Polyuridylic Acid Directed Binding of Phenylalanyl Transfer Ribonucleic Acid to Mammalian 40S Ribosomal Subunits*

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ABSTRACT: In the presence of polyuridylic acid, 40S ribosomal subunits from liver bind Phe-tRNA; 60S ribosomal subunits do not. Magnesium is required for Phe-tRNA to bind to 40S subunits, but not guanosine triphosphate or supernatant proteins. In most respects, binding of Phe-tRNA to mammalian 40S subunits was similar to that which has been reported to occur with bacterial 30S subunits, except that binding took place at 0° and was inhibited by sodium but not by tetracycline. The addition of 60S subunits to 40S subunits resulted in a threefold increase in Phe-tRNA binding and the formation of a small amount of di- and triphenylalanine.

Bacterial 30S ribosomal subunits bind synthetic polyribonucleotides and mRNA (Takanami and Okamoto, 1963); moreover, 30S subunits will bind the AA-tRNA specified by the template RNA (Suzuka *et al.*, 1965; Pestka and Nirenberg, 1966). In similar circumstances, AA-tRNAs do not bind to 50S subunits (Pestka and Nirenberg, 1966). However, the latter are essential for peptide-bond formation and indeed the peptide-bond-forming enzyme is an integral part of the large subunits (Monro, 1967).

Mammalian ribosomes will also bind AA-tRNA in the presence of a RNA template (Arlinghaus *et al.*, 1963). However, the subunit responsible had not been determined because there was no means of preparing active mammalian ribosomal subunits before Martin and Wool (1968) described a procedure. We have taken advantage of the method to study the role of ribosomal subunits in binding of AA-tRNA. We report here that the 40S subunit, in the presence of a RNA template, will bind the AA-tRNA specified by the polynucleotide.

Materials and Methods

Chemicals and Isotopes. Poly U was purchased from Miles Laboratories; *Escherichia coli* B tRNA and poly A from Schwarz BioResearch; [^{3}H]phenylalanine (5 Ci/mmole) and [^{3}H]lysine (3.96 Ci/mmole) from New England Nuclear; diphenylalanine and triphenylalanine from Cyclo Chemicals. Prior to use, reagent grade sucrose was treated with Norit A to remove material that absorbed in the ultraviolet region (Stirewalt *et al.*, 1967).

Preparation of Ribosomes and Ribosomal Subunits. Ribosomes were prepared from the livers of 150-g male Sprague–Dawley rats as described by Martin and Wool (1969).

Ribosomes were incubated with puromycin to remove nascent peptide and endogenous mRNA (Martin *et al.*, 1970) and then dissociated into subunits (Martin and Wool, 1968). The subunits (40 and 60 S) were separated by centrifugation on sucrose gradients in a Spinco SW-27 or Ti-15 rotor; the procedures are described in detail in a separate publication (Martin *et al.*, 1970). The 40S subunits were dialyzed overnight at 4° against 200 volumes of 0.01 M Tris-HCl (pH 7.5), 0.08 M KCl, and 0.005 M $MgCl_2$. The 40S subunits were stable in this buffer for at least 2 weeks if kept at 4°. The purity of the 40S subunits was estimated after analysis of the fraction itself on linear sucrose gradients and of the RNA extracted from the particles (Martin *et al.*, 1970); contamination with 60S subunits was generally less than 5%.

Preparation of [^{3}H]AA-tRNA. E. coli B tRNA was aminoacylated with [^{3}H]phenylalanine or [^{3}H]lysine and 19 nonradioactive amino acids (Wool and Cavicchi, 1967). The specific activities of preparations of [^{3}H]Phe-tRNA[1] varied from 390 to 805 cpm per μg of tRNA; the preparation used in each experiment is given in the figure captions. The specific activity of the [^{3}H]Lys-tRNA was 350 cpm/μg of tRNA. To estimate the concentration of tRNA, we assumed 1 mg to be equal to 22 A_{260} units at pH 7.0.

Determination of Binding of [^{3}H]Phe-tRNA to Ribosomal Subunits by Sucrose Gradient Centrifugation. Unless an exception is noted, the reaction mixture (0.5 ml) contained 0.01 M Tris-HCl (pH 7.5), 0.08 M KCl, and 0.0125 M $MgCl_2$ (buffer A), to which was added [^{3}H]Phe-tRNA, ribosomal subunits, and, where indicated, poly U. After incubation, the mixture was layered on to a 4.8-ml linear 15–30% sucrose gradient in buffer A and centrifuged at 60,000 rpm in a Spinco SW-65 rotor. The duration and temperature of centrifugation are given in the figure captions. After centrifugation, the contents of the tube were pumped through an ISCO Model UA-2 ultraviolet analyzer and 0.15-ml fractions were collected in glass vials. After adding a Triton–toluene

* From the Departments of Medicine, Biochemistry, and Physiology, University of Chicago, Chicago, Illinois 60637. *Received December 29, 1969.* The expenses of the research were met by a U. S. Public Health Service General Research support grant and an American Cancer Society Institutional award to the University of Chicago (J. J. C.) and by grants from the National Institutes of Health (AM-04842) and the John A. Hartford Foundation (I. G. W.).

[1] The abbreviations used are: [^{3}H]Phe-tRNA and [^{3}H]Lys-tRNA: unfractionated tRNA acylated with [^{3}H]phenylalanine or [^{3}H]lysine and 19 [^{1}H]amino acids. One A_{260} unit is the amount of material which would yield an absorbance of 1.0 at 260 mμ in a light path of 1.0 cm and a volume of 1.0 ml.

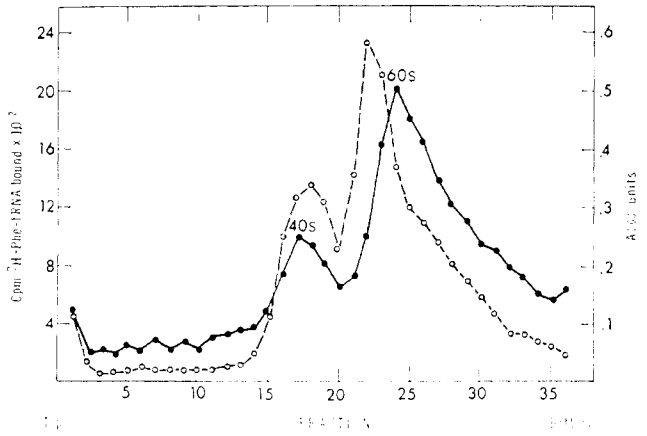

FIGURE 1: Binding of [^{3}H]Phe-tRNA to ribosomal subunits. Liver ribosomes (19.5 A_{260} units) were layered on a 4.8-ml linear 10–30% sucrose gradient in 0.05 M Tris-HCl (pH 7.5), 0.880 M KCl, 0.0125 M $MgCl_2$, 0.02 M 2-mercaptoethanol, and centrifuged at 50,000 rpm for 60 min at 28°. Fractions (0.12 ml) were collected and a portion (approximately 0.02 ml) was used to measure the absorbancy at 260 mμ; another portion (0.05 ml) was diluted to give a concentration of 0.01 M Tris-HCl (pH 7.5), 0.08 M KCl, 0.02 M $MgCl_2$ in a final volume of 1.0 ml, and 40 μg of [^{3}H]Phe-tRNA (805 cpm/μg) and 7.5 μg of poly U were added. An equal portion was treated in an identical manner except for the omission of poly U. The mixtures were incubated for 30 min at 30° then collected on nitrocellulose filters. The [^{3}H]Phe-tRNA bound in the absence of poly U was subtracted from the [^{3}H]Phe-tRNA bound in the presence of poly U. (●—●) Absorbancy at 260 mμ; (○---○) counts per minute [^{3}H]Phe-tRNA bound. Each 100 cpm is equivalent to 0.054 pmole of phenylalanine.

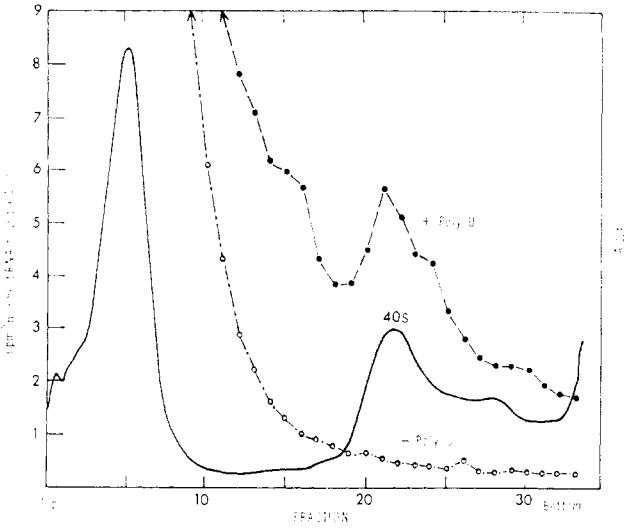

FIGURE 2: Binding of [^{3}H]Phe-tRNA to 40S ribosomal subunits. 40S subunits (3.9 A_{260} units) were incubated in buffer A with 80 μg of [^{3}H]Phe-tRNA (805 cpm/μg) and with or without 50 μg of poly U. After incubation for 15 min at 30°, the mixture was layered on a sucrose gradient. Centrifugation was for 2 hr at 4° and fractions were collected and analyzed. The material with absorbancy at 254 mμ at the top of the gradient is poly U and tRNA. (——) Absorbancy at 254 mμ; (●—●) counts per minute [^{3}H]Phe-tRNA in the presence of poly U; (○-·-○) counts per minute of [^{3}H]Phe-tRNA in the absence of poly U. Each 100 cpm is equivalent to 0.043 pmole of phenylalanine.

scintillation fluor (Patterson and Greene, 1965), the radioactivity of each fraction was determined in a Packard Tri-Carb spectrometer; the efficiency of the determination was 20%.

Determination of Binding of [^{3}H]Phe-tRNA to 40S Ribosomal Subunits on Nitrocellulose Filters. Unless an exception is noted, the reaction mixture (1.0 ml) contained 0.01 M Tris-HCl (pH 7.5), 0.08 M KCl, and 0.02 M $MgCl_2$ (buffer B), to which was added (in the amounts specified in the figure captions) poly U, [^{3}H]Phe-tRNA, and last of all 40S ribosomal subunits. Generally, incubation was for 30 min at 30°. After incubation, the tubes were placed in ice, diluted with 3 ml of ice-cold buffer B, poured quickly onto a nitrocellulose filter (Millipore filter; HAWP, 25 mm diameter, 0.45 μ pore size), and washed as described by Nirenberg and Leder (1964). The filters were dried in glass vials and, after addition of toluene scintillation fluid (4 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per l. of toluene), the radioactivity was measured in a Packard Tri-Carb spectrometer; the efficiency of the measurement was 16%. For each experiment, an identical assay was carried out without poly U; a correction was made for the radioactivity retained on the filters in the control. All values are the average of duplicate assays.

Determination of the Product Formed As the Result of Binding of [^{3}H]Phe-tRNA to Ribosomal Particles. Binding of [^{3}H]Phe-tRNA to ribosomal particles was carried out in the presence of poly U and the complex ([^{3}H]Phe-tRNA–poly U–ribosomal particle) was collected on a nitrocellulose filter. The filter was incubated in 2.0 ml of 1.0 N NH_4OH for 30 min at 37°; the procedure was repeated twice. (At least 95% of the radioactivity was eluted from the filter.) After lyophilizing the eluate, the sample was dissolved in 0.15 ml of 0.1 N NH_4OH and carrier mono-, di-, and triphenylalanine were added. The sample was applied to Whatmann No. 3MM filter paper and descending chromatography in *n*-butyl alcohol–concentrated ammonia–water (100:3:18, v/v) was carried out (Pestka, 1968). Mono-, di-, and triphenylalanine standards, run adjacent to the sample, were located with ninhydrin (0.25% ninhydrin in acetone). The area of the chromatogram containing the sample was cut into 1-cm strips and the radioactivity was eluted from the strips into glass vials with 1.0 ml of 0.1 N NH_4OH. After neutralizing the solution with acetic acid, a Triton–toluene scintillation fluor (Patterson and Greene, 1965) was added and radioactivity was measured in a Packard Tri-Carb spectrometer; the efficiency of the determination was 15%.

Results

Identification of the Ribosomal Subunit to Which [^{3}H]Phe-tRNA Is Bound. Sedimentation of mammalian ribosomes through a sucrose gradient containing a high concentration of potassium (0.88 M) results in dissociation of the ribosomes into 40S and 60S subunits (Martin and Wool, 1968). Subunit fractions taken from a sucrose gradient (Figure 1) were tested for the ability to bind [^{3}H]Phe-tRNA in the presence or absence of poly U. The fractions containing the 40S subunit bound [^{3}H]Phe-tRNA in the presence of poly U; so too did most of the 60S fractions although binding was skewed toward the lighter portion of the peak.

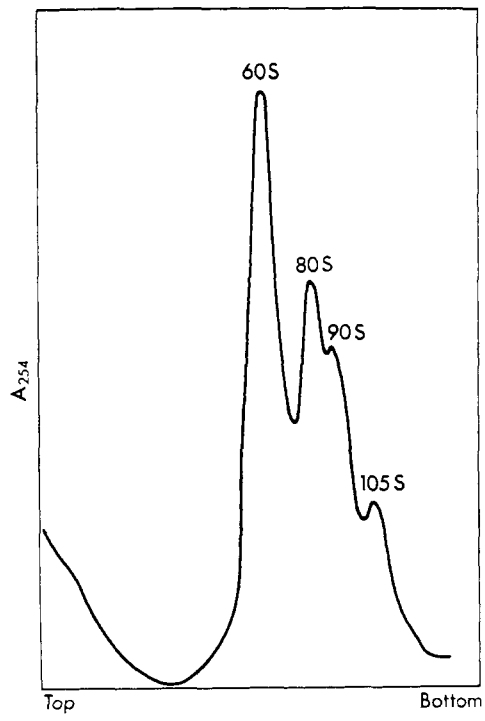

FIGURE 3: Analysis of the purity of the 60S ribosomal subunit fraction. Ribosomal particles (0.97 A_{260} unit), isolated from fractions 21–24 of a sucrose gradient similar to that shown in Figure 1, were layered on to a 4.8-ml linear 15–30% sucrose gradient in buffer A. After centrifugation at 60,000 rpm for 40 min at 28°, the contents of the tube were pumped through an ISCO Model UA-2 UV analyzer.

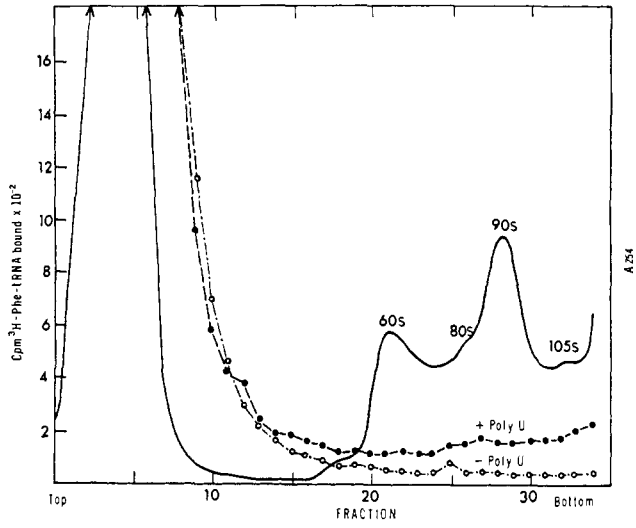

FIGURE 4: Failure of [³H]Phe-tRNA to bind to 60S ribosomal subunits. Ribosomal particles (6.8 A_{260} units) contaminated with only a small amount of 40S subunits were incubated in buffer A with 80 μg of [³H]Phe-tRNA (800 cpm/μg) and with or without 50 μg of poly U. After incubation for 15 min at 30°, the mixture was layered on a sucrose gradient. Centrifugation was for 35 min at 28° and fractions were collected and analyzed. (—) Absorbancy at 254 mμ; (●---●) counts per minute [³H]Phe-tRNA in the presence of poly U; (○-·-○) counts per minute of [³H]Phe-tRNA in the absence of poly U. Each 100 cpm is equivalent to 0.043 pmole of phenylalanine.

In a separate experiment 40S subunits were isolated and purity of the fraction was assessed by determination of the sedimentation coefficient of the RNA extracted from the particles; only 18S RNA was recovered. The 40S fraction could have been contaminated with no more than 5% of 60S subunits. The 40S subunits were incubated with [³H]Phe-tRNA and then layered on a sucrose gradient; radioactivity sedimented with the subunit provided poly U had been present in the incubation mixture (Figure 2). A component that sedimented faster than the 40S subunit also bound some [³H]Phe-tRNA; however, when we analyzed the material it too contained only 18S RNA. Moreover, the component does not appear if centrifugation is at 28°, it does if centrifugation is at 4° (as in Figure 2). We believe the particle to be either a compact or dimerized form of the 40S subunit which would account for its containing only 18S RNA and for its binding [³H]Phe-tRNA. A similar change in sedimentation of bacterial 30S ribosomal subunits has also been noted (Pestka and Nirenberg, 1966; Igarashi and Kaji, 1969).

We could not be certain from our first experiment whether [³H]Phe-tRNA was bound to the 60S subunit. However, isolation and analysis on a sucrose gradient of the trailing portion of the 60S peak, the portion that bound [³H]Phe-tRNA, gave evidence that it was contaminated with 40S subunits (Figure 3). The contamination was manifest in a complicated manner for no 40S subunits were actually found (Figure 3); rather the contamination was inferred from the presence of 80S ribosomes and of 105S particles. The 80S ribosomes, we presume, were formed from 40S subunits contaminating the 60S fraction because the potassium concentration used in the analytical gradient (Figure 3) was 0.08 M rather than the 0.88 M used in the original gradient

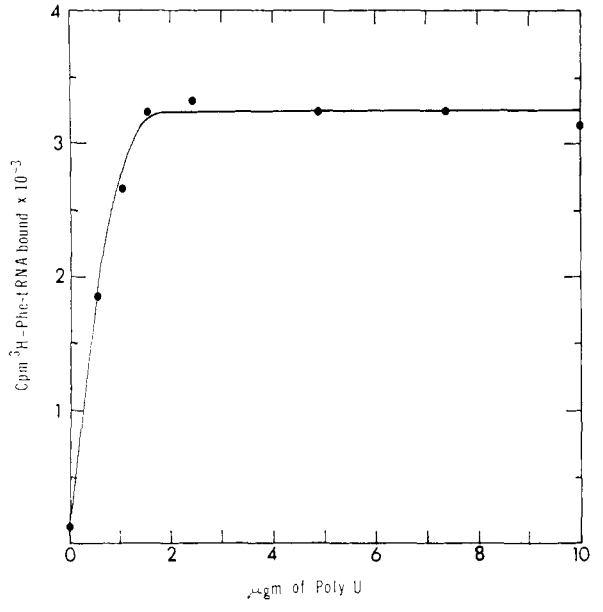

FIGURE 5: Effect of poly U concentration on binding of [³H]Phe-tRNA to 40S ribosomal subunits. 40S subunits (1.03 A_{260} units) were incubated in buffer B with 40 μg of [³H]Phe-tRNA (805 cpm/μg) and the amount of poly U indicated. Incubation was for 30 min at 30°. Each 100 cpm is equivalent to 0.054 pmole of phenylalanine.

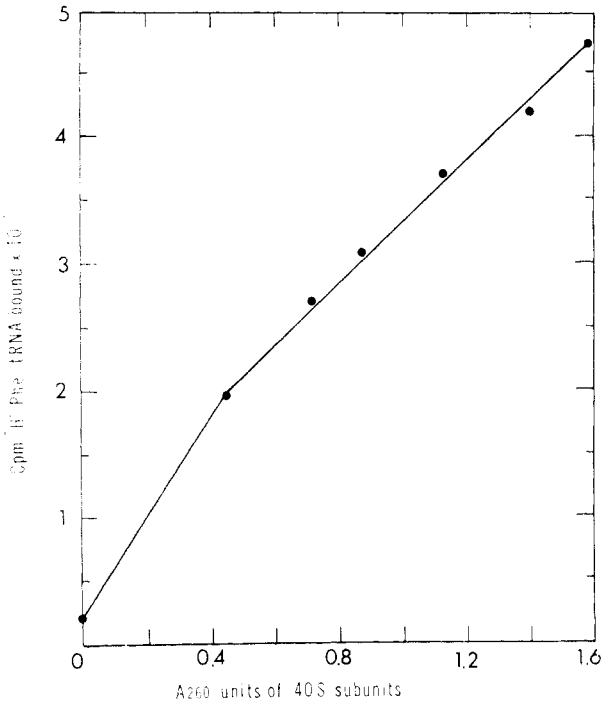

FIGURE 6: Effect of ribosome concentration on binding of [^{3}H]-Phe-tRNA to 40S ribosomal subunits. 40S subunits, in the amounts specified, were incubated in buffer B with 40 μg of [^{3}H]Phe-tRNA (640 cpm/μg) and 7.5 μg of poly U. Incubation was for 30 min at 30°. Each 100 cpm is equivalent to 0.054 pmole of phenylalanine.

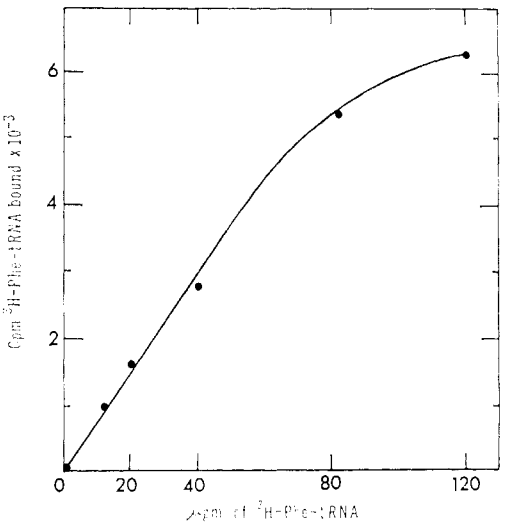

FIGURE 7: Effect of [^{3}H]Phe-tRNA concentration on binding of [^{3}H]Phe-tRNA to 40S ribosomal subunits. 40S subunits (1.03 A_{260} units) were incubated in buffer B with 7.5 μg of poly U and the concentration of [^{3}H]Phe-tRNA (805 cpm/μg) was indicated. Incubation was for 30 min at 30°. Each 100 cpm is equivalent to 0.054 pmole of phenylalanine.

(Figure 1). Ribosomes dissociate to subunits in 0.88 M potassium and reassociate at 0.08 M (Martin and Wool, 1968). The 105S particle is the result of the association of two 60S and one 40S subunit (Martin *et al.*, 1969); the 90S particle which also appears in the gradient (Figure 3) is a dimer of 60S subunits. We believe the [^{3}H]Phe-tRNA apparently bound to the 60S subunit in the first experiment (Figure 1) was in fact the result of contamination with 40S subunits. The contamination of the trailing portion of the 60S peak, to which most of the [^{3}H]Phe-tRNA was bound, most probably resulted from formation of a dimer of the 40S subunit that sediments with a coefficient of 55 in high concentrations of potassium (Martin *et al.*, 1970).

We have not been able to obtain pure 60S ribosomal subunits. However, if subunits are prepared in a zonal rotor (Spinco Ti-15), the 60S particles isolated from the portion of the 60S fraction that sediments fastest are contaminated with only small amounts of 40S subunits (Martin *et al.*, 1970). Such a preparation was preincubated with [^{3}H]Phe-tRNA and then layered on a sucrose gradient; virtually no radioactivity sedimented with the 60S subunits or the 90S particles (a dimer of the 60S subunit; Martin *et al.*, 1969) even in the presence of poly U (Figure 4).

Binding of [^{3}H]Phe-tRNA to 40S Ribosomal Subunits Measured on Nitrocellulose Filters. For convenience, the nitrocellulose filter assay (Nirenberg and Leder, 1964) was used to characterize binding of [^{3}H]Phe-tRNA to 40S subunits. The retention of AA-tRNA on nitrocellulose filters required its template RNA and 40S ribosomal subunits (Table I). In the presence of poly U, [^{3}H]Phe-tRNA was bound to 40S subunits but [^{3}H]Lys-tRNA was not. When the template was poly A, [^{3}H]Lys-tRNA was bound but not [^{3}H]Phe-tRNA.

If 1.03 A_{260} units of ribosomes were used, maximum binding of [^{3}H]Phe-tRNA to 40S subunits occurred with 1.5 μg of poly U (Figure 5). Poly U specific binding of [^{3}H]Phe-tRNA was proportional to the amount of 40S subunits over the range of 0.4–1.6 A_{260} units (Figure 6). In the circumstances of the assay (Figure 6), approximately 1.8 pmoles of [^{3}H]Phe-tRNA was bound per 70 pmoles of 40S subunits (1 A_{260} unit of 40S subunits is taken to be equal to 70 pmoles). Under identical assay conditions but with different preparations of [^{3}H]Phe-tRNA, as much as 3 pmoles of [^{3}H]Phe-

TABLE I: Polynucleotide Template Requirement for AA-tRNA to Bind to 40S Ribosomal Subunits.[a]

Incubation Mixture	cpm of [^{3}H]-Phe-tRNA Bound	cpm of [^{3}H]-Lys-tRNA Bound
Minus template	67	102
Plus poly U	2684	134
Plus poly U: minus ribosomes	52	
Plus poly A	48	230
Plus poly A: minus ribosomes		47

[a] To an incubation mixture containing buffer B were added: 0.78 A_{260} unit of 40S ribosomal subunits, 40 μg of [^{3}H]Phe-tRNA (390 cpm/μg) or 200 μg of [^{3}H]Lys-tRNA (350 cpm/μg), 7.5 μg of poly U or 12.5 μg of poly A. Incubation was for 30 min at 30°. Each 100 cpm is equivalent to 0.054 pmole of phenylalanine or 0.069 pmole of lysine.

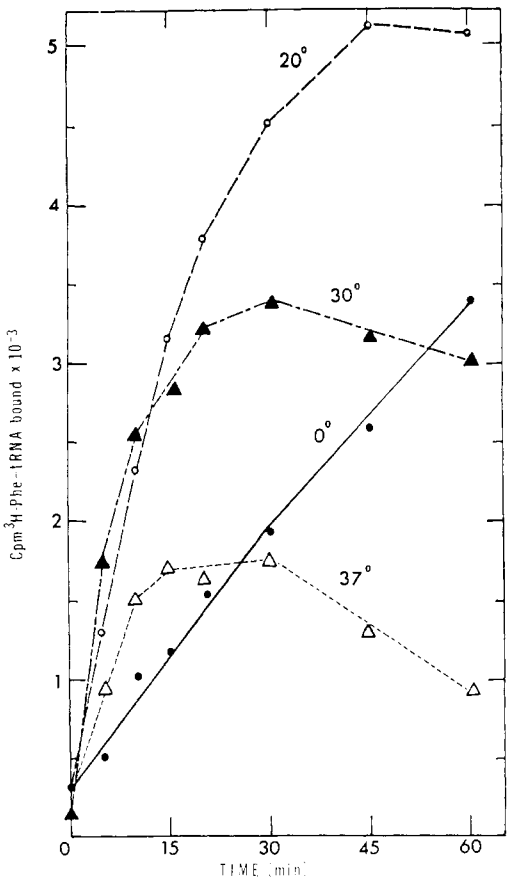

FIGURE 8: Effect of temperature on binding of [^{3}H]Phe-tRNA to 40S ribosomal subunits. 40S subunits (0.83 A_{260} unit) were incubated in buffer B with 40 μg of [^{3}H]Phe-tRNA (530 cpm/μg) and 7.5 μg of poly U. Incubation was for the time and the temperature specified. Counts per minute of [^{3}H]Phe-tRNA bound at: (●—●) 0°, (○--○) 20°, (▲-·-▲) 30°, and (△···△) 37°. Each 100 cpm is equivalent to 0.054 pmole of phenylalanine.

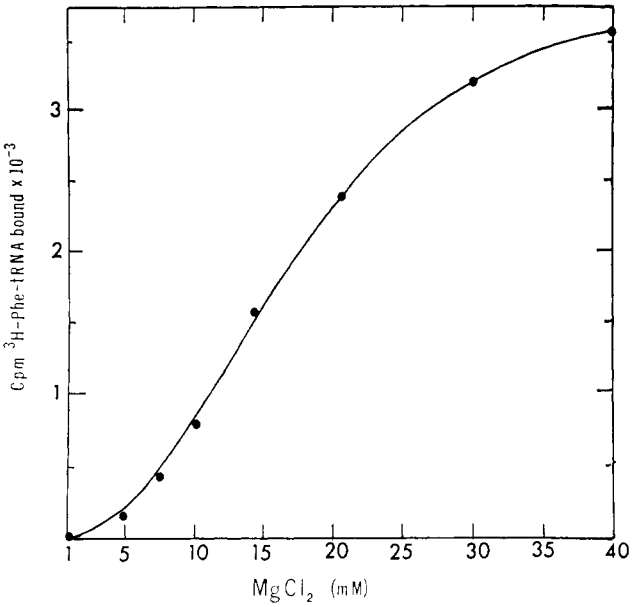

FIGURE 9: Effect of $MgCl_2$ concentration on binding of [^{3}H]Phe-tRNA to 40S ribosomal subunits. 40S subunits (0.88 A_{260} unit) were incubated with 40 μg of [^{3}H]Phe-tRNA (805 cpm/μg) and 7.5 μg of poly U in buffer containing 0.01 M Tris-HCl (pH 7.5), 0.08 M KCl, and the $MgCl_2$ concentration specified. Incubation was for 30 min at 30°. Each 100 cpm is equivalent to 0.054 pmole of phenylalanine.

tRNA was bound per 70 pmoles of 40S subunits; the variation was due to differences in degree of aminoacylation of tRNA. For any single preparation of [^{3}H]Phe-tRNA the amount bound to different preparations of 40S subunits did not vary more than 10%. The amount of [^{3}H]Phe-tRNA required for optimum binding to 1.03 A_{260} units of 40S subunits in the presence of 7.5 μg of poly U was greater than 120 μg (Figure 7). However, since binding was proportional to ribosome concentration with 40 μg of [^{3}H]Phe-tRNA (Figure 6), this was the concentration we used in most of the experiments.

We studied the effect of temperature on poly U directed binding of [^{3}H]Phe-tRNA to 40S subunits (Figure 8). Incubation for 45 min at 20° gave maximum binding of [^{3}H]Phe-tRNA. If incubation was at 30 or 37°, binding reached a peak after 30 min (albeit the amount bound was less than occurred at 20°), and decreased thereafter. Poly U directed binding of [^{3}H]Phe-tRNA to 40S subunits also occurred at 0°, indeed binding at 0° increased linearly for at least 60 min (Figure 8)—in contrast with bacterial 30S subunits which do not bind AA-tRNA at 0° (Suzuka *et al.*, 1965; Pestka and Nirenberg, 1966).

A precise evaluation of the effect of temperature is difficult because deacylation of AA-tRNA during the binding reaction increases as the temperature rises (Gatica *et al.*, 1966). The amount of AA-tRNA bound at 37° to bacterial 30S ribosomal subunits decreased as the time of incubation was extended because of deacylation (Pestka, 1967). Perhaps it is deacylation that accounts for the difference in binding at 20° and 30 or 37°, and for the decline in binding at 30 or 37° when the duration of incubation was greater than 30 min.

Magnesium was required for binding of [^{3}H]Phe-tRNA to 40S ribosomal subunits (Figure 9). Poly U directed binding increased with each increment in Mg^{2+} concentration up to the highest concentration tested (40 mM). Binding of [^{3}H]Phe-tRNA in the absence of poly U did not vary with Mg^{2+} concentration. The effect of Mg^{2+} concentration is similar to the results reported with 30S subunits from *E. coli* (Pestka and Nirenberg, 1966).

There was no absolute requirement for a monovalent cation for poly U directed binding of [^{3}H]Phe-tRNA to bacterial 30S ribosomal subunits but the addition of K^+ or NH_4^+ resulted in a twofold increase in binding in one study (Pestka and Nirenberg, 1966), although not in another (Igarashi and Kaji, 1969). We were unable to prepare active 40S subunits in a medium free of monovalent cations, and for that reason could not test whether there was an absolute requirement for monovalent cations. Poly U directed binding of [^{3}H]Phe-tRNA to mammalian 40S subunits was only slightly increased by increasing the NH_4^+ or K^+ concentration to 40 mM and binding was optimum over a broad range of NH_4^+ or K^+ concentrations (40–120 mM) (Figure 10); K^+ and NH_4^+ were equally effective in stimulating binding as was also the case when bacterial 30S ribosomal subunits were used (Pestka and Nirenberg, 1966). Concen-

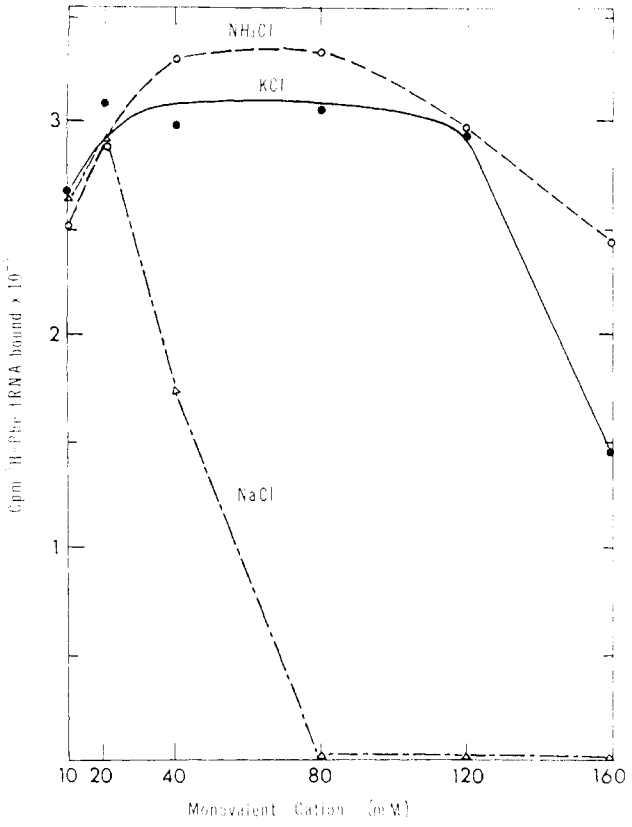

FIGURE 10: Effect of monovalent cation concentration on binding of [^{3}H]Phe-tRNA to 40S ribosomal subunits. 40S subunits (0.68 A_{260} unit) were incubated with 40 μg of [^{3}H]Phe-tRNA (805 cpm/μg) and 7.5 μg of poly U in a buffer containing 0.01 M Tris-HCl (pH 7.5), 0.02 M $MgCl_2$, and the monovalent cation indicated. KCl (10 mM) was present in the experiments with NH_4Cl or NaCl. Incubation was for 30 min at 30°. Counts per minute of [^{3}H]Phe-tRNA bound in the presence of: (●—●) KCl, (○--○) NH_4Cl, and (△-·-△) NaCl. Each 100 cpm is equivalent to 0.054 pmole of phenylalanine.

trations of Na^+ of 40 mM or greater inhibited binding to liver 40S subunits. Na^+ had no effect on poly U directed binding of [^{3}H]Phe-tRNA to bacterial 30S subunits (Pestka and Nirenberg, 1966).

Each increase in the concentration of Tris-HCl (pH 7.5) above 10 mM decreased poly U directed binding of [^{3}H]Phe-tRNA to 40S subunits (the decrease was 70% at 100 mM—the results are not shown); a similar observation was made with 30S subunits from *E. coli* (Pestka and Nirenberg, 1966). A sulfhydryl-protecting reagent (2-mercaptoethanol) was not necessary for poly U directed binding of [^{3}H]Phe-tRNA to 40S ribosomal subunits, nor did GTP increase binding. GTP did not stimulate binding of AA-tRNA to bacterial ribosomes either when binding was performed in the absence of supernatant enzymes (Kurland, 1966).

When deacylated tRNA was added before the 40S ribosomal subunits, binding of [^{3}H]Phe-tRNA was diminished (Table II). A similar effect of deacylated-tRNA has been noted with 70S ribosomes from *E. coli* (Nirenberg and Leder, 1964; Levin and Nirenberg, 1968).

Puromycin did not inhibit binding of [^{3}H]Phe-tRNA to 40S ribosomal subunits at concentrations (0.01–0.5 mM)

TABLE II: Effect of Deacylated tRNA on Poly U Directed Binding of [^{3}H]Phe-tRNA to 40S Ribosomal Subunits.[a]

Deacylated tRNA (μg)	cpm of [^{3}H]Phe-tRNA Bound (%)
0	2737 (100)
44	2115 (77)
132	1641 (60)

[a] 40S ribosomal subunits (1 A_{260} unit) were incubated in buffer B with 7.5 μg of poly U, 40 μg of [^{3}H]Phe-tRNA (390 cpm/μg), and the amount of deacylated tRNA specified. Incubation was for 30 min at 30°. Each 100 cpm is equivalent to 0.054 pmole of phenylalanine.

that abolish protein synthesis. The same result had been reported for bacterial 30S ribosomal subunits (Kaji *et al.*, 1966). Tetracycline inhibits poly U directed binding of Phe-tRNA to bacterial 70S ribosomes (Sarkar and Thach, 1968) and to 30S ribosomal subunits (Suzuka *et al.*, 1966). However, the antibiotic, in concentrations (0.001–0.1 mM) that inhibited bacterial ribosomes maximally, did not significantly reduce binding of [^{3}H]Phe-tRNA to mammalian 40S ribosomal subunits.

Reagents that react with sulfhydryl groups inhibit template-directed binding of AA-tRNA to reticulocyte ribosomes (McAllister and Schweet, 1968). Preincubation of liver 80S ribosomes with *N*-ethylmaleimide (5–30 mM) decreased subsequent binding of [^{3}H]Phe-tRNA by more than 90% (Table III). Pretreatment of 40S ribosomal subunits with *N*-ethylmaleimide diminished binding of [^{3}H]Phe-tRNA by only 50% (Table III). We cannot account for the difference.

Effect of 60S Ribosomal Subunits on Poly U Directed Binding of [^{3}H]Phe-tRNA to 40S Subunits. The 60S ribosomal subunit did not bind [^{3}H]Phe-tRNA in the presence of poly U (Figure 4). However, 60S subunits stimulated binding of [^{3}H]Phe-tRNA to 40S subunits (Figure 11). In calculating stimulation of binding to the 40S subunits by the 60S particles, a correction must be made for the contamination of the latter by the former. (There is binding to the 60S *fraction* when the assay is on nitrocellulose filters because of the presence of 40S subunits; Figure 11.) When the proper allowance was made for binding of [^{3}H]Phe-tRNA due to contamination, stimulation by 60S subunits was approximately threefold. Maximum stimulation of binding was noted when the ratio of 60S subunits to 40S subunits was 2:1 (A_{260} units). Bacterial 50S ribosomal subunits cause a twofold stimulation of binding of AA-tRNA to the 30S subunits (Suzuka *et al.*, 1966).

Product Formed As the Result of Binding of [^{3}H]Phe-tRNA to Ribosomal Particles. [^{3}H]Phe-tRNA was incubated with 40S ribosomal subunits in the presence of poly U and the complex was recovered from nitrocellulose filters. Chromatographic analysis showed only phenylalanine to be present on the ribosome (the results are not shown).

When [^{3}H]Phe-tRNA was incubated with 40S and 60S subunits in the presence of poly U and the complex was recovered and analyzed by chromatography, we found

TABLE III: Effect of *N*-Ethylmaleimide on Binding of [^{3}H]Phe-tRNA to 80S Ribosomes or 40S Ribosomal Subunits.[a]

N-Ethylmaleimide (mM)	cpm of [^{3}H]Phe-tRNA Bound to 80S Ribosomes (%)	cpm of [^{3}H]Phe-tRNA Bound to 40S Subunits (%)
0	5253 (100)	902 (100)
5	596 (11)	506 (56)
10	332 (6)	542 (60)
20	201 (4)	429 (48)
30	133 (3)	414 (46)

[a] Liver 80S ribosomes (1.06 A_{260} units/ml) or 40S ribosomal subunits (0.88 A_{260} unit/ml) were incubated in buffer B with *N*-ethylmaleimide. After incubation for 1 hr at 20°, 0.6 ml (0.64 A_{260} unit of 80S ribosomes or 0.54 A_{260} unit of 40S subunits) was added to 0.4 ml of buffer B which also contained 7.5 μg of poly U, 40 μg of [^{3}H]Phe-tRNA (640 cpm/μg) and 50 mM 2-mercaptoethanol; the latter was included to inactivate the unreacted *N*-ethylmaleimide. Incubation was for 30 min at 30°. Each 100 cpm is equivalent to 0.054 pmole of phenylalanine.

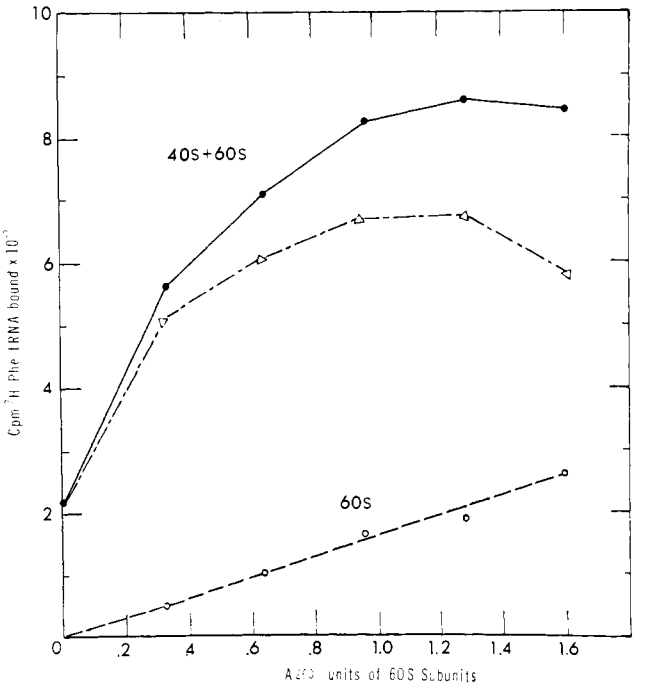

FIGURE 11: Effect of 60S ribosomal subunits on binding of [^{3}H]-Phe-tRNA by 40S ribosomal subunits. Various amounts of a 60S subunit fraction were incubated alone, or with 0.45 A_{260} unit of 40S subunits, in buffer B containing 40 μg of [^{3}H]Phe-tRNA (640 cpm/μg) and 7.5 μg of poly U. Incubation was for 30 min at 30°. (○---○) Counts per minute of [^{3}H]Phe-tRNA bound by the 60S subunit fraction; (●—●) counts per minute of [^{3}H]Phe-tRNA bound by 40S and 60S subunits; (△-·-△) counts per minute of [^{3}H]Phe-tRNA bound by 40S and 60S subunits minus the amount bound by the 60S subunit fraction alone. Each 100 cpm is equivalent to 0.054 pmole of phenylalanine.

primarily phenylalanine to be present on the ribosomes. Small amounts of di- and triphenylalanine were also detected, but the two accounted for less than 5% of the total radioactivity bound to the ribosomes (the results are not shown). The formation of phenylalanyl peptides by bacterial 70S ribosomes in the absence of supernatant enzymes has also been reported (Pestka, 1968).

Discussion

Mammalian 40S ribosomal subunits bind [^{3}H]Phe-tRNA in the presence of poly U; 60S subunits alone do not. In general, the requirements for binding AA-tRNA to liver 40S subunits are similar to those reported for bacterial 30S subunits (Pestka and Nirenberg, 1966). For example, binding is specified by a polynucleotide template and Mg^{2+} is required. However, binding to mammalian 40S ribosomal subunits differs in some minor ways: they can bind [^{3}H]Phe-tRNA at 0° whereas bacterial 30S subunits cannot (Suzuka *et al.*, 1965; Pestka and Nirenberg, 1966); and Na^+ (Pestka and Nirenberg, 1966) but not tetracycline (Suzuka *et al.*, 1966) inhibit binding of [^{3}H]Phe-tRNA to 40S subunits.

Since binding of AA-tRNA to 40S ribosomal subunits requires binding of a specific template RNA, it is possible that some of the variables we studied (for example, magnesium concentration) may primarily effect binding of template RNA and only secondarily binding of AA-tRNA.

Bacterial 50S subunits cause a twofold increase in binding of AA-tRNA to 30S subunits (Suzuka *et al.*, 1966). Mammalian 60S ribosomal subunits, on the other hand, produce a threefold increment in the amount of [^{3}H]Phe-tRNA bound to 40S particles. The ribosome is thought to have two tRNA binding sites (Warner and Rich, 1964; Bretscher and Marcker, 1966; Seeds *et al.*, 1967), and the results obtained from studies of AA-tRNA binding to bacterial ribosomal subunits have been interpreted as indicating that one molecule of tRNA can bind to the 30S subunit alone and that a second binding site is generated by combination of a 30S and 50S subunit to form a 70S ribosome (Suzuka *et al.*, 1966). However, the data do not rule out a number of other possibilities: That there are two tRNA binding sites on the small ribosomal subunit and that addi ion of the large subunit increases the affinity of the two sites for tRNA. That only a fraction of the small ribosomal subunits binds AA-tRNA, and that the large subunit merely increases the number competent to do so. That the large subunit will bind AA-tRNA but only in the presence of the small subunit. Finally, it is likely that some of the increment in AA-tRNA binding observed on addition of the large ribosomal subunit is due to formation of short peptides and protection of bound AA-tRNA from deacylation (Pestka, 1967). Our data does not permit us to infer the means by which addition of 60S to 40S subunits increases binding of AA-tRNA.

Radioactive di- and triphenylalanine are found on the ribosome when [^{3}H]Phe-tRNA is bound in the presence of 40S and 60S subunits; only phenylalanine is recovered when binding is to 40S subunits alone. The results accord with peptide-bond formation being a function of the 60S subunit and it is known that peptidyltransferase is an integral part of the bacterial 50S subunit (Monro, 1967). However, synthesis of triphenylalanine requires translocation as well as

peptide-bond formation. When poly U is used as a template for bacterial ribosomes, translocation occurs in the absence of GTP and the supernatant factor (translocase or factor G) which is generally required (Pestka, 1969). We can only presume from formation of triphenylalanine that translocation also occurred in our experiments even though no supernatant enzymes were added. The possibility that the subunits were contaminated with the factor cannot be excluded.

We have studied binding of [^{3}H]Phe-tRNA to mammalian 40S subunits in the absence of added supernatant proteins. Protein synthesis by animal ribosomes ordinarily requires a soluble protein (TF-I, aminoacyltransferase I) which catalyzes binding of AA-tRNA to a mRNA–ribosome complex in the presence of GTP (Arlinghaus *et al.*, 1964; Ibuki and Moldave, 1968; Lin *et al.*, 1969). It is possible that the binding of AA-tRNA we observed was due to residual aminoacyltransferase I on the 40S subunit, however, GTP was not required and the magnesium optimum was much greater than when binding is assayed using partially purified aminoacyltransferase I (J. J. Castles, unpublished results).

Acknowledgments

We thank Mr. Wilfred Saito and Mrs. Arlene Fox for expert technical assistance.

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