A New, Sensitive Determination of Phosphate

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Many published methods for the spectrophotometric determination of phosphorus have been based on the original method of Bell and Doisy (1). These methods generally depend on developing a specific blue color of the phosphomolybdate complex in the presence of reducing agents such as hydroquinone (1–3), 1,2,4-aminonaphtholsulfonic acid (4–6), and ascorbic acid (7–9).

Bartlett (6) improved the sensitivity of the widely used method of Fiske and SubbaRow (4). Heating at 100°C resulted in a 7.2-fold increase in sensitivity with a molar absorbance at 830 nm of 2.6×10^4 $M^{-1}cm^{-1}$ in comparison to $3.6 \times 10^3 \, M^{-1}cm^{-1}$ at 660 nm obtained with the other procedures.

Application of the method was limited however to the determination of total phosphorus as described by Bartlett (6): "The phosphorus heating method could not be used to assay inorganic orthophosphate in the presence of a number of phosphate esters which hydrolyzed under these conditions. The original Fiske and SubbaRow method was found most reliable for measuring inorganic phosphate when mixed with phosphate esters."

The enzymic activities of phosphatases, which release free phosphate from organic phosphate esters, are determined by measuring the liberated phosphate in the presence of the unreacted phosphate ester substrates. Phosphatases in tissue homogenates or preparations of subcellular particles may be made more accessible to substrate by adding solubilizing agents. However, application of the Fiske and SubbaRow method (4) to such enzymic assays often caused errors when Triton X-100 was used in the system. For instance Wattiaux and de Duve used 0.1% Triton X-100 in the incubation mixtures (10). They reported that "control experiments showed that the detergent was without inhibitory effect on any of the enzymes studied, but that it could interfere with the analytical procedures used in the phosphatase assays." In addition, Snoke and Nordlie (11) commented that "deoxycholate, cholate, Cetri-

mide and Triton X-100 all appear from these studies to be satisfactory activating agents for studies of the various activities of this multi-functional enzyme, although the last-mentioned compound occasionally produced mild turbidity in assay mixtures when the higher concentrations of this detergent were employed."

In checking the purity of subcellular fractions, we found it necessary to assay a variety of phosphohydrolases known to be selectively localized in different subcellular membranes (12). We noted increased turbidities in reaction mixtures compared to control samples, and wondered if the extent of the turbidity effect produced by Triton X-100 could be proportional to the concentration of the phosphomolybdate complex. If this proved to be true, the phenomenon probably could be used for the determination of inorganic phosphate.

EXPERIMENTAL

Reagents

Ammonium molybdate solution (2.5%): 2.5 gm $(NH_4)_6Mo_7O_{24}\cdot4H_2O$ (Baker analyzed reagent) was dissolved in a final volume of 100 ml 6 N H_2SO_4.

Triton X-100 solution (1%): 1 gm Triton X-100 (octylphenoxypoly-ethoxyethanol purchased from Sigma) was dissolved in distilled deionized water to give a final volume of 100 ml.

Phosphate standard solution (0.1 M): 13.8 gm $NaH_2PO_4\cdot H_2O$ (Mallinckrodt analytical reagent) was dissolved in distilled deionized water to give a final volume of 1 liter.

Apparatus

A Cary model 11 recording spectrophotometer and a Beckman model B spectrophotometer were used for the measurements.

Recommended Procedure

Increasing amounts of phosphate in 2.7 ml distilled deionized water were treated with 0.03 ml 1% Triton X-100. The contents of the tubes were thoroughly mixed using a Vortex mixer. Then 0.3 ml 2.5% ammonium molybdate was added to each separate tube and the contents were mixed immediately. After exactly 20 min, the absorbance of each solution was measured at 660 nm with the absorbance set at zero with a reference solution of distilled water. Correspondingly greater amounts of reagents were used for assays with final volumes of 6 and 9 ml when greater volume or lower sensitivity was desired. Increased sensitivity was

obtained by measuring the absorbance at either 510 or 550 nm, but the response was not linear over as wide a range of phosphate concentrations.

RESULTS

Figure 1 shows the absorbances from 450 to 750 nm in response to different phosphate concentrations. After examining the linearity in absorbance with respect to concentration, wavelengths of 660 nm and 510 nm were chosen for further experiments. Three procedures were standardized, ending in total volumes of 3, 6, and 9 ml for these two wavelengths. Figure 2 shows the results of these experiments. The in-
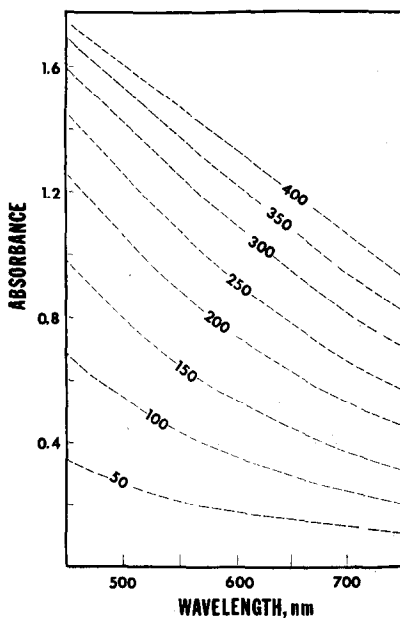

FIG. 1. Absorbance spectra for different initial phosphate concentrations. Total volume 10 ml; 0.1 ml 1% Triton X-100 added; amounts of phosphate in nmole per tube as indicated. Recording of each spectrum started at 750 nm, 8 min after adding 1 ml 2.5% ammonium molybdate, and completed at 450 nm 14 min after addition.

crease of absorbance was linear for different ranges of phosphate concentration depending on the final volume of the assay system. For instance, absorbance at 660 nm showed good linearity over a range of 30 to 230 nmoles of phosphate, with a value of 3.3×10^{-3} per nmole. The most sensitive value, 15.6×10^{-3}, was obtained at 510 nm with a 3 ml volume and was constant in the range of 10 to 50 nmoles of phosphate.
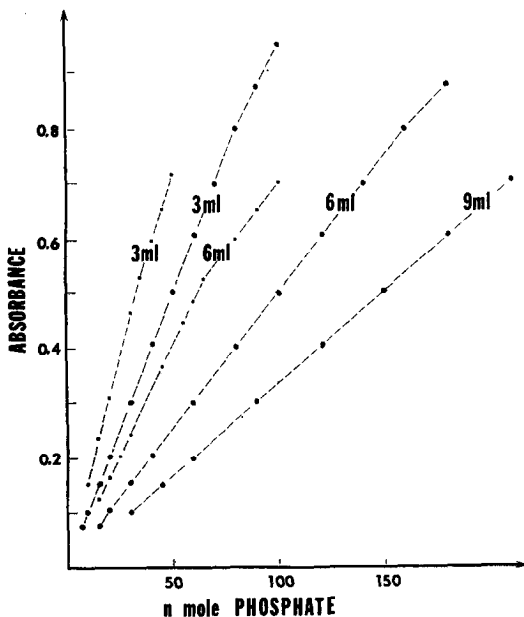
FIG. 2. Volume and wavelength changes in assays: (●) 660 nm, (■) 510 nm. Total volume as indicated. In all cases, absorbance readings made exactly 20 min after adding ammonium molybdate.

The influence of different concentrations of sulfuric acid is presented in Figure 3. The final concentration of $0.6\,N$ seemed quite satisfactory for further work and small variations above or below that level did not significantly alter the results.

The absorbancies resulting from different Triton X-100 concentrations are reported in Figure 4. Readings after different time intervals showed no difference between 25 and 45 min. The decreased absorbance after 200 min was associated with a greater flocculation of the particles. The
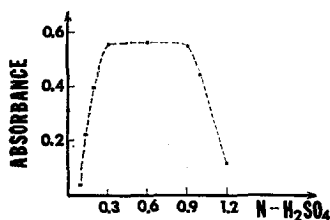

FIG. 3. Various sulfuric acid concentrations in assays. 200 nmole phosphate and 0.1 ml 1% Triton X-100 in 9.0 ml distilled deionized water containing varying concentrations of sulfuric acid were treated with 1 ml of 2.5% ammonium molybdate. Absorbance at 660 nm read after 8 min.
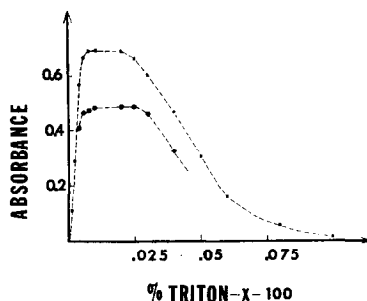
FIG. 4. Variation of Triton X-100 concentrations in assays. 200 nmole phosphate in 9.0 ml distilled deionized water containing varying amounts of Triton X-100 were treated with 1.0 ml ammonium molybdate (2.5% solution in 6 N H₂SO₄). Absorbance read after 25, 45 (■, upper curve), and 200 (●, lower curve) min.

results suggest that an optimal final concentration of 0.010 to 0.015% of Triton X-100 is desirable.

The effects of other detergents on the phosphomolybdate complex are presented in Table 1. Triton X-100 was the only material of those tested which gave a uniformly dispersed product.

The reproducibility of the method was indicated by repeating the phosphate determination three times under exactly the same conditions using a time of 8 min and seven different amounts of phosphate ranging from 100 to 400 nmoles in a final volume of 10 ml. The average absorbancies and standard errors per μmole phosphate were 2.71 $\pm$ 0.02, 2.69 $\pm$ 0.03, and 2.68 $\pm$ 0.03, respectively. The calculated error of these results is about 1% and approaches that expected in the volumetric procedures used.

Table 2 demonstrates an increase in absorbance with time for readings at different phosphate concentrations. The values increased, were then relatively constant between 15 and 25 min, and eventually dropped as

TABLE 1
Use of Other Detergents for Phosphate Assay
To 200 nmole of phosphate in 9 ml water, detergent was added to give final concentrations indicated below. Then 1 ml 2.5% ammonium molybdate was added and absorbance was read at 660 nm after exactly 10 min. ↓ indicates that a distinct sedimentation occurred.

% detergent added	Absorbance at 660 nm			
	Triton X-100	Cetrimide	Sodium laurylsulfate	Sodium deoxycholate
0.005	0.51	0.13	0	0.02
0.01	0.58	0.18↓	0	0.05↓
0.02	0.64	0.18↓	0	0.05↓
0.04	0.55	0.18↓	0	0.05↓

TABLE 2

Increase of Absorbance with Time

To varied amounts of phosphate (as indicated) and 0.1 ml Triton X-100 (1% solution in deionized water) in 9 ml water, 1 ml 2.5% ammonium molybdate was added. Absorbance at 660 nm was read after the times indicated.

Phosphate, nmoles	Absorbance at 660 nm				
	10 min	15 min	20 min	25 min	30 min
25	0.052	0.062	0.070	0.073	0.074
50	0.123	0.140	0.147	0.160	0.164
100	0.265	0.298	0.312	0.324	0.335
150	0.410	0.450	0.464	0.480	0.485
200	0.568	0.610	0.622	0.640	0.640
250	0.702	0.748	0.750	0.758	0.730
300	0.844	0.882	0.880	0.878	0.840
400	1.035	1.054	1.036	1.020	0.975

indicated earlier in Figure 4. The best time range to determine phosphate was therefore regarded to be between 15 to 25 min. For high accuracy and best reproducibility it seemed advisable to read the absorbance of each sample always at exactly the same time interval after adding ammonium molybdate.

DISCUSSION

The method described in this paper for the determination of phosphate is simple, uses wavelengths available on low-priced spectrophotometers, and reagents that are all stable at room temperature. The sensitivity is equal to or higher than that of the methods deescribed earlier, as shown in Table 3 in a comparison of the molar absorbancies. The values were calculated from the results presented by Bartlett (6).

Of the detergents tested, Triton X-100 showed a specific turbidity effect in relation to different concentrations of the phosphomolybdate complex. Sodium lauryl sulfate did not give any absorbance, cetyl trimethyl ammonium bromide (Cetrimide) precipitated the added ammonium molybdate, and sodium deoxycholate was insoluble in the acidic test solution.

TABLE 3

Comparison of Molar Absorbancies

Method	Wavelength, nm	Molar absorbance, M^{-1} cm^{-1}
Fiske and SubbaRow (4)	660	3.6×10^3
Bartlett (6)	830	2.6×10^4
New method	660	3.0×10^4
	510	4.7×10^4

The high sensitivity of the present method is available without the heating at 100°C required in the method of Bartlett (6). Assaying at room temperature may be important when determining free phosphate in the presence of organic phosphate esters. For instance, the following phosphohydrolases are used to determine the success of subcellular fractionations (10): glucose-6-phosphatase (EC 3.1.3.9), 5'-nucleotidase (EC 3.1.3.5), and acid phosphomonoesterase (EC 3.1.3.2). Under the conditions of the procedure described in this paper there was no unspecific or nonenzymic release of phosphate with these substrates. Although we encountered no difficulties, controls are obviously needed whenever the method is applied to diverse biological fluids. The method has now been successfully applied in a study of the distribution of rat liver acyltransferases among subcellular fractions (12).

SUMMARY

A new method for the determination of phosphorus using Triton X-100 instead of reducing agents is more sensitive than procedures described earlier. Organic phosphate esters do not appear to interfere with the determination of free phosphate under the conditions of the assay. All reagents are stable at room temperature and the absorbance is measured at convenient wavelengths.

ACKNOWLEDGMENT

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