Journal of Chromatography, 159 (1978) 33–46
Chromatographic Reviews

CHREV. 110T17

FRACTIONATION OF TRANSFER RIBONUCLEIC ACIDS BY CHROMATOGRAPHY ON NEUTRAL POLYSACCHARIDE MEDIA IN REVERSE SALT GRADIENTS

COLIN J. O. R. MORRIS

Department of Experimental Biochemistry, London Hospital Medical College at Queen Mary College, Mile End Rd., London E1 4NS (Great Britain)

CONTENTS

1. INTRODUCTION

Although about 80 transfer RNAs (tRNAs) have been isolated from a wide range of organisms (including an almost complete set for the 20 protein amino acids from *Escherichia coli*), their nucleotide sequences determined, and about 10 crystallized, a detailed three-dimensional structure is available for only one of these, yeast $tRNA^{Phe}$ (refs. 1 and 2). The other crystalline tRNA preparations have not yielded X-ray diffraction photographs of a quality adequate for high-resolution structural analysis. Various reasons have been advanced to account for this, but in my view a major contributory factor has been inadequate checking of the crystalline preparations for homogeneity. Several crystalline tRNA preparations which we have examined by high-resolution gel electrophoresis have shown gross inhomogeneity. Crystallinity is of little significance for homogeneity in the tRNA field, as the basic similarity in general structure among the different species makes co-crystallization very probable. In fact a crystalline preparation has been prepared from a mixed tRNA probably containing 30–50 individual species[3]. In the second place it is significant that yeast $tRNA^{Phe}$ is uniquely easy to purify, so that large amounts have been available for intensive purification and studies of the optimum conditions for crystallization.

The structural basis for the recognition of a specific tRNA by its cognate ligase remains unknown (for a review see ref. 4) and it is quite possible that different recognition mechanisms are operative in different tRNA-ligase systems. Although interchangeability at the ribosomal sites during protein synthesis imposes fairly rigid

constraints on the overall shapes and dimensions of the tRNA species, it now seems unlikely that much progress will be made with the tRNA-ligase recognition problem until the three dimensional structures of at least a majority of the set of tRNAs from one species are known.

As a first step towards this aim we have attempted to develop in this laboratory a general scheme for the isolation of a number of highly purified tRNA species from a mixed preparation made from yeast by the method of Holley[5]. This extract is very heterogeneous, and probably contains 50–60 individual species as well as partially degraded molecules formed during extraction. Fig. 1 shows a two-dimensional gel electrophoresis of a mixed tRNA preparation. At least 40 individual spots may be seen, many of which may be complex, while about 25 constitute major species.

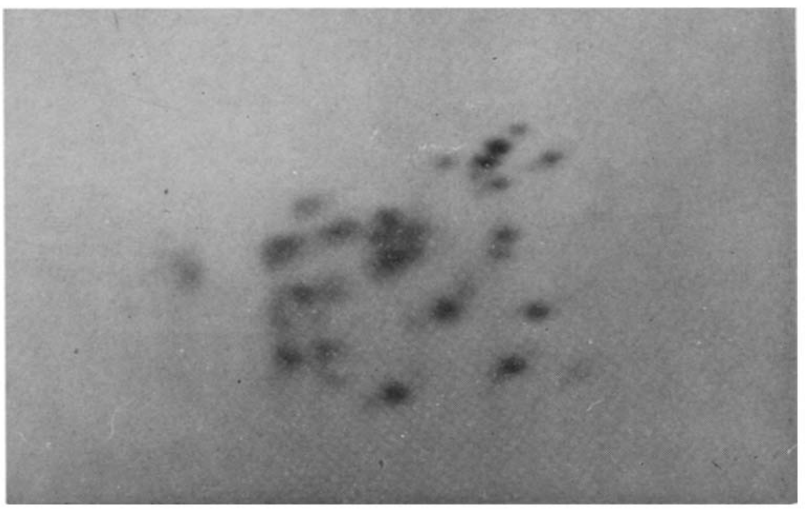

Fig. 1. Two-dimensional electrophoresis of mixed yeast tRNAs by the method of Varriccio and Ernst[7]. Start at top right. Horizontal dimension, Tris–borate–EDTA buffer (pH 8.3), 8 *M* urea. Vertical dimension, Tris–borate–EDTA buffer (pH 8.3).

It is evident that no single method can separate such a complex mixture of very similar components and that a sequential fractionation scheme will be required. The first stages of such a scheme are critical, as they should be relatively simple, capable of being scaled up to at least the gram scale, and if possible the initial fractions obtained should be suitable for introduction into the next stage without intermediate isolation of the solutes, a process which is invariably accompanied by losses and by the risk of enzymatic degradation. The main methods which have been used for tRNA separation are counter-current distribution, reversed-phase partition chromatography, ion-exchange chromatography and adsorption chromatography on materials such as hydroxyapatite and benzoyl-diethylaminoethyl (BD) cellulose. None of these satisfy all the criteria given above, and we have therefore studied in detail the method originally described by Holmes *et al.*[6]. This involves chromatography of the tRNA mixture on a column of Sepharose 4B in a gradient of decreasing $(NH_4)_2SO_4$ concentration in 10 m*M* acetate buffer (pH 4.25) containing 10 m*M* $MgCl_2$. The

method was originally applied to the fractionation of *E. coli* tRNAs, and has required modification for use with yeast tRNAs.

This paper presents an account of the optimization of the method, the subsequent use of a second stationary phase Ultrogel AcA 44, and of the separations achieved with the combined method.

2. MATERIALS AND METHODS

2.1. Materials

Sepharose grades 2B, 4B, 6B, 4B CL, Sephadex G-150 and Sephacryl S-200 were obtained from Pharmacia (London, Great Britain). Ultrogel grades AcA 34, 44 and 54 from LKB (Croydon, Great Britain). Bio-Gels P-2 and P-100 from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Spheron 1000 was a gift from Dr. Z. Prusík. Phenyl-Sepharose CL was a gift from Professor S. Hjertén. "Aristar" grade $(NH_4)_2SO_4$ (BDH, Poole, Great Britain) was used for the preparation of the salt gradients.

2.2. Chromatography

Gel chromatography was carried out in jacketed glass columns of diameters 0.6–2.3 cm and length 30–50 cm. Double-ended, plunger type columns were used for the larger diameters in order to minimize dead volumes. The columns were maintained at constant temperature during packing and operation. Sepharose chromatography was carried out in 10 m*M* acetate buffer (pH 4.25–4.30) containing 10 m*M* Mg^{2+} and 0.1% diethyl pyrocarbonate to act as a ribonuclease inhibitor. The column was packed with a suspension of the appropriate Sepharose grade in the acetate buffer and equilibrated with the same buffer containing 2.0 *M* $(NH_4)_2SO_4$ until the influent and effluent conductances were identical. The sample was dissolved in buffer and adjusted to 2.0 *M* $(NH_4)_2SO_4$ by addition of a 4.0 *M* solution of the salt in acetate buffer.

If a fraction was to be re-chromatographed directly, its electrolytic conductance was measured and solid $(NH_4)_2SO_4$ added to bring the concentration up to 2.0 *M*. The contributions of the other components of the buffer to the conductance were negligible. Sepharose columns were operated with a linear salt gradient from 2.0–0.5 *M* $(NH_4)_2SO_4$ in acetate buffer (pH 4.25) containing Mg^{2+}, while Ultrogel columns were operated in a linear gradient from 2.2–0 *M* $(NH_4)_2SO_4$ in the same buffer. Flow-rates were 11–12 ml/h·cm^2, and the total gradient volume was approximately 300 ml/cm^2 packed-bed surface area. Resolution appeared to be independent of column length above a minimum value. The tRNAs were recovered from selected fractions by exhaustive dialysis at 0° in Visking 18/32 cellulose tubing, concentration in a rotary evaporator and precipitation by the addition of two volumes of ethanol and about 4 drops of 3 *M* NaCl to the concentrate. The precipitates were stored overnight at 0°, collected by centrifugation, washed with ethanol and dried *in vacuo*.

2.3. Electrophoresis

Two-dimensional electrophoresis was carried out by the method of Varriccio and Ernst[7], in which migration in the first dimension is carried out in a 15 × 5% polyacrylamide gel containing 90 m*M* Tris–borate–EDTA buffer (pH 8.3) and 8 *M* in urea. The second migration is carried out in a 16 × 5% polyacrylamide gel slab,

using the same electrolyte without urea. Visualization of the tRNAs was achieved with the carbocyanine dye "Stains-all"[8].

One-dimensional electrophoresis was carried out on the long dimension of the LKB Multiphor gel electrophoresis apparatus, using the Tris–borate–EDTA buffer of Varriccio and Ernst[7] either with or without urea. Starts were made with 1×0.15 cm pieces of Schleicher & Schüll 2043a paper, wetted with solutions containing 0.5–2.0 μg tRNA, and dipped in liquid $16 \times 5\%$ polyacrylamide gel mixture immediately before laying on the starting position at the cathodic end of the gel slab. This starting method gave extremely good resolution of adjacent zones. Electrophoresis was carried out at constant power of 25 W (usually 750 V, 30–40 mA) at a coolant temperature of 10°.

2.4. Assay of amino acid acceptance activity

Assays were carried out as described by Hoskinson and Khorana[9] with minor changes as described by Gillam *et al.*[10] and by Holness and Atfield[11], using their cacodylate–ATP–Mg^{2+} buffer system, and incubating for 25 min at 38°. An aliquot was transferred to a paper disc, washed with 5% aqueous trichloroacetic acid, ethanol and ether, and radioactivity determined in a scintillation spectrometer, using a diphenylcarbazole–toluene scintillation fluid.

3. RESULTS

The effects of a number of changes in operating conditions were investigated in detail. The influence of pH was very marked. Insolubility in strong salt solutions precludes operation below about 3.3, as the isoelectric points of the tRNAs lie in the range 2.6–2.9 (ref. 12). The tRNAs are insufficiently retarded to give adequate resolution above pH 5. The operating pH is, however, critical for resolution in the latter part of the chromatographic profile (compare Fig. 6). Zones D and E were well resolved at pH 4.25, poorly resolved at pH 4.4, and merged into a single zone at pH 4.6. At pH 3.95, zone E although resolved from zone D trailed badly. The effect of pH in the Ultrogel system differed from that in the Sepharose system. In the former case changes of profile in the pH 4.0–4.6 range were more evident in the B and C region, changes in the latter part of the profile being less marked. The influence of pH on Ultrogel chromatography is obviously complex and requires further study.

The choice of chromatographic stationary phase was also very important. Fig. 2 shows that Sepharose 4B gave the best resolution under the standardized operating conditions, Sepharose 6B being only slightly worse, while Sepharose 2B and the cross-linked agarose gel Sepharose 4B CL were very inferior. Sephadex G-150, Sephacryl S-200 and the two beaded polyacrylamide gels Bio-Gel P-2 and P-100 gave virtually no fractionation. Phenyl-Sepharose 4B CL and Spheron 1000 gave some resolution but were inferior to Sepharose 4B. The composite agarose–polyacrylamide gel Ultrogel was however useful as shown in Fig. 3. In the manufacturers' AcA nomenclature the first numeral denotes the polyacrylamide content, while the second numeral indicates the agarose content. We examined only the 4% agarose grades AcA 34, 44 and 54. AcA 34 gave poor resolution with low retention. The less porous Ultrogel grades AcA 44 and 54 gave useful separations, similar to but distinct from those obtained with Sepharose 4B. Ultrogel AcA 44 proved to be very valuable for the further resolution of Sepharose 4B fractions.

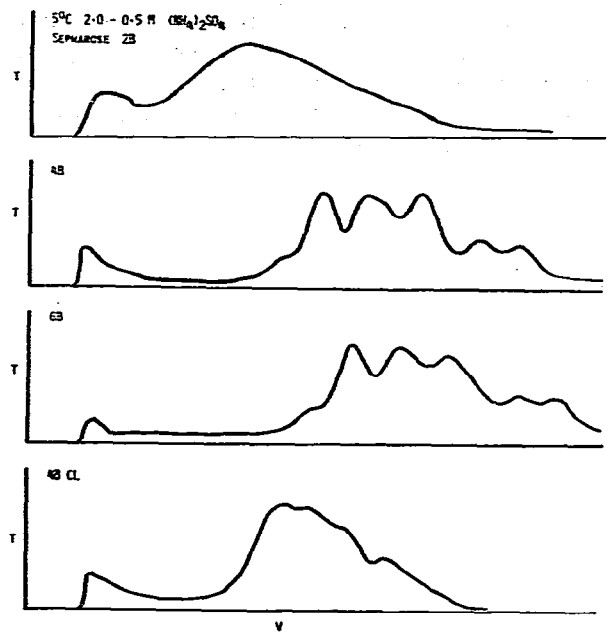

Fig. 2. Separation of mixed yeast tRNAs in a $(NH_4)_2SO_4$ gradient, 2.0–0.5 *M* on Sepharose 2B, 4B, 6B and 4B CL.

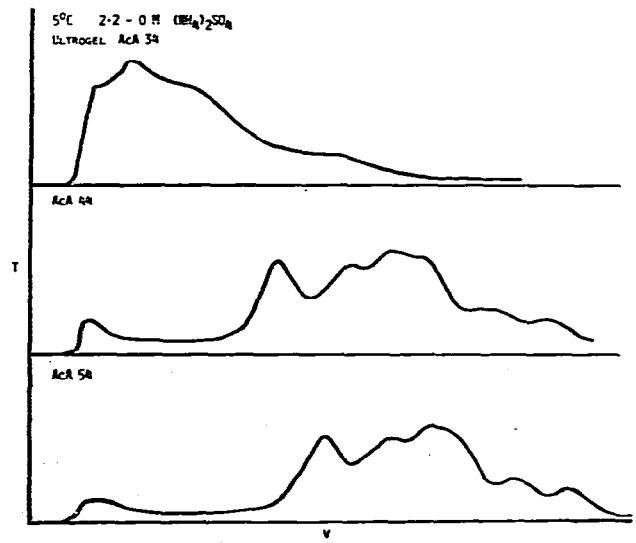

Fig. 3. Separation of mixed yeast tRNAs in a $(NH_4)_2SO_4$ gradient, 2.2–0 *M*, 5°, on Ultrogels AcA 34, 44 and 54.

The operating temperature also had a marked influence on these separations as shown in Fig. 4 for Sepharose 4B under the standard conditions at 5°, 10°, 20° and 35°. It is evident that both the retention volumes of individual zones and the distance between them decreases with increasing temperature, although the latter

effect is not uniform in the profile. These results are extremely important, since they indicate that the retention process is exothermic, and thus the free energy change of binding is primarily enthalpic rather than entropic. This in turn suggests that the binding mechanism is probably adsorption rather than hydrophobic interaction, a conclusion confirmed by the absence of hydrophobic sites in agarose. It will be shown later that this differential temperature effect may be used for fractionation.

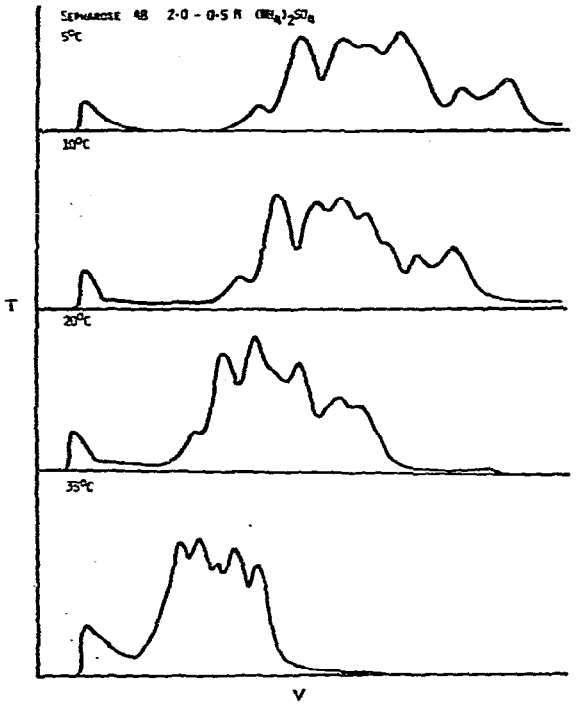

Fig. 4. Separation of mixed yeast tRNAs in a $(NH_4)_2SO_4$ gradient, 2.0–0.5 *M*, on Sepharose 4B at 5°, 10°, 20° and 35°.

Fig. 5 shows the similar temperature effect on retention by Ultrogel AcA 44, which in spite of the hydrophobic sites provided by the polyacrylamide content, also shows a negative temperature coefficient of binding. The even more hydrophobic phenyl-Sepharose 4B CL also has a negative temperature coefficient.

A typical separation of yeast tRNAs on Sepharose 4B under the standardized conditions of pH 4.25, 5°, and a flow-rate of 11–12 ml/h·cm^2 is shown in Fig. 6. This chromatographic profile was very reproducible over a wide range of column sizes and solute loads. Seven major zones are evident. The first zone PP, which is only slightly retarded, contains degraded RNA and will not be considered further here.

Fraction PreA was re-chromatographed twice on Sepharose 4B by the $(NH_4)_2SO_4$ concentration adjustment technique to reduce contamination with fraction A. The final chromatogram is shown in Fig. 7. This fraction contains high methionine acceptor activity, with minor contamination with arginine and valine acceptor activities. Gel electrophoresis (Fig. 8) confirms that this is a highly purified

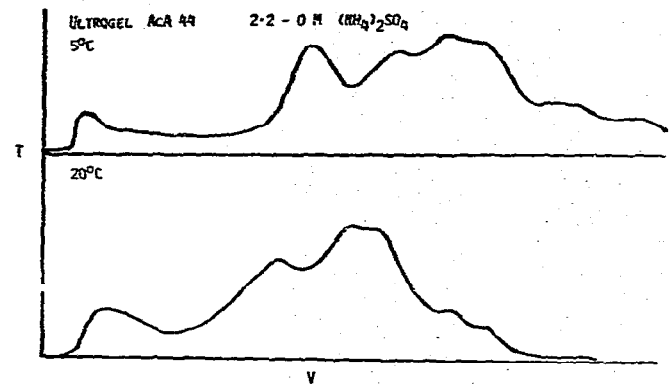

Fig. 5. Separation of mixed yeast tRNAs in a $(NH_4)_2SO_4$ gradient, 2.2–0 *M*, on Ultrogel AcA 44 at 5° and 20°.

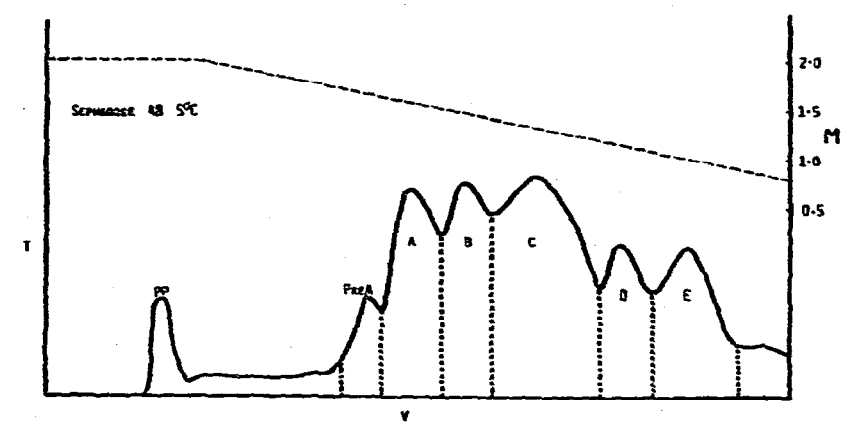

Fig. 6. Preparative separation of mixed yeast tRNAs on Sepharose 4B at 5° in a 2.0–0.5 *M* $(NH_4)_2SO_4$ gradient (— — —).

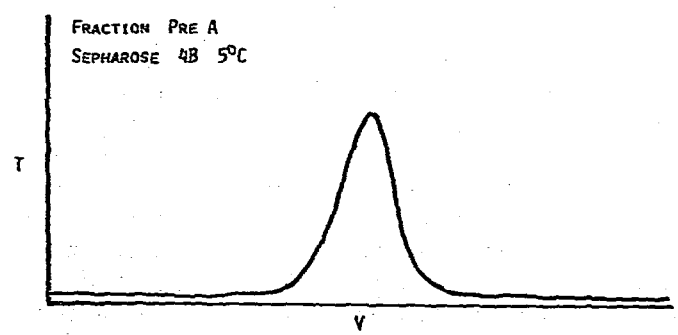

Fig. 7. Second re-chromatography of Fraction PreA on Sepharose 4B at 5° in a 2.0–0.5 *M* $(NH_4)_2SO_4$ gradient.

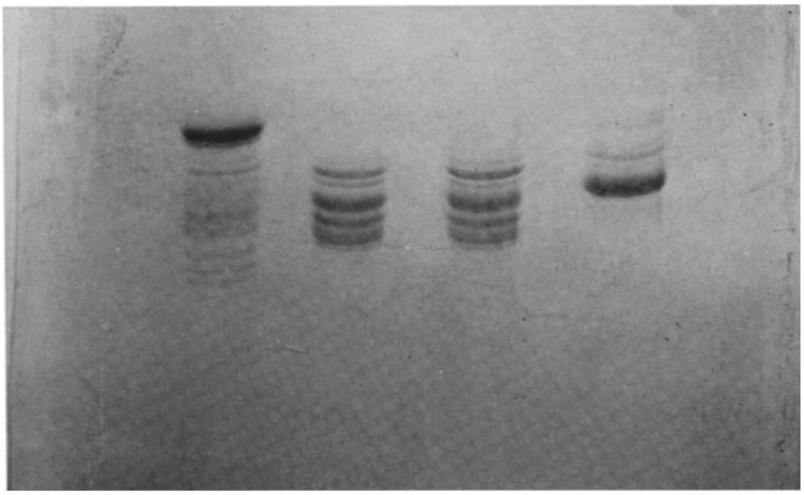

Fig. 8. Electrophoresis of Fractions PreA, A1 (front), A1 (rear) and A2 (left to right) at pH 8.3, 8 *M* urea.

$tRNA^{Met}$, which should readily be purified to homogeneity on an alternative chromatographic medium such as BD-cellulose[10].

Fraction A was re-run on Sepharose 4B to reduce contamination with fraction B, and then chromatographed on Ultrogel AcA 44 at 5° and in a 2.2–0 *M* $(NH_4)_2SO_4$ gradient to give two fractions A1 and A2 (Fig. 9). Fraction A1 had high tryptophan acceptor activity and substantial acceptor activities for threonine, arginine and valine. Fraction A2 had very high asparagine acceptor activity, with minor contamination with threonine, valine and lysine acceptor activity. This is confirmed by the gel electrophoresis of this fraction (Fig. 8). Samples from the front and rear of zone A1 together with a sample of zone A2 were run in this gel. Differences in acceptor activity and electrophoretic pattern through zone A1 appeared to be small. Contamination of the $tRNA^{Asn}$ in zone A2 was possibly due to traces of A1, and could probably be removed by re-chromatography on Ultrogel or in an alternative system.

Fraction B was extremely complex both in acceptor activities and electrophoretic pattern. It was enriched in glutamine, isoleucine and glycine acceptor activities but was refractory to further fractionation on either polysaccharide gel system.

Fraction C was also fairly complex, and could not be fractionated further on Ultrogel columns. It could however be separated into three fractions by making use of the temperature effect on Sepharose 4B. A fraction C obtained by an initial chromatography at 5° on Sepharose 4B was re-run after $(NH_4)_2SO_4$ concentration adjustment at 20° on the same stationary phase, with the results shown in Fig. 10. Fraction C1 was highly enriched in cysteine acceptor activity, which could be purified to homogeneity by chromatography on a reversed-phase partition system as shown by Holness and Atfield[11]. Fraction C2A was mainly enriched in tyrosine acceptor activity, but also contained substantial amounts of arginine, leucine and histidine acceptor activities. Fraction C2B also contained histidine and leucine acceptor activities. The electrophoretic patterns of the C fractions were clearly complex (Fig. 11).

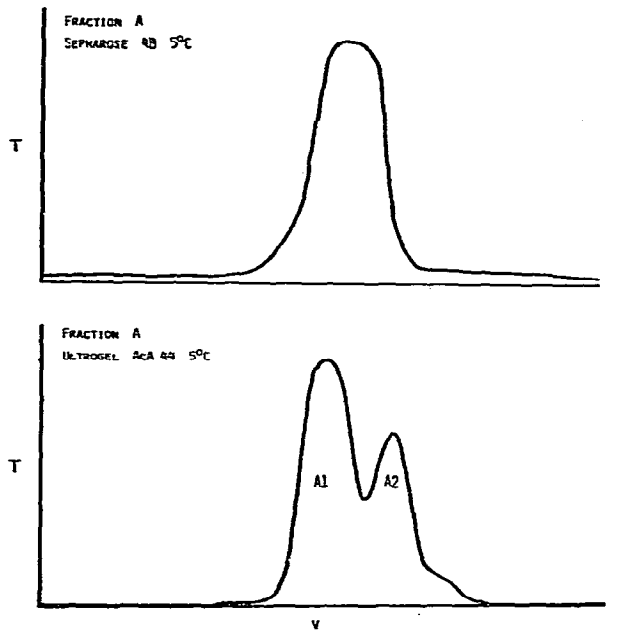

Fig. 9. Top: re-chromatography of Fraction A on Sepharose 4B at 5°, 2.0–0.5 *M* $(NH_4)_2SO_4$ gradient. Bottom: re-chromatography of purified Fraction A on Ultrogel AcA 44 at 5°, 2.2–0 *M* $(NH_4)_2SO_4$ gradient.

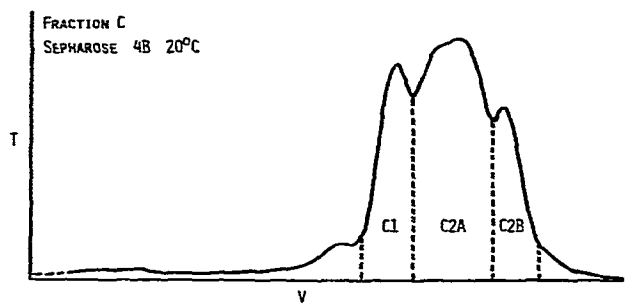

Fig. 10. Re-chromatography of fraction C on Sepharose 4B at 20°, in a 2.0–0.5 *M* $(NH_4)_2SO_4$ gradient.

Fraction D was less complex, and could be further separated on Ultrogel AcA 44 at 5° into two fractions D1 and D2 as shown in Fig. 12. Fraction D1 contains the major part of the histidine acceptor activity of the total tRNA mixture, but several minor contaminants including proline and lysine acceptor activities. Fraction D2 also contains high lysine and histidine activities. The electrophoresis diagram confirms that the last separation is relatively inefficient (Fig. 13).

Fraction E was relatively simple in composition and was re-run on Sepharose 4B at 5° to reduce contamination with Fraction D. It was further fractionated on Ultrogel AcA 44 at 5° in a 2.2–0 *M* $(NH_4)_2SO_4$ gradient to give three fractions as

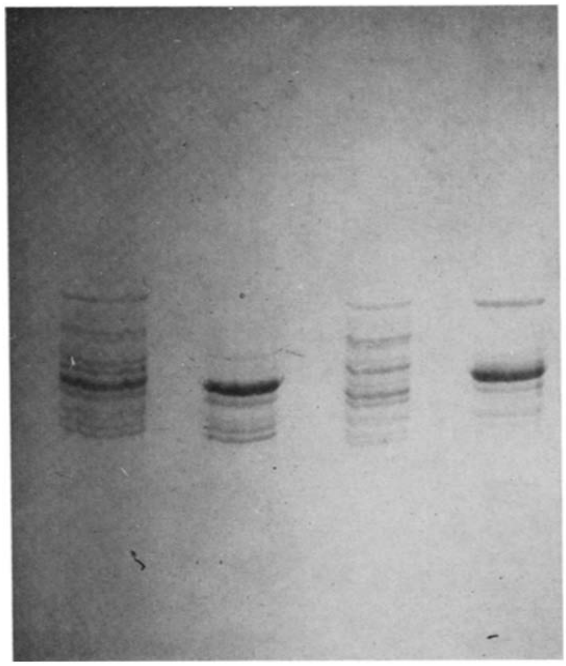

Fig. 11. Electrophoresis of (left to right) fractions C, C1, C2A and C2B at pH 8.3, 8 *M* urea.

shown in Fig. 14. Fractions E1 and E3 were relatively pure as demonstrated by the electrophoretic patterns shown in Fig. 15. Fraction E1 contains mainly proline acceptor activity with minor histidine acceptor activity. It should be relatively easy to purify to homogeneity. E2 is complex with high serine and proline acceptor activity, and minor alanine acceptor activity. The serine activity is probably associated with the three slow moving bands shown in Fig. 15. Fraction E3 appears to be virtually homogeneous by electrophoresis and amino acid acceptor assay. It is a $tRNA^{Ala}$ species, and could probably be purified from trace impurities by chromatography on BD cellulose. The results of these separations are summarised in Table 1.

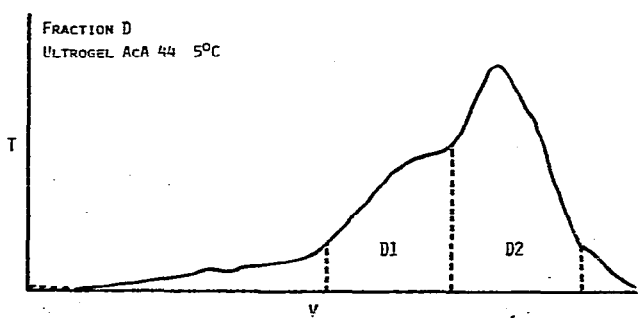

Fig. 12. Chromatography of fraction D on Ultrogel AcA 44 at 5°, in a 2.2–0.0 *M* $(NH_4)_2SO_4$ gradient.

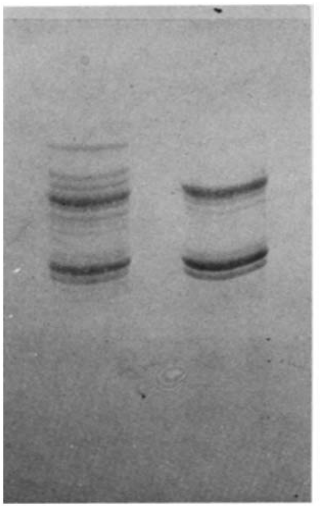

Fig. 13. Electrophoresis of (left to right) fractions D1 and D2 at pH 8.3, 8 *M* urea.

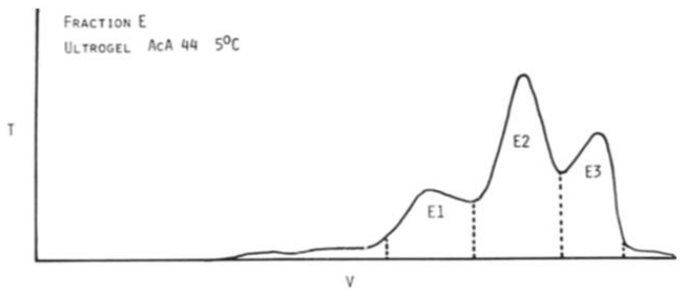

Fig. 14. Re-chromatography of purified Fraction E on Ultrogel AcA 44 at 5° in a 2.2–0 *M* $(NH_4)_2SO_4$ gradient.

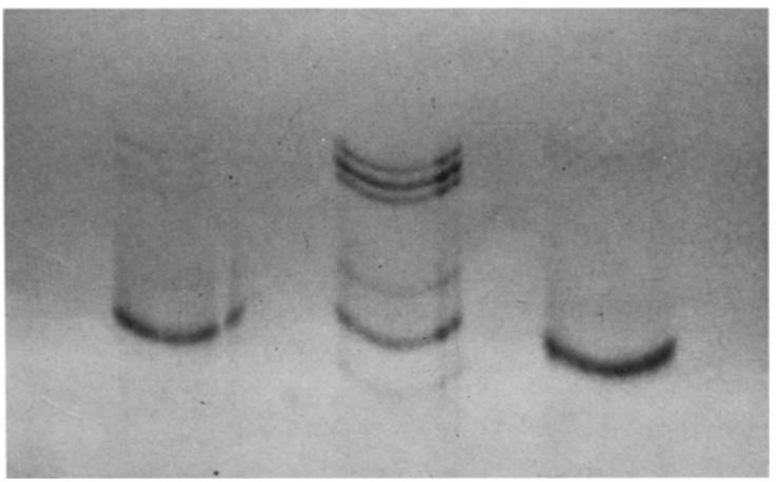

Fig. 15. Electrophoresis of (left to right) fractions E1, E2 and E3 at pH 8.3, no urea.

TABLE 1

AMINO ACID ACCEPTANCE OF SEPHAROSE AND ULTROGEL FRACTIONS

Glutamic and aspartic acid acceptor assays were unsatisfactory, and the results are not included in the Table. Results are graded from + (significant) to ++++ (very high acceptor activity).

Amino acid	*Fraction*											
	PreA	*A1*	*A2*	*B*	*C1*	*C2A*	*C2B*	*D1*	*D2*	*E1*	*E2*	*E3*
Alanine					+			+	+		+	++++
Arginine	+	+++				++						
Asparagine		++	++++									
Cysteine					++++	+						
Glutamine				+++	+	++						
Glycine				+++	+	++	+					
Histidine						++	++	+++	++	++		
Isoleucine				+++		+	++					
Leucine					+	++	++					
Lysine			+		+			+	++			
Methionine	++++	+		+				+				
Phenylalanine						+						
Proline								+	+	++++	++	
Serine						++	++		++		+++	
Threonine		+++	+									
Tryptophane		++++										
Tyrosine						+++	+					
Valine	+	++	+			+		+				

4. DISCUSSION

The relatively simple chromatographic system described here can provide relatively pure preparations of tRNAs for four acceptor activities, *viz.*, for alanine, asparagine, methionine and proline, and enriched concentrates for two further species, tryptophane and cysteine.

Its particular advantages for preparative purposes lies both in the ability to scale up with reproducibility of the chromatographic profile, and the almost unique facility with which chosen fractions can be re-chromatographed without desalting or isolation of the solutes, by simple adjustment of their salt concentrations to the chosen initial value (2.0 or 2.2 *M*). Ultrogel AcA 44 provides a useful alternative to Sepharose 4B for this purpose. This facility for re-chromatography makes it profitable to explore multiple re-runs on a single stationary phase as a means of eliminating minor impurities. This method has not been fully explored in the past, since the necessity for desalting and recovery of solutes between stages makes it more attractive to investigate alternative stationary phases. It must however be emphasized that the advantages of cascade operation apply equally to multiple re-chromatography on a single phase.

The mildness of the operating conditions is demonstrated by the fact that both Holmes *et al.*[6] and Nygard and Hulten[13] have been able to chromatograph aminoacyl tRNAs in the Sepharose 4B system with good recoveries.

The mechanism of tRNA chromatography on these neutral polysaccharide columns remains obscure. Molecular sieving effects appear to be unimportant, since although Sepharose 4B (exclusion limit $\approx 3 \cdot 10^6$) gives optimal separations, Ultrogel AcA 54 (exclusion limit $\approx 7 \cdot 10^4$) also gives good resolution. Sepharose 2B (exclusion limit $\approx 25 \cdot 10^6$) gives very poor resolution. Cross-linked gels appear to give poor separations both in the polygalactose series (Sepharose 4B CL) and in the polyglucose series (Sephacryl S 200). Satisfactory chromatography has in fact only been demonstrated with agarose derivatives with two free *cis* hydroxyl groups in a galactose unit. Since the exothermic nature of the retention process on both stationary phases has been demonstrated, either an adsorptive or a selective solubility retention mechanism appears to be indicated. The marked increase of the binding of the E fraction to Sepharose 4B over the narrow pH range 4.0–4.4 favours the latter mechanism as the rapidly increasing ionisation of the cytosine (pK_a 4.1) and adenosine (pK_a 3.8) amino groups in this pH range will tend to reduce the net charge on the tRNA molecule, and thus to reduce its solubility in strong salt solutions. Mixed tRNAs are in fact virtually insoluble in 2.0 *M* $(NH_4)_2SO_4$ in their isoelectric region of pH 2.6–2.9, even in the absence of gel media. However, since it has been demonstrated in this work that successful chromatography of the tRNAs is critically dependent on the nature of the gel stationary phase employed, it is very probable that both the extent and the steric configuration of the accessible internal surfaces within the gel bead are extremely important in the retention process. Unfortunately these factors are very difficult to assess independently.

5. ACKNOWLEDGEMENTS

I am indebted to my wife, Mrs. P. Morris, for the greater part of the experimental work on which this paper is based, and to K. W. M. Davy for carrying out the amino acid acceptance assays.

6. SUMMARY

A sequential scheme for the fractionation of yeast transfer RNAs by chromatography on the polysaccharide gel media Sepharose 4B and Ultrogel AcA 44 in reversed concentration gradients of ammonium sulphate is described and the optimum conditions worked out. Four species, $tRNA^{Met}$, $tRNA^{Asn}$, $tRNA^{Pro}$ and $tRNA^{Ala}$ are obtained in a highly purified form, while several other acceptor species are highly concentrated in the subfractions. The mechanism of this chromatographic system is discussed.

REFERENCES

1 G. J. Quigley, A. H. J. Wang, N. C. Seeman, F. L. Suddath, A. Rich, J. L. Sussman and S. H. Kim, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 4866.
2 J. E. Ladner, A. Jack, J. D. Robertus, R. S. Brown, D. Rhodes, B. F. C. Clark and A. Klug, *Proc. Nat. Acad. Sci. U.S.*. 72 (1975) 4414.
3 J. R. Fresco, R. D. Blake and R. Langridge, *Nature (London)*, 220 (1965) 1285.
4 R. W. Chambers, *Progr. Nucl. Acid Res. Mol. Biol.*, 11 (1971) 489.
5 R. W. Holley, *Methods Enzymol.*, 12A (1967) 596.
6 W. M. Holmes, R. E. Hurd, B. R. Reid, R. A. Rimerman and G. W. Hatfield, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 1068.
7 F. Varriccio and R. J. Ernst, *Anal. Biochem.*, 68 (1975) 485.
8 A. E. Dahlberg, C. W. Dingman and A. C. Peacock, *J. Mol. Biol.*, 41 (1969) 139.
9 R. M. Hoskinson and H. G. Khorana, *J. Biol. Chem.*, 240 (1965) 2129.
10 I. Gillam, S. Millward, D. Blew, M. Von Tigerstrom, E. Wimmer and G. M. Tener, *Biochemistry*, 6 (1967) 3043.
11 N. J. Holness and G. N. Atfield, *Biochem. J.*, 153 (1976) 429.
12 C. J. O. R. Morris, unpublished results.
13 O. Nygard and T. Hultin, *Prep. Biochem.*, 6 (1976) 339.