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HYDROXYAPATITE FOR CHROMATOGRAPHY

III. CATION AND pH EFFECTS ON FRACTIONATION OF tRNA FOR CRYSTALLIZATION

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SUMMARY

Hydroxyapatite (HA) prepared by a new method was applied to the rapid fractionation of *E. coli* tRNA. Elution at 5° with sodium or potassium phosphate, at two pH values, showed that the order of elution depended on both the buffer cation and the pH. These variations, and that due to temperature, were attributed to changes in tRNA structure which altered the pattern of exposed phosphate charges. The molarity at which tRNA started to elute was lowest at high pH, suggesting that HPO_4^{2-} was more effective than $H_2PO_4^-$ in displacing tRNA from HA. tRNA fractions from a column of benzoylated DEAE-cellulose were fractionated on HA to give nine species of purity 850–1600 pmoles per A_{260} unit, four of which could be crystallized.

INTRODUCTION

A number of published methods for making hydroxyapatite (HA) have claimed improvements in reproducibility, but commercial products based on such methods are often defective in flow-rate or binding properties or both[1]; this has led to rather less use of HA than might be expected from its unique binding mechanism, which makes it so useful when combined with other methods of purifying macromolecules. In Part II of this series[2] we have seen that variability can greatly be reduced by a fuller understanding of HA chemistry; an improved method has also been described in which flow-rate and resolution can be varied at will, while maintaining high affinity for the tRNA used as a test substance.

In this paper we describe fractionation of tRNA at two pH values, with each of two phosphate salts. We also shows how the use of HA combined with benzoylated DEAE-cellulose (BDC) can yield a number of tRNA species of sufficient purity to be crystallized for investigation by X-ray diffraction. Crystallization of two of these species has not previously been reported.

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MATERIALS AND METHODS

Column chromatography

HA used in this work was made in the course of developing the new method described in Part II[2], in which conversion of brushite to HA is carried out at neutral pH. Ammonia (later superseded by sodium hydroxide) was used for conversion, and the properties of the end product were similar to those of the general-purpose material (type GP) designed to give moderate resolution combined with a flow rate of 20–40 ml h^{-1} in a standard test (9 × 75 mm bed under a hydrostatic head of 250 mm at room temperature). BDC was from Boehringer (Mannheim, G.F.R.). Column eluates were continuously monitored for ultraviolet absorption and conductivity. Pooled fractions were concentrated on a small column of BDC at a loading limit of 5 mg tRNA per ml of bed, followed by step elution.

tRNA

Unfractionated tRNA, supplied by the Microbiological Research Establishment (Porton, Great Britain), had been extracted from *E. coli* K12 strain CA 265 by the method of Kirby[3] and further purified by phenol treatment and DEAE-cellulose chromatography[4]. A few batches showed irreversible aggregation associated with low amino acid acceptor activity, and these were rejected. None of the material showed evidence of hidden breaks (see below).

tRNA acceptor activity

We assayed by incubation on paper discs after Cherayil *et al.*[5] using the conditions of Nishimura *et al.*[6] and the modified washing procedure of Rubin *et al.*[7]. A crude enzyme preparation from *E. coli* MRE 600 was made following Muench and Berg[8]. ^{14}C-Labelled amino acids were from the Radiochemical Centre (Amersham, Great Britain). Appropriate tests were carried out to ensure a linear response, limited only by the quantity of tRNA. Inhibition by salts used in chromatography was measured using unfractionated tRNA.

Gel electrophoresis

15% polyacrylamide gels were made and stained according to Spencer *et al.*[9]. Gels were run at up to 1 W per tube (I.D. 7 mm) using buffer recirculation, the buffer reservoirs serving to cool the tubes. The normal medium was 0.005 *M* sodium acetate (pH 5.0), but to test for hidden breaks we used a formamide system similar to that of Staynov *et al.*[10] with 10% acrylamide, of which 5% was N,N'-methylene-bisacrylamide.

Crystallization

Purified tRNA was precipitated with ethanol, desalted by washing in 80% ethanol, resuspended in water at 2–10 mg ml^{-1}, centrifuged to remove contaminating particles and stored at −20°. Aliquots for crystallization were taken after heating, when necessary, to disperse aggregates.

Crystallization was carried out by vapour diffusion in divided polystyrene petri dishes (Sterilin, Teddington, Great Britain) sealed with vacuum grease. One half of each dish contained up to sixteen 10-μl droplets, made up from concentrated stocks of tRNA and salts. The other half contained a liquid reservoir whose composition

could be changed by pipetting through a grease-sealed port. These dishes were suitable for aqueous solutions and those containing ethanol and propanol; they were not suitable for dioxan. Dishes stored at 5° showed no loss of liquid over long periods, but those kept at higher temperatures required a humid atmosphere to prevent a slow drying out by diffusion through the plastic.

Crystallization was followed by inspection (without removing the lid) with a polarizing microscope. Samples for X-ray diffraction were mounted with droplets of mother liquor in thin-walled glass or quartz capillaries (Pantak, Windsor, Great Britain).

RESULTS AND DISCUSSION

Cation and pH effects

We ran HA columns at 5° with gradients of either sodium or potassium phosphate at various pH values (Fig. 1). The flow-rates were such that active fractions could generally be collected within 24 h of starting each run.

The major differences between the absorbance profiles in Fig. 1 are attributable to a limited number of changes in the elution order of species. The main peaks of $tRNA^{Arg}$ and $tRNA^{Lys}$ are considerably retarded by reduction of pH in sodium phosphate, but not in potassium. There are also many variations in the shapes of individual profiles which indicate that for final purification of a sub-species (there being several for each amino-acid accepting activity) it may be valuable to re-fractionate with a different cation and/or pH value.

The origin of these differences seems likely to lie in changes of secondary or tertiary structure in tRNA. The ionization of tRNA phosphates (many of which lie on the outside, accessible to HA) does not change over the pH range discussed, but changes in structure due to base residue interactions would alter the pattern of exposed phosphates. We have seen in Part I[1] that there is a correlation between the size of HA crystallites and the strength of binding to tRNA, suggesting that binding is maximised when close apposition of the maximum number of phosphates to the HA surface is achieved. Similar pH effects may be expected with proteins, though many of these probably have a more stable structure than tRNA.

Apart from specific shifts in elution position it is of interest to compare the salt concentrations at which tRNA elution begins under different conditions (Table I); certain tRNA species are always at the beginning of the profile and give a sharp initial rise in absorbance. The difference between sodium and potassium may be related to the ability of sodium to substitute for calcium in the HA lattice[11]. The most striking effect is, however, the variation with pH: between pH 5.5 and 7.5 there is a two- to threefold decrease in the molarity required to start elution. An even larger variation of this kind has been reported for acidic proteins by Bernardi *et al.*[12].

We suggest that this reflects a greater efficiency of HPO_4^{2-} compared with $H_2PO_4^-$ in competing for binding with calcium ions[12] or hydroxyl sites[13,14] on the HA surface. As the buffer pH increases, the proportion of HPO_4^{2-} also rises and a smaller overall phosphate molarity is needed for elution. A rough calculation, based on estimates of the proportions of the two ions (Table I) suggests that HPO_4^{2-} is at least three times as effective as $H_2PO_4^-$ in displacing tRNA.

It seems improbable that structural changes in proteins and tRNA would give

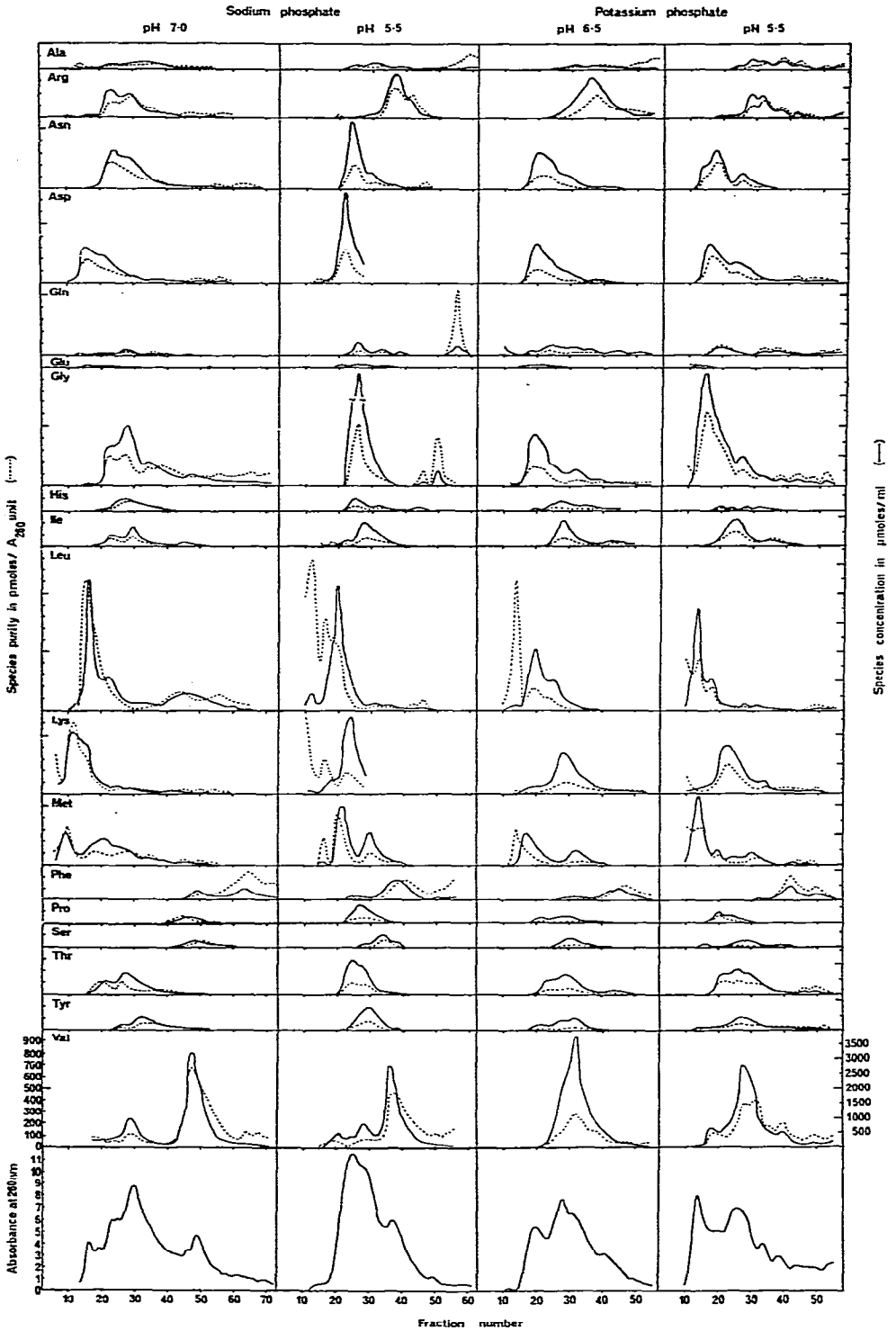


Fig. 1. Elution of *E. coli* tRNA from HA columns, run at 5° under 1.8 m hydrostatic head. Amino acid acceptance assays were carried out for 18 species (excluding cysteine and tryptophan). First column: 25 × 900 mm loaded with 100 mg tRNA, eluted with gradient of 2 × 500 ml 0.07–0.16 *M* sodium phosphate (pH 7.0), collecting 9.3 ml fractions at 33 ml h^{-1}. Second column: as first except for gradient 0.1–0.4 *M* sodium phosphate (pH 5.5), flow-rate 75 ml h^{-1}. Third column: 16 × 800 mm loaded with 40 mg tRNA, eluted with 2 × 200 ml 0.1–0.4 *M* potassium phosphate (pH 6.5), collecting 4.0-ml fractions at 11 ml h^{-1}. Fourth column: as third except for 0.1–0.4 *M* potassium phosphate (pH 5.5), flow-rate 22 ml h^{-1}.

TABLE I

ELUTION OF tRNA WITH PHOSPHATE BUFFERS

Columns of HA run at 5°; for further details see text and caption to Fig. 1.

*pH**	*Percent HPO_4^{2-}***	*Elution molarity at start of profile****	
		Na^+	K^+
5.5	5	0.17	0.23
6.0	12	0.15	—
6.5	32	0.11	0.12
7.0	61	0.10	0.10
7.5	84	0.09	0.08

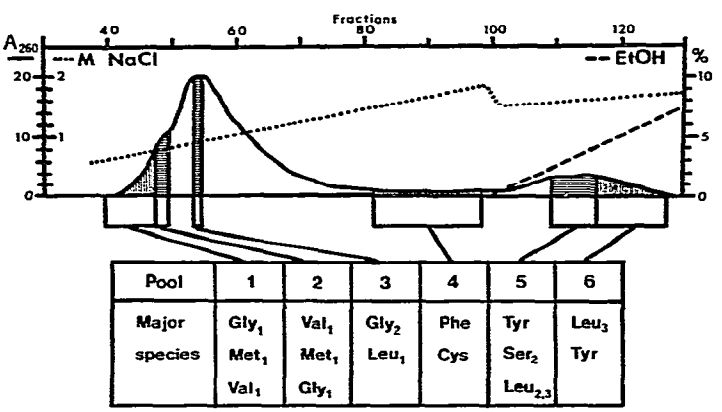
* Stock solutions made up at room temperature: pH 5.5–6.0 (0.4 *M*), pH 6.5 (0.3 *M*), pH 7.0–7.5 (0.2 *M*).

** Data from buffer tables for 0.1 *M*, 25°.

*** Estimated from conductivities.

similar effects in both; it is also inconceivable that changes in net charge are responsible, since for acidic molecules there would if anything be an increase in charge towards higher pH, with increased affinity for HA instead of the opposite.

Only limited comparisons are possible with the results of previous workers. Some[15–17] have fractionated *E. coli* tRNA with sodium phosphate at pH 6.8, but only at room temperature; several of their elution positions differ from ours, showing that temperature is another important variable. Others have used potassium phosphate at pH 6.8 and room temperature[18] or pH 5.8 at 0° (refs. 8, 17), but in the latter case the tRNAs were esterified to amino acids; it is already known[20] that this can change the elution position of a species. Yeast tRNA has also been fractionated[21–23] but the distribution of species is quite different to that in *E. coli*. It is not possible to compare our flow-rates with most of those reported, due to the pressure head not being recorded; where it was, our normalised flow-rates appear greater.



Pool	1	2	3	4	5	6
Major species	Gly_1 Met_1 Val_1	Val_1 Met_1 Gly_1	Gly_2 Leu_1	Phe Cys	Tyr Ser_2 $Leu_{2,3}$	Leu_3 Tyr

Fig. 2. Primary fractionation of *E. coli* tRNA on BDC at room temperature. 50 × 1000 mm column loaded with 4 g tRNA, eluted with 2 × 10 l 0.01 *M* $MgCl_2$ to (0.01 *M* $MgCl_2$ + 2 *M* NaCl), followed by 2 × 5 l (0.01 *M* $MgCl_2$ + 1.5 *M* NaCl) to (0.01 *M* $MgCl_2$ + 2 *M* NaCl + 20% ethanol). Fraction size 200 ml, flow-rate 500 ml h^{-1}. Pools used for refractionation on HA are shown with major species listed in order of decreasing purity.

Combination with BDC chromatography

Previous authors have used HA for purifying tRNA after primary fractionation by partition chromatography[8] or DEAE-Sephadex[16,17]. We chose BDC[24] for primary fractionation (Fig. 2) because of its high capacity and because tRNA can be fractionated in aqueous media without aminoacylation or chemical modification[25–27]. Fig. 3 illustrates a typical re-fractionation on HA to obtain the Leu_1 species[29]. The active fractions were subsequently pooled and run again on HA at pH 5.5 to obtain crystallizable material (see below). Other species were further purified by a second column of BDC (Table II). Tests for homogeneity and for hidden breaks were carried out by gel electrophoresis as in Materials and methods, and showed only minor cross-contamination and no detectable degradation.

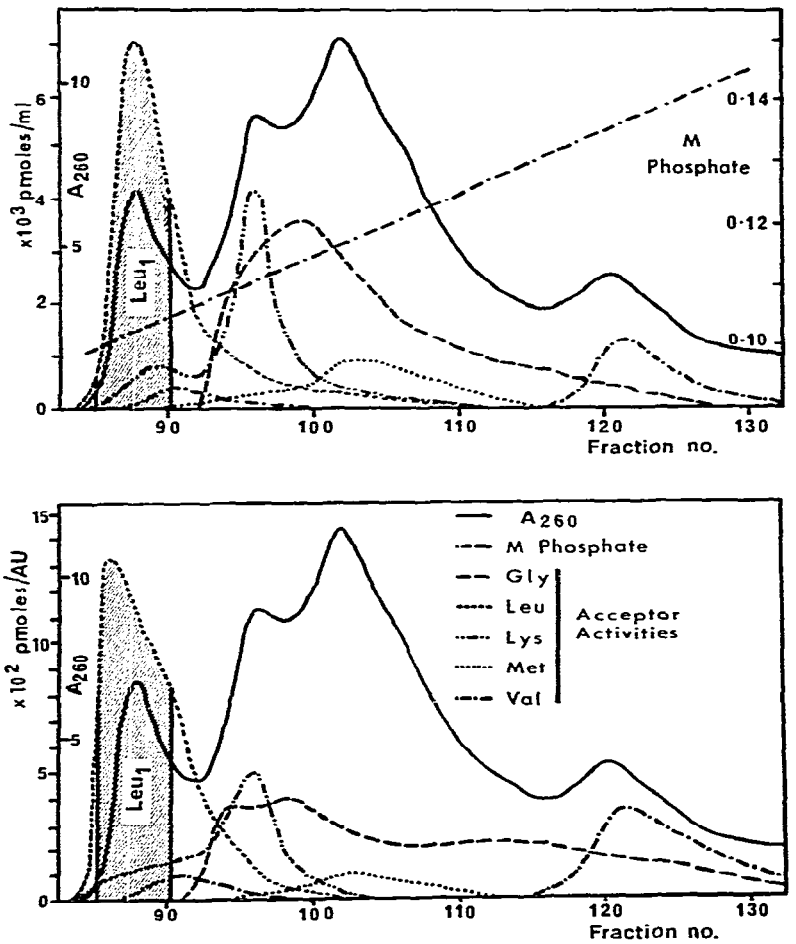


Fig. 3. Refractionation of pool 3 (Fig. 2) on HA at 5°. 25 × 1000 mm column loaded with 110 mg tRNA, eluted with gradient of 2 × 500 ml 0.06–0.16 *M* sodium phosphate (pH 7.0). Fraction size 5 ml, flow-rate 26 ml h^{-1}. Upper diagram, concentrations of major species; lower diagram, purities.

TABLE II

FRACTIONATION OF *E. coli* tRNA

Initial load, 4 g. Uncertainty in purities, ±50 pmoles per absorbance unit at 260 nm (A_{260}).

Species	*BDC fractionation (see Fig. 2)*			*HA fractionation (sodium phosphate, pH 7.0)*		*BDC re-fractionation (column 25 × 1000 mm)*		*Crystal form***
	Pool No.	*Yield (mg)*	*Purity (pmoles/A_{260})*	*Yield (mg)*	*Purity (pmoles/A_{260})*	*Yield (mg)*	*Purity (pmoles/A_{260})*	
Met_1**	1	180	500	11	1300	—	—	Plates
Gly_1	1	180	400	20	1000	6	1400	—
Val_1	2	195	750	21	1600	—	—	Prisms
Leu_1	3	200	150	13	1100	—	—	Prisms
Cys	4	165	200	32	600	10	1000	—
Phe	4	165	450	27	1300	—	—	Needles
Ser	5	150	150	9	900	—	—	—
Tyr	5	150	300	10	850	—	—	—
Leu_3	6	100	300	15	1500	—	—	—

* Met_1 crystallized at 25° from 4% 2-propanol with 5–10 m*M* $MgCl_2$, 5–10 m*M* sodium cacodylate (pH 6.0), 1–2 mg/ml tRNA, 0.5–4 m*M* spermine-Cl_4. For other species see caption to Fig. 4.

** Probably fMet.

Crystallization tests were as described under Materials and methods; the precipitants tried were $(NH_4)_2SO_4$, 2-propanol, ethanol and 2-methylpentan-2,4-diol at temperatures of 5°, 25° or 35°. The counterion used was Co^{2+}, Ni^{2+}, Mn^{2+}, spermidine or spermine together with varying amounts of Mg^{2+} and 5–10 m*M* sodium cacodylate (pH 6.0). The tRNA concentration was 1–2 mg ml^{-1}. Four tRNAs yielded crystals, some of which are illustrated in Fig. 4. The best ones were of the Leu_1 subspecies, whose crystallization has not previously been reported; further details will be published elsewhere[32].

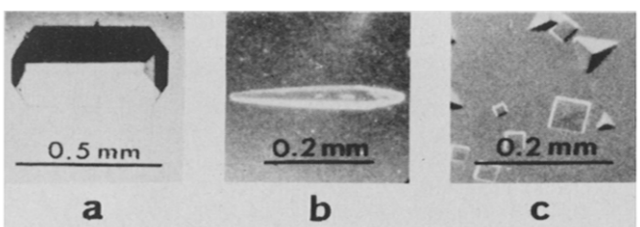


Fig. 4. tRNA crystals obtained by vapour diffusion at 5°. Droplets contained after equilibration 5–10 m*M* $MgCl_2$, 5–10 m*M* sodium cacodylate (pH 6.0) and 1–2 mg ml^{-1} tRNA, together with the components listed: (a) $tRNA_1^{Leu}$ with 2.5 *M* $(NH_4)_2SO_4$ + 2.5–10 m*M* $CoCl_2$ or $NiCl_2$; (b) $tRNA^{Phe}$ with 5% 2-propanol + 0.5–1 m*M* $CoCl_2$, $NiCl_2$, spermidine-Cl_3 or spermine-Cl_4; (c) $tRNA_1^{Val}$ with 2.5 *M* $(NH_4)_2SO_4$ + 5–10 m*M* $CoCl_2$ or $NiCl_2$.

None of the crystals diffracted X-rays well enough to enable structural analysis to proceed; this has unfortunately been a common experience of workers on tRNA[30], and may reflect an inherent flexibility in the structures of most of the species so far tested. Since there is an urgent need to obtain high-resolution data from tRNAs other than the one whose structure has been determined[31], fractionation by HA could be of

use in obtaining crystals from the numerous species not so far tested. Each organism has a unique tRNA population of at least 30 sub-species and so far nearly all work has concentrated on *E. coli* and yeast. The mild conditions used with HA would be additionally useful for purifying the rather labile complexes formed between tRNA and other macromolecules.

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