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SEPARATION OF PHOSPHOMOLYBDATE BY AFFINITY CHROMATOGRAPHY

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A new method of separating phosphomolybdate from phosphoric esters and anhydrides using a small column of polyvinylpolypyrrolidone is introduced. When a mixture of phosphate compounds and ammonium molybdate (1–3%, pH 3–5) is poured onto such a column, inorganic orthophosphate (Pi)[1] is selectively adsorbed onto the column material as phosphomolybdate, while other phosphate compounds, which do not react with molybdate, are drained through the column. Using radioisotopes, retention and recovery percentages were measured. At pH 3, 99.99% of Pi^{32} was retained in the column, while $97 \pm 1\%$ of ATP^{32} was recovered in the effluent. Retained Pi^{32} was eluted out later with 0.5 M ammonium hydroxide with a recovery percentage of $98 \pm 1\%$. Unlike other methods of separating phosphomolybdate, the separation was little affected by the presence of reducing agents. The use of disposable columns, which can be prepared easily, packed ahead of time, and stored for later use, makes the radioisotopes assay convenient and contamination-free.

INTRODUCTION

Various procedures have been developed for the specific separation of inorganic orthophosphate (Pi) from phosphoric esters and anhydrides. Beranblum and Chain (1) introduced a procedure of forming a complex from Pi and molybdate in an acidic aqueous solution followed by isobutanol extraction of the complex. Besides the fact that it is always unpleasant to use organic solvents, this method is time consuming and error is easily produced because of solvent droplets left in the aqueous phase. In order to solve these problems, Hagihara and Lardy (2) developed a procedure employing reversed phase chromatography to remove solvent soluble phosphomolybdate. However, the preparation of the column for this method is a very laborious procedure. Sugino and Miyoshi (3) reported that triethylamine is an effective and selective precipitant of phosphomolybdate.

[1] Abbreviations: AMB, ammonium molybdate; BSA, bovine serum albumin; DTT, dithiothreitol; MES, 2-(*N*-morpholino)ethanesulfonic acid; PCA, perchloric acid, Pi, inorganic orthophosphate; PVP, polyvinylpyrrolidone; PVPP, polyvinylpolypyrrolidone; TCA, trichloroacetic acid.

Common drawbacks of these three methods are (a) low pH (0.8–1.8) is required for an efficient separation, which tends to decompose acid-labile phosphate compounds in the presence of molybdate (4); (b) separation is strongly inhibited by reducing reagents; (c) the effect of reducing reagents could be overcome by the addition of bromine (2), but it is unpleasant to use bromine because of its toxic vapor.

In the course of developing a new assay procedure for Pi (5,6) using polyvinylpyrrolidone (PVP), which catalyzes the formation of phosphomolybdate from Pi and molybdate, we decided to use an immobilized form of PVP to adsorb phosphomolybdate. Since PVP is water soluble, we examined the possibility of using its cross-linked form, polyvinylpolypyrrolidone (PVPP), for the adsorption chromatography of phosphomolybdate. It was found that PVPP has a high affinity for phosphomolybdate, and that the use of a small column of PVPP permits almost complete separation of phosphomolybdate from other phosphate compounds. This method has numerous advantages over previously described methods: (a) the use of small columns makes the entire procedure easy; (b) for work with radioisotopes, the use of the disposable columns especially minimizes the danger of contamination; (c) this method is effective to pH 5, which considerably reduces the decomposition of labile phosphate compounds; (d) there is very little effect from various interfering agents; and (e) reducing reagents have little effect on the separation, thereby eliminating the need for toxic bromine, which is employed to oxidize the reduced form of phosphomolybdate (2).

MATERIALS AND METHODS

Chemicals

PVPP [the structure of the monomeric unit is shown in Fig. 1(A)], L-ascorbic acid, BSA, L-cysteine, glutathione (reduced form), and sodium tungstate were purchased from the Sigma Chemical Company (St. Louis, Missouri). Ammonium molybdate (AMB) was purchased from Fisher Scientific (Pittsburgh, Pennsylvania). DTT and MES were purchased from Calbiochem (La Jolla, California). Radioactive ATP^{32} and Pi^{32} were purchased from New England Nuclear (Boston, Massachusetts). Contaminating pyrophosphate in Pi^{32} was decomposed by boiling Pi in 1 N HCl (2,3). LSC-Complete (PPO-POPOP-toluene cocktail for the scintillation counting) was purchased from Yorktown Research (South Hackensack, New Jersey). BBS-3 (solubilizer for the scintillation counting) was purchased from Beckman Instruments, Inc. (Irvine, California).

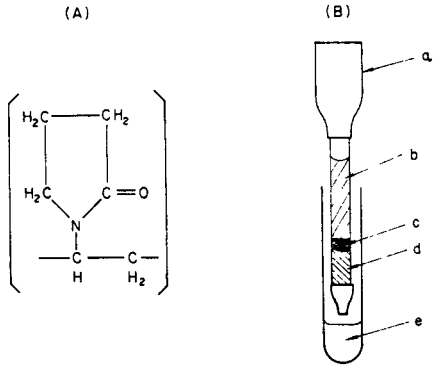

FIG. 1. (A) Structure of the monomeric unit of PVPP. (B) Separation of phosphomolybdate by a column of PVPP. Illustrations: *a*, column; *b*, solution of phosphomolybdate with slight yellow color; *c* a clear yellow band of phosphomolybdate adsorbed onto the column material (PVPP); *d*, PVPP column; *e*, colorless effluent.

Column and Packing

We used 0.7 cm (ID)×10 cm glass barrel Econo-Columns from Bio-Rad Laboratories (Richmond, California). PVPP was washed with 0.5 M HCl, then washed several times with water and dried overnight at room temperature. The dry washed PVPP was ground down with a pestle and mortar and 0.25–0.5 g was packed in the column to a height of 1–2 cm. The flow rate of solution through the column was usually 3–5 ml per h. This varied depending on the height and tightness of the column. The columns can be stored in the dry form indefinitely.

Measurement of Elution Percentage

We added 0.5 ml of 5 mM Pi^{32} with 1.5 ml of 5% AMB and 0.5 ml of acid (TCA, PCA, formic acid, or HCl; concentration depending upon the desired pH). From this separation mixture, an aliquot (0.02–0.2 ml) was transferred into a scintillation vial and added with a scintillation cocktail composed of 8 ml LSC-Complete and 2 ml aqueous solubilizer (1 part BBS-3 and 2 parts toluene), and the radioactivity was measured by a Beckman Scintillation Counter Model LS-150. The radioactivity of samples was 400,000 to 900,000 cpm. The remaining 2.3 ml of the separation mixture was poured onto the column immediately. After this solution

drained through, another aliquot (same volume as the previous aliquot) was taken out from the effluent and the radioactivity measured in the same way. The elution percentage was calculated from the ratio between the two radioactivities.

RESULTS AND DISCUSSION

Effect of pH on the Separation

The first problem to be solved in developing this method was to find the relation between pH and the separation of Pi. A range of pH from 1 to 7 was studied. TCA and PCA were used from pH 1 to 5, and MES buffer was used from pH 5.5 to 7. When pH of the separation mixture was below 5, a clear yellow band was observed in the top region of the PVPP column [Fig. 1(B)], indicating that the phosphomolybdate was adsorbed onto the column material. On the other hand, for pH above 5.5, no color was noticed at this region of the column. In agreement with these observations, the relation between pH and the elution percentage (Fig. 2) clearly shows that the separation is excellent (99.90–99.99%) below pH 5, but is very poor above 5.5. It was also found that the types of acid make no difference in the efficiency of the separation. Therefore, for the following experiments, pH of the separation was adjusted either by TCA or formate–HCl buffer. Although a greater separation was obtained with a lower pH, we have chosen pH 3 as a standard condition in order to reduce the decomposition of labile phosphate compounds (4).

Effect of Concentration of Ammonium Molybdate

Figure 3 shows the effect of the concentration of AMB on the separation of Pi. For 1 mM Pi, the concentration of AMB above 0.8% (7.2 mM) was required to produce the separation of 99.99%. For 4 mM Pi, 3% AMB (27 mM) was found to be needed to accomplish a similar separation. Of several AMB reagents distributed by different companies, the one from Fisher Scientific seems to give the best separation.

Effect of Interfering Reagents

One of the biggest problems in other separation methods is that the separation becomes very poor when phosphomolybdate is reduced (2). Since phosphorylation experiments using mitochondria or chloroplasts normally contain a variety of reducing agents, this poses a serious problem in

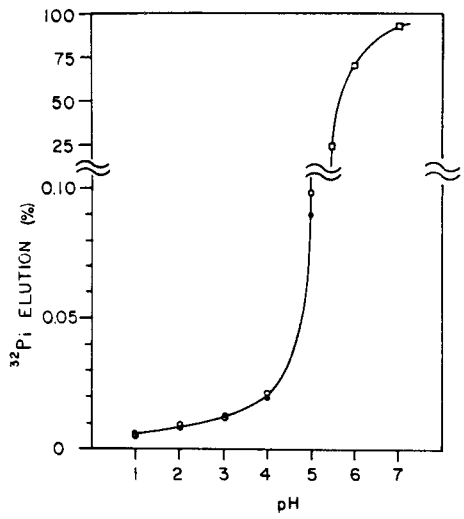

FIG. 2. Relation between the elution percentage of Pi and pH. Final concentrations of the chemicals in the separation mixture (volume, 2.3 ml) are 1 mM Pi^{32}, 1% AMB, and acid (or buffer) to give the desired pH. Symbols: filled circles, TCA; open circles, PCA; squares, 50 mM MES buffer. The dry height of the PVPP column is 2 cm; temperature, 4°C.

these studies. At present, use of bromine as suggested by Hagihara and Lardy (2) is the best way to cope with this problem, but bromine is unpleasant to use because it is volatile and the vapor is toxic. One of the advantages of the present method is that the separation is almost insensitive to normally used concentrations of reducing reagents. Even with 10 mM DTT or 30 mM ascorbic acid in the sample, which completely reduces phosphomolybdate and makes it a dark green-blue color, the separation of Pi above 99.93% was still obtained (Table 1). The effect of salt concentration is also minimal (Table 1).

Separation of ATP^{32} from Pi

In order to measure the recovery of ATP in this method, the sample containing 50 μM ATP^{32} and 1 mM Pi (nonradioactive) was added with AMB and acid (total volume 2.5 ml, temperature 4°C), and applied to a PVPP column. The recovery of ATP^{32} was 97 ± 1% following washing the column with a 5.0 ml washing solution containing AMB and acid. The

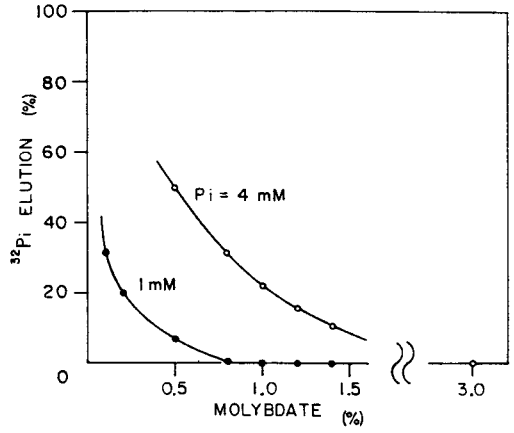

FIG. 3. Relation between the elution percentage of Pi^{32} and AMB concentration. TCA is used to adjust pH of the separation mixture to 3.0. Final concentrations of Pi and AMB in the separation mixture are shown in the figure. Filled circles, 1 mM Pi; open circles, 4 mM Pi; temperature, 4°C.

TABLE 1. **Effect of Interfering Reagents on the Elution Percentage[a] of Pi^{32}**

Interfering reagents[b]	Concentration in the sample					
	0	0.5 mM	10 mM	30 mM	0.3 M	1 M
Reductants[c]						
DTT	0.011	0.015	0.074	—	—	—
Ascorbic acid	0.011	0.011	0.012	0.021	—	—
Cysteine	0.011	0.011	0.013	—	—	—
Glutathione (reduced form)	0.011	0.011	0.011	—	—	—
Salt						
NaCl	0.011	—	—	—	0.016	0.039

[a] The separation mixture contains 1 mM Pi^{32} and 1% AMB. The pH of the separation mixture is adjusted to 3.0 by TCA. 2.3 ml of the separation mixture is applied onto the PVPP column (dry height 2 cm).
[b] 0.5 ml sample containing 5 mM Pi^{32} and the interfering reagent (the concentration as listed in the table) is mixed with AMB and acid to make the total volume of the separation mixture 2.5 ml.
[c] All reductants were used immediately after being dissolved into water.

sample containing 50 μM ATP (nonradioactive) and 1 mM Pi^{32} was handled the same way, and it was found that the amount of Pi^{32} washed out by the 5.0 ml washing solution was less than 0.002%.

Deproteinization

The effect of proteins was studied by using bovine serum albumin (BSA) as an example. If the sample contained 10 mg/ml of BSA, precipitates formed when AMB and acid were added, and the precipitates deposited on top of the PVPP column. The deposits did not interfere with the retention of Pi^{32}, but lowered the recovery of ATP^{32} to about 80%, probably due to the absorption. When a sample containing 10 mg/ml of BSA was deproteinized in TCA, mixed with AMB, and then applied to the column, the recovery was improved, but was still around 90%. It seems that a small amount of protein, which was not effectively deproteinized by TCA, was absorbed into the column and inhibited the complete washout of ATP^{32}. When such a sample was deproteinized in formate–HCl buffer (pH 3.0) containing 1% sodium tungstate (7), the deproteinization was improved to such a degree that both the retention of Pi and the recovery of ATP were completely unaffected.

Elution of Inorganic Phosphate

As seen in Fig. 2, the retention of Pi^{32} becomes abruptly poor when the pH of the separation mixture is raised above 5.5. This suggests that the retained Pi^{32} could be released from the column by raising the pH by an alkaline solution. Indeed, this is the case. It was found that the retained Pi^{32} could be eluted out by 5.0 ml of 0.5 M ammonium hydroxide with a recovery of $98 \pm 1\%$.

Separation Mechanism

The mechanism of separation of phosphomolybdate is unknown. It is probable that PVPP separates phosphomolybdate through aqueous–organic partitioning, because (a) phosphomolybdate is known to have a high partition coefficient to an organic phase (1), and (b) PVPP is known to adsorb aromatic compounds such as polyphenols (8). However, as shown in Table 1, phosphomolybdate can be adsorbed onto PVPP in the presence of a large amount of reducing reagents, which are known strongly to reduce the affinity of phosphomolybdate to an organic phase (2). Therefore, the aqueous–organic partitioning is not the only mechanism involved in the present method. It is quite possible that insoluble PVPP still maintains the catalytic

activity that noncross linked, soluble PVP demonstrated to form phosphomolybdate from Pi and molybdate. If this is the case, the separation mechanism is close to that of an affinity chromatography. It was found that PVPP has a weak cation-exchange property (9). Therefore, this property may also be involved in the separation.

STANDARD PROCEDURE

Solutions

1. Formate–tungstate buffer: 2% sodium tungstate, 250 mM formic acid, and enough HCl to bring the pH of the separation mixture to 3.0. (The concentration of HCl should be approximately 220 mM, but it should be increased when the sample contains a large amount of buffer.)
2. Molybdate solution: 5 g ammonium molybdate (Fisher Scientific) is dissolved in 100 ml water. Then the solution is filtered through no. 2 filter paper and stored in a plastic bottle at room temperature.
3. Washing solution: A mixture of one part water, one part formate–tungstate buffer, and three parts molybdate solution.
4. Eluting solution: 0.5 M ammonium hydroxide.

Separation

All separation procedures should be carried out below 4°C. A PVPP column is packed (height, 1 cm) and is prewashed by 2.5 ml of the washing solution. One part of the sample (the concentration of Pi^{32} should be less than 5 mM) is mixed with one part of the formate–tungstate buffer. After centrifugation, 1 ml of the supernatant is taken out and mixed with 1.5 ml of molybdate solution to form the separation mixture. After taking an aliquot (volume, 0.02 to 0.2 ml) from the separation mixture for the radioactivity measurement, the remainder of the separation mixture is applied to the column immediately, and the effluent is collected. When the solution drains through, 5 ml of the washing solution is added to the column and the effluent is collected. More than 99.98% of Pi^{32} is retained in the column, while phosphoric esters and anhydrides are almost completely recovered in the effluents ($97 \pm 1\%$).

Elution of Pi

After the washing is finished, retained Pi^{32} is eluted by 5 ml of the eluting solution with a recovery of $98 \pm 1\%$.

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