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Note

The anomalous behaviour of diphosphate anions in ion-exchange chromatography

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Ion-exchange chromatography is an important means of separating a series of phosphorus oxoanions[1]. Linear oligophosphates and lower phosphorus oxoanions have been separated by use of an exponential gradient-elution technique. In this laboratory, however, anomalous behaviour was observed in the anion-exchange chromatographic separation of linear oligophosphates. Hoff[2] found that orthophosphate anions are eluted from a Dowex 1-X8 resin column, giving two peaks for a sample solution at pH 4.7 and one peak for that at pH 8.0. He concluded that this chromatographic behaviour is based on the presence of the anions in different ionic states, *i.e.*, $H_2PO_4^-$ and HPO_4^{2-}. It is difficult to accept this explanation by reason of the rapid proton exchange between these anions and surrounding water molecules.

The present anomalous behaviour differs from the "carrier effect" that was observed in the separation of recoil ^{32}P oxoanions produced in neutron-irradiated solid inorganic phosphorus compounds[3,4]. The present authors examined the chromatographic behaviour of diphosphate anions and found that the anomalous effect was caused by the hydrolysis of diphosphate to give orthophosphate during the chromatographic run. The addition of EDTA to the eluent, however, is effective in securing the normal chromatographic behaviour of diphosphate anions.

EXPERIMENTAL

Reagents

Guaranteed-reagent grade tetrasodium diphosphate was used without further purification. Disodium dihydrogen diphosphate was prepared by suitably adjusting the acidity of an aqueous solution of tetrasodium diphosphate with acetic acid and recrystallizing by the addition of ethanol to the solution[5]. Sample solutions of these diphosphates were prepared freshly for each chromatographic run. Guaranteed-reagent grade sodium chloride was used without further purification and each eluent

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was prepared by dissolving a calculated amount of it in distilled water. The anion-exchange resin was Bio-Rad AG 1-X8, 100–200 mesh, chloride form.

Elution procedure

The resin was successively treated with adequate amounts of 3 and 0.5 *M* hydrochloric acid and distilled water. A gradient-elution and a simple elution method were employed. In the former method, a chromatographic column of 1.3 cm I.D. was filled with the resin to a height of 70 cm, and to a mixing bottle and a reservoir were added 750 ml of 0.12 *M* sodium chloride and 1000 ml of 0.35 *M* sodium chloride, respectively. In some instances, EDTA or sodium hydrogen carbonate was added to the eluent solution. In the simple elution method, a column of 1.0 cm I.D. was filled with the resin to a height of 40 cm and 0.2 *M* sodium chloride was used as eluent. After conditioning the resin bed with 0.12 *M* sodium chloride for the gradient elution and with 0.2 *M* sodium chloride for the simple elution, 0.5 or 1 ml of an aqueous solution of diphosphate (0.1–0.2 mg P/ml) was loaded on top of the resin bed. The flow-rate was kept constant in each elution, and unless otherwise indicated, was about 50 ml/h. The effluent was collected in 10-g fractions with an automatic fraction collector of the weight type.

Determination of phosphate content in the effluent fractions

A 1-ml volume of a molybdenum(V)–molybdenum(VI) reagent[6] was added to each test tube containing a fraction of the effluent, and the test tubes were heated at 95° for 1 h in a water bath. After adjusting the volume of the solution to 20 ml with distilled water, the absorbance was measured at 800 nm with a Hitachi-101 spectrophotometer.

Determination of orthophosphate in the presence of diphosphate

A solvent-extraction method[7] was applied to the determination of the orthophosphate content in a mixed solution of orthophosphate and diphosphate; 2 ml of 1% ammonium molybdate, 2 ml of 0.8 *M* sulphuric acid and 10 ml of isobutanol were placed in a stoppered test tube and a sample solution was added. After adjusting the volume of the solution to 30 ml with distilled water, the test tube was shaken vigorously for 1 min and allowed to stand to permit the separation of the organic and aqueous phases. To a 10-ml calibrated flask, 4 ml of the organic phase were transferred, 2 ml of 1% ascorbic acid and 2 ml of ethanol were added and the flask was allowed to stand in a thermostat at 40° for 30 min. The blue colour of reduced molybdophosphate was developed by this treatment. After cooling and adjusting the volume of the solution to 10 ml, the absorbance was measured at 800 nm.

Identification of phosphate species in the effluent

The chemical species of phosphate anions in the effluent were analyzed by the two methods described below.

Method I. Orthophosphate and the sum of ortho- and diphosphates contained in each fraction of the effluent were determined according to the methods described in the preceding sections.

Method II. The fractions of an anomalous and a normal portion of the effluent, *X* and *Y* in the elution diagram (see Fig. 4), were collected individually, concentrated

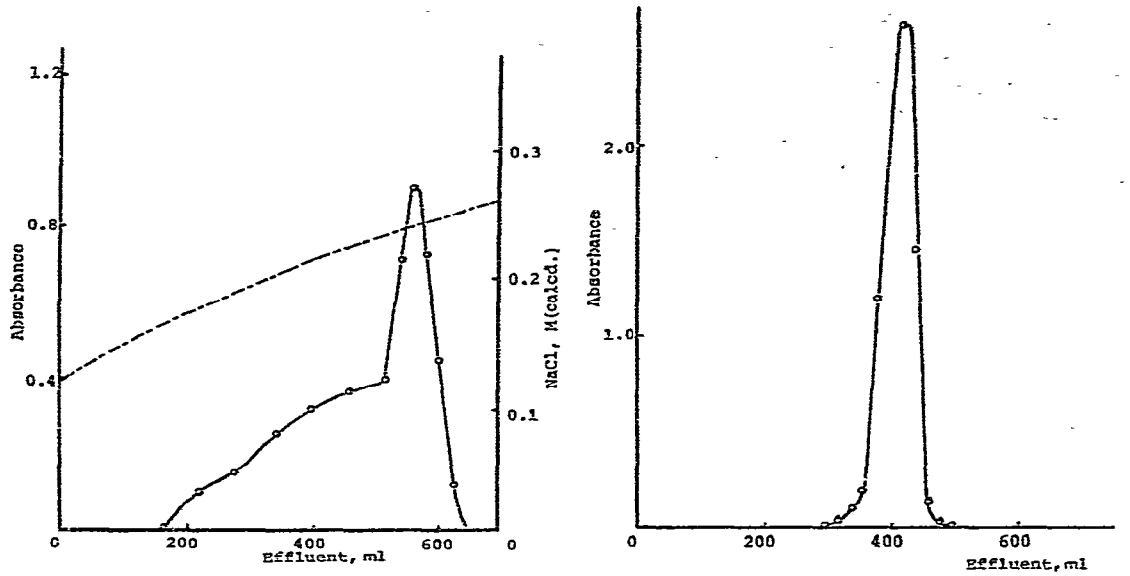

Fig. 1. Anomaly in the chromatogram of $Na_4P_2O_7$. Gradient elution; eluent, 0.12–0.35 *M* NaCl. ○—○, Elution curve; —·—·—, concentration of eluent.

Fig. 2. Effect of addition of EDTA to the eluent. Sample, $Na_4P_2O_7$; gradient elution; eluent, 0.12–0.35 *M* NaCl + $5.0 \cdot 10^{-3}$ *M* EDTA.

to a volume of 5 ml under reduced pressure at room temperature, desalted using gel chromatography (Sephadex G-10, column dimensions 3 × 50 cm), and concentrated further to 2 ml under reduced pressure at room temperature. These solutions were chromatographed by the gradient-elution method.

RESULTS AND DISCUSSION

Appearance of the anomaly in an elution diagram and its removal

Fig. 1 shows a typical elution diagram of tetrasodium diphosphate by the gradient-elution method. The anomaly in the elution diagram was observed to appear in front of the normal peak of diphosphate anions, and it also appeared when disodium dihydrogen diphosphate was eluted by the same method. A similar elution diagram was obtained by use of the eluent that was buffered with sodium hydrogen carbonate at pH 7. These results indicate that the difference between the hydrogen-ion concentrations in the sample solutions and in the eluents is not the primary factor in the occurrence of this anomaly. Furthermore, a similar anomaly was also observed when the simple elution method was employed. However, when the eluent contained EDTA, the anomaly disappeared and a normal elution diagram was obtained, as shown in Fig. 2. Consequently, in order to remove the anomaly it is sufficient to add EDTA to the eluent. Diphosphate anions were eluted with a smaller volume of the eluent in the presence of EDTA than in its absence, which suggests that EDTA anions are adsorbed competitively with diphosphate anions at the cationic sites of the resin phase.

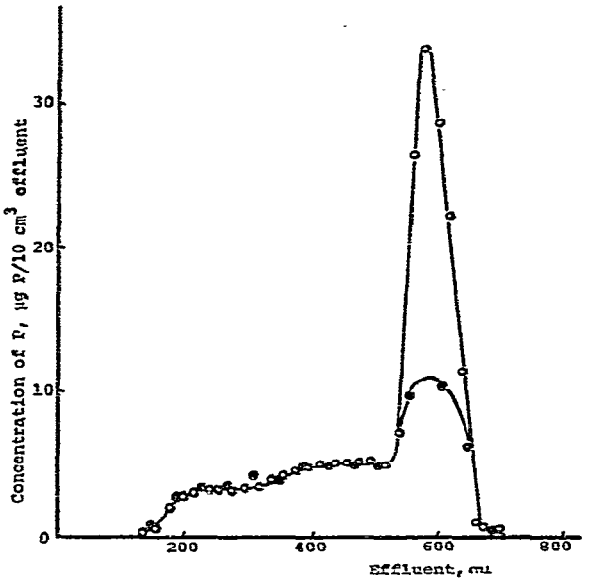

Fig. 3. Ortho- and diphosphate contents in the chromatogram of $Na_4P_2O_7$. Gradient elution; eluent, 0.12–0.35 *M* NaCl. ●—●, Orthophosphate; ○—○, ortho- + diphosphate.

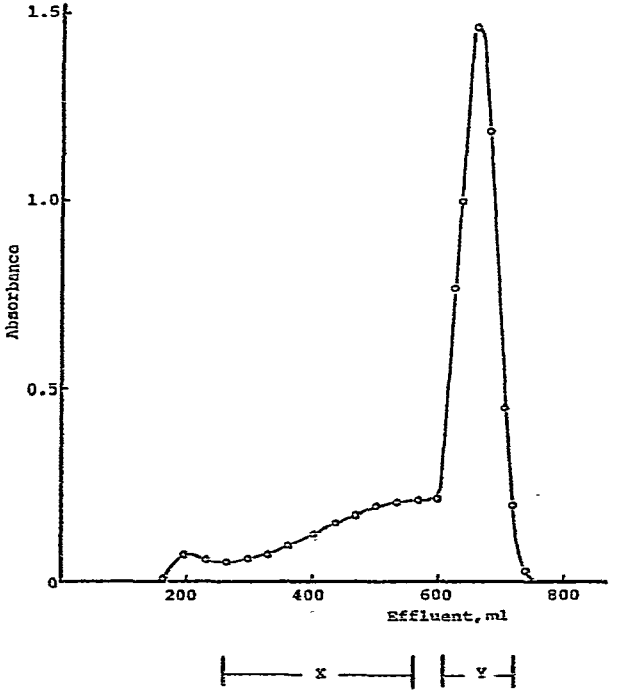

Fig. 4. Sampling of the anomalous and normal parts of the chromatogram of $Na_4P_2O_7$. Gradient elution; eluent, 0.12–0.35 *M* NaCl.

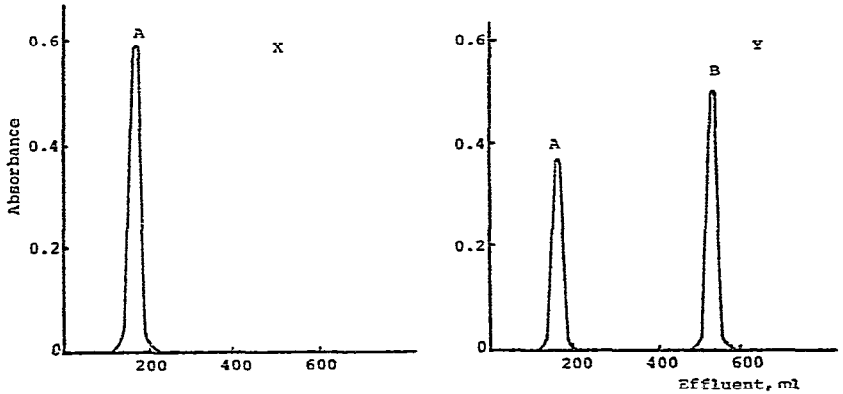

Fig. 5. Chromatograms of the phosphate species contained in fractions *X* and *Y*. Gradient elution; eluent, 0.12–0.35 *M* NaCl + $5.0 \cdot 10^{-3}$ *M* EDTA. A, orthophosphate; B, diphosphate.

Hydrolysis of diphosphate to give orthophosphate during chromatography

The appearance of the anomaly may be caused either by complex formation between diphosphate anions and metal-ion impurities contained in the eluent or by the hydrolysis of diphosphate to form orthophosphate during anion-exchange chromatography. If the former occurs, the chemical species of phosphate anions in the effluent should be retained as diphosphate anions, but if the latter, the presence of orthophosphate anions would be detected in the effluent. The determination of the chemical species of phosphate anions in the effluent is required in order to clarify the cause of the anomaly; the solvent-extraction method followed by colorimetric determination of phosphate anions was therefore carried out for this purpose. In the determination of orthophosphate by the solvent-extraction method, the absorbance of the orthophosphate extracted into the organic phase increased linearly with increase in its initial concentration in aqueous solution, and it was confirmed that the presence of diphosphate in aqueous solution did not affect the determination of orthophosphate by the present method. For the chromatographic run of tetrasodium diphosphate by the gradient-elution method, the amount of orthophosphate and the total phosphate contents were determined in each fraction of the effluent, as shown in Fig. 3. It is evident that the anomalous part of the elution diagram is composed substantially of orthophosphate anions, and, consequently, the hydrolysis of diphosphate anions during the run is suggested.

In order to confirm this result, the following further experiment was carried out. Two portions of the effluent of tetrasodium diphosphate, *X* and *Y*, were collected (Fig. 4), and treated by the concentration and desalting procedure described under Experimental. The phosphate anions contained in these two solutions were separated by gradient-elution anion-exchange chromatography, sodium chloride solution containing EDTA ($5 \cdot 10^{-3}$ *M*) being used as eluent. As shown in Fig. 5, the chromatogram of fraction *X* gave only one peak of orthophosphate anions, whereas that of fraction *Y* gave two peaks of ortho- and diphosphate anions. These results support the view that hydrolysis of diphosphate anions occurred during the above chromatographic run.

From a comparison of the results for the chromatographic separation of diphosphate with sodium chloride solution in the presence and absence of EDTA, it is concluded that the anomaly in the chromatogram is the consequence of the hydrol-

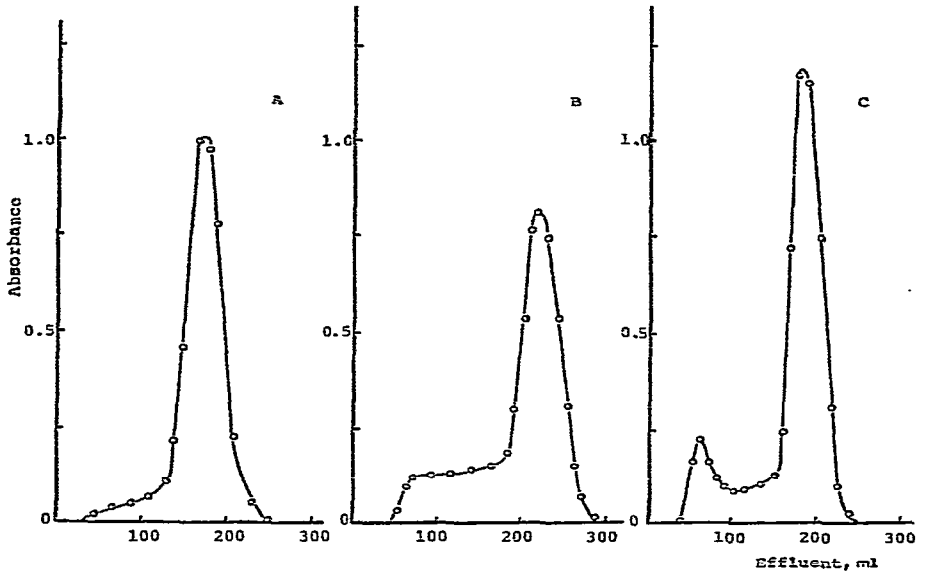

Fig. 6. Effect of the flow-rate on the chromatogram of $Na_4P_2O_7$. Simple elution; eluent, 0.20 *M* NaCl. Flow-rate (ml/min): A, 1.48; B, 0.63; C, 0.13.

ysis of diphosphate anions in the anion-exchange resin column. Further evidence to support this conclusion is the increase in the peaking of the anomalous part of the chromatogram with decrease in flow-rate of the eluent. As shown in Fig. 6, a clearly defined peak for orthophosphate anions was observed in the chromatographic run carried out with the lowest flow-rate. These observations are not consistent with knowledge concerning the hydrolysis of diphosphate anions in aqueous solution, the rate of which is expressed by first-order rate kinetics at a constant pH value. By using the data of the rate constant, $k = 1 \cdot 10^{-4}\ h^{-1}$ at pH 4.5 and 27° (ref. 8), one can calculate the mole fraction of the hydrolyzed diphosphate anions to be $1.4 \cdot 10^{-3}$ after a reaction time of 14 h. On the other hand, the data obtained in the present work indicate that the amount of diphosphate anions decreased to one half of its original amount after 13–14 h in the chromatographic runs. This unexpectedly fast hydrolysis seems to be due to catalysis by some metal-ion impurities present in the elution system employed. EDTA may act as a masking agent for such impurities by the formation of chelate compounds.

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