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THE FRACTIONATION OF YEAST tRNA AND PROTEINS ON NUCLEOSIDE DERIVATIVES OF CELLULOSE

SHIRLEY J. MORRIS

Max Planck Institut für medizinische Forschung, 69 Heidelberg 1, Jahnstr. 29 (G.F.R.)

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SUMMARY

A series of cellulose derivatives containing each of the major nucleosides has been prepared by the action of epichlorohydrin and nucleoside on cellulose. The chromatographic behaviour of yeast tRNA on the materials has been studied, and the results of variations in pH and of the presence or absence of magnesium ions are described. The behaviour of a mixture of yeast proteins and nucleic acids on some of the derivatives was also investigated, and the potentialities of these materials as chromatographic media for the purification of nucleic acids and proteins have been evaluated.

INTRODUCTION

For the investigation of the chemical properties of nucleic acids, *e.g.*, individual tRNAs, it is necessary to purify one or more components from a mixture of similar species. In the case of $tRNA_{Phe}$[1], and $tRNA_{fMet}$[2] from yeast, suitable yields and degrees of purity can be obtained by the use of BD-cellulose[3], but for the purification of other species it is usually necessary to use a combination of two or more different methods of fractionating. These methods at present include countercurrent distribution[4–6], chromatography on DEAE-cellulose[7], DEAE-Sephadex[8], ECTEOLA-cellulose[9], hydroxylapatite[10–12], methylated albumin on Kieselguhr[13,14] or silicic acid[15], reversed-phase chromatography[16], partition chromatography[17] and chemical or enzymatic modification of a species prior to fractionation[18,19].

Many of these methods are not suitable for large-scale fractionation; some result in alterations to the tRNA structure, as indicated by loss of amino acid acceptor activity, some give only poor separation between individual species and some chromatographic materials tend to decompose with time and release impurities into the eluate (*e.g.*, BD-cellulose[2] and methylated albumin on silicic acid[15]). Hence there is still a need for new chromatographic adsorbents which, to be effective, should have high capacity, be clean running, relatively easily prepared, and, in order to complement the ion-exchange methods generally in use, should depend on some other property for the selective adsorption and desorption of nucleic acids. It is probable that the interaction of the purine and pyrimidine bases of the tRNA molecules with similar bases linked to cellulose might form the basis of a useful fractionation technique for nucleic acids and, as many proteins have binding sites specific for nucleotide

derivatives, the materials might also be applicable to protein fractionation. Cellulose was chosen for the support material because of its availability in reproducible grades of quality and its ability to withstand changes of salt concentration, pH and hydrostatic pressure without gross changes in bed volume or flow characteristics. Previous reports of the fractionation of nucleic acids on cellulose which had been coupled with guanine[20], on polynucelotide celluloses[21], and on an adenosine derivative of cellulose[21] prepared by the method of Peterson and Sober[22], led us to investigate the properties of a series of nucleoside derivatives prepared by the latter method.

MATERIALS

Microcrystalline cellulose (Avicell), epichlorohydrin and uridine (Merck); inosine and *d*-thymidine (Waldorf AG); guanosine, adenosine and cytidine (Serva); and ^{14}C-labelled amino acids (Radiochemical Centre, Amersham, Great Britain), were laboratory-grade reagents and were used without further purification.

Brewer's yeast tRNA (Boehringer GmbH, Mannheim, G.F.R.) was treated with phenol to remove proteins, incubated at 37°, for 30 min, in 1.8 *M* Tris acetate of pH 8.0 to remove bound amino acids[17], and loaded on to a column of DEAE-cellulose (Whatman DE-52). After removal of low-molecular-weight polynucleotides with 0.2 *M* sodium chloride in 0.05 *M* Tris chloride, the tRNA fraction was eluted with 1.0 *M* sodium chloride. Two volumes of ethanol were added to the tRNA fraction and, after allowing the mixture to stand overnight at 0°, the resulting precipitate of tRNA was collected by centrifugation and re-dissolved in 0.01 *M* sodium acetate of pH 5.5 to give a final solution of *ca.* 905 A_{260} units/ml. This solution was stored at −30° until required for use.

An enzyme fraction containing aminoacyl-tRNA synthetases (E.C. subgroup 6.1.1.) was prepared from baker's yeast (Langemeier, Mettingen/Westf., G.F.R.) by homogenization in a Vibromühle (Bühler) with 0.01 *M* Tris chloride of pH 7.5, 0.02 *M* β-mercaptoethanol, 10% glycerol and DNAase, followed by centrifugation to remove cell debris, removal of RNA by precipitation with streptomycin sulphate and subsequent centrifugation, and the addition of ammonium sulphate (0.43 g/ml). The resulting precipitate was collected by centrifugation, dissolved in 0.01 *M* Tris chloride of pH 7.5, 0.01 *M* magnesium chloride, 0.05 *M* potassium chloride, 0.005 *M* β-mercaptoethanol and 10% glycerol, and was dialyzed for 24 h *versus* several changes of the same buffer mixture. After dialysis, the enzyme solution was divided into portions of 0.2–0.5 ml and stored at −30° until required for use. Enzyme preparations were thawed immediately before use and were not re-frozen; any thawed material not used within 1 h was discarded.

ASSAY FOR AMINO ACID ACCEPTOR ACTIVITY

Normally, column effluent (0.2 ml, containing 0.08–0.2 A_{260} units of total tRNA) was incubated at 37° for 15 min with aminoacyl-tRNA synthetases prepared as above (5 μl, saturating amounts) and specific ^{14}C-labelled amino acid solutions (0.1 ml). These solutions generally contained 50–400 μCi/μmole of [^{14}C]amino acid in 0.4 *M* Tris chloride of pH 7.5, 0.2 *M* ammonium chloride, 0.06 *M* magnesium chloride, 0.016 *M* ATP, 0.004 *M* CTP and 5.2%(v/v) β-mercaptoethanol, except

that for tyrosine, Tris chloride of pH 7.0 was used, for methionine, β-mercaptoethanol was replaced with 0.04 *M* glutathione and for cysteine, Tris chloride of pH 8.0 was used with cystine, reduced by 0.04 *M* dithiothreitol, in place of β-mercaptoethanol.

Incubation was terminated by the addition of 2 ml of 15% trichloroacetic acid at 0°. After being kept at 0° for 4–30 min, the precipitates were collected on Millipore (HAWP25) or glass-fibre filters (Whatman GF/C), washed with 5% trichloroacetic acid (3 × 5 ml), dried under an infrared lamp, placed in a scintillation vial with *ca.* 2 ml of toluene containing 0.5% PPO and 0.03% diMePOPOP, and counted in a Packard Tri-Carb 3320 liquid scintillation spectrometer.

ASSAY FOR AMINOACYL-t-RIBONUCLEIC ACID SYNTHETASES

The assay method was carried out essentially as described above, using 0.2–0.5 ml of column effluent, with yeast tRNA (0.1 ml containing *ca.* 1 A_{260} unit) and a mixture of [^{14}C]amino acids (0.1 Ci of Radiochemical Centre "Protein Hydrolysate", *ca.* 50 mCi/milliatom of carbon in 0.1 ml of the pH 7.5 buffer mixture).

PREPARATION OF CELLULOSE DERIVATIVES

Cellulose (100 g) was mixed thoroughly with 250 ml of ice-cold 10 *M* sodium hydroxide to give a stiff dough, which was kept in a stoppered flask at 0° for 45 min so as to promote mercerisation. Then a mixture of nucleotide (0.1 or 0.2 mole) (see Table I) in epichlorohydrin (100 ml) was added, and the mixture blended thoroughly so as to reduce lumps. The homogeneity of the final product depended to a great extent upon the efficiency of this blending. The mixture was allowed to stand overnight at room temperature, with the flask stoppered lightly. An exothermic reaction took place, with the evolution of brown fumes. Next day, the mixture was heated at 80° for 45 min, then washed successively with water, 2 *M* sodium chloride, 1 *M* sodium hydroxide, 1 *M* hydrochloric acid, 1 *M* sodium hydroxide and water until the washings were colourless. If necessary, lumps were reduced by pressing the paste through a coarse nylon household sieve at the sodium chloride washing stage. Fines were removed during the washing procedure by siphoning off the supernatant after settling, until material with a settling time of 5–10 min was obtained. The material was de-gassed in dilute acetic acid, suspended in 2 *M* sodium acetate of pH 5.5 and packed under a pressure of 1.5 kg/cm^2 into a chromatographic column (25 mm × 1 m). The column was equilibrated with 0.01 *M* sodium acetate of pH 5.5 until the conductivity of the effluent was the same as that on top of the column and the absorbance of the effluent at 260 nm was below 0.05. When not in use, the derivatives were stored at 4° in buffer, or in 2 *M* sodium chloride, under toluene in order to inhibit bacterial growth.

CHROMATOGRAPHIC METHODS

Yeast tRNA

All fractionations were performed at 4°. Initial experiments were performed on all cellulose derivatives to determine their reproducibility and elution characteristics. In general, 1000 A_{260} units in 1.1 ml of 0.01 *M* sodium acetate of pH 5.5 were loaded

TABLE I

CHROMATOGRAPHIC PROPERTIES OF THE NUCLEOSIDE-CELLULOSES PREPARED

Designation	*Nucleoside*	*Moles nucleoside per 100 g of initial cellulose*	*Capacity (A_{260} units of tRNA per 100 g initial cellulose)*	*Recovery(%) of tRNA (A_{260} units)*	*Elution range (M NaCl) of tRNA at pH 5.5*
A1	Adenosine	0.1	>1000	100	0.15–0.5
A2	Adenosine	0.2	>1000	80	0.25–0.5
C1	Cytidine	0.1	>1000	>80	0.15–0.75
C2	Cytidine	0.2	>1000	>80	0.2 –0.8
G1	Guanosine	0.1	300	90	0.02–0.1
G2	Guanosine	0.2	300	100	0.05–0.15
U1	Uridine	0.1	*ca.* 1000	>80	0.01–0.15
U2	Uridine	0.2	>1000	70	0.01–0.15
T1	*d*-Thymidine	0.1	400	>80	0.1 –0.25
T2	*d*-Thymidine	0.2	>1000	90	0.1 –0.35
I1	Inosine	0.1	350	>80	0.15–0.35
I2	Inosine	0.2	750	>80	0.01–0.2

on to the column, and elution was carried out with 0.01 *M* sodium acetate of pH 5.5 containing 0.02 *M* magnesium chloride until at least two void volumes of effluent had been collected. For all of the columns, one void volume was 80–130 ml. If overloading had occurred, elution with the initial buffer mixture was continued until the absorbance of the effluent at 260 nm returned to, and remained at, a value of less than 0.1, before elution with a sodium chloride gradient was started. After elution of the column with the initial buffer, a linear gradient of 0 to 1.0 *M* sodium chloride in a total of 1 l of 0.01 *M* sodium acetate of pH 5.5 and 0.02 *M* magnesium chloride was used; in some instances the gradient was extended to 2.0 *M* sodium chloride in a further 1 l of buffer. The absorbance of the effluent at 260 nm and its conductivity were monitored, and the molarity of sodium chloride in the tRNA elution region was found by comparison with a graph of conductivity *versus* molarity of sodium chloride in the buffer used. The chromatographic procedure was repeated, using chloride gradients indicated by the preliminary experiments, and analyses were performed for various species of tRNA by the method outlined above. After each fractionation, the cellulose derivatives were washed by passing 2 *M* sodium chloride through the column, and were then re-equilibrated with the starting buffer. For fractionations at pH 8.0 the column was first washed with 2 *M* Tris chloride of pH 8.0 and then equilibrated with 0.01 *M* Tris chloride of pH 8.0 and 0.02 *M* magnesium chloride.

For fractionations in the presence of EDTA, 0.01 *M* sodium acetate of pH 5.5 and 0.001 *M* EDTA were used for elution of the column before and after loading of the sample, and the sodium chloride gradient was also made up in this buffer; in order to minimize the reported loss of activity of tRNA on chromatography in the presence of EDTA[16], the collection tubes were charged with concentrated magnesium chloride solution so as to give a final concentration of 0.02 *M* magnesium chloride in the effluent collected.

Yeast cell extract

A 50-g amount of baker's yeast in 50 ml of 0.01 *M* Tris chloride of pH 7.5, 0.02 *M* β-mercaptoethanol and 10% glycerol was homogenized (Atomix) with 100 g of glass beads; after settling, the supernatant was decanted and the residue was re-homogenized with a further 50 ml of the buffer mixture. The combined supernatants were centrifuged for 60 min at 20000 *g* in an MSE HS-18 centrifuge to give a clarified supernatant (80 ml). This supernatant was divided into two 40-ml portions, one of which was placed on a column of A2 cellulose and the other on a column of C1 cellulose, each pre-equilibrated with the homogenization buffer. Elution was continued with 0.01 *M* Tris chloride of pH 8.0, 0.01 *M* magnesium chloride, 0.02 *M* β-mercaptoethanol and 10% glycerol until the absorbance of the eluate, at both 260 and 280 nm, returned to a low value; then a linear gradient of 0–2.0 *M* sodium chloride in the elution buffer mixture was started, the effluent was monitored for conductivity and for absorbance at 260 and 280 nm and the positions of aminoacyl ribonucleic synthetase activity were found by the above assay method.

RESULTS AND DISCUSSION

The cellulose derivatives and their elution characteristics are listed in Table I. It can be seen that their capacities for tRNA vary considerably, as do the elution

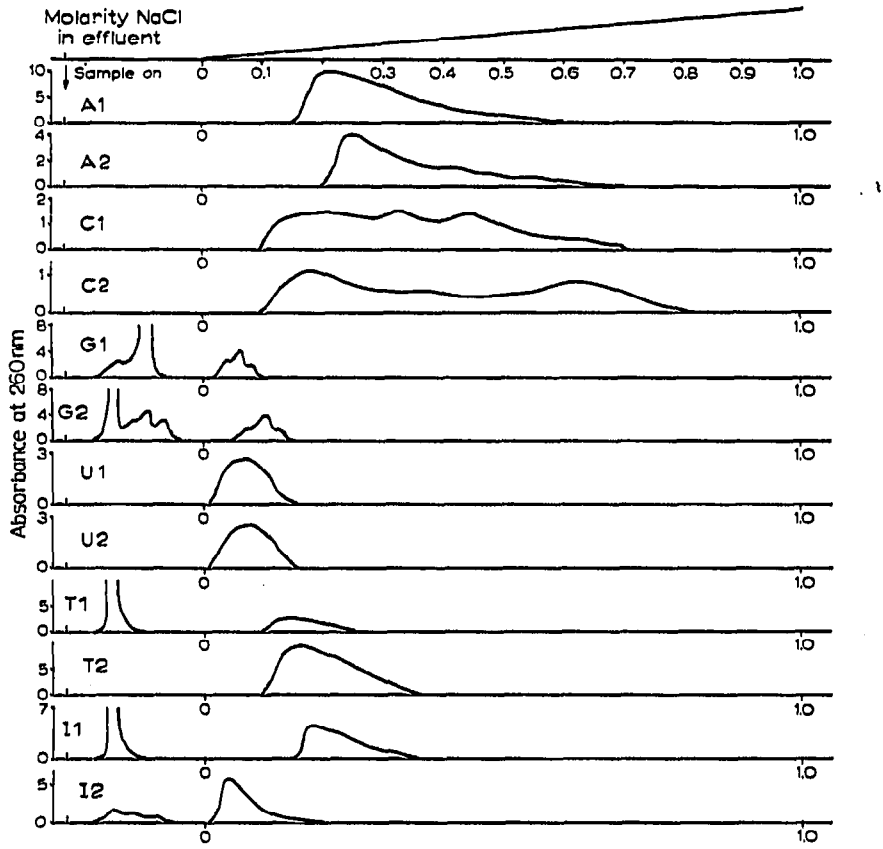

Fig. 1. Elution profiles for tRNA at pH 5.5 on the nucleoside derivatives. In each case, 1000 A_{260} units of yeast tRNA were loaded on to a column containing *ca.* 100 g of nucleoside derivative and eluted, first with 0.01 *M* sodium acetate of pH 5.5, 0.02 *M* magnesium chloride, then with a linear gradient of sodium chloride in the buffer mixture. ———, A_{260}.

ranges with a sodium chloride gradient. The recovery of UV absorbant material was satisfactory for all the columns run.

The elution profiles of tRNA at pH 5.5 for the whole series of cellulose derivatives are shown in Fig. 1; those for the adenosine and cytosine columns in particular indicate some degree of separation over the elution region. Analysis of fractions from the guanosine columns showed that there was partial separation of tRNA species, particularly within the overload peaks. As purine–pyrimidine hydrogen bonding, and hence adsorption of nucleic acid to the column material, should be enhanced by an increased salt concentration, equilibration and loading of the G2 column was repeated with initial buffers containing 1.0, 0.5, or 0.2 *M* sodium chloride; in each instance, the whole of the tRNA applied was eluted in the breakthrough volume of the column. Dilution of the tRNA solution prior to loading also had no effect on the retention properties. Although a cellulose derivative with a higher guanosine content might be a useful chromatographic material, the capacities of the existing guano-

sine derivatives were so low compared with those of adenosine or cytosine that their properties were not investigated further.

Similarly, the materials prepared from *d*-thymidine or from inosine were of low capacity and gave very little separation between individual species of tRNA, and so were not studied in detail.

Analytical results for the fractionation of tRNA, at pH 5.5 in the presence of magnesium on some of the derivatives are illustrated in Fig. 2. As shown by Table I and Figs. 1 and 2, the derivatives of adenosine, cytosine and uridine can adsorb useful amounts of tRNA, which can be recovered by a salt gradient under mild conditions; no losses in amino acid acceptor activity were observed, and recovery of material absorbing at 260 nm was usually 80–100%. The order of elution of individual species of tRNA varied for different derivatives; however, it is unlikely that purine–pyrimidine interactions play the major role in determining the order of elution, as adsorp-

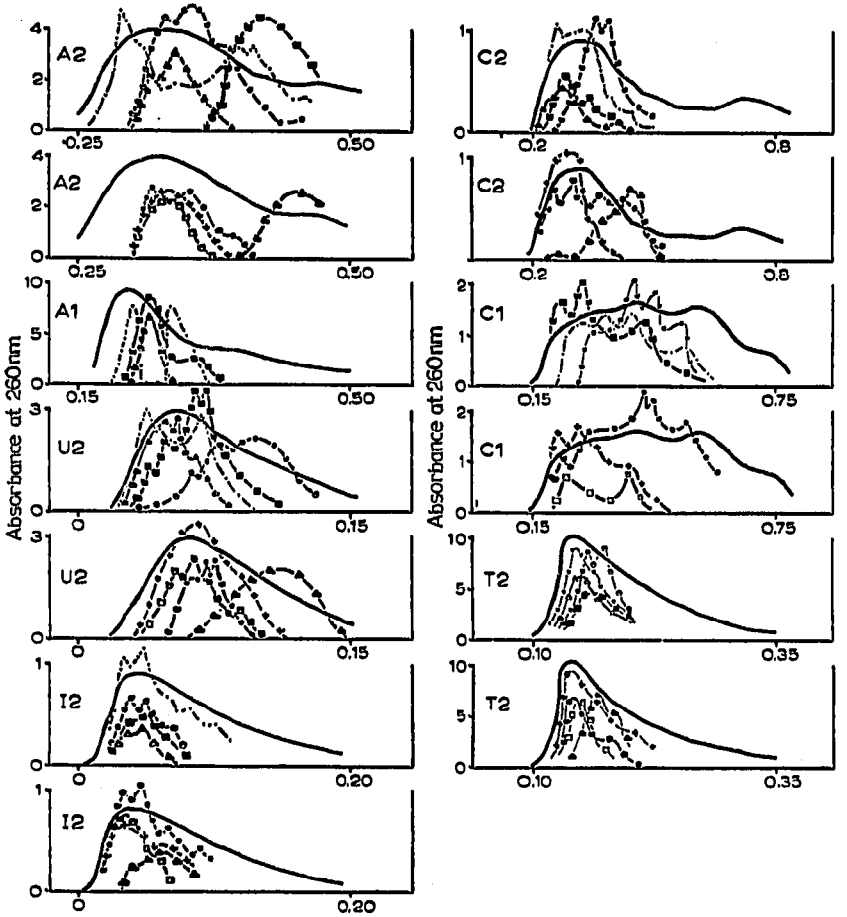

Fig. 2. Elution profiles for tRNA species at pH 5.5 on selected nucleoside derivatives *vs.* molarity of sodium chloride in the elution buffer (0.01 *M* sodium acetate of pH 5.5, 0.02 *M* magnesium chloride). ———, A_{260}; –·–·–,c.p.m. $tRNA_{Met}$; ○, c.p.m. $tRNA_{Phe}$; △, c.p.m. $tRNA_{Tyr}$; ■, $tRNA_{Lys}$; +, c.p.m. $tRNA_{Cys}$; ●, c.p.m. $tRNA_{Asp}$; □, c.p.m. $tRNA_{Asn}$; ▲, c.p.m. $tRNA_{His}$.

tion takes place at low salt concentrations and elution is accomplished by a positive salt gradient, the uridine and thymidine derivatives have different chromatographic properties and the relative elution positions for most of the fastest and slowest running species of tRNA on the adenosine and uridine derivatives are similar.

Chromatography at higher pH gave some changes in the elution pattern, but resulted in decreased affinity of the adsorbent for tRNA and also in broadening of individual tRNA peaks, and would therefore be of only limited use for purification.

Chromatography in the presence of EDTA resulted in virtually no separation between different species of tRNA, and hence the fractionation process must depend upon the maintenance of the tertiary structure of the tRNA molecule and interactions with some "exposed" bases or other parts. In this connection, it is perhaps noteworthy that $tRNA_{Phe}$ is usually eluted late in the sequence, as is also the case with BD-cellulose[3]; this may be due to the interaction of the unique base A*[3]. However, $tRNA_{His}$, which is also slow-running on nucleoside derivatives, elutes with the majority of tRNA species from BD-cellulose[3], while $tRNA_{Cys}$ and $tRNA_{Tyr}$, which run slowly on BD-cellulose[3], usually occur before, or in, the main peak eluted from the nucleoside derivatives; hence separations on BD-cellulose and on the nucleoside-celluloses are controlled by different factors and can be used to complement one another in the purification of several tRNA species.

Chromatography of the crude yeast extract (Fig. 3) resulted in fractionation of both proteins and nucleic acids, but the capacities of the columns were low, probably owing to the pH of the buffer used (a lower pH results in increased complex formation between tRNA and the synthetases, and also leads to precipitation of the synthetases and other enzymes). Although fractionation was obtained, the synthetases were not noticeably retarded relative to other proteins, hence their interaction with the cellulose derivatives is probably ionic in nature rather than due to a specific reaction at the active site.

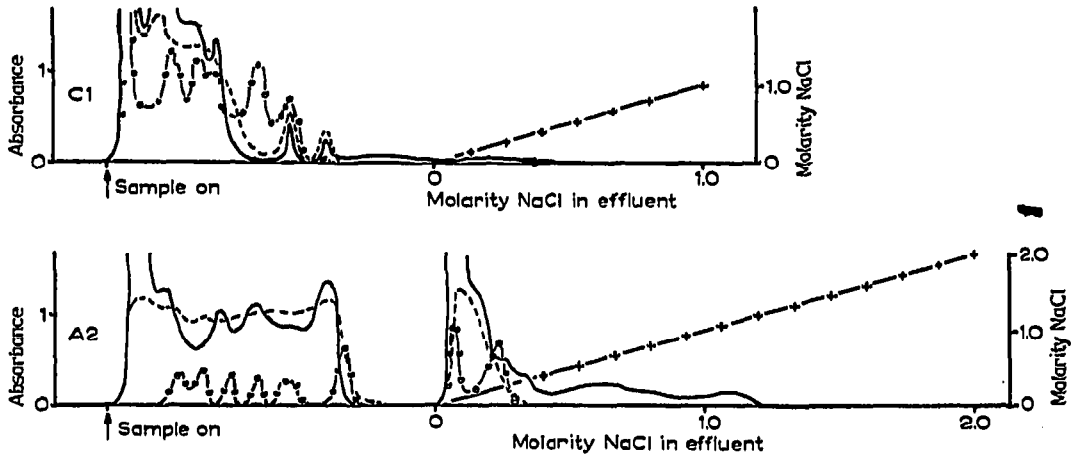

Fig. 3. Elution profiles for yeast crude cell extract on C1 and A2 columns. Elution buffer: 0.01 *M* Tris chloride of pH 8.0, 0.01 *M* magnesium chloride, 0.02 *M* β-mercaptoethanol, 10% glycerol; then a gradient of sodium chloride in the same buffer mixture. – – –, A_{260}; ———, A_{280}; ○, aminoacyl-t-ribonucleic acid synthetase activity; +, 1*M* NaCl.

The derivatives might be of use in the final steps of purification of a protein, but potentially their greatest promise is as materials for the fractionation of nucleic acids. The ease with which the derivatives can be prepared, together with the simplicity of operation of the columns and the reproducibility of the results, are further factors that indicate their suitability for general use as chromatographic media.

In particular, yeast $tRNA_{His}$, which is not well separated from most other tRNAs by current techniques[3,17,24] and which has so far not been obtained in a sufficiently pure state for detailed investigation, could potentially be obtained uncontaminated by other species, by successive chromatography on adenosine and uridine derivatives. This process can also be adapted to yield purified $tRNA_{Lys}$ and $tRNA_{Phe}$ as by-products.

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