CCXLIV. THE COLORIMETRIC DETERMINATION OF PHOSPHORUS IN THE PRESENCE OF INTERFERING SUBSTANCES.

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RECENTLY, the present authors [Davies and Davies, 1931] described a method for the elementary analysis of phosphorus in pure organic compounds, by a modification of the Briggs-Bell-Doisy method. It was observed during the course of the above investigation that it was necessary to standardise the conditions exactly, in order to ensure accuracy. At the same time, one of us was working with Dr J. H. Quastel, in the Biochemical Laboratory of the Cardiff City Mental Hospital, upon the presence of phosphatases in brain tissue. It was then noted that sufficiently large quantities of potassium pyrophosphate prevented the formation of the characteristic blue acid, due to the reduction of the yellow phosphomolybdic acid, resulting from the interaction between molybdic and orthophosphoric acids.

Fiske and Subbarow [1925], Vásárhelyi [1929], and King [1932] have shown that 1:2:4-aminonaphtholsulphonic acid, used in the place of quinol for the reduction of the yellow phosphomolybdic acid, diminishes the effect of many substances, such as sodium chloride, sodium nitrate and ammonium sulphate, all of which hinder the formation of the blue colour. Denis and Meysenbug [1922] found that in certain cases the original Bell and Doisy method gave anomalous results with both citrated and oxalated plasmas, and recommended that it was desirable to restrict the amount of anticoagulant when estimating the inorganic phosphate of blood. Whilst this paper was being written, Tschopp and Tschopp [1932] reported that a quantitative estimation of the orthophosphate ion by their modification of the colorimetric method was only possible when there were less than 30 mg. of ammonium oxalate, or 50 mg. of sodium citrate or 50 mg. of tartaric acid per 0.04 mg. of phosphorus present. They also discussed the effects of ferrous, ferric, cuprous and manganous ions and also of silicates and arsenates.

In the present work it has been found that a number of organic acids and their salts, which may often occur together with phosphorus-containing compounds in biological material, or may be added during biochemical manipulations, can, if present in sufficiently large quantities, entirely prevent the formation of the blue colour. Hence it has been felt necessary to investigate

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the maximum quantity of the interfering substance with which it is possible to estimate orthophosphate satisfactorily by the colorimetric method. When the amount of interfering substance is sufficiently large to affect the procedure, we have sought to modify our method to enable us to carry out the estimation accurately.

The effect of sodium and potassium pyrophosphate.

Table I illustrates the effect of increasing quantities of potassium pyrophosphate upon the estimation of 0.08 mg. of phosphorus present as potassium dihydrogen phosphate.

Table I. *The effect of potassium pyrophosphate.*

Tube No.	Mg. potassium pyrophosphate	Mg. phosphorus recovered	Percentage recovered	After 12 hours. Mg. phosphorus recovered
1	0	0.08	100	—
2	12.5	0.083	104	—
3	25	0.086	107.5	—
4	30	0.093	116	—
5	35	0.083	104	—
6	37.5	0.076	95	—
7	40	0.071	89	—
8	45	0.048	60	—
9	50	0.020	25	0.25
10	62.5	0.0	0	0.16
11	75	0.0	0	0.06
12	87.5	0.0	0	0.0
13	100	0.0	0	0.0

To each tube were added 1 cc. of potassium dihydrogen phosphate solution containing 0.08 mg. of phosphorus, increasing quantities of potassium pyrophosphate, 2 cc. of 5.5 *N* sulphuric acid, 2 cc. of 5 % ammonium molybdate, distilled water to about 12 cc. and 1 cc. of reducing agent (15 % sodium sulphite and 0.5 % quinol). The final volume was made up to 15 cc. with water and the contents of the tubes were allowed to stand for 30 minutes. At the end of this period the colour intensity in each tube was matched against an appropriate standard in a Klett top-reader colorimeter. It was so arranged that the colours of the standard and test solutions were almost identical.

Until a concentration of 2.5 mg. (No. 6) of potassium pyrophosphate per 1 cc. of reaction mixture was reached a more than theoretical recovery of orthophosphate was obtained. This was probably due to hydrolysis of the pyrophosphate in the acid solution producing orthophosphate, and perhaps to some extent slight contamination of our pyrophosphate sample. It should be noted, for example, in No. 4 in which the maximum increase in recovery of phosphorus was obtained, that this represented less than 0.3 % of the phosphorus content of the pyrophosphate present. Above concentrations of 2.5 mg. per cc. of reaction mixture the colour became less intense and finally disappeared at concentrations above 4 mg. The last column of Table I shows the effect of allowing the tubes to stand for 12 hours. This series gives further information concerning the nature of the hydrolysis of the pyrophosphate (see p. 2053).

The graphs, Fig. 1, show that the amount of sodium pyrophosphate necessary just to prevent the formation of the blue colour is proportional to the concentration of the ammonium molybdate reagent. The three series of experiments here figured only differ from those given in Table I in the quantities of ammonium molybdate added. Thus, using 1, 2 or 3 cc. of molybdate complete absence of colour commences when 2, 4 or 6 mg., respectively, of sodium pyrophosphate per cc. of the reaction mixture are added. These results indicate that the mechanism of the inhibition is one of complex salt formation between the alkali pyrophosphate and the ammonium molybdate. At con-

Fig. 1. Effect of variation of the amount of ammonium molybdate on the recovery of orthophosphate (0.08 mg. of phosphorus) in the presence of increasing amounts of sodium pyrophosphate. A, 1 cc., B, 2 cc., C, 3 cc. of 5 % ammonium molybdate.

centrations where complete absence of blue colour occurs, there is sufficient pyrophosphate present to use up the whole of the molybdate, so that no phosphomolybdate can be formed for ultimate reduction to the blue complex. The addition of more molybdate results in the reappearance of the blue colour.

Table II shows the effect of two different reducing agents and of increasing the hydrogen ion concentration on the recovery of orthophosphate from solutions containing increasing amounts of sodium pyrophosphate.

The tubes were made up as described for Table I, except for the acidities, which are given in the table, and the reducing agents, which are given below.

Reducing agent A. 1 cc. of quinol reagent. The colours were compared with the standards after 30 minutes with this reagent.

Reducing agent B. 0.5 cc. of 1:2:4-aminonaphtholsulphonic acid reagent (0.5 g. of the acid, 30 g. of sodium bisulphite and 6 g. of sodium sulphite made up to 250 cc. [King, 1932]). The colours were compared with the standards according to the directions given at the head of the table.

Under the standard conditions of acidity (2 cc. of 5.5 *N* H₂SO₄) it will be seen that with 1:2:4-aminonaphtholsulphonic acid as reducing agent rather more pyrophosphate was required to cause complete inhibition than with

Table II. *The effect of different reducing agents and of increased acidity.*

Tube No.	Mg. sodium pyrophosphate	Percentage recovery of phosphorus				
		Reducer A		Reducer B		
		2 cc.	4 cc.	Acidity in cc. 5·5 N H ₂ SO ₄ 2 cc. 2 cc. Read after 5 mins. After 20 mins.	4 cc. After 5 mins.	
1	5	106	< 50	—	—	< 50
2	10	106·5	Gradual decrease with no sharp break	100	—	Gradual decrease with no sharp break
3	15	112		—	115	
4	20	—		73	—	
5	25	112·5		—	116	
6	30	117	0	67	—	0
7	35	—		—	116	
8	50	83·5		—	114	
9	55	—		—	—	
10	60	0	—	—	—	—
11	65	—		—	96	
12	75	—		—	< 50	
13	85	—		0	—	
14	90	—		—	0	

quinol. The difference might possibly be ascribed to the greater sensitivity of the naphthol derivative as a reducing agent. Because of the increased time for which estimations with quinol have to be allowed to stand before comparison the hydrolytic process becomes apparent. This is also shown if the colours developed with reagent B are allowed to stand and then matched.

When the acidity is increased the weakening in the blue colour occurs with much smaller concentrations of pyrophosphate, but the point at which colour completely disappears is almost the same using either of the reducing agents. A similar decrease in the intensity of the blue colour is seen even in the absence of pyrophosphate when the acidity is increased, so that no importance can be attached to the above result.

It is only possible to estimate orthophosphate accurately by the colorimetric method when there are less than 10 mg. of alkali pyrophosphate present per 0·1 g. of ammonium molybdate. Also the reducing agent used should be one with which maximum colour intensity is reached rapidly (*e.g.* 1:2:4-aminonaphtholsulphonic acid), and the colour developed must be compared with the standard within 5 minutes of completing the addition of the reagents to the test solution. It is advisable to maintain the acidity at 2 cc. of 5·5 N sulphuric acid per 15 cc. of reaction mixture.

In the present case it is difficult to suggest the actual combining proportions of the molybdate and the pyrophosphate, because of the lack of accuracy in determining the end-point. For example, from Table II, column 5, tube 13, it is found that 85 mg. of sodium pyrophosphate combine with 100 mg. of ammonium molybdate, from which, by calculation, 0·3363 mols. of pyrophosphoric acid combine with 1 mol. of MoO₃. Compounds of pyrophosphates and tungstic acid have been described by Gibbs [1885] of the general formula

From the above calculation the following relation holds:

In respect, however, to the formulae of complex compounds of these types, it should be remarked that the degree of acidity of the reacting solutions has a very marked influence. Wu [1920] has drawn particular attention to this point.

Sodium glycerophosphate.

The effects of increasing quantities of sodium glycerophosphate on the production of blue colour were very similar to those described above with alkali pyrophosphate. Table III shows the interfering and hydrolytic effects in the acid solution. It was difficult to determine the exact point at which colour just disappeared because the colours were visibly increasing in intensity whilst they were being compared.

Table III.

Tube No.	cc. sodium glycerophosphate	Percentage of phosphorus recovered
1	0	100
2	0.5	118
3	0.75	118
4	1.0	118
5	1.1	95
6	1.2	Gradual decrease
7	1.5	decrease
8	1.9	0
9	2.2	No recovery after an interval of 10 mins.

Each tube contained 1 cc. of potassium dihydrogen phosphate solution containing 0.08 mg. of phosphorus; increasing amounts of sodium glycerophosphate solution (12.24 g. of $\text{Na}_2\text{C}_3\text{H}_7\text{O}_6\text{P}$, $5\text{H}_2\text{O}$ dissolved in water and made up to 100 cc., which then contained 0.04 mol. of the salt); 2 cc. of ammonium molybdate solution (6.1815 g. dissolved in water and made up to 100 cc., which then contained 0.005 mol. of the salt); 2 cc. of 5.5 *N* sulphuric acid; 0.5 cc. of 1:2:4-aminonaphtholsulphonic acid; and water to 15 cc. Colours were compared after 5 minutes. When allowed to stand for 12 hours, the contents of all the tubes became deeply coloured.

On the assumption that at the point at which the colour first disappears there are present exactly equivalent amounts of ammonium molybdate and sodium glycerophosphate to form a complex salt, it is possible to suggest the combining proportions of the two substances. By experiment, 1.9 cc. of sodium glycerophosphate solution combine with 2 cc. of ammonium molybdate solution from which, by calculation, 1.08 mols. of glycerophosphoric acid combine with 1 mol. of MoO_3 . A series of experiments was carried out using a much smaller concentration of acid (0.5 cc. of 5.5 *N* H_2SO_4 per 15 cc. of total mixture) and showed that the combining proportions were not materially affected.

In a recent paper, Fleury [1932] described a physical and a chemical method for determining complex formation between molybdic acid and alkali glycerophosphates in neutral solution. He also isolated crystalline complex salts from neutral solution of the following composition: $M_2C_3H_7O_6P$, $2.5MoO_3$, xH_2O .

It is possible to determine accurately 0.08 mg. of phosphorus present as alkali orthophosphate by the colorimetric method in the presence of less than 20 mg. of sodium glycerophosphate per 0.1236 g. of ammonium molybdate, provided the conditions of the estimation are those described for the accurate determination of phosphate in the presence of alkali pyrophosphate.

The effect of certain organic acids.

Table IV gives a list of certain organic acids which, in sufficient quantity, inhibit the formation of the blue colour due to the reduction of phosphomolybdic acid. In these cases hydrolysis with production of increasing quantities of orthophosphoric acid cannot take place. The point at which colour first disappears when adding increasing amounts of the inhibitor is not so obscure as in the previous cases. Moreover, the range over which accurate determinations of orthophosphate in the presence of these substances can be made is greater than the range in the presence of pyrophosphoric or glycerophosphoric acids.

Table IV.

Interfering acid	Maximum amount in mg. of the interfering acid with which 100 % recovery of phosphorus is possible	g.-mol. of interfering acid added when complete absence of colour is first observed	Combining proportions
Citric acid	30	3.6×10^{-4}	$0.5C_6H_8O_7, MoO_3$
Oxalic acid	50	8.0 "	$C_2H_2O_4, MoO_3$
Pyruvic acid	50	10.0 "	$1.5C_3H_4O_3, MoO_3$
Tartaric acid	90	10.4 "	$1.5C_4H_6O_6, MoO_3$
Malic acid	120	21.0 "	$3C_4H_6O_5, MoO_3$
Lactic acid	120	29.0 "	$4C_3H_5O_3, MoO_3$
Glycollic acid	250	64.0 "	$9C_2H_4O_3, MoO_3$

The corresponding quantity of ammonium molybdate present was 1.0×10^{-4} mol., whilst there were 0.08 mg. of phosphorus, and 2 cc. of the standard sulphuric acid. Aminonaphtholsulphonic acid was the reducing agent, and the total volume was finally 15 cc. All the solutions of the interfering substances were exactly neutralised with alkali before use.

A change of the reducing agent to quinol makes no alteration in the figures recorded in Table IV for citric acid. Similarly, with a change of the acidity to 0.5 cc. of the standard acid there is little difference in the figures.

In all cases the colours produced became darker on keeping. Even tubes which originally contained a slight excess of interfering acid became pale blue. Probably the organic acid-molybdate complex is reduced on preservation.

Many methods, both chemical and physico-chemical, have been suggested

for determining complex formation with molybdates. The actual isolation of the complexes is usually very difficult, since they are often non-crystallisable syrups. In general, the results of the various methods appear to be rather conflicting. Darmois [1923] gives a bibliography of previous work on the investigation of mixtures of molybdic acid and other substances. Further work in this direction has been carried out by Fleury [1932]. The experiments reported in the present paper furnish a new method for determining complex formation in acid solution between molybdates and other substances, and give the combining proportions of the two substances. An alkali orthophosphate and a reducing agent are used as indicator of complex formation, and, if the latter takes place, of the point at which all the molybdate is converted into the complex compound. A substance which interferes with the colour development in the manner described herein is probably one which combines with the molybdenum compound. When the interfering substance is in insufficient quantity a blue coloration, which disappears with an excess of the substance, develops. In Table IV combining proportions of molybdenum trioxide and organic acids obtained by this method are tabulated.

The following substances even in large amounts do not interfere with the colour development: formic, acetic, succinic, maleic, fumaric, and malonic acids, alcohol, glycerol, glycine, urea, glucose and fructose. We have not experimented with sufficient compounds to make a generalisation concerning the type of substance which would be likely to undergo complex formation with ammonium molybdate. However, in this connection it should be noted that all the substances given in Table IV contain the carboxyl group, and, in addition, all except oxalic acid contain a hydroxyl or a carbonyl group.

The competitive effects of orthophosphate and interfering substances for molybdate.

The effect of increasing the concentration of orthophosphoric acid in the presence of sufficient sodium citrate to prevent colour formation under standard conditions was investigated. Each tube contained 85 mg. of citric acid and increasing amounts of potassium dihydrogen phosphate. Colour was developed in the usual way. The colour intensity increased proportionately but did not at any time indicate theoretical recovery of orthophosphate. Table V gives the result of the experiment.

Table V. *The recovery of orthophosphate in the presence of sodium citrate.*

Tube No.	Orthophosphate added in mg. of phosphorus	Recovery in mg. of phosphorus
1	0.08	0
2	0.16	0.038
3	0.24	0.047
4	0.32	0.110
5	0.40	0.125

The results show the competitive effect of the orthophosphate and of the citrate for the ammonium molybdate. As the proportion of the phosphate to the citrate increases so the amount of phosphomolybdate increases, whilst the amount of the citric acid-molybdic acid complex must decrease. A complete explanation of the results is bound up with the relative stabilities of the two complex compounds. Reference to Table I, last column, shows a similar effect. When the contents of the tubes are kept for 12 hours, an increased amount of orthophosphoric acid is produced by reason of the hydrolysis of the pyrophosphate in acid solution. This is made evident by the appearance of a blue colour in tubes which originally contained sufficient pyrophosphate to inhibit the colour.

Similar competitive effects have been observed between citric acid and malic acid for ammonium molybdate. We are indebted to Dr J. H. Quastel for providing us with the following results.

Molecular rotations ($[M]_{5461} = \frac{10\alpha}{l.c}$, where α = the observed rotation, $l = 2$ dm. and c = the molar concentration per 1000 cc. of *l*-malic acid, at room temperature) of aqueous mixtures of ammonium molybdate with varying amounts of citric acid and malic acid are given in Table VI. The solution was made up of 10 cc. of 14.2 % ammonium molybdate solution, 1 cc. of glacial acetic acid, and 5 cc. of the mixture of malic and citric acids. The concentrations of malic and citric acids given in Table VI refer to this solution.

Table VI. *Molecular rotations of mixtures of ammonium molybdate, malic and citric acids.*

		Citric acid concentration				
		0	<i>M</i> /160	<i>M</i> /80	3 <i>M</i> /160	<i>M</i> /40
Malic acid concentration	<i>M</i> /160	+ 1096°	1888°	2096°	2400°	2400°
	<i>M</i> /80	1120	1688	1900	2000	2128
	<i>M</i> /40	1190	1550	1770	1894	—
	3 <i>M</i> /80	1177	1493	1659	—	—
	<i>M</i> /20	1195	1464	—	—	—

From Table VI it is evident that (1) the molecular rotation of the malo-molybdate complex increases with increase of the concentration of the malic acid to a maximum; (2) for a given concentration of malic acid, an increase in the concentration of citric acid in the mixture produces an increased molecular rotation which finally reaches a maximum. This shows the formation of a citro-malo-molybdate complex of higher rotation than the malo-molybdate complex; (3) for a given concentration of citric acid in the mixture, an increase in the concentration of malic acid reduces the molecular rotation.

These results indicate the replacement of malic acid by citric acid and *vice versa* from their complexes with ammonium molybdate. The trend of the reaction is governed by the relative concentrations of the combining substances. There is obviously competition by the two acids for the available valencies of ammonium molybdate.

Modified method of analysis.

In order to estimate orthophosphate in the presence of certain organic acids, Tschopp and Tschopp [1932] recommended that the latter be removed by oxidation with potassium permanganate. We suggest that the easiest method of obviating the difficulty arising from the presence of the interfering substance is to add sufficient ammonium molybdate to combine with the inhibitor and then the standard quantity to react with the orthophosphate. For example, with citric acid (neutralised with alkali) as inhibitor, 30 mg. are the maximum amount which can be allowed for theoretical recovery of orthophosphate, when using 2 cc. of standard ammonium molybdate (0.1236 g. of the salt). If, however, 50–70 mg. of citric acid are known to be present, then theoretical recovery of orthophosphate will be obtained by adding double the quantity of ammonium molybdate. This method requires no extra step in the method of analysis, but assumes a knowledge of the quantity of interfering substance present. The quantities of the inhibitor which interfere are large, and are much greater than those obtained under physiological or even pathological conditions. This means that they can only be present in such large amounts when they have been added during the course of experimental manipulation. Under these circumstances the amount of the interfering substance can be assessed. From the figures given in Table IV, a calculation can then be applied to find the amount of ammonium molybdate required. In spite of this, it is recommended that the amount of inhibiting substance, and consequently that of extra molybdate, should be kept as low as possible.

With those interfering compounds which through hydrolysis yield free orthophosphate in the acid solution, the above modification is of no value. The quantities present must be restricted to the maximum allowed.

SUMMARY.

1. Pyrophosphoric, glycerophosphoric, citric, oxalic, pyruvic, tartaric, malic, lactic and glycollic acids interfere with the quantitative determination of orthophosphate by the colorimetric method.

2. The maximum amounts of the interfering compounds which may be present for theoretical recovery of orthophosphate have been determined.

3. The anomaly is caused by complex formation between ammonium molybdate and the interfering substance. Besides forming complexes with ammonium molybdate, pyrophosphoric and glycerophosphoric acids cause further difficulties because they undergo hydrolysis producing orthophosphoric acid.

4. From a knowledge of the amount of interfering substance which is necessary just to prevent the formation of blue colour (from reduced phosphomolybdic acid), combining proportions of molybdate and interfering substance have been suggested.

5. By increasing the amount of molybdate, within certain limits, it is possible to estimate orthophosphate in the presence of larger amounts of the foreign substance than are given in (2).

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