Review of Phosphate Methodologies

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ABSTRACT

A description of the extensive literature on the methodologies for the determination of serum phosphate has been reviewed. The evolution of the various phases of the analytical techniques developed in the last century leading to the simplified, sensitive and accurate procedures of the present have been presented in some detail. A procedure involving a simple direct reaction for the determination of inorganic phosphate in serum using ascorbic acid as a reducing agent and a citrate-arsenite mixture as a stabilizing-sensitizing reagent, all in a semi-aqueous medium containing dimethylsulfoxide and the detergent Teepol 610 is recommended. Jaundice and mild hemolysis are noninterferents and turbidity from severe lipemia is easily overcome by reversing the sequence in which reagents are added because citrate binds molybdate in preference to phosphate. Thus, the serum blank and the reacted serum are identical in makeup thereby yielding an idealized correction for irrelevant absorption. Reaction characteristics and potential errors are included in the discussion of the procedure.

Introduction

The intricate involvement of phosphate in every aspect of human physiology and its highly diversified character makes the element unique in medicine and provides a challenge to analytical biochemists. It is, therefore, not surprising to find an extensive literature concerned with the determination of phosphate. However, the analysis for phosphate is by no means limited to the inorganic form, even though it is the usual form measured, since measurement also includes organic phosphate compounds, such as phospholipids, nucleic acids, nucleotides, glycolytic intermediates, phosphocreatine and 2,3-diphosphoglycerate which are often quantitatively determined by means of their phosphate content. In processing, they may be separated first by means of column chromatography, 14,100,107,122 thin-layer chromatography 84,101,141 or electrophoresis. 6,12 They may be extracted with organic solvents 49,114 or precipitated as a group. 131,148 After isolation, the organic portion is often destroyed by acid digestion and the inorganic phosphate released in the process is determined.

Furthermore, the activity of many phosphomonoesterases may be determined on the basis of their ability to hydrolyze certain organic phosphate substrates. Using the latter approach, a number of enzymes have been determined, including adenosine-
The Reaction

Gmelin, as quoted by Wu, was the first to precipitate phosphate as the yellow phosphomolybdate. Subsequently, the ratio of phosphate to molybdate in the precipitate was determined to be 1 P₂O₅ to 24 MoO₃, or 1 phosphate to 12 molybdates. The reaction in simplified form could be represented as follows:

\[ 12\text{Mo}^{VI} + \text{H}_3\text{PO}_4 + 24\text{H}^+ \rightarrow \text{H}_3\text{PMo}_{12}\text{O}_{40} + 12\text{H}_2\text{O} \]

An adequate acidity was considered to be an important facet of the reaction in which it was believed that condensation of molybdate into a complex heteropoly acid took place first followed by a combination of the acid with phosphate. More recent evidence seems to indicate that molybdate in strong acid solution exists as a dimer which then polymerizes and reacts with phosphate to form a duodecamolybdophosphoric acid (12-MPA) according to the following scheme:

\[ \text{PO}_4^{3-} \text{Mo}^{VI} \text{dimer} \rightarrow \text{Mo}^{V} \text{polymer} \rightarrow 12\text{MPA} \]

Upon reduction the heteropoly acid forms a blue product, often referred to as heteropoly blue. Mild reducing agents affect only some molybdate atoms leaving others still in the higher oxidation state.

According to Jakob and Kozlowski, molybdate could not be reduced beyond the pentavalent state in the presence of a mild reducing agent such as hydrazine sulfate. However, it was suggested by Berenblum and Chain that a strong reducer such as stannous chloride could reduce molybdate further. Since at lower acidity molybdate was reduced in the presence or absence of phosphate, the latter having an accelerating effect, it prompted some to suggest that phosphate acted as a catalyst in the reaction. However, this hypothesis has weakened considerably in recent years and new evidence indicates that the reaction is not catalytic. At higher acidity (pH < 0.7) phosphomolybdate alone is reduced and variation in the acidity determines the reaction rate only. Although the unreduced phosphomolybdate absorbs at 315 nm, it is the reduced blue form which is most frequently measured at either 700 nm or 840 nm.

Some Earlier Methods

As quoted by Wu, it was suggested by Sonnenschein in 1851 that the reaction between phosphate and molybdate be applied to the determination of phosphate, while Osmond in 1887 reduced phosphomolybdate with stannous chloride. In 1914 phenylhydrazine was employed by Taylor and Miller for the same purpose in their determination of inorganic phosphate in serum and urine. However, it was not until a
few years later that the literature concerning phosphate methodology started to grow rapidly.

In 1920, a method was described by Bell and Doisy\textsuperscript{17} in which hydroquinone was used as the reducing agent. It was suggested by these authors that phosphomolybdate was reduced in the reaction while molybdate remained at its higher valence state. The color was intensified by making the solution alkaline with sodium carbonate, so as little as 5 \( \mu \)g of P per dl could be detected by this technique. However, the instability of the color was a serious drawback in the technique.\textsuperscript{47,142} The method was modified by Briggs\textsuperscript{24,25} by changing the acid strength. It was observed that the presence of too much acid caused the rate of color formation to be greatly decreased while insufficient acidity led to the reduction of molybdate itself. Using the Briggs modification, it was noticed by Stanford et al\textsuperscript{123} that the color increased gradually at higher acid concentration but at the peak of intensity the color was unstable. Precisely 0.5N was chosen as the acid concentration for the final reaction mixture as a compromise between reasonable intensity and color stability.

While it was reported by Martland and Robison\textsuperscript{87} that a small variation in the acidity in the Briggs' method had no appreciable effect on the color, it was found by Man and Peters\textsuperscript{83} that the method was more sensitive to acid changes than the one described by Fiske and SubbaRow\textsuperscript{47}. The Briggs' modification was apparently unsuitable for Youngburg and Puchner\textsuperscript{146} who applied the original Bell and Doisy method to the determination of organic phosphorus in urine rather than the Briggs' modification and found the color in the original method to be more intense than in the Briggs' modification, a finding confirmed later by Woods and Mellon.\textsuperscript{143} Color instability and poor sensitivity were two analytical characteristics stressed by the early investigators. To cope with color instability, it was suggested by Baumann\textsuperscript{15} that the number of determinations performed at one time be limited to five. It was claimed by Benedict and Theis\textsuperscript{18} to have stabilized the color by placing the final mixture in boiling water for 10 minutes, a treatment also employed by Whitehorn.\textsuperscript{140} The heating step apparently improved the sensitivity to a large extent\textsuperscript{18} while the blank remained almost colorless.\textsuperscript{140} An objection to the heating step was described by Gomori\textsuperscript{58} on the ground that some organic phosphate compounds could hydrolyze under such strenuous conditions.

The Briggs' method was found by Fiske and SubbaRow\textsuperscript{47} to be subject to interferences by nitrates, chlorides, carbonates, silicates, etc., while the color development was erratic. A modification was proposed in which 1- amino-2-naphthol-4-sulfonic acid was substituted for hydroquinone. According to the authors, the change represented a substantial improvement over the Briggs' method, especially in the area of interferences. For example, the presence of as much as 25 mg of silicon could be tolerated, provided the color intensity was determined within a five-minute period. However, the presence of sodium chloride and potassium nitrate still retarded the reaction slightly while ammonium sulfate and trivalent iron interfered significantly.\textsuperscript{47}

Apparently sensitivity was sometimes inadequate, for it was suggested by the authors that a known amount of phosphate standard be added to samples containing low phosphate in order to increase the accuracy of measurements. The application of a heating step was not advocated and doubts were expressed whether or not such treatment could increase accuracy. In some of the numerous modifications of the Fiske-SubbaRow method, the heating step has been applied with some success. For example, the final mixture was heated for 90 minutes at 60° by Bessey and Lowry\textsuperscript{21} who claimed that the treatment increased the sensitivity. The mixture was heated for 20 minutes at 100° by Horecker et al.\textsuperscript{63} and others followed the same line of reasoning.
but varied the time of incubation; ten
minutes was used in some instances, and seven in others. The heating
effect applied to the Fiske-SubbaRow
method was studied by Bartlett who con­
cluded that the color developed completely
within four minutes at 100° and thereafter
remained stable at room temperature.

Among other objections, the method of
Fiske and SubbaRow had been criticized
for lack of sensitivity. The molar absorp­
tivity of 4,000 was similar to the one ob­
tained by using the Briggs' technique. In
addition, the method lacked linearity, rendered poor reproducibility and both the
color as well as the reagents lacked stability. Some found the method
difficult to use as three different molybdate
reagents had to be employed. Others liked the method because color de­
velopment was rapid, there was no in­
terference owing to silicates, the reagents
were stable, and several authors used the method as a referee procedure. On
the basis of quotations cited in the literature,
it is still the method of choice for many re­
searchers in the field of biochemistry.

In 1927, a method was described by
Kuttner and Cohen in which stannous chloride, a reagent previously em­
ployed by Osmond and Deniges, was used to reduce phosphomolybdic acid. Detailed studies were presented concerning the optimal conditions for the reaction, and the optimum acidity was reported in the final mixture to be in the range of 0.9 to 1.05N. Below this range, molybdate itself was reduced; above it, the rate of the color formation was decreased. At acidities of 1.2N or above, color formation was retarded and turbidity developed.

Similarly, the optimal range for molyb­
date concentration was established at 0.73 to 0.75 percent. There was a decrease in the color below this range while above it molybdate itself was reduced. The optimum concentration range for stannous chloride was 0.02 to 0.22 percent, while at concentrations above this range, molybdate itself was reduced. Nitrites, tartrates, hypochlorites and trichloroacetic acid interfered with the reaction, and hydrochloric acid decreased color stability and inhibited maximal color development. Silicates did not interfere at a 4 to 5 times greater concentration than phosphate.

The Kuttner-Cohen method was studied by Gomori whose main objection to the use of stannous chloride was the narrow range of acid tolerance and sensitivity to interferences. It was proposed by Gomori that methyl-p-aminophenol sulfate (also known as Metol, Enol, and Graphol) was the reducer. Objection was raised by Sum­mner to the high acidity needed in the stannous chloride reaction, and it was sug­gested replacing it with ferrous sulfate, while Woods and Mellon found turbidity de­velopment at low phosphate concentrations and color instability as main disadvantages. However, the main feature of the method was the excellent sensitivity, and the stannous chloride method was applied by Youngburg and Youngburg to the de­termination of inorganic phosphate and phospholipids in blood where it was found superior to other methods.

Methods Including Extraction

Although the sensitivity was largely improved, the methods discussed thus far were still subject to some form of interferences and the color was unstable. In an attempt to eliminate these shortcomings, the phosphomolybdic acid was extracted with isobutanol by Berenblum and Chain, thus leaving behind in the aqueous phase many of the interfering compounds including excess molybdate. Briefly, molybdate, sulfuric acid and isobutanol were added to the solution containing inorganic phosphate and the mixture was agitated for a minute. The organic extract was then washed with sulfuric acid solution and shaken with a stannous chloride reagent to reduce phosphomolybdate. The blue or­
ganic layer was washed into a volumetric
flask with ethanol, and the solution was diluted to volume with further washings.

A wide range of acidity, 0.05 to 1.5N, and a large excess of molybdate could be tolerated in the technique.\textsuperscript{20} A single extraction with isobutanol removed all of the phosphomolybdate with essentially no molybdate claimed to be present in the extract.\textsuperscript{81} The spectral characteristics of the color in butanol revealed two peak maxima, one at 625 nm and the other at 720 nm with corresponding molar absorptivities of 19,200 and 22,700 respectively.\textsuperscript{81} The presence of citrate and oxalate up to one mg per ml did not interfere but a larger amount of stannous chloride was required to overcome the interference of nitrite.\textsuperscript{81}

Although the Berenblum-Chain method was an improvement over previous methods, it also had shortcomings. The obvious problem was the complexity of the technique itself\textsuperscript{139} compounded by volume changes owing to partial solubility of isobutanol in water.\textsuperscript{86} Therefore, the method was modified by Martin and Doty\textsuperscript{86} by replacing isobutanol with a mixture of benzene-isobutanol and used an aliquot of the extract for the reduction step rather than the entire mixture. It was found by Schaffer et al\textsuperscript{115} that isobutanol caused high blank values and after experimenting with several higher carbon homologs, n-octyl alcohol was used for extraction. Poor reproducibility and color stability were obtained by Mozersky et al\textsuperscript{94} with the Martin and Doty modification and their own method was proposed.\textsuperscript{94,95} In addition, when the original method or the Martin-Doty modification was applied to inorganic phosphate determination in media containing highly acid-labile organic phosphate compounds such as phosphocreatine (PC) and adenosine triphosphate (ATP), there was a strong positive interference owing to the hydrolysis of PC and ATP.\textsuperscript{44,61,138}

To circumvent the problem, the Martin-Doty method was modified by Wahler and Wollenberger\textsuperscript{138} by lowering the acidity to 0.125N and extracting the complex with isopropyl acetate at 0° for 30 seconds. Either the absorbance of the unreduced phosphomolybdate was determined at 310 nm or the heteropoly acid was reduced with stannous chloride and its absorbance was determined at 710 nm. It was showed by those authors that under these conditions, no significant hydrolysis of organic phosphate compounds took place. However, it was pointed out by Kushmerick\textsuperscript{72} that large quantities of PC and ATP interfered in the modification by complexing molybdate and thus suppressed color formation. Also some samples of isopropyl acetate were found to absorb strongly at 310 nm.\textsuperscript{72} To overcome the color inhibition, the molybdate concentration was increased. To obtain a better yield of phosphomolybdate in the organic phase, the time of extraction was extended to 60 seconds by Kushmerick. The presence of extra molybdate did not affect ATP hydrolysis and PC was hydrolyzed to a very small extent.\textsuperscript{72}

According to Ennor and Stocken,\textsuperscript{44} PC and ATP undergo extensive acid hydrolysis in the Berenblum-Chain method. To minimize the effect, the time of phosphomolybdate extraction was limited to 10 seconds, thus decreasing the exposure time of PC and ATP to the action of acid. A 10-second extraction was also used by Yanagita\textsuperscript{145} but it was found necessary to lower the acidity to 0.1N. A 20-second extraction was used by Seraydarian et al\textsuperscript{120} for the determination of the inorganic phosphate content of sartorius muscle, but between 15 and 20 percent hydrolysis of PC was encountered. It was then found necessary to isolate the inorganic phosphate by precipitating it in the form of calcium phosphate from a weakly alkaline solution in order to avoid errors owing to acid hydrolysis of labile organic phosphate compounds.

However, the Martin and Doty method was applied by Szent-Gyorgyi and Holtzer\textsuperscript{127} without special precautions to the determination of adenosinetriphosphatase (ATPase) activity in muscle extracts, a medium known to contain a high concentration of PC and
ATP. According to Hansen et al., the Martin-Doty modification of the Berenblum-Chain method could lead to considerable PC hydrolysis unless the Wahler and Wollenberger extraction technique at 0° was used. A 40 percent reduction in the amount of phosphomolybdate extracted at 0° was found by Dreisbach. Fifteen seconds were used for extraction at 10° and it was claimed that no hydrolysis of either PC or ATP took place under those conditions. Since benzene was toxic, it was replaced with xylene and a mixture of xylene-isobutanol was used. It was reported by Penniall that a decrease was noticed in the extraction efficiency of phosphomolybdate into isobutanol-benzene at lower temperatures. Both the acid and the molybdate concentration were decreased in modification to lessen their effect on PC and ATP but the temperature was kept at 25°.

It was reported that many organic phosphate compounds, notably PC and ATP could undergo rapid acid hydrolysies and that this splitting process was accelerated by molybdate. Also, some organic compounds, such as citrate, oxalate and tartrate, were reported to bind molybdate. In order to avoid the catalytic effect on hydrolysis of molybdate, a modification of the Berenblum-Chain method was proposed by Marsh in which phosphomolybdate was extracted with butanol in 5 to 10 seconds, citrate added to bind excess molybdate and then the absorbance of the heteropoly acid determined directly at 310 nm without reduction. A similar technique had previously been described by Allen in which molybdate was masked from reduction by binding it with oxalic acid rather than citrate.

**Use of Ascorbic Acid as the Reducing Agent**

In order to prevent acid hydrolysis of organic phosphate compounds during color development, a method was proposed by Lowry and Lopez in which the pH was raised to 4, molybdate concentration was decreased to 0.1 percent, and ascorbic acid was employed as the reducing agent. At a pH between 0.9 and 2.8, the blank was very large, indicating reduction of molybdate, whereas at intermediate pH ranges from 0.4 to 0.9 and from 2.8 to 4.6, phosphomolybdate alone was reduced. A pH between 3.5 and 4.5 was selected because at the range of 2.8 to 3.5, the color developed erratically with time. A similar acid range dependence was observed when 1-amino-2-naphthol-4-sulfonic acid was used as the reducing agent except that the pH ranges were slightly different.

On the one hand, the rate of phosphomolybdate reduction increased with ascorbate concentration but at too high a concentration the color developed continuously with time, so a 0.1 percent ascorbate solution was employed. On the other hand, a decrease in molybdate concentration caused the color to develop more slowly.

One of the shortcomings of the Lowry-Lopez method was poor sensitivity and incomplete development of color which was not complete even after waiting for three hours. A modification was proposed in which the concentration of ascorbate was increased, the pH was lowered and the final mixture was incubated for one hour at 37°. The incubation step was applied previously by Ammon and Hinsberg who originally used ascorbate as the reducing agent in their own method but the acidity was much higher than in the method proposed by Lowry-Lopez and the mixture was incubated for seven minutes at 37°. A 40-minute incubation time at 50° was used by McClare and a sensitivity was found comparable to the one reported by Bartlett in his modification of the Fiske-SubbaRow method. A substantial increase in sensitivity was claimed by Lowry et al. who modified the technique by increasing the concentrations of acid, ascorbate, molybdate and lengthening the incubation time of the final
mixture to two hours at 37°. The modification resulted in an eight-fold increase in sensitivity over the Fiske-SubbaRow method.47

It would appear that a method which was specifically designed for inorganic phosphate determination in media containing acid-labile organic phosphate compounds was improved through modifications, but in the process of modification it sometimes became less suitable for the application for which it was intended. However, the original Lowry-Lopez method was particularly ill-suited for the purpose because at the low molybdate concentration used in the method the presence of such compounds as PC, ATP, PP and citrate would interfere significantly because they are known to form complexes with molybdate.2,34,64,88,72 Delays were reported in the color development using certain tissue extracts and it was suggested that an internal standard be included in such cases.78 Furthermore, it was reported by Peel and Loughman103 that the color was inhibited in the Lowry-Lopez method when the reaction was carried out in the presence of extracts containing sulfhydryl groups such as cysteine and glutathione. The inhibition was caused by the formation of complexes between sulfhydryl groups and copper where the presence of the metal in the reaction was believed essential in order for it to act as an intermediate electron carrier in the reduction of phosphomolybate by ascorbate.103 It was suggested,103 therefore, that an excess of the metal be added to the reaction mixture to overcome this inhibition. The accelerating effect of Cu⁺ on the reduction of phosphomolybdate by 1-amino-2-naphthol-4-sulfonic acid was also described.50 Others110 reported that sulfhydryl groups themselves could reduce phosphomolybate and suggested that formaldehyde be added to eliminate the error.

The interest in controlling conditions under which minimal acid-hydrolysis of organic phosphate compounds could take place was continued by Mokrasch,97 who devised a method in which acetate buffer was employed to control acidity and where most of the water was replaced with N,N-dimethyl-formamide. To offset complexation of copper by sulfhydryl groups, extra amounts of the metal were added. However, copper formed a yellow complex with ATP which absorbed strongly at 335 nm, the wavelength used in the method to measure the heteropoly blue complex. The molar absorptivity of the phosphomolybdic acid was 17,500 at 335 nm, but the presence of protein, NaCl and silicate interfered. Mozersky et al84 also experimented with organic solvents to prevent the acid hydrolysis of ATP in a system containing p-semidine as the reducing agent. Dimethylformamide and dimethylsulfoxide were employed but it was found that the solvents caused inhibition of phosphomolybdic acid formation.

To avoid some of the shortcomings described, a method was proposed13 in which moderately acidic conditions (pH 1.6) and a relatively low molybdate concentration (0.18 percent) were used. Residual molybdate remaining after the phosphomolybdate acid had been formed was masked by complexing molybdate with citrate. By this means, molybdate bound to citrate was not reducible nor could it react with any inorganic phosphate split off from organic phosphate compounds by acid hydrolysis. Sensitivity of the final color was increased by the addition of an arsenite solution to the system. The chromophore developed within 10 minutes and remained stable for hours. The molar absorptivity was over 17,000 at 700 nm and about 30,000 at 840 nm. The relatively high molar absorptivity made possible the employment of small samples which in effect decreased the amount of molybdate-complexing compounds present such as ATP and PP.

The method was also applied to the determination of alkaline phosphatase activity in serum,8 glucose-6-phosphatase in liver homogenates,9 phospholipids,5 nucleotide phosphate,12 nucleic acid phosphate6 and 51-
nucleotidase.\textsuperscript{11} It was also demonstrated that the molybdate concentration used in the method was of sufficient quantity to react with inorganic phosphate even in the presence of relatively large amounts of ATP or PP.\textsuperscript{7} However, a slight acid hydrolysis of PP was observed when the method was applied to the determination of pyrophosphatase activity.\textsuperscript{62,64} To obviate the problem, the pyrophosphate in the acid medium was stabilized by Woltgens and Ahsmann\textsuperscript{142} by complexing the excess PP with copper. It was found by Huxtable and Bressler\textsuperscript{65} that when the original method was applied to the determination of phosphate in the presence of 2.4 μM ATP, the color was no longer linear, presumably owing to complex formation between ATP and molybdate. The method was modified by replacing the ascorbate with l-aminoo-2-naphthol-4-sulfonic acid but leaving the arsenite-citrate in the system. Ascorbic acid was found by others to be the most satisfactory reducing agent,\textsuperscript{60} being able to produce in a short time the best molar absorptivity\textsuperscript{88} for the stable molybdenum blue complex. Since a modification of the original method is proposed in this review, the interferences and the conditions of the reaction will be detailed later.

Ascorbate was used by Crouch and Malmstadt\textsuperscript{30} as the reducing agent and a method was described based on the reaction rate of molybdenum blue formation under controlled conditions. Since at the initial stage of the reaction the rate at which color formed was directly proportional to phosphate concentration, an increase in the color during a specified time period was recorded as a function of inorganic phosphate concentration. The method as applied to the determination of phosphate in the presence of ATP was claimed to have no appreciable acid-hydrolysis under these conditions.\textsuperscript{31} However, since the phosphomolybdcic acid was formed in an acid solution and then the medium was neutralized for the reduction step, ATP and other organic phosphate compounds could still undergo hydrolysis during the acid developing stage of phosphomolybdate formation.

**Miscellaneous Methods**

Some reducing agents have certain characteristics in common which include acidity requirements and molybdate concentration. However, the intensity of color yield appears to be an individual feature of each reducing agent with stannous chloride and ascorbic acid yielding the highest molar absorptivities. Still another sensitive reducing agent which can be added to the latter two is n-phenyl-p-phenylenediamine.\textsuperscript{41} The compound itself turns blue upon oxidation, thus contributing to the color formed by the reduction of phosphomolybdate. The absorbance spectrum exhibits two maxima, the major one at 350 nm and the minor at 770 nm.\textsuperscript{41} The compound has been reported to be difficult to dissolve and unstable.\textsuperscript{69}

An interesting method for inorganic phosphate determination without the presence of an added reducing agent was proposed by Zinzadze.\textsuperscript{149} The technique was based on the ability of a mixture containing Mo(V) and Mo(VI) to produce a blue color in the presence of inorganic phosphate by self-reduction on heating. The method was subsequently modified\textsuperscript{116} and then automated.\textsuperscript{4} Several methods were proposed in which a reduction step was not included but instead the unreduced phosphomolybdate was measured in the ultraviolet region of the spectrum.\textsuperscript{37,137,138} In 1908 a method was described by Misson\textsuperscript{91} based on a yellow complex formation between phosphate, ammonium molybdate and vanadate having the structure\textsuperscript{91} $\text{PO}_4(\text{NH}_4)_3 \text{VO}_3(\text{NH}_4) \cdot 16\text{MoO}_3$ and absorbing strongly at 315 nm.\textsuperscript{90} The molybdovanadophosphoric acid (MVP) was reported as existing as a true solution and as being more stable than the molybdenum blue complex.\textsuperscript{52}

A desirable feature of the method was the low acidity (0.021 to 0.071N) required to produce maximum absorbance\textsuperscript{90} and lack of interference by silicon.\textsuperscript{137} The method was
applied to determination of phosphate in biological fluids after deproteinization with trichloroacetic acid followed by extraction of the complex with butanol.\textsuperscript{37} The method was applied by Parvin and Smith\textsuperscript{102} to the determination of inorganic phosphate in the presence of labile organic phosphate compounds including phosphocreatine and ATP and no acid hydrolysis was reported provided the extraction step was carried out immediately after the color developed. However, the presence of excessive amounts of ATP, PP and citrate did interfere by complexing molybdate.\textsuperscript{102,108} The interference by molybdate-complexing agents and reducing compounds was reported previously.\textsuperscript{109} The molybdovanadophosphate method was automated by Davies et al.\textsuperscript{35} and found to be more suitable than the stannous chloride method.

The method of Parvin and Smith\textsuperscript{102} was investigated by Van der Heiden and Desplanque\textsuperscript{134} who reported that when the reaction was carried out in the presence of TCA, a large blank resulted which they attributed to a possible TCA-molybdate complex formation. In addition, the effect of ammonium vanadate on the absorbance of the complex was studied and no change in absorbance was noticed whether or not vanadate was present. On the basis of the experimental data, it was concluded that the complex measured in the Parvin-Smith method was the phosphomolybdic acid not the molybdovanadophosphate complex.

An enzymatic method was introduced by Fawaz et al\textsuperscript{45} for inorganic phosphate determination based on the following sequence of reactions:

1. Glycogen + Inorganic phosphate (phosphorylase a) $\rightarrow$ Glucose-1-phosphate
2. Glucose-1-phosphate (phosphoglucomutase) $\rightarrow$ Glucose-6-phosphate
3. Glucose-6-phosphate + NADP (Glucose-6-phosphate dehydrogenase) $\rightarrow$ 6-phosphogluconate + NADPH + H\textsuperscript{+}

The absorbance of NADPH at 340 nm was then related to the concentration of inorganic phosphate. A similar method was published by Schulz et al\textsuperscript{117} and the technique was adapted for a centrifugal analyzer.\textsuperscript{106} The presence of ammonium sulfate decreased the rate of reaction,\textsuperscript{45} whereas amylase interfered by hydrolyzing glycogen.\textsuperscript{45,82} In the latter case, the interference could be obviated by preparing a protein-free filtrate, but trichloroacetic acid could not be used for this purpose because it inhibited the action of glucose-6-phosphate dehydrogenase.\textsuperscript{117}

Another enzymatic method was proposed by Guynn et al\textsuperscript{59} which included the following sequence of reactions:

1. Fructose-1,6-diphosphate (aldolase) $\rightarrow$ glyceraldehyde-3-phosphate + dihydroxyacetone phosphate
2. Glycer aldehyde-3-phosphate + NAD + P (glyceraldehyde-P dehydrogenase) $\rightarrow$ 1,3-diphosphoglycerate + NADH + H\textsuperscript{+}
3. 1,3-Diphosphoglycerate + MgADP + H\textsuperscript{+} (phosphoglycerate kinase) $\rightarrow$ 3-phosphoglycerate + MgATP
4. MgATP + D-fructose (hexokinase) $\rightarrow$ MgADP + fructose-6-P + H\textsuperscript{+}

A similar method was published by Scopes.\textsuperscript{118}

Most methods for inorganic phosphate determination in biological media include prior protein precipitation and the reaction is carried out in an aliquot of the protein-free filtrate or in a diffusate. A number of methods have been described in which the reaction was carried out in the presence of proteins. Protein precipitation in the presence of molybdate was prevented by alcalinization\textsuperscript{40,57,77} or by adding detergents.\textsuperscript{10,33,93} The reactions involved were based either on the reduction of phosphomolybdic acid by compounds such as o-phenylenediamine,\textsuperscript{93} ascorbate,\textsuperscript{13,57} p-methyl aminophenol sulfate,\textsuperscript{40} hydroquinone-ascorbate\textsuperscript{57} and 4-(methylamino)-phenylsulfate-potassium pyrosulfite,\textsuperscript{77} on the unreduced heteropoly acid which was measured at 340 nm\textsuperscript{33} or on a dye-binding
However, some of the methods were shown to suffer from interferences owing to the presence of bilirubin, lipemia and anything more than minimal hemolysis. An interesting approach was used by Itaya and Uii for the determination of phosphate based on a spectral shift principle where certain dyes bind phosphomolybdate giving rise to absorptions at wavelengths different from those at which the dyes alone absorb. It was found that the dye, Malachite Green, was the most satisfactory and colloid stabilizing detergents were employed in the system to prevent the colored complex from sedimenting. The sensitivity was 12 times higher than that obtained with the Martin-Doty method, and the presence of acid-labile organic phosphate compounds could be tolerated provided the temperature was kept below 5°C. However, at this low temperature, development of color became considerably slower. The method was applied to the determination of phosphate in deproteinized serum, and a similar technique utilizing Methyl Green 00 dye was automated without a dialysis step.

Interferences

Obviously, a great effort has been made in the last half century to improve inorganic phosphate methodology with particular emphasis laid on sensitivity and interferences. However, the biological media used for phosphate studies represent great diversification in matrix makeup since they include plasma, cerebrospinal fluid, urine, tissue homogenates and bone, with each having a specific composition which must be considered in selecting a method. A particular technique for phosphate determination suitable for plasma may not be applicable to tissue homogenates. It seems mandatory, therefore, to first establish whether or not the method would be subject to interferences in the medium under consideration and then to determine if the sensitivity is sufficient to maintain accuracy.

One of the frequently encountered interferences, irrespective of the type of medium employed, is due to the presence of silica. Although silica is not usually present in biological media in quantities sufficient to cause interference, the sample may be easily contaminated from the glass or by stationary phase material in cases where organic phosphate compounds are separated by thin-layer chromatography and the silica gel containing the spot is scraped off the plate for phosphate determination. Silica combines with molybdate to form a siliconomolybdate complex which is reduced to molybdenum blue in a fashion similar to phosphate, thus interfering in the phosphomolybdate reaction. However, the interference can be obviated in some instances by increasing the acid concentration in the reaction mixture as siliconomolybdate complex formation is inhibited at a low pH. Some also believe that when the sample is digested in acid by heating, silica may not interfere because in the process it is converted to an inert substance.

Another source of interference is the presence of compounds which complex molybdate such as citrates, oxalates and tartrates. Some of these carboxylic acids are used in blood specimens as anticoagulants or as buffers in the determination of phosphomonoesterases. In addition, it has been noted that molybdate also forms complexes with phosphocreatine, adenosine triphosphate, glucose-6-phosphate and pyrophosphate. An excess of molybdate can be added to overcome the inhibition, but molybdate in acidic solutions accelerates the hydrolysis of many organic phosphate compounds, thus one can easily substitute one form of interference for another. To diminish the effect of acid hydrolysis, citrate can be added to bind molybdate after the phosphomolybdate is formed, or the pH of the medium can be raised and the temperature lowered.

However, the very labile phosphocreatine seems to elude most efforts.
to control its stability. The problem is compounded when the analysis is performed in the presence of proteins. It is often necessary to prepare a protein-free filtrate before the reaction is carried out, a process which leads to somewhat prolonged exposure of organic phosphate compounds to the acids used for protein precipitation which can lead to acid hydrolysis. If the reaction is carried out in the presence of proteins, turbidity may develop owing to partial protein precipitation by molybdate\(^{47,111,150}\) and any attempt to eliminate the turbidity by centrifugation may lead to some loss of the phosphomolybdic acid which can adsorb to the precipitate.\(^{47,124}\) Protein interference can be eliminated by the addition of detergents.

Interferences owing to the presence of organic phosphate compounds can be eliminated in part by using a small sample size, thereby obviating the need for a large molybdate excess. In addition, it is often desirable to decrease the concentration of protein in order to avoid turbidity formation and perhaps decrease the effect of protein on the absorbance of the molybdenum blue formed.\(^{10}\) A sensitive method is needed for that purpose, where sensitivity can be defined in one way as the change in absorbance per unit concentration, where sensitivity refers to a fixed value at specified conditions, such as the wavelength, temperature and solvent medium, and where sensitivity is not increased by concentrating the color, using smaller reagent volumes or extracting into smaller solvent volumes. Although such measures may be useful in many cases they do not increase the true sensitivity of the reaction which is defined by its molar absorptivity.

The sensitivity of the phosphomolybdenum blue color or the depth of color generated depends to a large extent on the type of reducing agent employed. Stannous chloride and ascorbic acid appear to afford the most sensitivity. The dye binding techniques which do not utilize a reduction step but instead bind a dye to the unreduced phosphomolybdate complex are procedures which attain high molar absorptivities. However, these methods have not been extensively utilized and their relative merits can not be judged at this early time.

Direct Measurement of Serum Inorganic Phosphate

**PRINCIPLE**

Inorganic phosphate in serum is reacted directly with molybdate and the complex formed is reduced to the phosphomolybdous state by the action of ascorbic acid. The medium chosen is protective of turbidity formation by inclusion of a detergent whose function it is to keep all substances dispersed which might tend to precipitate. The medium is also sufficiently acid to permit the generation of enough heteropoly blue complex to afford a high molar absorptivity and allow the use of micro sized samples. The last reagent added involves a mixture of citrate and arsenite in dimethyl sulfoxide wherein all three ingredients appear to have important individual functions. Citrate binds excess molybdate to form a colorless complex and the excess molybdate is prevented thereby from reacting with inorganic phosphate that could be split from an organophosphate compound under the influence of the acid medium. Arsenite appears to enhance the color, although no definite mechanism for the reaction can be postulated at the moment. Dimethyl sulfoxide creates a semi-aqueous system which enables the reaction to exhibit the same spectrophotometric characteristics as are manifested in the presence of protein. The latter phenomenon is important in that it makes possible the use of non-proteinaceous standards to obtain accurate calibration curves which superimpose those derived from more difficult to prepare proteinaceous standards.

**REAGENTS**

*Stock phosphate standard (1 g per liter).* The stock reagent is prepared by dissolving 4.381 g of potassium dihydrogen
phosphate in phosphate free water and diluting it to one liter.

Working standards. The stock standard is diluted to prepare working standards of 20 to 80 mg per liter concentration in increments of 20 mg per liter, but including a 50 mg per liter standard for the everyday operation.

Ascorbic-sulfuric acid (ASA). Ascorbic acid (20.0 g) is dissolved in 300 ml of water in a one liter volumetric flask. Eight ml of concentrated sulfuric acid are added and the volume diluted to one liter with water.

Ammonium molybdate (0.5 percent). Five grams of ammonium molybdate tetrahydrate are dissolved in about 300 ml of water in a one liter volumetric flask. Then 20.0 ml of glacial acetic acid are added and the volume is diluted to one liter with water. Store in a plastic bottle.

Arsenite-citrate in DMSO (ACD). Twenty grams of anhydrous sodium arsenite are dissolved in about 500 ml of water in a one liter volumetric flask. Then 20.0 g of sodium citrate dihydrate are dissolved into the solution, followed by the addition of 20.0 ml of glacial acetic acid and 400 ml of dimethyl sulfoxide. The solution is cooled to room temperature and diluted to one liter with water.

Teepol dispersant solution. Fifty ml of Teepol 610° are diluted to one dl with water.

PROCEDURE

Fifty μl of serum are pipetted into a 13 x 75 mm tube followed by 1.0 ml of the ascorbic-sulfuric acid solution. An aliquot of a 50 mg per liter phosphate working standard is treated in the same fashion. The reagent blank is prepared similarly, except that water is substituted for serum. Teepol dispersant solution, 250 μl, is added and the mixture is vortexed immediately to redissolve the precipitate that forms only in the sample. Five hundred μl of the 0.5 percent molybdate reagent are added, the mixture is vortexed again and, after a one to three minute delay, 1.0 ml of arsenite-citrate solution is added with thorough mixing. About 10 minutes are allowed for full color development and the absorbances of the sample and the standard are determined against the reagent blank using a wavelength setting of either 700 or 840 nm. The latter wavelength, if available, increases the molar absorptivity of the measurement.

It is obvious that highly chylous or turbid serum specimens require a sample blank. This blank is easily prepared by reversing the order of addition of the molybdate and the arsenite-citrate reagents. That is, the ammonium molybdate reagent is added last, while the addition of arsenite-citrate precedes that of molybdate. The absorbance obtained for the sample blank is then subtracted from the absorbance obtained for the sample prepared by mixing the reagents in the regular order. This reverse system for a sample blank preparation is also optional for use against all samples at the discretion of the user.

DISCUSSION

The sequence of addition of reagents must be strictly adhered to, and it is necessary that the addition of each reagent be followed by thorough mixing. The procedure is not difficult, although a clear understanding of each step in the reaction is helpful. The detergent is added to keep protein molecules dispersed so no precipitate is formed when molybdate is introduced. The reaction between phosphate and molybdate must go to completion before citrate is added, therefore a short delay is required between the addition of the two reagents. When molybdate is added and the mixture thoroughly vortexed, a greenish-blue color develops gradually. At least a minute, but no longer than five minutes, should be allowed for the reaction to proceed before citrate is added. The reason for this is apparent when one understands that in this circumstance molybdate and phosphate react stoichiometrically within one minute, so adequate time should be allowed for the reaction to go
to completion. However, since molybdate reacts with citrate in preference to phosphate, it is obvious that if citrate were added too soon, either before the molybdate addition or even at the same time, no color could develop because citrate would have complexed all of the molybdate, leaving none to react with phosphate. If citrate was added after the five minute period, the stabilization of the final color would be prolonged. The presence of citrate in the reaction is necessary; if it was eliminated entirely, the color would develop continually and the solution would become cloudy.

Arsenite was reported to interfere in the phosphomolybdic acid formation by increasing the color and only 60 micrograms of arsenic per ml or less could be tolerated in the reaction. However, it was believed that arsenite was oxidized to arsenate and the latter formed an arsenomolybdate complex in a manner similar to that of phosphate. This mechanism can not apply to the reaction involved in the method proposed in this review because the blank would then have been extremely high since the arsenite is common to both sample and blank. What does seem to affect the blank is the molybdate concentration. For example, the absorbance of the blank is 0.04 when 0.5 percent molybdate concentration is used; it is increased to approximately 0.29 when the concentration is doubled.

Arsenite enhances the intensity of the chromophore, but the mechanism of its action is unknown at present. It is interesting to note that the enhancement of the color by trivalent bismuth was explained on the basis of its involvement in the phosphomolybdate complex. A similar mechanism was suggested for antimony whose presence also increased the phosphomolybdenum blue color. In view of these observations, it would be convenient to suggest a similarity between the action of arsenite and the other two elements, but a large excess of arsenite is necessary in the described system which is not the case when bismuth or antimony are used. When the concentration of arsenite is reduced by a factor of 10, the color increases slowly and reaches a peak after an hour rather than the usual 10 minutes. The latter observation leads to the inference that arsenite somehow participates in the complex formation but for some presently unknown reason, it must be present in large excess.

When dimethyl sulfoxide is excluded from the reaction, the absorptivity of the standard calibration curve containing protein is slightly different than the one observed for all aqueous standards. The presence of the solvent somehow eliminates the "protein effect", for when dimethyl sulfoxide is added, protein has no further influence on slope, enabling one to use conveniently aqueous standards for the calibration curve.

In case one intends to apply the method to the determination of phosphomonoestersases where either buffer compounds or the substrate employed are capable of complexing molybdate, the concentration of molybdate can be doubled in the reaction without compromising the accuracy of measurement, however, the blank will be higher. It should be stressed that acidity and reagent makeup have been worked out specifically for the direct determination of inorganic phosphate in serum. Any deviation from the described protocol, by introducing buffers at different molybdate concentration, may not be compatible with the conditions needed for the reaction. Therefore, an individual set of conditions may have to be established if such changes are needed.

Sulfuric and ascorbic acids are premixed for convenience. The most satisfactory grade of ascorbic acid was found to be the E. Merck brand.

**CALCULATION**

The calibration of inorganic phosphate by the described procedure is linear, so routine calculations can be made by absorbance ratios against a single standard. In the case of turbidity, a simple absorbance correction

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*Distributed by EM Laboratories, Elsford, NY.*
is applied as described under procedure and the corrected absorbance is then used in the calculation. A calibration curve should be established to ensure reliability of the slope used in the absorbance ratio calculation.

**SOURCES OF ERROR**

Contamination owing to traces of phosphate found on the glassware used for the determination can be a problem unless phosphate free detergents are used for washing. Since, normally, one determines approximately 2 μg of inorganic phosphorus in a 50 μl sample, a seemingly small contamination can lead to large errors.

A severely lipemic sample which remains turbid in the described system is of no consequence provided one resorts to the correction described under procedure. In the latter case, the sample blank is determined by reversing the sequence of addition of reagents. When the residual absorbance is subtracted from that of the sample, the difference represents the true absorbance of the chromophore itself. Bilirubin and hemoglobin do not interfere in the described process because they do not absorb at the wavelength used for measurements nor do they convert to interfering compounds which could absorb at those wavelengths.

**NORMAL RANGES**

Normal ranges reported in the literature are 2.5 to 3.0 mg per deciliter on the low side to 4.5 to 4.8 mg per deciliter on the high side. The values reported for children are higher and cover the range of 4.5 to 6.5 mg per deciliter. Our own adult range fits within the reported limits at 2.5 to 4.5 mg per deciliter.

**RÉSUMÉ OF CLINICAL INTERPRETATIONS**

There are several abnormal conditions in which serum hypophosphatemia occurs. Important among these are Addison's disease, familial hypophosphatemia, Fanconi syndrome, fevers of bacterial or viral origin, hepatic coma, hyperparathyroidism, hyperventilation, idiopathic steatorrhea, intestinal absorption impairment, kidney dysfunction, multiple myeloma, periodic paralysis, osteomalacia, renal tubular acidosis, rickets and renal rickets.

Important among the abnormal conditions in which serum hyperphosphatemia occurs are acromegaly, hypervitaminosis, hypoparathyroidism, lactic acidosis, pseudohypoparathyroidism and renal failure. 27, 119, 130

**References**

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