

THE COLORIMETRIC DETERMINATION OF PHOSPHORUS.

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The Bell and Doisy method¹ for the determination of phosphate, based on the reduction of phosphomolybdic acid by hydroquinone to a substance which is blue in the presence of sulfite, had one weakness, the rapid and irregular fading of the color in alkaline solution. By omitting the use of alkali, Briggs^{2, 3} has succeeded in eliminating this particular difficulty, but others, in some respects more serious, have at the same time been introduced. Briggs has suggested so many variations of his own method that no statement that could be made about any one of them would apply precisely to them all. We shall confine ourselves for the most part to the two (one for blood and the other for urine) described in his first paper on the subject.²

In the urine method, where ordinarily the greatest accuracy would be needed, the conditions during the synthesis of phosphomolybdic acid and its subsequent reduction by hydroquinone are distinctly different from those proposed by Bell and Doisy; chiefly, the concentration of hydroquinone called for in Briggs' directions is only $\frac{1}{50}$ as high. To counterbalance this to some extent, Briggs prolongs the reaction time from 5 to 30 minutes, but the color produced even then is certainly not more than four-fifths of the maximum. The reduction of phosphomolybdic acid by hydroquinone must be a reaction of at least the second order. To get proportionate readings with a bimolecular color reaction, one condition must absolutely be fulfilled—the reagent must be added in such large excess that the reaction runs an apparently unimolecular course. In Briggs' urine method, where this is not

¹ Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.

² Briggs, A. P., *J. Biol. Chem.*, 1922, liii, 13.

³ Briggs, A. P., *J. Biol. Chem.*, 1924, lix, 255.

the case, the deviation from proportionality with a solution one and two-thirds times the strength of the standard is as much as 3 per cent.

In the method which he first proposed for blood, Briggs did increase the speed of the reaction by using more of the reagents in proportion. Thus, he recommends 1 cc. of 0.5 per cent hydroquinone whether the final volume is to be 100 cc. (as with urine) or 10 cc. (as it ordinarily would be in the analysis of blood). The concentration of hydroquinone is hence ten times as high in the latter case; and the concentration of the acid molybdate reagent is similarly increased four times. Other things being equal, the proportionality should thereby be improved, but actually it is made distinctly worse. The reason is that the molybdic acid and hydroquinone, in the absence of phosphate, then give a brownish yellow color which is partly, though by no means wholly, discharged by sulfite.⁴ The blank so introduced has a colorimetric value equal to more than 10 per cent of the blood standard, and unless the standard and unknown are nearly equal the tints are not at all alike. Accurate matching under such conditions is very difficult, if not impossible. According to Briggs, this method gives exact results with solutions one and two-thirds times as strong as the standard, and also with less than half the standard quantity of phosphate; but in our experience the error in the former case is about 4 per cent, and in the latter fully 10 per cent.⁵

⁴ In the Bell and Doisy method this color is destroyed by adding alkali, and hence does not interfere.

⁵ More recently,³ in connection with the analysis of blood, Briggs proposes to intensify the color by means of two modifications, neither of which accomplishes that object. Since an excess of acid diminishes the color he aims, by using less of the acid molybdate reagent, to make it stronger,—but in this respect the two constituents of the reagent (molybdate and sulfuric acid) practically balance each other, and actually there is nothing gained. Postponing the addition of the sulfite until the reduction is well under way (his second alteration) does accelerate the development of color in the early stages, but at the end of 30 minutes it is no deeper than when sulfite is added at the start. The somewhat greater color which Briggs now gets, as compared with his original blood method, is the result of two quite different changes, on which he makes no comment. That is to say, he doubled the amount of hydroquinone, and likewise increased the standard from 0.025 to 0.03 mg. of phosphorus. The blank is not appreciably different in the modified procedure.

Since small amounts of hydroquinone reduce too slowly, and large amounts give a color with molybdic acid itself, it is clear that a really satisfactory method based on this principle calls for a reducing agent which is much more rapid. Such an agent, to be thoroughly acceptable, must bring about the practically complete reduction of phosphomolybdic acid within a reasonable period when added in a concentration too low to give a blank.

In our attempts to solve this problem, we have had in mind a method applicable to material of widely varying nature, and not merely the demands of ordinary blood and urine analysis. There the conditions are comparatively simple—besides the anticoagulant, the only interfering substances known to be important are the trichloroacetic acid with which the protein is precipitated and the sulfuric acid used for the digestion in total phosphorus determinations. While these things, as Martland and Robison⁶ have found, are not so harmless in the Briggs method as was at first supposed, minor alterations in technique suffice to make them so in so far as blood filtrates are concerned.⁷

The determination of total phosphorus in the presence of much organic matter is a somewhat different proposition, for ashing with sulfuric acid (aided by nitric acid or hydrogen peroxide) has its limitations. The quantity of sulfuric acid used must not be too large, otherwise the reduction of phosphomolybdic acid will be retarded; and it must not be too small, or there will be danger of loss of phosphate (variously ascribed to volatilization,⁸ to the formation of pyro- or metaphosphoric acid,¹ and to combination with the glass).^{6, 9}

Organic matter which gives rise to picric acid when digested by the so called Neumann method offers special difficulties—a completely colorless solution is then sometimes impossible to get without danger of overheating, and consequent loss of phosphate. In cases of this kind, and in fact when dealing with very refractory material of any sort, dry ashing is the only way left open. The most widely used reagent for this purpose—a mixture of sodium

⁶ Martland, M., and Robison, R., *Biochem. J.*, 1924, xviii, 765.

⁷ With respect to trichloroacetic acid, this is not always true. The point will be considered later.

⁸ Baumann, E. J., *J. Biol. Chem.*, 1924, lix, 667.

⁹ Whitehorn, J. C., *J. Biol. Chem.*, 1924-25, lxii, 133.

carbonate and potassium nitrate—causes hopeless complications in the subsequent steps of Briggs' method. In order to provide the proper acidity for the color reaction, the carbonate must first of course be neutralized. The most convenient, and practically the only feasible way to do this is by evaporation to dryness with a small excess of hydrochloric acid. The residue is then chiefly sodium chloride, with some nitrate, and analysis of this mixture with any accuracy (by Briggs' method) is precluded by the fact that chloride and nitrate both intensify the phosphate color, while nitrite (if any should be present) would have the opposite effect. Even with material that can be ashed with carbonate alone, erroneous results will be inevitable if the amount of carbonate exceeds about 0.3 gm.

Another source of error to be reckoned with, when the organic matter has been destroyed by alkali, is silicate. In 30 minutes, by Briggs' method, 1 mg. of Si gives as much color as 0.4 mg. of P. Some plant and animal tissues contain considerable silicate, and a rapid colorimetric phosphate method not affected by this substance might be found useful also in other fields than biochemistry.

In connection with the determination of total phosphorus in such materials as blood, regardless of the method of digestion, it is worthy of note that the reduction of phosphomolybdic acid is markedly retarded by ferric salts. Blood generally contains more iron than phosphorus; in some species four or five times as much—enough to introduce a serious error.

We have listed, in Table I, data which will serve to show, in a general way, how the development of color by hydroquinone is affected by the interfering substances which have been mentioned. Included there as well are the results of some observations with ammonium sulfate, which Rimington¹⁰ has already shown to modify the color. His data are presented only in the form of graphs, and from them it is impossible to judge the safe minimum concentration of this salt. Rimington's main concern was with the diminution of color intensity in strong ammonium sulfate solutions. The increase in color in more dilute solutions, which he passes over with slight comment, seems to us at least equally important.

¹⁰ Rimington, C., *Biochem. J.*, 1924, xviii, 1297.

If the departure from exact proportionality (amounting to a few per cent) were the only drawback, the Briggs method might still be made accurate enough by the use of appropriate corrections. But no such correction could be relied upon if the color does not

TABLE I.
Effect of Various Substances on Color Production in Briggs' Urine Method.
Volume, 100 cc. 0.5 mg. P. Time, 30 min. Standard set at 20 mm.

Substance added.	Reading.
	mm.
Sulfuric acid (additional), 0.1 N.....	20.3
0.2 ".....	20.5
0.3 ".....	20.9
Sodium chloride, 0.2 M.....	19.4
0.5 ".....	18.2
M.....	17.0
Potassium nitrate, 0.04 M.....	19.6
0.2 ".....	18.2
0.4 ".....	16.9
Sodium nitrite, 0.002 M.....	20.2
0.01 ".....	22.0
0.02 ".....	Yellow.
Ammonium sulfate, 0.05 M.....	19.5
0.25 ".....	18.1
0.5 ".....	17.7
Ferric chloride, 1 mg. Fe.....	20.4
5 " ".....	22.3
15 " ".....	30.0
Sodium silicate, 0.25 mg. Si.....	19.3
0.5 " ".....	18.8
2.0 " ".....	15.1

develop at the normal rate. We have given abundant evidence that many things do affect the rate, and many more undoubtedly could be found.

A reducing agent which would give the maximum color in 30 minutes (*i.e.*, at least 18 per cent more than hydroquinone, see

Table I), would help to only a limited extent. There would still be errors to contend with from the presence of substances which retard the color reaction. The first reducing agent that we found, capable of yielding about 20 per cent more color than hydroquinone in half an hour, was 5-aminosaligenin. By this time we had come to appreciate the tremendous advantage to be gained by the use of a reagent which would reduce phosphomolybdic acid (selectively of course) at a rate many times more rapid still. The necessity of waiting 30 minutes would be dispensed with, in the first place. But more important, a reducing agent which would normally give almost the maximum color within 5 minutes, let us say, would provide a means of determining (when working with unknown material) whether or not the color reaction is proceeding at the normal rate and when it is complete. In other words, the presence of any interfering substance would then be automatically revealed by a change in the colorimeter reading from one minute to the next. Finally, a very material increase in reduction rate offers the only hope of getting accurate proportionality of color over a wide range, without waiting several hours.

It is natural to expect that any reducing agent capable of meeting this criterion would itself be more or less unstable. Besides activity, therefore, the keeping qualities of the reagent had to be kept constantly in mind, as well as its solubility and the ease with which it can be prepared and purified. Two substances (2-chloro-4-methylaminophenol and 2, 4-diaminophenol) were found which, although better than anything that had been tried before, were still not entirely satisfactory from every standpoint.¹¹

At this juncture we tried an old sample of 1, 2, 6-aminonaphtholsulfonic acid (which was in stock and had been partially purified) with very encouraging results. Impure as it still was, it gave more color than anything that had been tested previously. A pure sample of this acid, made following the directions of Meldola,¹² gave about the same result. It acts so rapidly, in fact,

¹¹ Several other compounds have been tried. Monochlorohydroquinone and 2,5-dichlorohydroquinone are even slower than the unsubstituted phenol, while *p*-methylaminophenol and 2-chloro-4-aminophenol are not appreciably better. (For samples of a number of these products we are much indebted to Mr. W. G. Christiansen of the Department of Pharmacology.)

¹² Meldola, R., *J. Chem. Soc.*, 1881, xxxix. 47.

that the color intensity obtained by means of hydroquinone in half an hour is exceeded, in the presence of an equimolecular amount of the sulfonic acid, in a fraction of a minute; its activity as a reducing agent for this purpose must be more than 50 times as great.

1, 2, 6-Aminonaphtholsulfonic acid is fairly difficult to make. The preparation of one of its isomers (the 1, 2, 4 acid) has been described in full detail by Folin¹³ as a step in making his amino acid reagent; it is also on the market in crude form, and can be purified without much trouble. Inasmuch as the two isomers reduce phosphomolybdic acid with approximately equal speed, the more available one is naturally to be preferred.

The effect of those substances,¹⁴ most likely to be present in the analysis of biological material, which are known to retard the reduction of phosphomolybdic acid by this new reagent or to interfere in any other way, may be seen in Table II. In the experiments recorded there, the standard was in every case the same—0.4 mg. of phosphorus in a volume of 100 cc., containing 0.25 gm. of ammonium molybdate, 10 cc. of 5 N sulfuric acid, 0.6 gm. of sodium bisulfite, and 0.01 gm. of aminonaphtholsulfonic acid. The solution to be read against this standard contained between 0.2 and 1.6 mg. of phosphorus, besides the added foreign substance; the other reagents were the same as in the standard, except in certain special cases (indicated in the table) where a larger or smaller concentration of sulfuric acid was used. Readings were made at frequent intervals, beginning 2 minutes after adding the sulfonic acid, and continuing until there was no further change.

Allowing 5 minutes for the development of the color, or a little longer if the phosphate concentration is more than twice that in the standard, aminonaphtholsulfonic acid will generally give accurate results in the presence of at least ten times the amount of inhibiting material that would be permissible with hydroquinone. Moreover, when the safe limit has been exceeded, the fact will be made evident by a lack of constancy in the reading, and in some cases (ferric salts, ammonium sulfate, nitrite) also by a difference in tint between standard and unknown. We suggest that, when

¹³ Folin, O., *J. Biol. Chem.*, 1922, li, 386.

¹⁴ Recrystallized, when necessary to get them phosphate-free.

TABLE II.
Colorimetric Readings by Proposed Method in Presence of Interfering Substances.
 Standard (0.4 mg. P) set at 20 mm. Volume, 100 cc. Temperature, 25–27°.

H ₂ SO ₄ concentration.	0.5 N		0.6 N		0.7 N		0.5 N										0.0 N		0.3 N	
	Substance added.	0	0	0	0	0	^M NaCl	0.4 M KNO ₃	NaNO ₂		(NH ₄) ₂ SO ₄		Ferric chloride.			Na silicate.		20 cc. 20 per cent CCl ₃ COOH + 30 mg. potassium oxalate.†		
									0.005 M	0.02 M	0.25 M	0.5 M	2 mg. Fe.	8 mg. Fe.	15 mg. Fe.	Si:P= 50	Si:P= 200			
Time.																				
min.																				
2		39.8			39.9	40.0	39.5	39.6		40.5			39.7			39.3	39.5	39.9		
5		39.6			39.6	39.8	39.6	39.3		39.8	40.4		39.4		37.2	38.7	39.3	39.5		
10		39.6			39.4	39.6	39.7	39.4		39.7	38.9		39.6		37.1*	38.1	38.8	39.6		
20											38.7†					36.7	38.1			
0.8 mg. phosphorus (theoretical reading, 10.0 mm.).																				
2		10.2	10.2	11.2	10.3	10.3			10.4	10.7	14.2		11.1	12.4	10.2	10.2	10.2			
3		10.1	10.1	10.6	10.1	10.2			10.0	10.2	11.9		10.4	10.3	10.1	10.1	10.2			
4		10.1	10.1	10.3	10.1	10.1				10.1	10.6		10.3	10.1			10.1			
5		10.1	10.1	10.2	10.1	10.1			10.0*	10.1	10.2		10.2	9.9	10.0	10.0	10.1			
10				10.1							10.1		10.0*	9.8*	9.9	10.0				
20															9.7	9.9				

1.6 mg. phosphorus (theoretical reading, 5.0 mm.).

2	5.4	6.3	7.3	6.1	6.1	5.5	6.5	10.8	6.9	5.3	5.2	5.8
3	5.2	5.8	6.5	5.6	5.6	5.3	5.6	8.2	5.7	5.2	5.1	5.5
4	5.1	5.5	6.1	5.3	5.3	5.2	5.3	7.2	5.3	5.1	5.1	5.3
5	5.1	5.4	5.9	5.2	5.2	5.2	5.2	6.4	5.2	5.1	5.1	5.2
6		5.3	5.7	5.1				5.8	5.1	5.3		5.1
8		5.1	5.5	5.1		5.1	5.1	5.3				
10			5.4					5.1				
20			5.1							5.0	5.0	

* The solution has a slight color when no phosphate is present.

† The solution has a purple tint, and does not exactly match the standard.

‡ Roughly corresponding to the composition of the filtrate from blood containing 3 mg. potassium oxalate per cc.¹⁵

33

¹⁵ If oxalate did not penetrate the corpuscles, its concentration in the plasma would be much higher than in whole blood. In point of fact, the oxalate is about equally distributed between corpuscles and plasma. *E.g.*, 20 cc. of blood were mixed with 1 cc. of a solution containing 60 mg. of potassium oxalate (making the concentration in the mixture 2.86 mg. per cc.) and centrifuged. A sample of the plasma was precipitated with trichloroacetic acid as in the phosphate method, and the filtrate analyzed for oxalate following the general principles in common use for calcium estimations. 5 cc. of the filtrate (equivalent to 1 cc. of plasma) were found to contain 2.74 mg. of potassium oxalate.

working with material of uncertain nature, readings be taken after 5 and again after 10 minutes. If the two agree (assuming that the two solutions are practically alike in shade) there can be, as far as our experience goes, no doubt about the accuracy of the result. If, on the other hand, the reading has altered within that period, a third should be taken 5 minutes later. In case of any change occurring after 10 minutes, it is advisable to repeat the analysis with a smaller sample.¹⁶

That the numerous readings given in Table II might all represent the conditions finally adopted for the method here described, we were obliged, in order to avoid a delay of several months, to make them in warm weather (between 25 and 27°C.). The temperature coefficient of the color reaction as a whole happens to be distinctly high; a rise of 5° somewhat more than doubles the velocity. Except under extreme conditions, however, an allowance of 5 minutes is sufficient even at 20° if the reading is not less than 10 mm. At 20°, in the absence of interfering matter, 1.6 mg. of phosphorus (in a volume of 100 cc.) will take about 8 minutes to reach the proper reading. Agreement between consecutive readings, taken a few minutes apart, is perfectly reliable as a guide whatever the temperature may be.¹⁷

The superiority of aminonaphtholsulfonic acid is even more

¹⁶ We wish to warn specifically against attempting to make the conditions in standard and unknown more uniform by altering the former (*e.g.*, by adding trichloroacetic acid and oxalate in the analysis of blood filtrates). Nothing can be gained by this procedure unless the two solutions are nearly equal in their phosphate content, and very little then. The danger lies in the chance of forfeiting what might otherwise be learned by taking two or more successive readings on the same solution; for, if the intensity of the standard is increasing too, any change in the unknown will be that much less readily detected. The advantage, also, of a single standard which serves for every purpose is far from being insignificant.

¹⁷ Much time may be saved in special cases by the judicious use of warm water. For example, the trichloroacetic acid filtrate from a sample of nephritic plasma, when analyzed for inorganic phosphate in the customary manner (described below), gave a reading of 6.2. To a second sample of the same filtrate was added enough potassium oxalate to correspond to 3 mg. per cc. of plasma (in excess of that already present), followed by the usual reagents. Under these conditions, it would ordinarily take about half an hour to reach a constant reading. The unknown was therefore warmed for 5 minutes in a beaker of water kept at 35°. The reading was then 6.2, and underwent no further change.

pronounced in the case of solutions containing more than a trace of silicate. Partly because of the higher acidity¹⁸ which we use—higher than is safe with hydroquinone—there will be no error in the presence of 25 mg. of silicon to 1 mg. of phosphorus if the color is read within 5 minutes. When larger amounts of silica are present, accurate analyses can still be made (even with 200 times as much silicon as phosphorus) by adding to the unknown solution (before the molybdate) 1 cc. of 10 N sulfuric acid in case the solution is to be diluted to 100 cc., or a proportionate amount for smaller volumes. The total sulfuric acid concentration will then be 0.6 N, and the synthesis and subsequent reduction of silicomolybdic acid about one-eighth as fast as in 0.5 N acid.

Nitrite, in amounts too small to produce a noticeable error, will confer a yellowish tinge¹⁹ on the solution, easily perceived when examined in the colorimeter.²⁰ The permissible quantity of nitrite can be made considerably greater by using more bisulfite; if no yellow color can then be seen when making the reading, the result will be correct. In dealing with solutions containing much nitrite, the formation of gas bubbles offers another warning signal—in the colorimeter they make the solution appear gray by comparison with the standard. If the colorimeter cup is removed and gently rotated, the gas can all be liberated and accurate readings made, provided that enough bisulfite has been added.²¹

Sodium chloride and potassium nitrate both slightly retard the reduction. We have not attempted to establish definitely the upper limits that are safe in these two cases—it is sufficient that a good deal may be present without perceptible effect. For ashing purposes, as much as 5 gm. of a mixture of sodium carbon-

¹⁸ In our method the sulfuric acid concentration in the final mixture is 0.5 N; in Briggs' urine method about 0.27 N. At the latter acidity, aminonaphtholsulfonic acid will not give proportionate readings, while in 0.5 N acid hydroquinone will do so in the course of time (a matter of some hours). When the acidity is too low, either molybdic acid itself is reduced, or traces of silicate in the reagents interfere—we are not sure which is the more important factor.

¹⁹ A yellow color with various other naphthol derivatives has been used as a qualitative test for nitrite (*cf.* Vági, S., *Z. anal. Chem.*, 1925, lxvi, 14).

²⁰ With hydroquinone there is no such guide; the color intensity is affected by less nitrite than is required to modify the tint.

²¹ For each cc. of M nitrite present, the solution should contain about 3 cc. of 15 per cent bisulfite.

ate and potassium nitrate may be used (for quantities of phosphorus ranging from 0.2 to 1.6 mg.), and that is more than enough to cover ordinary needs.²²

Ammonium sulfate has a more pronounced effect than either of the salts just mentioned. Its presence, however, can be recognized by the purple shade of the solution, which appears when the concentration of ammonium sulfate is still too low to change the colorimeter reading.

The effect of ferric chloride is quite different from that of anything else that we have tested. In solutions which contain trivalent iron, the development of color could almost be called a "clock reaction." This feature is especially prominent in phosphate solutions much stronger than the standard, containing fairly large amounts of iron. There is then at first only a pale green color, which very gradually becomes deeper, though still green. After a latent period, which is longer the more iron there is present, the color suddenly becomes much more intense, and at the same time changes from green to blue. The time required for the reaction to reach any arbitrarily selected stage (*e.g.* the point where a printed letter observed through a given thickness of the solution becomes invisible) can be reproduced under uniform conditions within a few seconds. Once this final stage of the reduction has begun, the color reaction then proceeds with practically the same velocity that it would have shown if no iron had been added, as may be clearly seen from the experiment (Table II) with 1.6 mg. of phosphorus and 15 mg. of iron.

A cursory examination of Table II will make it evident that, in the presence of any substance which retards the color reaction, the time consumed in reaching the correct reading is longer the higher the phosphate concentration.²³ Although we have included

²² The nitrate, on ignition, is reduced to nitrite. It is assumed that most of the nitrous acid will be disposed of by evaporating with hydrochloric acid.

²³ This point might well be borne in mind in connection with the influence of foreign material on any colorimetric method. The mere fact that the color intensity of the standard is no different in the presence of a given substance has no bearing on what will happen if the solution is stronger or weaker than the standard. The truth of this is demonstrated in nearly every column of Table II.

solutions up to four times the strength of the standard in our tests, the reading of colors so far apart as that is not to be recommended as a routine practice, at least without special care in adjusting the zero points of the colorimeter. The proportionality range is nevertheless extremely wide, as color reactions go, and the knowledge that it does extend so far has in our hands prevented many unnecessary repetitions. By the use of small corrections, the accuracy of the reading can be made to compare favorably with that attainable in most colorimetric methods over a much narrower range.

We are not sure that the kind of colorimeter is immaterial. All the readings reported in this paper were obtained with the Bausch and Lomb "biological type," and with our instrument the correction is about 1 per cent when the reading is 10 mm., the same when it is 40 mm., and 2 per cent or slightly less for readings as low as 5 mm. That is, the averages of many consistent readings on solutions of the three strengths indicated are (to the nearest tenth) 10.1, 39.6, and 5.1 mm., respectively.

Having discussed what we have learned of the limitations of the method in the analysis of known mixtures, we may now proceed to its application to biological material, particularly to urine and to blood.

Solutions Needed.

10 N Sulfuric Acid.—450 cc. of concentrated sulfuric acid added to 1300 cc. of water.

Molybdate I.—2.5 per cent ammonium molybdate in 5 N sulfuric acid. Dissolve 25 gm. of the salt in 200 cc. of water. Rinse into a liter volumetric flask containing 500 cc. of 10 N sulfuric acid. Dilute to the mark with water and mix.

Molybdate II.—2.5 per cent ammonium molybdate in 3 N sulfuric acid. Prepared as above, but with only 300 cc. of 10 N sulfuric acid. (To be used only with blood filtrates in the determination of inorganic phosphate.)

Molybdate III.—2.5 per cent ammonium molybdate in water. As soon as any considerable amount of sediment (ammonium trimolybdate) has appeared in this solution, it should be discarded.

10 Per Cent Trichloroacetic Acid.—The quality of this reagent is of great importance. One brand that we have tried contains some unknown impurity which retards the color development to a most pronounced degree. Merck's U.S.P. product, which we now use, is free from any such contamina-

tion, but contains a trace of phosphate. The amount of this must be determined in each sample,²⁴ or else the reagent purified by distillation.⁶

Standard Phosphate (5 Cc. = 0.4 Mg. P).—Dissolve 0.3509 gm. of pure monopotassium phosphate in water. Transfer quantitatively to a liter volumetric flask, add 10 cc. of 10 N sulfuric acid, dilute to the mark, and mix. The standard keeps indefinitely.

15 Per Cent Sodium Bisulfite.—The solution must be free from turbidity before it can be used. Freshly prepared sodium bisulfite solutions may not filter clear, in which case 2 or 3 days standing (before filtering) will be necessary. Keep well stoppered.

20 Per Cent Sodium Sulfite.—Because it is likely to be of better quality, we prefer to use the crystalline sulfite ($\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$). Dissolve 200 gm. of this in 380 cc. of water. Remove any suspended matter by filtration, and keep stoppered.

0.25 Per Cent Aminonaphtholsulfonic Acid.—Dissolve 0.5 gm. of the dry powder (see next section) in 195 cc. of 15 per cent sodium bisulfite, add 5 cc. of 20 per cent sodium sulfite, stopper, and shake until dissolved. If the bisulfite solution is old, more than 5 cc. of sulfite will be needed—in that event add more sulfite 1 cc. at a time, shaking after each addition, until solution is complete. This reagent can be prepared in a few minutes (the powder need not be very accurately weighed), and if not left exposed to the air it should keep about 2 weeks. The solution is more stable the higher its acidity, hence no more sulfite should be added than is needed to dissolve the reducing agent.

1, 2, 4-Aminonaphtholsulfonic Acid.

This may be prepared from β -naphthol according to Folin's directions, with a single alteration. The final product, after washing with cold water,²⁵ still contains some colored material. To remove this, the crystals

²⁴ Arrange three tall beakers of 150 cc. capacity on a piece of white paper. Into one of these (A) put 100 cc. of water. In a second beaker (B) mix 85 cc. of water, 10 cc. of Molybdate I, and 4 cc. of 0.25 per cent aminonaphtholsulfonic acid; the result should be a solution practically as colorless as water, without a trace of blue (otherwise one or more of the reagents already added contains phosphate). To the third beaker (C) add 40 cc. of the trichloroacetic acid solution, 45 cc. of water, 10 cc. of Molybdate II, and 4 cc. of the sulfonic acid reagent, stirring thoroughly with a clean glass rod. Into B now run 1 cc. of a dilute phosphate solution containing 0.005 mg. of phosphorus per cc., and mix well. Proceed in the same way, adding 1 cc. of this phosphate solution at intervals of not less than 2 minutes, until B and C appear to have the same color when examined from above. The volume of phosphate solution which must be added to bring this about, multiplied by 0.05, is the correction (in mg. per 100 cc.) to be subtracted from the result in the analysis of blood.

²⁵ Folin, O., foot-note 13, p. 388, step 8.

on the filter, while still wet, should be further washed with alcohol as long as any color is extracted.

The reagent may also be obtained in satisfactory condition by one recrystallization of "technical" aminonaphtholsulfonic acid (Eastman Kodak Co.), as follows: Heat 1000 cc. of water to about 90°, and dissolve in it 150 gm. of sodium bisulfite and 10 gm. of crystalline sodium sulfite. To this mixture add 15 gm. of the crude sulfonic acid, and shake until all but the amorphous impurity has dissolved. Filter the hot solution through a large paper (about 32 cm.), cool the filtrate thoroughly under the tap, and add to it 10 cc. of concentrated hydrochloric acid. Filter with suction, wash with about 300 cc. of water, and finally with alcohol until the washings are colorless.

The purified sulfonic acid should be dried in air with the least possible exposure to light, then powdered and transferred to a brown bottle.

Determination of Inorganic Phosphate in Urine.

Description of Method.—Measure into a 100 cc. volumetric flask enough urine to contain between 0.2 and 0.8 mg. of inorganic phosphorus (usually 1 or 2 cc.).²⁶ Add water to bring the total volume to 70 cc., followed by 10 cc. of 2.5 per cent ammonium molybdate made up in 5 N sulfuric acid (Molybdate I), and 4 cc. of 0.25 per cent aminonaphtholsulfonic acid. After the addition of each reagent, the solution should be mixed by gentle shaking.

At the same time transfer to a similar flask 5 cc. of the standard phosphate solution (containing 0.4 mg. of phosphorus), 65 cc. of water, and the same reagents that were added to the urine sample. Dilute the contents of each flask to the mark, mix, and compare in the colorimeter after 5 minutes. With the standard set at 20 mm., 8 divided by the reading will give the inorganic phosphorus content of the sample in mg.

We have so far not encountered any urine in which the rate of reduction by the sulfonic acid is seriously retarded, but in case the reading falls below 10 mm., it should be controlled by a second reading taken a few minutes later. Unsatisfactory results with hydroquinone lead us to suspect that urine may sometimes contain interfering substances.

Results.—The above procedure has been checked against the titration method described some time ago by one of us²⁷ (based on

²⁶ As suggested earlier, accurate results can be obtained, with proper care, using as much as 1.6 mg. of phosphorus.

²⁷ Fiske, C. H., *J. Biol. Chem.*, 1921, xlv, 285.

the alkalimetric titration of MgNH_4PO_4 . The results (Table III) are all that could be hoped for.²⁸

Albuminous Urine.—According to Briggs, if turbidity appears on adding the molybdate reagent to the (diluted) urine sample,

TABLE III.
Analysis of Human Urine.

Urine No.	Volume per hr.	Titration method.				Colorimetric method.			
		Urine.	0.1 N HCl neutralized.	Inorganic P.	Inorganic P per hr.	Urine.	Reading.	Inorganic P.	Inorganic P per hr.
	cc.	cc.	cc.	mg.	mg.	cc.	mm.	mg.	mg.
1	109	10	2.49	3.86	42.1	1.0	20.7	0.386	42.1
						2.0	10.3	0.778	42.4
2	29	5	1.53	2.37	13.7	0.6	28.5	0.281	13.6
						1.0	17.1	0.468	13.5
3	24	5	2.67	4.14	19.9	0.4	24.2	0.331	19.9
						1.0	9.7	0.825	19.8
4	49	7	2.81	4.36	30.5	0.6	21.6	0.370	30.2
						1.0	13.0	0.616	30.2
5	40	7	2.66	4.13	23.6	0.6	22.7	0.352	23.5
						1.0	13.6	0.588	23.5
6	39	5	2.37	3.68	28.7	0.5	21.7	0.369	28.8
						1.0	10.9	0.734	28.6
7	18	5	2.40	3.72	13.4	0.5	21.8	0.367	13.2
						1.0	10.9	0.734	13.2

the precipitate is to be removed by centrifugation while the color is developing. The formation of a precipitate at this point must generally mean that protein is present—at least we know of no

²⁸ We have never been able to duplicate them by the Briggs procedure. Briggs compared his method with Bell and Doisy's on three samples of urine, and was evidently satisfied with discrepancies as large as 7 per cent. Errors of 3 per cent, in our experience, are common when hydroquinone is used, and sometimes the result may be much farther off.

other common constituent of urine that is likely to produce it²⁹—and in that event removing the suspended matter by centrifugation is most emphatically not the thing to do. No matter how slight the turbidity may be, if it can be thrown down at all by centrifuging, it will be seen to be intensely blue if our reducing agent has been used. Very roughly, 1 mg. of protein will precipitate 0.01 mg. of phosphorous (as proteinphosphomolybdate), and diminish the color of the supernatant fluid by that amount. No very large quantity of protein would be necessary to carry down every trace of phosphate, leaving the solution quite devoid of color.

As a matter of fact, our molybdic acid reagent is not a very delicate protein precipitant (far less so than phosphomolybdic acid), and with the majority of albuminous urines it would give no protein precipitate at all if phosphate were not present.

If the molybdate reagent causes any sign of cloudiness, the determination must be begun again with a new sample from which all protein has been removed. For this purpose, to an accurately measured amount of urine add four times its volume of 10 per cent trichloroacetic acid, stopper, shake, and filter through an ashless paper. 2 to 10 cc. of the filtrate (equivalent to 0.4 to 2 cc. of urine) should ordinarily be the right amount to take. In case the urine happens to be so dilute that more than 10 cc. of filtrate must be used, the analysis should be made by the slightly modified method outlined below for blood filtrates.

Determination of Inorganic Phosphate in Blood.

Anticoagulant and Protein Precipitation.—The inhibiting effect of such amounts of oxalate as might be used to prevent coagulation³⁰ was thought by Briggs to be avoided when the blue color is read in acid solution. The real reason, however, why his method is less sensitive than Bell and Doisy's to oxalate, etc., is that 30 minutes (instead of 5) are allowed for the development of color. The subsequent addition of carbonate—as in the Bell and Doisy

²⁹ Normal urine, if not previously diluted, may give a precipitate with the molybdate reagent. Turbidity not of protein nature is hence a possibility in the case of material very poor in phosphate (*e.g.* the urine of many Herbivora, or in special circumstances that of other animals), but we have not yet had occasion to observe it.

³⁰ Denis, W., and von Meysenbug, L., *J. Biol. Chem.*, 1922, lii, 1.

method—cannot make any difference, for the reduction of phosphomolybdic acid takes place in acid solution anyway.

Whether the necessary amount of oxalate, by itself, does or does not influence the color intensity is really of no particular moment. Blood filtrates, as prepared for this determination, always contain both oxalate (or some other anticoagulant) and trichloroacetic acid, and they affect the color reaction additively—a point of some importance which seems to have been wholly overlooked. Martland and Robison⁶ find, in fact, that trichloroacetic acid alone, in the concentration which Briggs' directions call for, is not without effect, and recommend the use of less; *i.e.*, enough to make the final concentration in the filtrate 2.5, instead of 4, per cent. However necessary this modification may be to give correct results, by Briggs' method, with phosphate solutions to which trichloroacetic acid has been added, it is unfortunately not safe to use with blood. Even 4 per cent trichloroacetic acid will sometimes fail to remove the protein completely, for we have often encountered turbidity on adding molybdic acid to such filtrates. The turbidity may be too slight to strike the eye in as small a volume of filtrate as 5 cc., except on careful examination against a dark background. But if a larger volume of the filtrate is treated in the usual way, definite turbidity is much too common an occurrence. Even if it cannot be clearly seen in 5 cc. of filtrate, it may still make the result too high by at least 0.2 mg. of phosphorus per 100 cc. of blood or plasma; at the same time it may so modify the shade that accurate matching is impossible. Diminishing the concentration of trichloroacetic acid to 2.5 per cent naturally does not improve the situation.

To be sure of quite protein-free filtrates, we have found it necessary to increase the trichloroacetic acid materially, so that the filtrate contains 8 per cent. Other advantages are then gained as well, for the filtration is several times as rapid, and the yield of filtrate greater.³¹ The effect of this additional acid alone on the rate of color development in our method would not be very serious, but since oxalate is present too the time may be undesirably prolonged. Here again we must insist that adding oxalate and trichloroacetic acid to the standard is not the way to get around the difficulty. Instead, we add to the blood (or plasma) filtrate—

³¹ Cf. Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liii, 253.

but *not* to the standard—a special molybdate reagent (Molybdate II) made up with less sulfuric acid. The acidity is then about the same in standard and unknown, and the color reaction proceeds quite rapidly enough. The combined effect of trichloroacetic acid and oxalate under these conditions is shown in the last column of Table II. Citrate has substantially the same effect as oxalate.

In view of the rise in inorganic phosphate³² that begins as soon as blood is laked,^{33, 34} the usual method of adding the protein precipitant to blood previously hemolyzed with water has had to be abandoned. Lawaczek³³ avoids this trouble by laking with hydrochloric acid (in conjunction with the Schenck method of precipitation); and Martland and Robison⁶ have ingeniously adapted the same principle by laking with dilute (1 per cent) trichloroacetic acid, which stops the enzyme action without precipitating protein. From the standpoint of eliminating the source of error mentioned, this is quite ideal, but it is cumbersome. Having had occasion, in the course of experiments on animals, to draw and precipitate blood with the greatest possible rapidity—in order to be free to go on with other operations—we were glad to find that laking is not necessary at all. Whole blood may be run directly into a solution of trichloroacetic acid with no danger of incomplete extraction.³⁵ The manipulation is then even simpler than in the old (and unreliable) method of laking first with water, for there is only one liquid (besides the blood) to be measured out, and that may be done before the blood is drawn. A number of comparisons with Martland and Robison's method of precipitation³⁶ have been made, without finding any difference. We shall

³² Caused by the enzymotic hydrolysis of phosphoric acid esters.

³³ Lawaczek, H., *Biochem. Z.*, 1924, cxlv, 351.

³⁴ Martland, M., Hansman, F. S., and Robison, R., *Biochem. J.*, 1924, xviii, 1152.

³⁵ This applies even to the most abundant crystalloid constituent of corpuscles (*i.e.* potassium), for the total base of corpuscle suspensions can be quantitatively extracted with trichloroacetic acid without laking (Fiske, C. H., unpublished experiments). It probably holds, therefore, for most substances in that category, and perhaps for all. To what extent the same is true with other protein precipitants we are not prepared to say. The high osmotic pressure of the trichloroacetic acid solution used is no doubt a factor in causing the corpuscles to expel their contents.

³⁶ Modified to make the final concentration of trichloroacetic acid 8 per cent.

give but one example—a specimen of blood which happened to be used for the investigation of some other points besides (Table IV).

One of these points is the question of the recovery of added phosphate. For this as the sole criterion of reliability for any method of blood analysis—as some consider it to be—we have not much respect.³⁷ It does, however, in this case have some bearing on the accuracy of the determination when the level is abnormally high (as in nephritic blood). 20 mg. of added phosphorus (in the

TABLE IV.
*Analysis of Dog Blood.**

Laked with 1 per cent trichloroacetic acid.†		Direct precipitation without laking.		Remarks.
Inorganic P.	Total acid-soluble P.	Inorganic P.	Total acid-soluble P.	
2.85	16.8	2.81 23.4	16.9 16.9 16.8 16.7	20 mg. P added.‡ 5 cc. filtrate ashed with 5 cc. 5 N H ₂ SO ₄ and 1 drop HNO ₃ . Same, but overheated.§ 1 cc. filtrate ashed with 1 cc. 5 N H ₂ SO ₄ and 1 drop HNO ₃ . Dry ashing.

* The blood was precipitated at a dilution of 1:5, the final concentration of trichloroacetic acid being 8 per cent. The figures in the table represent mg. of phosphorus per 100 cc. of blood.

† Martland and Robison's method.

‡ 10 cc. of blood were mixed in a small Erlenmeyer flask with 1 cc. of a KH₂PO₄ solution containing 2 mg. of phosphorus. As much of the mixture as possible was run into 39 cc. of 10 per cent trichloroacetic acid, and the residue in the small flask then thoroughly mixed with the precipitated blood.

§ As described in text.

form of KH₂PO₄) are quantitatively recovered by our precipitation method, even at a 1:5 dilution. The particular example cited in support of this (Table IV) is of interest in another way. 20 mg. of phosphorus were added; the recovery was 23.4 — 2.8 =

³⁷ A more reliable method of determining whether extraction is complete is to compare filtrates made from ordinary blood (*i.e.* with nothing added) at different dilutions, say 1:5 and 1:10. This we have done on numerous occasions; the result at the higher dilution is never any more.

20.6 mg., or 3 per cent too much. The excess, within the limit of error, is what should be expected when account is taken of the volume occupied by the protein precipitate. When blood containing 20 per cent of protein is precipitated at a dilution of 1:5, the concentration of the filtrate should be about 4 per cent too high. For ordinary comparative analyses this error may be neglected, but in some cases it must be taken into consideration and corrected for, or a higher dilution used.³⁸

In Table IV will be found also a few figures for total acid-soluble phosphorus. The result is in this case likewise no different when the laking is omitted.

Method for Inorganic Phosphate.—Transfer to an Erlenmeyer flask 4 volumes of 10 per cent trichloroacetic acid. While the flask is being gently rotated, run in 1 volume of blood,³⁹ plasma, or serum—as the case may be—from a pipette calibrated for delivery (not contents). Close the mouth of the flask with a clean, dry rubber stopper, and shake vigorously a few times. Filter through an ashless paper.⁴⁰

Measure 5 cc. of the filtrate into a tube graduated at 10 cc. or a 10 cc. volumetric flask. Add 1 cc. of 2.5 per cent ammonium molybdate in 3 N sulfuric acid (Molybdate II), and finally (after mixing) 0.4 cc.⁴¹ of the usual sulfonic acid reagent. Dilute to the mark and mix. The standard, to be prepared as nearly as possible at the same time, is identical with the standard used for urine (0.4 mg. of phosphorus in a volume of 100 cc., or 0.2 mg. in a 50 cc. flask with half as much of each reagent), so blood and urine may be read against the same solution.⁴² It should be noted that the molybdate reagent added to the standard is always the one containing 5 N sulfuric acid (Molybdate I), and is different from that used for the blood filtrate. The purpose of this, as stated

³⁸ *E.g.*, in calculating the composition of the corpuscles from analyses of whole blood and of plasma (which contains less protein).

³⁹ Oxalate is the most suitable anticoagulant. Use 2 (or at the most 3) mg. of potassium oxalate per cc. of blood.

⁴⁰ The mixture may be filtered at once.

⁴¹ 0.4 of the number of drops contained in 1 cc. is close enough.

⁴² With only 10 or 15 cc. of standard solution, as Briggs for example recommends, we are unable to see how the standard can first be matched against itself, as it must be always to get dependable results.

elsewhere, is to compensate for the high concentration of trichloroacetic acid in the filtrate.

The reading, as with urine, may be made in about 5 minutes, but it should be repeated a few minutes later if the color is particularly strong. To calculate the result in mg. of phosphorus per 100 cc. of blood or other fluid (the standard being set at 20 mm.), divide 80 by the reading. From the figure so obtained subtract the correction for any phosphate which the trichloroacetic acid may contain.

An inorganic phosphorus content of 2 mg. per cent is about the lower limit for convenient reading against the standard recommended, and a weaker color—such as would be obtained by using half as strong a standard—cannot be read so accurately. Hence we suggest, as perhaps the least objectionable arrangement when the phosphate content of the blood is very low, the addition of a known amount of phosphate to the filtrate. This may be done, when a low result can be anticipated, before introducing the reagents. Otherwise, if the reagents are already mixed with the blood filtrate and the color is seen to be unusually weak, phosphate may be added then, before diluting to the mark—the less delay of course the better, but any time within 5 minutes will do, provided that twice as long a period is allowed before the final reading. A suitable amount of phosphorus to add is 0.016 mg., or 1 cc. of a solution made by diluting 20 cc. of the regular standard to 100 cc. The calculation may then be made in the customary manner, and 1.6 mg. per cent subtracted from the result.

By way of illustration, the following check on this procedure may be mentioned. A solution of phosphate, oxalate, and trichloroacetic acid was prepared to simulate the filtrate from blood containing 0.40 mg. of inorganic phosphorus per 100 cc. The usual reagents were added to a 5 cc. sample of this solution and to the standard. 5 minutes later 0.016 mg. of phosphorus was added to the unknown. After 9 minutes, the corrected colorimeter reading was 38.0 mm. The calculation follows.

$80 \div 38.0 = 2.11$ mg. of phosphorus per 100 cc. From this must be subtracted a correction of 0.12 mg. for the phosphate in the trichloroacetic acid, as well as one of 1.6 mg. for the phosphate added in the course of the analysis. The final result becomes $2.11 - 0.12 - 1.60 = 0.39$ mg. per cent, instead of the theoretical figure 0.40.

Subsequent readings, made at intervals for the next half hour, showed no further change.

Determination of Total Phosphorus.

Destruction of Organic Matter.—Much has been written of the danger of loss of phosphate, in one way or another, in the course of the wet ashing process. The danger is without the slightest doubt a real one, at least in the case of material difficult to digest. But when no such difficulty presents itself (*e.g.* in ashing protein-free blood filtrates) we are convinced that the loss is brought about by trying to digest with so little sulfuric acid that the tube is nearly dry; and probably then by quite unnecessary overheating.

In making total phosphorus determinations by the colorimetric method in this laboratory, advantage has long been taken of the device which Martland and Robison⁹ now say is necessary for accurate results. That is, the entire amount of sulfuric acid which is later to be used in developing the blue color is added before the digestion is begun, and an ammonium molybdate solution which contains no sulfuric acid is substituted for the usual acidified reagent. If as much as 4 or 5 cc. of protein-free filtrate can be spared for the determination of total acid-soluble phosphorus in whole blood, the equivalent of about 0.7 cc. of concentrated sulfuric acid (5 cc. of 5 N) can then be used for ashing, with not the slightest danger of losing phosphate in the process. In the second total phosphorus determination recorded in Column 4 of Table IV, the mixture was heated much longer than necessary. After the solution had become entirely colorless, it was boiled for an additional period of 10 minutes, with the micro burner flame (1 cm. in length) just touching the bottom of the test-tube. The tube, throughout this time, was filled with sulfuric acid fumes to within 5 cm. of the top, and yet there was no loss. Substantially the same result was found with a 1 cc. sample of the same filtrate, which was ashed with only one-fifth as much sulfuric acid in a small test-tube, as described below. As a further check, one more analysis was made, this time by dry ashing with a fusion mixture. All four determinations gave the same result within the limit of error of the reading.

Method for Total Acid-Soluble Phosphorus in Blood.—5 cc. of the trichloroacetic acid filtrate should be used if possible. Boil this down, over a micro burner, in a large lipped Pyrex test-tube (200 by 25 mm.) with 5 cc. of 5 N sulfuric acid (or 2.5 cc. of 10 N) and a piece of quartz to prevent bumping. The bottom of the

tube should be about 2 cm. above the burner tip. As soon as charring can be seen, or fumes appear, turn the flame down very low, so that the mixture barely boils, and heat until there is no further blackening. Now add 1 drop of nitric acid so that it runs down the wall of the test-tube—it should not fall directly into the digestion mixture. If the color does not promptly disappear, add another drop of nitric acid in the same manner, and continue in this way until there is no color left. Nothing is gained by using a large amount of nitric acid. Ordinarily a single drop will be enough, and then about 30 seconds further boiling with the same low flame (to remove most of the nitric and nitrous acids left) will complete the ashing process.

Cool the tube under the tap, rinse the contents into a 50 cc. volumetric flask with 35 cc. of water, add 5 cc. of Molybdate III (2.5 per cent ammonium molybdate in water alone) and 2 cc. of the reducing agent. Dilute to the mark, and proceed as usual, reading against the standard that has been described before. 400 divided by the reading will give the desired result in mg. per 100 cc. of blood.

The analysis may be made with 1 cc. of filtrate, using 1 cc. of 5 N sulfuric acid. The procedure is then otherwise the same as that described above, except that the final dilution must be 10 cc. (instead of 50) and the reagents diminished in proportion. It is safer, in this case, to use a smaller test-tube (about 10 mm. in diameter) for the digestion.⁴³ The most probable cause of loss of phosphate is superheating at the edge of the meniscus, which should consequently be as far removed as possible from the source of heat. Digesting in 50 cc. flasks, as recommended by Martland and Robison,⁶ must certainly exaggerate the danger, and doubtless explains the trouble they experienced in trying to ash blood filtrates with a few drops of concentrated sulfuric acid.

Other Applications.

The colorimetric method having now been placed upon a sounder basis, it may be applied with safety to the analysis of any sort of

⁴³ To prevent excessive foaming, some stable and not too volatile inhibiting agent (*e.g.* phenyl ether) may be found helpful. It should be added from a tube drawn out to a fine capillary, so that the drops are very small. Only 1 drop should be used to start with, and no more added later unless the first drop has all been driven off before the foaming stage is passed.

biological material if the precautions stated are attended to. To give, in full detail, directions for meeting every possible contingency would take much space and is unnecessary.

In the determination of total phosphorus, for example, the main question in any case is how to ash most easily and safely—merely a matter of selecting one of the various wet and dry incineration methods that have long been used in gravimetric work. Urine, blood, lipid extracts, etc., may be treated according to the directions given for blood filtrates, except that more nitric acid will naturally be needed. Occasional checks by some dry ashing method will be found useful—in no other way can one's confidence in the wet method be maintained and its limitations clearly recognized.

Dry incineration as a routine practice also has its place. When large quantities of organic matter must be dealt with, it is not only more reliable but quicker. If there is a relatively large volume of water to be removed from the sample being analyzed, dry ashing is sometimes simpler anyway, for evaporation in an open dish may be less trouble than boiling off the water in a test-tube. With blood or plasma filtrates, the trichloroacetic acid should first be driven off on the water bath; the small amount of organic matter left can be destroyed by ignition with a few cgm. of sodium carbonate.

Nitrate must be added to more refractory material, and here no rigid rules can be laid down. In case the residue is more or less adhesive, as with urine, magnesium nitrate alone (1 cc. of 10 per cent) is very satisfactory. For tissues, or for comparatively pure products, it is not so good as the carbonate-nitrate fusion mixture of Berzelius, which was apparently first used for the quantitative analysis of biological products by Falck⁴⁴ in 1873.

The phosphate method we propose is so little affected by silicate that porcelain dishes may be used for the ignition. Our experience with them is so far limited to fusion mixtures of low melting point. For this reason, and because porcelain is not all alike, a blank should be run on every dish before using it for phosphorus determinations. The porcelain will be attacked to some extent, and silicate dissolved, but the subsequent evaporation with hydrochloric acid (to neutralize the carbonate) will

⁴⁴ Falck, F. A., *Ber. chem. Ges.*, 1875, viii, 12.

change most of it to insoluble silica, which is easily removed; the small amount remaining in solution we have never found to be enough to interfere. If any doubt exists upon this point, successive readings will decide it too, for silica enough to make the analysis inaccurate will cause a progressive growth in color intensity lasting many hours (see Table II).

Obviously, when nitrate fusions are made in platinum, as much as possible of the ash must be dissolved in water and transferred to glass or porcelain before adding hydrochloric acid.⁴⁵ The acid should be used to remove whatever undissolved material (calcium and magnesium phosphates) may be left adhering to the platinum dish. The entire solution, inclusive of the acid washings, is then evaporated to dryness in a beaker or a porcelain dish (covered with a watch-glass as long as carbon dioxide is evolved), the residue transferred with water to a volumetric flask, and the color developed as in any other case. Silica may be removed by centrifugation, if necessary, before the color is read.

Finally, we may mention a special problem which was always troublesome to handle by the older phosphate methods. This is the determination of traces of phosphorus in the presence of a large excess of carbonaceous matter. Outstanding examples are the determination of organic phosphorus in urine and the analysis of proteins containing very small amounts of phosphorus—whether an integral part of them or present as impurity. It is in cases of this kind that advantage may profitably be taken of the fact that the blue color is intensified by heating,^{46, 9} and here too aminonaphtholsulfonic acid is the best reagent known to us. We do not believe that the heating method can be made quite as accurate as the one with which this paper is concerned, and we use it only when the latter is excluded. The details will be published in due time.

⁴⁵ One apparent advantage of magnesium (or calcium) nitrate is the fact that ignition decomposes it to the oxide, not the nitrite. Evaporation with hydrochloric acid directly in the platinum dish will consequently cause no serious damage. We hesitate to recommend this as a regular thing, however. If the sample contains salts of sodium or potassium, some nitrite may be left undecomposed, and injury to the platinum in time may be appreciable.

⁴⁶ Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1924, lxi, 63.

THE COLORIMETRIC DETERMINATION OF PHOSPHORUS

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