Fractionation of Rat Liver Transfer Ribonucleic Acid. Isolation of Tyrosine, Valine, Serine, and Phenylalanine Transfer Ribonucleic Acids and Their Coding Properties*

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ABSTRACT: The transfer ribonucleic acid from normal rat liver has been fractionated by DEAE-Sephadex column chromatography and selected regions of the eluate further resolved either on reversed-phase columns or on benzoylated DEAE-cellulose columns. Excellent resolution of the transfer ribonucleic acids for at least 16 amino acids was obtained. Multiple transfer ribonucleic acid peaks were observed with most amino acids. Considerably purified preparations of the transfer ribonucleic acids for tyrosine, valine, serine, and phenylalanine were obtained. The codon recognition patterns of these mammalian transfer ribonucleic acids were studied in the ribosomal binding assay and the results are generally consistent with the "wobble mechanism" previously described with *Escherichia coli* and yeast transfer ribonucleic acids. An apparent redundancy of several transfer ribonucleic acids was also noted. The present methods should be useful for the preparation of purified mammalian transfer ribonucleic acids for composition and sequence studies. In addition, our data provide an extensive profile of transfer ribonucleic acids for normal liver which can, in subsequent studies, be compared with the transfer ribonucleic acid profiles of other mammalian tissues.

The elucidation by Holley and his coworkers (1965) of the nucleotide sequence of yeast alanine tRNA has been followed by rapid advances in the chemistry of other tRNAs. The nucleotide sequences of yeast serine (Zachau *et al.*, 1966a), tyrosine (Madison *et al.*, 1966), valine (Bayev *et al.*, 1967; Takemura *et al.*, 1968), and phenylalanine (RajBhandary *et al.*, 1968) tRNAs have already been published, as have the nucleotide sequences for *Escherichia coli* tyrosine (Goodman *et al.*, 1968) and *N*-formylmethionine (Dube *et al.*, 1968) tRNAs. These results indicate certain structural similarities and differences which provide important clues to the functional properties of these molecules (Zachau *et al.*, 1966b; Fuller and Hodgson, 1967).

Despite these advances, there have been relatively few attempts to purify or sequence individual tRNAs from mammalian cells, though the partial separation of mammalian tRNAs has been previously described (Apgar and Holley, 1962; Glebov *et al.*, 1965; Caskey *et al.*, 1967; Axel *et al.*, 1967; Taylor *et al.*, 1967; Holland *et al.*, 1967; Yang and Novelli, 1968). The possibility that changes in the structure and abundance of individual tRNAs may be an important aspect of cell differentiation and dedifferentiation has been suggested by a number of authors; see Weinstein *et al.* (1966a) and Axel *et al.* (1967) for a review of this subject. Recent studies from our laboratory also suggest that tRNA may be a critical target during chemical carcinogenesis (Axel *et al.*, 1967; Fink *et al.*, 1968). The present study was undertaken, therefore, to develop techniques for the purification of the tRNAs of normal rat liver and to provide an extensive profile of tRNAs for a normal tissue which can, in subsequent studies, be compared with the tRNA profiles of various tissues, including certain hepatic tumors.

The method of fractionation was derived from that used by Nishimura *et al.* (1967) for *E. coli* tRNA and, subsequently, for yeast tRNA (Takeishi *et al.*, 1967). DEAE-Sephadex column chromatography at pH 7.5 was used as the first step in the fractionation of rat liver tRNAs, and selected regions of the eluate were subsequently further resolved, either on reversed-phase columns of the types described by Kelmers and his coworkers (Kelmers *et al.*, 1965; Weiss and Kelmers, 1967), or on the benzoylated DEAE-cellulose column originally developed by Gillam *et al.* (1967) for yeast tRNA. These procedures provide excellent resolution of the rat liver tRNAs for at least 16 amino acids. Multiple tRNA peaks were observed with most amino acids, a finding consistent with the degeneracy and redundancy of tRNAs seen in other species (see Discussion).

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During the course of our studies, considerably purified preparations of tRNAs for tyrosine, valine, serine, and phenylalanine were obtained. These were examined in the ribosomal binding assay to determine their patterns of codon recognition. The results obtained are generally consistent with the "wobble hypothesis" of Crick (1966a,b), thereby indicating that factors governing the specificity of interaction between codon and anticodon in mammalian systems are similar to those previously elucidated for yeast and *E. coli* tRNAs.

The availability of purified tRNAs from normal rat liver makes it possible now to compare the chemistry of these tRNAs with that of tRNAs present in other tissues and other species.

Materials

DEAE-Sephadex A-50 (capacity 3.5 ± 0.5 mequiv/g and particle size 40–120 μ) was purchased from Pharmacia Fine Chemicals. Chromosorb W (dimethyldichlorosilane treated and acid washed, 100–200 mesh) was obtained from the Celite Division Johns-Manville Products Corp. Dimethyldilaurylammonium chloride (Aliquat 204) and methyltricaprylylammonium chloride (Aliquat 336) were gifts from the Chemical Division of General Mills, Inc., Kankakee, Ill. Benzoylated DEAE-cellulose, which had been prepared by the method of Gillam *et al.* (1967), was kindly supplied by Dr. Dieter Söll of Yale University. Tetrachlorotetrafluoropropane (Freon-214) was obtained from E. I. Du Pont De Nemours and Co., Inc., Wilmington, Del. Isoamyl acetate was reagent grade (Fisher) and liquified phenol was analytical reagent grade (Mallinckrodt). Disodium ATP was purchased from P-L Biochemicals, Inc. Poly U, poly A, poly (UG) (4:1), poly (AG) (3:1), poly (UA) (5:1), and poly (UC) (3:1) were purchased from Miles Laboratories. Poly (UC) (1:5), poly (UCA) (1:1:1), poly (UCG) (2.5:2.5:1), poly (AUG) (2.5:2.5:1), and poly (ACG) (2.5:2.5:1) were synthesized as previously described by Ishikura and Nishimura (1968). Chemically synthesized oligonucleotide triplets were kindly supplied by Dr. H. G. Khorana of the University of Wisconsin and Dr. T. Ukita of the University of Tokyo, to whom we are indebted. The preparation of rat liver tRNA is described under Methods. Protamine sulfate was purchased from Eli Lilly Co. Radioactive [^{14}C]amino acids were purchased from Schwarz BioResearch and had the following specific activities in millicuries per millimole: tyrosine (355), valine (160), serine (112), phenylalanine (355), methionine (110), threonine (120), lysine (240), histidine (305), glutamine (32), aspartic acid (110), glycine (67), alanine (96), leucine (170), isoleucine (158), tryptophan (22), and proline (130).

Methods

Preparation of Rat Liver tRNA. Two batches of rat liver tRNA were employed in the present studies. The first was a commercial preparation (General Biochemicals) which had been extracted from albino Wistar rats by a modification of the procedure of Brunngraber (1962) and stripped of endogenous amino acids by incubation at 37° for 10 min in glycine buffer at pH 8.5. The second batch of tRNA we prepared by the method of Delihas and Staehelin (1966). Sixty male albino Wistar rats, weighing 250–350 g, were fasted 18 hr and sacrificed by decapitation. The livers (combined wet weight, 560 g) were then processed for RNA exactly as described by Delihas and Staehelin (1966), and the tRNA was partially purified through the 1 M NaCl extraction procedure described by these authors. This material was then used, without further stripping of endogenous amino acids or additional purification, for column fractionation.

Preparation of Rat Liver Aminoacyl-tRNA Synthetase. Crude rat liver aminoacyl-tRNA synthetase was prepared by a modification of a previously published method (Axel *et al.*, 1967). Six male Wistar rats (each weighing 200–250 g and starved for 18 hr) were decapitated, the livers were excised, and the microsomal supernatant fraction (S-122) was prepared as previously described (Weinstein *et al.*, 1966b). The liver fractionation and all subsequent procedures were done at 4°. To each 100 ml of S-122 was added slowly, with mixing, 4.9 ml of 1% protamine sulfate and the suspension was stirred for 1 hr. The precipitate was removed by centrifugation at 16,000*g* for 10 min and discarded. A sufficient volume of saturated ammonium sulfate solution (adjusted to pH 7.5 with KOH) was added to the protamine supernatant fraction to yield a 70% saturated solution. The suspension was stirred for 30 min, and the precipitate was then harvested by centrifugation at 16,000*g* for 10 min. The precipitate was then suspended in a minimal volume (approximately 15 ml) of 0.1 M Tris-HCl (pH 7.2)–0.005 M $MgCl_2$, dialyzed for 3 hr against 4 l. of the same buffer, and a small amount of insoluble residue was removed by centrifugation. The enzyme fraction was divided into small aliquots and rapidly frozen in liquid nitrogen. It could be stored at −80° for at least 2 weeks without appreciable loss in activity. The enzyme contained less than 0.5% nucleic acid and was completely dependent upon the addition of tRNA when tested for amino acid acceptor activity. It displayed good activity for the 16 amino acids listed in Figure 1. However, for reasons that are not apparent, the activity for arginine and proline was quite variable between different preparations, and no activity was obtained for glutamic acid.

Assay of Amino Acid Acceptor Activity of tRNA. The assay system for determining the amino acid acceptor activity of rat liver tRNAs present in the column fractions is a modification of a system previously described for *E. coli* tRNAs (Nishimura *et al.*, 1967). The reaction mixture contained 0.01–0.03 ml of the column fraction, 10 μmoles of Tris-KCl (pH 7.5), 1 μmole of magnesium acetate, 1 μmole of KCl, 0.2 μmole of ATP, 0.01–0.02 μCi of ^{14}C-labeled amino acid, and 0.04 ml (approximately 1.4 mg of protein) of the aminoacyl-tRNA synthetase, in a total volume of 0.1 ml. The reaction mixture was incubated at 37° for 10 min. Aliquots (0.08 ml) were then applied to Whatman No. 3MM filter paper disks (diameter 24 mm).

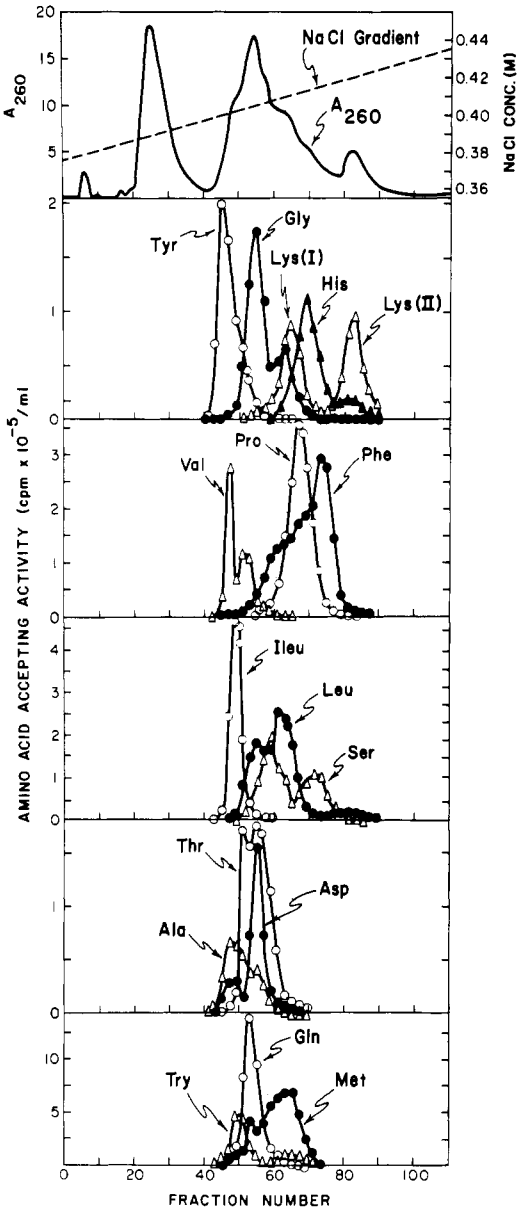

FIGURE 1: DEAE-Sephadex column chromatography of rat liver tRNA. The column (1 × 150 cm) was preequilibrated with 0.02 M Tris-HCl (pH 7.5), 0.0075 M $MgCl_2$, and 0.375 M NaCl. Commercial rat liver tRNA (3 ml) (see Methods) in water (total 3820 A_{260} units) was diluted to 12 ml with the starting buffer and applied to the column. A linear gradient elution was carried out using 1 l. of 0.02 M Tris-HCl (pH 7.5), 0.016 M $MgCl_2$, and 0.525 M NaCl in the reservoir, and 1 l. of 0.02 M Tris-HCl (pH 7.5), 0.0075 M $MgCl_2$, and 0.375 M NaCl in the mixing chamber. The flow rate was 10 ml/hr. Each fraction contained 7 ml of the effluent. In subsequent codon recognition studies, fraction 65 and 83 were used as sources of Lys_I and Lys_{II} tRNAs, respectively.

The disks were washed in cold 5% trichloroacetic acid, ethanol–ether, and finally ether, dried, and then assayed for radioactivity (counting efficiency 57%) in a Packard Tri-Carb liquid scintillation counter (Model 3375) by previously described methods (Nishimura *et al.*, 1967).

Assay of Aminoacyl-tRNA Binding to Ribosomes. Aliquots of certain column fractions were used for preparation of [^{14}C]aminoacyl-tRNAs to be used in the ribosomal binding assay. The reaction system was the same as that described above for the assay of amino acid acceptor activity, but was scaled up to a total volume of 1 ml. In some cases, the tRNA present in a column fraction was first concentrated as follows: two and one-half volumes of ethanol were added, the material was stored at −20° overnight, and the precipitate was harvested by centrifugation at 10,000 rpm and dissolved in a small volume of water. After charging with a [^{14}C]amino acid at 37° for 10 min, an equal volume (1 ml) of water-saturated phenol was added to the reaction system, the mixture was shaken at room temperature for 3 min, and then centrifuged for 5 min at 1500 rpm. The supernatant fluid was removed, mixed with 0.03 ml of 2 M sodium acetate buffer (pH 4.3), dialyzed against at least 300 volumes of 0.05 M sodium acetate buffer (pH 5.0)–0.002 M $MgCl_2$ at 4° overnight, then dialyzed against 300 volumes of 0.002 M $MgCl_2$ for 5 hr, and then stored at −20° until needed.

The ribosomal binding assay procedure described by Nirenberg and Leder (1964) was used. The reaction mixture (0.05 ml) contained 0.1 M Tris-HCl (pH 7.5), 0.05 M KCl, 1–2 A_{260} units of ribosomes, and 0.02 M magnesium acetate. [^{14}C]Aminoacyl-tRNA and polynucleotides or trinucleotides were added as specified in the legends. Incubation was carried out at 25° for 15 min, and samples were processed essentially as described by Nirenberg and Leder (1964).

Results

DEAE-Sephadex A-50 Column Chromatography of Rat Liver tRNA. Figure 1 presents the results obtained when 3820 A_{260} units of commercially prepared rat liver tRNA was fractionated by the DEAE-Sephadex procedure; 98% of the optical density was recovered. An optical density peak which accounted for approximately 30% of the total absorbance was eluted prior to the region of the tRNAs (fractions 20–40). This appears to be a low molecular weight contaminant, perhaps an oligonucleotide, since it was not precipitated by trichloroacetic acid and moved faster than tRNA when studied on polyacrylamide gel electrophoresis (Grossbach and Weinstein, 1968). Assay of the fractions for acceptance capacities for 16 amino acids indicated that individual tRNAs were eluted as rather sharp peaks which were reasonably well separated from each other. The presence of multiple forms of tRNA for a given amino acid was readily apparent (Figure 1). Additional studies indicated that a steeper gradient or a faster flow rate gave poorer resolution than that obtained in Figure 1.

Figure 2 indicates the results obtained when the same column procedure was applied to rat liver tRNA prepared by the hot phenol method of Delihas and Staehelin (1966). The optical density profile again revealed the presence of a low molecular weight contaminant which eluted prior to the region of tRNAs. The elution profiles of tRNAs for eight amino acids were grossly similar to those obtained in Figure 1. The

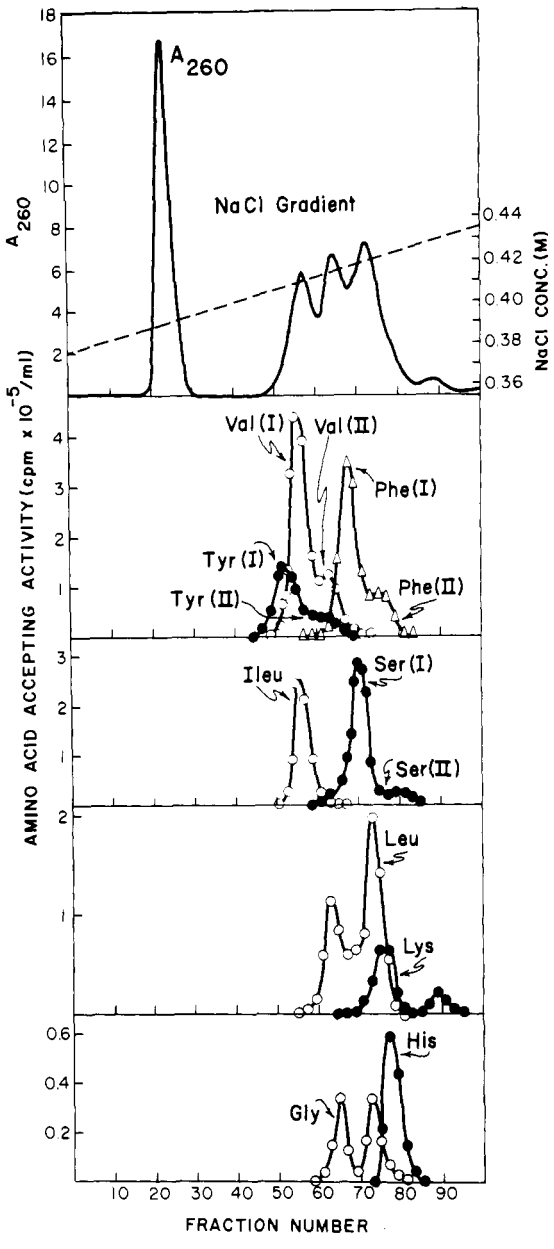

FIGURE 2: DEAE-Sephadex column chromatography of rat liver tRNA. The size of the column and the conditions for chromatography were the same as described in Figure 1. Rat liver tRNA (5 ml) prepared by the method of Delihas and Staehelin (see Methods) in water (total 1550 A_{260} units) was diluted to 20 ml by adding 15 ml of the starting buffer and applied to the column. In subsequent codon recognition studies, fraction 55 and a pool of fractions 61–65 were used as sources of Tyr_I and Tyr_{II}, respectively; fraction 66 and 77 for Phe_I and Phe_{II}; fraction 56 and 64 for Val_I and Val_{II}; fraction 72 and a pool of fractions 77–82 for Ser_I and Ser_{II}.

elution profile for phenylalanine tRNA, however, differed considerably between the two procedures. A comparison of Figures 1 and 2 also demonstrates certain quantitative differences in the tRNAs for tyrosine, serine, lysine, and glycine. Because the tRNA preparations used in Figures 1 and 2 were prepared by considerably different methods, the significance of these variations is not known at the present time.

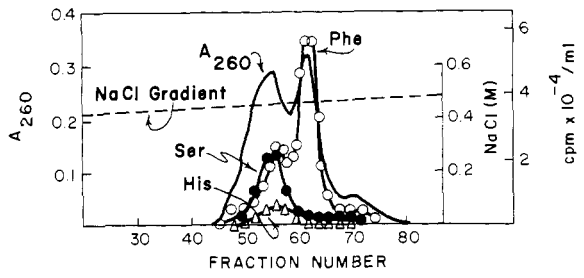

FIGURE 3: Purification of phenylalanine tRNA on a reversed-phase isoamyl acetate column. The column (0.3 × 150 cm) was previously washed with 0.02 M Tris-HCl (pH 7.5) and 0.4 M NaCl saturated with isoamyl acetate. Phenylalanine tRNA (24 A_{260} units) rich region obtained from a DEAE-Sephadex column (8 ml of the pooled fractions 71–77, Figure 1) was diluted to 14 ml with water and applied to the column. A linear gradient elution was carried out using 450 ml of 0.02 M Tris-HCl (pH 7.5), 0.008 M $MgCl_2$, and 0.6 M NaCl in the reservoir, and 450 ml of 0.02 M Tris-HCl (pH 7.5), and 0.4 M NaCl in the mixing chamber. Both buffers were saturated with isoamyl acetate. The flow rate was 3 ml/hr. A volume of 4 ml of effluent was collected/tube.

The results obtained in Figures 1 and 2 indicated that the DEAE-Sephadex procedure in itself had resulted in a 10- to 25-fold purification of tRNAs specific for tyrosine, phenylalanine, valine, serine, and lysine. It was of interest, therefore, to attempt further purification of these tRNAs.

Purification of Phenylalanine tRNA. The major phenylalanine tRNA peak obtained in Figure 1 was further purified on a reversed-phase isoamyl acetate column (Figure 3). This resulted in separation into two phenylalanine tRNAs. The minor component was still contaminated with serine and histidine tRNA. The major component was free of acceptance activity for the 15 other amino acids which we were able to test, and appeared relatively homogeneous from its elution profile. The total acceptance capacity of this material for phenylalanine was 38% of the theoretical.[1]

Purification of Tyrosine tRNA. Fractions corresponding to the major tyrosine tRNA peak, Tyr_I, obtained from the DEAE-Sephadex column (Figure 2) were pooled and further resolved on a reversed-phase Freon column (Figure 4). This yielded a peak (fraction 22) which was separate from the valine tRNA region, failed to accept all other amino acids tested, and had an acceptance capacity for tyrosine which was 46% of the theoretical.

Purification of Valine tRNA. The valine tRNA rich fraction obtained from a DEAE-Sephadex column (Figure 2) was further purified on a reversed-phase Freon column (Figure 5). This column resolved the material into three valine tRNA peaks ($Val_{I\text{-}A}$, $_{I\text{-}B}$, $_{I\text{-}C}$). $Val_{I\text{-}A}$ and $Val_{I\text{-}B}$ were well separated from isoleucine and tyrosine tRNAs. They did reveal small acceptance capacity for alanine, but this was less than 5% of the

[1] Assuming a theoretical acceptance capacity of a pure tRNA to be 1.67 mμmoles of amino acid /A_{260} unit of tRNA (Hoskinson and Khorana, 1965).

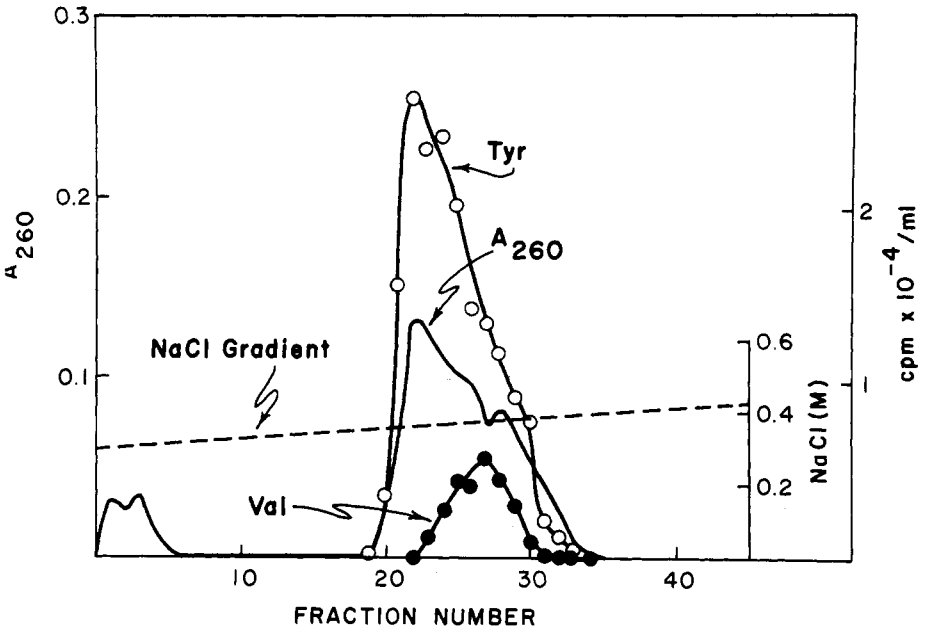

FIGURE 4: Purification of tyrosine tRNA on a reversed-phase Freon column. The column (0.3 × 150 cm) was previously washed with 0.02 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, and 0.3 M NaCl. The tyrosine tRNA rich region (5.6 A_{260} units) obtained from a DEAE-Sephadex column (7.5 ml of the pooled fractions 48–52, Figure 2) was mixed with 3.75 ml of water and applied to the column. 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 0.8 M NaCl in the reservoir, and 400 ml of 0.02 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, and 0.3 M NaCl in the mixing chamber. The flow rate was 8.5 ml/hr. A volume of 4.4 ml of the effluent was collected per tube. Fraction 22 was used as a source of purified tyrosine tRNA in subsequent studies.

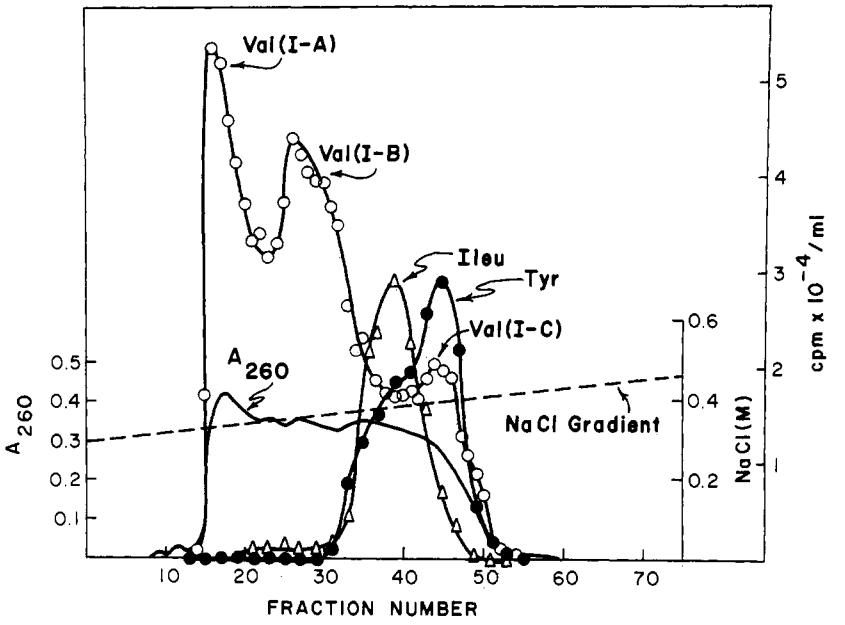

FIGURE 5: Purification of valine tRNA on a reversed-phase Freon column. The conditions for chromatography were the same as described in Figure 4. The valine tRNA rich region (35.6 A_{260} units) obtained from a DEAE-Sephadex column (9 ml of the pooled fractions 52–57, Figure 2) was mixed with 4.5 ml of water and applied to the column. A volume of 3 ml of effluent was collected per tube. In subsequent codon recognition studies, fractions 16–21, 25–32, and 43–48 were used as sources for $Val_{I\text{-}A}$, $Val_{I\text{-}B}$, and $Val_{I\text{-}C}$ tRNAs, respectively.

valine activity. Both $Val_{I\text{-}A}$ and $Val_{I\text{-}B}$ had 47% of the theoretical acceptance capacity for valine.

Figure 5 also demonstrates the heterogeneity of tyrosine tRNAs.

Purification of Serine tRNA. The serine and phenylalanine tRNA rich region obtained from a DEAE-Sephadex column (Figure 2) was further fractionated on a reversed-phase Freon column (Figure 6). This gave

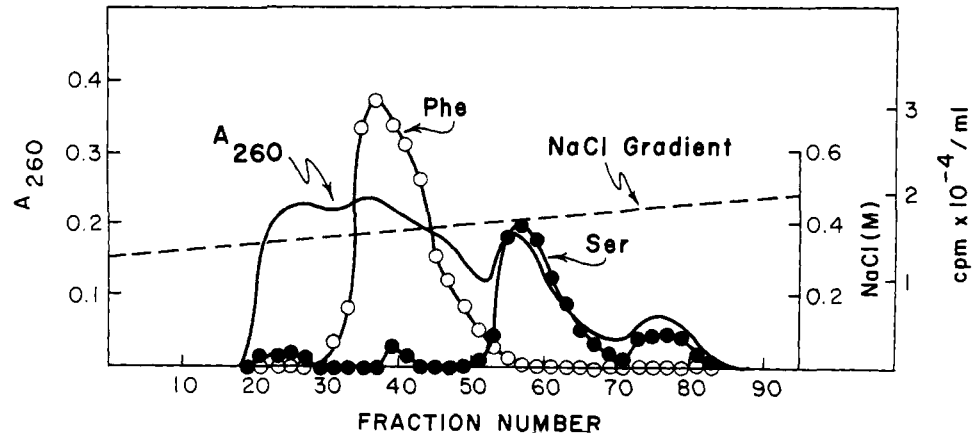

FIGURE 6: Purification of serine tRNA on a reversed-phase Freon column. The conditions for chromatography were the same as described in Figure 4. The serine tRNA rich region (27.8 A_{260} units) obtained from a DEAE-Sephadex column (5 ml of the pooled fractions 66–70, Figure 2) was mixed with 2.5 ml of water and applied to the column. A volume of 3 ml of effluent was collected per tube.

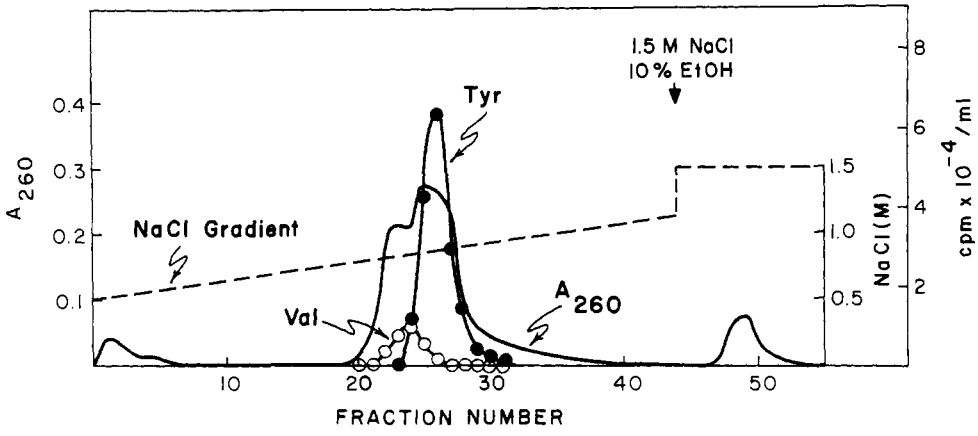

FIGURE 7: Purification of tyrosine tRNA on a benzoylated DEAE-cellulose column. The column (0.5 × 26 cm) was previously washed with 0.05 M sodium acetate buffer (pH 5.0) and 0.4 M NaCl. The tyrosine tRNA rich region (3.8 A_{260} units) obtained from a DEAE-Sephadex column (6.3 ml of the pooled fractions 48–52, Figure 2) were mixed with 3.2 ml of water. The pH of the solution was adjusted to 5.0 with 2 M acetic acid and the sample was applied to the column. A linear gradient elution was carried out using 100 ml of 0.05 M sodium acetate buffer (pH 5.0) and 1.5 M NaCl in the reservoir, and 100 ml of 0.05 M sodium acetate buffer (pH 5.0) and 0.4 M NaCl in the mixing chamber. The column was washed finally with 0.05 M sodium acetate buffer (pH 5.0) and 1.5 M NaCl containing 10% v/v of ethyl alcohol. A volume of 2.5 ml of effluent was collected per tube. The flow rate was 9 ml/hr.

good separation of phenylalanine and serine tRNAs. The serine tRNA displayed two components (serine$_{I\text{-}A, I\text{-}B}$). Serine$_{I\text{-}A}$ was free of acceptance capacity for the 15 other amino acids tested and had 40% of the theoretical acceptance capacity for serine.

Use of Benzoylated DEAE-cellulose for Purification of Tyrosine and Valine tRNAs. Figures 7 and 8 indicate results obtained when the tyrosine and valine tRNA regions obtained from a DEAE-Sephadex column (Figure 2) were further fractionated on benzoylated DEAE-cellulose columns. This procedure results in further purification of tyrosine tRNA (Figure 7), yielding a peak which had 56% of the theoretical acceptance capacity. Valine tRNA (Figure 8) was also well resolved by this procedure and gave a peak which had 47% of the theoretical acceptance capacity.

Assay of tRNAs for Codon Response. Because of the isolation of several amino acid specific tRNAs, as well as the separation of multiple forms of tRNAs for the same amino acid, obtained in the above studies, it was of interest to test the codon specificity of certain of these tRNAs. Previous studies indicated that the genetic code of mammalian cells (Weinstein, 1963; Marshall *et al.*, 1967; Caskey *et al.*, 1967), like that of *E. coli* (Crick, 1966b), is highly degenerate. We studied our isolated rat liver tRNAs in the ribosomal binding assay to determine whether the physical basis of degeneracy (Weisblum *et al.*, 1962, 1965; von Ehrenstein and Dais, 1963; Bennett *et al.*, 1965; Gonano, 1967), as well as the "wobble mechanism" by which a single tRNA recognizes more than one codon (Crick, 1966a,b; Kellogg *et al.*, 1966; Söll *et al.*, 1967; Söll and RajBhandary, 1967), previously elucidated with *E. coli* and yeast tRNAs, also applies to mammalian tRNAs.

Two lysine tRNAs, Lys$_I$ and Lys$_{II}$, were obtained from the DEAE-Sephadex column (Figure 1). When

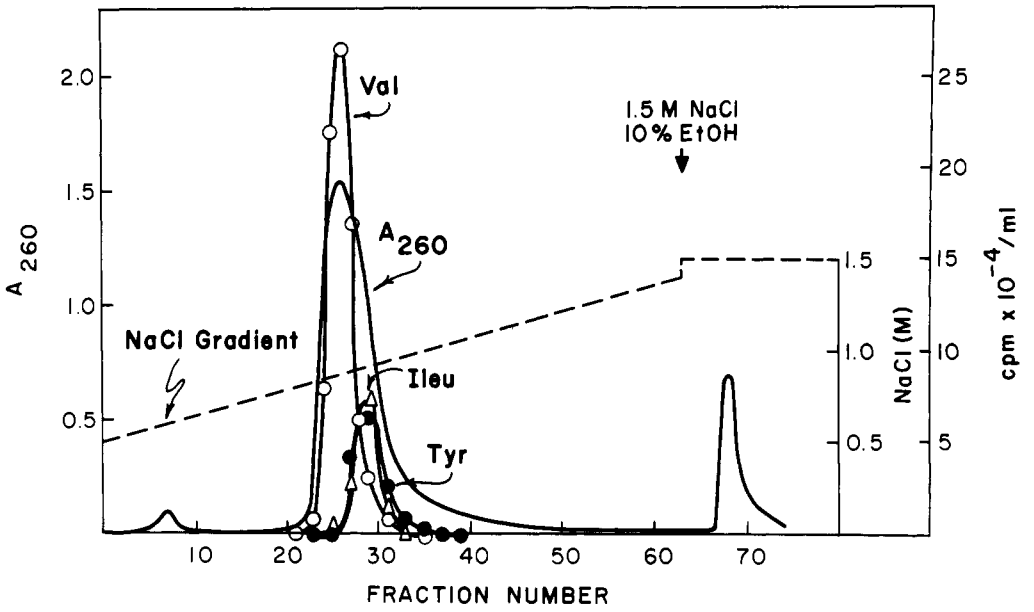

FIGURE 8: Purification of valine tRNA on a benzoylated DEAE-cellulose column. The conditions for chromatography were the same as described in Figure 7. The valine tRNA rich region (23.7 A_{260} units) obtained from a DEAE-Sephadex column (7 ml of the pooled fractions 52–57, Figure 2) was mixed with 3 ml of water. The pH of the solution was adjusted to 5.0 with 2 M acetic acid and the sample was applied to the column.

TABLE I: Codon Response of [^{14}C]Lys-tRNA Fractions.

Template	[^{14}C]Lys-tRNA Bound to Ribosomes	
	I	II
	Δμμmoles[a]	
Poly A	0.65	0.80
Poly (AG) (3:1)	0.62	0.80
	μμmoles	
None	0.14	0.38

[a] Δμμmoles represents the binding of [^{14}C]aminoacyl-tRNA to ribosomes in the presence of template minus binding in the absence of template. Reaction mixtures contained 3.20 μμmoles of [^{14}C]Lys-tRNA$_{I}$ (see Figure 1) or 3.43 μμmoles of [^{14}C]Lys-tRNA$_{II}$ (see Figure 1), 1 A_{260} unit of *E. coli* ribosomes, and 0.1 A_{260} unit of polynucleotides, as indicated. The remaining components of the binding assays, as well as the preparation of the [^{14}C]aminoacyl tRNAs, are described under Materials and Methods.

TABLE II: Codon Response of [^{14}C]Tyr-tRNA Fractions

Template	[^{14}C]Tyr-tRNA Bound to Ribosomes			
	I	II	I-A	I-B
	Δμμmoles[a]			
Poly (UA) (5:1)	0.71	0.21	0.36	1.38
Poly (UAC) (1:1:1)	0.76	0.21	0.32	1.18
UpApU	0.36	0.08	0.13	0.53
UpApC	0.15	0.03	0.06	0.24
	μμmoles			
None	0.26	0.04	0.12	0.51

[a] Δμμmoles as defined in Table I. Reaction mixtures contained 1.75 μμmoles of [^{14}C]Tyr-tRNA$_{I}$ (see Figure 2), 2.10 μμmoles of [^{14}C]Tyr-tRNA$_{II}$ (see Figure 2), 1.45 μμmoles of [^{14}C]Tyr-tRNA$_{I\text{-}A}$, or 5.03 μμmole of [^{14}C]Tyr-tRNA$_{I\text{-}B}$ (see Results for the source of Tyr$_{I\text{-}A}$ and Tyr$_{I\text{-}B}$) and 2 A_{260} units of *E. coli* ribosomes; 0.1 A_{260} unit of polynucleotides or triplets was added as indicated. For additional details, see Table I.

tested in the Nirenberg and Leder (1964) ribosomal binding assay, both of these responded equally to poly A and poly (AG) (Table I).

Two tyrosine tRNAs, Tyr$_{I}$ and Tyr$_{II}$, were obtained from the DEAE-Sephadex column shown in Figure 2. The codon responses of both of these (Table II) were compatible with the codon assignments UAU and UAC. When Tyr$_{I}$ was further fractionated on a reversed-phase Freon column, there was a suggestion of two components (see Figure 4). In a repeat of this procedure (not shown here), this separation was even more apparent. When these components, Tyr$_{I\text{-}A}$ and Tyr$_{I\text{-}B}$, were tested, both responded to the UAU and UAC codons (Table II). With all of these tyrosine tRNAs, the response to UAU was almost twice that obtained with UAC; there was no evidence that any of the fractions responded exclusively to either UAU or UAC.

In a separate experiment, the codon specificity of our most purified tyrosine tRNA, obtained by further fractionation of Tyr$_{I}$ on a reversed-phase column (see

TABLE III: Codon Response of Unfractionated *vs.* Purified [^{14}C]Tyr-tRNA.

Template	[^{14}C]Tyr-tRNA Bound to Ribosomes	
	Unfractionated	Purified
	$\Delta\mu\mu$moles[a]	
Poly (UA) (5:1)	0.26	2.43
Poly (UAC) (1:1:1)	0.19	2.11
UpApU	0.19	1.09
UpApC	0.08	0.57
Poly (UG) (4:1)	0	0.23
UpApA	0	0.19
UpApG	0	0.12
	$\mu\mu$moles	
None	0.11	0.46

[a] $\Delta\mu\mu$moles as defined in Table I. Reaction mixtures contained 4.01 $\mu\mu$moles of [^{14}C]Tyr-tRNA$_{unfractionated}$ (prepared by charging crude rat liver tRNA) or 6.30 $\mu\mu$moles of [^{14}C]Tyr-tRNA$_{purified}$ (see Figure 4) and 1 A_{260} unit of *E. coli* ribosomes; 0.1 A_{260} unit of polynucleotides or triplets was added as indicated. For additional details, see Table I.

Figure 4), was compared with that of unfractionated tRNA (Table III). Preferential response to the UAU and UAC codons was again apparent. It was of interest, however, that the quantitative response with the purified material was considerably greater than with unfractionated material. In addition, a small but significant response of the purified material was seen with poly (UG), UAA, and UAG. A similar decrease in codon specificity following purification has been seen with *E. coli* tyrosine tRNA (S. Nishimura, F. Harada, and Hirabayashi, unpublished studies). The precise explanation for this is not known but it may reflect: (1) chemical modification of the tRNA during purification, or (2) altered specificity due to the absence of competition by other tRNAs.

The two phenylalanine tRNAs, Phe$_I$ and Phe$_{II}$, obtained from the DEAE-Sephadex column shown in Figure 2, responded equally to the UUU and UUC triplets, as did unfractionated tRNA charged with [^{14}C]phenylalanine (Table IV).

Two valine tRNAs, Val$_I$ and Val$_{II}$, were obtained from the DEAE-Sephadex column (Figure 2). Val$_I$ was further fractionated into three components, Val$_{I\text{-}A}$, Val$_{I\text{-}B}$, and Val$_{I\text{-}C}$, on the reversed-phase Freon column (Figure 5). The codon responses obtained with these tRNAs are indicated in Table V. Unfractionated rat liver tRNA charged with [^{14}C]valine responded to the triplets GUU, GUC, GUA, and GUG, as predicted from the codon assignments in *E. coli* (Crick, 1966b). Val$_I$ also responded to all four of these codons. Val$_{II}$, however, while responding to the GUU, GUA, and GUG triplets, failed to respond to the GUC triplet. Consistent with this result were additional experiments

TABLE IV: Codon Response of [^{14}C]Phe-tRNA Fractions.

Template	[^{14}C]Phe-tRNA Bound to Ribosomes		
	Unfractionated	I	II
		$\Delta\mu\mu$moles[a]	
UpUpU	0.73	0.65	0.69
UpUpC	0.82	0.72	0.74
		$\mu\mu$moles	
None	0.23	0.23	0.18

[a] $\Delta\mu\mu$moles as defined in Table I. Reaction mixtures contained 9.58 $\mu\mu$moles of [^{14}C]Phe-tRNA$_{unfractionated}$ (prepared by charging crude rat liver tRNA), 1.89 $\mu\mu$moles of [^{14}C]Phe-tRNA$_I$ (see Figure 2), or 1.92 $\mu\mu$moles of [^{14}C]Phe-tRNA$_{II}$ (see Figure 2) and 1 A_{260} unit of *E. coli* ribosomes; 0.1 A_{260} unit of triplets was added as indicated. For additional details, see Table I.

(not shown here) indicating that Val$_I$ gave a good response to poly (UG) (4:1), poly (UCG) (2.5:2.5:1), and poly (UAG) (2.5:2.5:1); Val$_{II}$, however, while responding to the UG and UAG polymers, gave a negligible response to the UCG polymer. The three subfractions of Val$_I$, obtained from the reversed-phase Freon column, displayed important qualitative differences in codon recognition. Whereas Val$_{I\text{-}A}$ and Val$_{I\text{-}B}$ responded to all four triplets, Val$_{I\text{-}C}$ responded to GUU, GUC, and GUA but had only a negligible response to GUG.

The codon specificity of the two serine tRNAs, Ser$_I$ and Ser$_{II}$, obtained from a DEAE-Sephadex column (Figure 2), is described in Table VI. Ser$_I$ gave responses compatible with the codons UCU, UCC, UCA, and UCG. Ser$_{II}$, on the other hand, responded poorly to these triplets, and gave a polymer response compatible with the serine codons AGU and AGC. The likelihood that Ser$_I$ actually contains two species of serine tRNA is indicated by its subfractionation into two components on the reversed-phase Freon column (see Figure 6), but the codon specificity of each of these remains to be determined.

Discussion

The present study indicates that by applying the techniques of column chromatography on DEAE-Sephadex A-50, followed by column chromatography of selected regions on reversed-phase columns or on benzoylated DEAE-cellulose columns, it is possible to obtain excellent separation and purification of individual tRNAs from normal rat liver. The recovery of material from these columns, either as A_{260} units or as acceptance capacity for a given amino acid, was always greater than 75% and usually approached 100%. This indicates that no major tRNA components were lost, and that significant inactivation of acceptance activity did not occur during fractionation and purification.

TABLE V: Codon Response of [^{14}C]Val-tRNA Fractions.

Template	[^{14}C]Val-tRNA Bound to Ribosomes					
	Unfractionated	Val_I	Val_{II}	Val_{I-A}	Val_{I-B}	Val_{I-C}
			$\Delta\mu\mu$moles[a]			
GpUpU	0.94	3.26	0.67	2.83	3.28	4.16
GpUpC	0.30	1.45	0.04	1.20	1.29	1.68
GpUpA	1.80	2.06	3.25	1.61	1.86	2.20
GpUpG	1.63	4.36	1.26	5.75	2.27	0.38
			$\mu\mu$moles			
None	0.27	0.44	0.31	0.48	0.43	0.42

[a] $\Delta\mu\mu$moles as defined in Table I. Reaction mixtures contained 18.12 $\mu\mu$moles of [^{14}C]Val-$tRNA_{unfractionated}$ (prepared by charging crude rat liver tRNA), 13.81 $\mu\mu$moles of [^{14}C]Val-$tRNA_I$ (see Figure 2), 5.79 $\mu\mu$moles of [^{14}C]Val-$tRNA_{II}$ (see Figure 2), 11.78 $\mu\mu$moles of [^{14}C]Val-$tRNA_{I-A}$ (see Figure 5), 9.80 $\mu\mu$moles of [^{14}C]Val-$tRNA_{I-B}$ (see Figure 5), or 9.41 $\mu\mu$moles of [^{14}C]Val-$tRNA_{I-C}$ (see Figure 5) and 2 A_{260} unit of *E. coli* ribosomes; 0.1 A_{260} unit of triplets was added as indicated. For additional details, see Table I.

Evidence for the relative purity of the isolated tyrosine, valine, serine, and phenylalanine tRNAs was their lack of acceptance activity for other amino acids, and an acceptance capacity for a specific amino acid which was 40–55% of the theoretical acceptance capacity. The true acceptance capacity may actually be higher, since extensive studies on the optimal conditions for measuring acceptance capacity (by varying the temperature, substrate concentration, the ionic environment, or the use of more purified aminoacyl-tRNA synthetases) have not been done. Alternatively, it may be that a portion of the purified tRNA is inactive because of removal of a portion of the pCpCpA terminus (Lebowitz *et al.*, 1966) or a denatured conformation (Sueoka *et al.*, 1966; Adams *et al.*, 1967). Experiments are currently in progress to explore these possibilities.

As with *E. coli* and yeast tRNAs, rat liver tRNA displayed multiple components for a given amino acid: for tyrosine, 2 (possibly 3); glycine, 2; lysine, 2; histidine, 1 (possibly 2); valine, 4; phenylalanine, 2 (possibly 3); leucine, 2; serine, 3; threonine, 2; aspartic acid, 2; alanine, 2; tryptophan, 2; and methionine, 2. Proline, isoleucine, and glutamine gave single tRNA peaks. The number of peaks which we obtained represent a minimum estimate, since further fractionation may resolve additional components. Studies in progress, in fact, indicate that it is possible to resolve rat liver leucine tRNA into five components (also found by Dr. D. Novelli, personal communication), and methionine tRNA into at least three components by reversed-phase chromatography. Multiple tRNAs for a given amino acid have also been described in previous studies of mammalian tissues (Apgar and Holley, 1962; Glebov *et al.*, 1965; Axel *et al.*, 1967; Taylor *et al.*, 1967; Caskey *et al.*, 1967; Yang and Novelli, 1968). These findings are compatible with evidence that in mammalian cells, as in bacteria, degeneracy is a prominent feature of the genetic code (Weinstein, 1963; Marshall *et al.*, 1967; Caskey *et al.*, 1967). The alternative possibility that multiple peaks for certain amino acids may represent an experimental artifact is discussed in greater detail below.

The availability of isolated rat liver tRNAs made it possible for us to determine whether certain patterns of specificity by which a tRNA molecule recognizes codons in mRNA, previously established with *E. coli* and yeast tRNAs, also apply to higher organisms. Weisblum *et al.* (1962), employing *E. coli* tRNA, first established that in certain cases the physical basis for degeneracy is due to the existence of multiple types of tRNA for the same amino acid, with each type recognizing a distinct codon. An additional explanation for degeneracy has been provided by Crick (1966a,b) who has postulated (the "wobble hypothesis") that, in certain cases, an individual tRNA can recognize multiple codons, if these codons differ from each other only in the third position (3′ end) of the triplet. The "wobble hypothesis" states that, whereas the first and second positions of the codon pair with bases in the anticodon of tRNA by the usual base-pairing rules (A to U or G to C), the pairing of the third base in the codon with its corresponding base in the anticodon follows these rules: U in the anticodon will pair with either A or G in the codon, C in the anticodon with G in the codon, A in the anticodon with U in the codon, G in the anticodon with U or C in the codon, and I in the anticodon with either U, C, or A (Crick, 1966a,b). Studies on codon recognition by isolated *E. coli* and yeast tRNAs have yielded results which are generally in agreement with this hypothesis (Kellogg *et al.*, 1966; Söll *et al.*, 1967; Söll and RajBhandary, 1967).

In the present studies with isolated rat liver tRNA, both Lys_I and Lys_{II} responded to AAA and AAG (Table I); Tyr_{I-A}, Tyr_{I-B}, and Tyr_{I-C} responded to UAU and UAC (Table II); Phe_I and Phe_{II} responded to UUU and UUC (Table IV); and Ser_{II} responded to AGU and AGC (Table VI). These results are entirely consistent with the "wobble mechanism." The apparent response of Ser_I to the four codons UGU, UCC, UCA, and UCG (Table VI) does not fit the "wobble mechanism" but experiments in progress indicate that

TABLE VI: Codon Response of [^{14}C]Ser-tRNA Fractions.

Template	[^{14}C]Ser-tRNA Bound to Ribosomes		
	Unfractionated	I	II
		Δμμmoles[a]	
Poly (UC) (3:1)	1.68	2.92	0.29
Poly (UC) (1:5)	1.23	2.99	0.33
Poly (UCA) (1:1:1)	0.62	1.87	0.14
Poly (UCG) (2.5:2.5:1)	0.35	0.87	0.28
Poly (AGU) (2.5:1:2.5)	0.44	0.01	1.16
Poly (AGC) (2.5:1:2.5)	0.37	0.01	0.97
UpCpU	0.89	1.49	0.23
UpCpC	0.33	0.56	0.09
UpCpA	0.37	0.56	0.04
UpCpG	0.62	1.17	0.15
		μμmoles	
None	0.26	0.31	0.33

[a] Δμμmoles as defined in Table I. Reaction mixtures contained 23.38 μμmoles of [^{14}C]Ser-tRNA$_{unfractionated}$ (prepared by charging crude tRNA), 11.69 μμmoles of [^{14}C]Ser-tRNA$_I$ (see Figure 2), or 5.21 μμmoles of [^{14}C]Ser-tRNA$_{II}$ (see Figure 2) and 2 A_{260} units of *E. coli* ribosomes; 0.1 A_{260} unit of polynucleotides or triplets was added as indicated. For additional details, see Table I.

Ser$_I$ separates into two components on a reversed-phase Freon column (see Figure 6). It will be of interest to see whether the pattern of codon recognition for these two components resembles that obtained with *E. coli* serine tRNAs (Soll *et al.*, 1967; Ishikura and Nishimura, 1968) or whether one of these components will recognize the three codons UCU, UCC, and UCA. The latter pattern of recognition is seen with a purified yeast serine tRNA and is explained by the presence of inosine in the anticodon (Zachau *et al.*, 1966b). The results obtained with liver valine tRNAs (Table V) are difficult to interpret since the multiple valine tRNAs differ in their relative responses to similar codons and the subfractions may not be completely separated from each other. The response of Val$_{II}$ to GUA, GUG, and to a lesser extent, GUU, does not fit the "wobble mechanism." Mirzabekov *et al.* (1967) have reported the purification of three valine tRNAs from yeast and it is of considerable interest that one of these had a pattern of codon recognition identical with that of rat liver Val$_{II}$ tRNA. Kellogg *et al.* (1966) have described a valine tRNA from *E. coli* which also recognizes these three codons, and a similar finding can be seen in the data of Söll *et al.* (1966) with yeast tRNA. These findings suggest that an unusual base in the 5′ position of the anticodon, perhaps pseudouridine (Kellogg *et al.*, 1966; Mirzabekov *et al.*, 1967), can pair with either A, G, or U. Alternatively, it is possible that our Val$_{II}$ contains two components and further fractionation studies are required to clarify this point.

It is of interest that with phenylalanine, tyrosine, and lysine we have observed multiple tRNA fractions for the same amino acid which appear to be identical with respect to codon recognition. This apparent redundancy in tRNA has been previously described with both *E. coli* and yeast tRNAs (Söll *et al.*, 1967; Bergquist *et al.*, 1966, 1968). Its functional significance is not known, and our data do not exclude the possibility that it reflects an experimental artifact due to aggregation (Shleich and Goldstein, 1964), partial denaturation (Sueoka *et al.*, 1966; Adams *et al.*, 1967), or the possibility that some of our tRNAs are missing adenosine termini which are readded during our assay with the crude synthetase preparation (Lebowtiz *et al.*, 1966; RajBhandary *et al.*, 1968). The latter explanations cannot account entirely for the multiple forms, since multiple tRNA peaks for tyrosine and lysine were also observed when precharged rat liver tRNAs were chromatographed on methylated albumin kieselguhr (Axel *et al.*, 1967) or reversed-phase Freon columns (unpublished studies). Nor does it seem likely that these multiple peaks represent a mixture of mitochondrial (Barnett and Brown, 1967) and cytoplasmic tRNAs, since the extremely small amount of RNA present in mitochondria would either not appear in our chromatograms or, at most, give only very minor peaks. An additional explanation for the redundancy of certain tRNAs seen in the present study is that our strain of Wistar rats may be heterozygous with respect to chromosomal loci for specific tRNAs. On the other hand, redundancy for tRNAs apparently exists also in *E. coli* (Söll *et al.*, 1967) and in a haploid strain of yeast (Söll *et al.*, 1966). Indeed, it has been postulated that, in these organisms, the redundant copies may provide a source of tRNA suppressors (Söll *et al.*, 1966; Bergquist *et al.*, 1968). In addition, hybridization studies performed with *Drosophila* DNA suggest that there may also be redundancy of the cistrons for tRNA on the DNA (Ritossa *et al.*, 1966). We believe, therefore, that certain cases of redundancy seen in the present study also exist *in vivo*. Further studies are indicated to determine whether or not these redundant

copies play a role in cell regulation and differentiation in mammalian systems.

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