Effects of buffer cations on chromatography of proteins on hydroxylapatite

Three effects were noted when potassium and ammonium phosphates were used as eluting buffers instead of sodium phosphate in hydroxylapatite chromatography[1] of water-extractable soybean proteins: (a) relative areas under the chromatographic peaks varied; (b) degree of resolution of chromatographic fractions differed; and (c) similar fractions eluted at dissimilar phosphate buffer concentrations.

Fig. 1 shows gradient elution diagrams for water-extractable soybean proteins in which identical gradients of sodium, ammonium, and potassium phosphate buffers were used. A 1:10 (meal:water extraction ratio) extract of defatted meal was dialyzed against phosphate buffer, pH 7.6, ionic strength 0.5, which contained 0.01 *M*

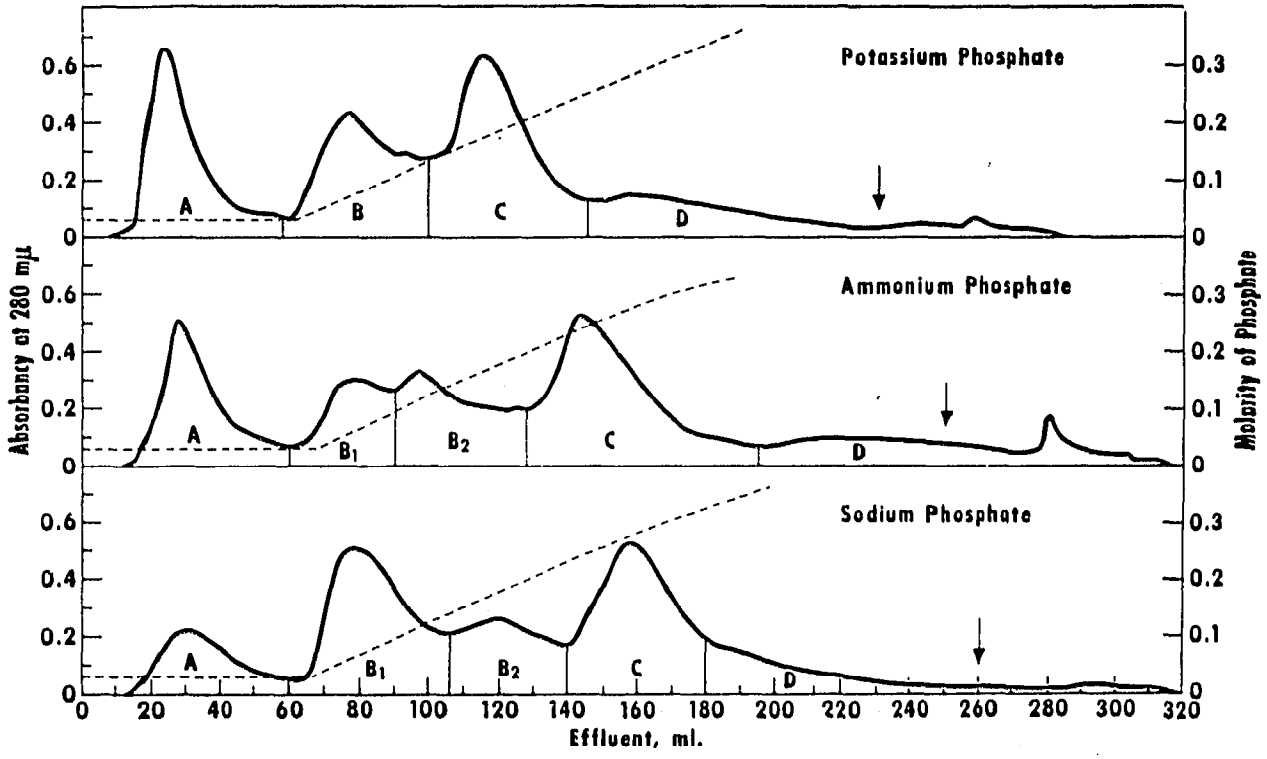

Fig. 1. Gradient elution of water-extractable soybean proteins on hydroxylapatite columns with potassium, ammonium, and sodium phosphate buffers at 25°. Vertical arrow indicates point where *M* phosphate buffer was added to complete elution. Solid curve is absorbancy at 280 mμ in 1-cm cell after 1:4 dilution. Dashed curve is elution gradient determined by conductance measurements. Respective column loads for potassium, ammonium, and sodium buffers were 193, 219, and 180 mg of protein.

mercaptoethanol[1]. The dialyzed solution was made 0.011 *M* with N-ethyl-maleimide to block protein sulfhydryl groups and to react with excess mercaptoethanol; the solution was again dialyzed against 0.03 *M* phosphate buffer, pH 7.6. After dialysis 10 ml of protein solution were placed on a 1 × 25 cm column of hydroxylapatite prepared by the method of ANACKER AND STOY[2] and pre-equilibrated with 0.03 *M* phosphate buffer, pH 7.6. Elution was begun with 320 ml of 0.03 *M* phosphate (pH 7.6) in a mixing chamber. After collecting 20 ml of effluent, a molar solution of

the corresponding buffer at pH 7.6 was added to the mixing chamber at the same rate that the buffer left it (the chamber).

Potassium phosphate eluted the proteins in four major fractions, designated A, B, C and D[1] (Fig. 1) and having the ultracentrifugal compositions shown in Table I. With ammonium phosphate, fraction A is smaller, fraction B is resolved into two parts (B_1 and B_2), and elution of C and D occurs at phosphate concentrations higher than with potassium phosphate. Sodium phosphate elution causes further reduction in the size of fraction A, shows greater resolution of B_1 and B_2, and elutes B_2 and C at phosphate concentrations even higher than with ammonium phosphate.

TABLE I

ULTRACENTRIFUGAL ANALYSIS[a] OF CHROMATOGRAPHIC FRACTIONS OBTAINED WITH DIFFERENT PHOSPHATE BUFFERS

Buffer cation	*Chromatographic fraction*	*Molarity of eluting buffer*[b]	*Total*,[c] %	*Ultracentrifugal composition*, %			
				2S	*7S*	*11S*	*15S + >15S*
——	Water-extractable proteins	—	100	25	39	25	11
Potassium	A	0.03	24	100[d]	—	—	—
	B	0.07	26	42	35	14	9
	C	0.17	33	7	11	68	14
	D	0.28	17	—	69	22	9
Ammonium	A	0.03	19	100[d]	—	—	—
	B_1	0.07	13	54	39	7	—
	B_2	0.12	18	40	45	15	—
	C	0.23	34	4	14	73	9
	D	0.40	16	—	76	17	7
Sodium	A	0.03	12	100[d]	—	—	—
	B_1	0.07	30	61	26	5	8
	B_2	0.18	16	49	36	15	—
	C	0.27	30	3	24	64	9
	D	~ 0.36	12	—	65	11	24

[a] Conditions as in ref. 1.
[b] Phosphate concentration at which fractions peaked on elution diagram.
[c] Estimated from areas under elution diagram.
[d] A mixture of two or more slow sedimentating components[1].

However, fraction D is not resolved from fraction C and elutes at a lower phosphate concentration than with ammonium phosphate. Although phosphate concentrations required for elution depend upon the buffer cation, the order of elution does not appear altered and the composition of the fractions does not vary greatly (Table I).

Similar results were obtained upon chromatographing bovine plasma albumin (Armour Lot P67502)* with sodium and potassium phosphates (Fig. 2). Albumin

* Reference to commercial products is for identification only and does not imply endorsement by USDA.

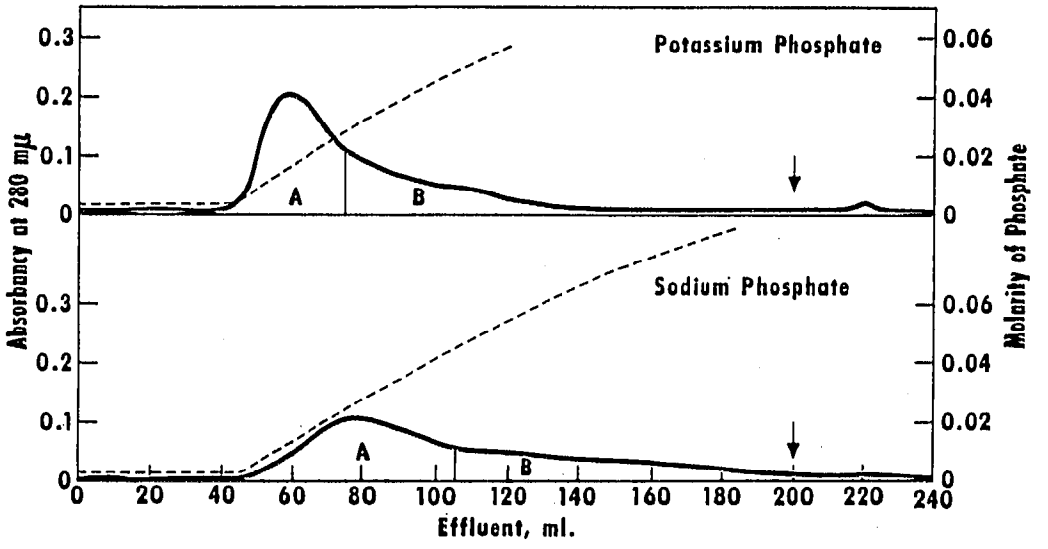

Fig. 2. Gradient elution of bovine plasma albumin from hydroxylapatite columns (1 × 25 cm) with sodium and potassium phosphate buffers, pH 7.6, at 25°. Vertical arrow indicates where 0.25 *M* phosphate at pH 7.6 was added. Solid curve is absorbancy at 280 mμ in 1-cm cell after 1:4 dilution. Dashed curve is elution gradient determined colorimetrically[3]. Column loads were 41 mg.

samples were adsorbed to the columns from 0.003 *M* phosphate, pH 7.6, and eluted by adding 0.25 *M* phosphate, pH 7.6, to 320 ml of 0.003 *M* phosphate in the mixing chamber. Separation into two fractions, A (60 %) and B (40 %), is indicated. Respective concentrations of phosphate at the maxima of the A fractions were 0.015 and 0.025 *M* for potassium and sodium, respectively. After chromatographing albumin with sodium phosphate, the column was treated with *M* potassium phosphate and equilibrated with 0.003 *M* potassium phosphate. Chromatography of another albumin sample with potassium phosphate gave an elution pattern similar to the upper diagram of Fig. 2, and fraction A peaked at 0.014 *M* phosphate. The effect of buffer cations on the chromatographic behavior of plasma albumin therefore appears reversible.

Ultracentrifugal analysis of the original albumin sample indicated 84 % monomer and 16 % dimer. After chromatography with potassium phosphate buffer, fractions A and B (Fig. 2) were dialyzed until free of salts and freeze-dried. Ultracentrifugal analysis of fraction A indicated 83 % monomer and 17 % dimer, whereas fraction B contained 71 % monomer, 16 % dimer, and 12 % of a faster material (possibly a trimer). Clear-cut separation into monomer and dimer[4] did not occur, but a separation tendency is indicated. Because the presence of trimer in fraction B indicates that aggregation occurred, probably the amount of monomer may have been higher in the samples before freeze-drying.

To our knowledge, effects of buffer cations on chromatography of proteins on hydroxylapatite as described here have not been reported previously. However, such effects were anticipated[5,6]. Hydroxylapatite exchanges calcium ions in the crystal lattice for sodium and potassium ions to different extents[7]. This exchange reaction suggests that different buffer cations form crystal surfaces having varying binding strengths for proteins, thereby accounting for the effects reported. A survey of the literature shows that sodium, potassium, and ammonium buffers have been used

individually or in combinations with one another in chromatography of proteins on hydroxylapatite; often it is difficult to ascertain which buffer cation was used. Our results show the importance of knowing exactly which buffer cation is used.

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Gas chromatographic analysis of ethylene and some fluoroethylenes

It was of interest in a recent investigation[1] to separate the C_2-olefius ethylene, 1,1-difluoroethylene and tetrafluoroethylene. Silica gel and silver nitrate–ethylene glycol packings show excellent separational properties for hydrocarbons and olefins[2,3], and in the present work these materials have been used to separate the C_2-olefins.

Using silica gel alone it is found that 1,1-difluoroethylene is separated from the ethylene and tetrafluoroethylene peak, while silver nitrate–ethylene glycol separates ethylene from the fluoroolefins. The quantitative separation of all three olefins is achieved by using both column materials in series.

Experimental and results

Silver nitrate in diethylene glycol on a firebrick support was purchased from the Perkin-Elmer Co., as was the silica gel. A Perkin-Elmer gas chromatograph (154-C) was used.

The silver nitrate phase was packed into a 12 ft. length of $^1/_4$ in. O.D. aluminium tubing and coiled, while the silica gel was contained in a 3.3 ft. Pyrex glass column;

TABLE I

Column used	*Retention times (min)*		
	C_2H_4	CF_2CH_2	C_2F_4
Silver nitrate–ethylene glycol	12.5	7.0	7.0
Silica gel	14.2	19.0	14.2
Silver nitrate–ethylene glycol plus silica gel	30.8	25.6	21.6