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REVERSE SALT GRADIENT CHROMATOGRAPHY OF tRNA ON UNSUBSTITUTED AGAROSE

I. VARIATIONS IN ELUTION PROFILE AND EVIDENCE FOR TWO FRACTIONATION MECHANISMS

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SUMMARY

E. coli tRNA was fractionated by the application of ammonium sulphate reverse gradients to Sepharose 4B. Variations in elution profile were partly attributable to differences between batches of Sepharose. The profile also varied with column length and gradient parameters. This suggests the existence of two distinct mechanisms which do not separate different tRNAs in the same sequence. The first mechanism, believed to be interfacial precipitation, releases tRNAs progressively as the salt concentration is reduced. A second mechanism introduces adsorptive retardation in which molecules lag behind the solvent. This process, widely believed not to be important in the chromatography of macromolecules with multiple binding sites, is in the present case mainly responsible for the improved resolution of peaks on passage down a long column. Isocratic (constant-salt) fractionation is also feasible. The Sepharose batch variation affects the second mechanism more than the first.

INTRODUCTION

Since the first reports of protein and tRNA fractionation by reverse salt gradients on unsubstituted agarose (Sepharose) there has been controversy about the binding mechanism involved. There have also been unexplained differences in behaviour between published accounts of its use. Since Sepharose carries few charges, ionic interactions are generally thought to be unimportant except in work at very low ionic strengths[1]. Gel filtration is assumed to be inoperative because the solute lags behind the solvent, and because the exclusion limit of the Sepharose used is always much higher than the molecular weights of the molecules being fractionated. Holmes *et al.*[2], working with *E. coli* tRNA, presumed by analogy with earlier work with proteins on valine-substituted Sepharose[3] that only hydrophobic interactions were involved. They were unable to explain on this basis why binding appeared negligible at pH 7.5 compared with that at pH 4.5. Other results from work with acidic proteins

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at pH 6.6 (ref. 4) were also interpreted as favouring hydrophobic interactions, on the grounds that the method required high concentrations of structure-forming anions such as sulphate.

Work with polynucleotides[5] also led to the conclusion that hydrophobic interactions were dominant above pH 4, though under more acid conditions the irreversible binding of poly(C) suggested that precipitation had occurred. However, von der Haar[6] proposed that the binding of proteins to Sepharose was due not to hydrophobic bonding but simply to interfacial salting out, precipitation being induced by contact with the gel surface; this can occur at a lower salt concentration than that required in free solution. Such effects were observed long ago in the chromatography of proteins on other materials[7].

A similar conclusion was reached by Morris[8], who fractionated yeast tRNA on unsubstituted Sepharose and other media under a wide variety of conditions. He found that the binding strength decreased markedly with rise in temperature, and argued that this made hydrophobic interactions unlikely. He also pointed out that unsubstituted agarose does not contain strongly hydrophobic regions. Morris concluded that "either an adsorptive or a selective solubility retention mechanism appears to be indicated". This does not of course rule out the possibility of multiple weak interactions with the methyl substituents that are present even in unmodified Sepharose.

Our interest in the high-salt Sepharose fractionation of tRNA arose from the fact that the order of elution of species is different from that found with other comparably gentle methods[9]. We started by following the procedure of Holmes *et al.*[2] using Sepharose 4B, which gives a better separation of species than other agarose-based media[8]. We found, however, that under standard conditions of pH and temperature the general form of the profile depended markedly on the length of the column and other parameters. The results presented here suggest that, at least for tRNA, there are two distinct separation processes: one due to interfacial salting-out and the other to differences in migration rate after release from the precipitate. There are also considerable variations between different batches of Sepharose. In Parts II[10] and III[11] the origin of these variations is discussed.

MATERIALS AND METHODS

Sepharose 4B was purchased from Pharmacia. Batches used in the present work were identified as in Table I, the date of manufacture being ascertained where possible from the manufacturer. tRNA from *E. coli* K12 strain CA 265 was obtained from the Microbiological Research Establishment (Porton, Great Britain).

Columns were operated under a hydrostatic head of 2 m in a room kept at 5 ± 2°C. For work with Sepharose batches C, D, E and F the columns were also insulated to keep the temperature variations within ±0.5°C. Eluent conductivity and ultraviolet absorbance were continuously monitored, and amino-acid acceptor activities determined as described elsewhere[9].

All solutions contained 10 m*M* sodium acetate buffer, pH 4.5, with 10 m*M* magnesium chloride and 1 m*M* EDTA (solution A)[2]. A rise in the apparent pH to *ca.* 4.9 was observed on addition of ammonium sulphate to 1 *M* or higher, but in view of uncertainties about the reliability of the pH electrode under these conditions no

TABLE I

BATCHES OF SEPHAROSE 4B

Designation in text	*Manufacturer's Lot No.*	*Year of manufacture*
A	Not recorded	Before 1977
B	Not recorded	Before 1977
C	Not recorded	Before 1977
D	8237	1975
E	5326	1978
F	11801	1979

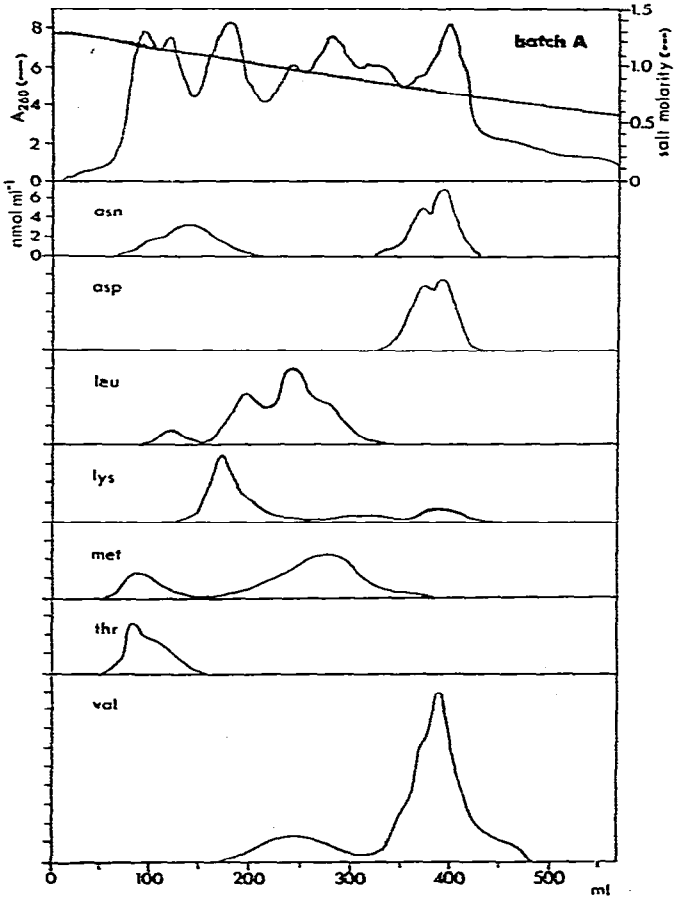

Fig. 1. Chromatography of *E. coli* tRNA on Sepharose 4B. A small volume of solution containing 150 mg tRNA (including *ca.* 25% water) was applied to a 1000 × 16 mm column and eluted with a 2 × 500-ml reverse gradient of ammonium sulphate from 1.3 *M* to zero in solution A. The flow-rate was 9 ml h^{-1}. In this and similar figures, the volume plotted on the horizontal axis is measured from the start of gradient elution from the base of the column. For further details see Materials and methods.

subsequent adjustment was made. The effect was unrelated to the grade of ammonium sulphate used, which in most of the work was "ultrapure" material from Schwarz/Mann. Impurities in this salt normally give an opposite shift in the pH; acetate buffer is also expected to show a slight depression of pH on addition of neutral salt. A comparable shift in pH was not observed when sodium chloride was used.

RESULTS AND DISCUSSION

Batch variations

In our early experiments we followed Holmes *et al.*[2] in using a reverse gradient at pH 4.5 starting at 1.3 *M* ammonium sulphate. We noted that after application of tRNA in solution a zone of light precipitation could be seen over the first few centimetres of the bed; this dispersed on application of the reverse salt gradient. Thus interfacial salting-out seemed certain to be at least one of the factors involved. The results obtained with *E. coli* tRNA (Fig. 1) showed good separation of species; the major peak of valine acceptance showed a particularly high purity of *ca.* 50%. The shape of the absorbance profile was not, however, the same in detail as that obtained (also with *E. coli* K 12) by the earlier workers, nor with that reported by others[12] for

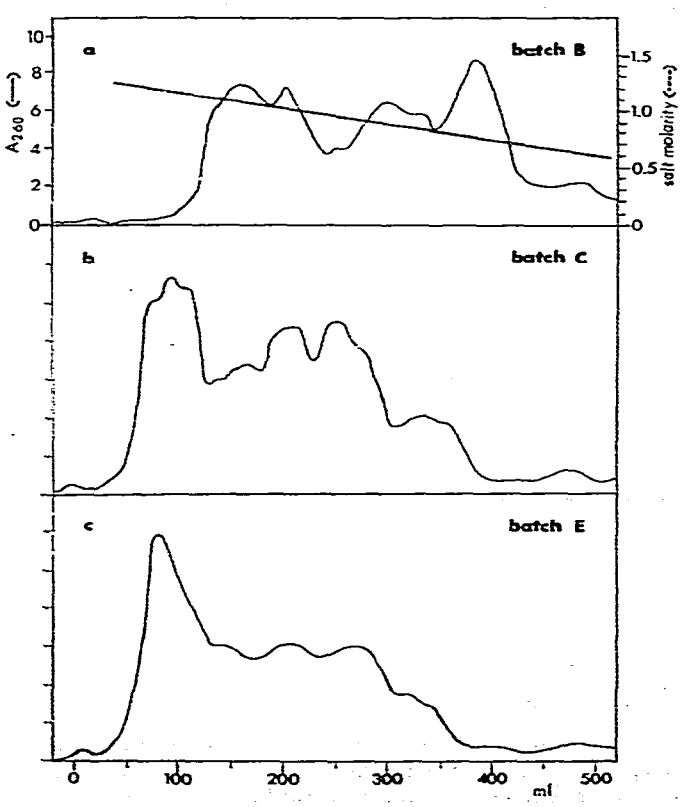

Fig. 2. Variations between batches of Sepharose 4B. Conditions were similar to those in Fig. 1 except that flow-rates were up to 30 ml h^{-1}.

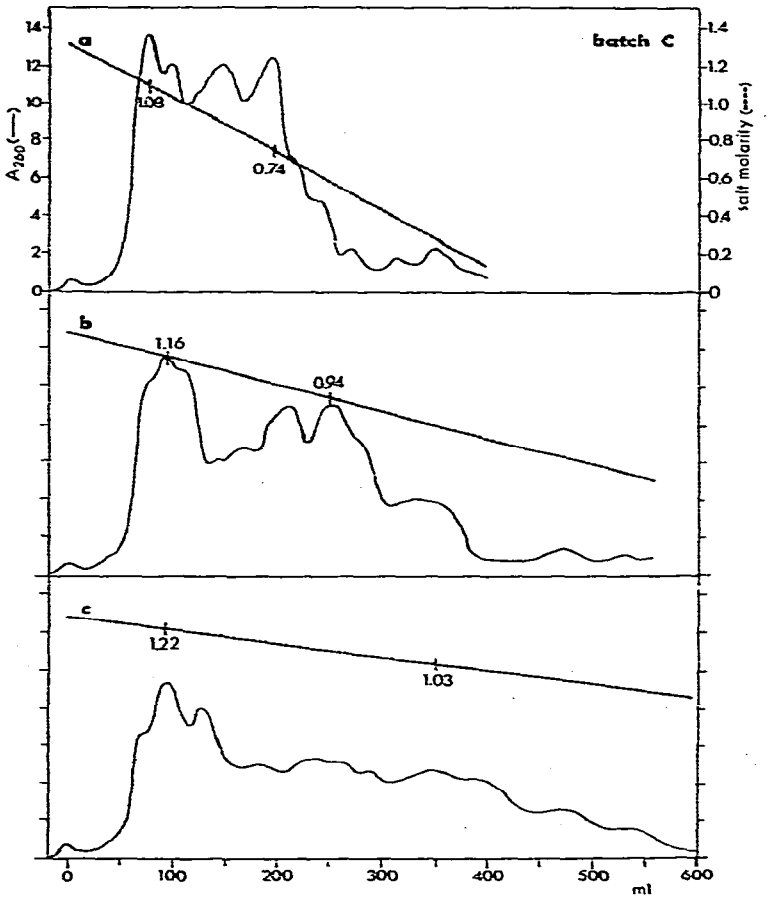

Fig. 3. Variations with gradient slope. Conditions were similar to those in Fig. 1, except that in (a) the gradient was 2 × 250 ml and in (c) it was 2 × 1000 ml. Flow-rates were 15 ml h^{-1}.

E. coli MRE 600. There were also some variations in the assay results; the leucine profile of Fig. 1 was not, for instance, the same as that in Fig. 4 of Holmes *et al.*[2]. Hjertén *et al.*[13] have reported similar discrepancies.

It became apparent after further work that these variations could partly be attributed to differences between the properties of different batches of Sepharose. Fig. 2 shows profiles obtained with three other batches under conditions similar to those of Fig. 1. Tests showed that a given profile was reproducible, even when different columns were prepared from the same batch. The flow-rate was not critical. However, no two batches gave exactly the same profile; both the distribution of peaks and the overall range of elution were different for each batch.

Variations with gradient slope and column length

We also found considerable variations in profile with the volume of the gradient. Fig. 3 illustrates this for batch C. Fig. 3a shows that a steeper gradient (obtained by halving the volume) did not simply compress the profile of Fig. 3b; there

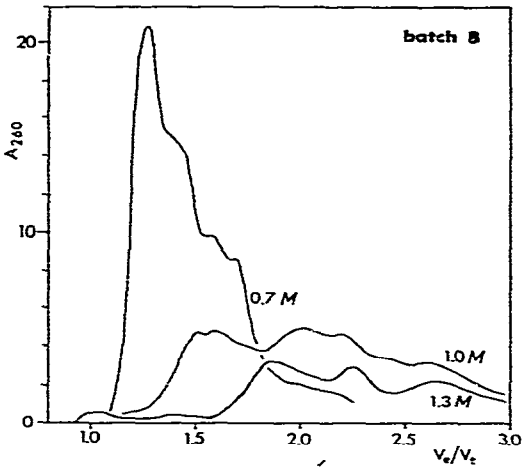

Fig. 4. Isocratic elution of *E. coli* tRNA from Sepharose 4B. A 150-mg sample was applied in a volume of 4 ml to a 1000 × 16 mm column and eluted with a constant concentration of ammonium sulphate as indicated. V_t is the volume of liquid in the column and V_e the volume collected after application of the sample; thus, unretarded material emerges at $V_e/V_t = 1$. Flow-rates were up to 20 ml h^{-1}.

was a redistribution of peaks, and also a shift in the overall range of elution. With the steeper gradient, elution occurred at lower salt concentrations than in Fig. 3b. Conversely, making the gradient more shallow as in Fig. 3c gave elution at higher salt concentrations. Doubling the column length while keeping the gradient volume the same had a similar effect (not illustrated) to halving the gradient volume. Halving or doubling the sample load had minor effects on the shape of the profile but did not change the elution range.

All this suggested that tRNA released from the column matrix lagged progressively behind the solvent as it moved down the column. We shall refer to this as *adsorptive retardation;* there is partition between the immobilized state when bound to the matrix and the free state when travelling with the solvent, and under given conditions an equilibrium distribution between the two states*. Such a mechanism is, of course, the basis of the classical chromatography of small molecules, but it is often stated[14,15] that with macromolecules having multiple strong binding sites there is usually an abrupt transition between high and low distribution ratios. Binding is thus expected to be an "all-or-nothing" process, and molecules once released should suffer little further retardation. Gradient elution is commonly employed to release sequentially the components in a mixture, rather than using isocratic elution at contant salt concentration.

Direct evidence of adsorptive retardation on Sepharose was obtained by isocratic elution at various salt concentrations. Fig. 4 (obtained with batch B, as in Fig. 2a) shows that a substantial fraction of the sample travelled at less than half the rate of the solvent; the higher the salt concentration, the greater the retardation. As expected, more material was released at the lower salt concentrations. What is most interesting is the existence of discrete peaks, suggesting that this might be a useful alternative method of fractionating tRNA.

* The effect is sometimes called "finite-adsorption equilibrium" to distinguish it from "tight adsorption".

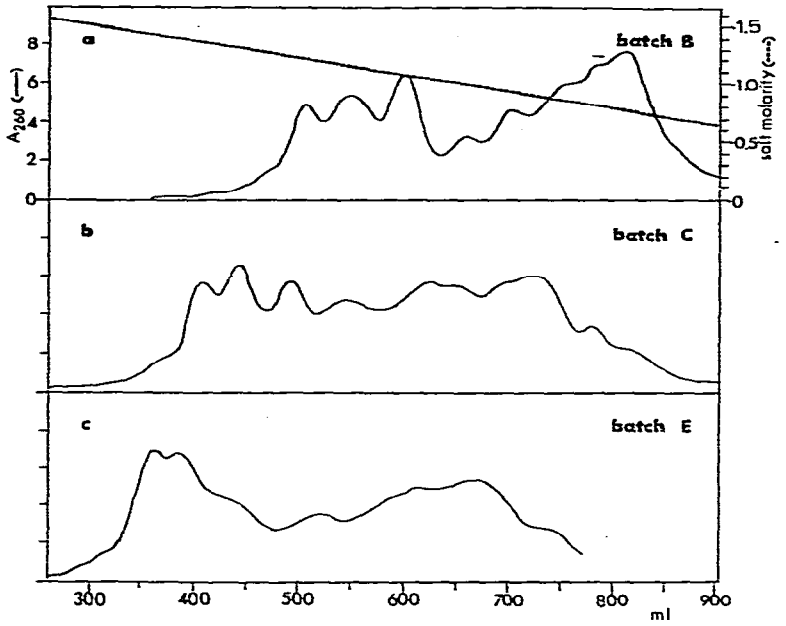

Fig. 5. Batch variations for a 2 × 750 ml reverse gradient of ammonium sulphate from 2.0 *M* to zero. Conditions otherwise similar to those in Fig. 2.

In view of this evidence for retardation it seemed likely that, although very little material eluted with the sample solvent when the gradient started at 1.3 *M* salt, it was not in fact all bound to the matrix at this salt concentration. The experiments illustrated in Fig. 2 were therefore repeated using gradients starting at 2.0 *M*. At this molarity some tRNA precipitated even before addition to the column. Fig. 5 shows that quite different profiles were then obtained; in particular, the large early peak given by batch E in Fig. 2c was resolved into multiple peaks by the more extended gradient in Fig. 5c. Evidently much of the material applied at 1.3 *M* salt was never fully immobilized on the column. Furthermore, even the later regions of the elution profile were not identical in the two cases. This suggested that there might be at least two distinct mechanisms at work, one responsible for "tight binding" and the other for adsorptive retardation after release, and that the various amino-acid accepting species of tRNA were not affected in the same way by the two mechanisms.

To attempt a separation of the two effects we compared elution from a short column with that from a much longer one, and conducted a limited number of assays for amino-acid acceptance. The same batch of Sepharose was used for each column. Elution was, as expected, much earlier in the gradient from the shorter column, and resolution was inferior to that obtained with the longer column. Results for the long column (Fig. 6b) were broadly similar to those shown in Fig. 1 for another long column, even though the batch and the gradient were not the same. However, for the short column (Fig. 6a and unpublished data for other acceptors), although most species eluted in a similar way there were some significant differences. Thus, although threonine acceptors eluted early from both long and short columns, the major valine acceptor eluted earlier in the short-column profile and the leucine profiles were quite different.

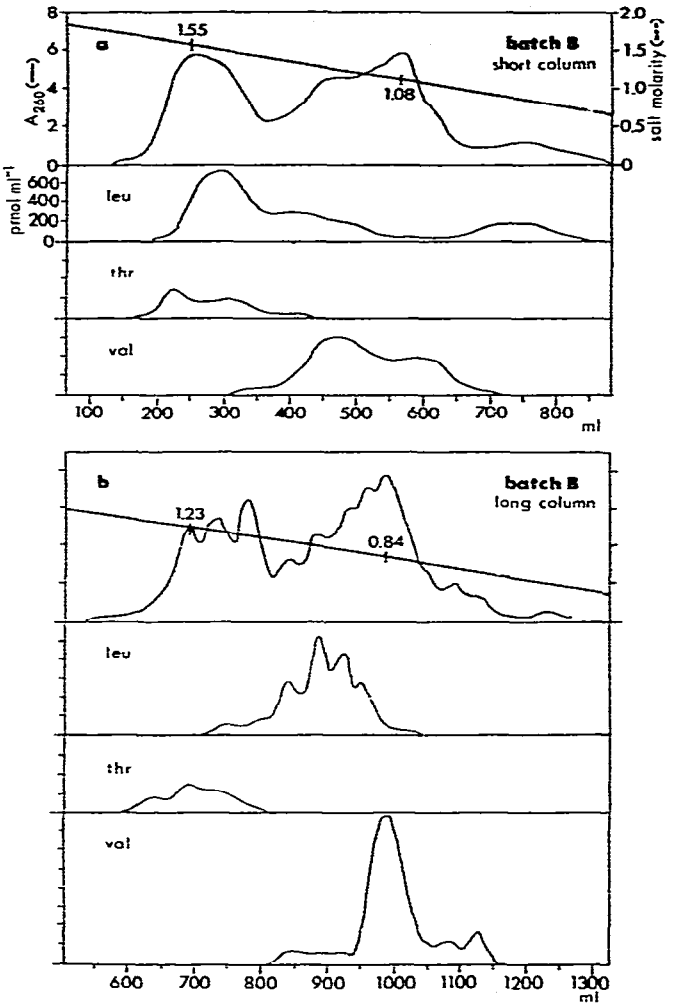

Fig. 6. Chromatography of 150 mg *E. coli* tRNA on Sepharose 4B, using 2 × 750 ml reverse gradients of ammonium sulphate from 2.0 *M* to zero. (a) Column, 100 × 16 mm; flow-rate, 72 ml h^{-1}. (b) Column, 1000 × 16 mm; flow-rate, 7 ml h^{-1}.

There are at least five isoacceptors for leucine[16], and only a full anticodon assay would show exactly what redistribution had occurred, but it is clear that the short and long columns did not retard all acceptors in the same sequence. There must therefore be some differences between the two mechanisms at work; with a single mechanism, changes in column length alone would not be expected to cause a redistribution of peaks.

This conclusion has some interesting corollaries. It must to some extent be possible to change the order of elution of species simply by varying the length of the column; if a species X is released ahead of species Y, it may still be overtaken by Y in its passage down a long column if Y suffers a lesser retardation after release. With a shorter column, however, X will emerge ahead of Y. Similarly, the application of a

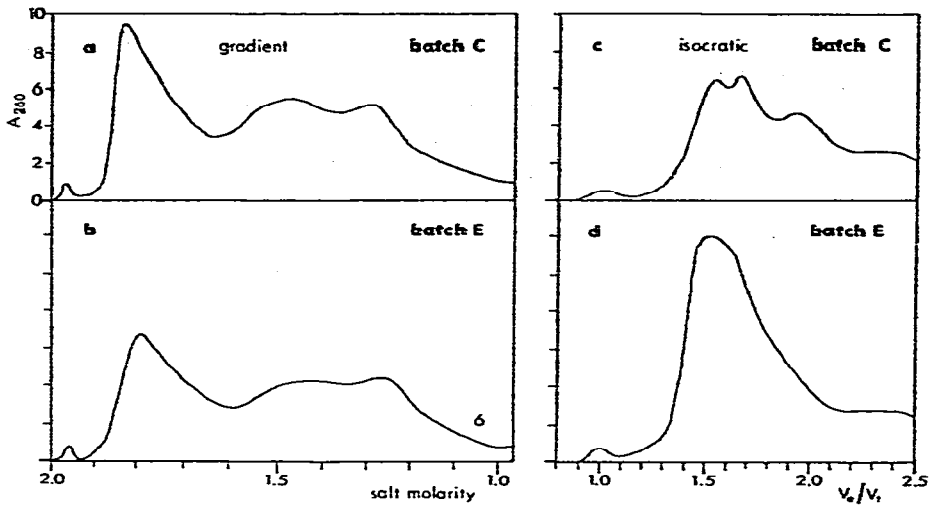

Fig. 7. Comparison of isocratic elution at 1.3 *M* ammonium sulphate, as in Fig. 4, with reverse gradient elution from 100 × 16 mm column as in Fig. 6.

gradient starting at a value lower than the limiting release molarity could change the order of elution, as suggested by comparing Figs. 2 and 5. Simply by varying parameters such as these one might achieve a higher degree of purification by successive refractionation. Yet another variant would be to follow isocratic elution by the application of a reverse gradient to the same column.

Further evidence for two distinct mechanisms is provided by Fig. 7. Between the two batches chosen there was little difference between the profiles obtained when a reverse gradient was applied to a short column: release from tight binding was similar in each case. With isocratic elution, however, batch C retarded more strongly than batch E, and gave a better resolution of peaks. This difference in adsorptive retardation is consistent with the long-column profiles of Fig. 5b and c.

The difference between the isocratic profiles in Fig. 7 is not simply attributable to a shift in the salt concentration associated with a given profile. Experiments at higher salt concentrations (not illustrated) with batches E and F, both of which gave a single peak at 1.3 *M* salt, showed more adsorptive retardation but failed to give the degree of resolution into multiple peaks that was characteristic of the other batches (Figs. 4 and 7, and unpublished data).

For the sake of completeness it would have been useful to perform gradient fractionation under conditions where the precipitation effect was negligible; one would then expect no variation of profile with column length. Unfortunately such experiments are not feasible, because to ensure the absence of precipitation one would have to start the gradient at a salt concentration well below the 1.3 *M* used in our initial experiments. Fig. 4 indicates that under these conditions, even for the exceptionally retarding batch B, the partition retardation would be so reduced in magnitude as to give inadequate resolution of peaks.

CONCLUSION

It is now clear that the differences between profiles obtained under different conditions are traceable to two causes: firstly a considerable batch variation in the range over which elution occurs, and secondly a surprisingly high degree of adsorptive retardation. This has previously obscured the fact that much of the tRNA is released from tight binding at higher salt concentrations than the values often used at the start of a reverse gradient.

The mechanism of fractionation seems to be a combination of two effects. There is evidently interfacial precipitation and sequential release of species by the reverse gradient, but subsequent adsorptive retardation has a profound effect on the profile and contributes much to the resolution of overlapping peaks. The variation between batches appears to lie much more in the adsorptive retardation than in the release from tight binding.

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