RAPID COLORIMETRIC METHODS FOR THE DETERMINATION OF PHOSPHORUS IN URINE AND BLOOD.

By RICHARD D. BELL and EDWARD A. DOISY.

(*From the Biochemical Laboratories of Harvard Medical School, Boston, and Washington University School of Medicine, St. Louis.*)

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Several years ago, Taylor and Miller (1914) described a method for the determination of phosphates depending upon precipitation as ammonium phosphomolybdate and colorimetric estimation of the precipitated molybdenum by reduction with phenylhydrazine. Although this method has been quite extensively used, and other reducing agents have been proposed, the observation does not seem to have been made previously that certain reducing agents will reduce phosphomolybdic acid without affecting molybdic acid. By taking advantage of this selective reduction, the molybdenum present as phosphomolybdic acid can be colorimetrically determined in the presence of an excess of molybdic acid, avoiding the necessity of isolating the ammonium phosphomolybdate.[1]

We have found that hydroxylamine and many phenols have this selective reducing action in weakly acid solution at room temperature and we have selected hydroquinone as the most suitable reducing reagent. The color produced by the reduction

[1] After this paper was prepared for publication, we were shown the proof of a paper recently published by Dr. Wu in this *Journal*, in which he refers to this specific reduction and proposes a tentative method for the determination of phosphate. While we knew that Dr. Wu was investigating phosphotungstic and phosphomolybdic acids, we were unacquainted with the details of his work and have derived no information from it. We demonstrated the reduction of phosphomolybdic acid and the non-reduction of molybdic acid by hydroxylamine in the presence of Dr. Wu several months before his paper was sent in for publication and stated that we intended to develop methods for the determination of phosphate on this basis. We, therefore, do not feel bound by his implied reservation of this field.

of the phosphomolybdic acid by the hydroquinone is intensified by making the solution alkaline and a greenish shade due to the presence of quinone is removed by the addition of sulfite. The alkali (carbonate) and the sulfite are added in one solution.

If hydroquinone is added to a weakly acid solution of molybdic acid, no phosphate being present, the mixture will be perfectly colorless when treated with alkaline sulfite, but, if phosphate is present, phosphomolybdic acid is formed and reduced giving a blue color. As little as 0.005 mg. of phosphorus as phosphate gives a distinct blue color in a volume of 100 cc. The reaction is the same as in the phenol determination of Folin and Denis (1915), except that in our case there is an excess of phenol, and in theirs an excess of phosphomolybdic acid.

In the actual determination, the solution to be determined is placed in one volumetric flask and a standard solution of potassium phosphate in another. Distilled (phosphate-free) water is added to make both solutions to the same volume, and molybdic acid and hydroquinone solutions are added to both. After standing 5 minutes, the alkaline sulfite solution is added, the flasks are made up to volume, and the colors compared in the colorimeter in the usual manner after further standing of 5 to 10 minutes. It is possible that not all the phosphomolybdic acid is reduced by this process, but the color is strictly proportional to the amount of phosphate present, over a wide range of concentration. The unknown and the standard should have nearly the same acidity, volume, and temperature during the reduction with hydroquinone in acid solution, since variation in these factors has some effect on the final depth of color.

We have developed methods for the determination of inorganic, organic, and total phosphorus in urine and inorganic and total acid-soluble phosphorus in whole blood, plasma, and serum, using the solutions given below.

Molybdic Acid Solution.—50 gm. of pure ammonium molybdate are dissolved without heat in 1 liter of phosphate-free normal sulfuric acid. 5 cc. of this solution, treated with an equal amount of the hydroquinone solution and, after standing 5 minutes, with 25 cc. of the carbonate-sulfite solution, should give an absolutely colorless mixture. If the ammonium molybdate contains phosphate, it may be purified as follows: 150 gm. of

ammonium molybdate are dissolved in 1 liter of water and added to 375 cc. of concentrated nitric acid (specific gravity 1.42) which has been diluted to 1 liter. 200 gm. of ammonium nitrate are dissolved in this mixture and the whole is allowed to stand several days in a warm place. After the precipitated ammonium phosphomolybdate is filtered out, the filtrate is treated with two volumes of alcohol and then with ammonia until only faintly acid to litmus. The molybdate separates completely in a few minutes and is filtered out with suction, washed with 50 per cent alcohol, and dried.

This solution keeps indefinitely. The formation of a small amount of sediment does no harm; it may be removed by decantation.

Hydroquinone Solution.—20 gm. of pure hydroquinone are dissolved in 1 liter of phosphate-free water and 1 cc. of concentrated sulfuric acid is added. On standing, this solution becomes colored due to the formation of some quinone. A moderate amount of color does no harm as the quinone is reduced in the determination by the alkaline sulfite solution. The solution should be kept tightly stoppered to reduce the amount of oxidation by the air.

Carbonate-Sulfite Solution.—To 2,000 cc. of a 20 per cent solution of Na_2CO_3 are added 75 gm. of sodium sulfite dissolved in 500 cc. of water. The mixture is filtered. The presence of a moderate amount of phosphate in this solution does no harm since phosphomolybdates are not formed in alkaline solution.

Stock Solution of Monopotassium Phosphate.—Pure monopotassium phosphate is finely ground and exposed in a desiccator over sulfuric acid for several days. Of this dry preparation, 4.394 gm. are dissolved and made up to 1 liter with phosphate-free water in an accurate volumetric flask. 1 cc. of this solution contains 1 mg. of phosphorus. The solution should be preserved with chloroform and kept tightly stoppered.

Standard Phosphate Solution for Urine.—Of the above stock solution of phosphate, 50 cc. are accurately measured into a 500 cc. volumetric flask and made up to volume with phosphate-free water. This solution should also be preserved with chloroform. If the solution becomes turbid or if molds appear, a new solution should be made up. 5 cc. of this solution contain 0.5 mg. of phosphorus.

Dilute Standard Phosphate Solution for Blood.—5 cc. of the stock phosphate solution are made up to 1,000 cc. and preserved with chloroform in the same manner as the standard solution for urine. 5 cc. of this solution contain 0.025 mg. of phosphorus.

Urine.

Inorganic Phosphate.—1 to 5 cc. of urine, according to concentration, are accurately measured into a 100 cc. volumetric flask and 25 cc. of phosphate-free water added. In a similar flask are placed 5 cc. of the standard phosphate solution for urine, also with 25 cc. of water. To both flasks are added 5 cc. of the molybdic acid solution and 5 cc. of the hydroquinone solution. After standing 5 minutes, 25 cc. of the carbonate-sulfite solution are added and the flasks made up to volume and mixed. The solutions are read after 5 to 10 minutes. While the color is not absolutely permanent, we have found that the standard and the unknown fade at almost exactly the same rate, and that readings made at the end of an hour give the same result as those made at the end of 5 minutes. However, the solution in the cup of the colorimeter does not fade at the same rate as that in the flask, and both cups of the colorimeter should be filled at the same time whenever a comparison is to be made.

If the urine is read against the standard set at 20 mm. the calculation is as follows:

$$\frac{10}{\text{Reading} \times \text{cc. urine used}} = \text{Gm. inorganic P per liter}$$

If an insufficient amount of water is added to the urine before adding the molybdic acid, certain urinary constituents are precipitated by the phosphomolybdic acid leading to slightly high results.

Since 1 to 4 cc. of urine are diluted to 100 cc. during the determination, the color of the urinary pigments seldom interferes with the determination. We have even determined urines containing bile by the above process. If the color of the urine is troublesome, it may be removed by adsorption. For this purpose we have found certain samples of "activated carbon," prepared by the National Carbon Company of Cleveland, to be satisfactory.

A carbon to be used for this purpose should be carefully tested by comparing determinations on the standard solution with and without treatment with carbon, to insure that phosphate is neither added nor removed.

Total Phosphorus.—1 cc. of urine is measured with an Ostwald pipette into a hard glass test-tube and 6 to 8 drops of concentrated sulfuric acid, 1 cc. of concentrated nitric acid, and a piece of quartz are added. The tube is cautiously heated until nitrous fumes no longer come off and the remaining drops of sulfuric acid are colorless. Care should be taken to avoid evaporating to dryness or pronounced overheating which may cause loss of phosphoric acid.[2] The remaining few drops of sulfuric acid are transferred to a 100 cc. volumetric flask with about 25 cc. of phosphate-free water. Into a similar volumetric flask, containing 4 to 6 drops of concentrated sulfuric acid, are measured 2, 3, or 5 cc. of the standard phosphate solution for urine, the amount being determined by the result found for inorganic phosphate. Having been brought to the same volume with phosphate-free water, the standard solution and urine are treated and compared as in the determination of inorganic phosphate. If the urine is compared with the standard set at 20 mm., the calculation is as follows:

$$\frac{2 \times \text{cc. standard used}}{\text{Reading}} = \text{Gm. total P per liter}$$

Organic Phosphorus.—As pointed out by Taylor and Miller (1914), it is not permissible to estimate organic phosphorus by subtracting inorganic from total phosphorus since the amount of organic phosphorus is so small. They recommend precipitation of the inorganic phosphate with barium and determination of the organic phosphorus in the filtrate. The technique is as follows: 20 cc. of the urine are measured into a 25 cc. volumetric flask, made just alkaline with powdered barium hydroxide, filled to the

[2] We have not found that the addition of cane sugar as recommended by Bloor has any influence on the amount of phosphorus obtained by our method. It is possible that phosphoric acid compounds of the type mentioned by Bloor might give the full amount of color by our method and yet fail to be precipitated by his strychnine molybdate reagent. On the other hand, pyro- and *m*-phosphoric acids do not form phosphomolybdic acid and so escape determination by our method.

mark with water, and filtered. The precipitate contains barium sulfate and phosphate. To remove the excess of barium, 20 cc. of this filtrate are measured into another 25 cc. flask, made just acid with dilute sulfuric acid, filled to volume, and filtered. An excess of sulfuric acid is to be avoided since it affects the final acidity of the determination. 1 cc. of this second filtrate is equivalent to 0.64 cc. of the original urine.

We have made a few determinations of organic phosphorus in the following manner: 10 cc. (equivalent to 6.4 cc. of urine) of filtrate prepared as above are evaporated with 8 to 10 drops of concentrated sulfuric acid to about 2 cc., and 2 cc. of concentrated nitric acid are added. The digestion is carried out as described under "Total phosphorus" and the residue transferred to a 25 cc. flask with 10 cc. of distilled water. In another similar flask are placed 5 cc. of the dilute standard solution for blood (equivalent to 0.025 mg. of P), 4 to 6 drops of concentrated sulfuric acid, and 5 cc. of water. To both flasks are added 1 cc. of the molybdic acid solution and 2 cc. of the hydroquinone. After 5 minutes, 10 cc. of the carbonate-sulfite solution are added, and the flasks made up to volume and read as usual, except that it is better to set the standard at 30 or 40 mm. instead of 20 mm. If the urine is read against the standard set at 40 mm., the calculation is as follows:

$$\frac{1{,}000}{\text{Reading} \times 6.4} = \text{Mg. organic P per liter}$$

Urines high in organic phosphorus may be read against a stronger standard. It is better, for most urines, to use the dilute standard described above, rather than to increase the amount of urine filtrate used, since the smaller the amount of urine, the easier is the digestion.

Determinations of organic phosphorus should be done on fresh urines, since, as pointed out by Mathison (1909), the values decrease on standing.

We have also determined the organic phosphorus in urine by precipitating the inorganic phosphate with magnesium citrate as described below, and estimating the phosphorus in the filtrate. The values thus obtained are slightly higher and probably more accurate than those obtained by the barium precipitation, but the

determination is not so convenient and requires at least 8 hours for complete precipitation.

Accuracy.—We attempted to check the results secured on urines by these methods against gravimetric determinations of inorganic phosphate. 50 cc. portions of urine were precipitated with magnesium citrate according to the technique of Mathison (1909) and filtered after standing over night. The paper and precipitate were dried over night at room temperature and weighed. The precipitate was then brushed off the paper and the paper reweighed as suggested by Jones (1916).[3]

Table I gives the values for inorganic phosphate obtained by this gravimetric process, by our colorimetric method, and by the uranium titration, using ferrocyanide as indicator. The results by the uranium titration are usually distinctly higher than by the other two methods. The colorimetric and gravimetric methods both give good duplicate determinations, but the colorimetric values are higher.

That the difference between the results by the gravimetric and colorimetric methods is due to too low results by the former, is indicated by the values in Table II. The determinations in this series were done just as in the first series except that the filter papers, after reweighing, were extracted with dilute sulfuric acid and the phosphate thus recovered was determined colorimetrically. The first column under gravimetric determination in Table II gives the result calculated from the weight of precipitate obtained, the second column gives the additional amount calculated from the precipitate adhering to the filter paper, and the third column gives the sum of these two. In this second series, the results by the colorimetric and the corrected gravimetric methods are in much better agreement than in the first series. The results by the uranium titration tend to be higher than those by the other methods, and this is especially true in the urines containing albumin and bile.

Although the filter paper (7 cm., Schleicher and Schull's No. 589, black ribbon) was as small as could be conveniently used, examination of the column of corrections shows that quite large

[3] This method of determination was suggested to us by Dr. C. H. Fiske of this department, to whom we are also indebted for other valuable suggestions.

TABLE I.

Inorganic Phosphate as P per Liter.

Urine.	Gravimetric.	Colorimetric.	Uranium.
	gm.	*gm.*	*gm.*
1. Normal mixed.		0.135 0.135	0.145
2. " "		0.119 0.119	0.127
3. " "	0.5568 0.5564	0.555 0.553	0.562
4. " "	0.2388 0.2384	0.253 0.255	0.262 0.260
5. " "	0.1356 0.1356	0.143 0.142	0.145 0.146
6. " "	0.2899 0.2899	0.294 0.294	0.310 0.307
7. Sugar 1 per cent; $FeCl_3$+++.	0.3954 0.3954	0.421 0.416	0.415 0.414
8. " 2 " " $FeCl_3$+++.	0.1911 0.1915	0.216 0.218	0.213 0.210
9. No sugar; $FeCl_3$+++.	0.3706 0.3713	0.406	0.392 0.389
10. Albumin +++.	0.4408 0.4417	0.455	0.445 0.450
11. Normal mixed.	0.3000 0.3012	0.308	0.313
4a. "	0.2850	0.284	
8a. Sugar present.	0.1918	0.211	0.209

TABLE II.

Inorganic Phosphate as P per Liter.

Urine.	Gravimetric.			Colorimetric.	Uranium.
	Weighed.	Correction.	Corrected value.		
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
12. Normal mixed.	0.5739	0.0028	0.5767	0.584	0.564
	0.5693	0.0073	0.5766		0.570
	0.5703	0.0056	0.5759		
13. Sugar 2.3 per cent; $FeCl_3$++; albumin +.	0.2315	0.0090	0.2405	0.235*	0.263
					0.269
14. Sugar 0.1 per cent; $FeCl_3$−.	0.2169	0.0068	0.2237	0.227	0.225
	0.2134	0.0096	0.2230		0.224
14a. Same as No. 14, 2 days later.	0.2399	0.0084	0.2483	0.244	
15. Sugar 0.2 per cent; $FeCl_3$+++.	0.1244	0.0010	0.1254	0.124	0.127
	0.1254	0.0007	0.1261		
16. Sugar 1.5 per cent; $FeCl_3$++.	0.1840	0.0048	0.1888	0.188	0.193
	0.1858	0.0035	0.1893		0.195
17. Sugar 0.4 per cent; $FeCl_3$−; bile ++.	0.4649	0.0192†	0.4841	0.485	0.515
	0.4700	0.0167†	0.4867		
18. Normal mixed.	0.2599	0.0035	0.2634	0.258	0.269
	0.2599	0.0035	0.2634	0.260	0.271
19. " "	0.1524	0.0030	0.1554	0.157	0.162
	0.1532	0.0025	0.1557	0.158	0.162
20. " "	0.1636	0.0018	0.1654	0.166	0.169
	0.1641	0.0015	0.1656	0.167	0.170

* This value was obtained after clearing with carbon. The urine contained much mucus and was very turbid.

† The precipitate in this urine was extremely fine so that some of it passed through the filter. This was collected by centrifuging, and is included in the correction.

and variable amounts of precipitate adhere so firmly that they cannot be brushed loose. The amount of this adhering precipitate varies with different urines, though it is usually nearly the same in duplicate determinations, and it is perhaps dependent on variations in crystal size and shape in different urines. The urines having the largest corrections are those containing albumin and bile. When dealing with 50 cc. of urine, it is evident that satisfactory duplicate determinations by weighing magnesium ammonium phosphate are no guarantee of accuracy. However, the variations obtained with different urines and the low correction in certain urines (Nos. 15 and 20, Table II) suggest that with other material the adhering precipitate may be much less.

Blood.

For the determination of the inorganic and acid-soluble phosphorus in blood we have made use of trichloroacetic acid. The acid ammonium sulfate used by Bloor (1918) cannot be conveniently used with our method on account of the large amount of salts in the filtrate. The tungstic acid filtrates of Folin and Wu (1919) give consistently low results, probably due to the formation of phosphotungstic acid from the tungstic acid reagent and the inorganic phosphate of the blood and combination of this substance with the protein precipitate. The acetic-picric acid filtrates of Greenwald (1916) cannot be used for the determination of inorganic phosphate by our method on account of the interference of the color of the picric acid. Acetic-picric acid filtrates can be used for the determination of the total acid-soluble phosphorus if the picric acid is driven off as described by Greenwald and the residue digested with nitric and sulfuric acids. We have always found trichloroacetic acid to be free from phosphorus, but all samples should be tested.

We have found that a dilution of 1:5 is sufficient for normal plasma and gives the same result as a dilution of 1:10. If the plasma is suspected to be high, or if whole blood is used, we recommend a dilution of 1:10. 5 cc. of plasma are mixed with 15 cc. of distilled water in a 25 cc. volumetric flask and 5 cc. of 20 per cent trichloroacetic acid added with shaking. The flask is filled to the mark with water, mixed, and after 10 minutes is

filtered through a phosphate-free (acid-washed) filter. If whole blood is used, 5 cc. are laked by mixing with 35 to 40 cc. of distilled water in a 50 cc. volumetric flask and 5 cc. of 20 per cent trichloroacetic acid are added with shaking. The flask is made up to volume, and filtered after 10 minutes.

Inorganic Phosphate.—To 10 cc. of the trichloroacetic acid filtrate in a 25 cc. volumetric flask are added 1 cc. of the molybdic acid solution and 2 cc. of the hydroquinone solution. At the same time, molybdic acid and hydroquinone are added to a similar flask containing 10 cc. (equivalent to 0.05 mg. of P) of the dilute standard phosphate solution for blood, and either 1 or 2 cc. of 20 per cent trichloroacetic acid, according to whether a dilution of 1:10 or 1:5 has been used. After 5 minutes, 10 cc. of the carbonate-sulfite solution are added, and the flasks are made up to volume and mixed. The colors are compared after 5 to 10 minutes. If the unknown is read against the standard set at 20 mm., the calculation is

$$\frac{100}{\text{Reading}} = \text{Mg. P per 100 cc. (if 1:10 dilution was used)}$$

or

$$\frac{50}{\text{Reading}} = \text{Mg. P per 100 cc. (if 1:5 dilution was used)}$$

It may be necessary to use a stronger standard or less filtrate in bloods containing much phosphorus. The proper standard and amount of filtrate may be roughly estimated before the determination is made. A 5 cc. mark is made on two small similar test-tubes. In one of these tubes is placed 1 cc. of the dilute standard phosphate solution for blood and in the other, 1 cc. of filtrate. About 10 drops of molybdic acid and hydroquinone solutions are added to both tubes, and after a few minutes the tubes are filled to the mark with carbonate-sulfite solution. The amount of color may be roughly gauged and the strength of standard and amount of filtrate decided upon. Serum or plasma may be roughly estimated in the same manner before removing the proteins in order to judge the proper dilution to use.

results. After standing 24 hours, the values for inorganic phosphate and total acid-soluble phosphorus are nearly identical. One plasma, containing 9.6 mg. of acid-soluble phosphorus per 100 cc., gave the following values for inorganic phosphate: 30 minutes after filtering, 8.8 mg.; 18 hours after filtering, 9.4 mg. Another plasma, containing 3.5 mg. of acid-soluble phosphorus,

TABLE III.

Inorganic and Total Acid-Soluble Phosphorus as P per 100 Cc.

Specimen.	Inorganic phosphate.	Acid-soluble phosphorus.	
		Trichloro-acetic acid.	Acetic-picric acid.
	mg.	*mg.*	*mg.*
1. Plasma, human	3.5	5.0	
2. " "	3.9*	4.0	
3. Whole blood, beef	4.9	8.3	
	5.1	8.6	
4. " " "	5.9	11.6	
5. Plasma, human (hemolysis)	9.0	10.5	
6. " " (diabetic)	4.4	5.1	
7. " " "	4.4*	4.4	
8. " " "	4.5	5.0	
9. " " "	4.8†	4.7	4.8
10. " " (hemolysis)	8.8	9.8	9.6
11. Whole blood, beef	5.1	10.3	10.3
12. Plasma, human (diabetic)	3.2	3.6	3.5
13. " beef	8.0*	8.1	8.0
14. Same as No. 13 with added phosphate (40 mg. per 100 cc.)	47.7		

* These specimens were several days old when analyzed.

† The filtrate from this specimen stood 24 hours before the determination was carried out.

gave the following values for inorganic phosphate: 30 minutes after filtering, 3.2 mg.; 90 minutes after filtering, 3.4 mg.; 5 hours after filtering, 3.4 mg.

Total Acid-Soluble Phosphorus.—The total acid-soluble phosphorus is determined in a manner similar to the total phosphorus in urine. 10 cc. of the trichloroacetic acid filtrate, in a hard glass test-tube, are evaporated down to about 2 cc. with 6 to 8 drops of concentrated sulfuric acid and a piece of quartz. 1 cc.

of concentrated nitric acid is added and the digestion continued until all the nitric acid has been driven off and the remaining drops of sulfuric acid are clear. The residue is transferred to a 25 cc. volumetric flask with about 10 cc. of distilled water and treated as under inorganic phosphate. The standard to be used is determined by the value previously found for inorganic phosphate. 4 to 6 drops of concentrated sulfuric acid are added to the standard to balance that used in the digestion but no trichloroacetic acid is added, since all of this substance is volatilized during the digestion.

Results.—Table III gives some values obtained by these methods. The agreement between the trichloroacetic acid and picric acid filtrates is satisfactory. The ratio of inorganic to total acid-soluble phosphorus is about that usually found by other methods.

SUMMARY.

Rapid colorimetric methods are described for the determination of inorganic, organic, and total phosphorus in urine. While not so accurate as suitable gravimetric determinations, these methods are much more convenient and are sufficiently accurate for many purposes. The method for inorganic phosphate appears to be more accurate and rapid than the usual uranium titration.

Methods are also described for the determination of inorganic and acid-soluble phosphorus in blood.

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