Journal of Chromatography, 499 (1990) 153–163
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1638

HIGH-PERFORMANCE ADSORPTION CHROMATOGRAPHY OF TRANSFER RIBONUCLEIC ACIDS AND PROTEINS ON 2-μm SPHERICAL BEADS OF HYDROXYAPATITE

INFLUENCE OF SODIUM CHLORIDE AND MAGNESIUM IONS ON THE RESOLUTION

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SUMMARY

The influence of sodium chloride and magnesium chloride on the adsorption of tRNA and proteins on a high-performance liquid chromatographic column of 2-μm spherical hydroxyapatite beads was investigated. The resolution of ^{14}C-labelled aminoacyl-tRNA isoacceptors was improved in the presence of sodium chloride. Inclusion of magnesium chloride in the buffers led to a separation of two tRNA species that could not be fractionated with or without sodium chloride in the eluting buffers (the original properties of the column were lost, however, and could not be regenerated by simply returning to magnesium chloride-free phosphate buffer). Also, the adsorption of some proteins was affected when salt was included in the buffers. For instance, the elution order of proteins could be changed by choosing an appropriate concentration of sodium chloride. This finding might be utilized to facilitate the purification of certain proteins.

INTRODUCTION

Spherical beads of hydroxyapatite have been developed recently for use in high-performance liquid chromatographic (HPLC) systems. We have previously described some basic properties of this bed material, and we also briefly discussed the effects of including sodium chloride in the eluting phosphate buffer in connection with the purification of γ-globulin from whole serum[1]. The general influence of sodium chloride on the adsorption of proteins on hydroxyapatite has been studied by Gorbunoff[2,3] and Gorbunoff and Timasheff[4], but has not in our knowledge been used for the fractionation of ribonucleic acids. In this paper we report some further investigations on the application of this technique to both protein and tRNA. In addition, as magnesium ions are known to change the three-dimensional structure of tRNA[5], the effect of including magnesium chloride in the eluting buffers was investigated.

0021-9673/90/$03.50

EXPERIMENTAL

The chromatographic equipment included a Model 2152 HPLC controller, a Model 2150 HPLC pump and a Model 2210 recorder (LKB, Bromma, Sweden), a Model 786 variable-wavelength detector (Micromeritics, Norcross, GA, U.S.A.), a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.), a Microcol TDC 80 fraction collector (Gilson, Villiers le Bel, France) and a Model LS 2800 liquid scintillation system (Beckman, Irvine, CA, U.S.A.).

The high-performance hydroxyapatite columns (100 mm × 7.5 mm I.D.) were a gift from Toa Nenryo Kogyo (Tokyo, Japan). DEAE-Sepharose and Sepharose 6B were obtained from Pharmacia (Uppsala, Sweden), sodium chloride from KEBO Lab. (Stockholm, Sweden), sodium dihydrogenphosphate, disodium hydrogenphosphate, potassium dihydrogenphosphate, dipotassium hydrogenphosphate, potassium chloride, magnesium chloride, sodium acetate and hydrochloric acid (37%) from E. Merck (Darmstadt, F.R.G.), L-[U-^{14}C]leucine (340 mCi/mmol), L-[U-^{14}C]valine (265 mCi/mmol) and L-[U-^{14}C]phenylalanine (536 mCi/mmol) from New England Nuclear (Dreieich, F.R.G.), the xylene-based scintillation liquid (Quickszint 212) from Zinsser Analytic (Maidenhead, U.K.), tris(hydroxymethyl)aminomethane (Tris), chicken egg albumin and lysozyme from Sigma (St. Louis, MO, U.S.A.) and chymotrypsinogen A from Pharmacia (Uppsala, Sweden). Human serum albumin and human transferrin were gifts from KabiVitrum (Stockholm, Sweden).

Preparation of bulk tRNA

Bulk tRNA was prepared from *Escherichia coli* MRE 600 as described by Zubay[6]. After deacylation at pH 9.0 in 1 *M* Tris–HCl for 1 h, high-molecular-weight RNA was removed by chromatography on Sepharose 6B[7].

Preparation of aminoacyl-tRNA ligase

A crude extract of aminoacyl-tRNA ligase was prepared from *Escherichia coli* B according to Muench and Berg[8]. The preparation was stored at −70°C in portions of 0.1 ml.

Preparation of [^{14}C]aminoacyl-tRNA isoacceptors

Pools of specific tRNA isoacceptors, obtained from chromatography on cross-linked agarose beads[9] (see Fig. 1), were labelled with their cognate ^{14}C-labelled amino acids. The reaction mixture contained 1 volume (100–200 μl) of the chosen tRNA pool, 1 volume of amino acid mixture (see Table I), 2 volumes of distilled water and 0.01 volume of ligase. After incubation at 37°C for 20 min, the reaction was interrupted by addition of 0.25 volume of 1 *M* sodium acetate buffer (pH 4.5).

The solution obtained was diluted with an equal volume of 0.02 *M* sodium acetate (pH 4.5) and applied to a DEAE-Sepharose column (20 mm × 10 mm I.D.), equilibrated with 20 m*M* sodium acetate (pH 4.5). The column was then washed with the equilibration buffer, containing 0.3 *M* sodium chloride, until UV-absorbing material (unreacted amino acids, ligase, ATP, etc.) ceased to appear in the effluent. The tRNA isoacceptor was finally eluted with the same buffer, containing 1 *M* sodium chloride. The eluted tRNA fraction was precipitated with 2 volumes of 95% ethanol, then vacuum dried and stored at −20°C.

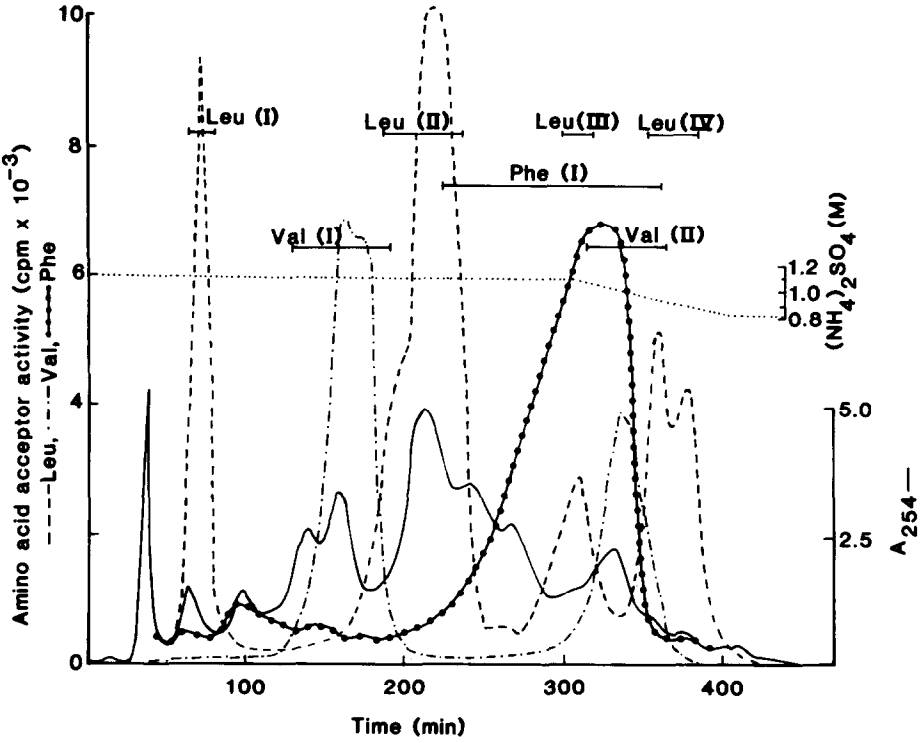

Fig. 1. Fractionation of *E. coli* tRNA by HPLC on cross-linked agarose beads[9]. Aliquots of each fraction were assayed for leucine, valine and phenylalanine acceptance. The fractions corresponding to each activity peak were pooled as indicated: Leu(I), Leu(II), Leu(III), Leu(IV), Val(I), Val(II) and Phe(I). tRNA in each pool was labelled with its cognate ^{14}C-labelled amino acid prior to hydroxyapatite chromatography (see Figs. 3–6).

Radioactivity measurements

Scintillation liquid (3.0 ml) was carefully mixed in a scintillation tube with 0.9 ml of each fraction collected in the agarose and hydroxyapatite chromatography experiments, and counted in a scintillation system for 10 min. The radioactive decay in counts per minute (cpm) was calculated and plotted on the chromatograms.

TABLE I

COMPOSITIONS OF AMINO ACID MIXTURES

HEPES = N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid; ATP = adenosine 5'-triphosphate; DTT = dithiothreitol; 2-ME = 2-mercaptoethanol.

Amino acid	*[^{14}C]Amino acid (μM)*	*0.4 M buffer*		*Concentration (mM)*					
		Type	*pH*	*Mg^{2+}*	*K^+*	*NH_4^+*	*ATP*	*DTT*	*2-ME*
Leucine	3.08	HEPES	8.0	40	–	24	16	–	80
Valine	5.68	Sodium cacodylate	7.1	40	40	–	8	–	–
Phenylalanine	1.50	HEPES	7.1	40	–	20	16	4	–

RESULTS

Influence of sodium chloride on the adsorption of bulk tRNA on hydroxyapatite

A solution of bulk tRNA (27 A_{260} units) was applied to the column and eluted immediately with a gradient in phosphate concentration from 0.044 to 0.102 *M* at pH 6.8 over 180 min. Fractions of 1.0 ml were collected and measured for radioactivity. The experiment was then repeated with 0.1 *M* sodium chloride included in all buffers.

The absorbance pattern at 260 nm (see Fig. 2) was altered when sodium chloride was added to the buffers, and showed that some peaks became narrower and that the resolution seemed to be improved. The recovery was 99% in the presence and 100% in the absence of salt, as determined by absorbance measurements and the "cut-and-weigh" technique.

HPLC of ^{14}C-labelled aminoacyl-tRNA isoacceptors on hydroxyapatite in the absence and presence of sodium chloride

In order to investigate the influence of sodium chloride on the separate aminoacyl-tRNA isoacceptors, a series of [^{14}C]aminoacyl-tRNA isoacceptors were prepared as described above (see Experimental).

The vacuum-dried isoacceptor was dissolved in 30 µl of 5 m*M* sodium phosphate buffer (pH 6.8). Of this sample, 29 µl were applied to the hydroxyapatite column and eluted immediately with a phosphate gradient from 0.044 to 0.102 *M* over 180 min. Fractions of 1.0 ml were collected and analysed for radioactivity. A similar experiment was then performed with 0.1 *M* sodium chloride added to the buffers.

This procedure was repeated for each of the aminoacyl-tRNAs used. Also, when an aminoacyl-tRNA appeared in several peaks on chromatography on cross-linked agarose beads (see Fig. 1), each peak was studied separately on the hydroxyapatite column.

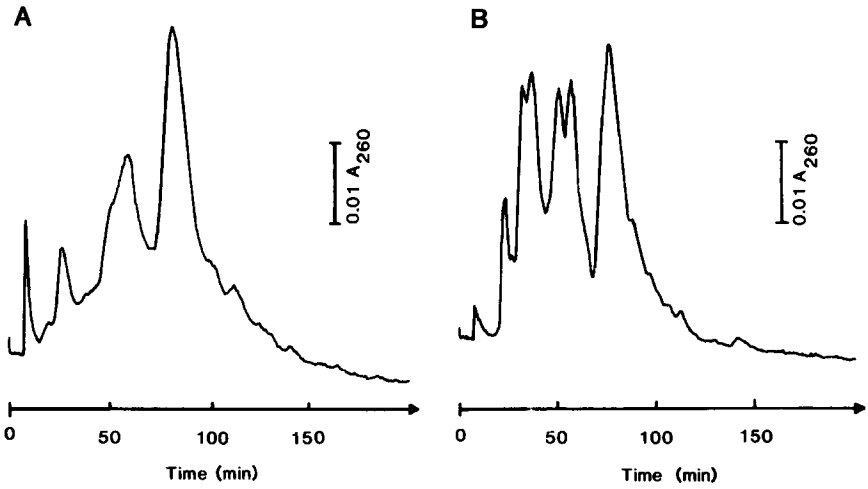

Fig. 2. Influence of sodium chloride on the adsorption of tRNA on hydroxyapatite. Samples, bulk tRNA (0.7 A_{260} units); volume, 29 µl; column dimensions, 100 mm × 7.5 mm I.D.; flow-rate, 0.4 ml/min. Elution: (A) a gradient from 0.044 *M* sodium phosphate buffer (pH 6.8) to 0.102 *M* over 180 min; (B) as in A, but the buffers also contained 0.1 *M* sodium chloride.

Following the course described above, four [^{14}C]leucyl-, one [^{14}C]phenylalanyl- and two [^{14}C]valyl-tRNA isoacceptor pools (see Fig. 1) were used to investigate the influence of sodium chloride on the adsorption to hydroxyapatite (see Figs. 3–5).

The resolution seemed to be improved for all [^{14}C]aminoacyl-tRNAs when 0.1 *M* sodium chloride was included in the buffers, with the possible exception of the second [^{14}]valyl-tRNA sample [Val(II) in Fig. 1] and the two [^{14}C]leucyl-tRNA samples [Leu(I) and Leu(II) in Fig. 1], which appeared more heterogeneous in the absence of salt (see Figs. 3 and 4). The fourth [^{14}C]leucyl-tRNA [Leu(IV)], which in the sodium chloride-free chromatography experiment seemed to consist of a single component, could be resolved easily into two distinct peaks when sodium chloride was present in the buffers (see Fig. 3).

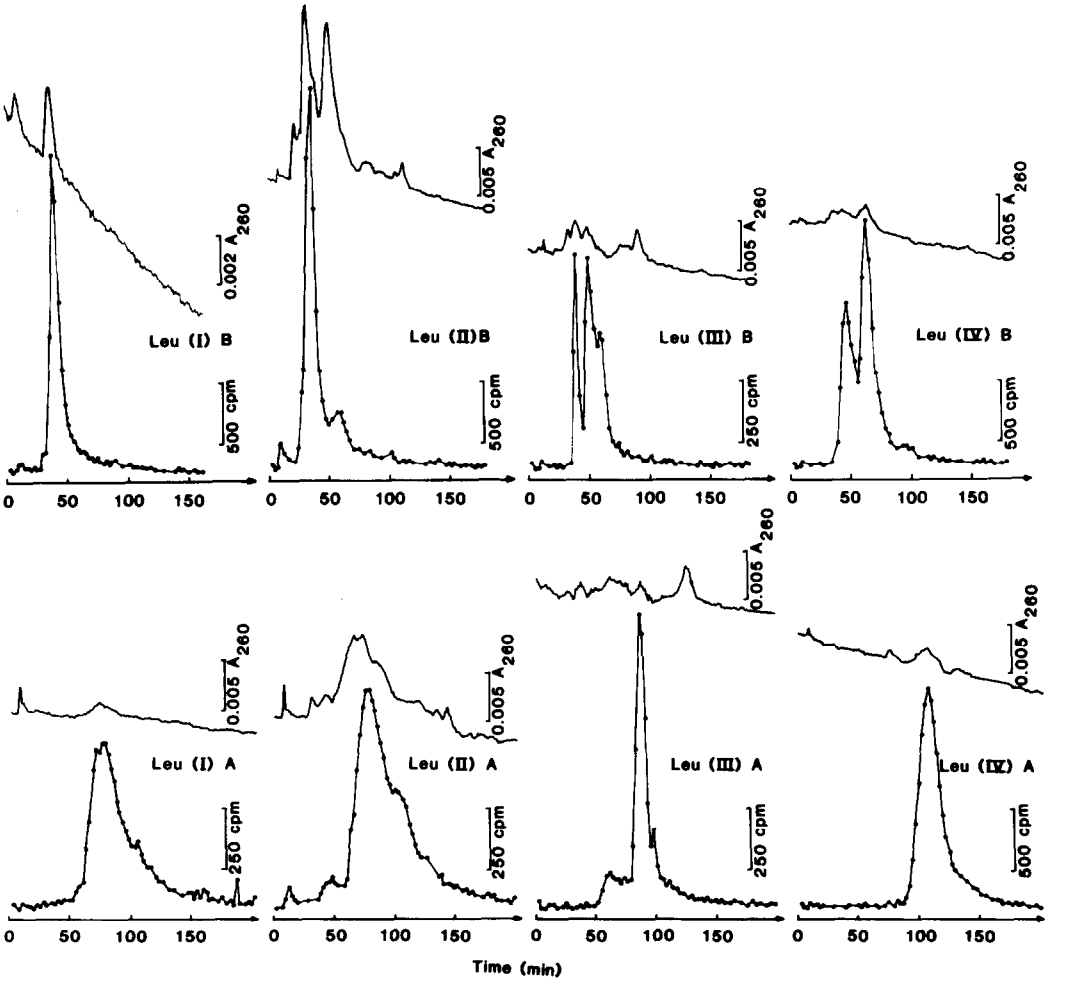

Fig. 3. HPLC of ^{14}C-labelled leucyl-tRNA isoacceptors on hydroxyapatite in (A) the absence and (B) the presence of 0.1 *M* sodium chloride. Samples, [^{14}C]leucyl-tRNA isoacceptors [Leu(I)–Leu(IV) in Fig. 1]. Conditions and elution (A and B) as in Fig. 2.

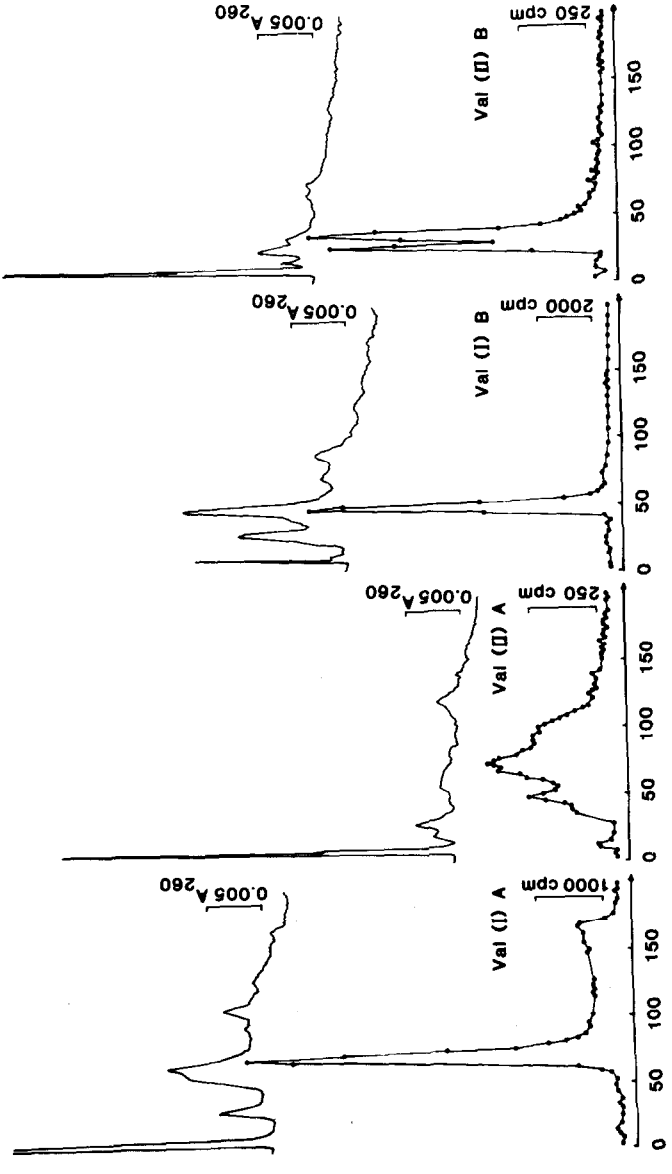

Fig. 4. HPLC of ^{14}C-labelled valyl-tRNA isoacceptors on hydroxyapatite in (A) the absence and (B) the presence of 0.1 *M* sodium chloride. Samples, [^{14}C]valyl-tRNA isoacceptors [Val(I) and Val(II) in Fig. 1]. Conditions and elution (A and B) as in Fig. 2.

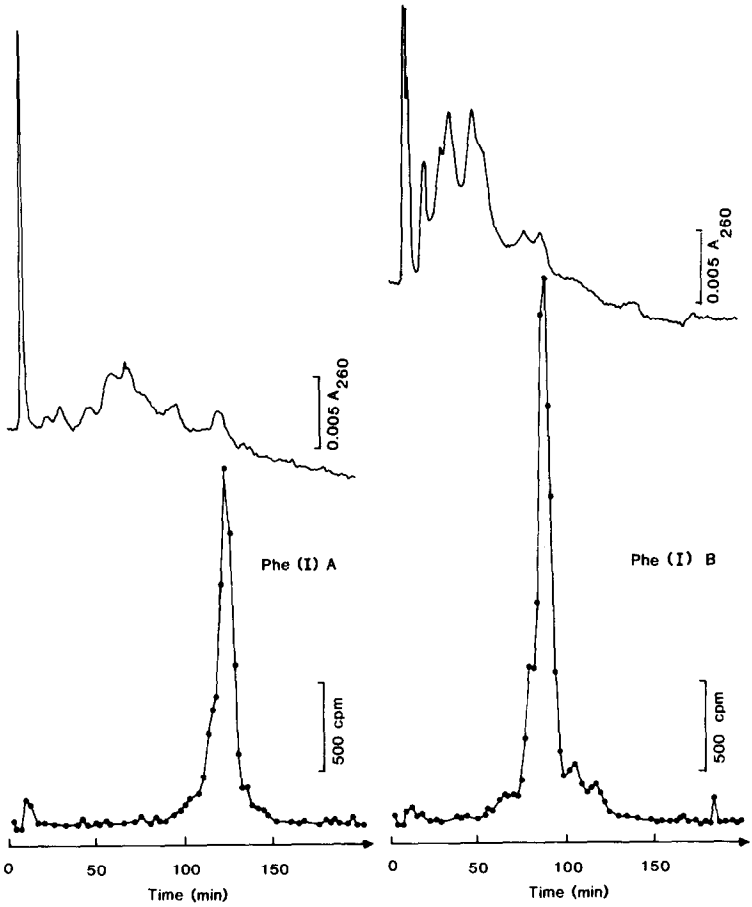

Fig. 5. HPLC of ^{14}C-labelled phenylalanyl-tRNA isoacceptors on hydroxyapatite in (A) the absence and (B) the presence of 0.1 *M* sodium chloride. Sample, [^{14}C]phenylalanyl-tRNA isoacceptors [Phe(I) in Fig. 1]. Conditions and elution (A and B) as in Fig. 2.

HPLC of ^{14}C-labelled valyl-tRNA on hydroxyapatite in the presence of magnesium chloride

As magnesium ions are known to influence the three-dimensional structure of tRNA[5], the adsorption of tRNA on hydroxyapatite might be expected to change on inclusion of magnesium chloride in the buffers (magnesium might also interact with the phosphate ions in the hydroxyapatite and thereby change its chromatographic properties).

To examine this, the [^{14}C]valyl-tRNA isoacceptor I [Val(I) in Fig. 1] was applied again and eluted from the hydroxyapatite column, but in this experiment the sodium chloride was replaced with 10 m*M* magnesium chloride.

The apparently homogeneous peak observed in both the absence and presence of sodium chloride (Fig. 4) was split into two distinct peaks when 10 m*M* magnesium chloride was included in the phosphate buffers (see Fig. 6).

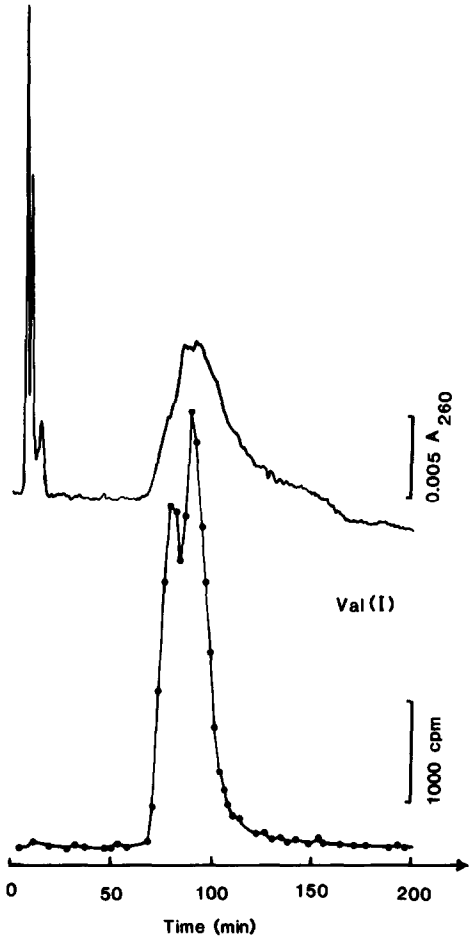

Fig. 6. HPLC of ^{14}C-labelled valyl-tRNA isoacceptors on hydroxyapatite in the presence of 10 m*M* magnesium chloride. Sample, [^{14}C]valyl-tRNA isoacceptors [Val(I) in Fig. 1]. Conditions as in Fig. 2. Elution, gradient of sodium phosphate buffer (pH 6.8), including 10 m*M* magnesium chloride, from 0.044 to 0.102 *M* over 180 min. The chromatogram in the absence of magnesium chloride is shown in Fig. 4.

Influence of sodium chloride and potassium chloride on the adsorption of proteins on hydroxyapatite

Chloride ions at moderate concentrations are known to elute mainly basic proteins from hydroxyapatite columns[2–4]. If chloride ions were included at non-eluting concentrations in the eluting buffer, one could expect the elution order of the proteins to change. To investigate this, the hydroxyapatite column was equilibrated with 0.01 *M* phosphate buffer (pH 6.8). A sample consisting of 90 μg of ovalbumin, 90 μg of human transferrin, 340 μg of human serum albumin, 16 μg of lysozyme and 21 μg of chymotrypsinogen A dissolved in 120 μl of the equilibration buffer was applied to the column and allowed to be adsorbed for 10 min. The proteins were then eluted with a gradient in phosphate concentration from 0.01 to 0.19 *M* over 90 min.

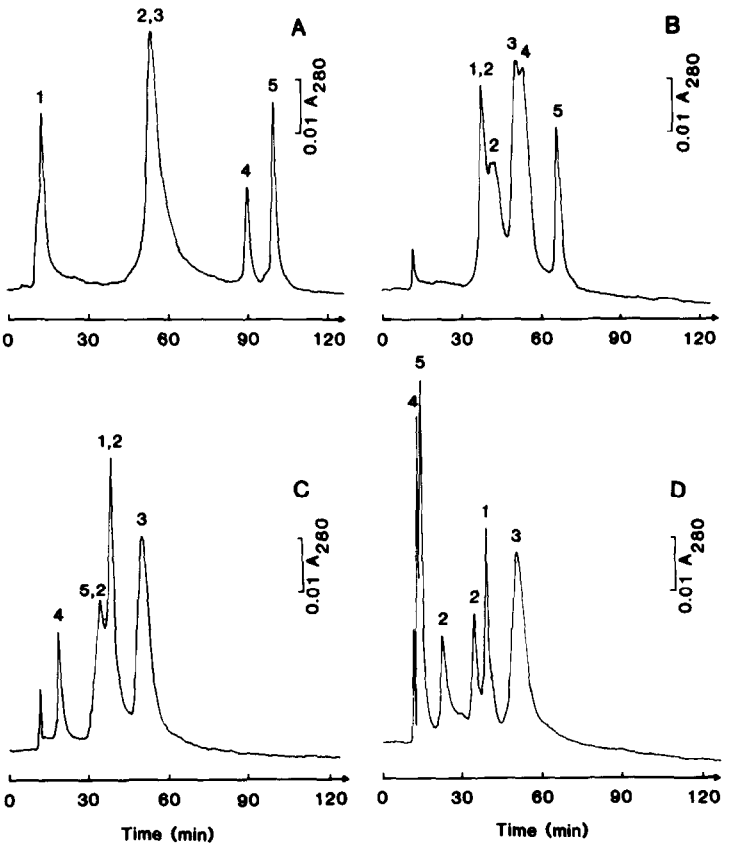

Fig. 7. HPLC of a model protein mixture in the presence of increasing sodium chloride concentrations: 1 = chicken egg albumin (90 μg); 2 = human transferrin (90 μg); 3 = human serum albumin (340 μg); 4 = lysozyme (16 μg); 5 = chymotrypsinogen A (21 μg). Sample volume, 120 μl; column dimensions, 100 mm × 7.5 mm I.D.; flow-rate, 0.3 ml/min. Elution: (A) 0.01 *M* sodium phosphate buffer (pH 6.8) for 28 min, then a gradient from 0.01 to 0.19 *M* over 90 min; (B), (C) and (D) as in A, but the buffers also included 0.1, 0.2 and 0.5 *M* sodium chloride, respectively.

This procedure was followed four times, using sodium phosphate containing 0.0, 0.1, 0.2 and 0.5 *M* sodium chloride. In order to investigate the influence of the cation, the experiment was repeated using potassium phosphate and potassium chloride.

As expected, the two basic proteins, lysozyme (p*I* 10.5–11) and chymotrypsinogen A (p*I* 8.8–9.6), showed markedly decreased adsorption on inclusion of sodium chloride in the buffers (Fig. 7). Human transferrin, although fairly acidic (p*I* 5.2–6.2), was also eluted earlier at increasing chloride concentrations. On the other hand, chicken egg albumin (p*I* 4.6) was adsorbed more strongly when chloride ions were included. Finally, the elution of human serum albumin (p*I* 5.8) was more or less independent of the chloride concentration in the buffers.

Only minor differences in the elution patterns were seen on using sodium or potassium as cations.

DISCUSSION

In HPLC of any kind it is generally preferable to keep the elution conditions as simple as possible in order to maximize reproducibility. Sometimes, however, it can be of advantage to utilize an additional parameter to manipulate the appearance of the chromatogram.

We have shown that although sodium chloride alone cannot desorb tRNA from a hydroxyapatite column, it does have the attractive property of improving the separation of tRNA compared with elution by sodium phosphate buffers alone. A general narrowing of the eluting zones was observed (Figs. 2–5), and sometimes an apparently homogeneous peak split into two parts [Leu(IV) in Fig. 3] when sodium chloride was present in the buffers. This zone-narrowing effect may be attributed to a reduction in the number of ways tRNA can be adsorbed on hydroxyapatite. The sodium chloride concentration (0.1 *M*) was found to be suitable in our experiments, but may not be the optimum concentration for all separations.

Magnesium ions are known to alter the three-dimensional structure of tRNA[5] and should also compete with the calcium ions of the hydroxyapatite for the phosphodiester bridges of the ribonucleic acids. In addition, it should interact with phosphate groups on the column, and might therefore be expected to influence the adsorption of tRNA on hydroxyapatite. We have demonstrated here that a valyl-tRNA isoacceptor appearing homogeneous in both the presence and absence of sodium chloride [Val(I) in Fig. 4] could be resolved into two distinct components when magnesium chloride was included in the buffers (Fig. 6). Unfortunately, the column could not be regenerated by washing with phosphate buffers not containing magnesium chloride, and thereby lost its original properties.

Inclusion of sodium chloride in the eluting phosphate buffer has previously been used for the specific purification of γ-globulin from whole serum[1,10]. In this work we further demonstrated this phenomenon by separating a model protein mixture with increasing concentrations of sodium chloride in the eluting buffers. As expected, the adsorption of the basic proteins lysozyme (p*I* 10.5–11) and chymotrypsinogen A (p*I* 8.8–9.6) was lowered considerably in the presence of sodium chloride[2] but, surprisingly, that of human transferrin (p*I* 5.2–6.2) was also reduced. Chicken egg albumin (p*I* 4.6), on the other hand, was bound more strongly to the column when sodium chloride was included in the buffers, whereas human serum albumin (p*I* 5.8) was hardly affected. By an appropriate choice of chloride and phosphate concentrations in the eluting buffers, one might be able to elute selectively one specific protein out of several proteins adsorbed on the column (*e.g.*, γ-globulin from whole serum as in ref. 1) or to elute all but one particular protein from the column. An example of the latter possibility might be human serum albumin in Fig. 7D, provided that the eluting phosphate concentration is chosen properly. This cannot be achieved with only sodium phosphate in the eluting buffer (see Fig. 7A).

The reproducibility was high for both the tRNA and the protein experiments.

ACKNOWLEDGEMENTS

This work was financially supported by the Swedish Natural Science Research Council and the Knut and Alice Wallenberg, Carl Trygger and Bengt Lundqvist Memorial Foundations.

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