
The use of a dipolar ion-exchanger for the fractionation of transfer ribonucleic acid

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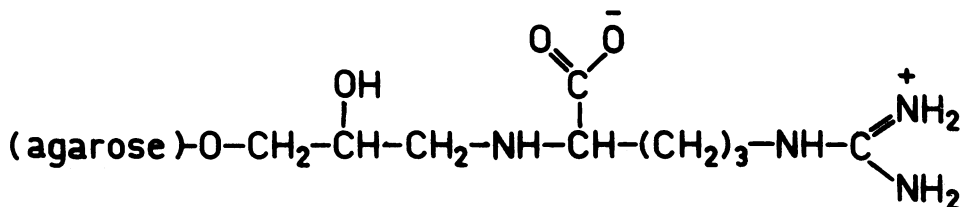
ABSTRACT

Transfer ribonucleic acid is well fractionated on columns of arginine-agarose, whose properties appear in general to be similar to those of DEAE-Sephadex. However, the amino acid acceptor species are separated into sharper peaks and in several instances, notably for methionine, glycine, serine, leucine and aspartate accepting tRNAs from Escherichia coli, isoaccepting species are well resolved. In the case of methionine accepting tRNA from E. coli the tRNA_m^{Met} species is eluted before the tRNA_f^{Met} species and since it is also eluted prior to the bulk of the tRNA it is obtained in a high degree of purity. By comparing the properties of columns of arginine-agarose and its methyl ester in which the carboxylate anion is blocked, it is seen that the carboxylate ion plays a role in the fractionation of the tRNA^{Met} species.

INTRODUCTION

During the past decade much attention has been devoted to the isolation of purified species of tRNA, and among the most popular methods to emerge for this purpose are those using anion-exchange chromatography, and anion-exchange chromatography which also incorporates a secondary effect. DEAE-cellulose¹ and DEAE-sephadex^{2,3} have been used for the separation of several species, the latter being particularly useful for the purification of tRNA^{Met} from Escherichia coli². However, neither of these methods efficiently separates isoaccepting tRNA species. Of the methods used in which anion-exchange is combined with a secondary effect, benzoylated DEAE-cellulose chromatography⁴ and reversed-phase chromatography, particularly RPC5⁵, have been used extensively.

Porath and Fornstedt⁶ have described the use of arginine-agarose, which is prepared simply in a two step reaction by coupling arginine to sepharose 6B using epichlorohydrin, for the fractionation of plasma proteins. They termed it a "Dipolar ion-exchanger" because of its zwitterion structure which is shown below.



Arginine-agarose

They pointed out that arginine-agarose has properties similar to DEAE-Sephadex, but has the advantage that the bed volume is not subject to such large alteration with alteration in ion concentration since in dipolar ion-exchangers the distribution of ions rapidly reaches equilibrium.

Here it is shown that arginine-agarose behaves similarly to DEAE-Sephadex for the fractionation of tRNA. The role of the carboxylate anion in the fractionation has been investigated. Although cation exchangers themselves have not proved to be successful for the fractionation of tRNA, it was of interest to see whether the carboxylate anion when introduced as a secondary effect would influence the pattern of fractionation. A report on the use of arginine-agarose for the purification of tRNA_m^{Met} and tRNA_f^{Met} has been published previously⁷. This paper gives a more comprehensive description of the properties of arginine-agarose for the fractionation of tRNA.

MATERIALS AND METHODS

Chemicals were of analytical grade and obtained from B.D.H. Chemicals Ltd., Poole, Dorset, except where stated otherwise. L-[¹⁴C]-labelled amino acids were obtained from The Radiochemical Centre, Amersham, U.K. and had the following specific activities (mCi. mmole⁻¹): alanine, 156; arginine, 318; aspartic acid, 229; glycine, 109; glutamine, 48; histidine, 327; leucine, 331 methionine, 54; phenylalanine, 513; serine, 153, tyrosine, 513; valine, 260. Escherichia coli strain M.R.E. 600 frozen cell were obtained from Whatman Biochemicals Ltd., wheat germ (grade 1) was a gift from Rank Hovis McDougall Flour Mills Ltd., and white rats bred in this department originated from Carworth Europe CFHB strain. Sepharose 4B and 6B were obtained from Pharmacia Fine Chemicals AB, Sweden. E. coli tRNA was a generous gift from Dr. S. Nishimura.

Arginine-agarose

Arginine-agarose (Arginine-Sepharose) was prepared by coupling L-arginine on to Sepharose 6B through epichlorohydrin according to the method described by Porath and Fornstedt⁶. Elemental analysis (kindly performed by Mr. D. Newman of the Department of Organic Chemistry of this University) gave a nitrogen composition of 3.71%.

Arginine methyl ester-agarose

A methyl ester of arginine-Sepharose was prepared by coupling arginine methyl ester onto a carboxyl derivative of Sepharose 4B in a manner similar to the method used by Cuatrecasas⁸ for the insolubilisation of estradiol. Carboxyl-Sepharose (5g), prepared by reacting 6-aminocaproic acid with cyanogen bromide (Eastman Kodak Ltd.) activated Sepharose, was washed thoroughly and suspended in 20ml distilled water containing 0.75g arginine methyl ester (Sigma Chemical Co. Ltd., London). The suspension was adjusted to pH 4.7 with N HCl, and 100mg of water soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] (Sigma, London) dissolved in 1 ml H₂O was added over 5 min. at room temperature. After 30 min. a further 100mg of the carbodiimide was added over 5 min. and the suspension was stirred overnight at room temperature. The gel was washed with M NaCl to remove excess reagents and urea and then washed thoroughly with H₂O.

Rat liver tRNA

Rat livers were homogenised in 0.3% bentonite suspension at 0 - 4°C, and the RNA was extracted by treatment with phenol at room temperature for 15 min. After precipitation of the total RNA with ethanol, tRNA was extracted with M NaCl solution and deacylated by incubation in 0.2M Tris.HCl pH 9.0 for 2hr at 37°C. This crude tRNA dissolved in buffer A (20mM Tris.HCl pH 7.5, 10mM MgCl₂) containing 0.1M NaCl was applied to a DEAE cellulose (Whatman DE 32) column, 4 x 5cm. Following elution of the column with 0.25M NaCl in buffer A, the tRNA was eluted with 0.75M NaCl in buffer A and precipitated from solution by addition of 2 volumes of ethanol at -20°.

Wheat germ tRNA

Crude wheat germ tRNA was obtained by a procedure similar to that for rat liver tRNA, but the DEAE cellulose step was omitted.

Aminoacyl-tRNA synthetases

1) E. coli. A crude mixture of aminoacyl-tRNA synthetases was prepared from strain M.R.E. 600 (Whatman Biochemicals) by a method similar to that described by Nirenberg⁹. After dialysis of the S-100 fraction, nucleic acid was removed by streptomycin sulphate precipitation, and solid ammonium sulphate was added to the supernatant at pH 7.5. The precipitate

formed between 0.3 and 0.7 saturation was collected and dialysed against standard buffer.

2) Rat liver. Crude aminoacyl-tRNA synthetases from rat liver were obtained according to the method of Nishimura and Weinstein¹⁰.

3) Wheat germ. The crude mixture of synthetases was prepared in a manner similar to that described for the preparation of a wheat germ S-30 fraction¹¹ with additional steps to remove ribosomes (centrifugation at 125,000g) and nucleic acid (precipitation with protamine sulphate).

Assay of amino acid acceptor activity of tRNA.

The aminoacylation assay of tRNA with E. coli synthetases was performed according to the method of Hoskinson and Khorana¹². The assay mixture consisted of 25mM Tris.HCl pH 7.5, 5mM MgCl₂, 0.5mM EDTA, 2.5mM ATP 2.5μM L-[¹⁴C]-amino acid, a sample of tRNA and an appropriate amount of the crude synthetase. Assays for the aminoacylation of tRNA using the crude synthetases from rat liver or wheat germ were performed similarly except that the pH of the reaction mixture was 7.8.

Preparation of L-[¹⁴C]-methionyl-tRNA

Crude E. coli tRNA was aminoacylated with L-[¹⁴C]-methionine using the crude E. coli synthetase as described above in the assay procedure, but with scaling up to give a total reaction volume of 2.5ml. The reaction was terminated by the addition of a tenth volume of M sodium acetate pH 4.5. After treatment with phenol, the aminoacyl-tRNA was recovered from the aqueous phase by precipitation with ethanol, dialysed against 5mM sodium acetate pH 4.5, and stored at -20°C.

Chromatography was performed in glass columns at atmospheric pressure, the eluent being pumped from the bottom of the column. The absorption of the eluent was monitored at 254 nm in a flow cell (LKB Uvicord II or Isco UA4) or on some occasions the absorption of individual fractions was measured in a Pye-Unicam 1800 spectrophotometer.

RESULTS

A preliminary study showed that on small columns of arginine-Sepharose (1 x 15cm) crude E. coli tRNA was retained at low salt concentrations, but was eluted at higher salt concentrations. Experiments were performed at 23°C and 4°C. Figs. 1(a) and 1(b) show the elution profiles (Transmittance at 254nm) obtained when approximately 1mg of tRNA was eluted at 23°C and 4°C respectively, using a concentration gradient of NaCl in buffer A. It will be seen that at 23°C most of the material was eluted between 0.42 and 0.65M NaCl whereas at 4°C the material was eluted between slightly higher

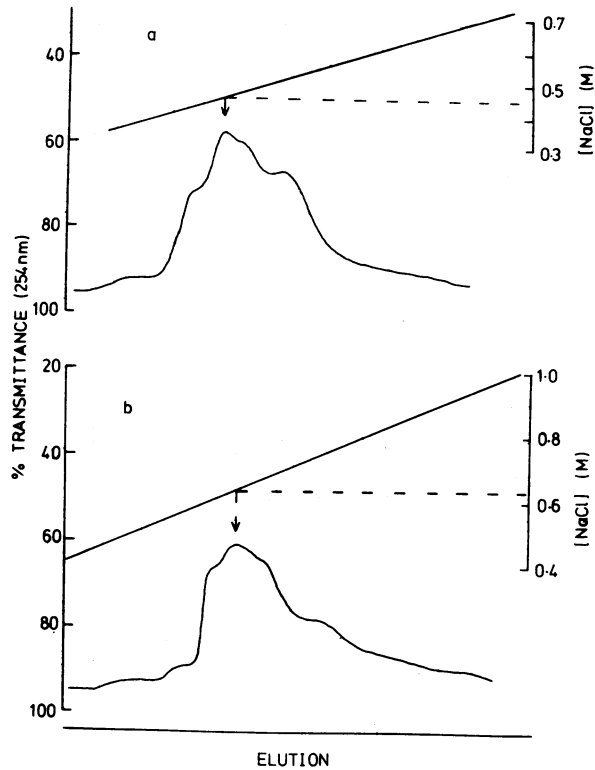


Fig 1: Effect of temperature on the elution of E. coli tRNA from arginine-agarose: a, 23°C; b, 4°C.

salt concentrations (0.58 - 0.80M NaCl). In both cases approximately 85-90% of the material added to the column was eluted within the concentration ranges indicated above. Very little material was eluted either by increasing the salt concentration to 2M or by elution with 1M NaCl-ethanol (4:1 v/v), but with one column volume of 1M NaOH tightly absorbed material was eluted. It was judged that the elution profile of the tRNA obtained at 23°C was better than that at 4°C and further experiments of chromatography of tRNA on arginine-Sepharose were carried out at room temperature.

50mg of crude E. coli tRNA was fractionated on a column of arginine-Sepharose (1.75 x 45cm). After addition of the tRNA (dissolved in buffer A) to the column, elution was carried out with a linear gradient of 0.3 to 1.0M NaCl in buffer A (total volume 1.2l) at a flow rate of 23ml.hr⁻¹. Fractions of 2ml were collected and the E₂₆₀ of every fifth fraction was measured. These fractions were also assayed for their acceptor activity of several

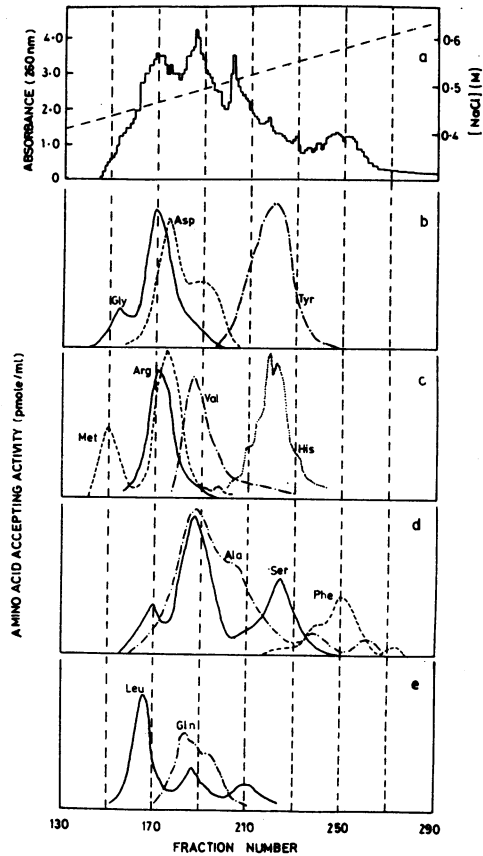


Fig. 2: Fractionation of *E. coli* tRNA on arginine-agarose.

amino acids. Fig. 2a shows the absorbance profile (E_{260}) and Figs. 2(b)-(e) the amino acid acceptor activities, which are drawn on an arbitrary scale for convenience of the drawing of the figure.

The actual amino acid accepting activities of the peak fractions of individual amino acids are given in Table 1, where they are compared with the amino acceptor activities found in the crude tRNA.

It will be seen that the amino acid acceptor activities (pmol. amino acid accepted/ E_{260} unit of tRNA) of several species of tRNA have been increased considerably. The greatest increase in activity is for the first peak of methionine acceptor activity which is the $tRNA_m^{Met}$ species⁷. In this one chromatographic step it is obtained in greater than 50% purity. Considerable purification of histidine (13 fold) and tyrosine (12 fold) acceptor activity

Table 1

Amino Acid Acceptor	Peak Fraction Number	Amino Acid pmole/ml	Acceptor Activity pmole/E ₂₆₀	Crude tRNA Pmole/E ₂₆₀
Alanine	186	299	80	20
Arginine	171	821	235	38
Aspartate 1	175	416	128	46
Aspartate 2	190	236	76	
Glycine 1	154	225	180	31
Glycine 2	169	509	196	
Glutamine	183	320	86	33
Histidine	219	298	284	22
Leucine 1	166	563	173	45
Leucine 2	185	135	36	
Leucine 3	207	59	24	
Methionine 1	150	450	692	42
Methionine 2	175	950	310	
Methionine 3	197	98	42	
Phenylalanine	240	77	59	32
Serine 1	170	143	41	30
Serine 2	185	270	72	
Serine 3	225	137	125	
Tyrosine	221	299	230	19
Valine	186	738	174	33

is achieved also in this one chromatographic step. It will be seen that arginine-Sepharose is able to separate isoaccepting tRNA species. For example aspartate and glycine accepting tRNAs are separated into two species, whereas serine, leucine and methionine accepting tRNAs are separated into three species. In the latter three cases, the three isoaccepting species are well separated into individual peaks. Other species of tRNA also show the presence of more than one species. Besides its major peak alanine accepting tRNA is found in two minor slower eluting peaks, glutamine accepting tRNA has a pronounced slower moving shoulder, and phenylalanine accepting tRNA has a shoulder preceding the major peak and a slower moving minor peak.

An examination of the ability of arginine-Sepharose to fractionate a mammalian tRNA (rat liver) and a plant tRNA (wheat germ) was also carried out. Crude rat liver tRNA (3840 E₂₆₀ units) in buffer A was applied to a column of arginine-Sepharose (80 x 115cm) equilibrated in buffer A containing 0.35M NaCl. The sample was eluted with a linear gradient of 0.35 to 0.75M NaCl in buffer A (total volume 1.6 l). 4ml fractions were collected at a flow rate of 22ml/hr, and the E₂₆₀ of every third fraction was measured. The elution profile seen in Fig. 3 shows that the bulk of the tRNA is eluted

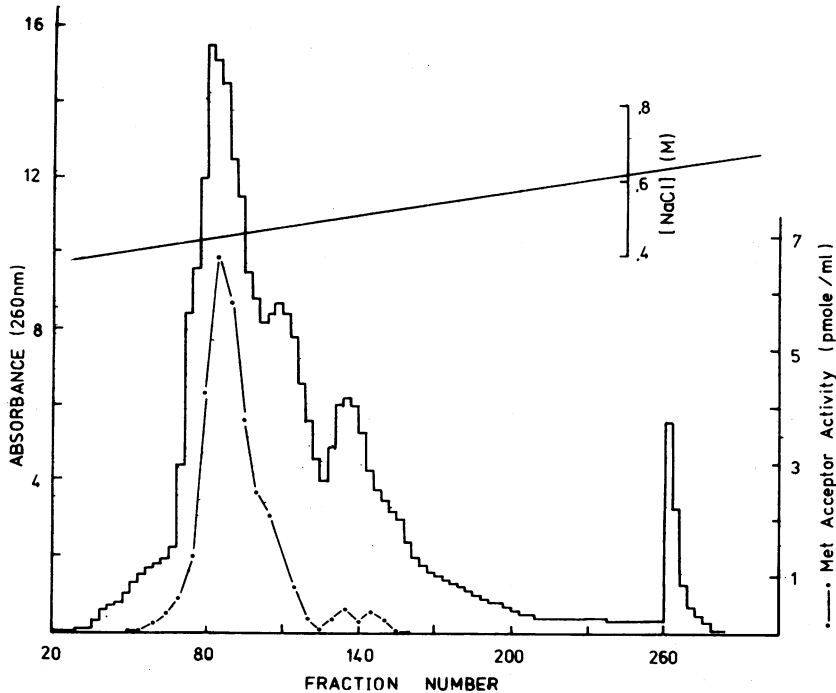


Fig. 3: Fractionation of rat liver tRNA on arginine-agarose.

In a similar experiment using the same size of column and chromatographic conditions, crude tRNA (768 E_{260} units) from wheat germ was fractionated and the E_{260} profile is shown in Fig. 4.

between 0.44 - 0.53M NaCl. Over the whole concentration range quantitative recovery of material was achieved.

It will be seen that the elution obtained for rat liver and wheat germ tRNA are very similar to each other, but differ from the E. coli tRNA elution pattern, in particular in the range of salt concentration required to elute the tRNA from the columns. Assay for the methionine accepting activity however shows that the methionine acceptor tRNA distribution differs in rat liver and wheat germ fractionations. In the case of the rat liver tRNA the methionine acceptor activity is eluted virtually as a single peak with no obvious separation of a formylatable species. (The percentage of methionine acceptor tRNA in the peak, which is formylatable is similar to the percentage found in unfractionated tRNA (35%)). On the other hand the methionine accepting tRNA from the wheat germ tRNA fractionation is resolved into at least two and possibly three species. Peak I represents 14.4% and peak II 74.9%

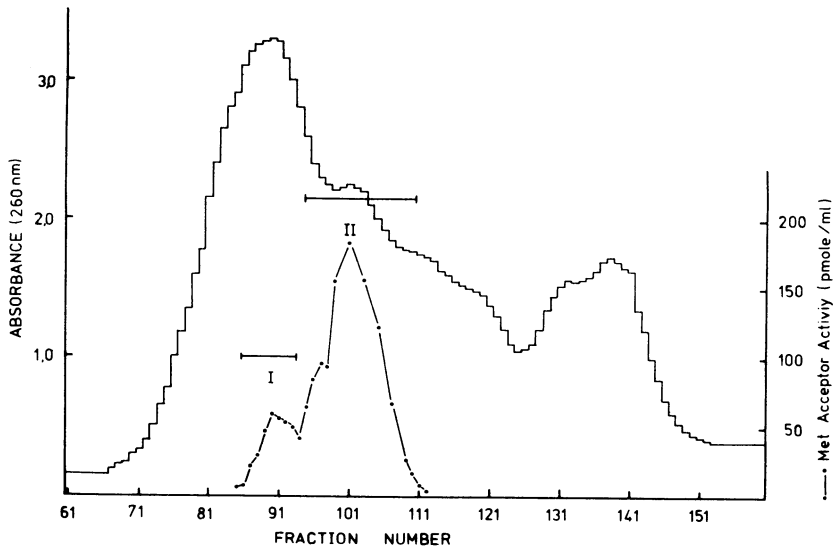


Fig 4: Fractionation of wheat germ tRNA on arginine-agarose.

of the total methionine acceptor activity applied to the column. Attempts to determine if the methionine acceptor tRNA in the peaks could be formylated were unsuccessful. Earlier it had been found that methionyl tRNA in the crude wheat germ tRNA could not be formylated using an *E.coli* enzyme preparation. This negative result was not due to inactive enzyme since the methionyl-tRNA of *E. coli* and rat liver were formylated to 61% and 35% respectively. An enzyme preparation from a blue-green alga, *Anacystis nidulans* was found to formylate *E. coli* methionyl-tRNA to 43% and wheat germ methionyl-tRNA to the much lower level of 8.3%. Previously Ecarot and Cedergren¹³ had observed 13% formylation of wheat germ Met-tRNA_i^{Met} with an enzyme preparation from *A. nidulans*.

In order to determine the role, if any, of the carboxyl groups of the arginine-Sepharose, experiments were designed in which chromatography was carried out on a column of arginine methyl ester-Sepharose in which the carboxyl groups are protected. We were unable to prepare arginine methyl ester linked to sepharose via epichlorohydrin since the ester group is labile to the basic reaction conditions. Therefore arginine methyl ester was linked to Sepharose by a different method. Here 6-aminocaproic acid was linked to cyanogen bromide activated sepharose⁸ and to this derivative arginine methyl ester was coupled using a water soluble carbodiimide. For these experiments the properties of the columns were examined by fractionation of crude *E. coli*

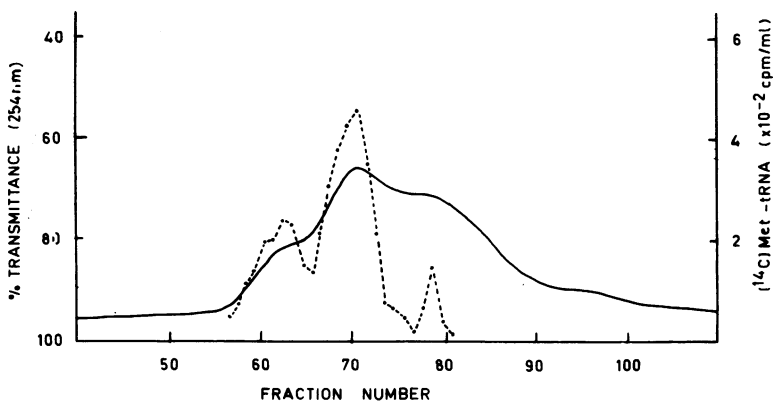


Fig. 5: Fractionation of E. coli [¹⁴C]-Met-tRNA on arginine-agarose at pH 5.0

tRNA of which the methionine accepting species were previously charged with L-[¹⁴C]-methionine. The E₂₅₄ of the eluent was monitored and the radioactivity of the fractions was determined. Methionine-charged crude tRNA was run firstly on arginine-Sepharose. 25 E₂₆₀ Units dissolved in 2.5ml 0.35M NaCl in buffer B (20mM sodium acetate, pH 5.0, 10mM magnesium chloride) was loaded onto an arginine-Sepharose column (0.8 x 20cm) previously equilibrated with 0.35M NaCl in buffer B. The sample was eluted with a linear gradient of 0.35M - 0.75M NaCl in buffer B at 4° (total volume 250ml). Aliquots from each fraction were dispensed onto filter paper discs and the [¹⁴C]-Met-tRNA was precipitated with cold TCA for radioactivity determination as in the case of [¹⁴C]-amino acid acceptor activity assays. The results are seen in Fig. 5, which show that the E₂₅₄ profile and the fractionation of the methionine accepting tRNA is similar to the pattern obtained with uncharged crude tRNA. This validates the use of methionine charged tRNA in these experiments for comparison of the different arginine-Sepharose derivatives as chromatographic media. Similar fractionations of methionine-charged crude tRNA was performed using arginine methyl ester-Sepharose and arginine methyl ester-Sepharose which had been treated previously with 0.1N NaOH at 37° for 1 hour in order to remove the methyl ester groups. This treatment increased the carboxylate ion concentration of the gel from 1.8μ equ./10ml to 11.1μ equ./10ml. In these experiments [¹⁴C]-methionine-charged crude tRNA was eluted from the columns (0.8 x 20 cm) with a linear gradient of 0.43M - 0.75M NaCl in buffer B at 4°C (200ml total volume).

Comparison of Figs. 6a and b shows that when arginine methyl ester-Sepharose is used as the chromatographic medium [Fig. 6a], although the E₂₅₄ profile

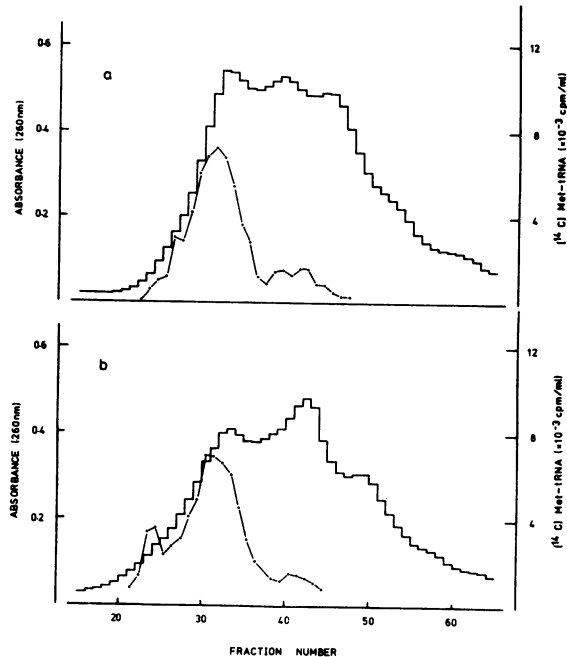


Fig. 6: Fractionation of *E. coli* [^{14}C]-Met-tRNA on (a) arginine methyl ester-agarose; and (b) alkali treated arginine methyl ester-agarose.

is not too dissimilar to that seen previously with arginine-Sepharose, the pattern of [^{14}C]-Met tRNA has changed and the forerunning peak has virtually disappeared. However, after removal of the methyl ester groups from the arginine methyl ester-Sepharose, chromatography of methionine charged crude tRNA gives a pattern of [^{14}C]-Met tRNA [Fig. 6b] similar to that seen with arginine-Sepharose (Fig.5). Three distinct peaks of radio-activity are seen, of which one appears before the bulk of the E_{260} absorbing material is eluted.

DISCUSSION

Arginine-agarose has free guanidino groups ($\text{pK}_a = 12.5$) and carboxyl groups ($\text{pK}_a 2.8$). Within the pH range usually used for transfer RNA fractionation (pH 5-8) both these groups will be ionised and therefore available for ion-exchange. For the fractionation of nucleic acids the major contributory factor would be expected to be the anion exchange between the anion forming the salt with the guanidino group and the phosphate of the nucleic acid, similar to the effect obtained with the diethylaminoethyl groups linked to either cellulose or Sephadex. However, a possible secondary contribution by the carboxylate anion needed to be examined. Transfer RNA

is retained by columns of arginine-agarose at low salt concentrations and can be removed quantitatively by elution with sodium chloride at a concentration above $0.4M$.

When crude *E. coli* tRNA is fractionated on arginine-agarose, the order of elution of the species of tRNA is somewhat similar to that obtained using DEAE-Sephadex². However, the absorption pattern is spread over a wider salt concentration gradient, and the size of column and gradient volume is considerably less. Also packing of the columns at the higher salt concentrations does not occur, and therefore there are no flow-rate problems. Fractionation of *E. coli* tRNA on arginine-agarose differs from that on DEAE-Sephadex however, in that several of the specific amino acid accepting tRNAs are separated into a number of different isoaccepting species, for example those of glycine, aspartate and most notably methionine. Of greatest interest in the latter case is the fact that the tRNA^{Met}_m species is eluted before a major and minor species of tRNA^{Met}_f. Details of this separation have been reported elsewhere⁷. In this previous report we noted that the elution of the tRNA^{Met}_m species before tRNA^{Met}_f was in contrast to results obtained with either BD-cellulose or RPC 5 where the reverse is the case, and with DEAE Sephadex where these isoaccepting species are not separated.

One major difference between arginine-agarose and the other anion-exchangers to which it has been compared is its possession of a carboxylate anion. In order to determine whether this carboxylate anion contributes to some of the unique fractionation properties of arginine-agarose a comparison was made of the fractionation of [¹⁴C]-methionine charged crude tRNA on arginine-agarose and on a derivative in which the carboxylate ion was blocked. Arginine-amide was not available for linkage to Sepharose in a manner similar to that used for arginine, and for reasons already outlined in the Results section it was necessary to couple the available arginine methyl ester by a different procedure. The use of this arginine methyl ester-agarose derivative is not without limitations. Since the coupling reaction is not completely quantitative there is a low level of residual carboxyl groups which might influence the chromatographic separation. However, a qualitative difference is sufficient to show whether the carboxyl groups play any role in tRNA separation. When crude tRNA acylated with [¹⁴C]-methionine was run on arginine-agarose (arginine linked via epichlorohydrin at pH 5.0 the absorbance pattern and distribution of tRNA^{Met} (as shown by radioactivity) was found to be very comparable with that obtained previously using unacylated crude tRNA. This showed that the addition of methionine to the tRNA^{Met} had little or no

effect on its behaviour on the column. Also the change in pH from 7.5 to 5.0 did not affect the separation properties of the system. When [^{14}C]-methionyl tRNA was chromatographed under identical conditions on a column of the methyl derivative only one major peak of radioactivity was obtained, and this gave an indication that the carboxylate anions influence the fractionation of the isoaccepting species of at least one tRNA (tRNA^{Met}). The peak obtained using the methylated derivative was wider than expected and showed a hint of a forerunning shoulder. However, this may be due to residual carboxylated anions which originate from incomplete reaction between arginine methyl ester and the carboxyl-alkyl-agarose. The results obtained above show a comparison between two substituted derivatives of agarose whose preparation involved a different procedure, and therefore it might be argued that the differences in chromatographic behaviour are due to differences in the linkages between the arginine or arginine methyl ester and the agarose. Therefore the methyl groups of the arginine methyl ester-agarose were hydrolysed under mildly alkaline conditions, and this new arginine-agarose was used for the fractionation of crude [^{14}C]-methionyl tRNA. Here a radioactive pattern with two clearly distinguishable peaks, resembling that of arginine-agarose prepared by the previous method was obtained. This change in chromatographic property must be due to the reintroduction of ionisable carboxyl groups, and it is suggested that there is a secondary interaction, albeit weak, between the bases of tRNA and the carboxylate anions of the column when tRNA is fractionated on arginine-agarose.

The elution profiles obtained when crude tRNA from rat liver and wheat germ were fractionated on arginine-agarose were quite dissimilar to that obtained with crude E. coli tRNA. This is to be expected since it is well known that tRNAs from different organisms differ in their structure¹⁴.

However, it is interesting to note that the elution profiles of the eukaryote tRNAs are remarkably similar, which may indicate a closer structural resemblance between these tRNAs than between either of the eukaryote tRNAs and E. coli tRNA. Whereas the methionine accepting tRNA from E. coli was eluted early from arginine-agarose, the methionine accepting tRNA from both rat liver and wheat germ was eluted with the bulk of the tRNA. This compares with results obtained on DEAE-Sephadex, where it is found that methionine accepting tRNA from E. coli elutes before the bulk of the tRNA² whereas methionine accepting tRNA from rat liver elutes very much later with the bulk of the tRNA sample (Smith, A. E. and Marcker, K. A., personal communication).

Besides arginine-agarose Porath described other "dipolar-ion exchanges" for the separation of plasma proteins, which included lysine-agarose. This material has also been examined as a media for the fractionation of tRNA. However, it was found that the tRNA was eluted as a single peak at a salt concentration of about 0.1M. It would appear therefore that the tRNA is only loosely held by this material. Lysine-sepharose because it binds nucleic acids much less strongly than other anion exchanges and arginine-agarose has a promising use for the separation of different size classes of RNA (Jones and Jay, unpublished results).

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