Coding Properties of Methyl-Deficient Phenylalanyl Transfer Ribonucleic Acid from *Escherichia coli**

Robert Stern,† Fabio Gonano,‡ Erwin Fleissner, and Uriel Z. Littauer§

ABSTRACT: The biological function of methyl-deficient phenylalanyl transfer ribonucleic acid and fully methylated normal phenylalanyl transfer ribonucleic acid was compared in several systems. Ribosome binding assays demonstrated that the methyl-deficient phenylalanyl transfer ribonucleic acid was markedly less efficient than normal phenylalanyl transfer ribonucleic acid in binding to polyuridylic acid or to a copolymer of uridylic and cytidylic acids. This difference was observed over a wide range of magnesium ion concentrations, and in the presence of streptomycin. The ribosome-dependent binding assay showed that the relative ratio of binding to a copolymer of uridylic and cytidylic acids compared to that found for polyuridylic acid was the same for normal and methyl-deficient phenylalanyl transfer ribonucleic acid. Similarly, no difference in the level of error was found for normal and methyl-deficient transfer ribonucleic acid when examined in a messenger ribonucleic acid and transfer ribonucleic acid dependent phenylalanine incorporating system supplemented with polyuridylic acid or a copolymer of uridylic and cytidylic acids. The ability of the various phenylalanyl transfer ribonucleic acid fractions to transfer labeled phenylalanine into the α chain of rabbit reticulocyte hemoglobin was examined. In the *in vitro* hemoglobin-synthesizing system, tryptic peptides derived from the normal and methyl-deficient phenylalanyl transfer ribonucleic acid fractions were indistinguishable on Dowex 50 column chromatography. It was also observed that the *Escherichia coli* phenylalanyl transfer ribonucleic acid species were able to insert phenylalanine into all phenylalanine containing peptides of the α chain of rabbit hemoglobin.

Methionine-starved Phe-tRNA[1] can be separated into four distinct components on MASA[2] column chromatography (Littauer *et al.*, 1966; Stern and Littauer, 1968) and by countercurrent distribution (Fleissner, 1967). This has made it possible to circumvent the difficulty found in studying the function of methyl-deficient tRNA using methionine-starved tRNA. These preparations contain an approximately equal mixture of normal and methyl-deficient species which obscures subtle differences which might be due to the methyl-deficient species alone. The MASA column has enabled us to compare the response of normal and methyl-deficient species of $tRNA^{Phe}$ with a number of synthetic polynucleotides in the ribosome-dependent binding assay, in a mRNA–tRNA-dependent amino incorporating system and in the transfer of amino acids into polypeptide in a rabbit reticulocyte *in vitro* hemoglobin-synthesizing system (Jacobson, 1966).

Materials and Methods

Uniformly labeled L-[^{14}C]phenylalanine (0.32 mCi/μmole) was obtained from Schwarz BioResearch Inc. Methylated albumin was prepared by the method of Mandell and Hershey (1960) using crystalline bovine serum albumin fraction V from the California Foundation for Biochemical Research. Poly U was purchased from Miles Laboratories, Inc. Poly U,C (1:2) and poly U,C (1:1) were prepared with purified polynucleotide phosphorylase from *Micrococcus lysodeikticus*. The synthetic polynucleotides were purified by the method of Singer *et al.* (1963). The copolymers obtained had the following base composition following alkaline hydrolysis and Dowex 1-X2 formate chromatography: poly U,C (1:2), 31% U and 69% C; poly U,C (1:1), 51% U and 49% C. The concentrations of the polynucleotides were determined by measuring the total organic phosphate (Chen *et al.*, 1956). *E. coli* G-15 RC^{rel} Met^- His^- Biotin$^-$ was obtained from Dr. G. Stent. *E. coli* A-19 RC^{rel} Met^- RNase I^- and *E. coli* Q-13 (a mutant deficient in RNase I and with an altered polynucleotide phosphorylase) were obtained from Dr. S. Spiegelman. *E. coli* MRE-600 RNase I^- was obtained from Dr. H. E. Wade and *E. coli* $K_{12}W6$ RC^{rel} Met^- was obtained from Dr. E. Lederberg.

Methylated Albumin Column Chromatography. The prep-

* From the Department of Biochemistry (R. S. and U. Z. L.), Weizmann Institute of Science, Rehovot, Israel, and the International Laboratory of Genetics and Biophysics (F. G.), Naples, Italy, and the Sloan-Kettering Institute for Cancer Research (E. F.), New York, New York. *Received August 18, 1969.* Supported in part by agreement No. 455114 from the National Institutes of Health, U. S. Public Health Service. This is the third publication in this series dealing with studies on the fractionation of tRNA on MASA columns. Publication No. 2 in this series is *Biochemistry 8*, 313 (1969).

† Special postdoctoral fellow of the National Cancer Institute, Public Health Service fellowship (1-F3-CA-28, 111-01).

Present address: Cell Biology Section, Laboratory of Biochemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Md. 20014.

‡ Present address: Istituto di Patologia Generale, Universita Degli Studi di Modena, Modena, Italy.

§ To whom inquiries should be addressed.

[1] In this paper methionine-starved tRNA refers to the tRNA extracted from a relaxed methionine-requiring mutant of *E. coli* (A-19, $K_{12}W6$, or G-15) grown in a medium deficient in methionine. Methyl-deficient tRNA refers only to those species which contain few or no methyl groups. Methionine-starved tRNA is an approximately equal mixture of methyl-deficient tRNA and fully methylated normal tRNA.

[2] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: MAK, methylated albumin–kieselguhr; MASA, methylated albumin–silicic acid.

aration of the MASA column is described in an earlier publication (Stern and Littauer, 1968). The indicated fractions (Figure 2) were pooled, dialyzed overnight against 10^{-3} M NaCl, lyophilized to a volume of 5 ml, and treated with pronase, followed by phenol extraction; the tRNA was precipitated twice with salt and ethanol and reacylated as described in the preceding communication (Stern and Littauer, 1968). In some experiments, the lyophilized fractions were treated only with phenol, precipitated twice with ethanol and NaCl, and reacylated with the indicated amino acid.

The MAK columns were prepared according to the method of Yamane and Sueoka (1963) with some modification. Kieselguhr (10 g) was suspended in 50 ml of 0.05 M sodium phosphate buffer (pH 6.8) followed by boiling for 2 min and cooling of the suspension. Then 3.0 ml of 1% methylated albumin solution in water was added slowly with stirring. The suspension was stirred for 10 min at 4°. A layer of glass beads was placed at the bottom of a jacketed column (25 mm i.d.). The MAK suspension was poured onto the glass bead layer and packed by air pressure. A suspension of 1 g of kieselguhr in 10 ml of phosphate buffer was placed on this layer as a protective covering layer.

The column was washed with 200 ml of 0.2 M sodium chloride in 0.05 M sodium phosphate buffer (pH 6.8) (initial buffer) and was then ready for use. The column temperature was maintained at 16°. The tRNA was dissolved in 20 ml of the initial buffer, applied to the column, which was then washed with an additional 50 ml of initial buffer. A 380-ml gradient was used consisting of 190 ml each of 0.2 and 1.1 M sodium chloride in 0.05 M sodium phosphate buffer (pH 6.8) and 2.5-ml fractions were collected. The chromatography was completed in approximately 5–8 hr. Each receiving tube contained 0.2 ml of 1 M sodium acetate buffer (pH 4.0) to prevent hydrolysis of the aminoacyl-tRNA bond before measurement of acid-precipitable counts could be made. Processing of the column fractions was the same as that described for the MASA column.

Ribosome-Dependent Binding Assay. Polynucleotide-directed binding of phenylalanyl-tRNA to ribosomes was performed according to the method of Nirenberg and Leder (1964). The reaction mixture (0.05 ml) contained 0.1 M Tris-HCl buffer (pH 7.2), 0.05 M KCl, magnesium acetate, polynucleotide, ribosomes, and [^{14}C]Phe-tRNA as indicated. The reaction was started with [^{14}C]Phe-tRNA and incubation was carried out at 24° for 20 min as detailed by Leder (1968). Three kinds of early log-phase ribosomes were used during the course of these experiments. Ribosomes were prepared from *E. coli* MRE-600 according to the method of Nirenberg (1963). *E. coli* B ribosomes were isolated from an extract preincubated at 37° with 50 μg/ml of puromycin (Gilbert, 1963), and ribosomes from *E. coli* Q-13 were isolated and washed according to the method of Kurland (1966). Ribosomes were stored frozen in liquid air until used. Ribosomes once thawed were not refrozen.

Assay of Polyphenylalanine Formation. The participation of normal and methyl-deficient tRNA in phenylalanine incorporation into polyphenylalanine was assayed as described by Littauer and Milbauer (1965).

In Vitro Hemoglobin Synthesis. Ribosomes from rabbit reticulocytes were used to synthesize hemoglobin *in vitro*. The preparation of ribosomes, the transfer of phenylalanine from acylated tRNA fractions into hemoglobin, chain

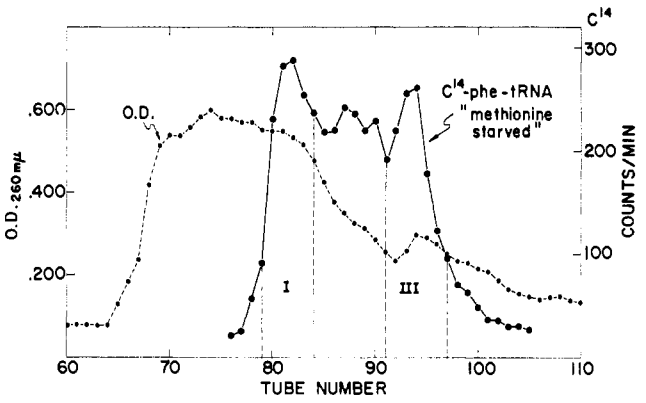

FIGURE 1: MAK chromatography of methionine-starved [^{14}C]-Phe-tRNA. Methionine-starved tRNA (1.8 mg) from *E. coli* G-15 was acylated, the Phe-tRNA was isolated, and MAK chromatography was carried out as described under Materials and Methods. Radioactive material precipitable by trichloroacetic acid in 0.2-ml portions was measured in a liquid scintillation counter. Fractions I and III were pooled, dialyzed against 1 mM NaCl, and lyophilized. The tRNA fractions were dissolved in 0.57 ml of 0.01 M Tris-HCl buffer (pH 7.4) and 0.5 ml of pronase (preincubated for 90 min in 0.01 M Tris-HCl, pH 7.4; Revel and Littauer, 1965) was added (2 mg/ml). The mixture was incubated at 37° for 5 hr and treated three times with 1.0 ml of 70% phenol and 0.1 ml of chloroform. The tRNA was precipitated from the aqueous phase with NaCl–ethanol, dissolved, and reprecipitated. The pellet was washed once with ethanol–ethyl ether (1:1), dried *in vacuo*, and dissolved in 0.2 ml of H_2O before use for biological assays.

separation, and digestion with trypsin were performed, according to previously published methods (Dintzis, 1961; Weisblum *et al.*, 1965; Gonano, 1967). Ion-exchange chromatography on Dowex 50-X8 (H^+) of the soluble α-chain peptides was done according to the method of Jones (1964). Following the fractionation, aliquots were assayed with ninhydrin by the method of Moore and Stein (1954). Additional aliquots were dried in a counting vial redissolved with 0.2 ml of Hyamine and counted with toluene-based scintillation fluid in a Packard Tri-Carb scintillation spectrometer.

In vivo labeled homoglobin was isolated by incubating intact rabbit reticulocytes with [^{14}C]phenylalanine (Dintzis, 1961).

The countercurrent distribution fractions were identical with those described by Fleissner (1967).

Results

Resolution of Phe-tRNAs. MAK column chromatography resolves methionine-starved Phe-tRNA into three components (Figure 1). It has been established previously (Revel and Littauer, 1965) that the first peak (I) and the third peak (III) correspond to methyl-deficient and normal Phe-tRNA components, respectively. Peak II is probably a mixture of normal and starved species and was not examined for its biological properties.

MASA column chromatography of methionine-starved tRNA resolves four peaks of Phe-tRNA (Figure 2). Peaks a and b represent methyl-deficient tRNA and peak d chromatographs with normal Phe-tRNA. The identity of peak c remains uncertain. Normal Phe-tRNA contains a major peak and a variable minor peak which precedes the major peaks

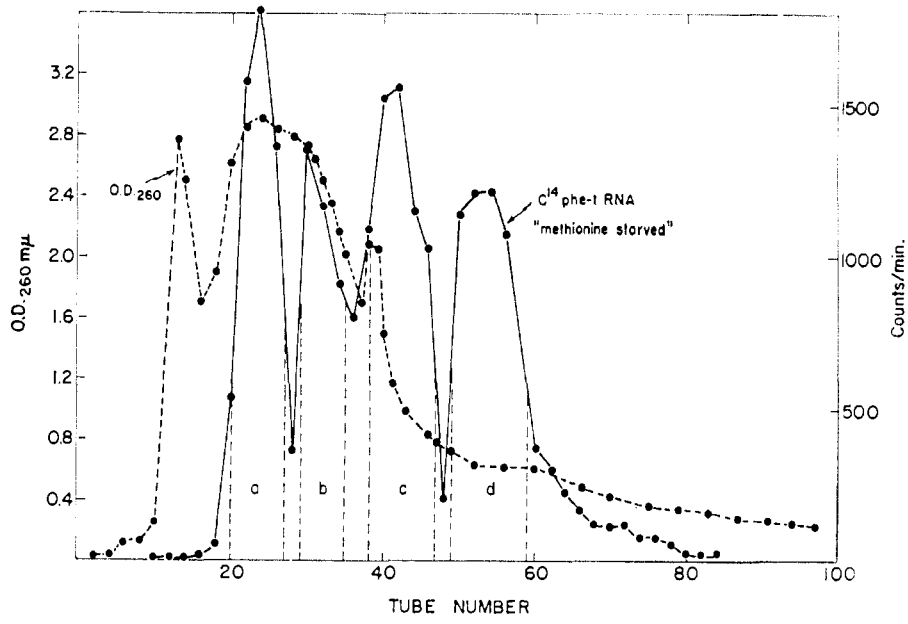

FIGURE 2: MASA column chromatography of methionine-starved [^{14}C]Phe-tRNA from *E. coli* A-19. [^{14}C]Phe-tRNA (18 mg) was dissolved in 10 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.4 M NaCl. This mixture was applied to a MASA column (2 × 12 cm) which was then washed with 25 ml of 0.05 M sodium acetate buffer (pH 5.5)–0.8 M NaCl. The RNA was eluted with a linear gradient formed by 200 ml each of 0.8 and 1.15 M NaCl in 0.05 M sodium acetate buffer (pH 5.5). Fractions (3.3 ml) were collected at 16° over a period of 8 hr. Aliquots were removed for the measurement of the A_{260} and acid-precipitable radioactivity. Fractions were taken from the center of the regions labeled a, b, c, and d, pooled, and treated as described under Figure 1.

TABLE I: Binding Properties of Normal and Methyl-Deficient Phenylalanyl-tRNA.[a]

Source of Phe-tRNA	% Input Bound/0.1 μg of Polynucleotide		Poly UC:poly U × 100	% Rel Binding Compared with Normal tRNA Fraction	
	Poly U	Poly U,C		Poly U	Poly U,C
MASA a	14.5	1.30	9.0	58.0	47.1
b	18.4	2.04	11.1	73.6	73.9
c	18.7	2.42	12.9	74.8	87.7
d	25.0	2.76	11.0	100	100
Countercurrent distribution 1	15.6	1.26	8.1	56.3	30.0
2	22.8	2.90	12.7	82.3	69.0
3	26.8	2.96	11.0	96.7	70.5
4	27.7	4.20	15.2	100	100
MAK I	16.6	3.00	18.1	63.6	63.8
III	26.1	4.70	18.0	100	100
Unfractionated methionine-starved tRNA	20.7	2.05	9.9	74.2	69.7
Unfractionated normal tRNA	27.9	2.94	10.5	100	100

[a] The value of per cent Phe-tRNA bound to ribosomes when 0.1 μg of polynucleotide was added to the reaction mixture is given in columns 2 and 3. The values for the MASA and countercurrent distribution fractions were extrapolated from the data in Figures 4 and 5. The other values were extrapolated from similar binding assays. The per cent relative binding of Phe-tRNA to ribosomes in the presence of poly U and poly U,C compared with normal Phe-tRNA taken as 100 is given in columns 5 and 6. *E. coli* Q-13 ribosomes were prepared by the method of Kurland (1966) and the assays were carried out at a magnesium concentration of 0.022 M. For MAK column fractionation *E. coli* G-15 methionine-starved tRNA was used. The same bacterial strain was used for the isolation of unfractionated normal and methionine-starved tRNA.

in MASA elution patterns (Stern and Littauer, 1968). Peak c did not coelute with the minor Phe-tRNA peak in double label experiments when normal and methionine-starved tRNA were chromatographed together (*cf.* Figure 2 in Stern and Littauer, 1968). The assignments of the MASA column peaks were verified by rechromatography on MAK columns, on which the nature of the peaks had already been established in an earlier communication (Littauer and Revel, 1966).

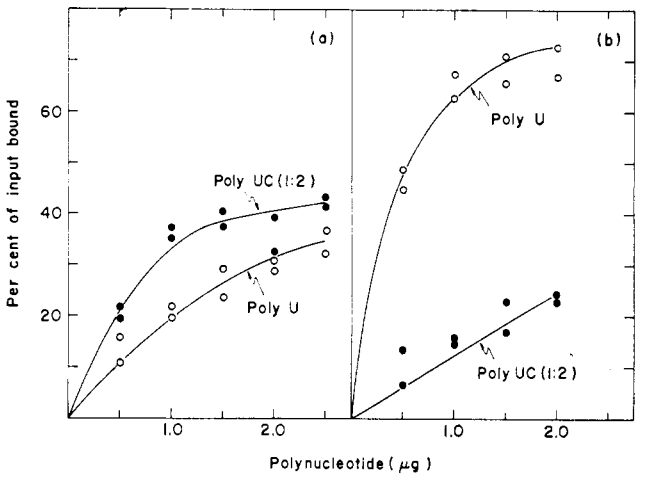

FIGURE 3: Millipore filter binding assays with normal *E. coli* [^{14}C]-Phe-tRNA taken from MASA column fractions, with and without pronase pretreatment following column chromatography. Methionine-starved *E. coli* A-19 Phe-tRNA was chromatographed on a MASA column and yielded a profile similar to the one illustrated in Figure 2. (a) The fractions from the center of peak IV corresponding to normal Phe-tRNA were pooled and treated as described under Materials and Methods. With dialysis, lyophylization, phenol extraction, and precipitation with ethanol and NaCl followed by reacylation with phenylalanine. In the case of Figure 3b, a pronase digestion step was inserted following lyophylization. Ribosome-dependent binding assays were performed as described under Materials and Methods. Results are plotted as % of input [^{14}C]Phe-tRNA bound to ribosomes as a function of concentration of the indicated polymers, poly U or poly U,C (1:2). Phe-tRNA (0.4 $\mu\mu$mole) was added to the reaction mixture in (a) corresponding to 212 cpm and 2.0 $\mu\mu$moles in (b) corresponding to 994 counts/min. The ratio of binding poly UC:poly U was 170% in (a) and 14% in (b). The magnesium concentration was 0.017 M. The *E. coli* ribosomes (2.0 A_{260}) were from MRE-600 cells prepared as described by Nirenberg (1965).

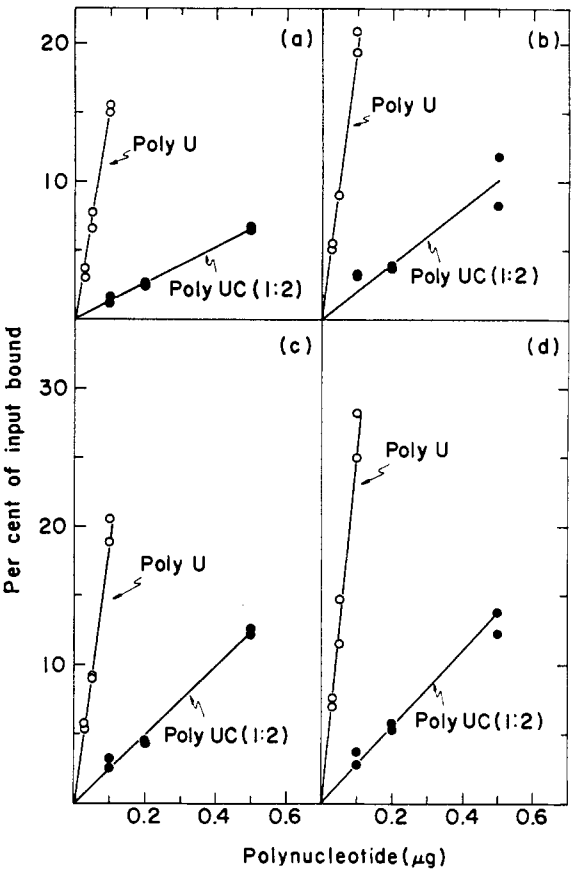

FIGURE 4: Millipore filter binding assays with Phe-tRNA fractions taken from MASA column fractions. Results are plotted as per cent of input Phe-tRNA bound to ribosomes as a function of concentration of the indicated polymers, poly U or poly U, C (1:2). Methionine-starved *E. coli* G-15 Phe-tRNA was chromatographed on a MASA column similar to the one shown in Figure 2. The phenylalanine acceptance for each of the column fractions were, for fractions a, b, c, and d, respectively, 0.380, 0.555, 1.170, and 1.220 mμmoles per mg of RNA The micromicromoles of Phe-tRNA added to each reaction mixture ranged from 3.60 to 3.93. The magnesium concentration was 0.022 M. (a) Fraction a, (b) fraction b, (c) fraction c, and (d) fraction d. Ribosomes were prepared by the method of Kurland (1966).

Countercurrent distribution resolves methionine-starved $tRNA^{Phe}$ into four peaks (I, II, III, and IV) which correspond to the four Phe-tRNA peaks (a, b, c, and d) obtained by chromatography on MASA columns (Fleissner, 1967).

Ribosome-Dependent Binding Studies. Traces of methylated albumin were eluted with the MASA and MAK column fractions during the course of chromatography. This phenomenon was observed even with columns which had been used repeatedly. The presence of this basic polycation gave spurious and unreproducible results in experiments on the coding properties of Phe-tRNA. When the MASA fractions were treated with phenol alone in an attempt to remove the methylated albumin, the relative binding to poly U,C to that found for poly U varied between 42 and 170%. This binding ratio decreased considerably following proteolytic treatment of the fractions with pronase and reextraction with phenol (Figure 3). Moreover, there was very little variation from one experiment to another following pronase treatment. This procedure appeared to remove the tightly bound contaminating basic protein material and its miscoding effect. This was demonstrated by the similarity in response (Table I) between the MASA column treated fractions (Figure 4) and the Phe-tRNA fractions obtained by countercurrent distribution (Figure 5). The latter fractions had had no contact with methylated albumin. It should be noted that proteolysis with pronase was accompanied by aminoacyl-tRNA hydrolysis, which necessitated phenol extraction, isolation of the tRNA, and reacylation.

The efficiency of binding of the four MASA column Phe-tRNA fractions was examined (Table I). Peak a (methyl deficient) was found to be less efficient in binding to poly U or poly U,C than peak d (normal). In addition, the binding responses of peak b and c were higher than that of peak a. This difference in efficiency was somewhat more pronounced with poly U,C than poly U, thereby reducing slightly the apparent poly U,C:poly U response to a value of 9% (Figure 4). Similar coding responses were obtained with the four Phe-tRNA fractions separated by countercurrent distribution (Figure 5).

The poly U,C:poly U ribosomal binding ratio was high for both MAK column Phe-tRNA peaks (Table I). However, these results may have been spurious since it was observed that the binding efficiency to poly U,C was higher in the MAK

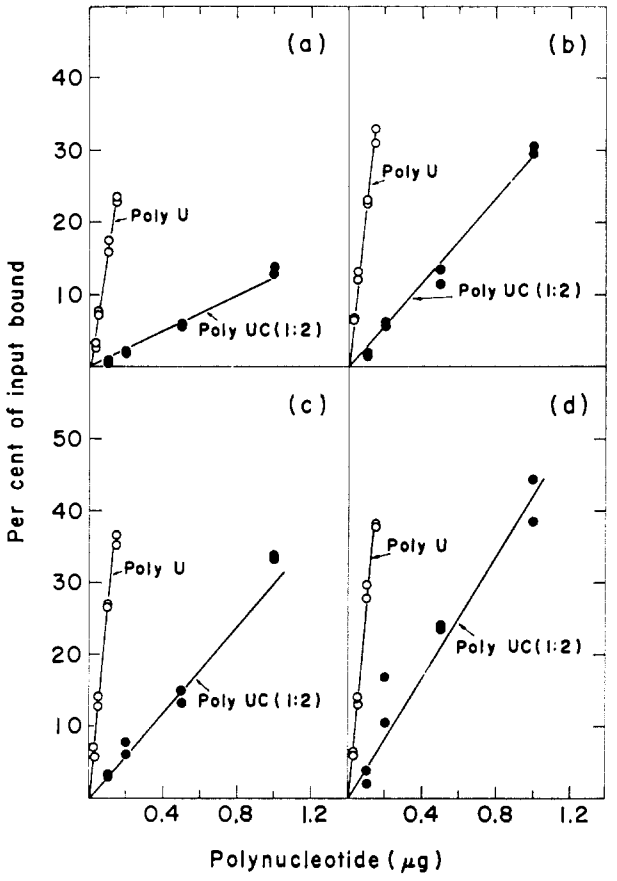

FIGURE 5: Millipore filter binding assays with Phe-tRNA fractions taken from countercurrent distribution. Results are plotted as per cent of input Phe-tRNA bound to ribosomes as a function of concentration of the indicated polymers, poly U or poly U,C, (1:2). Countercurrent distribution of *E. coli* K_{12} W6 methionine-starved tRNA was performed as described by Fleissner (1967). Phenylalanine acceptance for each of the fractions taken from countercurrent were, for fraction I, II, III, and IV, respectively, 0.48, 1.00, 1.00, and 2.30 mμmoles per mg of tRNA. The μμmoles of phe-tRNA added to each reaction mixture ranged from 3.7 to 4.4. The magnesium concentration was 0.022 M and *E. coli* Q-13 ribosomes prepared as described by Kurland (1966) were used in the assay. (a) Fraction I, (b) fraction II, (c) fraction III, and (d) fraction IV.

column fractions than in unfractionated normal and methionine-starved Phe-tRNA. It may be that a column contaminant was enhancing binding of Phe-tRNA to poly U,C. It should be noted that the tRNA derived from MAK column fractions was less than 0.1 mg. Although this material underwent proteolytic treatment with pronase to remove methylated albumin, it is conceivable that traces of this basic protein were still causing an increased response to poly U,C. Fractions from MASA columns contained relatively lower amounts of methylated albumin and contained more tRNA which was easier to carry through the manipulations of proteolytic digestion and extraction with phenol.

We attempted to repeat our earlier results (Revel and Littauer, 1966) on the binding and transfer properties of MAK fraction I and III. We found that the relative poly U,C: poly U response in the binding assay varied from 10 to 36% for both fractions. We were unable to decrease these variations from experiment to experiment. In any event, there was no indication to lead us to believe that fraction I had a higher binding ability to poly U,C than fraction III. It is therefore concluded that the results obtained with MAK column fractions at that time were probably due to methylated albumin contamination.

The ribosomal binding response and the efficiency of binding were examined at a number of Mg^{2+} concentrations (Table II). Increasing the Mg^{2+} ion concentration elevated the binding of Phe-tRNA to poly U but had very little effect on binding to poly U,C. Thus, with unfractionated normal Phe-tRNA the binding to poly U increased from 9 to 37% as the Mg^{2+} concentration increased from 10 to 30 mM. A similar effect was observed with unfractionated methionine-starved Phe-tRNA, but with this preparation the efficiency of binding remained consistently lower, throughout the range of Mg^{2+} concentrations. The increased binding to poly U affected, therefore, the poly UC: poly U ratio, which decreased from 22 to 7% for both the normal and methionine-starved Phe-tRNA species.

Streptomycin is a known miscoding agent (Davies *et al.*, 1964). The effect of streptomycin on the coding fidelity of normal and methionine-starved Phe-tRNA was compared. At 22 mM Mg^{2+}, streptomycin (0.12–1.2%) had no effect on the relative poly U,C: poly U binding ratio. Moreover, at the concentration tested methionine-starved Phe-tRNA was less efficient (70%) than normal Phe-tRNA.

Comparison of Ribosomes. To test whether ribosomes could participate in the fidelity of the reading of the genetic message, we tested various preparations of ribosomes in measuring the binding response of the MASA Phe-tRNA fractions. Ribosomes were isolated from 100,000*g* supernatant of an *E. coli* B extract preincubated with puromycin (Gilbert, 1963), from *E. coli* MRE-600 cells as described by Nirenberg (1963), or from the ammonium sulfate washed ribosomes of *E. coli* Q-13 (Kurland, 1966). In each case similar results were obtained. It should be noted that the more thoroughly washed ribosomes prepared by the Kurland (1966) method were superior in reproducibility and in efficiency of binding.

The Incorporation of Phenylalanine with Various tRNA Fractions into Polyphenylalanine. The participation of normal and methyl-deficient $tRNA^{Phe}$ fractions in ribosome-mediated transfer of phenylalanine into poly phenylalanine was studied. The assay system consisted of labeled phenylalanine, tRNA, and puromycin-treated ribosomes supplemented with poly U or poly U,C (1:1). Streptomycin-treated supernatant served as the enzyme source; this system was dependent on the addition of tRNA. The synthesis of polyphenylalanine was measured as incorporation of [^{14}C]phenylalanine into hot trichloroacetic acid precipitatable material. Table III summarizes the results of these experiments. No major difference could be detected in the response to the two polymers between normal and unfractionated methionine-starved tRNA, nor between the four peaks of tRNA from the MASA column or from the countercurrent distribution fractionation. The tRNA recovered after MASA column chromatography showed a slightly lower transfer activity. The decrease affected the various MASA tRNA fractions equally and did not appear to effect the ratio of response to poly U,C (1:1) over that found for poly U.

In addition, transfer assays were repeated in the presence of a range of Mg^{2+} concentrations (0.0047–0.0177 M). No marked differences were observed among the four MASA

TABLE II: Effect of Magnesium Ions on the Binding Properties of Normal and Methionine-Starved Phe-tRNA.[a]

Source of Phe-tRNA	Mg^{2+} Concn (mM)	% Input Bound/0.1 μg of Polynucleotide		Poly U,C:Poly U × 100	% Rel Binding Compared with Normal tRNA	
		Poly U	Poly U,C		Poly U	Poly U,C
Unfractionated methionine-starved tRNA	10	5.7	1.25	21.9	61.3	63.1
	16.6	14.4	2.05	14.2	81.3	90.7
	22	20.7	2.05	9.9	74.2	69.7
	30	29.0	2.05	7.1	78.4	75.9
Unfractionated normal tRNA	10	9.3	1.98	21.3		
	16.6	17.7	2.26	12.8		
	22	27.9	2.94	10.5		
	30	37.0	2.70	7.3		

[a] The same procedure was used as described in Table I except the magnesium ion concentration was varied as described in column two. The tRNA samples were isolated from *E. coli* G-15 cells.

TABLE III: Incorporation of Phenylalanine with Various tRNA Fractions into Polyphenylalanine.[a]

Source of tRNA	tRNA		Incorporation Dependent upon (μμmoles)		Poly UC:Poly U × 100
	Input (μg)	Sp Act. (μμmoles/μg)	Poly U	Poly U,C	
MASA a	0.43	0.45	1.7	0.93	55
b	0.59	0.29	2.0	1.4	70
c	0.52	0.55	1.6	1.1	69
d	0.435	0.82	2.2	1.4	64
Countercurrent distribution 1	0.64	0.48	4.2	2.0	48
2	0.33	1.00	2.4	1.6	67
3	0.32	1.00	2.0	1.5	75
4	0.168	2.00	2.4	1.9	79
MAK I	0.51	0.82	1.6	0.77	48
III	0.41	0.94	1.8	1.2	67
Unfractionated methionine-starved tRNA	0.50	0.68	3.4	2.1	62
Unfractionated normal tRNA	0.50	0.76	3.9	2.7	69

[a] The amount of tRNA in the assay mixture is shown in column 2. The tRNA concentrations are expressed as their capacity to accept phenylalanine (micromicromoles per microgram of RNA) and shown in column three. The amount of phenylalanine incorporated into hot 5% trichloroacetic acid insoluble material in the presence of poly U or poly U,C is shown in columns 4 and 5, and the ratios of this incorporation in column 6. The reaction mixture for polyphenylalanine formation (0.1 ml) contained 10 μmoles of Tris-HCl buffer (pH 7.5), 36 mμmoles of ATP, 15 mμmoles of GTP, 250 mμmoles of ammonium phosphoenolpyruvate, 2 μg of pyruvate kinase, 0.33 mμmole of L-[^{14}C]phenylalanine (300 μCi/μmole), 90 mμmoles of mercaptoethanol, and 14 μg of ribosomes (*E. coli* B 70S ribosomes isolated by sucrose gradient centrifugation from a crude extract preincubated for 10 min with 50 μg/ml of puromycin at 37° (Gilbert, 1963)), tRNA as indicated, 3.6 μg of poly U or 12.5 μg of poly U,C (1:1), and 37 μg of protein of *E. coli* B streptomycin supernatant (Daniel and Littauer, 1965). The final concentration of Mg^{2+} and NH^{4+} was adjusted with $MgCl_2$ and ammonium acetate to 17.7 and 179 mM, respectively. The reaction mixtures were incubated 60 min at 30°; 6.0 ml of 5% trichloroacetic acid and 1.0 mg of albumin were added. The precipitates were washed twice with cold trichloroacetic acid, suspended for 15 min at 90° in 5% trichloroacetic acid, filtered onto Millipore filters, and counted. The data presented were obtained after substraction of the control values in the absence of tRNA (1 μμmole of polyphenylalanine). For the MASA column and countercurrent distribution fractionation methionine-starved tRNA was isolated from *E. coli* A-19 and *E. coli* $K_{12}W6$ cells, respectively. For MAK column fractionation *E. coli* G-15 methionine-starved tRNA was used. The same bacterial strain was used for the isolation of unfractionated normal and methionine-starved tRNA.

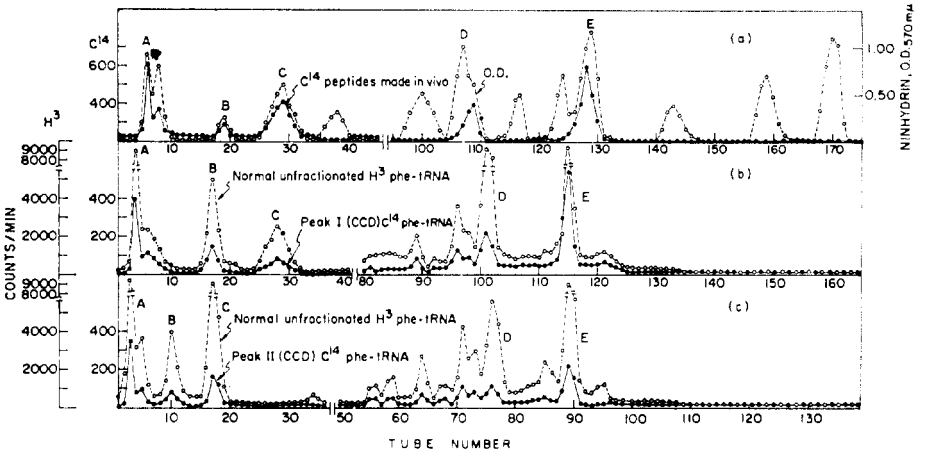

FIGURE 6: Elution profile of peptides derived from tryptic digests of rabbit hemoglobin α chain on columns of Dowex 50 (H^+). Hemoglobin was synthesized and labeled *in vivo* (a) and *in vitro* (b and c). The labeled hemoglobin was synthesized in intact reticulocytes (*in vivo*) in the presence of [^{14}C]phenylalanine and isolated, and the chains were separated according to Dintzis (1961). The α chain was digested with trypsin and chromatographed on Dowex 50 (H^+) column (Gonano, 1967). Fractions of 5 ml were collected and aliquots of 0.5 ml were assayed for ninhydrin. Fractions of 3 ml were dried in counting vials and redissolved with 0.2 ml of Hyamine, and 15 ml of a standard toluene scintillation mixture was added (a). (○—○) Ninhydrin and (●—●) [^{14}C]phenylalanine. In the radioactive profiles of tryptic digests of rabbit hemoglobin α chains labeled *in vitro* (b and c), label was incorporated into hemoglobin using [^{3}H]phenylalanine attached to normal unfractionated Phe-tRNA of *E. coli* (○—○) and [^{14}C]phenylalanine attached to the methyl-deficient Phe-tRNA of peak I from countercurrent distribution b and the methyl-deficient Phe-tRNA of peak II (c), (●—●). The rabbit reticulocytes cell-free system was prepared according to Gonano (1967) with some modification. For the transfer of labeled aminoacyl-tRNA into hemoglobin a 3-ml reaction mixture was used containing 30 μmoles of $MgCl_2$, 6 μmoles of [^{12}C]phenylalanine, 1 μmole each of the 19 amino acids (minus phenylalanine), "pH 5" pellet 200–300 A_{260} units (Gonano, 1967), 300 μmoles of Tris-HCl buffer (pH 7.4), 300 μmoles of KCl, 3 μmoles of GTP, 6 μmoles of ATP, 40 μmoles of mercaptoethanol, 2 μmoles of phosphoenolpyruvate, 100 μg of pyruvate kinase, 140 μg of rabbit tRNA and in (b) unfractionated normal [^{3}H]Phe-tRNA containing 650,000 cpm and [^{14}C]Phe-tRNA from countercurrent distribution I 30,800 cpm and in (c) unfractionated normal [^{3}H]Phe-tRNA containing 475,000 cpm and [^{14}C]Phe-tRNA from countercurrent distribution II 8460 cpm. The reaction was followed by removing 0.05-ml aliquots at 0, 5, 15, and 30 min for assay of radioactive material in protein; 20–25% of the radioactive label had been inserted into peptide linkage by 30 min in each case. After 30 min at 37° the mixture was acidified to pH 5 and the precipitate was removed. Hemoglobin was isolated from the supernatant, and the α chain was separated from the β chains and further treated as for part a. Fractions of 5 ml were collected and aliquots of 3 ml were counted in a scintillation counter. (^{3}H and ^{14}C incorporations in counts per minute are plotted in the figure. The peaks are lettered according to the phenylalanine-containing peaks synthesized *in vivo* in (a). The chemical identification of the phenylalanine-containing peptides is reported in Table IV. In (b) and (c) the ninhydrin profile was omitted for clarity, and is similar to (a).

column fractions, countercurrent distribution fractions, MAK column fractions, or between unfractionated normal and methionine-starved Phe-tRNA.

Transfer of Phenylalanine from Phe-tRNA Fractions into Specific Peptides of Hemoglobin. A rabbit reticulocyte cell-free system was used to test whether differences in the specificity of the various normal and methyl-deficient Phe-tRNA fractions could be observed in the synthesis of proteins from a natural messenger.

In a preliminary experiment, rabbit hemoglobin was labeled uniformly with [^{14}C]phenylalanine in intact reticulocytes. Figure 6a demonstrates the separation of the tryptic peptides of the α chain of rabbit hemoglobin on a Dowex 50 column. Five [^{14}C]phenylalanine-containing peptides (A, B, C, D, and E) were observed (Table IV).

In order to test the specificity of the normal and methyl-deficient Phe-tRNA fractions, *in vitro* incorporation experiments were carried out and the phenylalanine peptides were analyzed for radioactivity. To ensure identical conditions for comparison, fully methylated normal unfractionated [^{3}H]Phe-tRNA was used as an internal standard by mixing it with (1) [^{14}C]Phe-tRNA of peak I from countercurrent distribution and (2) with [^{14}C]Phe-tRNA of peak II from countercurrent distribution. Each mixture was incubated and analyzed separately. Comparison of the results in Figure 6a with that of 6b,c shows that all five phenylalanine-containing peptides are labeled with ^{3}H derived from normal unfractionated [^{3}H]Phe-tRNA. Figure 6b,c also demonstrates that the methyl-deficient [^{14}C]Phe-tRNA countercurrent distribution fractions (I and II) were able to incorporate phenylalanine into all the potential peptides. It was found that countercurrent distribution fractions of Phe-tRNA inserted phenyl-

TABLE IV: Chemical Identification of the Phenylalanine-Containing Peptides of the α Chain of Rabbit Hemoglobin.

Peak[a]	Tryptic Peptide[b]
A	αT_{14}
B	αT_{12}
C	αT_5
D	αT_{6a}
E	αT_{6b}

[a] Peak letters refer to the letters of Figure 6a, indicating the peaks of tryptic peptides containing radioactive phenylalanine synthesized *in vivo* using intact rabbit reticulocytes and eluted from the Dowex 50 column. [b] Tryptic peptides derived from the α chain of human hemoglobin according to Baglioni (1961) and von Ehrenstein (1966).

alanine more efficiently into hemoglobin peptides than the MASA column fractions. This suggested that contaminating interfering material from the MASA column might still be present in these fractions despite pronase and phenol treatments of these tRNAs. It should be noted that a high level of unlabeled phenylalanine was present in the rabbit hemoglobin-synthesizing system, to diminish possible back-reaction to free amino acid and mixing of radioactive labels and subsequent reincorporation. In addition, it is unlikely that such a reaction was occurring since heterologous mammalian phenylalanyl-tRNA synthetase is unable to recognize $tRNA^{Phe}$ from *E. coli* (Doctor and Mudd, 1963). For the same reason enzymatic exchange of phenylalanine between the Phe-tRNAs (without going through the amino acid pool) is excluded. In Figure 6, the small peaks preceding peak D in (b) and (c) may be due to contamination with the β chain due to imperfect chain separation. It is important to point out that the ^{3}H and ^{14}C peaks are symmetric in each case and that no significant difference can be demonstrated.

Discussion

In an earlier publication it was reported (Revel and Littauer, 1966) that in the binding and transfer assays, the relative response to poly U,C over that for poly U was higher with methyl-deficient Phe-tRNA (fraction 1 from a MAK column) that with normal Phe-tRNA (fraction III from a MAK column). These results differ from those obtained in the present report using MASA and countercurrent distribution fractions. The variable results obtained with MAK column tRNA fractions were now shown to arise from methylated albumin contamination. Our present results indicate that methyl-deficient and normal $tRNA^{Phe}$ could not be distinguished in their relative response to poly U,C over that for poly U in the ribosome binding assay and in the transfer assay. These results agree with those of Fleissner (1967). It should be noted that the random polymer poly U,C was used. Therefore, it cannot be determined which of the possible U and C containing ordered triplets elicited the observed response.

In the transfer assay no difference could be detected over a wide range of Mg^{2+} concentrations. Similarly, in the *in vitro* hemoglobin-synthesizing system no difference could be detected between the normal and the methyl-deficient Phe-tRNA in their ability to insert phenylalanine into hemoglobin peptides. The polyphenylalanine and hemoglobin assays can only detect gross differences between normal and methyl-deficient Phe-tRNA. On the other hand, the binding reaction of Phe-tRNA to a polynucleotide ribosome complex seems to be a more sensitive assay. Indeed, the only difference which we were consistently able to detect was in the efficiency of binding which was much lower in the case of methyl-deficient Phe-tRNA. The efficiency of binding was more than twice as high for normal Phe-tRNA than that found for peak a of the MASA column, or peak I of the countercurrent distribution. This same observation was made by Fleissner (1967).

The sequence of $tRNA^{Phe}$ from *E. coli* has been recently elucidated by Barrel and Sanger (1969). The $tRNA^{Phe}$ was found to contain three methylated nucleotides: ribosylthymidylic acid, 7-methylguanylic acid, and 2-thiomethyl-6-isopentenyladenylic acid. The modified adenylic acid is adjacent to the anticodon and may well be important for efficient binding to mRNA–ribosome complexes. This assumption is supported by the studies of Gefter and Russell (1969) on su^{+}_{III} $tRNA^{Tyr}$ which also contains 2-thiomethyl-6-isopentenyladenylic acid near the anticodon. It was found by these authors that a species of su^{+}_{III} $tRNA^{Tyr}$ that has an unmodified residue of adenylic acid adjacent to the anticodon shows a reduction in its ability to support *in vitro* protein synthesis and is defective in its ability to bind to ribosomes. It is therefore possible that the marked reduction in binding of methyl-deficient $tRNA^{Phe}$ to a polynucleotide–ribosome–complex found in the present studies may arise from a lack of methylation of the 2-thio-6-isopentenyladenylic acid residue. On the basis of MASA and polyacrylamide chromatography it was suggested (Littauer and Stern, 1967; Stern *et al.*, 1969) that aminoacylated $tRNA^{Phe}$ has a different configuration from unacylated $tRNA^{Phe}$. It was also indicated that the conformation of tRNA plays a role in the resolution of tRNAs on MASA column chromatography. The observation that methyl-deficient Phe-tRNA has a profoundly different profile on this column from normal Phe-tRNA suggests that methylation may play a role in stabilizing the integrity of the normal aminoacylated $tRNA^{Phe}$ configuration. The postulated effect of methylated bases on the configuration of Phe-tRNA may in turn be related to the lowered binding efficiency found in the present investigation.

The role of the methylated bases in tRNA has been studied in several laboratories and reviewed recently (Littauer and Revel, 1966). A requirement for the normal complement of methylated bases in tRNA for specific codon recognition has been demonstrated in the case of Leu-tRNA from *E. coli* (Capra and Peterkofsky, 1968). Recent data by Shugart *et al.* (1968) indicate that the initial velocities of tRNA aminoacylation are much lower for the methyl-deficient $tRNA^{Phe}$ species than for the normal species. In one other case has the aminoacyl-tRNA synthetase been implicated as important in relation to the full complement of methyl groups of tRNA. A heterologous leucyl-tRNA synthetase from yeast is able to distinguish between normal and methyl-deficient *E. coli* $tRNA^{Leu}$ (Peterkofsky, 1964). It may emerge finally that no single function of the methyl groups of tRNA will be revealed. Depending upon their nature and their position in the tRNA chain the role of the methyl groups will be found to differ from one species of tRNA to another, and in each case will cause subtle modifications of one or another of the functions of tRNA in protein synthesis, kinetics of aminoacylation, efficiency of binding to ribosomes, or alterations in coding response.

Acknowledgment

The excellent technical assistance of Mrs. Louise Ellman-Zutra and Mr. David Giveon is gratefully acknowledged.

References

Baglioni, C. (1961), *Biochim. Biophys. Acta 48*, 392.

Barrel, B. G., and Sanger, F. (1969), *Fed. European Biochem. Soc. Letters 3*, 275.

Capra, J. D., and Peterkofsky, A. (1968), *J. Mol. Biol. 33*, 591.

Chen, P. S., Jr., Toribara, T. T., and Warner, H. (1956), *Anal. Chem. 28*, 1756.

Daniel, V., and Littauer, U. Z. (1965), *J. Mol. Biol. 11*, 692.
Davies, J., Gilbert, W., and Gorini, L. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 883.
Dintzis, H. M. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 247.
Doctor, B. P., and Mudd, J. A. (1963), *J. Biol. Chem. 238*, 3677.
Fleissner, E. (1967), *Biochemistry 6*, 621.
Gefter, M. L., and Russell, R. L. (1969), *J. Mol. Biol. 39*, 145.
Gilbert, W. (1963), *J. Mol. Biol. 6*, 374.
Gonano, F. (1967), *Biochemistry 6*, 977.
Jacobson, K. B. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 719.
Jones, R. T. (1964), *Cold Spring Harbor Symp. Quant. Biol. 29*, 297.
Kurland, C. G. (1966), *J. Mol. Biol. 18*, 90.
Leder, P. (1968), *Methods Enzymol. 12*, 725.
Littauer, U. Z., and Milbauer, R. (1965), *in* Symposia on RNA Structure and Function, Tuppy, H., Ed., New York, N. Y., Pergamon, p 9.
Littauer, U. Z., and Revel, M. (1966), *in* Symposium on Genetic Elements, Properties and Function, Shugar, D., Ed., New York, N. Y., Academic, p 315.
Littauer, U. Z., Revel, M., and Stern, R. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 501.
Littauer, U. Z., and Stern, R. (1967), *in* Symposium on Structure and Function of Transfer RNA and 5S-RNA, Zachau, H. G., Organizer, Universitetsforlaget, Oslo, Federation of European Biochemical Societies, p 93.
Mandell, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1*, 66.
Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 907.
Nirenberg, M. W. (1963), *Methods Enzymol. 6*, 17.
Nirenberg, M. W., and Leder, P. (1964), *Science 145*, 1399.
Peterkofsky, A. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1233.
Revel, M., and Littauer, U. Z. (1965), *Biochem. Biophys. Res. Commun. 20*, 187.
Revel, M., and Littauer, U. Z. (1966), *J. Mol. Biol. 15*, 389.
Shugart, L., Novelli, G. D., and Stulberg, M. P. (1968), *Biochim. Biophys. Acta 157*, 83.
Singer, M. F., Jones, O. W., and Nirenberg, M. W. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 392.
Stern, R., Ellman-Zutra, L., and Littauer, U. Z. (1969), *Biochemistry 8*, 313.
Stern, R., and Littauer, U. Z. (1968), *Biochemistry 7*, 3469.
von Ehrenstein, G. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 705.
Weisblum, B., Gonano, F., von Ehrenstein, G., and Benzer, S. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 328.
Yamane, T., and Sueoka, N. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 1093.