Journal of Chromatography, 174 (1979) 123–130

CHROM. 11,749

pH DEPENDENCE OF THE DISTRIBUTION COEFFICIENTS OF MONOMERIC OXO ANIONS OF PHOSPHORUS IN GEL CHROMATOGRAPHY WITH TIGHTLY CROSS-LINKED GELS

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(Received January 23rd, 1979)

SUMMARY

Sodium phosphinate, sodium phosphonate and potassium dihydrogen orthophosphate were chromatographed on a Sephadex G-10, G-15 or G-25 column with six kinds of eluents containing different alkali metal halides at various pH values. The distribution coefficient (K_d) on a tightly cross-linked gel column varies significantly depending on the pH of the eluent and is effectively insensitive to the kind of the eluent used. The pH profile of the K_d value is characteristic of the corresponding oxo acid of phosphorus and resembles closely its pH titration curve. The pH dependence of the K_d value of the oxo anion is discussed in terms of the distribution of the species in different dissociation states, which depends on the pH of the eluent.

INTRODUCTION

Gel chromatography is well known as a method for separating solutes depending on their size[1]. However, it has been found that a chromatogram is sometimes affected by side effects such as an electrostatic and a hydrophobic interaction when relatively small molecules or ions are chromatographed with aqueous eluents on tightly cross-linked gel columns[2,3]. The gel chromatographic behaviour of many kinds of oxo anions of phosphorus has been examined extensively[4–9] and is regarded as a case without such side effects.

Ueno *et al.*[9] found that linear polyphosphates are eluted in order of decreasing degree of condensation on an appropriately cross-linked gel column, *e.g.*, Sephadex G-25. Moreover, the separation of monomeric, dimeric and trimeric oxo acids of phosphorus was achieved on a Sephadex G-25 column, regardless of the oxidation states of the phosphorus atoms[10]. In contrast, the separation of oxo acids of phosphorus with the same degree of condensation has been considered to be impossible in spite of some attempts based on their different dissociation states[11].

It has been observed, however, that the distribution coefficients (K_d) of several kinds of organic acids, bases and ampholites are considerably dependent on the pH of the eluent when using Sephadex G-10 or porous polystyrene copolymers[12–14].

Therefore, the present work was undertaken in order to investigate the effect

of the pH of the eluent on the K_d values of salts of three monomeric oxo acids of phosphorus (phosphinate, phosphonate and orthophosphate) on tightly cross-linked gel columns (Sephadex G-10, G-15 and G-25). It was found that the K_d values of the oxo acids vary significantly depending on pH, and that the pH profiles of the K_d values on the Sephadex G-10 and G-15 columns are characteristic of the corresponding oxo acid.

EXPERIMENTAL

Sample solutions

All reagents used were of guaranteed reagent grade from Wako (Osaka, Japan) or Nakarai Chemicals (Kyoto, Japan), unless otherwise stated. Phosphorus-32, obtained from the Japan Atomic Energy Research Institute (Tokyo, Japan), was used as a tracer for orthophosphoric acid. Sample solutions were prepared by dissolving $NaH_2PO_2 \cdot H_2O$ (abbreviated to P^1), $Na_2HPO_3 \cdot 5H_2O$ (P^3) or KH_2PO_4 (P^5) in the eluents. The sample concentration was maintained constant at $2 \cdot 10^{-3}$ *M* in all chromatographic runs.

Solutions of Blue Dextran 2000 (Pharmacia, Uppsala, Sweden; 0.25%) and tritiated water (Radiochemical Centre, Amersham, Great Britain) were used as standard materials with $K_d = 0$ and 1, respectively.

Eluents

The eluents used were 0.1 *M* lithium chloride, sodium chloride, potassium chloride, caesium chloride, potassium fluoride and potassium bromide solutions of various pH. In the pH range 1.5–4, the pH values of the eluents were adjusted with hydrochloric acid, in the pH range 4–6 with 0.1 *M* acetic acid–0.1 *M* sodium acetate buffer, in the pH range 6–9 with 0.1 *M* hydrochloric acid–0.1 *M* sodium 5,5′-diethylbarbiturate buffer, and in the pH range 9–12 with sodium hydroxide or potassium hydroxide solution. The buffer concentration in the eluents was maintained at $5 \cdot 10^{-3}$ *M*.

Columns

Sephadex G-10, G-15 (dry particle size 40–120 μm) and G-25 (20–80 μm) (Pharmacia) were used as bed materials. The dry gel particles were suspended in the eluent and allowed to swell overnight. The supernatant was decanted to remove undesirable fine particles and the suspension of the gel, exposed to supersonic waves, was deaerated under reduced pressure before use. The slurry of the prepared gel was poured into a vertical glass tube with a porous polystyrene disc at the bottom (Shoei Works, Tokyo, Japan). A small disc of filter-paper was placed on top of the gel bed to settle the bed surface. The dimensions of the gel bed were 93.5 × 1.5 cm. The eluent was passed through the column until the pH of the effluent was identical with that of the eluent.

Procedure for elution

The column was water-jacketed and its temperature was kept at 21 ± 0.5° by circulating thermostated water during elution. A 1-ml volume of the sample solution was applied on the top of bed just as the last few drops of the eluent soaked into the bed. The sample solution was allowed to soak into the gel and then the top of the bed

was washed with several portions (*ca.* 0.5 ml) of the eluent. After the eluent vessel had been attached to the top of the column, the elution was allowed to proceed at a constant flow-rate of 30, 40 or 60 ml/h with a peristaltic pump (Tokyo Rikakikai, Tokyo, Japan). Within this range of flow-rates no significant alteration in the elution profile was observed. The effluent was collected in fractions of *ca.* 1 ml with an LKB Ultrorac 7000 fraction collector. Some fractions were chosen arbitrarily and their volumes were measured so that the fraction volume could be determined accurately.

Concentrations of the oxo anions of phosphorus were determined colorimetrically with molybdenum(V)–molybdenum(VI) reagent (830 nm)[15,16]. Radioactivities of ^{32}P-labelled P^5 anions were measured with a low-background automatic GM counter (Aloka LBC 22B) after the effluents had been dried in stainless-steel planchets. Blue Dextran 2000 was determined colorimetrically at 630 nm. The activities of tritiated water were measured with a liquid scintillation spectrometer (Packard 3320).

Calculation of the K_d value

The K_d value is defined by the equation

$$K_d = (V_e - V_0)/(V_t - V_0)$$

where V_t is the total volume excluding the volume of the gel matrix, V_0 is the void volume outside the gel and V_e is the elution volume of the sample[17]. The elution volumes of tritiated water and Blue Dextran 2000 were used as V_t and V_0 values, respectively.

RESULTS AND DISCUSSION

Effect of cross-linkage of the gel matrix

Fig. 1 shows the pH dependence of K_d values obtained with 0.1 *M* sodium chloride solution as the eluent. The K_d values of P^1, P^3 and P^5 on a Sephadex G-25 column are almost identical and constant over the pH range 2–10, as reported by Ueno *et al.*[8].

However, the characteristic pH profiles of the K_d values were observed on both Sephadex G-10 and G-15 columns; the K_d values of P^1, P^3 and P^5 change significantly with the pH of the eluent, and the pH profiles of the K_d values are similar to the pH titration curves of the corresponding oxo acid. This result obtained on the G-10 and G-15 columns suggests that the K_d value is dependent on the distribution of dissociated species of the oxo acids of phosphorus in the eluent, which depends on the pH of the eluent. It is also supported by the fact that the relationships between the average charges of P^1, P^3 and P^5 and pH, demonstrated in Fig. 1d, closely resemble the pH profiles of their K_d values shown in Fig. 1b and c.

On the other hand, the pH independence of the K_d values on Sephadex G-25 can be interpreted in terms of its pore size being too large to differentiate among the dissociated species of the oxo acid of phosphorus. It seems to be the result of an increase in the negative charge of the gel matrix that the K_d values, which should be constant for P^1 and P^3 anions, decrease at pH values above 11. The details are discussed later (see *pH dependence of K_d values*).

Further experiments were performed on the G-10 column only.

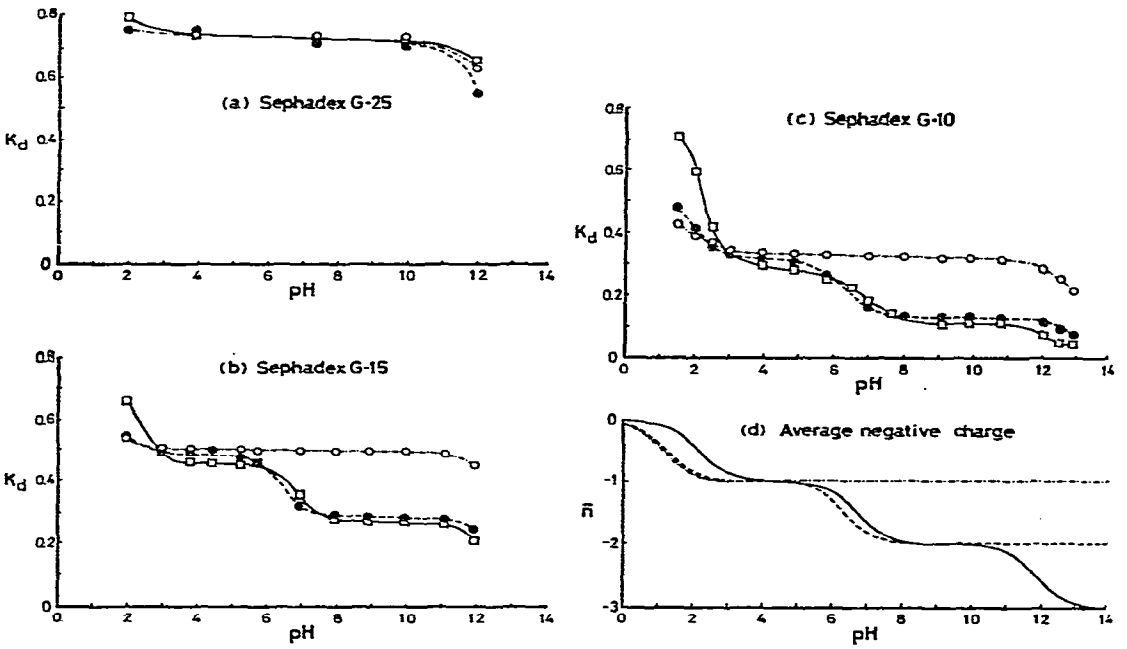

Fig. 1. pH dependence of K_d values of P^1, P^3 and P^5 on Sephadex G-10, G-15 and G-25 and their average negative charges as a function of pH. Eluent: 0.1 *M* NaCl at various pH values. $\bar{n}$ was calculated according to eqn. 5 using $pK_1 = 1.12$ for P^1, $pK_1 = 1.20$ and $pK_2 = 6.36$ for P^3 and $pK_1 = 2.10$, $pK_2 = 6.71$ and $pK_3 = 11.8$ for P^5. ○, P^1; ●, P^3; □, P^5.

Effects of buffer and ionic strength

Ueno *et al.*[8] chromatographed the sixteen oxo acids of phosphorus on a Sephadex G-25 column with 0.1 *M* potassium chloride solutions containing different sort of buffer, and found that the K_d values of all oxo anions with the eluent buffered with borate were lower than those with eluents containing phthalate–sodium hydroxide, Tris and ammonium chloride–ammonia buffers, where the K_d values were almost identical. They proposed that the buffering agent for the eluent should be selected carefully.

In order to examine the effect of buffering agents, ^{32}P-labelled P^5 was eluted with 0.1 *M* sodium chloride solution containing hydrochloric acid–sodium 5,5'-diethylbarbiturate or potassium dihydrogen orthophosphate–sodium hydroxide buffer at a concentration of $5 \cdot 10^{-3}$ *M* at pH 7 and 8. The K_d values of P^5 with the use of these two buffer solutions were 0.175 and 0.179 at pH 7 and 0.133 and 0.130 at pH 8, respectively. The values obtained at the same pH agree well, within experimental error. Hence, the buffer anions used in this study seem not to be adsorbed on the dextran gel to the extent of disturbing the gel chromatographic separation mechanism, although the effect of acetic acid–sodium acetate buffer was not examined.

The ionic strength of the eluent varied slightly from 0.10 to 0.13 because hydrochloric acid, sodium hydroxide or the buffer solution is added to adjust the pH of the eluent. The effect of the ionic strength on the K_d values of P^1, P^3 and P^5 was examined at pH 4 (adjusted with hydrochloric acid) and 10 (adjusted with sodium hydroxide solution), at which the K_d values are not affected by pH variations, a

shown in Fig. 1c. The results are shown in Fig. 2. When the ionic strength changes from 0.10 to 0.13, the K_d values increase only by 4 and 9% at pH 4 and 10, respectively. Therefore, it is concluded that the pH profiles of the K_d values shown in Fig. 1b and c are not attributable to changes in ionic strength.

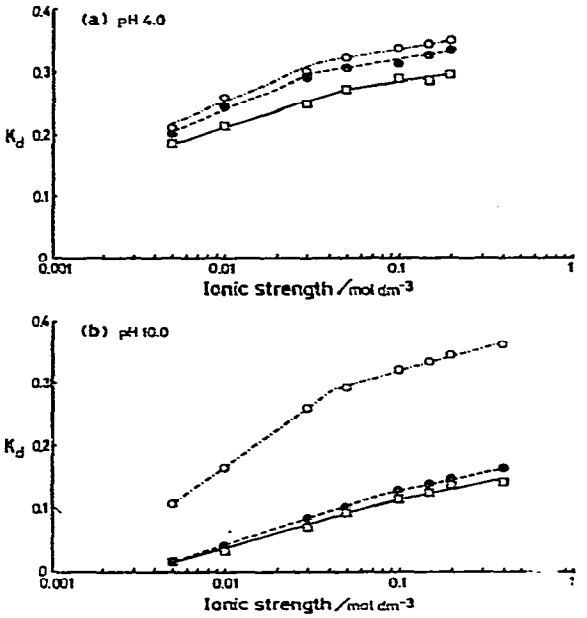

Fig. 2. Effect of ionic strength on K_d values of P^1, P^3 and P^5 at pH 4 and 10. Gel: Sephadex G-10. Eluent: NaCl solutions of various concentrations. ○, P^1; ●, P^3; □, P^5.

The tendency for K_d values to increase with increasing ionic strength could be explained by the decrease in ionic exclusion[6], but it is not yet clear whether or not the changes of slopes around $\mu = 0.03$–0.08 are due to an alteration of the solute species (*e.g.*, ion pair formation) and/or of the properties of the gel matrix.

Effect of the eluent

If the pH dependence of the K_d values demonstrated in Fig. 1b and c on the Sephadex G-10 and G-15 columns is ascribed only to the distribution of the species in the different dissociation states of the oxo acids of phosphorus in the eluent, the pH profiles of the K_d values will be independent of the type of eluent, because an eluent generally acts only to mask the electrostatic repulsion between the oxo anions and the negative charge of the gel matrix. Therefore, P^1, P^3 and P^5 were also eluted with 0.1 *M* lithium chloride, potassium chloride, caesium chloride, potassium fluoride and potassium bromide solutions at various pH values on the G-10 column. The results are shown in Fig. 3.

With potassium fluoride solution as eluent, only the pH profile of P^5 was obtained in the limited pH range from 7 to 12, because a glass column is attacked slowly by the acidic solution and colorimetry with molybdenum(V)–molybdenum(VI)

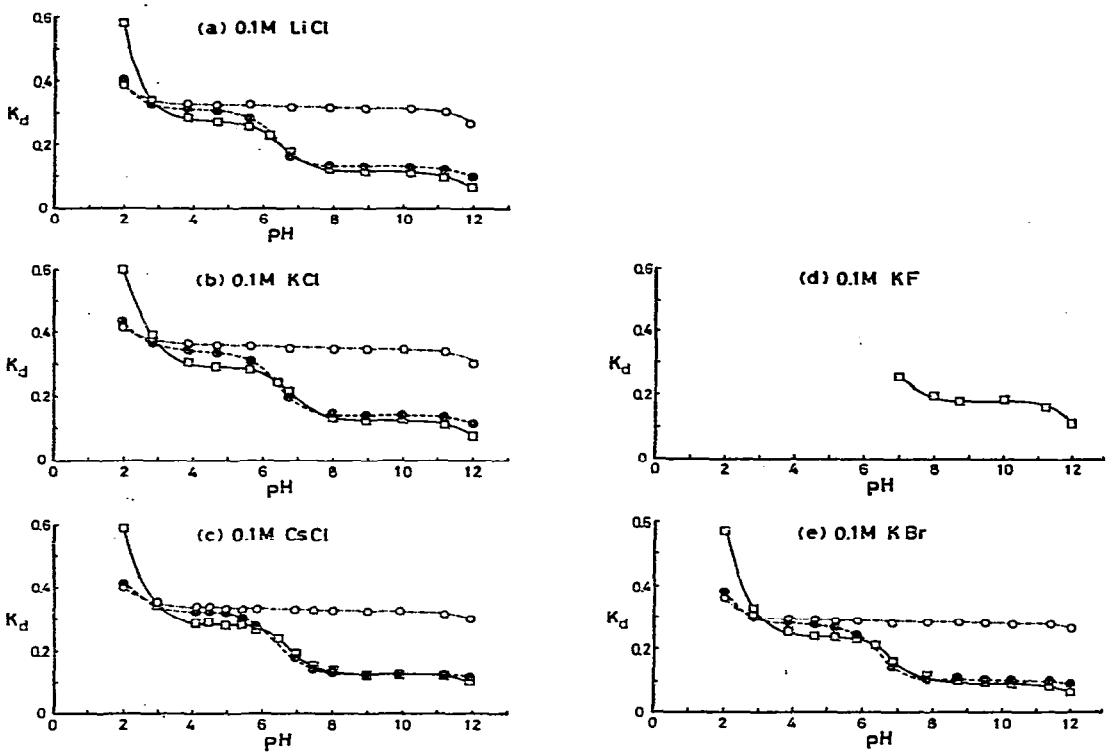

Fig. 3. Effect of eluents on pH dependence of K_d values of P^1, P^3 and P^5. Gel: Sephadex G-10. Eluent concentration: 0.1 *M*. ○, P^1; ●, P^3; □, P^5.

reagent is difficult because of interference from the silicate ions dissolved from the column[18].

Each oxo anion of phosphorus shows a characteristic pH profile that is independent of the type of eluent, although the K_d value itself is somewhat affected by the eluent.

pH dependence of K_d values

All of the pH profiles of the K_d values seem to have the same number of inflexion points as the dissociation constants of the corresponding oxo acid of phosphorus, although the pH profiles below pH 2 and above pH 12 were not obtained because of the properties of the gel matrix.

The pH values at inflexion points around pH 6–7 and the differences in K_d values between the plateaus before and after the inflexion (ΔK_d) are given in Table I. Those pH values for P^3 and P^5 on both the G-10 and the G-15 columns are independent of the type of eluent used. The average values on G-10 are 6.40 ± 0.08 for P^3 and 6.78 ± 0.10 for P^5, and these correspond with the pK_2 values (the negative logarithm of the second dissociation constant), which have been reported as 6.36 for P^3 and 6.71 for P^5 at an ionic strength of 0.1 mol/dm^3 at 25° and 20°, respectively[19]. This excellent agreement supports the following suggestions about the pH dependence of the K_d values of the oxo acids of phosphorus.

The K_d value obtained dynamically by a column method has been accepted as the parameter representing the thermodynamic equilibrium in gel chromatography, because this is usually equal to the static value obtained by a batch method[8]. According

TABLE I

pH VALUES AT INFLEXION POINTS AROUND pH 6–7 AND ΔK_d FOR P^3 AND P^5

*Eluent**	P^3		P^5	
	pH	ΔK_d	*pH*	ΔK_d
LiCl	6.32	0.16	6.70	0.14
NaCl	6.43	0.17	6.87	0.15
	6.38**	0.18**	6.85**	0.17**
KCl	6.38	0.19	6.87	0.14
CsCl	6.45	0.18	6.77	0.13
KBr	6.43	0.16	6.68	0.13

* Concentration of the eluents was 0.1 *M* in each instance.
** Values on the Sephadex G-15 column.

to the plate theory, this means that the solute equilibrates instantaneously between the liquid phase and the gel phase on a plate.

An oxo acid of phosphorus on one plate of the column dissociates so that a particular distribution of the species in different dissociation states is attained at a particular pH of the eluent. Although the liquid phase on the plate is displaced successively because of its movement, the distribution is invariable at a constant pH, because inorganic acids protonate and deprotonate very rapidly.

The overall K_d value would depend on the K_d contributions of the individual species existing in the eluent. Therefore, it should also depend on the distribution of dissociative species of the acid, which is dependent on the pH of the eluent.

The above interpretation of the pH dependence of K_d value is proved by comparing the pH profile of the K_d values with that of average negative charge ($\bar{n}$) of the oxo acid, which is obtained as follows. A *n*-protic acid of phosphorus, H_nP, dissociates successively according to the reactions

$$H_nP \rightleftharpoons H^+ + H_{n-1}P^- \quad (1)$$

$$H_{n-(i-1)}P^{(i-1)-} \rightleftharpoons H^+ + H_{n-i}P^{i-} \quad (2)$$

$$HP^{(n-1)-} \rightleftharpoons H^+ + P^{n-} \quad (3)$$

The *i*th successive dissociation constant, K_i, is represented by the equation

$$K_i = \frac{[H^+]\,[H_{n-i}P^{i-}]}{[H_{n-(i-1)}P^{(i-1)-}]} \quad (4)$$

Then $\bar{n}$ at any hydrogen ion concentration is defined by the equation

$$\bar{n} = \frac{K_1[H^+]^{n-1} + 2K_1K_2[H^+]^{n-2} + \ldots + iK_1K_2\ldots K_i[H^+]^{n-i} + \ldots + K_1K_2\ldots K_n}{[H^+]^n + K_1[H^+]^{n-1} + \ldots + K_1K_2\ldots K_i[H^+]^{n-i} + \ldots + K_1K_2\ldots K_n} \quad (5)$$

The $\bar{n}$ values of the oxo acids of phosphorus at varying pH were calculated according to eqn. 5 and are plotted as a function of pH in Fig. 1d.

The close similarity of the pH profiles of the K_d values to those of the average charges of the corresponding acids demonstrates that the pH dependence of the K_d values on Sephadex G-10 and G-15 results from the distribution of the different dissociation states, which depends on the pH of the eluent.

Moreover, the ΔK_d values on Sephadex G-10 shown in Table I are almost identical for the respective oxo acids: 0.16–0.19 for P^3 and 0.13–0.15 for P^5. This

suggests that there is a relatively small interaction among the oxo anions of phosphorus, the eluent ions and the gel matrix. It seems to be due to the different degree of cross-linkage of the gel matrix that the value for P^5 on Sephadex G-15 differs somewhat from that on G-10.

Sephadex gels, consisting of cross-linked polysaccharide, have small amounts of negative charges due to carboxylic groups fixed to the gel matrix[20]. Neddermeyer and Rogers[6] have shown that a concentration of background electrolyte higher than 0.01 *M* is sufficient to eliminate the ionic exclusion on the gel matrix. As the concentration of electrolytes in the eluents was sufficiently large in this study, the K_d values for P^1 should be kept constant over the pH range 3–11. However, the K_d values in this pH range decrease slightly on increasing the pH of the eluents, as shown in Fig. 1b and c and Fig. 3. Similar tendencies are observed over the pH ranges 3–5 and 8–10 for both P^3 and P^5. These phenomena reveal that the ionic exclusion is not completely eliminated under the experimental conditions used here. The ionic exclusion becomes more significant at pH values above 11. This fact might be attributed to an increase in negative charge due to proton dissociation from alcoholic hydroxide groups fixed to the gel matrix.

The pH profiles of K_d values for the monomeric oxo acids of phosphorus suggest that mixtures of these oxo acids will be separated into their components by elution at appropriate pH values by gel chromatography using a Sephadex G-10 or G-15 column, *i.e.*, P^5 would be isolated from P^1 and P^3 at pH $\approx$ 2 and P^1 from P^3 and P^5 at pH $\approx$ 10. A report on these separations is in preparation.

ACKNOWLEDGEMENTS

The authors thank Professor S. Ohashi and Dr. N. Yoza of Kyushu University for valuable discussions and encouragement, and Mrs. T. Suzuki, Mr. H. Inoue and Miss H. Horie for technical assistance.

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