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A Robotics-Based Automated Assay for Inorganic and Organic Phosphates

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Phosphate analyses are fundamental to a broad range of biochemical applications involving inorganic phosphate and organic phosphoesters such as phospholipids, phosphorylated proteins, and nucleic acids. A practical automated method utilizing robotics is described in this report. Five colorimetric methods of phosphate analyses based on formation of a phosphomolybdate complex and compatible with the automated assay were tested, and the fundamental chemistry is discussed. The relative sensitivities are malachite green > crystal violet > quinaldine red > ascorbate reduction > antimony-modified ascorbate reduction, although only a fourfold improvement was observed in going from the modified ascorbate procedure to malachite green. Malachite green was selected to optimize the assay because this dye provided the highest sensitivity. However, where color stability and low blanks are more important than sensitivity, the ascorbate reduction and quinaldine red methods were found to be better choices than malachite green. Automation using a robotic liquid-handling system substantially reduces the labor required to process large arrays of samples. The result is a sensitive, nonradioactive assay of inorganic phosphate with high throughput. A digestion step in an acid-resistant 96-well plate was developed to extend the assay to phosphate esters. The robotic-based assay was demonstrated with inorganic phosphate and a common phospholipid, phosphatidylcholine. © 1999 Academic Press

Key Words: **phosphate assay; inorganic phosphorous; automation; robotics; phosphomolybdate; malachite green; crystal violet; quinaldine red; phospholipid; organophosphate.**

The use of robotics permits the rapid processing of a large number of samples in a highly reproducible manner. Automated systems utilizing robotics have been used in DNA isolation (1), labeling (2), and sequencing (3); for drug screening (4); and in combinatorial chemistry (5). Colorimetric phosphate assays, which can be carried out with inexpensive reagents in relatively few steps, are also well-suited to automation. An automated phosphate analysis has the potential for widespread applications in biological studies involving hydrolysis of phospholipids by phospholipases, quantitation of nucleic acids and phosphorylated proteins, as well as for inorganic phosphate determination. In addition, a sensitive automated phosphate assay is of interest to those involved in the testing of natural waters and wastewaters, where nonrobotic automated procedures for measuring phosphate contamination have been available since the initial reports in the mid 1960s (6–8).

Our interest in developing a sensitive nonradioactive assay of the organophosphate products formed by phospholipases C and D has led us to develop a robotic automated assay for inorganic phosphates and phosphate esters. This assay is compatible with any of the colorimetric methods based on the formation of a phosphomolybdate complex, such as cationic dye binding and ascorbate reduction. The present study is, to our knowledge, the first report of an automated phosphate assay utilizing robotics.

MATERIALS AND METHODS

Reagents and Materials

Ammonium heptamolybdate · 4 H_2O, crystal violet, sodium ascorbate, Tergitol NP-35, and dipalmitoylphosphatidylcholine were from Sigma (St. Louis, MO); potassium antimony tartrate was from Aldrich (Milwaukee, WI); sulfuric acid, malachite green oxalate, quinaldine red, and polyvinyl alcohol (98% hydrolyzed, average MW 16,000) were from Fisher Scientific (Pittsburg, PA); perchloric acid and nitric acid were from J. T. Baker (Phillipsburg, NJ); and sodium

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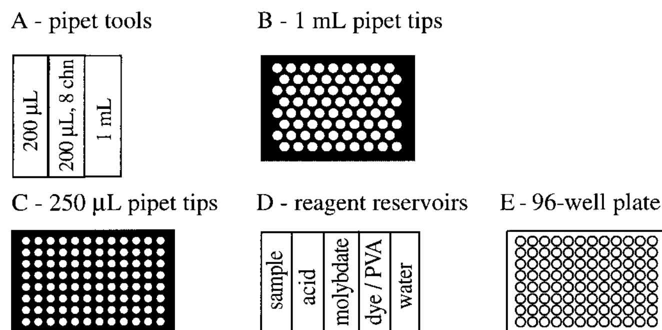

FIG. 1. General layout of the automated liquid handling system worksurface used in the malachite green phosphate assay. Three pipet tools were used as indicated in A. The pipet tips are located in holders B and C, and the reagent reservoirs in D. Additions of sample, sodium phosphate or dipalmitoylphosphatidylcholine (DPPC), to the 1-mL wells in reaction plate E and the addition of water to the DPPC prior to digestion were carried out with the single-channel 200-μL pipet tool. The eight-channel 200-μL pipet tool was used to add water to the DPPC after digestion and to add the molybdate and dye reagents to both the sodium phosphate and DPPC samples. For the addition of water to the sodium phosphate and the addition of acid digestion mixture to the DPPC, the 1-mL single-channel pipet tool was selected. A fresh pipet tip of appropriate volume was used for each transfer of liquid. For the assay of sodium phosphate, reagent reservoirs containing sodium phosphate sample, molybdate in acid, dye/PVA solution, and water were used. In the predigestion phase for DPPC, reservoirs for the DPPC sample, acid digestion mixture (labeled acid in diagram), and water were used. In the postdigestion phase for DPPC, the acid digestion mixture and DPPC sample were removed and replaced by reservoirs containing molybdate in water and dye/PVA solutions.

phosphate ($NaH_2PO_4 \cdot H_2O$) and sodium molybdate were from Mallinckrodt (Paris, KY). A 96-well Teflon microplate (well capacity, 1 mL) was machined from a virgin Teflon block [8.9 × 13.0 × 3.8 cm (Laird Plastics, West Palm Beach, FL)] by the University of Oregon Science Services Machine Shop.

Assays

Malachite green assay of sodium phosphate (9). All manipulations in the malachite green assays were carried out using a Beckman Biomek 2000 Laboratory Automation Workstation. The work surface is diagrammed in Fig. 1. Sodium phosphate (0–75 μL, 100 μM) was added to one column of a standard 96-well microplate using a 200-μL capacity single-channel pipet tool, and the total volume in each well was brought to 500 μL with deionized water using a 1-mL-capacity single-channel pipet tool. Using a 200-μL-capacity eight-channel pipet tool, 172 μL of 28 mM ammonium heptamolybdate in 2.1 M H_2SO_4 was added, followed by 128 μL of a mixture containing 0.35% polyvinyl alcohol and 0.76 mM malachite green. A blue–green color developed in the wells containing phosphate; blanks were yellow. After 20 min at room temperature, absorbance measurements were made at 610 nm using a Titertek Instruments, Inc. (Huntsville, AL), Multiskan 310C plate reader equipped with a 610-nm filter.

Crystal violet assay of sodium phosphate (10). To 0–7 nmol of sodium phosphate in 168 μL of deionized water were added (with vortex mixing) 100 μL of 1% polyvinyl alcohol, 200 μL of 0.1 M sodium molybdate, 132 μL of a 0.76 mM solution of crystal violet in deionized water containing 0.35% polyvinyl alcohol, and 200 μL of 1.96 M HNO_3. Phosphate-containing samples were violet in color; blanks were green. Absorbance values were measured at 560 nm, 40 min after the addition of nitric acid.

Quinaldine red assay of sodium phosphate (11). To 0–10 nmol sodium phosphate in 315 μL of deionized water were added 5 μL of 3.15M H_2SO_4 and 320 μL of an aqueous mixture containing 0.1 mg/ml quinaldine red and 0.14% polyvinyl alcohol. Addition (with vortex mixing) of 160 μL of 7 mM ammonium heptamolybdate in 1.25 M H_2SO_4 resulted in the formation of a pink quinaldine red–phosphomolybdate complex. Blanks were essentially colorless. Absorbances were measured at 525 nm, 15 min after the addition of molybdate.

Ascorbate reduction assay of sodium phosphate (12, 13). Eighty microliters of 3.15 M H_2SO_4 and 160 μL of 20 mM ammonium heptamolybdate were added to 12 × 75-mm glass culture tubes, followed by the addition of 0–12 nmol sodium phosphate in 480 μL of deionized water. The solutions were mixed and then 80 μL of a freshly prepared 10% solution of sodium ascorbate in water was added with vortexing. The tubes were placed in a 37°C water bath for 1.5 h, followed by cooling in cold tap water for 5 min. A blue color developed in phosphate-containing tubes with intensity dependent on phosphate concentration. Absorbance values at 820 nm were read using a Beckman DU-40 spectrophotometer.

Modified ascorbate reduction assay of sodium phosphate (14). First, a 20-ml stock solution containing 1.4 mL concentrated H_2SO_4, 2.6 mg potassium antimony tartrate, and 112 mg ammonium heptamolybdate · $4H_2O$ was prepared. Then 11.2 mg of sodium ascorbate was dissolved in 2 mL of the stock solution to form the combined reagent (which is not stable indefinitely and should be used within several hours of being prepared). Sodium phosphate (0–12 nmol) in 667 μL of deionized water was added (with vortex mixing) to 12 × 75-mm glass culture tubes containing 133 μL of combined reagent. Complete development of a blue color in tubes containing phosphate occurred within 15 min at room temperature. Absorbance values were read at 880 nm.

Malachite green assay of phosphate in dipalmitoylphosphatidylcholine (DPPC).[2] To prepare a 100 μM stock solution of DPPC as vesicles in water, 100 μl of a 10 mM DPPC/$CHCl_3$ solution was added to a glass vial and the $CHCl_3$ evaporated off under a stream of dry nitrogen. Ten milliliters of deionized water was then added to the vial, vortexed, and then sonicated using a Branson Model 1210 bath sonicator for 5 min. Volumes between 0 and 75 μL of this stock solution were pipetted into wells of a Teflon 96-well microplate using the 200-μL single-channel pipet tool. (At the time these experiments were initiated, 96-well plates made of glass were unavailable; however, recently glass multiwell plates have become commercially available from suppliers such as Aldrich.) Four hundred microliters of 0.9 M H_2SO_4/0.16 M $HClO_4$ was then added to each well with the 1-mL pipet tool, and the microplate was placed on a hot plate and heated at 120°C for 18 h (15). Following digestion, 400 μL of deionized water, 172 μL of 28 mM ammonium heptamolybdate, and 128 μL of a mixture containing 0.35% polyvinyl alcohol and 0.76 mM malachite green were added in turn, all with the 200 μL eight-channel pipet tool. DPPC-containing wells were blue–green. Three hundred microliters from each well was transferred to either a clear-bottom polystyrene 96-well plate for plate reader absorbance measurements or clear poly(methyl methacrylate) cuvettes for spectrophotometer absorbance measurements. Absorbances were then measured at 610 nm.

RESULTS

Determination of Optimal Experimental Conditions for the Dye-Enhanced Phosphate Assay

Color development in the cationic dye-enhanced phosphate assay is dependent on having appropriate concentrations of sulfuric acid, ammonium heptamolybdate, and dye as well as a stabilizer to prevent fading due to aggregation of dye-phosphomolybdate complexes. The experiments described here were carried out in 0.45 M sulfuric acid, a concentration which has previously been shown to be optimal for color development in the malachite green phosphate assay (9) and very close to the concentration selected for this and other dyes by Soyenkoff (11). At this concentration of sulfuric acid, malachite green bound to phosphomolybdate is blue–green in color with an absorption maximum at 610 nm. Unbound dye solution is yellow, contributing little absorbance at 610 nm and thus resulting in low absorbance values for blanks. The optimal concentrations of ammonium heptamolybdate and malachite green were established by varying the concentrations of each reagent while holding the other constant. Figure 2A shows the effect on absorbance at 610 nm of samples containing 6 nmol of sodium phosphate and blanks with varying concentrations of ammonium heptamolybdate and a constant malachite green concentration of 120 μM. Color in the blanks did not change significantly with molybdate concentration and appeared to result almost entirely from the malachite green. With 6 nmol of phosphate present, absorbance at 610 nm increased with increasing concentrations of molybdate up to approximately 4 mM ammonium heptamolybdate where a plateau was observed. The plateau extended from 4 to 9 mM where the absorbance began to increase again. The center of this region (6 mM heptamolybdate) was chosen for subsequent assays. Figure 2B shows the dependence of the absorbance on malachite green concentration in the presence of 6 mM heptamolybdate. The absorbance increased rapidly with increasing concentrations of malachite green until reaching a plateau at a concentration of approximately 50 μM. On the other hand,

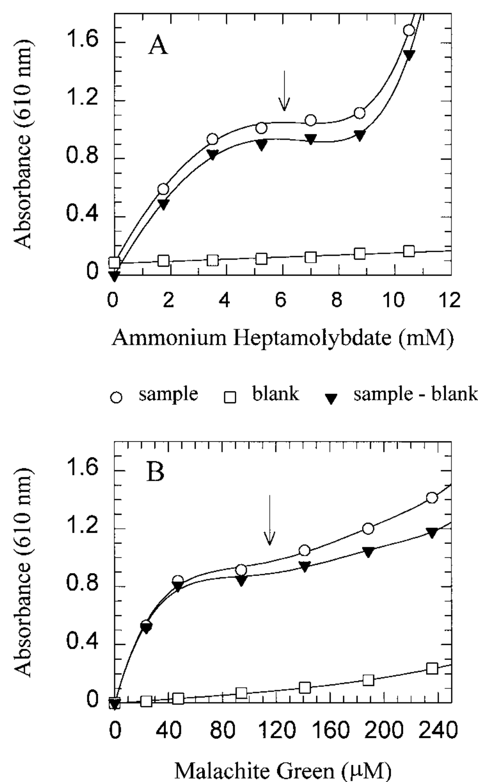

FIG. 2. Optimization of experimental conditions in the malachite green phosphate assay. (A) The concentration of ammonium heptamolybdate was varied, while the malachite green concentration was maintained at 120 μM. (B) The concentration of malachite green was varied while that of ammonium heptamolybdate was fixed at 6 mM. All samples (experimental and blanks) were 0.45 M in sulfuric acid. Experimental samples contained 6 nmol sodium phosphate and blanks contained none. Arrows indicate conditions used for subsequent malachite green phosphate assays.

[2] Abbreviations used: DPPC, dipalmitoylphosphatidylcholine; PVA, polyvinyl alcohol.

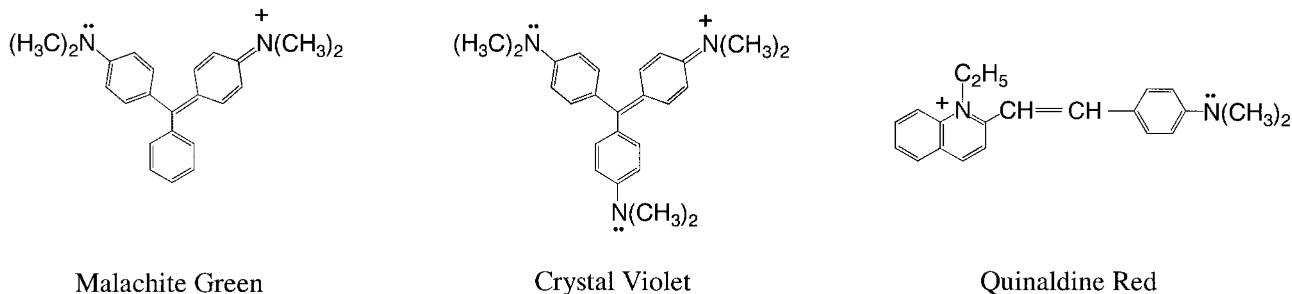

FIG. 3. Structures of three organic dyes which enhance the sensitivity of phosphate analyses. Malachite green, crystal violet, and quinaldine red are all cationic dyes examined in the early work of Soyenkoff (11).

absorbance observed with the blanks rose steadily as the malachite green concentration increased. In order to optimize sensitivity, while avoiding excessively high blanks, 120 μM malachite green was used in all assays. Color stability of the malachite green–phosphomolybdate complex was also examined. When no stabilizer was present and the phosphate concentrations were low (6 nmol was phosphate present in the assay mix, corresponding to a concentration of 4.3 μM), no significant color deterioration was observed. However, as the phosphate concentration increased in the unstabilized sample, the color was more prone to fading (data not shown). We have found that addition of small amounts of the frequently used stabilizer polyvinyl alcohol (9) to the malachite green before mixing with the phosphomolybdate complex provided increased stability for higher phosphate concentrations when absorbance measurements were made 10–40 min after addition of the malachite green–polyvinyl alcohol mixture. By 60 min there was a noticeable reduction in color resulting in reduced sensitivity of the assay. Comparable stabilization was obtained with the nonionic polyglycol ether surfactant Tergitol (NP-35), which appears to be closely related to the Sterox detergent, whose use as a stabilizer was described in earlier reports (16, 17).

Comparison of Phosphate Assays

We compared the malachite green phosphate assay with assays utilizing two other dyes, quinaldine red (11) and crystal violet (10), and two widely used colorimetric procedures based on the formation of molybdenum blue (12–14). The structures of the dyes are shown in Fig. 3. As with the malachite green assay, all of these procedures are based on formation of a phosphomolybdate complex at low pH. The assays not involving dyes involve formation of molybdenum blue by reduction with either sodium ascorbate alone (12, 13) or in the presence of potassium antimony tartrate (14). Using these five different assays, standard curves of absorbance vs nanomoles of phosphate were prepared as shown in Fig. 4. Each of these assays demonstrated a linear relationship between absorbance and phosphate concentration, although a deviation from linearity was observed with the quinaldine red assay at low phosphate concentrations. Based on the slopes of the curves in Fig. 4, the two assays utilizing the formation of molybdenum blue were similar in sensitivity though the modification using potassium antimony tartrate was slightly less sensitive. The quinaldine red assay was approximately twice as sensitive as the assays based on the formation of molybdenum blue, produced very low (A = 0.008) blanks and had the best color stability of the cationic dyes tested. The most sensitive assays of those tested were obtained with the triphenylmethane dyes, crystal violet, and malachite green, although we encountered serious problems with color stability and nonlinear standard curves when using crystal violet. The relative sensitivities of the malachite green, crystal violet, quinaldine red, ascorbate, and modified ascorbate methods were 4.3:3.4:2.2:1.2:1.0.

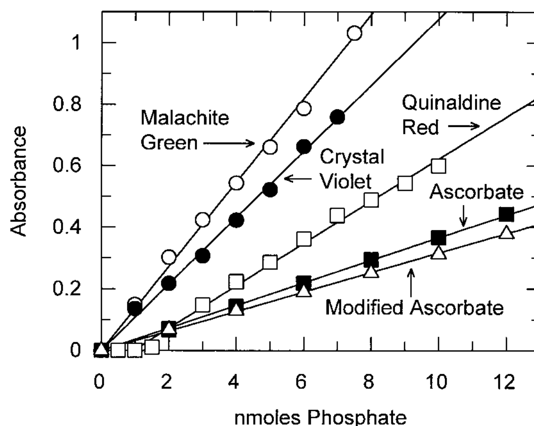

FIG. 4. Comparison of the sensitivities of five phosphate analysis methods based on the formation of the phosphomolybdate complex. Absorbance was measured at 610 nm in the malachite green assay, 560 nm in the crystal violet assay, 525 nm for quinaldine red, 820 nm in the standard ascorbate reduction method to form molybdenum blue, and 880 nm in the antimony-modified ascorbate reduction procedure.

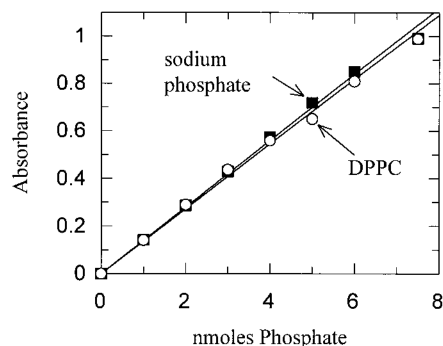

FIG. 5. Data illustrating the linearity and sensitivity of the automated malachite green phosphate assay. The curves for inorganic phosphate and for an equivalent amount of the organic phosphodiester (DPPC) are in excellent agreement, indicating that the automated digestion step is quantitative.

Automated Phosphate Analysis of a Phospholipid

In order to broaden the range of possible applications for the automated phosphate assay, we carried out a phosphate analysis on the phospholipid DPPC. Since the assay is capable of detecting only orthophosphate, it was necessary to first carry out an acid digestion of the DPPC, liberating orthophosphate. This step was performed in a reusable 96-well plate machined from Teflon in order to avoid chemical reaction between the acid and the plate. Sulfuric acid is non-volatile and remained in the wells of the plate after digestion. Thus, the sulfuric acid in the DPPC assay was added directly after addition of the sample, a modification of the sodium phosphate assay, where the sulfuric acid was added with the molybdate. The calibration curve that resulted from this assay (Fig. 5) is nearly identical to the standard curve obtained with sodium phosphate. The excellent agreement of these curves demonstrates the suitability of the automated assay for analysis of compounds containing organic phosphates or polyphosphates.

DISCUSSION

Our objective in this study was to devise an automated colorimetric phosphate assay utilizing a 96-well plate format and robotics. As a starting point, we chose the reaction between orthophosphate and ammonium heptamolybdate, which, under acid conditions, results in the formation of a phosphomolybdate complex (18–20). Although the phosphomolybdate complex is the basis for nearly every colorimetric phosphate assay in use today, the chemistry involved is rarely discussed in the phosphate analytical literature. Since it is advantageous to understand the fundamentals involved when the conditions of the assay are selected, we include a short discussion of the acid–base and complexation chemistry here.

It is well known that upon addition of acid, molybdenum(VI) salts form numerous polymolybdate(VI) structures (18–21). When phosphate is present, heteropolyanions are formed in which the phosphate group is surrounded by a cage of MoO_6 octahedra with shared corners. These are also known as heteropolyacids, phosphomolybdate complexes, Keggin structures, and one subclass of heteropoly oxometallates. The various species present in solution have been studied, although not usually in the range of concentrations of interest in a phosphate assay (22–24). Many equilibria are involved, and the heteropolyanion formed depends on the pH and concentrations of molybdate and phosphate. A representative reaction is shown in Fig. 6, in which 12 molybdates surround one phosphate to form the complex. The crystal structures of this complex and related complexes have been determined (25–28). In Fig. 6, the phosphate reactant is shown as phosphoric acid rather than phosphate since the $pK_a = 2.1$ for the first ionization of phosphoric acid, and the pH is roughly 0.5 at a sulfuric acid concentration of 0.40 M. Likewise, the molybdate is shown as molybdic acid since the $pK = 3.6$ for the first ionization of molybdic

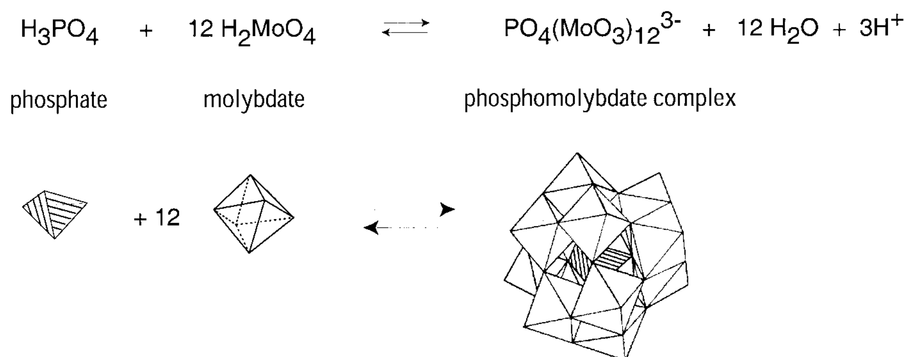

FIG. 6. Phosphomolybdate complex formation. A representative equilibrium is shown here; the individual phosphate and molybdate subunits combine to form the heteropoly complex (17, 20, 24). The malachite green dye then binds to the heteropoly complex. The phosphate is tetrahedral. The molybdate is shown as an octahedral structure to illustrate the components of the complex. However, the molybdate is likely to be tetrahedral as a monomer, forming an octahedral structure in the complex.

acid (22). Alternatively, the molybdic acid may exist as an octahedral hydrate at this pH (21). Two essential facts are relevant to the design of the assay: (i) Strongly acidic conditions are required for the stability of the complex, and (ii) sufficient molybdate must be present to supply 12 molybdates for each phosphate. Excess molybdate is required to drive the equilibrium to the right so that essentially all of the phosphate is converted to the phosphomolybdate complex. For example, in the malachite green assay (using 6 mM ammonium heptamolybdate) of a solution containing 12 μM phosphate, the molybdate:phosphate ratio is 3500:1.

Once formed the colorless phosphomolybdate complex can be converted to a colored complex by several different procedures. Many of these rely on reduction of the phosphomolybdate complex resulting in the formation of molybdenum blue. A number of reducing agents have been used for this purpose: a mixture of sulfite and aminonaphtholsulfonic acid (29), stannous chloride (30), ascorbic acid (12, 13), and ascorbate in the presence of bismuth (31) or antimony (14). A somewhat less-sensitive procedure relies on the formation of a yellow vanadomolybdophosphoric acid, which occurs when the phosphomolybdate complex is formed in the presence of vanadate (32).

The sensitivity of the colorimetric assay can be enhanced by addition of a cationic dye to the phosphomolybdate complex. In a landmark paper published in 1947, Soyenkoff (11) observed the color change of malachite green, crystal violet, and quinaldine red in the presence of phosphomolybdic acid and devised a sensitive colorimetric assay based on quinaldine red. Soyenkoff selected this dye over malachite green and crystal violet because he observed it to have the lowest blanks and greatest color stability under the conditions of his assay. Since this early paper, many variations of the dye-enhancement method have been reported. The most frequently used dye is malachite green (33–36). Crystal violet, on the other hand, has been used only infrequently (10) and quinaldine red is rarely cited.

The cationic dyes that have proven most useful for colorimetric phosphate assays are pH indicator dyes having pK_as slightly above the pH used for the formation of the phosphomolybdate complex. Malachite green, crystal violet, and quinaldine red all meet these criteria. With each of these dyes, only the form of the dye normally present at the higher pH is detected bound to the complex. In the case of malachite green [pK_a $\sim$ 1.2 (37–39)], a solution of the dye is yellow in 0.45 M sulfuric acid (the concentration of acid in the assay with pH $\sim$0.5), but above pH 2 it is blue–green. Adding molybdate to the solution does not change the color. However, when phosphate is added, the phosphomolybdate complex forms and the solution turns blue–green. The color change is caused by the electrostatic binding of the blue–green form (but not the yellow form) of malachite green to the anionic phosphomolybdate complex. Quinaldine red and crystal violet behave similarly, although the colors are different. Other cationic dyes, notably rhodamine B (which has a molar absorption coefficient several times higher than that of malachite green), does not undergo a significant color change when a complex is formed with phosphomolybdate (40). Thus, when using this dye, it is necessary to separate unbound dye from that bound to the complex before optical measurements can be made (40). The separation and washing steps would complicate the automation, so we did not consider rhodamine B further.

As with the dye-binding assay, the two ascorbate-based assays examined in this study are capable of being automated using the procedure described here. However, the absorbance maxima (>800 nm) are out of the range of many commercial plate readers. Moreover, as shown in Fig. 4, the malachite green assay is approximately fourfold more sensitive in the detection of orthophosphate than either the ascorbate or the modified ascorbate assays. Improvements of a factor of 10 or 20 are sometimes claimed for malachite green procedures (13, 33). However, the comparison is usually with the Fiske and Subbarow method (29), which, although important historically, was replaced long ago by more sensitive methods. To our knowledge, the largest gains in sensitivity in colorimetric phosphomolybdate-based phosphate assays were described in Soyenkoff's paper in 1947 (11). When modern methods of phosphate analysis are compared, the differences in sensitivity are relatively modest (Fig. 4). The dye-binding and ascorbate reduction methods are sufficiently similar that the choice of method will depend on the application. Each has advantages and disadvantages. The dye-binding assays have the advantage of sensitivity, but they often have lower color stability. Dye binding decreases the net negative charge on the phosphomolybdate complex, and thus reduces its solubility. Therefore, a stabilizer is required in order to prevent precipitation. No stabilizer is required in the ascorbate reduction and modified-ascorbate reduction methods. In flow injection analysis, for example, a reduction method may be preferable over a dye-binding assay (41). The modified ascorbate reduction method and the dye-binding methods require no heating for color development, whereas the ascorbate reduction method requires an hour and a half at 37°C. In our hands the modified ascorbate procedure gave low blanks, comparable to those obtained with the quinaldine red assay and significantly lower than with the ascorbate reduction method.

Once the desired variation of the phosphate assay has been selected, it can be automated using robotics as described in this report. A significant amount of

time (several hours) is required to create and test the initial program that controls the robot. However, once the program is functional, it can be quickly and easily modified to alter the assay conditions. Automation of the assay substantially reduces the length of time required to carry out an assay. For processing of large numbers of samples, automation of the assay virtually eliminates mistakes in addition of reagents and transfer of samples. Finally, the accuracy obtained with the automated assay is at least comparable to that of a manual assay.

In summary, an automated phosphate assay utilizing robotics has been developed which removes much of the tediousness of present phosphate assays. In addition, five different colorimetric assays based on the phosphomolybdate complex were compared. All five can be adapted to automation. When sensitivity is the important criterion, the malachite green assay, or a similar assay based on quinaldine red, has an advantage over the other colorimetric phosphate assays tested. Finally, an additional digestion step was added in order to extend the new automated assay to include not only inorganic (i.e., orthophosphate) but more complex phosphate compounds such as polyphosphates and organically bound phosphates.

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