Continuous-Flow Determination of Serum Inorganic Phosphate with a Single Reagent—The Vanadomolybdate Method Re-evaluated

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The merits of the vanadomolybdate method in the determination of inorganic phosphate are highly underestimated with regard to the simplicity of the method and the stability of both the reagents and the color that is formed. The absorption curve of the phosphovanadomolybdate complex, with its maximum at 335 nm, extends into the visible range of the spectrum. This permits measurements with inexpensive tungsten-lamp colorimeters. On-stream dialysis is best done in a nitric acid medium, 0.15 mol/liter. Paralleled by the change in the $\text{H}_2\text{PO}_4^-/\text{H}_3\text{PO}_4$ ratio, appreciable protein binding and poor dialysis efficiency are seen at lower acid concentrations (pH > 1.0). Optimum reaction-mixture concentrations of vanadium and molybdenum appeared to be respectively 0.2 and 5 mmol/liter up to 3 mmol of phosphate per liter, in a final acid medium of 0.2 mol/liter, concentrations considerably lower than those used in some studies published earlier. Color development with the stable combined reagent is complete after only 20 s at room temperature and the color is stable for at least 2 h. Figures on precision and accuracy demonstrate the reliability of the method.

Most methods for determining inorganic phosphate are based on the phosphate-complexing behavior of molybdate in an acid environment. Usually this phosphomolybdate complex is then reduced to molybdenum blue, which can be measured photometrically at some wavelength between 650 and 750 nm. The reduction has to be done under carefully controlled conditions, because the excess molybdate may itself be reduced to molybdenum blue. Many authors have been concerned about the instability of certain of the reducing agents used, and have either changed their nature or added stabilizers to them, or both (1–5).

A much less-well established sort of chemistry is represented by the simultaneous action of molybdenum and vanadium by which a yellow complex is formed with phosphorus, a reaction first described by Mission in 1908 (6). The reagents used in this reaction reportedly are stable, but the method is troubled with spectrophotometric nonlinearity (7–9). Also, a relatively high—and therefore for biological samples unfavorable—acidity is said to be required (10, 11).

To our knowledge, only two applications based on this method have appeared in the literature of the last five years (9, 12).

In this study we investigated a variety of factors affecting the vanadomolybdate method. A reagent with much lower reactant concentrations in comparison to former studies (7, 9, 12, 18) has been used. Moreover, the combined reagent is stable indefinitely and has linear spectrophotometric characteristics. A relatively low acidity can be used. The final procedure will be presented as a continuous-flow method.

Materials and Methods

Apparatus. During developmental work spectrophotometric measurements were made with a Zeiss PMQ II/M4Q III spectrophotometer. Spectral absorbance curves were generated by a Spectronic 200 ultraviolet recording spectrophotometer (Shimadzu/Bausch & Lomb).

Reagent. During development of the method separate vanadate and molybdate reagents were used. Once the final procedure had been established, a combined reagent was used: 75 mg of ammonium metavanadate and 3 g of ammonium heptamolybdate tetrahydrate are dissolved in diluted nitric acid (20 ml of concd HNO$_3$ per liter; 1.4 kg/liter) by first pouring about 800 ml of distilled water onto the solid salts. The solution initially is intensely yellow. After it is stirred for about 15 min, 20 ml of concentrated HNO$_3$ is added, which causes the yellow color to fade away. After complete dissolution the mixture is diluted to 1 liter. This reagent is stable indefinitely.

Continuous-flow system. Figure 1 shows the flow diagram; the system is composed of single-channel components manufactured by Cenco (Breda, The Netherlands). "Teepol," 1 ml/liter, is used as a wetting agent in both the donor and recipient streams.

Results and Discussion

Spectrum. Figure 2 shows the spectral characteristics of the phosphovanadomolybdate complex. For com-
parison, the spectrum of the unreduced phosphomolybdate complex has also been depicted, together with the blank absorbance, which appears to be identical for the two systems. The absorption maximum at 335 nm is at the same position for both. Therefore most sensitive measurements evidently should be made at or near this wavelength. Unfortunately, the blank absorbance at this wavelength region is so high that measurements can only be made with dual-beam spectrophotometers. Therefore the number of papers that deal with measurements of either the molybdate complex (14, 15) or the vanadomolybdate complex (8) at short wavelengths is limited. Recall that the reduced molybdenum blue complex, besides its fairly flat absorption maximum at 725 nm, also shows a sharp absorbance peak at 335-nm, in the near-ultraviolet region, which has about fourfold the absorptivity as that at 725 nm (1).

Because more of the spectrum of the vanadomolybdate complex extends into the visible range than is true for the unreduced molybdate complex, measurements can be made with tungsten-lamp colorimeters. We decided to use in all our experiments a wavelength near 400 nm (405 nm), because the difference between sample and blank absorbance appears to be maximum in this region.

Sensitivity. If a wavelength of 405 nm is used there are repercussions on sensitivity. The molar absorptivity, measured under the final conditions, appeared to be 2.3 \( \times 10^6 \) mol\(^{-1} \) cm\(^{2} \) at 405 nm, whereas at 335 nm this value is as high as 19 \( \times 10^6 \) mol\(^{-1} \) cm\(^{2} \), compared to a value of 15 \( \times 10^6 \) mol\(^{-1} \) cm\(^{2} \) for the unreduced molybdate complex at 335 nm. In Table 1 these values are compared to those obtained with other methods.

Acid and acid strength. In the original vanadomolybdate method nitric acid was used (6). As an alternative, sulfuric acid plus nitric acid (12), perchloric acid (7), and hydrochloric acid (8) have been used without noticeable difference. Therefore the choice of the acid seems to be arbitrary. Because dilution of the serum with nitric acid, in the concentration range studied, caused the least trouble with turbidity, we decided to use nitric acid in all further experiments. The comparative studies aimed at finding the best acid concentration have been focused on the range 0–0.7 mol/liter final acid concentration. All comparisons were made with a

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**Table 1. Comparison of Frequently Used Methods for Determination of Inorganic Phosphate**

<table>
<thead>
<tr>
<th>Type of reaction</th>
<th>Acidity, mol/liter</th>
<th>Effective acidity (mol/liter) ( \times 1 )</th>
<th>Sensitivity (absorptivity) ( \times 10^6 ) mol(^{-1} ) cm(^{2} )</th>
<th>Wavelength nm</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Semidine reduction</td>
<td>0.45</td>
<td>4.5</td>
<td>11.5 ( \times 10^6 )</td>
<td>770</td>
<td>(1)</td>
</tr>
<tr>
<td>Sn(^{2+} ) reduction</td>
<td>2.3</td>
<td>2.0</td>
<td>31 ( \times 10^6 )</td>
<td>660</td>
<td>(2)</td>
</tr>
<tr>
<td>Fe(^{3+} ) reduction</td>
<td>0.8</td>
<td>22.2</td>
<td>3.5 ( \times 10^6 )</td>
<td>660</td>
<td>(3)</td>
</tr>
<tr>
<td>o-Phenylenediamine reduction</td>
<td>1.4</td>
<td>7.0</td>
<td>11.3 ( \times 10^6 )</td>
<td>725</td>
<td>(4)</td>
</tr>
<tr>
<td>Arsenite reduction</td>
<td>0.45</td>
<td>4.4</td>
<td>17.7 ( \times 10^6 )</td>
<td>700</td>
<td>(5)</td>
</tr>
<tr>
<td>Spectral shift with Malachite Green</td>
<td>0.8</td>
<td>0.8</td>
<td>93 ( \times 10^6 )</td>
<td>660</td>
<td>(16)</td>
</tr>
<tr>
<td>Unreduced phosphomolybdate</td>
<td>0.45</td>
<td>1.7</td>
<td>14.4 ( \times 10^6 )</td>
<td>340</td>
<td>(15)</td>
</tr>
<tr>
<td>Vanadomolybdate</td>
<td>0.62</td>
<td>1.6</td>
<td>?</td>
<td>420</td>
<td>(9)</td>
</tr>
<tr>
<td>Vanadomolybdate</td>
<td>0.2</td>
<td>0.45</td>
<td>2.3 ( \times 10^6 )</td>
<td>405</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19.2 ( \times 10^6 )</td>
<td>335</td>
<td></td>
</tr>
</tbody>
</table>

* All data obtained are from works cited, after recalculation into the same units. Effective acidity is expressed as mol/liter acid to which the sample is exposed, multiplied by the time of exposure (t).
Vanadium and molybdenum concentrations. We studied the influence of final vanadium concentration over the range 0–4.5 mmol/liter. Even without vanadium there is some net absorbance, which most likely originates from the phosphomolybdate complex (cf. Fig. 2). With a vanadium concentration of 0.15 mmol/liter the net absorbance is greatest. A further increase evidently only increases blank absorbance. We decided to use a vanadium concentration of 0.2 mmol/liter in the final procedure. On varying the final molybdenum concentration we found that a region of constant net absorbance was obtained with a concentration of about 4 mmol/liter. Thus in the final procedure we decided to use a molybdenum concentration of 5 mmol/liter. When our reactant concentrations are compared with those in other studies (Table 2), they are seen to be the lowest ones reported except for the values of Michelsen (8). The latter system, however, appeared to be linear only up to 30 μmol of phosphate per liter. The lowest vanadium and molybdenum concentrations that can be used are fixed by the highest phosphorus concentration that is to be determined. Therefore, if with our system a linearity up to 0.1 mmol of phosphate per liter (corresponding to 3 mmol of the sample per liter in the final automated procedure) is desired, our reactant concentrations are also near the lowest possible, within the restrictions set by the acidity.

Our results confirm the findings of Gee and Dietz (17), who calculated a 1:1 phosphorus/vanadium ratio in the complex. Our results are also consistent with their stated minimum ratio of molybdenum to phosphorus of 15:1.

Color development and color stability. In the final procedure, color development is complete after only 20 s and the color is stable: after 2 h an increase in net absorbance of only 1% was observed.

Diluent. When continuous-flow analysis is used, it is assumed that standards and test samples behave identically. If they do not, serious inaccuracy may result. There has been some controversy in the literature as to final phosphate concentration of 0.10 mmol/liter, a concentration that reflects the final concentration in the automated procedure of a sample containing 3 mmol/liter, when 10% of the phosphate is dialyzed. As can be seen from Figure 3, the optimum acid concentration depends on the concentration of the other reactants. A region of constant absorbance is situated between 0.05 and 0.20 mol/liter HNO₃ if the final vanadium and molybdenum concentrations are not less than 0.2 and 5 mmol/liter, respectively. Within these restrictions, it appeared that an acidity of 0.2 mol/liter was also the lowest concentration that gave a relatively low blank absorbance. Therefore we used this concentration for all further studies, and also adopted it in the final automated procedure. Henry (10, 11) states that the vanadomolybdate method yields serum phosphate values that are 0.1 mmol/liter too high. This possibly might be caused by “the higher acidity employed.” Indeed, this might also have been the case in some of the older vanadate studies. However, as Table 1 shows, comparison with other frequently used phosphate methods reveals a very favorable acid concentration for our method, especially when the duration of sample exposure to acid is taken into account. The relatively mild conditions of our finally adopted procedure can also be illustrated by the fact that analysis of 10 mmol/liter solutions of ATP, β-glycerophosphate, or pyridoxal phosphate resulted in phosphate concentrations of respectively 0.05, 0.01, and 0.10 mmol/liter. These values correspond to 0.5, 0.1, and 1.0% of the parent solutions. So if there is any hydrolysis at all, it is less than 1%, even if the absence of all contaminating inorganic phosphate in the parent solutions is assumed.

**Table 2. Comparison of Variables in Other Vanadomolybdate Methods**

<table>
<thead>
<tr>
<th>Acid</th>
<th>Acid mol/liter</th>
<th>Vanadium mol/liter</th>
<th>Molybdenum mmol/liter</th>
<th>Phosphorus mmol/liter</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl</td>
<td>0.07</td>
<td>0.08</td>
<td>1.6</td>
<td>0.03</td>
<td>(8)</td>
</tr>
<tr>
<td>HNO₃</td>
<td>0.53</td>
<td>2.14</td>
<td>28.3</td>
<td>0.05</td>
<td>(18)</td>
</tr>
<tr>
<td>H₂SO₄/HNO₃</td>
<td>0.35</td>
<td>1.5</td>
<td>39</td>
<td>0.1*</td>
<td>(12)</td>
</tr>
<tr>
<td>H₂SO₄/HNO₃</td>
<td>0.62</td>
<td>1.2</td>
<td>28</td>
<td>0.1*</td>
<td>(9)</td>
</tr>
<tr>
<td>HNO₃</td>
<td>0.2</td>
<td>0.2</td>
<td>5</td>
<td>0.1</td>
<td>Present study</td>
</tr>
</tbody>
</table>

*Estimated and supposed to be of the same order as in the present study.
the proper diluent for the sera during the on-stream dialysis procedure in the phosphate determination. It has been recognized earlier that diluting in and dialyzing against NaCl solution (9 g/liter) resulted in poor and erratic dialysis (12, 19–22). Pragmatic approaches to this problem have led to the use of various acid diluents (12, 20), concentrated NaCl solution (21), or formate or acetate buffers with pH values ranging from 4 to 5 (19, 21). In most recently published methods an acid diluent is used. The problem can best be illustrated with the experiment shown in Figure 4. A serum with a known target value was serially diluted 0-, 2-, 4-, and 8-fold and analyzed with the proposed method according to the flow pattern of Figure 1, except for the variation of the final acid strength in the donor sample stream. A strong dependence on the final acid strength in obtaining the appropriate target value is demonstrable, this dependence being larger with higher protein concentrations. With a protein content of 5 g/liter no difference is observed between the acid concentrations used. It is therefore easy to postulate that at higher protein concentrations a considerable fraction of the phosphate is protein bound, thereby preventing efficient dialysis. To confirm and extend this explanation, we examined the percentage analytical recovery of phosphate added to albumin solutions of different concentrations. The results (Figure 5) again show the strong relation between protein content and analytical recovery on the one hand and the final acid strength on the other hand. For the 30 g/liter solutions the corresponding pH value of each dialysis mixture has also been depicted. Obviously, the percentage recovery is strongly related to the pH of the dialysis mixture. Albumin, as the most important protein component involved in serum, has its isoelectric point at pH 4.7. Below this pH it carries a net positive charge and can bind phosphate, whether in the H₂PO₄⁻ form or in the HPO₄²⁻ form. Therefore the ratio of un-ionized orthophosphoric acid to primary orthophosphate has to be taken into account. When a pKᵣ of orthophosphoric acid of 2.0 is assumed, this ratio is in a 10:1 preference for H₃PO₄ over H₂PO₄⁻ at a pH of 1.0. So the results support the view that phosphate has to be predominantly in its un-ionized form (pH ≤ 1) if there is to be no protein binding. This has been accomplished in our final procedure, where serum and standards are diluted with an equal volume of 0.3 mol/liter HNO₃. A sound theoretical basis has now been given to the pragmatic solutions used in earlier studies.

**Linearity, precision, carryover.** As discussed before, we ascertained that absorbance and concentration are linearly related in the physiologically important range up to 3 mmol/liter, which condition had been set by the choice of reactant concentrations.

The percentages of steady state in the 1:1 sample/wash mode with 40, 60, and 100 samples per hour were respectively 96, 88, and 81%. With these sampling rates the percentages of carryover were respectively 0.8, 1.0, and 1.2%.

Within-day precision was determined in the final working mode (1:2 sample/wash ratio; 40 samples per hour) by making 25 replicate determinations on a pooled serum that had a mean phosphate concentration of 1.22 mmol/liter. The CV was 1.0%. Day-to-day precision was established by determination of two different unassayed control sera (“Autonorm” high and normal; Nyegaard, Oslo) at 12 consecutive times. The CV’s were 2.4 and 2.9%, with means of 2.40 and 1.35 mmol/liter, respectively.

**Accuracy.** Accuracy is defined as the agreement between the best estimate of a quantity and its true value (23). The difficulty is the assessment of the true value, because no definitive method is yet available for phosphate. In most cases, the method in question is therefore compared with the best method available by assaying many sera by both methods. Alternatively, sera with assigned reference values are sometimes assayed with the proposed method. The problem remains as to how to define an acceptable reference method giving no bias with the true value. With this in mind, we decided to follow another approach to assess accuracy. We had the opportunity to analyze eight different sera that had previously been assayed in the Dutch Quality Control Survey by some 150 laboratories. There is good evidence that the truncated mean values (assessed by eliminating the values outside the 3 SD limits) established in this.

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**Figure 4.** Dependence on the protein content and the acid strength during dialysis in attaining the target value of a control serum. All concentrations given are final ones.

**Figure 5.** Recovery of added phosphate to albumin samples of different concentrations in relation to the final acid strength during the dialysis procedure. Albumin concentrations given are final ones in the dialysis mixture.
way closely approach the true values (24). Results of this determination with the proposed method are shown in Figure 6 for the eight sera, covering the range 1.0–2.6 mmol/liter. Again we could demonstrate the strong dependence on the acid strength used during the dialysis procedure. Mean systemic biases with the mean values for the three acidities tested were respectively −0.4, −2.7, and −7.4% for 150, 100, and 50 mmol/liter final HNO₃ concentrations. Thus, in the finally adopted procedure we obtained excellent agreement with the mean (or true) values.

Study on Interferences

Anticoagulants. A possible methodological interference caused by anticoagulants was studied by analysis of sera before and after addition of the particular anticoagulant. Two concentrations were used. The lower one resembled that normally occurring in plasma, the higher concentration was twice as great. The anticoagulants studied were sodium citrate-2H₂O, NaF, lithium heparin and disodium EDTA. Of these anticoagulants only citrate interfered with the analysis: with a concentration of 19 g/liter a mean negative error of 5.1% was found. This value increased to 11.9% for the higher concentration. So the use of citrated plasma has to be avoided. However, this is not a drawback that is peculiar to this method; all other methods based on a reaction with molybdate are so affected. It is found earlier that citrate and phosphate compete for binding with the molybdate ion, and the former reportedly is bound in preference to phosphate (5).

Chromogens. Thanks to the presence of an on-stream dialysis procedure, interference from normally or therapeutically occurring chromogens can be expected to be minimal. Nevertheless, a possible interference by hemolysis or bilirubin has been studied. Hemolysis was studied by adding increasing amounts of a washed and hemolyzed erythrocyte concentrate to a specimen of pooled serum. A small but detectable positive error was found, amounting to 0.02 mmol/liter phosphate for every 0.1 mmol of hemoglobin per liter (160 mg/100 ml). Likewise, bilirubin, added in increasing quantities to a pooled normal serum, gave a positive error equivalent to only 0.01 mmol of phosphate per liter for every 150 μmol of bilirubin per liter.

Drugs. Since the phosphovanadomolybdate complex is measured at 405 nm, it can be imagined that drugs or their metabolites with a yellow color might interfere with the determination. For this reason increasing amounts of salicylazosulfopyridine (Salazopyrine®) were added to serum samples up to concentrations therapeutically encountered (80 mg/liter). In spite of the intense yellow color, there was no interference.

Phenothiazine compounds reportedly cause negative errors in the phosphate determination (25). Therefore we also tested chlorpromazine in concentrations in serum of 2 mg/liter. Here, too, no influence was observed. Therefore it can indeed be assumed that in the dialysis step drugs are sufficiently removed so that in the reaction mixture not enough is present to cause errors.

Turbidity. A possible influence of turbid sera was investigated by means of a dilution technique. The specimens to be studied were first analyzed undiluted, then analyzed diluted two-, four- and eightfold with the

Table 3. Four Serum Specimens, Varying in Appearance from Slightly Turbid (I) to Extremely Turbid (IV), Analyzed Both Undiluted and Diluted with the 1.0 mmol/liter Standard Solution in the Range as Shown.

<table>
<thead>
<tr>
<th></th>
<th>Undiluted</th>
<th>1 → 2</th>
<th>1 → 4</th>
<th>1 → 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found</td>
<td>Found</td>
<td>Found</td>
<td>Found</td>
</tr>
<tr>
<td>mmol/liter</td>
<td>Expected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1.40</td>
<td>1.20</td>
<td>1.20</td>
<td>1.10</td>
</tr>
<tr>
<td>II</td>
<td>1.15</td>
<td>1.08</td>
<td>1.08</td>
<td>1.04</td>
</tr>
<tr>
<td>III</td>
<td>1.28</td>
<td>1.14</td>
<td>1.14</td>
<td>1.07</td>
</tr>
<tr>
<td>IV</td>
<td>1.12</td>
<td>1.06</td>
<td>1.03</td>
<td>1.03</td>
</tr>
</tbody>
</table>
1.0 mmol/liter phosphate standard solution. From the results (Table 3) it is clear that turbid sera can be used in determination of inorganic phosphate by the proposed method.

Summarizing, we conclude that (a) the method presented may be regarded as an improvement for those laboratories that are only equipped with simple flow-through colorimeters; (b) the manifold is extremely simple and the liability to errors is minimized by use of a single, stable reagent; (c) the reaction conditions are mild, thus preventing hydrolysis of labile organic phosphates; and (d) analyses of control sera reveal a high degree of accuracy.

References