On the Nature of Transfer Ribonucleic Acid Isolated from Polyphenylalanyl Transfer Ribonucleic Acid*

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ABSTRACT: Transfer ribonucleic acid liberated from polyphenylalanyl transfer ribonucleic acid by alkaline deacylation had very little phenylalanine acceptor capacity. Because of this observation and because no concrete evidence was available that transfer ribonucleic acid of polyphenylalanyl transfer ribonucleic acid is transfer ribonucleic acid specific for phenylalanine, studies on the nature of transfer ribonucleic acid from polyphenylalanyl transfer ribonucleic acid were undertaken. It was found that the isolated transfer ribonucleic acid from polyphenylalanyl transfer ribonucleic acid behaved almost identically with phenylalanine transfer ribonucleic acid upon sucrose density gradient centrifugation, reversed-phase chromatography, Sephadex column chromatography, and methylated albumin kieselguhr column chromatography. When transfer ribonucleic acid was released by puromycin from polyphenylalanyl transfer ribonucleic acid on the ribosomes it retained most of its acceptor capacity. From these observations we conclude that transfer ribonucleic acid of polyphenylalanyl transfer ribonucleic acid is phenylalanine transfer ribonucleic acid. Therefore, the failure of transfer ribonucleic acid liberated from polyphenylalanyl transfer ribonucleic acid by alkaline deacylation to accept phenylalanine is apparently due to inactivation of the transfer ribonucleic acid during the isolation process.

In preceding studies we reported that tRNA isolated from polyphenylalanyl-tRNA had very little acceptor capacity for phenylalanine (Kuriki and Kaji, 1967a,b). To explain this observation we suggested two possibilities. The first was that the tRNA in polyphenylalanyl-tRNA was inactivated during the isolation procedure. The other possibility was that the tRNA of polyphenylalanyl-tRNA was different from the usual tRNA which accepts amino acids. To distinguish between these two possibilities, we compared various characteristics of tRNA isolated from polyphenylalanyl-tRNA with those of $tRNA^{Phe}$. We concluded that tRNA of polyphenylalanyl-tRNA was indeed $tRNA^{Phe}$, thus eliminating the second possibility. When [^{14}C]phenylalanyl-tRNA was bound to the complex of poly U and ribosomes in the absence of soluble enzymes, the bound tRNA could be released from ribosomes by washing with a buffer containing 10^{-4} M Mg^{2+}. This bound tRNA will be called loosely bound tRNA. On the other hand, polyphenylalanyl-tRNA bound to ribosomes could not be released by this procedure (Gilbert, 1963). This bound polyphenylalanyl-tRNA will be called tightly bound tRNA.

Experimental Section

Preparation of Cell Extracts. Escherichia coli was grown and cell extracts were prepared as described previously (Kuriki and Kaji, 1967a,b). Other material such as tRNA from *E. coli*, and aminoacyl-tRNA, were the same as those of the preceding paper. The preparation of the soluble protein fraction (fraction A) containing aminoacyl-tRNA transfer factor and aminoacyl-tRNA synthetase has been described previously (Momose and Kaji, 1965). Preparation of tRNA mixture lacking $tRNA^{Phe}$ was according to the method of Kelmers *et al.* (1965). The mixture of tRNA and other soluble ribonucleic acids were purchased from General Biochemicals and called sRNA in this communication.

The specific activities of radioactive materials used in this paper were: [^{14}C]phenylalanine, 355 μCi/μmole; and [^{3}H]-phenylalanine, 1000 μCi/μmole. Counting efficiency was about 10^6 cpm/μCi for ^{14}C, 2×10^5 cpm/μCi for ^{3}H, and 1.85×10^6 cpm/μCi for ^{32}P. Millipore filter paper used had a pore size 0.45 μ.

Preparation of [^{32}P]tRNA. For the preparation of ^{32}P-labeled tRNA, *E. coli* B was grown in a medium containing the following in grams per liter: NaCl, 5.4; KCl, 3.0; NH_4Cl, 1.1; $CaCl_2$, 0.011; $MgCl_2$, 0.095; $FeCl_2$, 0.00162; and glucose, 0.2. In addition, it contained 0.1 M Tris-HCl (pH 7.4), 0.16 mM Na_2SO_4, 0.1 mM KH_2PO_4, and 20 mCi of [^{32}P]KH_2PO_4 (carrier free). When the culture reached an absorbancy of 145 Klett units, the cells were harvested and washed with a buffer containing 0.005 M Tris-HCl (pH 7.4) and 0.01 M magnesium acetate. The cells were then suspended in 9 ml of above buffer and mixed with 1 ml of 10% sodium dodecyl sulfate and the mixture was incubated at 30° for 30 min. After incubation, 10 ml of phenol was added to the mixture and RNA was extracted in the aqueous layer. The aqueous layer was treated with three 10-ml portions of phenol. The phenol layer was extracted with water again and the aqueous phases were combined (3.6 ml).

The RNA solution (1.2 ml) was layered on the top of a 25-ml linear sucrose gradient (5–20%) containing 0.1 M NaCl and 0.005 M Tris-HCl (pH 7.4). The tube was centrifuged in a Beckman–Spinco SW25 rotor at a speed of 25,000 rpm for 20 hr. The 1-ml fractions were collected from the top of the gradient. The portions containing 4S RNA were removed

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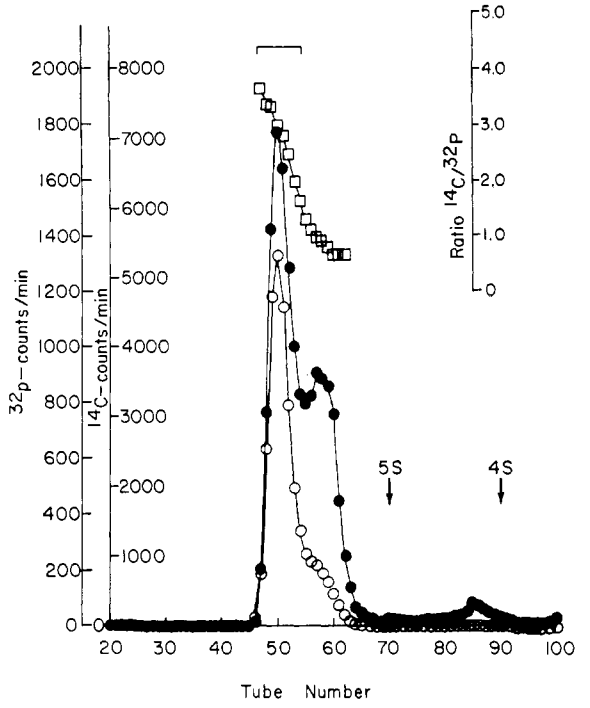

FIGURE 1: Sephadex column chromatography of [^{14}C]polyphenylalanyl-[^{32}P]tRNA. The conditions of the experiment are described in the text. (●—●) ^{32}P radioactivity; (○—○) ^{14}C radioactivity; (□—□) the ratio of ^{14}C/^{32}P.

and dialyzed against 4 l. of 0.086 M KCl, 0.017 M magnesium acetate, and 0.005 M Tris-HCl (pH 7.4) overnight at 4°. This preparation was called [^{32}P]tRNA.

Preparation of [^{32}P]tRNA Enriched with [^{32}P]tRNA^{Phe}. To enrich the [^{32}P]tRNA preparation with tRNA^{Phe}, the specific binding of phenylalanyl-tRNA to ribosomes was used. The reaction mixture (5.4 ml) for the binding of [^{32}P]tRNA contained the following in micromoles per milliliter: Tris-HCl (pH 7.1), 146; magnesium acetate, 20; β-mercaptoethanol, 7; and KCl, 66. In addition, it contained 944 μg of 70S ribosomes, 180 μg of poly U, and 0.05 ml of the crude [^{32}P]tRNA (277 μg, 7.97 × 10^8 cpm) prepared as in the preceding section. This mixture was incubated for 30 min at 30° and mixed with 72 ml of buffer I containing 0.02 M magnesium acetate, 0.05 M KCl, and 0.1 M Tris-HCl (pH 7.1). The mixture (77.4 ml) was divided into 24 portions and each was poured through a Millipore filter (pore size 0.45 μ). The ribosomes on the Millipore filter were washed with three 3-ml portions of buffer I containing 5 × 10^{-2} M KCl, 0.02 M magnesium acetate, and 0.1 M Tris-HCl (pH 7.1). The bound tRNA was then eluted from the filter with four 0.5-ml portions of the same buffer containing 10^{-4} M magnesium acetate (buffer II). To the combined eluates (48 ml) was added 50 ml of phenol saturated with water and the mixture was shaken. The aqueous phase was dialyzed against 6 l. of 0.086 M KCl, 0.017 M magnesium acetate, and 0.005 M Tris-HCl (pH 7.4). After 15-hr dialysis the solution was lyophilized and the lyophilized material was dissolved in 2.5 ml of water, dialyzed against the same buffer, centrifuged for 10 min at 15,000*g*, and the pellet was discarded. The final solution contained 4 × 10^7 cpm of [^{32}P]tRNA. This preparation was called [^{32}P]tRNA^{Phe}. The distribution of ^{32}P radioactivity through the steps of the above procedure is shown in Table I. It should be pointed out that the dilution of the specific activity of [^{32}P]tRNA due to the nonlabeled tRNA^{Phe} which was initially present on the ribosomes was negligible (0.55%).

TABLE I: Distribution of ^{32}P Radioactivity during the Process of Preparation of [^{32}P]tRNA^{Phe}.

Steps	Total cpm
Growth medium for *E. coli*	2.51 × 10^{10}
E. coli cells	2.34 × 10^{10}
RNA fraction after phenol extraction	5.33 × 10^9
4S fraction after sucrose density centrifugation	1.50 × 10^9
tRNA^{Phe}	4.12 × 10^7

Preparation of Crude [^{14}C]Polyphenylalanyl-[^{32}P]tRNA. The reaction mixture (1.8 ml) for formation of [^{14}C]polyphenylalanyl-tRNA contained the following in μmoles/1 ml: Tris-HCl (pH 7.8), 140; KCl, 66; β-mercaptoethanol, 7; magnesium acetate, 20; ATP, 3.3; GTP, 0.33; phosphoenolpyruvate, 66. In addition it contained the following components per 1 ml: 1.6 μCi of [^{14}C]phenylalanine, 1.7 × 10^7 cpm of ^{32}P-labeled tRNA^{Phe}, 33 μg of pyruvate kinase, 170 μg of well-washed ribosomes, 266 μg (protein) of fraction A, and 200 μg of poly U. The reaction mixture was incubated at 30° for 30 min. After the incubation, 9 ml of buffer I was added and the mixture was divided into three equal parts. Each part was filtered through the Millipore filter. The ribosomes on the Millipore filter were washed with four 4-ml portions of buffer I and four 3-ml portions of buffer II. The ribosome-bound polyphenylalanyl-tRNA was then eluted from each Millipore filter with four 0.5-ml portions of 0.5% sodium dodecyl sulfate solution. The eluate was lyophilized and the dried material was dissolved in 1 ml of water.

Sephadex Column Purification of [^{14}C]Polyphenylalanyl-[^{32}P]tRNA. The crude [^{14}C]polyphenylalanyl-[^{32}P]tRNA preparation (1.0 ml), 9.51 × 10^5 cpm of ^{32}P and 1.98 × 10^6 cpm of ^{14}C, was placed on a Sephadex G-100 column (0.8 × 220 cm) which had been equilibrated with 0.1 M NaCl, 0.05 M sodium acetate (pH 5.6), and 0.5% sodium dodecyl sulfate. The elution of peptidyl-tRNA was carried out with the same buffer at the flow speed of 0.8 ml/tube per 15 min. Aliquots (15 μl) of each fraction were used for radioactivity measurements. The elution profile of peptidyl-tRNA is shown in Figure 1. It is noted in this figure that the ratio of ^{14}C to ^{32}P was progressively less toward later fractions. This indicates that the length of polyphenylalanine determined the position at which the peptidyl-tRNA was eluted. The peptidyl-tRNA fractions were pooled and lyophilized. The dried material was dissolved into 2 ml of water and mixed with 4 ml of ethanol. The precipitate was dissolved in 0.5 ml of 0.1 M NaCl–0.5% sodium dodecyl sulfate. The above procedure is a modification of the method reported by Gilbert (1963).

Purification of Polyphenylalanyl-tRNA by Sucrose Density Gradient Centrifugation. For purification of polyphenylalanyl-tRNA the peptidyl-tRNA fraction (0.2 ml) containing 1.04 × 10^5 cpm of ^{32}P and 9.55 × 10^4 cpm of ^{14}C was placed on a

TABLE II: Radioactivity of [^{32}P]tRNA prepared from [^{14}C]-Polyphenylalanyl-[^{32}P]tRNA.

Preparation[a]	Radioactivity (cpm) ^{32}P	^{14}C
1. Fraction from Sephadex column before Pronase	2.97×10^5	1.07×10^6
2. Fraction from Sephadex column after Pronase	2.46×10^5	
3. Fraction from sucrose density gradient centrifugation before Pronase	2.6×10^4	4.2×10^4
4. Fraction from sucrose density gradient centrifugation after Pronase	1.6×10^4	
5. Fraction from sucrose density gradient centrifugation before Pronase	5.22×10^4	4.78×10^4
6. Fraction from sucrose density gradient centrifugation after Pronase	4.35×10^4	

[a] Preparations 1 and 2 contained 5 mg of sRNA as carrier and preparations 3 and 4 contained 0.2 mg of sRNA and 0.05 mg of tRNA^{Phe} as prepared by the method of Kelmers *et al.* (1965). Fractions 5 and 6 contained 0.8 mg of sRNA mixture free of tRNA^{Phe}. Preparation 2 was used for the experiment shown in Figure 3 and preparation 4 was used for the experiments in Figures 4 and 6. Preparation 6 was used for the experiment shown in Figure 5.

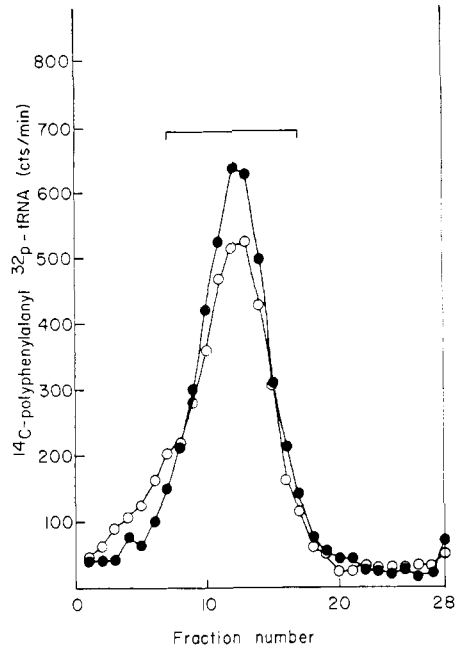

FIGURE 2: Sucrose density gradient centrifugation of [^{14}C]polyphenylalanyl-[^{32}P]tRNA. A solution containing [^{14}C]polyphenylalanyl-[^{32}P]tRNA was subjected to sucrose density gradient centrifugation as described in the text. After the centrifugation, four drop fractions were collected from the bottom of the tube and 10 μl of each fraction was used for radioactivity measurement. (●—●) ^{32}P radioactivity; (○—○) ^{14}C radioactivity.

5–10% linear sucrose gradient containing 0.5% sodium dodecyl sulfate, 0.001 M Tris-HCl (pH 7.1), and 0.1 M NaCl. The tube was centrifuged in a Beckman–Spinco SW50 rotor at 40,000 rpm for 20 hr. The distribution of ^{14}C and ^{32}P radioactivity is shown in Figure 2. It is noted in this figure that the distribution of [^{14}C]polyphenylalanine closely followed the distribution of ^{32}P indicating that major portions of radioactivity in this preparation represents [^{14}C]polyphenylalanyl-[^{32}P]tRNA. As indicated previously (Kuriki and Kaji, 1967a,b) [^{14}C]polyphenylalanine by itself sediments much slower than tRNA and thus one can conclude that most of the ^{14}C radioactivity was bound to [^{32}P]tRNA. The peak radioactive fractions were lyophilized and the dried material was dissolved in 1 ml of distilled water. To this solution was added 0.8 mg of carrier tRNA mixture lacking tRNA^{Phe}. The RNA was then precipitated by the addition of two volumes of ethanol and the precipitate was dissolved in 1 ml of water. The procedure of precipitation and suspension was repeated four times and the final precipitate was dissolved in 1 ml of 0.1 M Tris-HCl (pH 7.8).

Pronase Treatment of [^{14}C]Polyphenylalanyl-[^{32}P]tRNA. The [^{14}C]polyphenylalanyl-[^{32}P]tRNA (1 ml) prepared in the preceding sections was mixed with 0.1 ml of solution containing 0.5 mg of the mixture of tRNA. To this mixture was added 0.1 ml of a solution containing 0.5 mg of Pronase (obtained from Calbiochem) in 0.1 M Tris-HCl (pH 7.8). The reaction mixture was incubated at 37° for 60 min. At the end of the reaction, 1 ml of phenol was added and the aqueous layer was saved. To the aqueous layer two volumes of ethanol were added and the precipitate was dissolved in 1 ml of 0.1 M Tris-HCl (pH 8.0). To remove the amino acid attached to the [^{32}P]tRNA, the mixture was further incubated at 37° for 60 min and the RNA was precipitated again by the addition of two volumes of alcohol. The precipitate was suspended in 1 ml of 0.1 M Tris-HCl (pH 7.4) and the process of precipitation and resuspension was repeated several times. The final precipitate was dissolved in 1 ml of 0.3 M NaCl, 0.05 M Tris-HCl (pH 7.4), and 0.01 M $MgCl_2$. The radioactivity of various preparations of [^{32}P]tRNA obtained from [^{14}C]polyphenylalanyl [^{32}P]tRNA is shown in Table II.

Assay of tRNA^{Phe}. A typical reaction mixture for the assay of the tRNA^{Phe} contained the following in μmoles/1 ml: Tris-HCl (pH 7.8), 357; magnesium acetate, 17; β-mercaptoethanol, 14; ATP, 7.1; and phosphoenolpyruvate, 14.2. In addition it (1 ml) contained 36 μg of pyruvate kinase, 0.28 mg of fraction A, and 6.1 μCi of [^{3}H]phenylalanine or 0.71 μCi of [^{14}C]phenylalanine. For the assay of tRNA^{Phe} in the eluate from the complex of polyphenylalanyl-tRNA and ribosomes, 0.5 ml of the eluate was mixed with 0.14 ml of the above reaction mixture. The mixture was incubated for 30 min at 30° and trichloroacetic acid was added at the end of the incubation period to a final concentration of 10%. The mixture was poured through a Millipore filter and the filter was washed three times with 5% trichloroacetic acid. The [^{3}H]phenylalanyl-tRNA retained on the Millipore filter was counted. The assay of loosely bound phenylalanyl-tRNA was carried out as described previously (Kuriki and Kaji, 1967a,b).

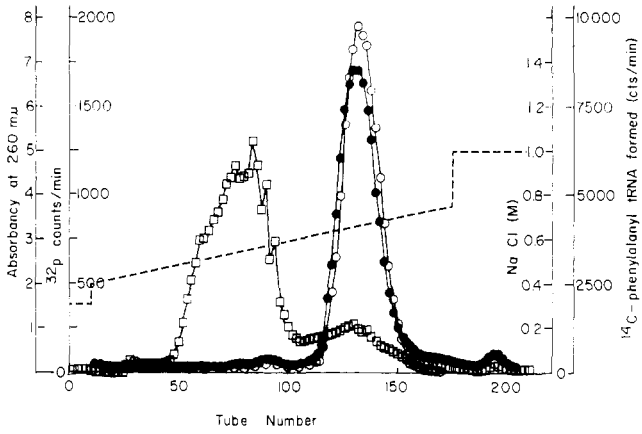

FIGURE 3: Reversed-phase column chromatography of [^{32}P]tRNA isolated from [^{14}C]polyphenylalanyl-[^{32}P]tRNA. A solution (10 ml) containing 2.46 × 10^5 cpm of the [^{32}P]tRNA isolated from polyphenylalanyl-tRNA as described in the text and 146 mg of commercially available sRNA in 0.3 M NaCl, 0.05 M Tris-HCl (pH 7.4), and 0.01 M $MgCl_2$ was placed on a column (1.4 × 230 cm) prepared as described by Kelmers *et al.* (1965). The column was washed with 100 ml of buffer saturated with isoamyl acetate containing 0.05 M Tris-HCl (pH 7.4), 0.01 M $MgCl_2$, and 0.3 M NaCl, and the sRNA was eluted from the column with a linear gradient of NaCl in the above buffer. The gradient was established by use of 1000 ml of 0.75 M NaCl in the buffer in the reservoir and 1000 ml of 0.4 M NaCl in the same buffer in the mixing chamber. Fractions of 12 ml were collected in a tube every 7 min. A 2.5-ml aliquot of each fraction was mixed with 5 ml of cold ethanol and the mixture was kept at 0° for several hours. The RNA precipitated was collected on a Millipore filter and ^{32}P radioactivity was measured. For the assay of $tRNA^{Phe}$ in each fraction, 0.1 ml of each fraction was mixed with 0.03 ml of the reaction mixture for the assay of $tRNA^{Phe}$ as described in the text. [^{14}C]Phenylalanyl-tRNA formed in 0.1 ml of the total mixture was measured (Mans and Novelli, 1960; Bollumn, 1959). (□—□) Absorbancy at 260 mμ; (●—●) ^{32}P radioactivity; (○—○) [^{14}C]phenylalanyl-tRNA formed per 0.1 ml of the reaction mixture; (- - - -) NaCl concentration of each fraction.

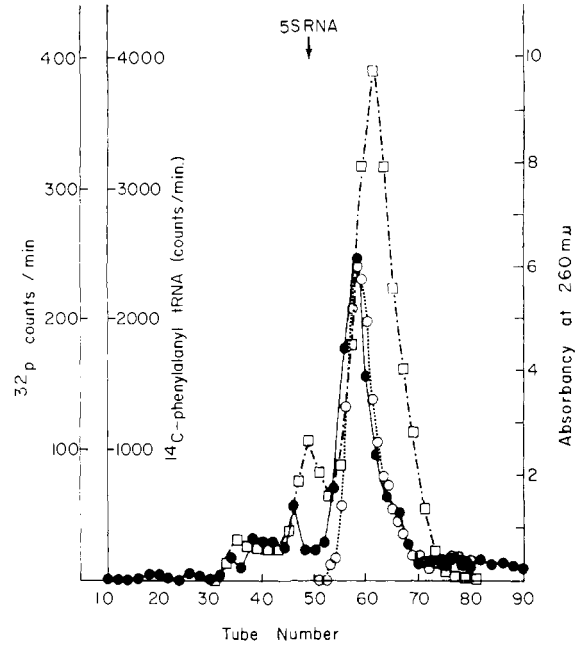

FIGURE 4: Sephadex gel filtration of [^{32}P]tRNA obtained from [^{14}C]-polyphenylalanyl-[^{32}P]tRNA. A solution (1 ml) containing 3570 cpm of the [^{32}P]tRNA isolated from polyphenylalanyl-tRNA and 10 mg of sRNA in 0.1 M NaCl and 0.01 M sodium acetate (pH 5.6) was placed on a Sephadex G-100 column (0.8 × 210 cm) and eluted with a buffer containing 0.1 M NaCl and 0.01 M sodium acetate (pH 5.6). A twenty-drop fraction was collected in a tube every 20 min. This method of gel filtration is a modification of that of Schleich and Goldstein (1966). A 1-ml aliquot of each fraction was mixed with 2 ml of ice-cold ethanol and kept standing at 0° for several hours. The RNA precipitated was collected on a Millipore filter and ^{32}P radioactivity was counted. For the assay of $tRNA^{Phe}$ in each fraction, 0.2 ml of the fraction was assayed as described in the text using [^{14}C]phenylalanine. [^{14}C]Phenylalanyl-tRNA formed in 0.1-ml aliquot of the total mixture was counted. (●—●) ^{32}P radioactivity; (○—○) ^{14}C radioactivity; (□—□) absorbancy at 260 mμ.

Results

Behavior of [^{32}P]tRNA Isolated from Polyphenylalanyl-tRNA upon Reversed-Phase Column Chromatography. It has been shown that reversed-phase column chromatography separates *E. coli* $tRNA^{Phe}$ from most of other tRNA (Kelmers *et al.*, 1965; Kelmers, 1966). It appeared therefore that this fractionation procedure was best suited for deciding whether tRNA of polyphenylalanyl-tRNA was $tRNA^{Phe}$ or an entirely different kind of tRNA-like material. In the experiment shown in Figure 3 ^{32}P-labeled tRNA from polyphenylalanyl-tRNA was mixed with crude commercially available tRNA and the mixture was subjected to reversed-phase column chromatography. Each fraction was assayed for $tRNA^{Phe}$ and the distribution of ^{32}P-labeled tRNA was also determined. In confirmation of the reports of Kelmers *et al.* (1965) $tRNA^{Phe}$ was eluted away from the bulk of tRNA which was represented by a large peak of ultraviolet-absorbing material. The ^{32}P radioactivity followed closely the distribution curve of $tRNA^{Phe}$. These results suggest that [^{32}P]tRNA isolated from [^{14}C]polyphenylalanyl-tRNA has the primary nucleotide sequence identical with $tRNA^{Phe}$ but is modified during the isolation so that it loses its phenylalanine acceptor capacity.

Sephadex Column Chromatography of [^{32}P]tRNA Isolated from Polyphenylalanyl-tRNA. In our previous communication a possibility was raised that the tRNA of peptidyl-tRNA might be 5S RNA (Kuriki and Kaji, 1967a,b). This possibility, though attractive, was unlikely because of the observation that polylysyl-tRNA binding to ribosomes was stimulated by the addition of poly A (Rychlik, 1966). The experiment shown in Figure 4 eliminated this possibility. In this experiment, [^{32}P]tRNA isolated from [^{14}C]polyphenylalanyl-tRNA and commercially available tRNA mixture was mixed and placed on a Sephadex G-100 column. The distribution of $tRNA^{Phe}$ was measured by assaying each fraction for the content of $tRNA^{Phe}$. Again, ^{32}P radioactivity closely followed the distribution of $tRNA^{Phe}$ except for the minor components which were eluted earlier. The fact that the peak of ^{32}P radioactivity coincided with the peak of $tRNA^{Phe}$ supports the concept that tRNA of polyphenylalanyl-tRNA was $tRNA^{Phe}$. Whatever the modification which might have occurred during the isolation procedure to inactivate its acceptor capacity is not serious enough to alter the behavior on Sephadex G-100 column. It is noted in this figure that $tRNA^{Phe}$ was eluted slightly earlier than the bulk of tRNA. A similar observation was reported with yeast tRNA (Lindahl and Fresco, 1967). At any rate, no appreciable ^{32}P radioactivity was found at the position where 5S RNA was eluted.

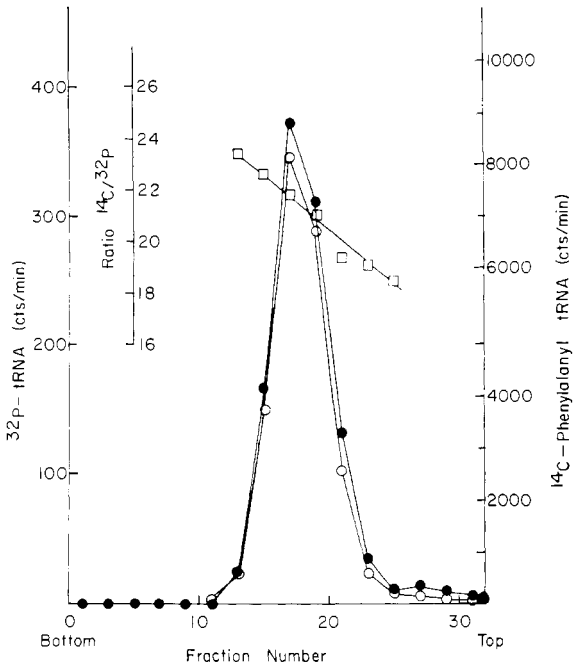

FIGURE 5: Sedimentation behavior of [^{32}P]tRNA obtained from [^{14}C]polyphenylalanyl-[^{32}P]tRNA. A solution (0.11 ml) containing 5170 cpm of the [^{32}P]tRNA isolated from polyphenylalanyl-tRNA as described in the text and 0.2 mg of sRNA mixture in 0.01 M potassium acetate (pH 5.6) was placed on top of a 5-ml linear sucrose gradient (5 ~ 20%) in 0.06 M KCl, 0.01 M Tris-HCl (pH 7.2), and 10^{-4} M magnesium acetate. The tube was centrifuged at a speed of 40,000 rpm in a Spinco SW50 rotor for 21 hr at 3°. After the centrifugation, two drop fractions were collected from the bottom of the tube. Each fraction (0.12 ml) was mixed with 0.04 ml of the reaction mixture for the assay of tRNA^{Phe} as described in the text. [^{14}C]Phenylalanyl-tRNA formed and ^{32}P radioactivity in 0.1 ml of the total reaction mixture was measured simultaneously. (●—●) ^{32}P radioactivity; (○—○) [^{14}C]phenylalanyl-tRNA formed; (□—□) the ratio of [^{14}C]/[^{32}P].

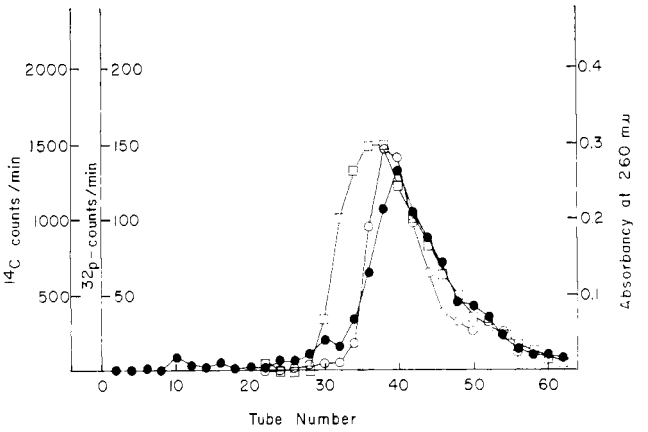

FIGURE 6: Methylated albumin column chromatography of [^{32}P]-tRNA obtained from [^{14}C]polyphenylalanyl-[^{32}P]tRNA. A solution (2.5 ml) containing 1.64×10^4 cpm of [^{32}P]tRNA isolated from polyphenylalanyl-tRNA and 54 μg of tRNA^{Phe} and 133 μg of commercially available sRNA mixture in 0.1 M NaCl and 0.05 M sodium phosphate buffer (pH 6.7) was placed on a methylated albumin column (1 × 2 cm) as described (Sueoka and Yamane, 1962). The column was washed with 10 ml of 0.1 M NaCl–0.05 M sodium phosphate (pH 6.7) and the RNA was eluted from the column with a linear gradient of NaCl. The gradient was established by use of 18 ml of 1.1 M NaCl in 0.05 M sodium phosphate (pH 6.7) in the reservoir and 18 ml of 0.1 M NaCl in the same buffer in the mixing chamber. Fractions (0.8 ml) were collected in each tube. A 0.1-ml aliquot of each fraction was used as in Figure 3 for the assay of ^{32}P radioactivity. For the assay of tRNA^{Phe} in each fraction, 0.04 ml of each fraction was mixed with 0.12 ml of the tRNA^{Phe} assay mixture as described in the text. [^{14}C]Phenylalanyl-tRNA formed in 0.1 ml of the total reaction mixture was measured. (●—●) ^{32}P radioactivity; (○—○) [^{14}C]phenylalanyl-tRNA formed in the reaction mixture; (□—□) absorbance at 260 mμ.

Sucrose Density Gradient Centrifugation and Methylated Albumin Kieselguhr Column Chromatography of [^{32}P]tRNA Isolated from [^{14}C]Polyphenylalanyl-[^{32}P]tRNA. Additional evidence that the tRNA of polyphenylalanyl-tRNA is tRNA^{Phe} was obtained by the sucrose gradient centrifugation technique (Kaji and Tanaka, 1967). In the experiment shown in Figure 5, [^{32}P]tRNA isolated from [^{14}C]polyphenylalanyl-[^{32}P]tRNA sedimented to the same position as tRNA^{Phe}. However, the ratio of ^{14}C to ^{32}P progressively decreased toward the top of the tube indicating that tRNA^{Phe} sedimented slightly faster than [^{32}P]tRNA.

A similar conclusion can be drawn from the experiment with the methylated albumin kieselguhr column (Figure 6). The tRNA^{Phe} was eluted toward the end of the peak of the tRNA mixture in confirmation of the original report of Sueoka and Yamane (1962). The [^{32}P]tRNA isolated from [^{14}C]polyphenylalanyl-[^{32}P]tRNA behaved similarly to tRNA^{Phe}.

Phenylalanine Acceptor Capacity of tRNA Liberated from Ribosome-Bound Polyphenylalanyl-tRNA by the Action of Puromycin. The results described in the preceding sections suggested that tRNA from polyphenylalanyl-tRNA is tRNA^{Phe} but was modified somewhat during the isolation procedure resulting in a slight difference in its behavior in various tests for identity such as reversed-phase column chromatography and sucrose density gradient centrifugation. Damage to the tRNA during the isolation procedure was perhaps responsible for its inability to accept phenylalanine. If this notion is correct, one might expect that native active tRNA^{Phe} may be liberated from polyphenylalanyl-tRNA by the action of puromycin. This was indeed the case, as shown in Figure 7. In this experiment, complexes of poly U, polyphenylalanyl-tRNA, phenylalanyl-tRNA, and ribosomes were isolated. As a control, complexes of tRNA^{Phe}, poly U, and ribosomes were prepared by an identical method. These complexes were incubated separately in the presence and absence of puromycin. At the various time intervals the mixture was poured through the Millipore filter and the ribosomes, bound peptidyl-tRNA, and tRNA^{Phe} were isolated on the Millipore filter. The tRNA^{Phe} and phenylalanyl-tRNA^{Phe} were then eluted with buffer II (containing 10^{-4} M Mg^{2+}). As described previously any polyphenylalanyl-tRNA which did not react with puromycin will not be eluted by this buffer. As shown in this figure, in the presence of puromycin the "loosely bound" tRNA^{Phe} which can be eluted with buffer II increased during the period of incubation with puromycin. When the complex of phenylalanyl-tRNA, ribosomes and poly U was made, however, puromycin did not increase the loosely bound tRNA^{Phe} because polyphenylalanyl-tRNA was absent from the ribosomes in this case. In confirmation of our preceding reports the amount of loosely bound tRNA^{Phe} was reduced during polyphenylalanine formation.

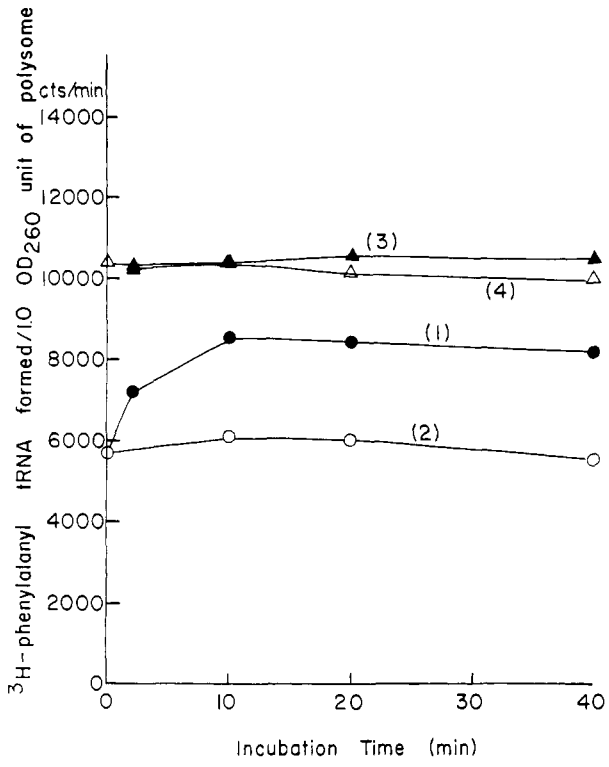

FIGURE 7: Phenylalanine acceptor capacity of tRNA liberated from ribosome-bound polyphenylalanyl-tRNA by the action of puromycin. The reaction mixture for the formation of polyphenylalanine was as described in the text except that it contained 5 μCi of [^{14}C]phenylalanine, 2.5 mg of sRNA, 4.0 mg of ribosomes, and 1.12 mg of fraction A but not [^{32}P]tRNA^{Phe} in a total volume of 0.9 ml. The mixture was incubated at 30° for 30 min and chilled to 3°. Three 0.25-ml portions of the aliquot were placed on three 5-ml gradients of sucrose (5–20% in 140 mM Tris-HCl, pH 7.8), 66 mM KCl, 20 mM magnesium acetate, and 7 mM β-mercaptoethanol. The tubes were centrifuged for 60 min at 37,000 rpm in a Spinco SW-50 rotor. After the centrifugation three drop fractions were collected from the bottom. The complex of polyphenylalanyl-tRNA, ribosomes, and poly U in the fractions 1–10 (counting from the bottom) was pooled and used in the experiment. This pooled polysome fraction was called polysome A. As a control, a similar reaction mixture except for the omission of ATP, GTP, phosphoenolpyruvate, pyruvate kinase, [^{14}C]phenylalanine, and fraction A was prepared and subjected to the same sucrose density gradient centrifugation. The polysome fraction from this control mixture was called polysome B. The reaction of the polysomes with puromycin was carried out in four separate tubes. Tube 1 contained 3 ml of polysome A (9.66 $A_{260\,m\mu}$ units, containing 5.49 × 10^5 cpm of [^{14}C]phenylalanine), 0.01 ml of 0.1 M magnesium acetate, and 0.5 ml of 5 × 10^{-3} M puromycin. Tube 2 was the same as tube 1 but without puromycin. Tube 3 was the same as tube 1 except that 3 ml of polysome B (8.8 260-mμ absorbancy unit) was used. Tube 4 was the same as tube 3 but without puromycin. Total volume of all four tubes was adjusted to 3.5 ml. The mixture was incubated at 30° and a 0.7-ml aliquot was taken at the time intervals indicated in the figure. The aliquots were poured through a Millipore filter and washed with three 0.5-ml portions of buffer II (containing 10^{-4} M magnesium acetate). The filtrate as well as the washings were carefully collected. (Total volume was 2.2 ml.) The amount of tRNA^{Phe} in the eluate and the washing were assayed as described in the method. [^{3}H]Phenylalanyl-tRNA formed in 0.5 ml of the eluate was plotted against the incubation period of the polysomes with or without puromycin. (○—○) Polysome A, no puromycin; (●—●) polysome A, plus puromycin; (△—△) polysome B, no puromycin; (▲—▲) polysome B, plus puromycin.

Exchangeability of Tightly Bound [^{32}P]tRNA. Since the tRNA of polyphenylalanyl-tRNA is apparently tRNA^{Phe}, the tightly bound tRNA of polyphenylalanyl-tRNA should be exchanging constantly with incoming new tRNA^{Phe} as the polyphenylalanine chain elongates. The experiments shown in Figure 8 were carried out to test this possibility. Polyphenylalanine formation was allowed to proceed in the presence of ^{32}P-labeled tRNA and an excess of nonlabeled tRNA was added halfway through the reaction. The tightly bound ^{32}P-labeled tRNA representing polyphenylalanyl-[^{32}P]tRNA decreased sharply after the addition of nonlabeled tRNA. On the other hand, the amount of tightly bound polyphenylalanyl-tRNA increased during this process. These observations indicate that the tRNA^{Phe} bound to polyphenylalanine is constantly exchanging with incoming tRNA^{Phe} and that the addition of an excess of nonlabeled tRNA chases out the ^{32}P radioactivity bound to the ribosomes. The data in C show that the extent of chasing of tightly bound ^{32}P radioactivity from the ribosomes depends upon the amount of nonlabeled tRNA added to the reaction mixture.

Discussion

Despite the widely accepted hypothesis that tRNA is bound to a growing polypeptide, no solid experimental evidence has supported this notion. Using crude mixtures of [^{32}P]tRNA, Gilbert (1963) observed that RNA is bound to the growing polyphenylalanine, but the experiment did not prove that the RNA was tRNA^{Phe}. The data indicating that polylysine is bound to RNA through its terminal adenosine (Bretcher, 1963) was suggestive but did not prove that the RNA is tRNA^{Lys}. Using reticulocyte ribosomes it has been shown that a small phenylalanine peptide is linked to RNA through adenosine, but this observation again does not prove that the RNA is tRNA^{Phe} (Shaeffer *et al.*, 1968). On the other hand, the report by Rychlik (1966) that polylysyl-tRNA can be bound to ribosomes in the presence of poly A indicates that sRNA linked to polylysine contains the nucleotide sequence corresponding to the triplet of adenine nucleotide. However, a control which indicates that in the presence of poly A other peptidyl-tRNA does not bind was missing in Rychlik's experiment.

The present investigation was prompted by our previous observation that tRNA isolated from polyphenylalanyl-tRNA was inactive as phenylalanine acceptor tRNA. A series of experiments described in this paper using various fractionation techniques for nucleic acid established that [^{32}P]tRNA isolated from polyphenylalanyl-[^{32}P]tRNA behaved like tRNA^{Phe} in all the tests performed. A slight difference in the behavior of this RNA from tRNA^{Phe} in these tests could be attributed to possible damage during the isolation procedure which results in the inactivation of the acceptor activity. It is also possible that the Pronase treatment and subsequent deacylation at pH 8.0 did not remove all the phenylalanine from the [^{14}C]polyphenylalanyl-[^{32}P]tRNA. Since phenylalanyl-tRNA (Kaji and Tanaka, 1967) and polyphenylalanyl-tRNA (Kuriki and Kaji, 1967a,b) sediment slower than tRNA^{Phe}, this possibility is strengthened by the observation that [^{32}P]tRNA from polyphenylalanyl-tRNA sediments slightly slower than tRNA^{Phe}.

Further evidence that RNA of polyphenylalanyl-RNA is tRNA^{Phe} is that this RNA can accept phenylalanine if it is

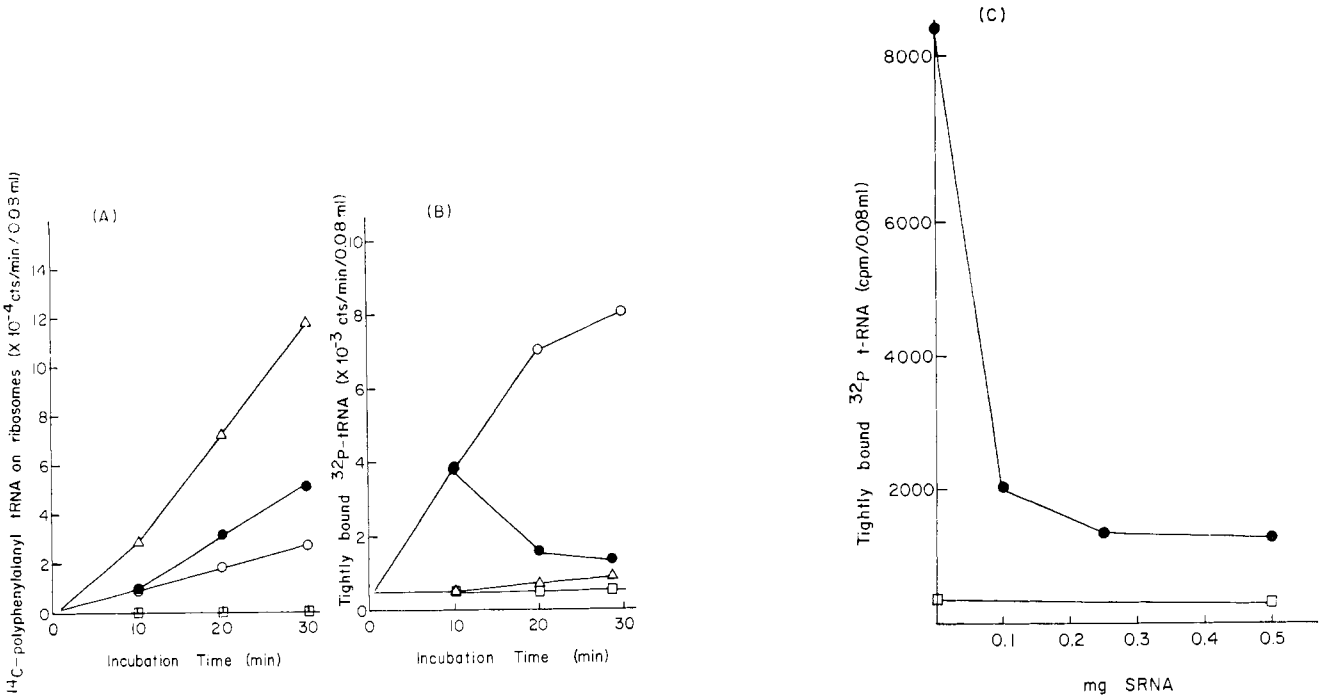

FIGURE 8: Exchangeability of tightly bound [^{32}P]tRNA with unbound tRNA. The complete reaction mixture for polyphenylalanine was as described in the text except that it (0.3 ml) contained 10 μg of pyruvate kinase, 4 μCi of [^{14}C]phenylalanine, 32 μg of fraction A, 120 μg of poly U, 1.98 $\times$ 10^5 cpm of [^{32}P]tRNA^{Phe}, and 68 μg of ribosomes. The mixture was incubated at 30°. At the various time intervals 0.08 ml of the reaction mixture was taken and mixed with 3 ml of buffer I. The mixture was then poured through a Millipore filter, and the paper was washed with three 3-ml portions of buffer I followed by three 3-ml portions of buffer II. The tightly bound [^{32}P]tRNA as well as [^{14}C]polyphenylalanyl-tRNA on the ribosomes were counted. (A) [^{14}C]Polyphenylalanyl-tRNA bound to ribosomes. (B) [^{32}P]tRNA tightly bound to the ribosomes. (○—○) Complete reaction mixture; (●—●) complete reaction mixture, but additional sRNA mixture was added at 10 min; (△—△) complete reaction mixture but additional sRNA mixture was added at zero time; (□—□) ATP, GTP, phosphoenolpyruvate, pyruvate kinase, [^{14}C]phenylalanine, and fraction A were omitted from the complete reaction mixture. In part C the tightly bound [^{32}P]tRNA at 20 min after the onset of the reaction was plotted against the amount of nonlabeled sRNA added at 10 min after the reaction started. (□—□) Poly U omitted from the reaction mixture.

removed from polyphenylalanine by puromycin on the ribosomes. It is noteworthy that tRNA^{Phe} released from polyphenylalanyl-tRNA in the absence of added enzymes stays on ribosomes. This suggests that some enzymatic mechanism exists to remove the deacylated tRNA from the ribosomes. Preliminary evidence suggests that such a mechanism does indeed exist and this will be reported elsewhere. The fact that tightly bound [^{32}P]tRNA of polyphenylalanyl-tRNA can be exchanged with incoming phenylalanyl-tRNA during polypeptide synthesis unequivocally ruled out the possibility that a special kind of RNA such as 5S RNA exists which functions only to hold the growing polypeptide to the ribosomes (Kuriki and Kaji, 1967a,b). Although data presented in this paper indicate that tRNA of polyphenylalanyl-tRNA is tRNA^{Phe}, the question remains as to why this tRNA does not accept phenylalanine. A control experiment showed that the procedure of isolation of tRNA from polyphenylalanyl-tRNA was mild enough to preserve most of the acceptor capacity of tRNA. It appears therefore that linking with polyphenylalanine renders tRNA^{Phe} more susceptible to alkaline treatment. It has been reported that in some cases tRNA exists as an inactive form and it can be converted into an active form by a variety of treatments (Ishida and Sueoka, 1967; Muench, 1966; Lindall *et al.*, 1966). Thus, the inactive tRNA^{Phe} isolated from polyphenylalanyl-tRNA has been exposed to 0.02 M Mg^{2+} and 0.002 M EDTA at 60 or 80° for various times with the hope that activation of inactive tRNA^{Phe} may take place. Under the conditions we used no appreciable activation of the tRNA^{Phe} was observed. Despite these unsuccessful attempts to activate the tRNA^{Phe} isolated from polyphenylalanyl-tRNA, the possibility that tRNA changes its configuration by linking with polypeptide is being examined.

Acknowledgments

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Subfractionation and Polyacrylamide Gel Analysis of Liver Ribonucleic Acid from Normal and Glucocorticoid-Treated Rats*

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ABSTRACT: A new subfractionation procedure for the separation and study of ribonucleic acid has been devised. Rat livers were fractionated into the nuclear, free polysomal, and total reticular fraction, and ribonucleic acid was extracted by means of phenol. The cytoplasmic fractions were extracted first at pH 6.0 and 0° in the presence of 0.05 M Tris-acetate containing 0.1% sodium lauroyl sarcosinate plus 1 mg/ml of bentonite; second at pH 8.3 and 0° with 0.05 M Tris-acetate, containing 2.5% sarcosinate plus 1 mg/ml of bentonite; and third with the same components, but at 40°. The ribonucleic acid from the second and third extractions showed high specific radioactivity on pulse labeling and appeared to contain species of molecular weight greater than that of the 28S ribosomal ribonucleic acid. Nuclear ribonucleic acid was extracted once at pH 6.0 and 0° in 0.05 M Tris-acetate containing 0.1% sarcosinate plus 1 mg/ml of bentonite, then with the same medium, but at 65°. The specific activity of the latter fraction was 10–30 times that of the former.

Ribonucleic acid from all fractions was analyzed as to molecular weight and labeling distributions by means of polyacrylamide gel electrophoresis. Gel profiles indicated (1) the presence of a rapidly labeled ribonucleic acid species migrating with the 18S ribosomal ribonucleic acid present in reticulum, but not in polysomes; (2) the existence of other nonribosomal ribonucleic acids in the cytoplasm of rat liver cells with molecular weights from 5×10^5 to $>10^7$ which also become rapidly labeled.

Utilizing a double-label technique, ribonucleic acid was analyzed to detect selective induction(s) of any species by triamcinolone, a synthetic highly effective glucocorticoid. No such induction was detected in studies ranging from 1 to 2.5 hr after hormone administration.

During recent years many aspects of the mode of action of glucocorticoid hormones have become established. Beginning with studies on glycogen deposition and gluconeogenesis, continued refinements and innovations in technique have permitted the demonstration that following hormone administration to an appropriate animal, tissue, or cell culture system, increases occur in the intracellular concentrations of certain enzymes concerned with protein catabolism and with glucose synthesis from amino acid precursors (Feigelson and Greengard, 1962; Kenney, 1962; Segal and Kim, 1965). These rises in enzyme concentration have been shown to be due to increases in the rate of synthesis of the enzyme involved, rather than to decreases in its rate of degradation (Kenney, 1962; Segal and Kim, 1963, 1965; Knox *et al.*, 1964; Schimke *et al.*, 1964).

Recent studies suggest that the hormone may be acting at the level of transcription. The relevant observations include (1) increases in the transcriptive ability of chromatin from hormone-treated systems (Bonner *et al.*, 1968; Chambon *et al.*, 1968; Stackhouse *et al.*, 1968); (2) increases in the translational capabilities of RNA synthesized in nuclei (or nuclear systems) from treated cells (Dukes *et al.*, 1966; Schmid *et al.*, 1967); (3) alterations in the DNA–RNA hybridization characteristics of RNA from treated animals (Drews and Brawerman, 1967); (4) sensitivity of enzyme inductions to agents which interfere with DNA-directed RNA synthesis (*i.e.*, actinomycin D and other antibiotics) (Greengard and Acs, 1962; Kvam and Parks,

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