LXXII. THE ESTIMATION OF PHOSPHORUS AND MAGNESIUM.

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I. Phosphorus.

In the estimation of phosphorus, the highest accuracy, undoubtedly, is to be attained by the use, in conjunction with a delicate micro-balance, of some gravimetric method in which the phosphorus is precipitated as a compound of which it forms only a small percentage. Gravimetric methods, however, are apt to be long and troublesome. Particularly is this the case when the amount of substance to be estimated is very small, so that slightly incomplete drying of the precipitate, for example, may introduce a very considerable percentage error into the final result. Although the colorimetric method described by Bell and Doisy [1920]—especially in the modified form introduced by Briggs [1922]—is rapid and convenient, we have had difficulty in obtaining uniformly accurate results by its use, except in the case of inorganic phosphorus. The main sources of error in the estimation of acid-soluble and total phosphorus have been pointed out by Martland and Robison [1924]. They reduce the amount of sulphuric acid used in the ignition since they find that the amount used by Briggs, with that added in the molybdate solution, may bring the total acidity near, or even above, the limit of safety. Further, they allow the ignition to continue for as short a time as possible, to prevent loss of phosphorus by absorption by the glass.

Another source of error, which applies to estimations of inorganic phosphorus, is introduced by the trichloroacetic acid, which these authors find to contain an impurity, which itself gives a blue colour with the Briggs reagent. This error is serious only when the colorimetric readings are made after long standing, and the interfering substance may be removed by distilling the trichloroacetic acid. Although, by incorporating these modifications in the method, accuracy may be attained, we desired to avoid, if possible, the use of colorimetry, and therefore turned our attention to volumetric methods.

It has long been customary, in determining the phosphorus content of steel, etc., to precipitate the phosphorus as ammonium phosphomolybdate, to dissolve the precipitate in standard sodium hydroxide solution, and to titrate the excess alkali with standard acid. This method, which was originated

by Pemberton [1893], is very well known. The amounts of phosphorus dealt with, however, have been much greater than those present in the small volume of blood available for analysis, and it was therefore our first task to see whether the method was sufficiently delicate to estimate with reasonable accuracy the 0·05 mg. of inorganic phosphorus present in 2 cc. of normal blood. This, once the appropriate conditions of precipitation and titration had been determined, was found to be the case; it was possible to estimate, within 3 %, 0·05 mg. of phosphorus contained in 20 cc. of fluid. A second series of experiments showed that exactly the same conditions with respect to quantity of precipitant, etc., sufficed for the estimation of six times that concentration of phosphorus with, indeed, somewhat greater accuracy. These results (Table I) showed that the method was capable of estimating the inorganic phosphorus in 2 cc. of blood through a range extending from below to far above the normal value. For total, or acid-soluble phosphorus, even less blood would be required.

Table I. *Estimation of phosphorus in a solution of pure potassium dihydrogen phosphate.*

Weight of phosphorus in mg.		Error
Present	Found	%
0·05	0·0514	3
	0·0485	3
	0·0505	1
	0·0497	0·6
0·10	0·0999	—
	0·101	1
	0·101	1
	0·0995	0·5
0·15	0·150	—
	0·152	1
0·20	0·198	1
	0·199	0·5
	0·199	0·5
	0·201	0·5
0·30	0·298	0·6
	0·305	1·6
	0·302	0·6
	0·297	1

There remained a further important point to be determined. Bell and Doisy [1920] have pointed out that in their blood filtrate the inorganic phosphate increases on standing, becoming, after the lapse of a few hours, equal to the acid-soluble phosphorus, owing to the gradual hydrolysis in acid solution of the organic phosphorus-containing substances present. Martland and Robison [1924], on the other hand, have carried out experiments which appear to show that the hydrolysis is enzymic and practically ceases after the addition of trichloroacetic acid. Hence they recommend that the laking of the blood and the precipitation of proteins by trichloroacetic acid (1 %) be carried out as quickly as possible, whilst Bell and Doisy consider it essential that the whole estimation be carried out at once. In face of these contradictory results, we considered it necessary to determine with what rapidity

the estimation must be carried out. For our experiments on the point to have any force, however, the precipitation of ammonium phosphomolybdate must be complete in a few minutes so that the estimation may be carried out rapidly; and equally, on longer standing, there must be no further precipitation—*e.g.* of ammonium tetramolybdate or of molybdic acid. That this condition is amply satisfied is shown by the results recorded in Table II, from which it appears that the precipitation of phosphorus from a solution of potassium phosphate is complete in 10 minutes, and is not increased when the reaction mixture is allowed to stand for as long as 14 hours. The second part of Table II gives the results of our experiments on the time factor in the case of blood. It will be seen that they confirm the results of Martland and Robison [1924], showing that, whilst it is necessary to precipitate the blood proteins as rapidly as possible after laking, the amount of inorganic phosphate in the protein-free acid filtrate undergoes little or no change on standing.

Table II.

(*a*) *Estimation of phosphorus in a solution of pure potassium dihydrogen phosphate.*

Time allowed for precipitation	Weight of phosphorus in mg.	
	Present	Found
5 mins.	0·20	0·12
10 „	0·20	0·199
15 „	0·20	0·198
	0·20	0·201
20 „	0·20	0·201
	0·20	0·201
30 „	0·20	0·199
60 „	0·20	0·201
14 hours	0·20	0·201
	0·20	0·199

(*b*) *Estimation of inorganic phosphorus in blood.*

	mg. P/100 cc.
I. Blood laked and proteins precipitated at once. Inorganic phosphorus estimated in filtrate at once. 15 mins. allowed for precipitation of ammonium phosphomolybdate	3·86
Inorganic phosphorus estimated at once. 2 hours allowed for precipitation of ammonium phosphomolybdate	3·86
Inorganic phosphorus estimated after allowing protein-free filtrate to stand 2 hours at room temperature. 15 mins. for precipitation of ammonium phosphomolybdate	3·90
II. Blood laked and allowed to stand 1 hour at room temperature. Proteins then precipitated and inorganic phosphorus estimated in filtrate at once. 15 mins. for precipitation of ammonium phosphomolybdate	4·60

Still further preliminary experiments with known amounts of phosphorus showed that the accuracy of the method was not affected by the presence of such substances as are either found normally in blood and urine or are added in preparing them for analysis. There was no loss of phosphorus during ignition with sulphuric and nitric acids for the removal of organic matter (Table III).

Table III.

(*a*) *Potassium dihydrogen phosphate added to urine. Inorganic phosphorus estimated.*

Weight of phosphorus added in mg./100 cc.	Weight of phosphorus found in mg./100 cc.	% of added phosphorus recovered
—	63·0	—
32·2	95·8	101·8
61·7	124·2	99·2
105·5	168·0	99·5

(*b*) *Salts etc. added to a solution of pure potassium dihydrogen phosphate.*

	Weight of phosphorus in mg.	
Substance added	Present	Found
Sodium sulphate (2·5 g.)	0·10	0·099
Trichloroacetic acid (0·4 g.)	0·20	0·199

(*c*) *Solution of pure potassium dihydrogen phosphate, with added cane sugar ignited with the sulphuric-nitric acid mixture.*

Weight of phosphorus in mg.	
Present	Found
0·10	0·101
0·20	0·199

Method.

20 cc. of the liquid to be examined were measured into a 100 cc. beaker, and 1 cc. concentrated nitric acid and 3 cc. 32 % ammonium nitrate were added. The beaker was heated to 70–75° on the water-bath, and, after the addition of 5 cc. 3·5 % ammonium molybdate, was re-heated to that temperature. It was then allowed to stand and cool. After not less than 10 minutes —we adopted 15 as the standard time—the liquid was filtered through paper pulp which should be free from scraps of unpulped paper. We have found that the presence of such scraps hinders very considerably the complete solution of the precipitate in the standard alkali. In the filtration, only a moderate suction should be used. The precipitate was then washed, first with a 2 % solution of nitric acid, and finally with a 2 % solution of potassium nitrate until the washings were free from acid. Usually, two washings with acid and four with potassium nitrate were sufficient, using 20 cc. for each washing. The paper pulp and precipitate were then returned to the beaker, the funnel and glass rod being washed with the potassium nitrate solution, and not with distilled water, which decomposed the precipitate giving high values. The precipitate was then dissolved in a known volume of standard sodium hydroxide, and the excess alkali determined by titration with standard acid.

The indicator used in the titration was phenolphthalein, and it was therefore necessary to use CO_2-free alkali. Moreover, since shaking to dissolve the precipitate in the open air inevitably caused the absorption of an appreciable —and varying—amount of CO_2, it was necessary to carry out the titration in

a CO_2-free atmosphere. The acid and alkali (CO_2-free) were kept in paraffined bottles from which they were siphoned into burettes by means of the arrangement described by Cole [1920]. The delivery tubes of the burettes passed through holes in a rubber stopper which fitted the mouth of the beaker used in the precipitation. Through this stopper passed also a third, small, burette, from which the indicator was added, and two glass tubes by means of which a stream of air was drawn through the beaker during the titration. Carbon dioxide was removed from the air by means of soda-lime. The air inlet tube reached almost to the bottom of the beaker so that the air current not only removed CO_2 from the potassium nitrate solution used to wash in the precipitate, but kept the liquid well stirred. The beaker containing the precipitate was placed in position and the air current was started. Two minutes sufficed to remove CO_2 from the potassium nitrate solution and from the air in the beaker. 10 cc. $N/70$ sodium hydroxide were then added from the (20 cc. capacity) burette. The precipitate dissolved readily unless the quantity of ammonium phosphomolybdate was too great, in which case a second 10 cc. were added. As soon as the precipitate was completely dissolved, a drop of phenolphthalein was added, and the excess alkali was titrated with $N/70$ sulphuric acid.

1 cc. $N/70$ sodium hydroxide used in dissolving the ammonium phosphomolybdate was equivalent to 0·0198 mg. phosphorus. The factors given in the literature vary enormously, for though the precipitate is here referred to as ammonium phosphomolybdate its composition is not by any means constant but appears to depend on the conditions under which it is formed. Whatever its actual composition may be, however, a large number of determinations has shown that it does not vary when the reasonably elastic conditions we prescribe are adhered to. The factor given above is based on our own work.

By the use of still more dilute standard solutions it would, no doubt, be possible to achieve greater accuracy in dealing with the very small amounts of inorganic phosphorus met with in normal blood, but we chose $N/70$ solutions for two reasons. Firstly, they cover adequately the required range, since with 0·05 mg. phosphorus the maximum error is only 3 % and the accuracy increases with increasing amounts of phosphorus up to 0·30 mg. Secondly, we employ solutions of this strength for other routine purposes such as the estimation of non-protein nitrogen and urea in blood.

Since the work described in this paper was completed, our attention has been called to a somewhat similar method for the estimation of phosphorus in urine and faeces [Richards and Godden, 1924]. These authors, however, deal with amounts of phosphorus vastly greater than those available in blood analysis. To overcome the difficulty of absorption of CO_2 by the alkali, they dissolve the precipitated ammonium phosphomolybdate in standard alkali (0·5 N), boil, add a known volume of standard acid in excess of that required for neutralisation and boil to remove CO_2. After cooling, the solution is titrated with standard alkali. Not only is this procedure longer than our

method of titration in a CO_2-free atmosphere, but it does not appear to us to be equally satisfactory for work on the micro scale, since it does not give so rigorous an exclusion of carbon dioxide.

Preliminary treatment of blood and urine.

Urine. 5 cc. urine were diluted to 250 cc. with distilled water. For *inorganic phosphorus*, 20 cc. of this solution were used and precipitated direct. For *total phosphorus*, 20 cc. were evaporated to small bulk in a wide boiling tube. 2 cc. of a mixture of three parts of concentrated nitric acid and one part of concentrated sulphuric acid were then added, and the mixture heated until nitrous fumes ceased to be evolved and white fumes of sulphur trioxide were just beginning to appear. When necessary, more nitric acid was added and heating continued in the same way until, after removal of the nitric acid, the residual solution was colourless. This was diluted with about 5 cc. distilled water and neutralised (to methyl orange) with concentrated sodium hydroxide solution. The neutral solution was then transferred quantitatively to a 100 cc. beaker for precipitation. The total volume with the washings was about 25 cc. *Organic phosphorus* was estimated by difference, but where the increased accuracy of a direct determination is required, the procedure of Taylor and Miller [1914] may be carried out. To 20 cc. urine in a 25 cc. flask they add powdered barium hydroxide until the liquid is just alkaline, make up the volume to 25 cc. with water and filter. To 20 cc. of the filtrate in another 25 cc. flask, they add dilute sulphuric acid until the liquid is slightly acid, fill up to the mark with water, and filter from the precipitated barium sulphate. 1 cc. of this second filtrate is equivalent to 0·64 cc. of the original urine. For the volumetric determination of organic phosphorus it should be diluted and treated as described above for total phosphorus. The determination of inorganic or organic phosphorus should be carried out on fresh urine.

Blood. For the determination of *total phosphorus* in blood, 0·5 cc. was treated with 2 cc. of the sulphuric-nitric acid mixture as described for urine. For *inorganic* and *acid-soluble phosphorus* 5 cc. blood were laked as quickly as possible by adding to 30 cc. distilled water in a 50 cc. flask. 5 cc. of a 25 % solution of trichloroacetic acid were added at once, and the liquid was diluted to 50 cc. After standing for 5 minutes it was filtered. 20 cc. of the filtrate (equivalent to 2 cc. blood) were used for the direct precipitation of the inorganic phosphorus; 10 cc. were treated as described for total phosphorus in urine for the determination of the total acid-soluble phosphorus.

A few typical results with blood and urine are given in Table IV. The colorimetric method used for comparative purposes was that of Briggs [1922] as modified by Martland and Robison [1924].

Table IV.

(1) *Blood.*

Phosphorus; mg./100 cc.					
Inorganic		Acid soluble		Total	
Colorimetric	Titrometric	Colorimetric	Titrometric	Colorimetric	Titrometric
3·75	3·76	26·60	26·40	34·4	34·8
3·40	3·33	23·64	23·59	—	—
4·40	4·43	22·20	22·10	—	—
3·28	3·23	25·20	25·00	—	—
2·96	2·99	22·20	22·08	—	—
3·35	3·34	23·40	22·97	—	—
3·28	3·31	26·67	26·83	35·8	35·5
(2) *Urine.*					
69·65	69·53	—	—	72·36	72·20
41·66	41·85	—	—	43·55	43·68

II. Magnesium.

Many of the methods at present in use for the estimation of magnesium in blood and serum depend on the estimation—usually colorimetric—of the phosphorus content of ammonium magnesium phosphate. It is therefore obvious that our method for the determination of phosphorus affords a means of estimating magnesium titrometrically. The average normal magnesium content of blood serum is 2·5 mg. per 100 cc.; 2 cc. serum thus contain 0·05 mg. magnesium, which corresponds to 0·129 mg. phosphorus, and is therefore well within the range of our method.

In dealing with serum, calcium is first precipitated as described by Kramer and Tisdall [1921]. 2 cc. serum are added to 2 cc. water in a 15 cc. centrifuge tube; 1 cc. of a saturated solution of ammonium oxalate is added, and the volume made up to 6 cc. with distilled water. After standing for half an hour, the precipitated calcium oxalate is removed by centrifuging. From 5 cc. of the supernatant liquor, magnesium is precipitated by the method of these authors. 1 cc. of a solution of ammonium phosphate and 2 cc. concentrated ammonia (0·880) are added and the mixture is allowed to stand overnight. (The solution of ammonium phosphate is prepared by dissolving 25 g. di-ammonium hydrogen phosphate in 250 cc. water, adding 25 cc. concentrated ammonia, allowing to stand overnight, filtering, boiling off excess ammonia, and diluting to 250 cc. For use, 20 cc. of this solution are diluted to 100 cc.) Next morning, the liquid is centrifuged and the precipitate washed by centrifuging three times with 5 cc. ammonia solution (10 cc. concentrated ammonia in 100 cc.). The precipitate is then dissolved in 5 cc. 2 % nitric acid; the solution is transferred quantitatively to a 100 cc. beaker, and the phosphorus estimated as usual. 1 cc. $N/70$ sodium hydroxide is equivalent to 0·0151 mg. magnesium. Table V gives a few results from standard solutions of magnesium sulphate and from blood serum.

Table V. *Estimation of magnesium.*

(1) In a solution of pure magnesium sulphate mg. magnesium per 100 cc.		(2) Found in serum mg. magnesium per 100 cc.
Present	Found	
1·25	1·22	I. 2·41
	1·23	II. 2·38
2·50	2·46	III. 2·49
	2·47	IV. 2·40
5·00	4·95	V. 2·52
	4·93	

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