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REVERSE SALT GRADIENT CHROMATOGRAPHY OF tRNA ON UNSUBSTITUTED AGAROSE

II. FURTHER STUDY OF BINDING MECHANISMS

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SUMMARY

The existence of two mechanisms in the fractionation of tRNA on Sepharose 4B, using reverse gradients of ammonium sulphate, was confirmed by comparing yeast and *E. coli* tRNAs, and by extending the range of conditions used. The difference in elution range between the two kinds of tRNA is attributable mainly to variations in tight binding rather than adsorptive retardation. Variations of pH or of temperature produce not only changes in peak distribution, but overall shifts in the elution profile. The shifts correlate closely with changes in the solubility of tRNA in free solution, confirming that tight binding is associated with interfacial precipitation. On the other hand the degree of adsorptive retardation, as indicated by the difference between elution profiles from long and short columns, is quite similar under all conditions. It is if anything slightly greater at 25°C than at 5°C, implying a binding mechanism analogous to hydrophobic bonding. Binding-equilibrium studies suggest that the effect is related to the formation of a monolayer of tRNA on the agarose threads of the matrix. Experiments with hydroxyapatite also demonstrate adsorptive retardation, comparable in degree to that observed with Sepharose. This indicates that the effect may be much more important in ion-exchange chromatography than is normally assumed.

INTRODUCTION

In Part I[1] it was concluded that two mechanisms are involved in the fractionation of tRNA by reverse gradients of ammonium sulphate on Sepharose 4B. The first mechanism, believed to be interfacial precipitation, releases different tRNAs sequentially as the salt concentration is reduced. The second, which contributes largely to the high resolution of peaks obtainable by this technique, is a previously unsuspected adsorptive retardation of the kind more usually associated with the single-site binding of small molecules. The two mechanisms do not separate tRNAs in exactly the same sequence, so that they appear to be different in origin.

Any explanation of these effects must take account of reports[2,3] that the bind-

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ing of tRNA to Sepharose is much reduced at pH values away from the normally used value of 4.5, and that resolution appears to deteriorate as the temperature is raised[3]. In Part I[1] the elution profiles were all obtained at the same values of pH and temperature, so that possible variations in tRNA structure did not complicate the issue. In the present paper two sources of tRNA are compared, the range of conditions is extended, and elution profiles are compared with the solubility curves for tRNA in free solution.

MATERIALS AND METHODS

Sepharose 4B was purchased from Pharmacia. Batches denoted A, B, C etc. in the present paper were as in Table I of Part I[1]. *E. coli* tRNA was as used earlier[1]. Yeast tRNA (depleted of $tRNA^{Cys}$) was the gift of Professor C. J. O. R. Morris. Buffered solutions were adjusted in pH before addition of ammonium sulphate, which was "ultrapure" material from Schwarz/Mann. For work at 5°C, pH 7.5, solutions containing Tris–HCl were adjusted to pH 7.0 at room temperature to allow for the expected variation with temperature.

Solubility measurements were carried out at 5°C, using a water-bath regulated to ±0.5°C. Samples were made up at room temperature to a total volume of 5 ml and left overnight in the water bath; they were then centrifuged for 15 min at about 700 *g*. The supernatant concentration of tRNA was determined by spectrophotometry at 260 nm, diluting with 0.2 *M* ammonium sulphate and assuming a specific absorptivity ($A_1^{1\%}$) of 200.

Sepharose binding was measured in a similar way by adding to the mixture an aliquot from a settled slurry of Sepharose, previously equilibrated in a column with a solution containing 2 *M* ammonium sulphate and the appropriate buffer. The suspension was dispensed with a wide-bore volumetric pipette, specially calibrated to deliver the required volume; this was necessary because Sepharose tended to stick to the walls. Instead of standing in a water bath, tubes were left overnight at 5°C on a tube rotator (Stuart Scientific, Croydon, Great Britain) operating at *ca.* 60 rpm, and centrifuged as described above. From the measured volume of Sepharose after centrifugation, the concentration of agarose in the gravity-settled slurry was estimated to be 2.1 ± 0.2%.

RESULTS AND DISCUSSION

Comparison of yeast and E. coli tRNAs

Fig. 1 compares the fractionation of tRNAs from these two different organisms, using both long and short columns but the same batch of Sepharose throughout. It proved necessary to start the reverse gradient as high as 3.0 *M* ammonium sulphate because yeast tRNAs were less tightly bound, and also because the batch of Sepharose used bound all tRNAs rather less strongly than that used for the profiles in Fig. 6 of Part I[1].

The long-column profile of Fig. 1d differs markedly from that obtained by Morris[3] at pH 4.25, but in view of what is now clear about batch variations and other factors this is not very surprising. A more striking feature is the overall difference between tRNAs from yeast and *E. coli*; yeast tRNAs elute earlier in the gradient, and

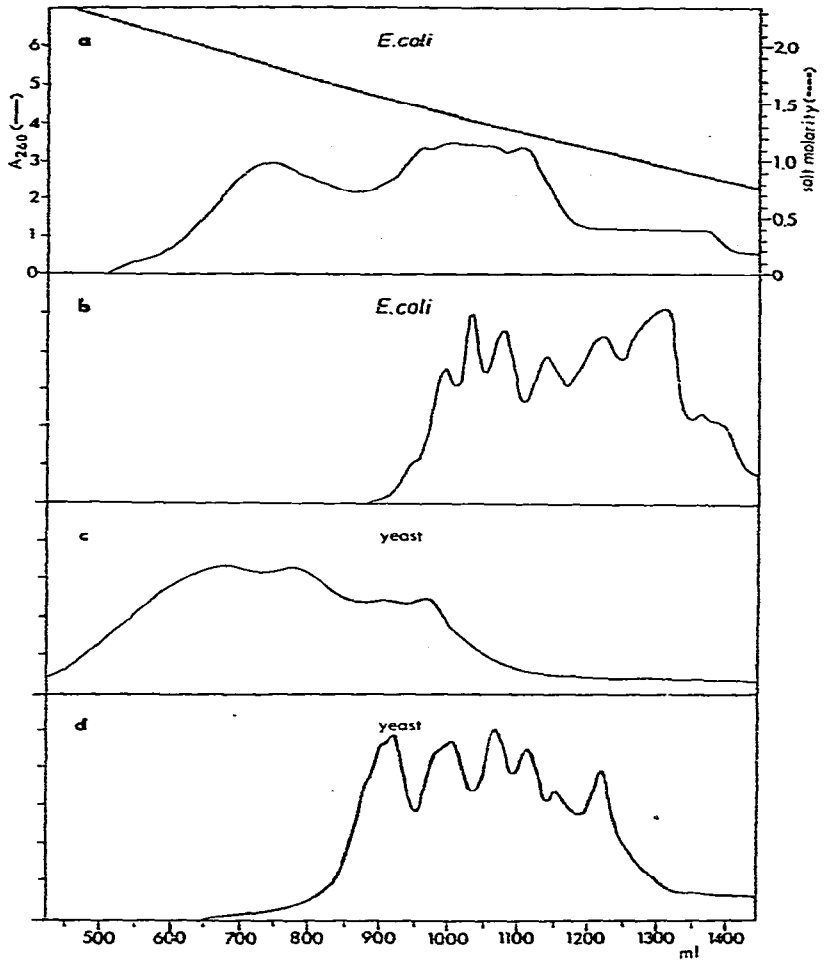

Fig. 1. Comparison of yeast and *E. coli* tRNA chromatography on Sepharose 4B (batch C) at 5 ± 0.5°C. All gradients were 2 × 1000 ml of ammonium sulphate from 3.0 *M* to zero; solutions also contained 10 m*M* magnesium chloride, 1 m*M* ethylenediaminetetraacetate (EDTA) and 10 m*M* sodium acetate buffer, pH 4.5. In (a) and (c) the column was 100 × 16 mm, in (b) and (d) 830 × 16 mm. Loads were 150 mg and flow-rates 15 ml h^{-1}. The plotted salt gradient applies to all experiments, and the volume shown is measured from the start of gradient elution from the bottom of each column.

Fig. 1 suggests that this is due mainly to a difference in the degree of tight binding rather than in the adsorptive retardation after release. The latter effect is equally strong in both cases.

Variations in profile with pH and temperature

Fig. 2 illustrates short- and long-column elution profiles of *E. coli* tRNA at 5°C, pH 4.5; 5°C, pH 7.5; and 25°C, pH 4.5. The apparent pH of the eluate in the second case was *ca.* 6.5 at 5°C, but this may have been an electrode artifact associated with the high concentration of salt[1]. The release of tRNA from the short column occurred earlier in the reverse gradient at pH 7.5 than at pH 4.5, and much earlier at

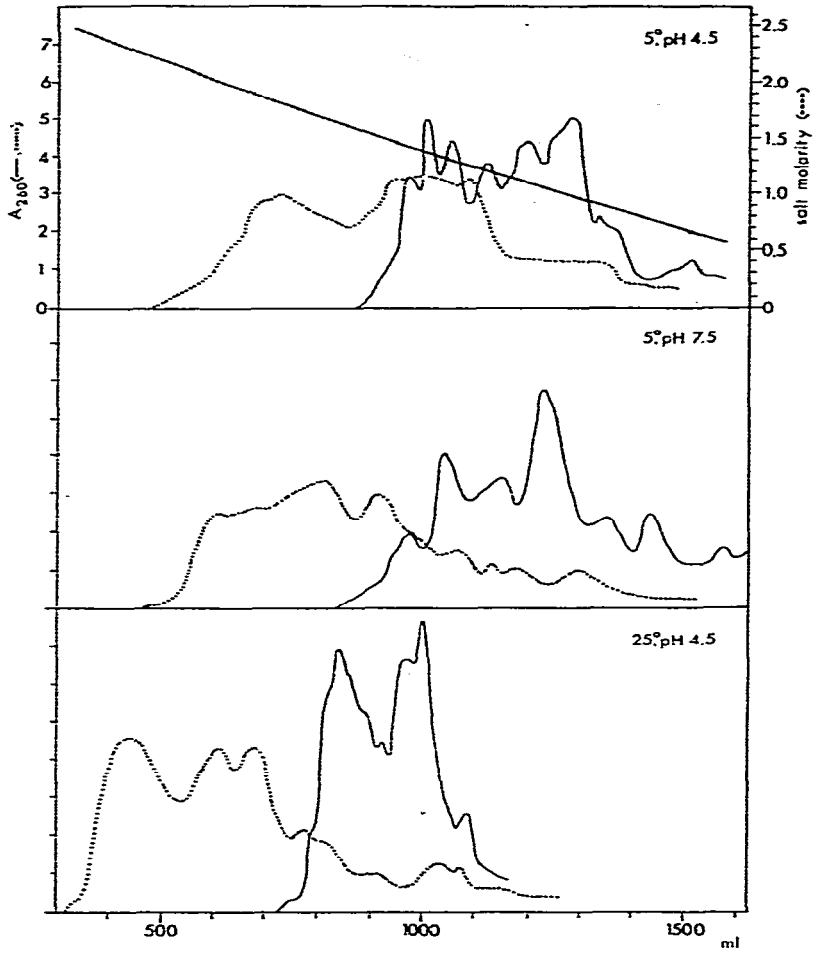

Fig. 2. Chromatography of *E. coli* tRNA on Sepharose 4B (batch C) under different conditions. Temperatures were controlled within ±0.5°C. In each case the dotted line represents elution from a short column (*ca.* 100 × 16 mm) and the solid line, elution from a longer one (*ca.* 800 × 16 mm). Conditions were otherwise as in Fig. 1, except that for pH 7.5 the buffer was 10 m*M* Tris–HCl.

25°C than at 5°C. However, the degree of adsorptive retardation after release, as shown by the difference between long- and short-column profiles, was similar in all three cases; indeed, the separation of the two kinds of profile was somewhat greater at 25°C than at 5°C. This reinforces the conclusion[1] that there are two distinct binding mechanisms at work.

Fig. 2 shows that resolution is not greatly impaired by elevated pH or temperature, provided that the gradient starts at a sufficiently high salt concentration. The failure of Holmes *et al.*[2] to obtain any fractionation at pH 7.5 is now seen to arise from a combination of two factors; firstly their gradient started at 1.3 *M* salt, so that interfacial precipitation did not occur; and secondly, they used (for these experiments only) a relatively short column of 150 × 15 mm, so that adsorptive retardation was not able to develop. The observations of Morris[3] with yeast tRNA may be similarly

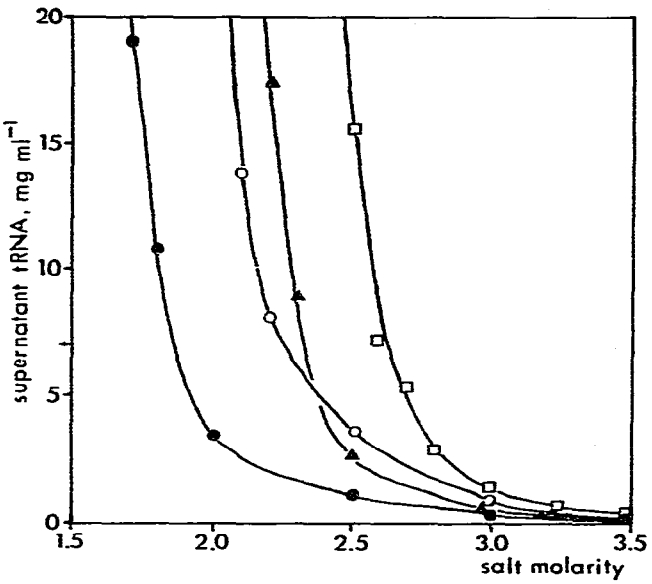

Fig. 3. Solubility of tRNA in ammonium sulphate. Solutions were as in Fig. 1; ●, *E. coli* tRNA, pH 4.5, 5°C; ○, yeast, pH 4.5, 5°C; ▲, *E. coli*, pH 7.5, 5°C; □, *E. coli*, pH 4.5, 25°C.

explained; his gradients all started at 2.0 *M* salt, which was probably too low for optimum resolution under some of the conditions used.

Solubility and binding studies

Fig. 3 illustrates measurements at two pH and temperature values of the solubilities in free solution of *E. coli* and yeast tRNAs, as a function of ammonium sulphate concentration. Because the tRNA from each organism contained a large variety of amino-acid accepting species it did not show a unique solubility; the amount left in solution at a given salt concentration depended on the total concentration of tRNA. The points plotted correspond to the onset of visible precipitation. It is clear that at pH 4.5 and a given salt concentration, yeast tRNA is considerably more soluble than material from *E. coli*; also for *E. coli* tRNA, the solubility is greater at pH 7.5 than at pH 4.5, and much greater at 25°C than at 5°C.

All these results are consistent with the hypothesis that a solubility-related process is important in the binding of tRNA to Sepharose, for the elution from short columns (where interfacial precipitation is presumed to be dominant) follows the same pattern of behaviour. Results reported here and in Part I[1] show that elution of *E. coli* tRNA is earliest at 25°C, pH 4.5 and latest at 5°C, pH 4.5, while similar intermediate behaviour is shown by *E. coli* tRNA at 5°C, pH 7.5 and by yeast tRNA at 5°C, pH 4.5.

The variations with pH are probably due to the combined effects of changes in ionization[4] and consequent changes in conformation[5]. However, comparison of Fig. 3 with the corresponding elution profiles (Figs. 1 and 2) shows that Sepharose induces binding at much lower salt concentrations than those at which tRNA precipitates from free solution. This is confirmed by Fig. 4, which illustrates the equilibria at-

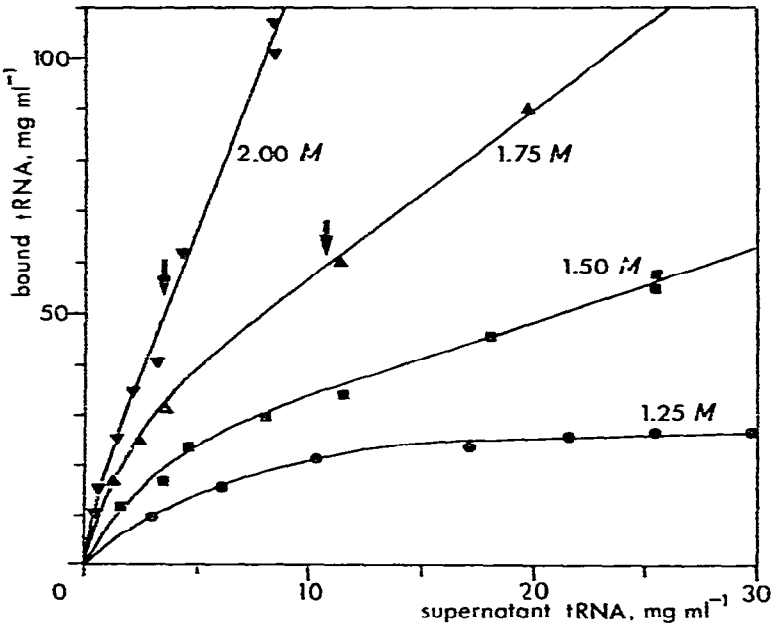

Fig. 4. Binding of *E. coli* tRNA to Sepharose 4B (batch E) at 5°C. All solutions contained pH 4.5 buffer and other salts as in Fig. 1. The ordinate represents the excess tRNA bound by 1 ml settled volume of Sepharose suspension. Arrows indicate the points at which precipitation begins in free solution (Fig. 3).

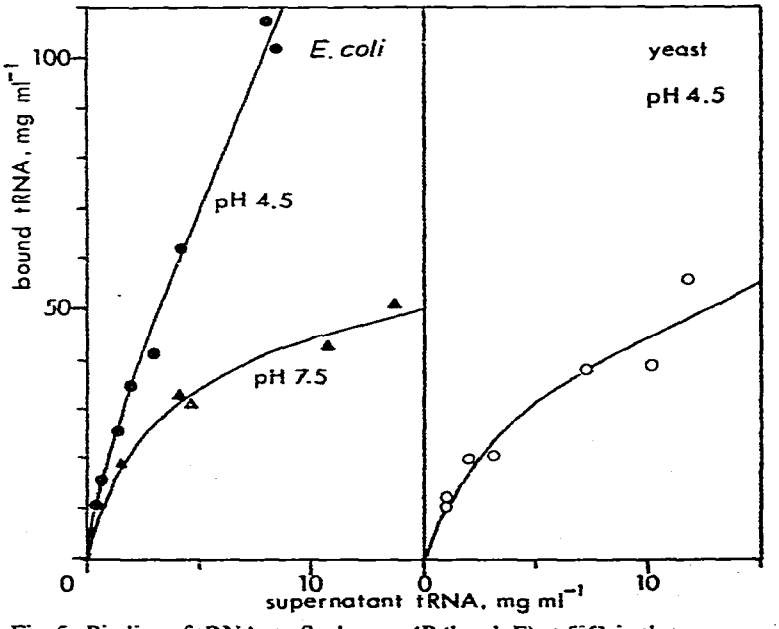

Fig. 5. Binding of tRNAs to Sepharose 4B (batch E) at 5°C, in the presence of 2.0 *M* ammonium sulphate. For further details see Fig. 4.

tained when Sepharose beads are added to solutions of tRNA and ammonium sulphate at pH 4.5. Strong binding occurs even at 1.25 *M* salt, where tRNA is highly soluble in free solution.

Fig. 5 compares the binding in 2.0 *M* salt of *E. coli* tRNA at pH 4.5 and pH 7.5 with that of yeast tRNA at pH 4.5. The last two curves resemble that obtained with *E. coli* tRNA at pH 4.5 and the lower salt concentration of 1.5 *M* (Fig. 4); it appears that a similar family of binding curves applies to each set of conditions.

At pH 4.5 and 1.25 *M* salt, the binding behaviour of *E. coli* tRNA (Fig. 4) approximates to the Langmuir isotherm expected when a monolayer of homogeneous solute attaches to the surface of a stationary phase. At all higher salt concentrations there is no plateau; such behaviour could be taken to indicate multi-layer adsorption, or more than one type of adsorption site, or a range of solute affinities for the substrate[6]. It seems almost certain that at the higher salt concentrations the curves are influenced by interfacial precipitation, but the different effects are not separable.

The origin of adsorptive retardation

Figs. 4 and 5 suggest that a Langmuir adsorption equilibrium, involving no more than a monolayer of tRNA on Sepharose, may be associated with the observed adsorptive retardation. Interfacial precipitation could differ from this because it involved intermolecular interactions between successive layers of tRNA, as well as direct interactions between tRNA and Sepharose. Molecules released from the multilayer precipitate could still then be susceptible to monolayer binding or retardation further down the column. It would thus be likely that tRNAs released together from the precipitate did not suffer the same subsequent retardation.

This hypothesis is strengthened by a numerical calculation from the binding data of Fig. 4. At 1.25 *M* salt, the binding levels off at a value of about 27 mg ml^{-1}. Since the slurry of Sepharose used in these experiments was about 2.1% (w/v) in agarose (see Materials and methods), the binding within a bead of 4% gel would be 51 mg ml^{-1}. If we accept the model for agarose structure proposed by Laurent[7], a 4% gel is composed of fibres with an average radius of 2.56 nm, and 1 ml of gel contains a fibre length of $2.36 \cdot 10^9$ m. Assuming a tRNA molecular weight of $2.6 \cdot 10^4$, this implies that the plateau region corresponds to a binding of one molecule for every 2.0 nm of agarose thread. This is roughly consistent with the known dimensions of tRNA[8], if one assumes that molecules are close-packed in linear arrays along the threads. An alternative calculation gives a thread surface area of 32 nm^2 per molecule.

Regarding the nature of the binding forces involved, we must reconsider the notion that something analogous to "hydrophobic bonding" is responsible. The idea was largely discounted by Morris[3] on the grounds that the observed binding became weaker as the temperature was raised, whereas hydrophobic forces are normally expected to become stronger. However, the data discussed here indicate that this is largely a consequence of changes in tRNA solubility, which influences the release of molecules from interfacial precipitate. The degree of adsorptive retardation *after* release is, as we have seen, at least as great at 25°C as at 5°C.

The binding of tRNA to agarose may be analogous to that occurring between the double helices of the agarose gel; in both cases, alterations in water structure could induce free energy changes favouring cohesion[9]. In agarose, the rigidity of the helices implies the absence of a configurational entropy term that favours solution[10].

Such effects (which could affect the balance either way) have been invoked to explain why flexible polynucleotides are less strongly bound to a substrate than more ordered molecules[11], but for tRNA it is reasonable to assume that under high-salt conditions (which are known to favour crystallization) the structure is stable.

It is significant that hydrophobic column substituents are known to enhance the binding of tRNA[12]; although agarose is hydrophilic, it does contain alternating residues of D-galactose and a more hydrophobic anhydrogalactose[13].

Comparison with hydroxyapatite

Since the use of reverse salt gradients is much less common than ion-exchange chromatography in positive-slope gradients, it was of interest to test whether adsorptive retardation was important in such methods. Comparable experiments were therefore performed with high-resolution hydroxyapatite[14], in which tRNA was bound at low ionic strength and displaced by a positive gradient of phosphate buffer[15]. The results led to the conclusion that adsorptive retardation was significant here as well:

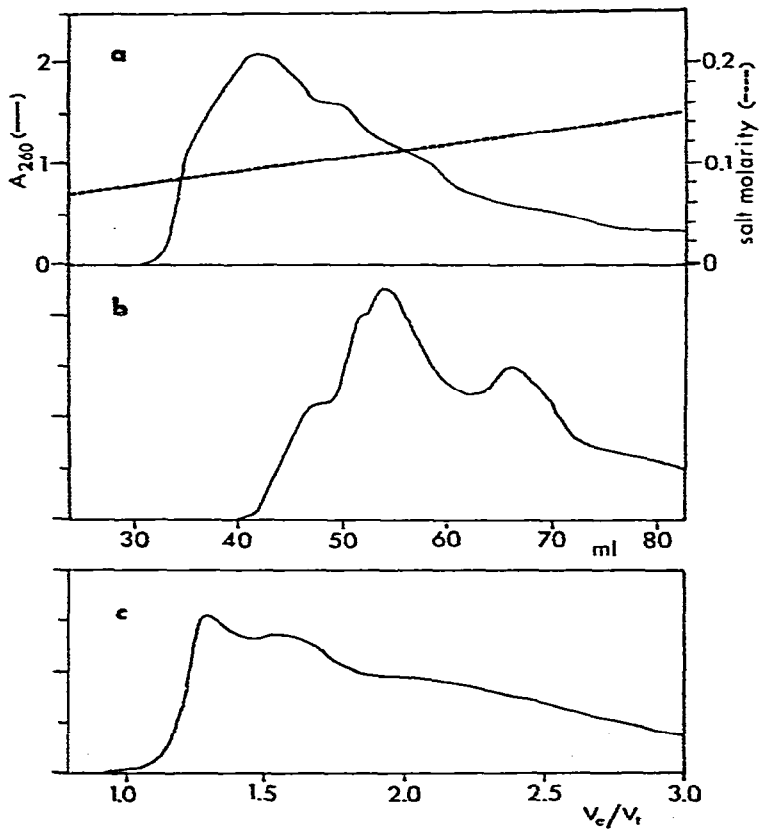

Fig. 6. Chromatography of *E. coli* tRNA on hydroxyapatite at 5°C. A solution containing 4 mg tRNA was applied, and eluted with sodium phosphate buffer, pH 7.0. (a) Gradient 2 × 60 ml of 0 to 0.2 *M* phosphate, column 30 × 9 mm; (b) as above but column 280 × 9 mm; (c) isocratic elution at 0.11 *M* from column 280 × 9 mm. Flow-rate, 3 ml h^{-1}. In (a) and (b) the volume plotted is measured from the start of gradient elution from the bottom of the column; in (c), V_t is the volume of liquid in the column and V_e the volume collected after application of the sample.

the profile for a long column was delayed compared with that of a short one and showed better resolution (Fig. 6a and b), while an isocratic run (Fig. 6c) showed retardation and apparent fractionation comparable to that found with Sepharose. This suggests that the theoretical arguments for neglecting adsorptive retardation in ion-exchange methods are of doubtful general validity. The binding of tRNA to hydroxyapatite should be a classic example of multi-site attraction; there is also evidence that binding is strongest when the linear dimensions of hydroxyapatite microcrystals approach those of a tRNA molecule[15]. The present results are, however, consistent with the empirical tradition among biochemists that even with gradient elution it is generally better to use a long, narrow column to obtain maximum resolution.

There are in fact precedents from the early history of ion-exchange chromatography, mentioned briefly in reviews[16], for the observation of partition retardation. In the development of cellulose derivatives for column chromatography Sober and coworkers[17,18] and Peterson[19] reported that when protein mixtures were applied to a column, some components were separable by elution at a constant salt concentration; most, however, required the application of a gradient of salt concentration and pH. The use of gradients subsequently became general in this field.

CONCLUSION

The unique character of the profile obtained under a given set of conditions shows the remarkable versatility of reverse salt gradient chromatography, and a better understanding of the underlying mechanisms should enable the method to be used to greater advantage. There remains an uncertainty about the origin of the batch variation, which affects mainly the adsorptive retardation effect. This effect seems on present evidence to be associated with Langmuir adsorption to the surfaces of agarose threads, by some mechanism analogous to hydrophobic bonding that presumably involves changes in the water layers surrounding substrate and solute.

The observed batch variations in adsorptive retardation (Part I[1], Fig. 7) could arise from either chemical or physical variations in the structure of the agarose gel. Although they might also arise from variable retention of the emulsifier used in manufacture, this seems to be ruled out by the persistence of the effects during repeated re-use of the material. The studies reported in Part III[20] were designed to test a number of possible causes.

The importance of adsorptive retardation in ion-exchange fractionation on hydroxyapatite raises the question of how general such an effect may be in the chromatography of large molecules, and there is clearly scope here for further study.

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