouring off as described for calcium on blood. Dissolve the combined calcium and magnesium precipitates in a little hydrochloric acid approximately 0.2 N. Develop the color as described for magnesium on blood. Compare with a standard containing 0.10 mg. of P = 0.0784 mg. of magnesium.

Results reported before (9) by a similar technique in which CaCO$_3$ and MgNH$_4$PO$_4$ were precipitated at the same time were a little high, due no doubt to the formation of some calcium phosphate. Results by the technique described here give results which check gravimetric determinations within ±2 per cent.

Determination of Total Base.

Total base has been determined recently by Van Slyke and coworkers (10) as sulfate. All other acids except phosphoric acid were expelled by heating with excess sulfuric acid; the phosphoric acid present was converted to m-phosphoric acid, a monovalent acid. The base bound by phosphoric acid was determined separately and added to that computed from the residue of sulfates.

A convenient procedure suggested by that above is to add an excess of phosphoric acid to the combined sulfates. Then by
heating at high temperature drive off all the sulfuric acid and some of the excess phosphoric acid. The excess of phosphoric acid is titrated and the total phosphates are determined colorimetrically. After reducing the results to equivalent normal solutions the total base is obtained by difference.

The actual technique as applied to a urine is: Transfer 5 cc. of a clear acid urine to a platinum crucible and evaporate cautiously to dryness. Moisten with a drop or so of concentrated H$_2$SO$_4$ and ignite gently. Cool, moisten with sulfuric acid, and again ignite. Repeat if the ash is not white, but avoid heating to redness at this stage. Add 0.2 cc. of a phosphoric acid solution equivalent to approximately 50 mg. of phosphorus. Heat over a free flame, cautiously at first, until the SO$_3$ has been driven off and the phosphoric converted to m-phosphoric acid. Then heat so that the whole crucible is kept at a dull red heat for 40 to 60 seconds. Cool, add 10 cc. of standardized 0.1 N H$_2$SO$_4$. Cover with a watch-glass and heat 2 hours on a water bath, adding water if necessary. Transfer to a small flask and titrate with standard 0.1 N NaOH using methyl red as indicator and as a color for the end-point, a similar flask containing the same amount of water and methyl red, and about 10 mg. of pure K$_2$HPO$_4$. Then transfer the titrated contents to a 100 cc. volumetric flask, dilute with water to the mark and mix. Transfer 10 cc. of this solution to a 200 cc. volumetric flask. To a similar flask transfer 5 cc. of the stock K$_2$HPO$_4$ solution equivalent to 5 mg. of P. To each add 25 cc. of the molybdate solution, 20 cc. of the hydroquinone solution, and dilute with water to 200 cc. After a half hour compare in the colorimeter.

A very intense green color is obtained for comparison so that the addition of sulfite is unnecessary.

The phosphoric acid solution used is that obtained by mixing 3 volumes of 85 per cent phosphoric acid and 1 volume of concentrated H$_2$SO$_4$. After the precipitated calcium has settled, the phosphoric acid content was determined roughly to be about 50 mg. of P for 0.2 cc.

**Calculation.**—The normal equivalent of the 10 cc. of 0.1 N H$_2$SO$_4$ used to accelerate solution and hydrolysis is subtracted from the normal equivalent of the titration. This value is then subtracted from that obtained by dividing the total mg. of
phosphorus by 31.04. This answer is, of course, cubic centimeters of normal base per 5 cc. of urine.

Three determinations by this procedure on 30 mg. of Na + 5 mg. of Ca, equivalent to 15.5 cc. of 0.1 N base, gave 15.13, 15.62, and 15.60 cc. of 0.1 N base. Duplicate determinations on a urine by this procedure gave 124 and 130 cc. of 0.1 N base per 100 cc.; by the method of Van Slyke, 124 and 125 cc. of 0.1 N base.

SUMMARY.

Improved technique is given for the colorimetric determination of small amounts of inorganic phosphate. Procedures are given for the application of the colorimetric phosphate method to the determination of various phosphorus compounds of the blood, for calcium and magnesium, and for total base.

BIBLIOGRAPHY.

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SOME APPLICATIONS OF THE COLORIMETRIC PHOSPHATE METHOD

A. P. Briggs

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