RAT LIVER PHENYLALANINE tRNA: COLUMN PURIFICATION AND FLUORESCENCE STUDIES

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Within the past three years the primary structures of 7 transfer RNAs, obtained from E. coli or yeast, have been published (Holley et al., 1965; Zachau et al., 1966; Madison et al., 1966; Bayev et al., 1967; RajBhandary et al., 1968a; Goodman et al., 1968; Dube et al., 1968). Although several authors have speculated that individual tRNAs may play an important role in cell differentiation, there are relatively few studies on individual tRNAs from higher organisms. This laboratory has been engaged, therefore, in the purification of individual tRNAs from mammalian cells. In a study to be reported elsewhere (Nishimura, S. and Weinstein, I. B., manuscript submitted for publication), rat liver tRNAs for 16 amino acids were resolved on DEAE-Sephadex, reverse phase, or benzoylated DEAE-cellulose columns. The present report describes a rapid method for the isolation of rat liver phenylalanine tRNA, and indicates that the material contains an unidentified fluorescent constituent which can be used in monitoring the purification. Our studies on fluorescence were prompted by the report of RajBhandary et al. (1968b) that yeast phenylalanine tRNA contains an unidentified nucleoside (Y) which is fluorescent. During the course of these studies Dudock et al. (1968) reported the purification and nucleotide sequence of wheat germ phenylalanine tRNA. It is of interest that this tRNA also contains the Y nucleoside and

has a fluorescence spectrum identical to that which we obtained with rat liver phenylalanine tRNA (Dudock, B. S., personal communication).

MATERIALS AND METHODS

Benzoylated DEAE-cellulose (BD-cellulose), prepared by the method of Gillam et al. (1967), and C^{14}-L-amino acids were purchased from Schwarz BioResearch. BD-cellulose columns and reverse phase freon (RPC-freon) columns were prepared as previously described (Gillam et al., 1967; Weiss and Kelmers, 1967) and were run at room temperature. Additional details are given in the Legends.

To obtain rat liver tRNA, male Hotzmann rats (250-300 gm) were fasted 18 hours, decapitated, and their livers promptly frozen in liquid nitrogen. RNA was extracted as described by Delihas and Staehelin (1965) with the following modifications: all phenol extractions were done at 20°, rather than 60°C, and the tRNA obtained after the 1 M NaCl extraction was used, without further purification, for column chromatography. Crude rat liver aminoacyl-tRNA synthetase was prepared as previously described (Axel et al., 1967) but with the following modification: prior to the 70% ammonium sulfate precipitation step, 0.05% protamine sulfate was added to the microsomal supernatant fraction to completely precipitate any endogenous tRNA.

Fluorescence spectra were measured at 20°C in an Aminco-Bowman Spectrophotofluorometer equipped with a 150 Watt Zenon Arc Lamp, Model No. 281188. Absorbance was determined on a Zeiss Spectrophotometer Model PMQ II in 1 ml, 1 cm light path at neutral pH.

The reaction mixture for assaying amino acid acceptor activity of tRNA contained: 0.01-0.03 ml of the column fraction, 10 μmoles of Tris-HCl (pH 7.5), 1 μmole of magnesium acetate, 1 μmole of KCl, 0.2 μmole of ATP, 0.02-0.05 μc of C^{14}-L-phenylalanine (311 mc/mmole), and 0.04 ml (approximately 1.4 mg of protein) of aminoacyl-tRNA synthetase, in a total volume of 0.1 ml. Assays were incubated at 37° for 10 minutes. Aliquots (0.08 ml) were processed on Whatman 3MM filter paper disks and counted according to previously described methods (Nishimura et al., 1967).

RESULTS

Gillam et al. (1967) originally found that, when uncharged yeast tRNA is applied to BD-cellulose, most of the material was eluted with a NaCl gradient but the phenylalanine tRNA remained tightly bound to the column and required 10% ethanol in 1.5 M NaCl for elution. In subsequent experiments (vid infra) we obtained similar results with rat liver tRNA, suggesting a method of rapid purification.

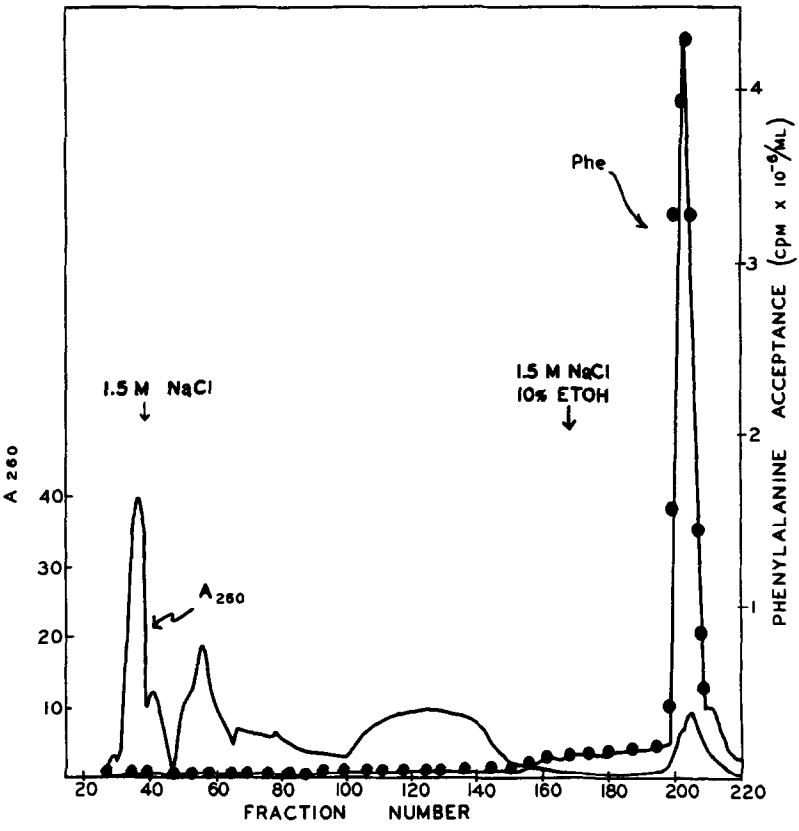

Fig. 1. BD-cellulose column chromatography of rat liver tRNA. The column (4 x 46 cm) was equilibrated with 0.4 M NaCl, 0.05 M sodium acetate (pH 5.0) and 0.01 M $MgCl_2$, and 18,180 A_{260} units of rat liver tRNA in 100 ml of the initial buffer were applied. The column was then eluted stepwise with: 500 ml of the initial buffer; 2,500 ml of 1.5 M NaCl, 0.05 M sodium acetate (pH 5.0), 0.01 M $MgCl_2$ and, finally, 1,000 ml of 10% ethanol in 1.5 M NaCl, 0.05 M sodium acetate (pH 5.0), 0.01 M $MgCl_2$. The flow rate was 1 ml/min and fractions were approximately 11 ml. All fractions were assayed for absorbance at 260 mμ, and phenylalanine acceptance capacity as described under Methods.

Fractions 50-100 (pool 1), 101-160 (pool 2), and 199-217 (pool 3) were combined, the tRNAs precipitated with 2 volumes of ethanol (-20°C), and the precipitates resuspended in water.

Figure 1 indicates our results when uncharged rat liver tRNA was eluted stepwise, with NaCl and then NaCl plus ethanol, from a BD-cellulose column. The material eluted in the ethanol region contained only 6% of the total A_{260} units, but essentially all of the phenylalanine tRNA. Additional studies (not shown here), employing a gradient of 0.4 to 1.5 M NaCl, demonstrated that rat liver tyrosine tRNA eluted at approximately 0.8 M NaCl, histidine tRNA at approximately 0.9 M NaCl, and precharged tyrosine tRNA at approximately 0.95 M NaCl. Precharged phenylalanine tRNA eluted somewhat later in the ethanol region than uncharged phenylalanine tRNA.

Assays of amino acid acceptance capacity indicated that the BD-cellulose procedure had resulted in a seventeenfold enrichment for phenylalanine tRNA (Table I).

Table I. Purification of rat liver phenylalanine tRNA and its fluorescence

tRNA	Fluorescence at 430 mμ (Arbitrary Units)	Phenylalanine Acceptance (μμmoles/A_{260} unit)
Unfractionated tRNA	2	27
BD-cellulose pool 1	0	4
" " " 2	3	7
" " " 3	16	464
RPC-freon fraction 48	50	1112

The sources of these tRNAs are described in Figs. 1 and 2. They were centrifuged at 30,000 xg for 45 minutes to remove turbidity, adjusted to 0.65 A_{260} units/ml, and their fluorescence measured with excitation at 310 mμ. Additional details are described under Methods.

An additional 2.4-fold purification was achieved by chromatography on a RPC-freon column (Fig. 2 and Table I). The phenylalanine tRNA peak obtained from this column had an acceptance capacity of 1112 μμmoles of phenylalanine per 1 A_{260} unit (Table I) which is 67% of the theoretical, assuming that a pure and fully active tRNA will accept 1666 μμmoles of amino acid per 1 A_{260} unit of tRNA (Hoskinson and

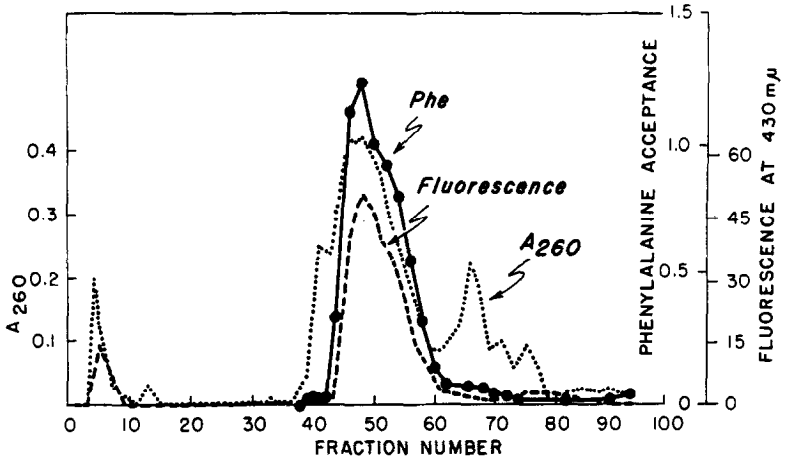

Fig. 2. Further purification of phenylalanine tRNA on a reverse phase freon column. The column (0.3 x 150 cm) was equilibrated with 0.02 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 0.15 M NaCl, and 182 A_{260} units of the phenylalanine tRNA region obtained from a BD-cellulose column (pool 3, Fig. 1) were mixed with 7 ml of water and applied. A linear gradient elution was carried out using 400 ml of 0.02 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$ and 0.65 M NaCl in the reservoir; 400 ml of 0.02 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$ and 0.15 M NaCl in the mixing chamber. The flow rate was 10 ml/hr. A volume of 7.2 ml of effluent was collected per fraction. Fractions were assayed for absorbance at 260 mμ, C^{14}-phenylalanine acceptance, and for fluorescence at 430 mμ with excitation at 310 mμ as described under Methods. Fluorescence intensity is expressed in arbitrary units.

Khorana, 1965). In addition, this material was free of acceptance activity for at least 16 other amino acids.

Yeast phenylalanine tRNA contains an unidentified nucleoside (Y) which is adjacent to the anticodon and fluoresces (RajBhandary et al., 1968b). It seemed likely to us that the Y residue might account for the high affinity of yeast phenylalanine tRNA for BD-cellulose and that rat liver tRNA, having a similar affinity for BD-cellulose, might also contain the Y residue. We undertook, therefore, to demonstrate by fluorescence the presence of Y in intact rat liver phenylalanine tRNA. Figure 3 indicates that our purified material does have strong fluorescence with an emission maximum at 430 mμ and an excitation maximum at 310 mμ Dudock (personal communication) has independently observed a similar excitation and emission maximum for the fluorescence of wheat germ phenylalanine tRNA. Table I

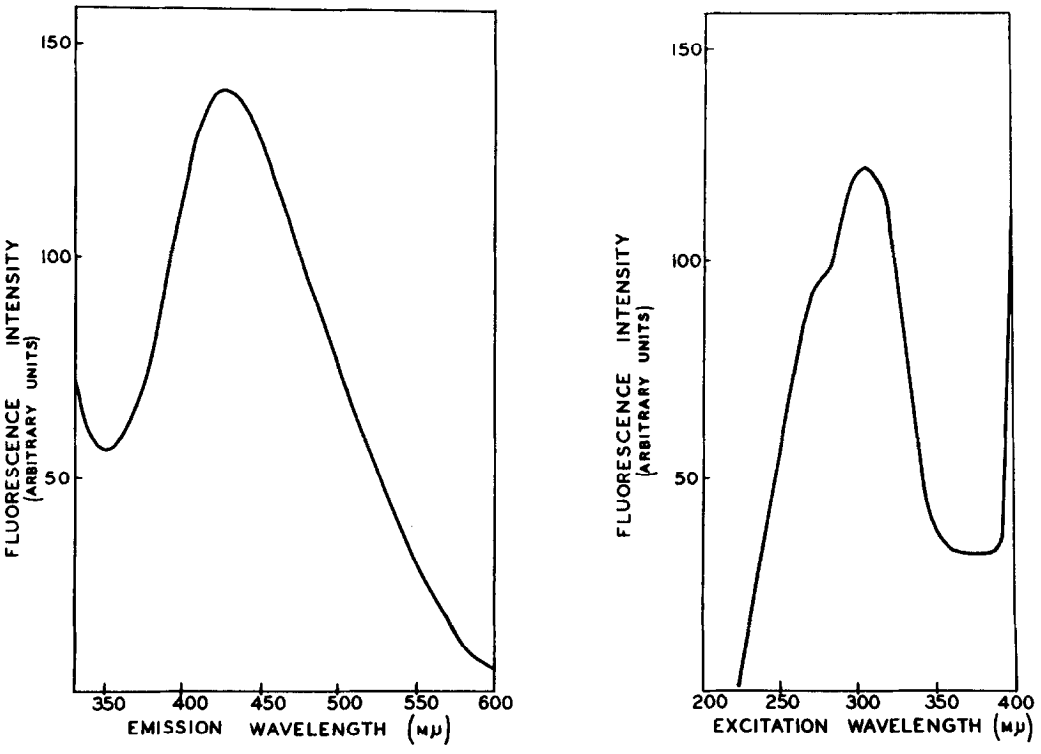

Fig. 3. Fluorescence spectrum of rat liver phenylalanine tRNA. The tRNA (fraction 48, Fig. 2) was studied at 0.6 A_{260} units/ml in the column elution buffer.
(A) Emission spectrum with excitation at 310 mμ.
(B) Excitation spectrum with emission measured at 430 mμ.

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indicates that fluorescence at 430 mμ is a useful guide during the purification of phenylalanine tRNA. Figure 2 also indicates concordance between fluorescence intensity and the acceptance capacity for phenylalanine across the phenylalanine tRNA peak. The significance of the small fluorescent peak at the beginning of the gradient (Fig. 2) is not known at the present time.

DISCUSSION

The present study suggests that phenylalanine tRNA of rat liver, like that of yeast and wheat germ, contains an unidentified nucleoside (Y). The precise nature of Y is not known at the present time, but the unusual affinity which these three tRNAs have for BD-cellulose suggests that Y contains a highly hydrophobic substitu-

ent (perhaps a polycyclic derivative). Definitive isolation and characterization of this residue from mammalian and other tRNAs are required and may shed light on its physiologic significance. With respect to function, it is of interest that a modified purine adjacent to the anticodon has been observed in several tRNAs. In the case of yeast serine (Zachau et al., 1966) and tyrosine tRNAs (Madison et al., 1966), this residue is N^6-(γ,γ-dimethylallyl)-adenosine, a compound which has cytokinin activity. It will be of interest to know, therefore, whether the Y residue also has an important biologic activity separate from its presence in tRNA. The fractionation of rat liver tRNA on DEAE-Sephadex yields at least two phenylalanine tRNA peaks (Nishimura and Weinstein, unpublished studies). Studies are in progress to determine whether these differ in their content of the fluorescent residue.

The two-step method of purification of rat liver phenylalanine tRNA, described in the present study, yields material which has 67% of the theoretical acceptance activity. Additional studies are required to determine whether this is due to partial inactivation of our material or residual contamination with other tRNAs. We tend to favor the former possibility, since our material failed to demonstrate acceptance activity for other amino acids. If this is the case, then these procedures should be useful for preparing material suitable for nucleotide sequence studies.

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