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ENZYMATIC DETERMINATION OF PHOSPHATE IN CONJUNCTION WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

DANA K. MORGAN and NEIL D. DANIELSON*
Department of Chemistry, Miami University, Oxford, OH 45056 (U.S.A.)
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SUMMARY

A selective assay for orthophosphate in complex matrices was developed based on the nucleoside phosphorylase catalyzed conversion of inosine and orthophosphate to hypoxanthine. The enzyme reaction using only 0.28 units/assay was allowed to proceed for 30 min before quenching. Separation of inosine and hypoxanthine was performed by reversed-phase high-performance liquid chromatography. Quantitation of the hypoxanthine peak was found to be linear with orthophosphate up to about 30 μg/g. A detection limit of 0.75 ppm could be otbained after dialysis of the commercial enzyme. Interference studies showed that the enzymatic assay unlike the colorimetric molybdate-blue technique was essentially unaffected by complex matrices such as serum, urine, polyphosphates, and phosphoesters.

INTRODUCTION

Orthophosphate is found in a wide variety of samples having complex compositions. Monitoring of phosphate in natural water samples is important since phosphorus is thought to be a limiting nutrient in the growth of algae. High phosphate concentrations will lead to excessive algae growth in a lake causing eutrophication[1]. To minimize this possibility, the routine determination of phosphate in treated sewage effluents and other water samples must be carried out[2]. The determination of free phosphate can also be important for various clinical and biological studies. The amount of phosphate is of diagnostic significance with regard to renal failure[3]. Phosphate often acts as a product or reactant in methods involving enzyme regulation[4].

The standard assay for phosphate involves reaction with the molybdate ion in acid to form phosphomolybdate which is then reduced to the molybdate-blue species for measurement by colorimetry[2,5,6]. Although this method is sensitive down to the low ppm level, sample matrices such as surfactants have been known to interfere severely[7,8]. In addition, the low pH required for the molybdate-blue method can cause the hydrolysis of polyphosphates[9] or phosphoesters[10] to orthophosphate. Methods of sample cleanup, like extraction[7,8] and chromatography[11], can eliminate the interference; however, they are time-consuming and usually sensitivity is lost.

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To increase selectivity for the assay of phosphate, several enzymatic methods have been developed. Some of the methods use coupled systems involving up to 4 enzymes[10,12]. To eliminate the need for multienzyme reaction schemes, a two-enzyme method involving nucleoside phosphorylase (NP) and xanthine oxidase (XO) was developed by Hwang and Cha[13].

$$\text{Phosphate} + \text{inosine} \xrightarrow[Mg^{2+}]{NP} \text{ribose-1-phosphate} + \text{hypoxanthine}$$

$$\text{Hypoxanthine} + 2\,H_2O + 2\,O_2 \xrightarrow{XO} \text{uric acid} + 2\,H_2O_2$$

The concentration of phosphate was obtained by measuring the absorbance of uric acid at 293 nm. The major problems with this coupled enzyme system were the excessive reaction time of 60 min and the large blank value of almost 0.4 absorbance units. The latter drawback was probably due to various organic compounds in the sample absorbing in the UV region. In addition, use of this method for the determination of phosphate in a variety of matrices has not been demonstrated.

To eliminate the problems associated with the enzymatic methods but to keep the selectivity, we have investigated the possibility of using only the NP-catalyzed reaction to determine phosphate. The inosine and hypoxanthine were separated by reversed-phase high-performance liquid chromatography (HPLC) and the amount of hypoxanthine produced was related to the phosphate concentration. Comparison of this HPLC method for the determination of orthophosphate in complex matrices such as polyphosphates, detergents, and biological systems with the colorimetric molybdate-blue method without sample cleanup was also investigated.

EXPERIMENTAL

Apparatus

The HPLC system was assembled of various components as follows. The high-pressure Milton Roy Model 396 pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) was modified with a tee-configuration pulse dampener consisting of a 52 cm × 4.6 mm stainless-steel tube. The injector, equipped with a 130 μl sample loop, was a Rheodyne Model 7010 with a loop filler port Model 7011 (Rheodyne, Berkeley, CA, U.S.A.). The separations were acquired with a 5 cm × 4.6 mm I.D. precolumn and a 15 cm × 4.6 mm I.D. working column. Both stainless-steel columns were packed with 10 μm LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.) using a Model 10-600-30 pneumatic amplifier pump (SC Hydraulic Engineering, Los Angeles, CA, U.S.A.) and a high-pressure slurry packer (Alltech, Deerfield, IL, U.S.A.). An Altex Model 153 UV detector, constant wavelength (254 nm) (Altex, Berkeley, CA, U.S.A.) was used to monitor the column effluent. Peaks were recorded with a Fisher Recordal Model 5000 (Houston Instruments, Austin, TX, U.S.A.). In addition, the chromatograms were digitized by an Apply 2+ minicomputer (Apple, Cupertino, CA, U.S.A.) equipped with a 12-bit analogue-to-digital converter (Interactive Microware, State College, PA, U.S.A.).

All spectrophotometric work was performed on a Hewlett-Packard Model 8450 UV–VIS spectrophotometer equipped with a Model 9872B *x–y* plotter (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Chemicals

All water used was triply distilled. Tris(hydroxymethyl)aminomethane (Tris) was supplied by Aldrich (Milwaukee, WI, U.S.A.). Didodecyldimethylammonium bromide, trioctylpropylammonium iodide, and *m*-aminophenol were obtained from Eastman Kodak (Rochester, NY, U.S.A.). *m*-Aminophenol was recrystallized from diethylether before use. Benzalkonium chloride, inosine, nucleoside phosphorylase (orthophosphate ribosyltransferase; E.C. 2.4.2.1), phosphocreatine, fructose-6-phosphate, and pentasodium tripolyphosphate were all obtained from Sigma (St. Louis, MO, U.S.A.). All chemicals were reagent grade or the highest purity available and stored as recommended.

Procedure

Kinetic study. A reagent solution containing 0.4 units/ml of nucleoside phosphoryclase, 9.85 m*M* inosine, 3.85 m*M* of magnesium sulfate and 0.4 mg/ml *m*-aminophenol was made in a 0.1 *M* Tris buffer at pH 7.4. Samples were prepared by adding 0.15 ml of the reagent solution to 0.85 ml of a 78 ppm phosphate solution. The samples were allowed to react for various time intervals and then stopped by placing the reaction vessels into boiling water for 2 min. After cooling, the samples were injected into the HPLC system.

Optimization of enzyme concentration. A sample solution containing 54 ppm phosphate was prepared and 1.4 ml of this sample solution was mixed with various amounts of the previously described reagent solution. The total volume was brought to 2 ml with the addition of 0.1 *M* Tris buffer, pH 7.4. A constant reaction time of 30 min followed by 2 min in boiling water was used. After cooling the HPLC separations were carried out.

Calibration curve and real samples. Both standard and real samples were analyzed by the following procedure. A reagent solution of 20.7 m*M* inosine, 6.06 m*M* magnesium sulfate, 0.4 units/ml nucleoside phosphorylase and 0.4 mg/ml *m*-aminophenol was prepared in 0.1 *M* Tris buffer, pH 7.4. A 0.7-ml aliquot of this reagent solution was added to a 2-ml volumetric flask, which was then filled to the mark with the sample of interest. After mixing by inversion of the flask, the solution was then allowed to react for 30 min in a constant temperature bath at 20°C. The reaction was stopped at the end of 30 min by placing the volumetric flask into boiling water for 2 min. As many as 7 samples were prepared and reacted at one time. After cooling, the samples were chomatographed.

Colorimetric method. The molybdate-blue method using L-ascorbic acid as the oxidizing agent was used as previously described[2] however all the volumes were reduced by two. A constant sample size of 3 ml was used.

Calculations. Software, similar to that of Perone[14], was written for the Apple computer to integrate the chromatographic peaks and calculate peak areas. The computer was also used to do all least squares curve fitting and statistical calculations.

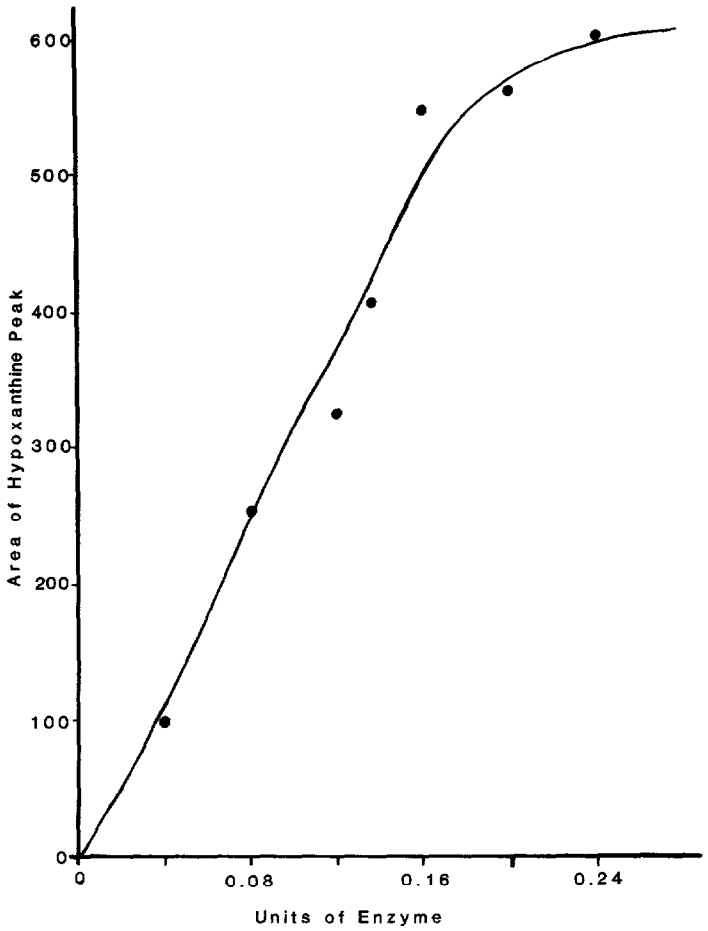

Fig. 1. Area of the hypoxanthine peak as a function of the amount of enzyme. The phosphate concentration was held constant at 38 ppm.

RESULTS AND DISCUSSION

A mobile phase of 0.1 *M* Tris buffer, pH 7.4 at a flow-rate of 1.8 ml/min was found to effectively separate inosine from hypoxanthine on the C_{18} column. To help compensate for any loss in column efficiency and maintain good accuracy, an internal standard, *m*-aminophenol, which eluted after the hypoxanthine but before the inosine, was used in the assay procedure.

The reaction conditions for nucleoside phosphorylase with respect to pH, temperature, amount of enzyme, and time were all considered for optimization. Previously, Park and Agarwal[15] have shown that the enzyme is stable throughout the temperature range of 13–40°C and has a relatively broad pH optimum between 6.5 and 8.0. Based on this information, a pH of 7.4 and a temperature of 20°C was chosen for our work. To determine the appropriate units of enzyme needed per assay, the reaction product, hypoxanthine, was measured with respect to the units of enzyme. These results, as

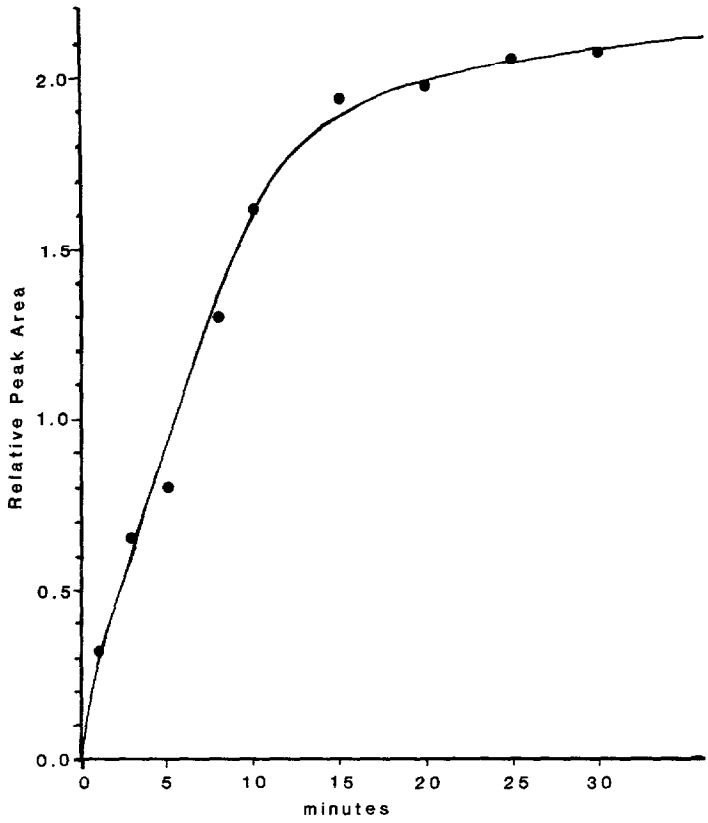

Fig. 2. Relative peak area, hypoxanthine/*m*-aminophenol, as a function of reaction time. The phosphate concentration was held constant at 66 ppm.

shown in Fig. 1, indicated the product increased linearily with the enzyme amount up to about 0.16 units. The plot also showed that the amount of product formed was relatively constant at values greater than 0.20 units. To minimize error associated with the amount of enzyme, the value of 0.28 units was used for all subsequent reactions. Fig. 2 shows the relative peak area with respect to time of the reaction. The relative peak area was defined as the area of the hypoxanthine peak divided by the area of the internal standard peak, *m*-aminophenol. As can be seen in Fig. 2, a plateau of maximum product formation occurred between 20 and 30 min. A reaction time of 30 min was used for all future samples.

Fig. 3 shows a typical chromatogram for a standard 42-ppm phosphate solution and hypoxanthine peaks resulting from various phosphate samples after reaction with the enzyme. The small peak that was seen for the blank could be attributed to residual phosphate in the enzyme. The calibration curve, shown in Fig. 4, had a slope of 0.043 $\pm$ 0.002 and an intercept of 0.124 $\pm$ 0.033 with a correlation coefficient of 0.998. Linearity up to 30 ppm was observed. Relative standard deviation of triplicate runs was 10% or less. The detection limit, twice the signal of the blank, was determined to be 1.5 ppm. Dialysis of the commercial enzyme in 0.1 *M* Tris buffer, pH 7.4, for 24 h was

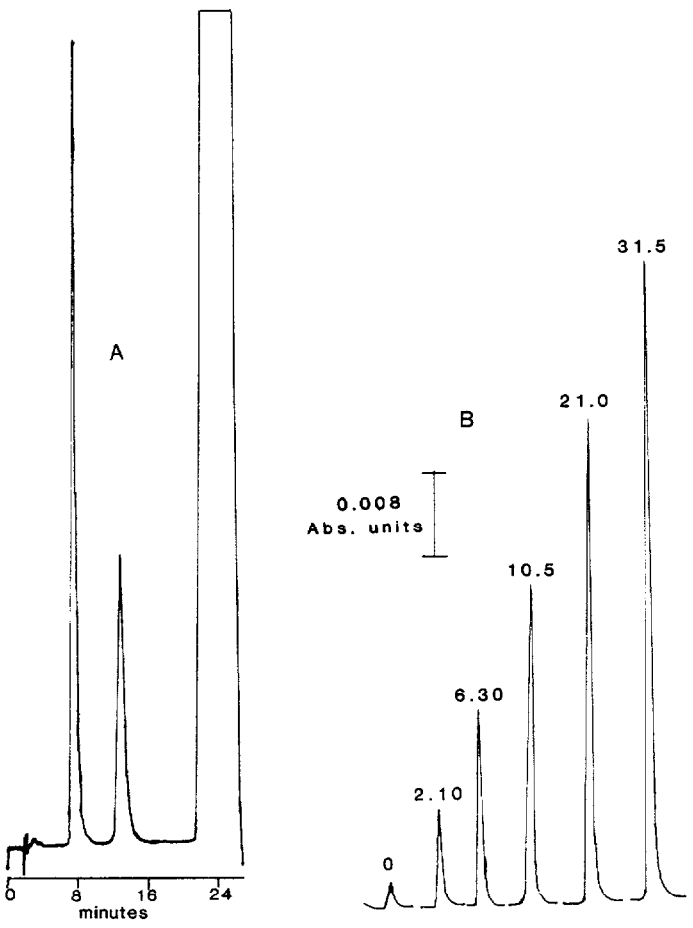

Fig. 3. (A) Sample chromatogram of a 42 ppm phosphate sample. Absorbance units full scale, a.u.f.s. = 0.08. Peaks: 1 = hypoxanthine; 2 = *m*-aminophenol; 3 = inosine. (B) Hypoxanthine peak for various phosphate concentrations in ppm (indicated above each peak).

found to reduce the blank and therefore the detection limit by at least a factor of 2. A detection limit (twice the blank) of 2.7 ppm was established from the calibration curve of the coupled NP–XO enzymatic method[13].

To characterize the accuracy of the HPLC method, a comparison was made between the enzymatic method and the molybdate-blue method. Fig. 5 is a plot of phosphate concentration given by the enzymatic HPLC method *vs.* that found by the molybdate-blue method. The line fit to these 6 points had a slope of 0.999 ± 0.046 and an intercept of 0.421 ± 1.222 with a correlation coefficient of 0.996.

To compare the selectivity of the enzymatic method with that of the molybdate-blue method, interference studies were conducted for both methods. Very little similar comparison work has been reported in the literature. The first study was the effect of tripolyphosphate in the sample (Fig. 6). The molybdate-blue method showed a positive deviation with increasing amounts of tripolyphosphate. The reason

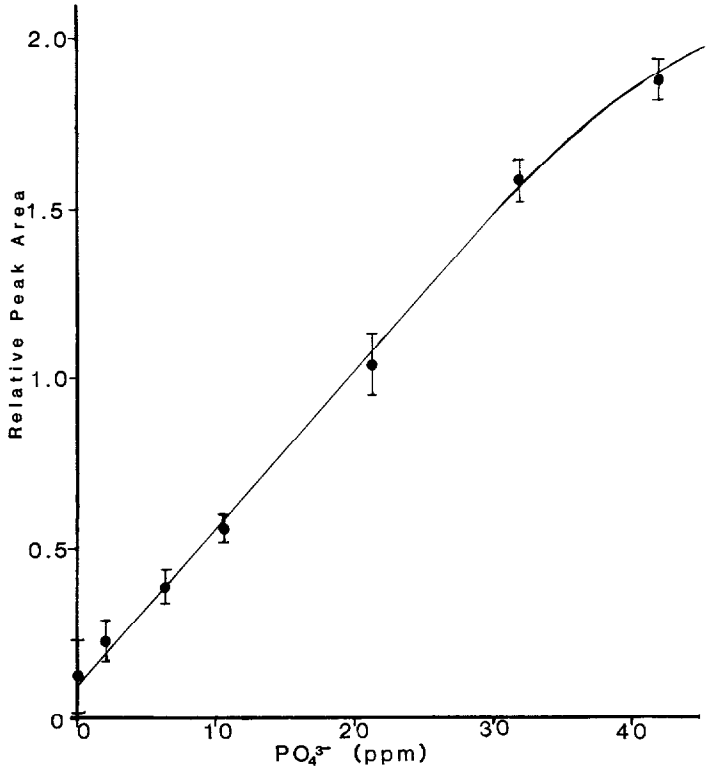

Fig. 4. Calibration curve of the enzymatic–HPLC method. Each point represents a mean of 3 determinations. Error bars represent the standard deviation.

for this positive deviation can be attributed to the acid hydrolysis of tripolyphosphate to orthophosphate[9]. The 1.5 pH value of the sulfuric acid solution used to form the phosphomolybdate was actually quite high compared to previous published methods. The enzymatic method also showed a slight positive deviation due probably because the tripolyphosphate was only practical grade in purity (90%). It is likely a major impurity could be orthophosphate.

One type of real sample that contains both tripolyphosphates and surfactants, in quantities as large as five times the orthophosphate concentration, is detergent[16]. The detergent that was used contained about 15% sodium tripolyphosphate and 1.9% of an anionic surfactant. The chromatograms of the detergent and the assayed detergent (Fig. 7) showed that phosphate was present in the sample. The quantity of phosphate estimated from a calibration curve using the enzymatic method was 5%, close to the 4% value of orthophosphate reported on the product's label. This sample could not be directly analyzed by the molybdate-blue method because of the hydrolysis of the sodium tripolyphosphate. Anionic surfactants did not appear to interfere with the molybdate-blue method at values less than 2500 ppm[7] or our enzymatic method. However, cationic surfactants such as benzalkonium chloride were found to cause a negative deviation for both methods. The interference was twice as great in the molybdate-blue method as the enzymatic method. The interference was significant with surfactant con-

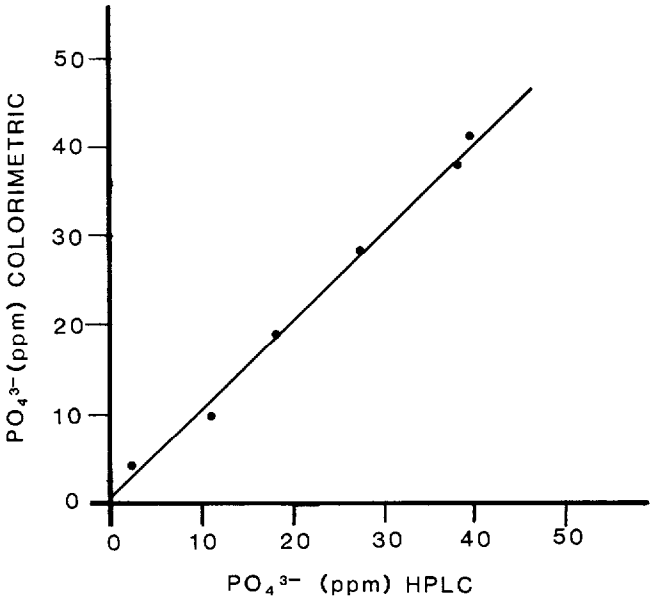

Fig. 5. Concentration of phosphate obtained by the colorimetric method *versus* the concentration obtained by the enzymatic–HPLC method.

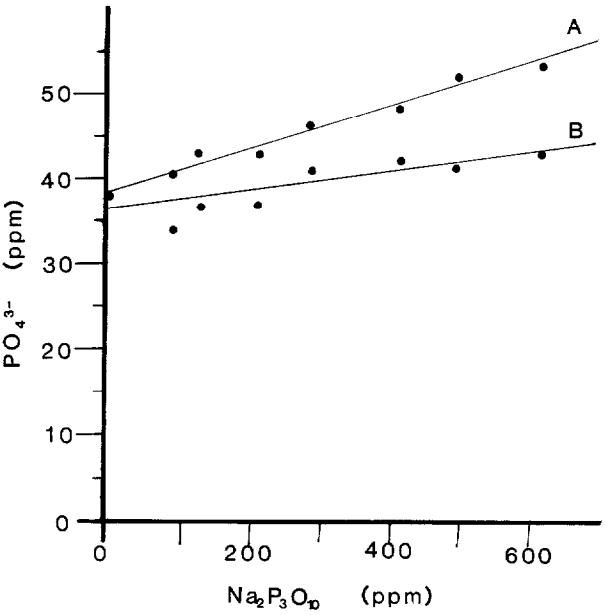

Fig. 6. Phosphate concentration obtained by the colorimetric (A) and the enzymatic–HPLC method (B) as a function of tripolyphosphate concentration. Each sample contained 38 ppm phosphate.

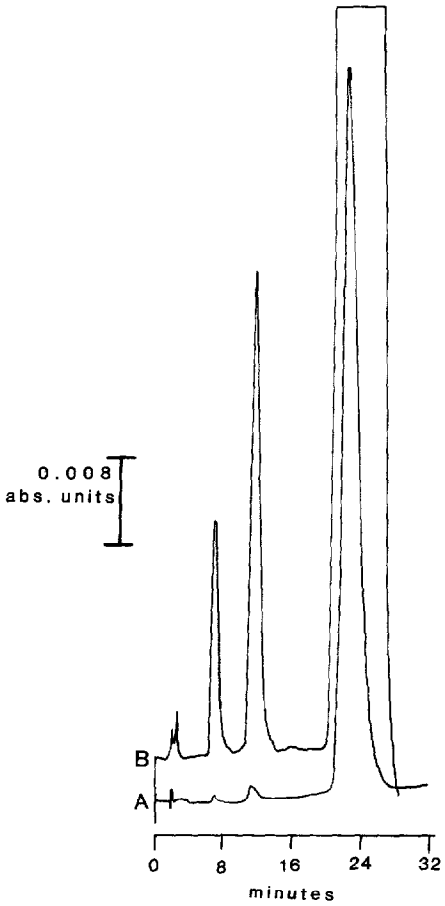

Fig. 7. Chromatograms of a real detergent sample before (A) and after (B) reaction with the enzymatic solution. a.u.f.s. = 0.08.

centrations as small as 50 ppm for both methods. Although it is known that surfactants can denature enzymes[17], the fact that only cation surfactants interfere implies ion-pairing with the anionic phosphate ion prevented the enzymatic reaction[18]. A pretreatment step involving the use of a cation-exchange column to adsorb the benzalkonium ion and allow the phosphate anion to pass through would be recommended.

In addition, many biological samples such as serum and urine are difficult matrices for phosphate determination by the molybdate-blue method without a prior cleanup step. Phosphate in urine and serum were assayed by the enzymatic method (Fig. 8) and estimated to be 1200 and 100 ppm, respectively. Both values were found to be within the acceptable range of 500 to 1500 ppm for urine and 75 to 145 ppm for serum. The small peaks co-eluting with hypoxanthine and *m*-aminophenol were considered insignificant for clinical diagnostic tests and could be subtracted out with the aid of a computer. For these biological matrices, the combination of a C_{18} precolumn and an aqueous mobile phase undoubtedly caused pretreatment of the sample by adsorbing hydrophobic substances such as protein and some aromatics. When the same

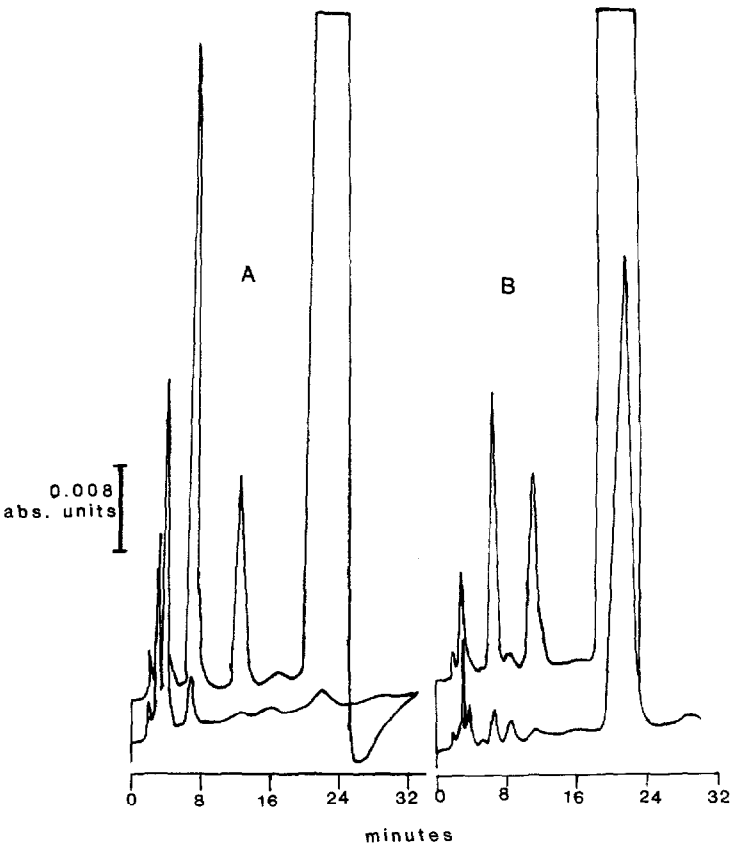

Fig. 8. Chromatograms of a diluted (× 150) urine sample before and after the enzyme reaction (A). Chromatograms of a diluted (× 10) serum sample before and after reaction (B). In both cases the lower chromatogram is before reaction. a.u.f.s. = 0.08.

samples were analyzed by the molybdate-blue method, the serum gave similar results but the urine indicated significantly higher levels of phosphate most likely caused by the matrix.

A serious interference by phosphocreatine with the molybdate-blue method is indicated in Fig. 9, graph A. The increase of absorbance with phosphocreatine was linear with a slope of about 0.114 ± 0.008. The reason for this increase in measurable phosphate was due to the acid-catalyzed release of orthophosphate from the phosphocreatine. The presence of phosphocreatine had essentially no effect on the enzymatic–HPLC method (Fig. 9, graph B). This line had a slope of only 0.008 ± 0.005. Similar studies were done with fructose-6-phosphate and no major interference was seen for either method. The standard free energy of hydrolysis for phosphocreatine and fructose-6-phosphate are −10.30 and −3.80 kcal, respectively[4], explaining the greater lability of the former compound as compared to the latter. These values also represent a fairly moderate range of free energies for phosphorylated compounds. Chemicals such as phosphoenolpyruvate having a standard free energy of −14.80 kcal

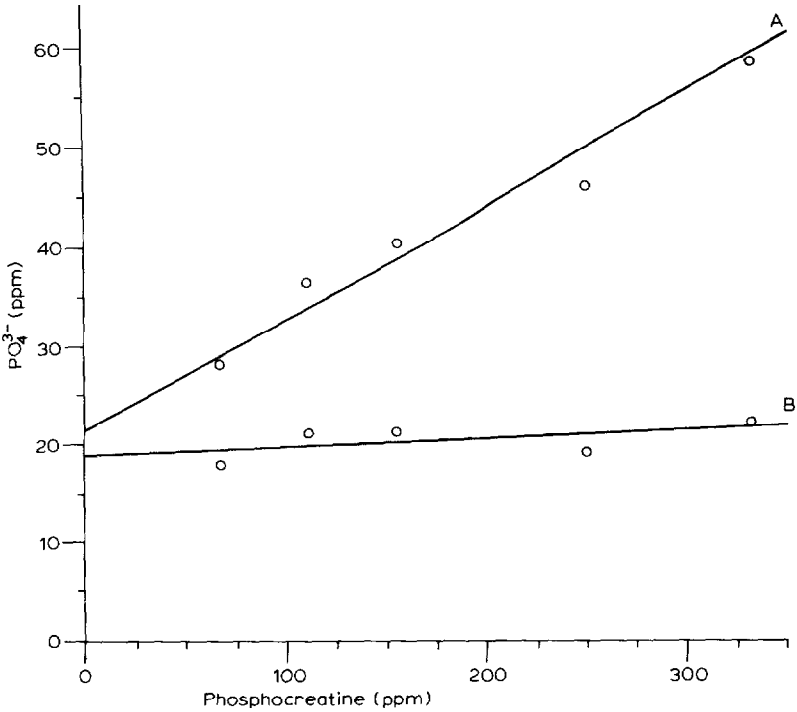

Fig. 9. Phosphate concentration obtained by colorimetric (A) and enzymatic—HPLC (B) methods as a function of phosphocreatine concentration. Each sample contained 19 ppm phosphate.

would be expected to interfere with the molybdate test while those like glucose-6-phosphate with a value of −3.30 kcal would not.

This enzymatic–HPLC method for phosphate has been shown to be generally free of matrix problems that can effect the molybdate-blue method. Besides requiring only one enzyme, our assay was found to have a lower detection limit than that reported for the NP–XO method. With precise timing, a shorter enzyme reaction time could be used without a dramatic loss in sensitivity. In addition, the presence of a valve between the precolumn and the analytical column to shunt the inosine peak to waste should allow an increase in sample throughput.

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