Journal of Chromatography, 515 (1990) 563–572
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1781

High-performance liquid chromatography of transfer ribonucleic acids on spherical hydroxyapatite beads

II. Effects of pH and sodium chloride on chromatography

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ABSTRACT

The effects of pH and sodium chloride on the high-performance liquid chromatography of transfer ribonucleic acids (tRNAs) on spherical hydroxyapatite beads were investigated. Binding of tRNAs on the beads was strengthened by increasing the pH of the mobile phase (phosphate buffer, pH 6.4–8.0), a phenomenon which is opposite to the retention of proteins on the beads. By using phosphate buffer of pH 8.0 instead of pH 6.8, the resolution of tRNAs was improved significantly; as many as ten times more theoretical plates were calculated with the use of the former buffer. Addition of sodium chloride to the phosphate buffer has a bifunctional effect on the retention of tRNAs: elution of $tRNA^{f\text{-}Met}$ and $tRNA^{Val}$ was retarded whereas that of $tRNA^{Phe}$ was facilitated.

INTRODUCTION

High-performance liquid chromatography (HPLC) on spherical hydroxyapatite (HAP) beads is a useful method for the purification and analysis of biomacromolecules such as proteins and nucleic acids[1–3]. The beads have been successfully applied to the

0021-9673/90/$03.50

purification of mouse monoclonal antibodies from ascitic fluid[4], a protease from the venom of the king cobra[5] and the separation of two isomers of human tumour necrotic factor[6]. Recently, chromatography of tRNAs on HAP beads has been reported[7,8]. Both reports demonstrated that the HPLC of tRNAs on HAP beads gave good resolution and a high recovery of tRNAs (>90%). The column was also useful for the separation of isoacceptors of tRNAs. In an attempt to improve the resolution of tRNAs by HPLC on HAP beads, we examined the effects of pH and NaCl on resolution in comparison with the previously described method in which tRNAs were analysed in a phosphate buffer at pH 6.8.

EXPERIMENTAL

tRNAs

Purified *Escherichia coli* tRNAs (Val, Lot No. 97F-0079-1; f-Met, Lot No. 128F-01371; and Phe, Lot No. 88F-01691) were purchased from Sigma (St. Louis, MO, U.S.A.). Total tRNA ($tRNA^{Total}$) was prepared from *E. coli* K12 and *Bacillus subtilis* W168 as described by Zubay[9]. Purified tRNA was dissolved in 5 m*M* phosphate buffer (pH 8.0) at about 0.5 mg/ml and was stored at 5°C. $tRNA^{Total}$ was also dissolved in the same buffer at a concentration of about 1.5–2 mg/ml. The concentration of tRNA was determined spectrophotometrically using $A_{1\,cm}^{1\%} = 20$ at 260 nm.

Determination of amino acid-accepting activity

The preparation of crude *E. coli* aminoacyl-tRNA synthetase and the determination of the amino acid-accepting activity of tRNA with ^{14}C-labelled amino acids were carried out as described by Nishimura *et al.*[10].

Columns

Packed columns (10 × 0.75 cm I.D. and 10 × 2.14 cm I.D.) of spherical beads of HAP were obtained from Toa Nenryo Kogyo (Tokyo, Japan). The small-bore column packed with 2.2-μm HAP beads was used for routine analysis and the larger bore column packed with 5-μm beads for semi-preparative use.

Apparatus

A Shimadzu LC-7A liquid chromatograph with a two-pump gradient system was used for the analytical column. Elution of nucleic acids was monitored at 260 nm with an SPD-6A UV detector and recorded with a Chromatopak C-R4A. Samples were introduced into the column from a SIL-6A automatic sample injector. The semi-preparative column was eluted with a CCPM prep-pump (Tosoh, Tokyo, Japan) and the eluate was monitored with a Model 875-UV detector (JASCO, Tokyo, Japan) with a 1-mm light-path cell.

Elution

Elution of tRNA from the column was performed with a linear gradient of phosphate buffers of various pHs between solvents A (5 m*M*) and B (300 m*M*). The gradient was started immediately after sample injection, but its action is delayed by 15 min when the analytical column was eluted at a flow-rate of 0.5 ml/min. The

analytical column was usually elutd at a flow-rate of 0.5 ml/min (back-pressure *ca.* 20 kg/cm^2). The semi-preparative column was eluted at 5.0 ml/min (*ca.* 5 kg/cm^2); 5-ml fractions of eluate were collected by a fraction collector for determination of the respective amino acid-accepting activity. Chromatography was carried out at room temperature.

RESULTS

Effects of pH on the retention of tRNAs on the HAP column

Fig. 1 shows a comparison of typical elution profiles of a mixture of equal amounts of purified *E. coli* tRNAs (f-Met, Val and Phe) on the HAP column when the column was developed using the same linear gradient (15–78% B in 60 min) of phosphate ion concentrations but at different pHs (6.8 and 8.0). It is evident that binding of tRNAs to the HAP column was greater at the higher pH. All three tRNAs were separately eluted from the column within a narrow range of phosphate ion concentrations (*ca.* 20–40% B; 60–120 m*M*) at pH 6.8. However, a higher and wider range of phosphate ion concentrations (*ca.* 40–70% B; 120–210 m*M*) was necessary to elute these tRNAs at pH 8.0. In addition to this finding, tRNA^{Phe}, which was eluted from the column as a single peak at pH 6.8, was completely divided into two distinct peaks, tentatively named Phe-I and Phe-II, at pH 8.0; a peak that eluted just before tRNA^{Val} at pH 6.8 was also split into several minor peaks, which were eluted between

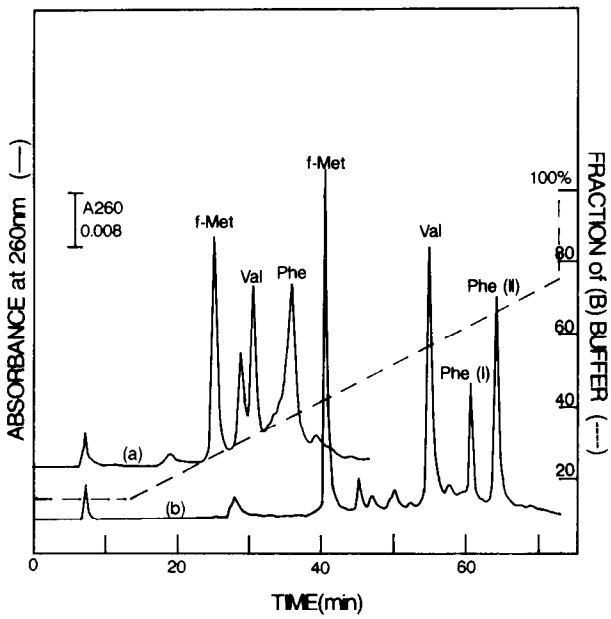


Fig. 1. Chromatography of a mixture of *E. coli* tRNAs on the analytical column (10 cm × 0.75 cm I.D.) of HAP at different pH. A mixture of purified *E. coli* tRNAs (f-Met, Val and Phe; 3.5 μg each) was eluted from the column at a flow-rate of 0.5 ml/min by a gradient of phosphate ion concentration (15–78% B in 60 min) at (a) pH 6.8 and (b) pH 8.0. Buffer A, 5 m*M* phosphate buffer (pH 6.8 or 8.0); buffer B, 300 m*M* phosphate buffer (pH 6.8 or 8.0).

the peaks of $tRNA^{f\text{-}Met}$ and $tRNA^{Val}$ in the analysis at pH 8.0. Moreover, each tRNA was eluted more sharply from the column at the higher pH in spite of the elution with the same gradient of phosphate ion concentrations.

Fig. 2 shows the relationship between the pH of the mobile phase and the retention times of the three tRNAs on the HAP column when a mixture containing equal amounts was eluted with the same gradient of phosphate ion concentrations from 15 to 78% B in 60 min at different pHs. An effect of pH on the retention of tRNAs on HAP beads was evident. The binding of tRNAs on the HAP beads was strengthened with, but not proportionally, to the increase in pH of the eluent. Incidentally, the separation of $tRNA^{Phe\text{-}I}$ and $tRNA^{Phe\text{-}II}$ and the split peak of $tRNA^{Val}$ mentioned above also became clearer at higher pH. As phosphate buffer possesses a weak buffering action above pH 8.0, experiments above pH 8.0 were not performed.

The efficiency of the HAP chromatography of tRNA between pH 6.8 and 8.0 was determined by comparing the theoretical plate numbers of the column. For this purpose, the gradient of phosphate ion concentration was adjusted so that corresponding tRNA molecules would elute at a similar retention time at both pH values. The resulting chromatograms and theoretical plates (N) of the column are shown in Fig. 3. Regarding the elution of $tRNA^{f\text{-}Met}$ and $tRNA^{Val}$, as many as ten times more theoretical plates were calculated for the column at pH 8.0 than that at pH 6.8; about six times more theoretical plates were also calculated with the $tRNA^{Phe\text{-}II}$ peak at pH 8.0. The results clearly demonstrate that a higher pH is advantageous for the HPLC of tRNAs on HAP beads.

Chromatography of $tRNA^{Total}$ of E. coli and B. subtilis

Under the conditions described above, we analysed *E. coli* and *B. subtilis* $tRNA^{Total}$ on the analytical HAP column in phosphate buffer at pH 8.0. Fig. 4a shows a typical elution profile of *E. coli* $tRNA^{Total}$ on the analytical HAP column with an optimized linear gradient (40–75% B in 60 min) of phosphate ion concentrations at pH 8.0; the elution profile of the mixture of equal amounts of purified tRNAs (f-Met,

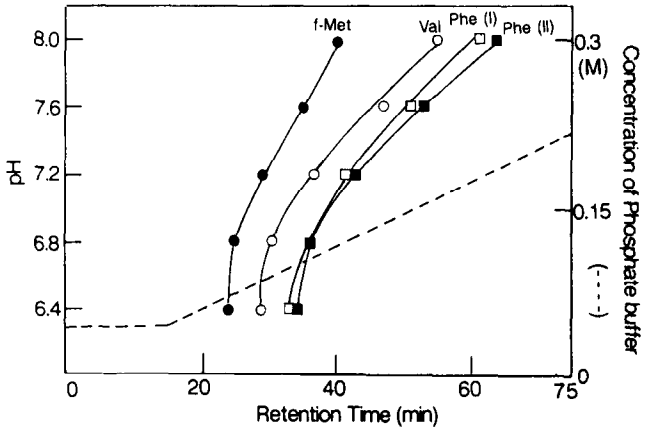


Fig. 2. Effects of pH on the retention of tRNAs on HAP. A mixture of equal amounts of *E. coli* tRNAs was chromatographed on the analytical column with the same linear gradient of phosphate ion concentration as in Fig. 1 at various pHs; retention times of the tRNAs were plotted against pH of the phosphate buffer.

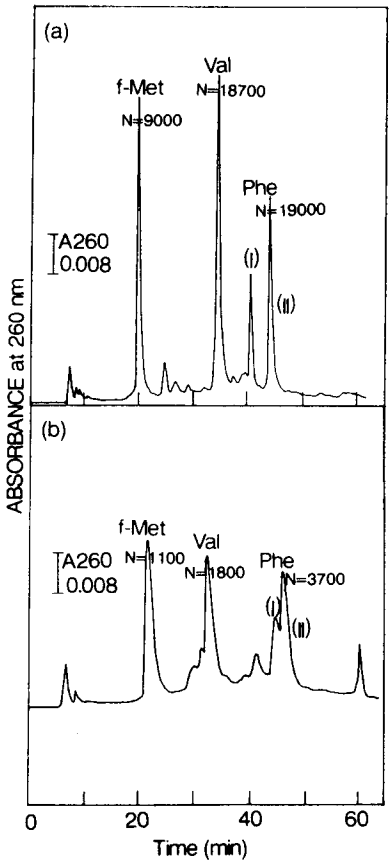


Fig. 3. Comparison of elution profiles of mixtures of equal amounts of *E. coli* tRNAs at pH 8.0 and 6.8. (a) A mixture of *E. coli* tRNAs (the same as in Fig. 1) was eluted from the analytical HAP column at a flow-rate of 0.5 ml/min with a linear gradient of phosphate ion concentration (40–78% B in 45 min, buffer A 5 m*M*, buffer B 300 m*M*) at pH 8.0. (b) The same mixture as in (a) was eluted from the HAP column at a flow-rate of 0.5 ml/min with a linear gradient of phosphate ion concentration (23–43% B in 45 min, buffer A 5 m*M*, buffer B 300 m*M*) at pH 6.8.

Val and Phe) analysed under the same conditions is shown in Fig. 4b. As expected, the resolution of the $tRNA^{Total}$ on the analytical column was improved considerably by eluting the column at pH 8.0 when compared with the chromatogram shown in the previous paper[7], in which phosphate buffer of pH 6.8 was used for elution. When the $tRNA^{Total}$ was analysed at pH 6.8[7], the elution profile revealed peaks that were widely spread and irregularly shaped, and only ten significant peaks could be recognized; in contrast as many as twenty significant peaks were observed in the chromatography at pH 8.0. Therefore, we analysed $tRNA^{Total}$ of *B. subtilis* under the same conditions. As shown in Fig. 5, the resolution of $tRNA^{Total}$ of *B. subtilis* on the column was also improved by eluting the column with phosphate buffer of pH 8.0.

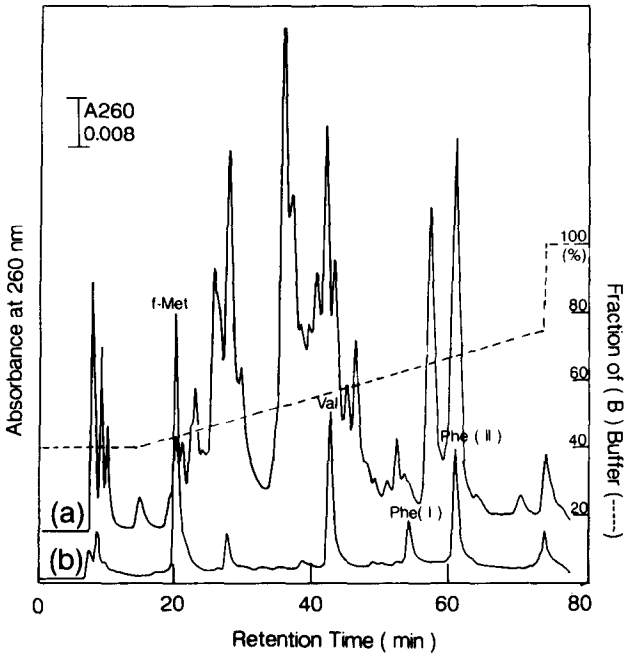


Fig. 4. Chromatography of *E. coli* $tRNA^{Total}$ and a mixture of equal amounts of purified *E. coli* tRNAs on the analytical HAP column. (a) 50 μg of *E. coli* $tRNA^{Total}$ were applied to the analytical HAP column and eluted with a 60-min linear gradient of phosphate ion concentration from 40 to 75% B (buffer A 5 m*M*, buffer B 300 m*M*; pH 8.0) at a flow-rate of 0.5 ml/min. (b) A mixture of equal amounts of *E. coli* tRNAs was chromatographed under the same conditions as in (a).

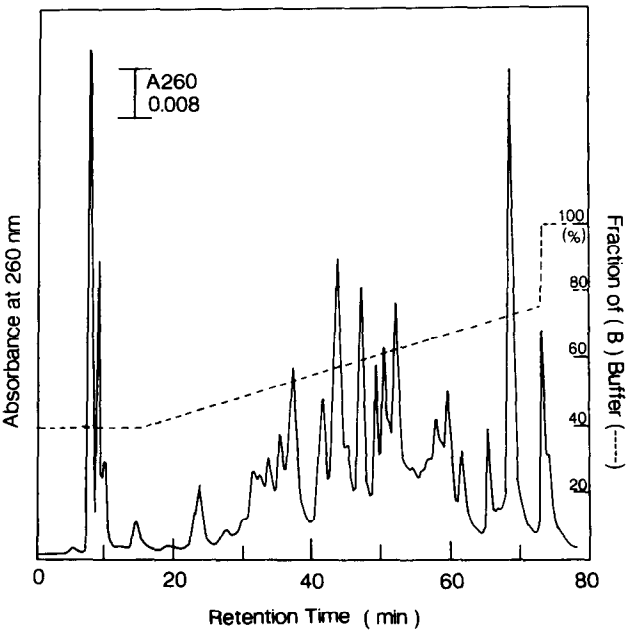


Fig. 5. Chromatography of *B. subtilis* $tRNA^{Total}$ on the analytical HAP column. 40 μg of $tRNA^{Total}$ of *B. subtilis* were chromatographed under the same conditions as in Fig. 4.

Chromatography of E. coli tRNA^{Total} on the semi-preparative column and determination of amino acid-accepting activity of several tRNAs

Fig. 6 shows typical elution profile of *E. coli* tRNA^{Total} on the semi-preparative column (10 cm × 2.4 cm I.D.) eluted with optimized phosphate ion concentration at pH 8.0. The elution profiles of the accepting activity of six amino acids in the eluate are also shown. The retention of tRNAs on this column was weaker than that on the analytical column and a phosphate ion gradient of 25–55% B at pH 8.0 was optimum for elution of tRNA^{Total}. The elution profile of tRNA^{Total} monitored at 260 nm (top) was not as good as that obtained with the analytical column (see Fig. 4) as the column was packed with 5-μm beads instead of 2.2-μm beads for the analytical column. As expected, the elution of tRNA from the semi-preparative column was also clearly improved in comparison with the previous chromatography performed at pH 6.8. tRNAs such as tRNA$^{f\text{-}Met}$, tRNA^{Val} and tRNA^{Phe} contained in the tRNA^{Total} were eluted from the column separately at the earlier, middle and later parts, respectively, of the gradient, as expected from the results on the analytical column (see Fig. 1).

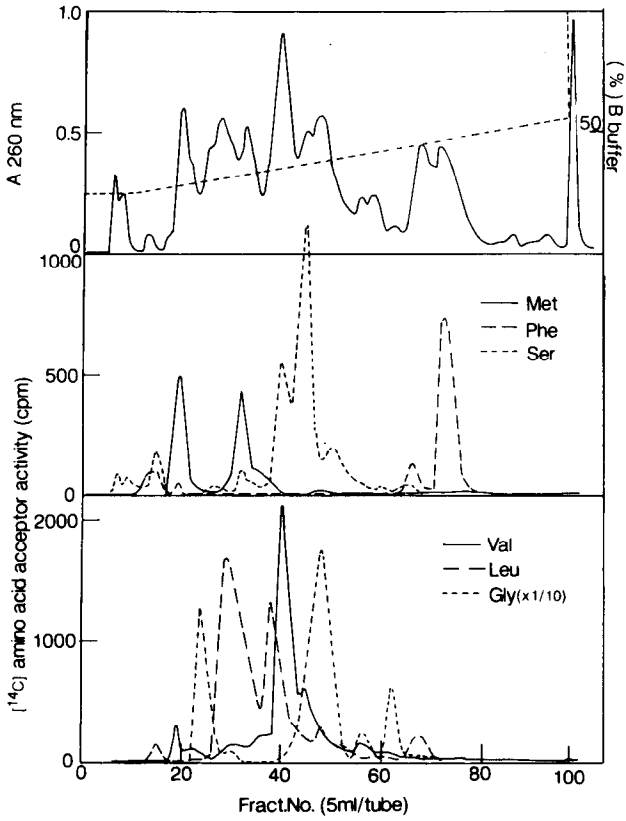
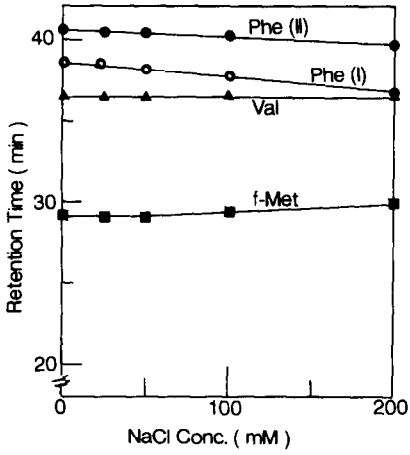


Fig. 6. Chromatography of *E. coli* tRNA^{Total} on the semi-preparative HAP column. *E. coli* tRNA^{Total} (7 mg) was applied to the semi-preparative column (10 cm × 2.14 cm I.D.) of HAP equilibrated with 25% B and eluted with a 90-min linear gradient of phosphate ion concentration from 25 to 55% B (buffer A 5 m*M*, buffer B 300 m*M*; pH 8.0) at a flow-rate of 5 ml/min. Fractions of 5 ml were collected and A_{260} (top) and amino acid acceptor activities of the fractions (middle and bottom) were determined.

Two methionine-accepting activity peaks were eluted from the column, which appears to indicate separation of $tRNA^{f\text{-}Met}$ and $tRNA^{Met}$ in the preparation. Judging from the retention times of these peaks, the former methionine-accepting peak was considered to be $tRNA^{f\text{-}Met}$. The elution of two phenylalanine-accepting activities in the later part of the gradient corresponded to the elution of isoacceptors of $tRNA^{Phe\text{-}I}$ and $tRNA^{Phe\text{-}II}$ observed in the purified preparation of $tRNA^{Phe}$, as pointed out earlier. In addition, it was noted that two or more peaks were found for some amino acid-accepting activities, which indicates the presence and separation of isoacceptors of each tRNA in the preparation.

Effects of sodium chloride on chromatography at pH 8.0

It has been shown[7,8] that the resolution of tRNAs, and also aminoacyl-tRNAs, can be improved by the inclusion of sodium chloride in the phosphate buffer eluent at pH 6.8. Therefore, we analysed the effect of sodium chloride on the HAP chromatography of tRNAs at pH 8.0. As demonstrated in Figs. 7 and 8, addition of sodium chloride to the phosphate buffers had a significant effect on the retentions of the tRNAs on HAP; elution of $tRNA^{Phe\text{-}I}$ and $tRNA^{Phe\text{-}II}$ was facilitated by the addition of sodium chloride whereas that of $tRNA^{f\text{-}Met}$ and $tRNA^{Val}$ was retarded. No obvious change in the widths of the tRNA peaks was observed either with or without



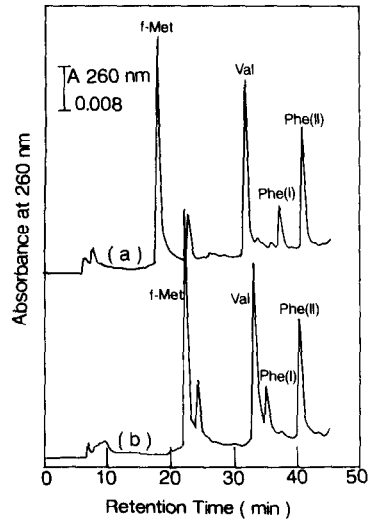


Fig. 7. Effect of NaCl on the retention of tRNAs on hydroxyapatite. A mixture of equal amounts of *E. coli* tRNAs was eluted from the analytical column at a flow-rate of 0.5 ml/min with a 30-min linear gradient of phosphate ion concentration from 15 to 83% B at pH 8.0. Retention times of the tRNA peaks were plotted against the concentration of NaCl included in the buffers. Buffer A, 5 m*M* phosphate buffer (pH 8.0) containing various concentrations of NaCl (0, 25, 50, 100, 200 m*M*); buffer B, 300 m*M* phosphate buffer containing NaCl at the same concentrations as in buffer A.

Fig. 8. Chromatography of a mixture of equal amounts of purified *E. coli* tRNAs (a) without and (b) with 0.2 *M* NaCl. The mixture of purified tRNAs was eluted from the analytical column at a flow-rate of 0.5 ml/min with a 30-min linear gradient of phosphate ion concentration from 40 to 80% B at pH 8.0 in or absence (a) or presence (b) of 0.2 *M* NaCl. Buffer A, 5 m*M* phosphate buffer (pH 8.0) with or without 0.2 *M* NaCl; buffer B, 300 m*M* phosphate buffer with or without 0.2 *M* NaCl.

sodium chloride, as demonstrated in Fig. 8. In this connection, $tRNA^{Total}$ of *B. subtilis* was chromatographed at pH 8.0 in the presence and absence of 0.2 *M* sodium chloride. The results indicated that there is no advantage in adding sodium chloride to the buffers for the chromatography of tRNAs on spherical HAP beads in phosphate buffer at pH 8.0.

DISCUSSION

tRNAs were retained more strongly on the beads with an increase in the pH of the mobile phase (phosphate buffer), which is opposite to the retention of proteins on the beads[11]. We also observed that λ-phage DNA would bind more strongly to the beads at pH 8.0 than pH 6.8 (unpublished observation). These findings indicate that the interactions between the calcium site of HAP and the phosphate group of tRNAs are strengthened at higher pH, probably owing to the ionization of the phosphate group in tRNAs and/or conformational changes of the tRNA molecules. The improvement in the resolution of tRNAs may be attributed to strong binding of tRNAs to the beads, as chromatography at higher concentrations of phosphate buffer may decrease the non-specific interactions of tRNAs on the beads and aid the selective elution of the individual tRNAs. In addition, the use of a steep phosphate ion gradient also aided the sharp elution of the peaks.

Inclusion of sodium chloride in the phosphate buffer eluent showed bifunctional effects on the retention of tRNAs on the HAP: elution of $tRNA^{f\text{-}Met}$ and $tRNA^{Val}$ was retarded whereas that of $tRNA^{Phe\text{-}I}$ and $tRNA^{Phe\text{-}II}$ was facilitated. In this connection, it is noteworthy that inclusion of sodium chloride in the phosphate buffer also had a bifunctional effect on the retention of proteins on the HAP beads; it weakened the binding of highly basic proteins such as lysozyme and chymotrypsinogen A on the beads whereas it strengthened the retention of highly acidic proteins such as chicken egg albumin[8]. Sodium chloride strengthened the binding of substances that have relatively weak interactions with HAP such as strongly acidic proteins and $tRNA^{f\text{-}Met}$, whereas it reduced the strong interactions between HAP and basic proteins and $tRNA^{Phe\text{-}I}$ or $tRNA^{Phe\text{-}II}$. Further investigations are necessary to clarify such bifunctional effects of sodium chloride on the retention of biomacromolecules on HAP. In the expectation of such bifunctional effects, rechromatography in the presence of sodium chloride might be effective for the purification of certain tRNAs.

The semi-preparative column retained tRNAs weakly compared with the retention of tRNAs on the analytical column. This may be due to the difference in the sintering temperature of HAP. Preliminary experiments indicated that protein retention on HAP was also affected by the sintering temperature. HAP prepared at higher temperatures showed a lower binding activity with acidic proteins such as bovine serum albumin, whereas binding of basic proteins such as lysozyme and cytochrome *c* on the HAP was hardly affected by the sintering temperature. Considering these results, the sintering temperature might have affected the properties of the calcium sites of the HAP. Incidentally, the effects of sintering temperature on the binding capacity of HAP for protein were demonstrated by Inoue and Ohtaki[12] and their results seem to be in agreement with our observations.

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