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THERMAL CHROMATOGRAPHY OF LYSINE-SPECIFIC TRANSFER RIBONUCLEIC ACID FROM *ESCHERICHIA COLI* B

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SUMMARY

Two *Escherichia coli* B $tRNA^{Lys}$ isoacceptors have been separated by thermal chromatography on hydroxyapatite demonstrating the potential of this approach in the fractionation of single-stranded polynucleotides. Elution temperatures were reproducible and depended primarily on the cation present and its concentration. Cesium phosphate was a more effective eluent than sodium phosphate.

The integrity of the polynucleotide following thermal chromatography was examined by ultracentrifugation in a 60% dimethyl sulfoxide solvent, electrophoresis on polyacrylamide gels containing urea and oligonucleotide mapping. Adsorption to hydroxyapatite did not noticeably increase phosphodiester bond breakage compared with that in solution. Minimal, if any, rupture of covalent linkages was detected under the conditions of preparative separation of the isoacceptors. Several observations suggest that only one $tRNA^{Lys}$ was transcribed by the *E. coli* B cell.

INTRODUCTION

Thermal chromatography on hydroxyapatite has been hitherto studied with regard to the behavior of double-stranded nucleic acids[1,2]. Applications were primarily in the separation of denatured, single-stranded polynucleotides from their native parental forms (reviewed by Bernardi[3]). A study of single-stranded nucleic acid appeared of interest especially with the prospects of providing an additional principle for fractionation and establishment of homogeneity. $tRNA^{Lys}$ from *Escherichia coli* B was chosen as the subject since: (1) It assumes a high degree of conformational structure under the usual temperature conditions. (2) "Melting" was expected over a narrow temperature range[4]. (3) Only two isoacceptors are present[5–7]. (4) Methods for separation of the isoacceptors available at the inception of this study[6] were technically difficult and time consuming.

Here it is shown that, using simple equipment, thermal chromatography will separate the isoacceptors within several hours without noticeable damage to covalent bonds. Differences in the effect of temperature on the elution from hydroxyapatite of higher-molecular-weight single-stranded nucleic acids have been observed indepen-

dently[8]. Taken together, these studies indicate that thermal chromatography is potentially useful in fractionating other single-stranded polynucleotides as well.

MATERIALS AND METHODS

Materials

Cesium phosphate. Cesium dihydrogen phosphate and dicesium hydrogen phosphate were obtained from Rocky Mountain Research, Denver, Colo., U.S.A.

Hydroxyapatite. Bio-Gel HT and HTP were from Bio-Rad Labs., Richmond, Calif., U.S.A.

E. coli B tRNA. *E. coli* B tRNA was purchased from Schwarz/Mann, Orangeburg, N.Y., U.S.A.

Highly radioactive [^{32}P]tRNA^{Lys}. [^{32}P]tRNA^{Lys} was routinely obtained by phenol extraction of *E. coli* B grown in a low-phosphate medium in the presence of carrier-free $H_3{}^{32}PO_4$. Unlabeled tRNA was added during a second phenol extraction. The schedule of subsequent treatments included extraction with 1 *N* sodium chloride, stripping of esterified amino acids (pH 8.9, 37°, 90 min), elution from BD-cellulose[9] with buffered (10^{-2} *M* sodium acetate pH 4.5, 10^{-2} *M* magnesium chloride, 10^{-3} *M* mercaptoethanol) 1 *N* sodium chloride, and charging with lysine using a highly purified synthetase followed by phenoxyacetylation. The RNA was then applied again to BD-cellulose equilibrated with 0.3 *N* sodium chloride and washed with 50–100 ml of this solvent and 150–200 ml of 1 *N* sodium chloride both buffered as above. A similarly buffered 100-ml linear gradient between 1 *N* NaCl and 2.5 *N* NaCl 25% ethanol was subsequently used to elute the RNA. Following precipitation with ethanol the RNA was again stripped as above to remove the derivatized amino acid, precipitated with ethanol and stored in water at −20°.

Thermal chromatography

The following procedure was adapted from Miyazawa and Thomas[1]. A simple jacketed glass column, 1.0 cm in diameter, was maintained at the desired temperature by means of water circulating from a Haake constant-temperature bath. Bio-Gel HT was washed three times with 0.08 *M* sodium phosphate (diluted from a 1 *M*, pH 6.8 stock solution), 1% methanol, 0.02% sodium azide[6] also 10^{-3} *M* in mercaptoethanol (sodium phosphate buffer), and the fines were removed. Bio-Gel HTP was treated similarly except that it was first suspended overnight at room temperature in sodium phosphate buffer or in the cesium phosphate buffer that was used during the adsorption of the RNA. Cesium phosphate buffers contained equal concentrations of the mono- and dihydrogen salts[2] as well as methanol, sodium azide and mercaptoethanol as above. The column was packed at 25° by addition to 2 ml of buffer of a freshly prepared hydroxyapatite slurry allowing free flow of the buffer. In sodium phosphate such columns retained approximately 10 mg tRNA per ml bed volume at 25°.

tRNA in sodium phosphate buffer diluted two-fold or in a cesium phosphate buffer (usually 1 ml) was loaded onto the column, washed in with 1 ml of the same and allowed to equilibrate for 60–90 min at 25°. Elution was with 8–10 ml buffer prewarmed to the desired temperature and allowed to equilibrate in the column for another 5–10 min. Flow-rates were approximately 15 ml/h and usually not more than 20 ml/h. Operation of the column may be interrupted overnight and even for several days.

Gentle stirring, when the column was clogged, had no adverse effects. Radioactivity was determined in a scintillation counter either on an aliquot in Bray's fluid or by Čerenkov radiation without dilution[10].

Analytical ultracentrifugation

Analytical ultracentrifugation was performed in the AN-D rotor of the Beckman Model E centrifuge equipped with schlieren optics.

Freshly prepared samples in a 60% (aq., v/v) dimethyl sulfoxide 0.1 *N* sodium chloride solvent also containing sodium cacodylate pH 7.0, $I = 0.1$, diluted twenty-fold, were centrifuged at 25° at 60,000 rpm. Whenever necessary, insoluble material was removed by centrifugation at room temperature just before the run.

RESULTS AND DISCUSSION

Thermal elution profile of $tRNA^{Lys}$

Two major components were revealed as expected (Fig. 1). In several runs in which $tRNA^{Lys}$ from two different cell growths was eluted with sodium phosphate buffer[1], the maxima for peaks I and II were 86° and 76°, respectively and did not usally deviate by more than ±2° from these values. Elution may not have been exhaustive at each step. Yet the reproducibility of the profile including some of the finer details indicated that only minor carry over into subsequent fractions occurred. Material of peak I was degraded by pancreatic ribonuclease (5 μg/ml, pH 7.0, 37°, 10 min) thus excluding extensive contamination with host DNA eluting at a similar temperature[1]. That peak II was a distinct species, even when largely overshadowed by peak I, (see below) was demonstrated by re-chromatography of the corresponding fractions. These eluted at a temperature characteristically lower than peak I, traces of which were still discernible (Fig. 2).

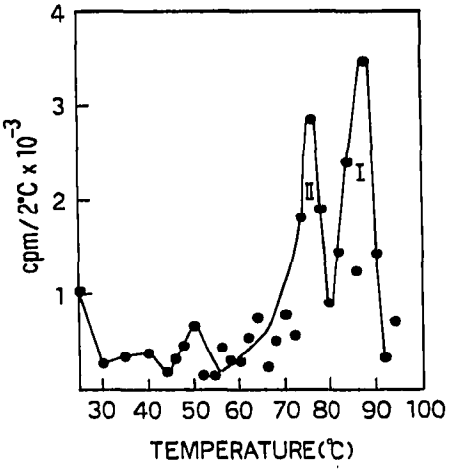

Fig. 1. Thermal elution of [^{32}P]$tRNA^{Lys}$ with sodium phosphate. Hydroxyapatite HT (1.5 × 1.0 cm) was used as described in Materials and methods, except allowing free flow of the eluent (22, 20, 30 ml/h at 54°, 70° and 88°, respectively). The small peak eluting at 50° was probably due to degradation and is not characteristic.

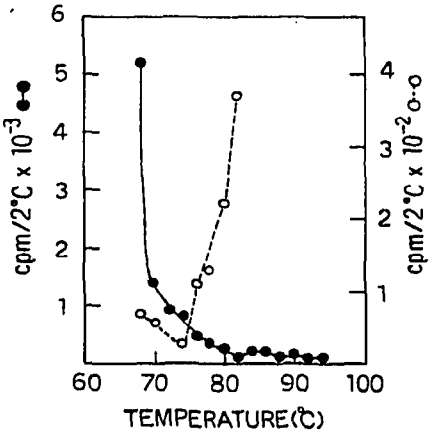

Fig. 2. Re-chromatography of Peak II. [^{32}P]tRNA^{Lys} eluting initially between 68° and 76°, in 40 ml sodium phosphate buffer, was diluted with 40 ml water and readsorbed to a thermal Bio-Gel HT column (2.0 × 1.0 cm). Following elution with 8 ml of sodium phosphate buffer at 25° releasing 21% of the total counts the temperature was raised to 68° and elution continued as indicated. ○---○, Initial; ●---●, re-chromatography.

The behavior of *B. subtilis* DNA[2], described while this study was in progress, suggested that cesium phosphate might be a more effective eluent than the sodium salt used so far. This expectation was borne out as shown in Table I since at 25° usually no more than 5% of the counts were eluted by the 80 m*M* sodium phosphate buffer. Furthermore, elution profiles were similar but appeared displaced towards lower temperatures (Fig. 3). In a number of profiles obtained between 20 m*M* (peak at 72°, shoulder at 60°) and 50 m*M* cesium phosphate (where profiles were difficult to obtain and most counts eluted below 45°), the extent of displacement usually increased with the cesium phosphate concentration in the eluent. Although not studied systematically, elution temperatures appeared insensitive to the concentration of cesium phosphate (1 or 10 m*M*) and occasional presence of $5 \cdot 10^{-4}$ *M* EDTA in the

TABLE I

ELUTION OF [^{32}P]tRNA^{Lys} WITH INCREASING CONCENTRATIONS OF CESIUM PHOSPHATE

[^{32}P]tRNA^{Lys} (approximately 21,000 cpm) in 10 ml of 8 m*M* cesium phosphate buffer was adsorbed to a 2.0 × 1.0 cm Bio-Gel HTP column equilibrated with same. Elution with 8 ml of buffer, cesium phosphate concentrations increasing as indicated.

Cesium phosphate (mM)	*tRNA^{Lys} eluted*	
	cpm	*% of total*
20	934	8.6
30	2033	18.8
40	2806	26.0
50	2242	20.1
60	1970	18.3
70	809	7.5

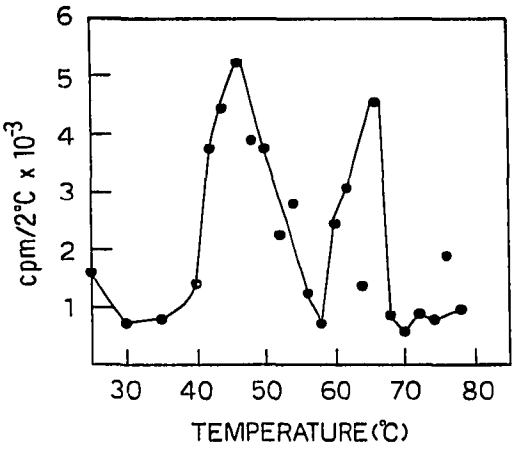

Fig. 3. Thermal elution of [^{32}P]tRNA^{Lys} with cesium phosphate. Adsorption in 10 m*M* cesium phosphate buffer onto Bio-Gel HTP (1.5 × 1.0 cm) equilibrated with the same. Elution with 40 m*M* cesium phosphate buffer.

adsorption buffer and to whether or not the RNA had been previously denatured (60°, 5 min, rapid cooling) in the same. Presumably, during the run the RNA rearranges to assume a conformation dictated by the eluent and then rapidly re-equilibrates with the column.

The behavior of tRNA^{Lys} during thermal chromatography thus resembles that of native *B. subtilis* DNA[2] in some respects. Assuming that the T_m is in the range of that of unfractionated *E. coli* B tRNA[4] and that elution requires a considerable degree of melting, it appears that hydroxyapatite similarly stabilizes the RNA towards denaturation when sodium is the cation. Also, cesium phosphate is a more effective eluent than sodium phosphate. These similarities are not surprising considering the high degree of conformational structure present in tRNA. The contributions towards this behavior of double-stranded and highly stacked single-stranded regions are not known.

Integrity of the polynucleotide

It was of interest whether adsorption to hydroxyapatite endangered the phosphodiester backbone. To avoid masking of hidden breaks, the solvent for analytical ultracentrifugation included dimethyl sulfoxide. A solvent denaturation profile[11] (25°, 260 nm) of *E. coli* B tRNA (not shown) was in good agreement with those obtained at wavelengths other than the main absorption peak[12,13] whether or not the RNA was pre-treated with EDTA (10^{-3} *M*, pH 7.0, 60°, 5 min, rapid cooling). This indicated that denaturation was complete even in the presence of bound divalent ions when dimethyl sulfoxide was 60% (v/v) as used here.

Thus, following mock treatment approximating that required for preparative separation of components I and II using sodium phosphate as the eluent, *E. coli* B tRNA appeared intact and no less homogeneous than the control (Fig. 4a, b). That phosphodiester bonds, and most other covalent bonds, stayed intact during the pre-

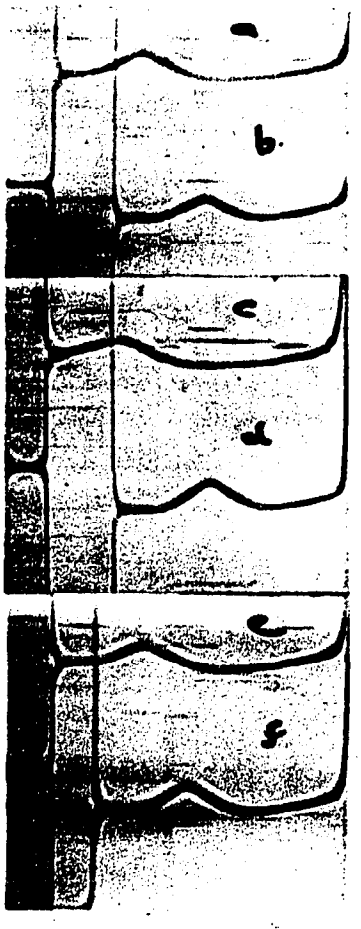

Fig. 4. Sedimentation of *E. coli* B tRNA following thermal chromatography. 10–40 mg *E. coli* B tRNA were adsorbed onto 1.5–4.0 × 1.0 cm columns of Bio-Gel HTP and eluted as described in Materials and methods. Following elution with 10 ml of sodium phosphate buffer at 95° the RNA was precipitated with cold ethanol, taken up in water, and the insoluble material (mostly phosphate) was removed by centrifugation. Cesium phosphate samples were adsorbed in 10m*M* buffer onto Bio-Gel HTP equilibrated with the same. The column was then washed with 10 ml of 40m*M* buffer at 25° and eluted at 74° with the same. Dialysis overnight against two 1000-ml changes of 10^{-3} *M* sodium acetate pH 4.5, 10^{-3} *M* mercaptoethanol in the cold followed and the RNA again precipitated adding sodium chloride to 50 m*M* or sodium acetate pH 4.5 to 20 m*M* and two volumes cold ethanol. In the controls, an equivalent amount of untreated tRNA was dissolved in 10 ml of eluent and then precipitated and dialyzed as described. (a) Mock preparative treatment (sodium phosphate). The column was maintained at 68° and 78° for 90 min each prior to elution. (b) Control. (c) Extended heating (sodium phosphate). 8 h at 94°. (d) Control. (e) Mock preparative treatment (cesium phosphate). Column maintained at 40° and 58° for 90 min each prior to elution. (f) Control. The concentration of tRNA during ultracentrifugation was approximately 5.5 mg/ml in (a) and (b), 4 mg/ml in (c) and (d), and 5 mg/ml in (e) and (f). Centrifugation time: (a) and (b), 230 min; (c) and (d), 241 min; (e) and (f), 252 min.

parative separation of the isoacceptors is apparent also from two-dimensional oligonucleotide maps[14]. Pancreatic ribonuclease digests of peak I material (Fig. 5) appeared the same as those of unchromatographed [^{32}P]tRNA^{Lys}. Non-specific hydrolysis on the column would have resulted in the appearance of free adenosine 2′,3′-phosphate and guanosine 2′,3′-phosphate but none was detected. It is estimated that 0.1–0.2 mole per mole tRNA of either nucleotide would have been revealed readily. Extensive heating on the column resulted in partial degradation, the RNA now sedimenting in the ultracentrifuge as a slower, asymmetric peak (Fig. 4c, d). Thermal hydrolysis under comparable conditions but in aqueous solution[15] was expected to cause approximately 0.7 and 3.0 mole phosphodiester bond breaks per mole tRNA during the mock preparative and the extended heating treatments, respectively.

When sodium was replaced with cesium as the cation and temperatures were reduced accordingly in a mock preparative procedure, again the integrity of the polynucleotide backbone appeared preserved. This was deduced from the sedimentation pattern (Fig. 4e, f) and confirmed by polyacrylamide gel electrophoresis under

Fig. 5. Peak I pancreatic ribonuclease fingerprint. [^{32}P]tRNA^{Lys} was adsorbed onto a 2.0 × 1.0 cm Bio-Gel HTP column equilibrated with sodium phosphate buffer as described in Materials and methods. The column was then eluted with the same at 66° (10 ml), 78° (20 ml) and 81° (10 ml). The fraction eluted subsequently with 20 ml at 94° was dialyzed overnight against 4 l of cold 10^{-2} *M* sodium acetate pH 4.5, 10^{-3} *M* β-mercaptoethanol. Following adsorption to a (1.0 × 0.5 cm) DEAE-cellulose column equilibrated with 0.1 *N* sodium chloride, the RNA was washed with another 50 ml of the same and then eluted with 1 ml of 1 *N* sodium chloride and precipitated with cold ethanol. Encircled spots are not representative having appeared prior to thermal chromatography and in this preparation only.

denaturing conditions[16] (75–100 μg tRNA, 10-cm gel). Essentially the same electropherograms were observed whether or not the RNA had been subjected to cesium phosphate thermal chromatography. In either case no components were detected with mobilities up to six times that of the main tRNA front. The overall conclusion is that adsorption of tRNA to hydroxyapatite *per se* was without drastic effects, if any, on the susceptibility to hydrolysis of phosphodiester bonds compared with that in solution.

The oligonucleotide pattern in Fig. 5 indicated that most base residues remained intact under preparative conditions even though some minor change may have gone undetected. However, even after extended heating on the column (essentially as in Fig. 4c) of *E. coli* B tRNA followed by renaturation[17], lysine acceptances[18] were as high as 44% of the controls. Most probably this included the charging of reassociated fragments generated by limited phosphodiester bond breakage (see above). Renaturation is often incomplete[19] and the conditions used[17] may not have been optimal for $tRNA^{Lys}$. Presumed representative of the upper limits of damage, these observations suggest that functional groups required for biological activity largely remain unharmed even under such drastic conditions. From the reproducibility of the elution profiles, especially those obtained with sodium phosphate involving prolonged exposures to high temperature, it is surmised that functional groups critical to the conformation of the RNA and its interaction with hydroxyapatite are not jeopardized.

Freshly prepared [^{32}P]$tRNA^{Lys}$ usually consisted mostly (estimated at 90%) of component I. Upon repeated use of the same preparations the proportion of peak II progressively increased in profiles obtained both with sodium phosphate and cesium phosphate. A similar observation[20] was made using polyacrylamide gel electrophoresis[16]. [^{14}C]Lysine *E. coli* B tRNA that had been handled considerably less travelled as one peak only in that system. Also, reversed-phase chromatography[7] of [^{32}P]$tRNA^{Lys}$ pre-incubated at 47° for 2 h at pH 7.0, conditions that presumably maximize the effects of repeated handling, similarly led to considerable enrichment in the minor component (not shown). Most probably then, only one $tRNA^{Lys}$ species was transcribed both under the present and the commercial growth conditions. The second isoacceptor resulted from subsequent modifications of as yet unknown nature.

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