Specificity of the Interaction of Aminoacyl Ribonucleic Acid with a Protein–Guanosine Triphosphate Complex from Wheat Embryo*

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ABSTRACT: A new assay for functionally correct conformations of transfer ribonucleic acid is described. The assay depends upon the interaction of aminoacyl transfer ribonucleic acid with protein–guanosine triphosphate complexes, a reaction which is a necessary intermediate step in protein biosynthesis. With protein–guanosine triphosphate complexes from wheat embryo and aminoacyl transfer ribonucleic acid from *Escherichia coli* and yeast, it was shown that this interaction requires unblocked aminoacyl transfer ribonucleic acid in a native conformation. *N*-Acetyl or nitrous acid deaminated aminoacyl transfer ribonucleic acid or free transfer ribonucleic acid is unable to interact, as measured either by release of protein–guanosine triphosphate complexes from nitrocellulose filters or by competition with aminoacyl transfer ribonucleic acid. Partially purified denatured leucine transfer ribonucleic acid of yeast also does not react, demonstrating the need for correct transfer ribonucleic acid conformation in addition to an unblocked aminoacyl group. The apparent dissociation constant for *E. coli* aminoacyl transfer ribonucleic acid is 8×10^{-8} M. Since neither tetracycline nor the tetranucleotide TpψpCpGp affect the interaction although both inhibit the binding of aminoacyl transfer ribonucleic acid to ribosomes, it is suggested that these two compounds must act at some subsequent step in the binding reaction. The specificity shown by this interaction is consistent with a possible functional role in the exclusion of N-acylated aminoacyl transfer ribonucleic acid from binding to ribosomes at the A site and interfering with the orderly progress of peptide-chain elongation.

The mechanism by which amino acids are added to a growing polypeptide chain is currently thought to require four enzymes, GTP, and AA-tRNA. Two of these enzymes, identified as the T factors in *Escherichia coli*, have been recently shown to specifically bind GTP (Allende *et al.*, 1967; Gordon, 1967). Binding requires the presence of both T factors, T_u and T_s (Ertel *et al.*, 1968a; Skoultchi *et al.*, 1968), although only T_u participates in the complex, T_s being required in a catalytic role (Ertel *et al.*, 1968b). In a subsequent reaction, AA-tRNA interacts with this complex to give a new complex consisting of T_u, GTP, and AA-tRNA (Ravel *et al.*, 1968). These two complexes are readily distinguishable by the fact that the T factor–GTP complex is adsorbed on nitrocellulose membranes while the AA-tRNA–T_u–GTP complex is not (Gordon, 1968; Ravel *et al.*, 1967). Evidence that these reactions are related to protein synthesis are the facts that (a) T factors are known to be required for the enzymatic binding of AA-tRNA to ribosomes in a reaction stimulated by GTP (Ertel *et al.*, 1968a; Skoultchi *et al.*, 1968; Lucas-Lenard and Haenni, 1968; Ravel, 1967), (b) the factor–GTP complex interacts with AA-tRNA but not with unacylated tRNA (Skoultchi *et al.*, 1968; Ertel *et al.*, 1968b; Gordon, 1968; Ravel *et al.*, 1967), and (c) the AA-tRNA–T_u–GTP complex is an intermediate in the binding of AA-tRNA to the ribosome (Ravel *et al.*, 1968; Lucas-Lenard and Haenni, 1968).

The reaction of AA-tRNA with the T factor–GTP complex thus provides a new and useful assay for investigating the way in which chemically induced structural changes affect the functional properties of tRNA. The present experiments were begun in order to assess the specificity requirements for this reaction and thus its usefulness as an assay of tRNA function.

In this report we describe first some properties of a GTP-binding protein from wheat embryo and then experiments utilizing this system which examine the subsequent reaction of the GTP–protein complex with AA-tRNA. Our results show that the amino group of the amino acid attached to tRNA must be free for interaction with the factor–GTP complex to take place. If the amino group is blocked by acetylation, or if it is removed by nitrous acid treatment or by removal of the entire amino acid, no reaction occurs. In addition to a free amino group, the correct conformation of the tRNA is necessary. Thus the denaturable Leu-tRNA described by Fresco and his associates (1966) does not react when tested in the denatured conformation although interaction is very effective when Leu-tRNA is in the native state.

Materials and Methods

[^{3}H]GTP (1 Ci/mmole) and other radioactive nucleotides, *E. coli* tRNA, baker's yeast tRNA, and RNase-free sucrose were obtained from Schwarz BioResearch. AA-tRNA of *E. coli* charged with [^{14}C]phenylalanine (100 mCi/mmole) plus 19 other [^{12}C] amino acids was prepared by previously described methods (Conway, 1964). [^{14}C]Leu-tRNA of yeast was pre-

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pared using the enzyme preparation described by Lindahl *et al.* (1967a) in a reaction mixture containing 100 mM Tris (pH 7.5), 20 mM KCl, 10 mM MgOAc, 0.67 mM EDTA, 10 mM ATP, 0.03 mM [^{14}C]leucine (40 mCi/mmole), and 19 other [^{12}C]-amino acids, enzyme, and 6 mM RNA nucleotide. Before incubation, the tRNA was treated at 60° for 5 min in 140 mM Tris (pH 7.5), 20 mM MgOAc, 100 mM KCl, and 1.4 mM EDTA to renature any denatured species in the commerical tRNA preparation. Fully charged RNA was isolated after 40-min incubation at 35° by phenol extraction and repeated alcohol precipitation, and finally dialyzed against 10 mM cacodylate buffer–1 mM EDTA (pH 7.0) in the cold.

Modified AA-tRNAs. Stripped tRNA was prepared from *E. coli* AA-tRNA either by treatment with 1 M Tris (pH 9.0) for 2 hr at 25° (Sarin and Zamecnik, 1964) or by treatment at pH 10.2 with dilute NH_3 for 30 min at 37°. RNA was recovered by alcohol precipitation. *N-Acetyl-AA-tRNA* was prepared from *E. coli* AA-tRNA by acetylation with acetic anhydride as described by Haenni and Chapeville (1966). Paper electrophoresis at pH 2.0 showed that acetylation was complete, at least for the [^{14}C]phenylalanyl-tRNA marker. This preparation was functionally intact as measured by its ability to bind effectively to wheat ribosomes. We thank Marta Gatica for the preparation and analysis of this compound. *AA-tRNA^{AC}* was prepared by stripping the *N*-acetylamino acid from *N*-acetyl-AA-tRNA by incubation for 2 hr at 30° with 1 M Tris (pH 9.0). After isolation of the tRNA^{AC}, it was recharged with a complete mixture of amino acids. Stripping was 93% effective as measured by loss of [^{14}C]phenylalanine and recharging to 110% of the previous level was achieved. AA-tRNA^{AC} was re-isolated by phenol extraction and ethanol precipitation. *Deamino-AA-tRNA* was prepared by nitrous acid treatment of *E. coli* AA-tRNA essentially as described by Hervé and Chapeville (1965) except that the pH was adjusted to 6.0 and reaction was carried out at 0° for 2 hr in the presence of 5 mM Mg (F. Chapeville, personal communication). Analysis of the reaction product after alkaline hydrolysis at pH 12 for 3 hr showed that all of the [^{14}C]phenylalanine marker had reacted and more than 85% had been converted into a compound with an R_F like phenyllactic acid in the solvent 1-butanol–acetic acid–water (78:5:17) (Hervé and Chapeville, 1965). Conditions stronger than these are reported not to deaminate the terminal adenosine of tRNA (Chousterman *et al.*, 1966) nor to impair the ability of phenyllactyl-tRNA to bind to ribosomes and make polypeptide (Hervé and Chapeville, 1965; Chapeville, personal communication). *AA-tRNA^{HONO}* was prepared from *E. coli* tRNA treated with nitrous acid as described above for deamino-AA-tRNA. After isolation of the tRNA^{HONO} it was charged with a complete mixture of [^{12}C]amino acids containg [^{14}C]phenylalanine as a marker. Charging was achieved to 89% of a control tRNA, tRNA^{C}, previously incubated at pH 6, 0°, 2 hr, 5 mM Mg but in the absence of nitrous acid. AA-tRNA nucleotide concentration was determined from the A_{260} in 10 mM MgOAc–3 mM Tris (pH 7.5) using an ϵ(P) of 6.8×10^3 (J. Ofengand, unpublished results), and correcting for an assumed 70% aminoacylation of the preparation. All AA-tRNA dilutions were made in assay buffer (see below).

GTP Binding Protein. Supernatant protein was prepared from wheat embyro extracts as described previously (Allende and Bravo, 1966) and the fraction precipitating between 40 and 80% saturation with ammonium sulfate was used. For improved stability it was stored in 50% glycerol–1 mM dithiothreitol at −15°; 1 mg = 2.32 A_{280} units (ratio A_{280}/A_{260} = 0.7).

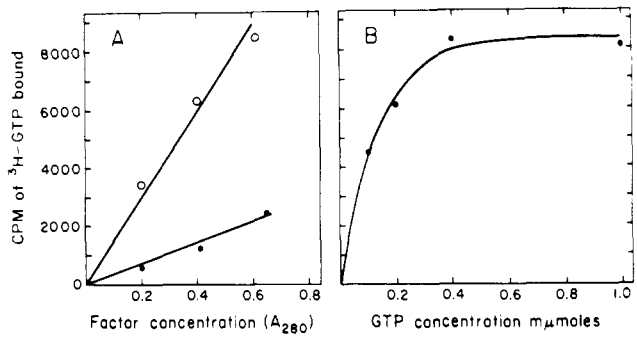

FIGURE 1: Effect of factor and GTP concentration on complex formation. (A) Assays were performed as described in Methods except that incubation was for 10 min at 0° (open circles) or 5 min at 0° with addition of 120 mμmoles of [^{3}H]GTP and incubation for an additional 5 min (filled circles). (B) The standard assay was used with 0.23 A_{280} unit of a more active preparation of factor and variable amounts of [^{3}H]GTP as indicated. The values have been corrected by subtracting the counts retained on the filter in the absence of factor; 133 cpm bound = 1 μμmole of GTP.

TpψpCpGp tetranucleotide was isolated from yeast tRNA after complete digestion with T_1 RNase. DEAE-Sephadex chromatography in 7 M urea, first at pH 7.6 and then at pH 2.7, was sufficient to free the compound from all but trace impurities (J. Ofengand and C. Henes, unpublished results). The final salt-free product had an average chain length of 3.5 as determined by the ratio of terminal to total phosphorus and the following nucleotide composition: T, 1.0, ψ, 1.1, C, 1.3, G, 1.3, U, 0.4; A, 0.1.

Binding of [^{3}H]GTP to Wheat Enzyme and Reaction with AA-tRNA. Reaction mixtures consisted of assay buffer (10 mM Tris (pH 7.5), 50 mM NH_4Cl, 10 mM MgOAc, and 10 mM dithiothreitol), 3 μM [^{3}H]GTP, and 0.34 A_{280} unit of enzyme in a total volume of 205 μl. After 5-min incubation at 0°, AA-tRNA or excess [^{3}H]GTP was added in volumes up to 20 μl. After incubation for 10 min more at 0°, the reaction was stopped by dilution with 2 ml of cold assay buffer and immediately passed through a prewashed Millipore filter (HA, 0.45 μ) under suction. The filter was washed three times with 4 ml of assay buffer, dissolved in 10 ml of Bray's solution (Bray, 1960), and counted at 6% efficiency. There was no [^{14}C]amino acid contribution. For reproducible results it was necessary to start the first reaction by adding enzyme rather than GTP and to filter the reaction mixtures immediately after dilution. The amount of GTP used was saturating.

Results

Properties of the GTP–Protein Interaction. The results shown in Figure 1 illustrate the formation of the GTP–protein complex and its dependence upon the concentrations of protein and GTP. At limiting concentrations of GTP (0.1 mμmole), 25% of the added nucleotide was bound to the protein. The reaction was extremely rapid even at 0°, reaching 90% of the final value in 1 min. Approximately 25% of the radioactivity bound after 5-min incubation could not be removed by subsequent incubation with a 200-fold excess of cold GTP (see also Table III). This nonexchangeable radio-

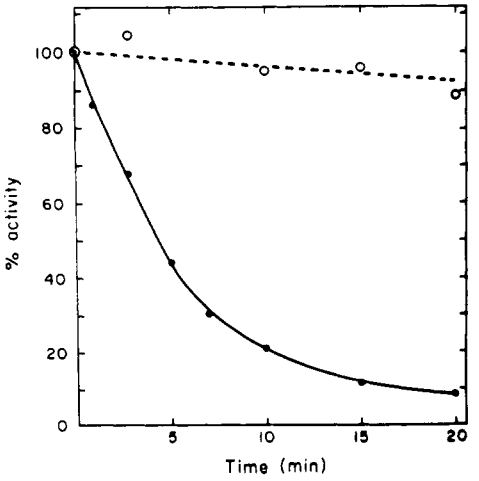

FIGURE 2: Inactivation of nucleoside triphosphate binding factors at 42°. A factor preparation containing approximately 2 mg/ml of protein was incubated at 42° in the buffer used for GTP binding assays. At the times indicated two 0.1-ml aliquots were withdrawn and chilled. One series of aliquots was assayed for binding of [^{3}H]-GTP (filled circles) and the other for [^{3}H]ATP (open circles). The zero time aliquots that had not been heated at 42° bound 47 μμmoles of GTP and 10.7 μμmoles of ATP, respectively. These amounts were considered the 100% values for the two activities.

activity, however, could be released from the protein by treatment with 5% trichloroacetic acid, mild alkali (pH 11), or EDTA (0.2 M) and thus appears to bind in a noncovalent manner.

The formation of the complex was not inhibited by the presence of ribonuclease, deoxyribonuclease, T_1 ribonuclease, or 10^{-3} M fusidic acid, an antibiotic recently shown to specifically inhibit AA-tRNA translocation (Tanaka *et al.*, 1968; Pestka, 1968). This amount of fusidic acid inhibited 80% of the aminoacyl transfer reaction in wheat extracts.

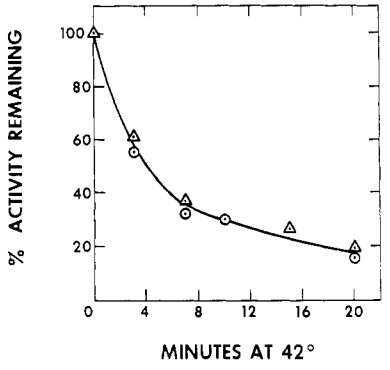

FIGURE 3: Heat inactivation of GTP binding activity and aminoacyl transfer activity. GTP binding protein was heated at 42° for the indicated times in standard assay buffer. GTP binding activity (△) was assayed as described in Methods. Aminoacyl transfer activity assays (⊙) were performed by means of a poly U directed synthesis of polyphenylalanine starting from Phe-tRNA as described by Allende and Bravo (1966) with limiting amounts of transfer enzyme except that the ribosomes were further purified by resedimentation through 0.5 M NH_4Cl, 0.01 M $MgCl_2$, and 0.01 M Tris-HCl buffer (pH 7.5). The pellet was then resuspended in 0.5 M sucrose, 0.05 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 0.025 M KCl, and centrifuged at 30,000*g* to eliminate aggregates. The supernatant fraction containing the ribosomes was stored at −15°; 100% transfer activity was 446 cpm (2.0 μμmoles) transferred into polyphenylalanine and 100% GTP binding activity was 2054 cpm (15.4 μμmoles) retained on the filter.

TABLE I: Nucleotide Specificity of Binding to the Supernatant Factor.[a]

Expt	Radioactive Nucleotide (mμmoles)	Nonradioactive Addn (mμmoles)	μμmoles of Radioactive Complex
1	[^{3}H]GTP (0.4)		31.5
	[^{3}H]GTP (1.0)		35.7
	[^{3}H]ATP (0.4)		9.6
	[^{3}H]ATP (1.0)		14.6
	[^{3}H]CTP (1.2)		1.5
	[^{3}H]UTP (0.6)		0.7
2	[^{14}C]GTP (0.4)		38.8
	[^{14}C]GTP (1.0)		42.4
	[^{14}C]GDP (0.4)		26.4
	[^{14}C]GDP (1.0)		45.6
	[^{14}C]GMP (0.4)		3.6
	[^{14}C]GMP (1.0)		4.7
3	[^{3}H]GTP (0.4)		27.0
	[^{3}H]GTP (0.4)	ATP (10)	26.0
	[^{3}H]GTP (0.4)	CTP (10)	27.5
	[^{3}H]GTP (0.4)	UTP (10)	24.0
	[^{3}H]GTP (0.4)	GDP (10)	7.3
	[^{3}H]GTP (0.4)	GMP (10)	28.0
4	[^{3}H]GTP (0.4)		38.5
	[^{3}H]GTP (0.4)	GMP-PCP (10)	29.6

[a] The formation of complex in the presence of approximately 150 μg of factor protein was assayed by measuring the retention of radioactivity on nitrocellulose membranes in the standard manner described in the text.

The results summarized in Table I demonstrate the specificity of the interaction between the wheat supernatant protein and several nucleotides. It is clear that of the four common nucleotide triphosphates GTP was bound most efficiently. However, ATP binding to a material retained on the Millipore was considerable. From the data in expt 3, however, it would appear that the binding of ATP was independent of the GTP interaction since ATP did not inhibit GTP complex formation. In addition heat inactivation studies demonstrated considerable differences between the stability of the factors that bind the two nucleoside triphosphates. The GTP binding factor was much less stable to heating at 42° (Figure 2). Of the guanosine derivatives, both GTP and GDP were effectively bound to the factor but GMP showed no interaction. 5′-Guanylylmethylenediphosphonate, an analog of GTP described by Hershey and Monro (1966), was a rather poor inhibitor.

Involvement of the GTP Binding Protein in the Process of Peptide Synthesis. Several lines of evidence relate this GTP binding to the process of polypeptide synthesis.

First the rate of inactivation of the GTP binding activity at

TABLE II: Effect of GTP Binding Protein on Aminoacyl Transfer.[a]

Transfer Enzyme	μμmoles of Phe Transferred
(1) Crude factor	0.81, 0.89
(2) Part 1 heated for 15 min at 42°	0.22, 0.16
(3) Purified GTP binding protein	0.14, 0.13
(4) Parts 2 + 3	1.09, 0.99

[a] Heat treatment of GTP binding protein and assay of transfer activity was carried out as described in Figure 3. Purified GTP binding protein was obtained by passage of the untreated factor (line 1) over a DEAE-cellulose column that had been equilibrated with 10 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, and 0.5 mM dithiothreitol. GTP binding activity was not retained in the column under these conditions and therefore could be recovered in the first column volume. A constant amount of crude factor was present in tubes 1, 3, and 4.

TABLE III: Effect of AA-tRNA and Excess GTP on E–GTP Complex.[a]

Expt	Additions	% GTP Bound[b]
1	No additions	100
	Plus AA-tRNA	36
	Plus [^{1}H]GTP	19
2	No additions	100
	Plus [^{1}H]GTP at zero time	0
	Plus [^{1}H]GTP	29
	Plus [^{1}H]GTP and AA-tRNA	31
	Plus AA-tRNA	52
3	No additions	100
	Plus [^{1}H]GTP	21
	Plus [^{1}H]GTP and AA-tRNA	25

[a] E–GTP complex formation and reaction with AA-tRNA or GTP was carried out as described in Methods except that incubation was for 10 min at 0°. Where indicated, additions were made after 5 min at 0° and incubation was continued for an additional 5 min; 75 mμmoles of [^{1}H]GTP (125-fold excess over [^{3}H]GTP) and 455 mμmoles of AA-tRNA nucleotide (a large excess, see Figure 5) were added to 0.2-ml reaction volumes. [b] 100% corresponds to 50.8, 38.5, and 68.3 μμmoles of GTP bound for expt 1, 2, and 3, respectively, with different enzyme preparations.

42° is the same as the rate of loss of aminoacyl transfer activity from the same preparation (Figure 3).

Second, as shown in Table II, transfer activity of the heated enzyme can be restored by the addition of a partially purified preparation of GTP binding protein which by itself has negligible transfer activity. Thus the purified heat-labile (Figure 3) GTP binding protein can substitute for the heat-labile component of the transfer system. The crude factor must also contain a heat-stable component of the transfer system that is removed by passage over DEAE in the preparation of the purified GTP binding protein.

Third, like the *E. coli* T factor–GTP complex, this factor–GTP (E–GTP) complex can also react with AA-tRNA resulting in the decrease of filter-bound GTP (Table III). Experiment 1 shows that two-thirds of the total GTP bound could be converted into a filterable form after reaction with excess AA-tRNA. This experiment also illustrates the nonexchangeability of 20% of the bound GTP seen in Figure 1A. By contrast, if excess unlabeled GTP was added at zero time, no labeled GTP could be detected in complex formation (expt 2). This table shows that the bound GTP nonreactive with excess AA-tRNA is made up of a part which is exchangeable with unlabeled GTP and a second part which is not. Thus in each experiment addition of unlabeled GTP reduced the bound GTP to a lower level than was reached with excess AA-tRNA, and AA-tRNA plus [^{1}H]GTP were no more effective than [^{1}H]GTP alone. In subsequent experiments, the amount of GTP bound in the presence of excess AA-tRNA has been treated as background and subtracted from the total amount bound. Note that more than half of this background is GTP nonexchangeable material.

The time course of the further reaction of E–GTP complex formed in a 5-min incubation was examined. Both the reaction with AA-tRNA and with unlabeled GTP was over by 5 min and stayed constant for at least 20 min. This result is in agreement with that reported for the *E. coli* complex (Gordon, 1968), but contrasts with the need for incubation at 30° reported for the *Bacillus stearothermophilus* complex (Skoultchi *et al.*, 1968). The time course of E–GTP formation was also studied in terms of the ability of the complex to subsequently react either with AA-tRNA or unlabeled GTP. It was considered possible that although GTP binding is over in 1 min, formation of a complex capable of reaction with AA-tRNA might take longer. However, varying the first incubation from 5 to 15 min had no effect on the extent of the second 10-min incubation with either AA-tRNA or [^{1}H]GTP.

As in the *E. coli* system, the decrease in the retention of GTP on the filter caused by the presence of AA-tRNA is accompanied by the appearance of transfer activity in the filtrate (Figure 4). Filtrates of control tubes containing the highest amounts of AA-tRNA used but no protein had no transfer activity (data not shown). Moreover, the appearance of transfer activity in the filtrate does not represent breakdown of the E–GTP complex induced by AA-tRNA since isolation of the GTP–enzyme complex by gel filtration on Sephadex G-50 columns demonstrated that AA-tRNA rather than inducing a breakdown of the complex, interacts with it and has a stabilizing effect. Thus columns run in the absence of AA-tRNA or in the presence of deacylated tRNA yielded 38,500 and 39,800 cpm of [^{3}H]GTP complex, respectively, while the same material run in the presence of [^{14}C]phenylalanyl-tRNA gave 74,300 cpm of [^{3}H]GTP complex.

Specificity of the AA-tRNA Reaction. The quantitative nature of the reaction of AA-tRNA with E–GTP complex is shown in Figure 5A. The nature of the concentration dependence suggests that the reaction is a simple equilibrium be-

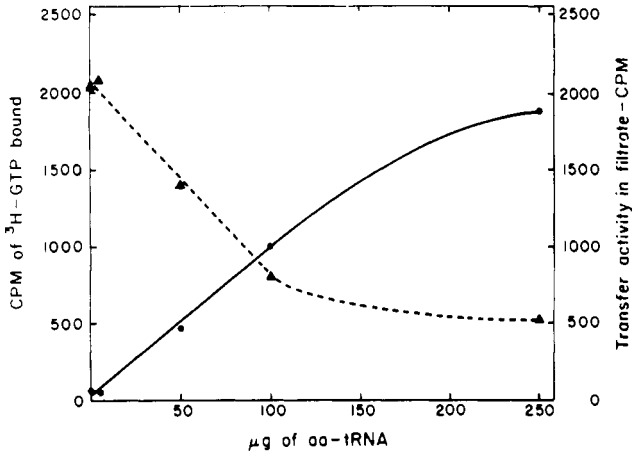

FIGURE 4: Effect of AA-tRNA on the retention of the GTP–factor complex on Millipore filters. The binding of [^{3}H]GTP (▲) was assayed in the standard manner with 0.15 mg of enzyme protein and the indicated amounts of *E. coli* AA-tRNA except that the 200-μl reaction mixtures were diluted with only 1 ml of the standard buffer and the filtrate was removed before washing with the same buffer. The filters were then counted for [^{3}H]GTP retention; 0.5-ml aliquots of the initial filtrates were assayed for aminoacyl transfer activity (●) as described in Figure 3; 2000 cpm of [^{14}C]phenylalanine transferred was equivalent to 9.0 μμmoles of amino acid polymerized and 2000 cpm of [^{3}H]GTP retained was equivalent to 15.0 μμmoles of nucleotide.

TABLE IV: Competition between AA-tRNA and Modified AA-tRNAs for Reaction with E–GTP Complex.[a]

Modified AA-tRNA	mμmoles of Nucleotide	% GTP Bound	Theory (% Bound) No Competition	Theory (% Bound) Complete Competition
N-Acetyl-AA-tRNA	13	50	52	84
	21	54	39	83
	34	43	25	83
Deaminated AA-tRNA	13	56	52	84
	21	44	39	83
	34	41	25	83
tRNA (Tris)	15	60	52	86
	25	50	39	85
	40	32	25	85
tRNA (pH 10)	15	62	52	86
	25	49	39	85
	40	30	25	85

[a] E–GTP complex formation and reaction with AA-tRNA was carried out as described in Methods. In order for both AA-tRNA and competitor to be presented to the E–GTP complex simultaneously, a mixture of modified AA-tRNA and AA-tRNA was prepared and aliquots were added to the E–GTP complex formed by 5-min preincubation at 0°. Reactions were stopped after 10 min further incubation by filtration. The ratios of competitor AA-tRNA to AA-tRNA were: *N*-acetyl-AA-tRNA and deaminated AA-tRNA, 5:1; stripped tRNA (both Tris and pH 10), 5.9:1. The values given in column 2 refer to competitor RNA nucleotide. In each case unmodified AA-tRNA was also present. The method for obtaining the theoretical values given in the last columns is described in the text.

tween complex and AA-tRNA of the type: E–GTP + AA-tRNA $\rightleftharpoons$ E–GTP–AA-tRNA. Since the total E–GTP complex in the system is determined by the amount of protein added when the [^{3}H]GTP concentration is saturating, the equilibrium reaction can be described by the simple relation: $(\text{E–GTP}_{max}/\text{E–GTP} = 1 + (\text{AA-tRNA})/K_d$, where K_d is the dissociation constant and $(\text{E–GTP})_{max}$ is the amount of [^{3}H]-GTP bound in the absence of AA-tRNA. Thus a suitable plot of the data should give a straight line with slope equal to $1/K_d$. Figure 5B shows the result obtained when the data of Figure 5A are replotted in this form after correction of the total AA-tRNA added for the amount bound in the new complex. On the assumption that this simple treatment is adequate a dissociation constant of 8×10^{-8} M can be calculated and illustrates the tightness of the binding of AA-tRNA in this reaction.

Figure 5A also shows the specificity of the reaction. Thus *N*-acetyl-, deamino-, or deacylated AA-tRNA are all unable to change the filtration properties of the E–GTP complex. However, since the reasons for the filterability of the E–GTP–AA-tRNA complex are unknown at present, it was thought possible that only the *filtration* properties of the complex might require a free amino group, but not necessarily the ability to *form* the complex. To test this, a competition experiment was set up between the modified AA-tRNAs and the untreated AA-tRNA. It was reasoned that if the modified AA-tRNAs made a complex that was retained on the Millipore filter, the presence of excess modified AA-tRNA in the presence of AA-tRNA should result in increased retention of bound GTP over that found in the presence of AA-tRNA alone. However, even with a five- to sixfold excess of modified AA-tRNA, there was no evidence for any competition (Table IV). The theoretical value for "no competition" is that expected for the given amount of AA-tRNA alone, calculated from the curve of Figure 5A. That for "complete competition" is calculated on the expectation that both modified and untreated AA-tRNA compete equally and randomly for E–GTP complex. Thus the per cent reacted can be calculated from the curve of Figure 5A, and then corrected for the fact that the fraction of complex made with modified AA-tRNA will still adsorb to the filter.

Another possible artifact in this experiment could arise if the chemical treatments used produced an alteration in the tRNA itself. This was considered possible despite the fact that the modified AA-tRNAs could still participate in ribosome binding and polypeptide synthetic reactions. In order to test this point, the modified amino acid was removed from its tRNA and the putative modified tRNA recharged with amino acids and tested in the E–GTP reaction. The *N*-acetylamino acid was readily removed by chemical hydrolysis and amino acids could be recharged to the same extent as before. This AA-tRNA^{AC} was active in the E–GTP complex reaction

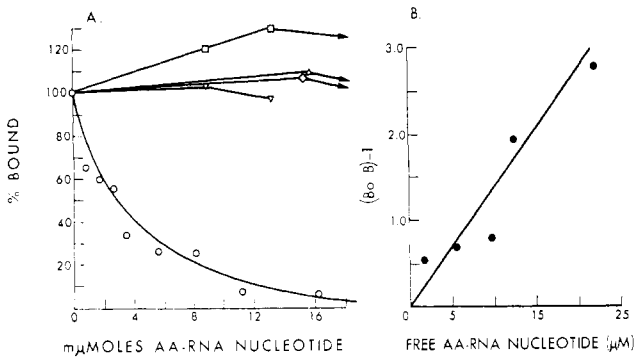

FIGURE 5: Ability of AA-tRNA and modified AA-tRNA to react with E–GTP complex. (A) Unmodified AA-tRNA, ○; *N*-acetyl-AA-tRNA, □; deamino-AA-tRNA, ▽; Tris buffer deacylated AA-tRNA, △; pH 10 deacylated AA-tRNA, ◊. Formation of E-GTP complex and reaction with AA-tRNA or modified AA-tRNA was carried out as described in Methods. The 100% bound value (19.3 μμmoles of GTP) was obtained by subtracting the [^{3}H]GTP remaining bound to the filter after reaction with a large excess of AA-tRNA (54 and 90 mμmoles) from the amount bound in the absence of additions. This amounted to 34% of the total, of which 53% was also nonexchangeable with excess unlabeled GTP (see also Table III). (B) The first five points of part A were analyzed as described in the text. AA-tRNA bound in the complex was calculated from the amount of [^{3}H]GTP removed from the filter with the assumption of an average chain length of 83 for the tRNA and a 1:1 stoichiometry between AA-tRNA and GTP in the complex. B_0 and B refer to $(E\text{–}GTP)_{max}$ and (E–GTP), respectively. K_d was calculated from the slope of the line to be 8.3×10^{-8} M.

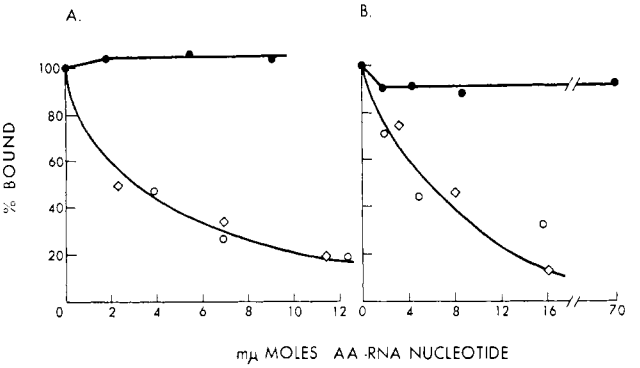

FIGURE 6: Ability of AA-tRNA and modified AA-tRNA to react with E–GTP complex. Preparation of materials and assay procedure is described in Methods. (A) Deamino-AA-tRNA, ●; AA-tRNA^{HONO}, ○; AA-tRNA^{C} (control RNA), ◊. (B) *N*-Acetyl-AA-tRNA, ●; AA-tRNA^{AC}, ○; unmodified AA-tRNA, ◊.

(Figure 6B). It proved difficult to completely hydrolyze the nitrous acid treated AA-tRNA samples under conditions that would not effect the phosphodiester bonds, so tRNA^{HONO} was made by direct treatment of tRNA with nitrous acid. This material could be fully charged with amino acids when compared with a suitable control (see Methods) and was also able to react efficiently with the E–GTP complex (Figure 6A).

The conclusion from these experiments is that the free amino group of the amino acid is necessary for the reaction of AA-tRNA with E–GTP complex.

Ability of TpψpCGp to Substitute for AA-tRNA. Other experiments have shown that the tetranucleotide TpψpCpGp is able to specifically inhibit the nonenzymatic binding of Phe-tRNA to poly U–ribosome complexes (J. Ofengand and C. Henes, unpublished results). It was of interest, therefore, to see if the effect could also be shown at the level of binding of AA-tRNA to E–GTP complex. It is clear from Figure 7, however, that TpψpCpGp is completely without effect on E–GTP complexes either when added alone or in competition with AA-tRNA even at molar concentrations 3000 times higher than the AA-tRNA.

Effect of Tetracycline. Since tetracycline inhibits the binding of AA-tRNA to the A site on the ribosome (Gottesman, 1967; Sarkar and Thach, 1968), presumably the same place where the T_u–AA-tRNA–GTP complex binds, it was of interest to see if tetracycline would block the formation of this complex. As shown in Table V, at levels known to be inhibtory in the wheat embryo system (J. Allende, unpublished observations), there was no inhibition of the reaction of AA-tRNA with E–GTP.

Conformational Requirements. The need for a correct conformation of AA-tRNA as well as for a free amino group was shown by an experiment using the denaturable RNA^{Leu} of yeast that has been described by Fresco *et al.* (1966). This particular RNA species was used since it can be stabilized in both native and denatured forms with its amino acid attached. Separation of this species from the nondenaturable Leu-tRNAs was achieved by Sephadex chromatography following the procedures of Lindahl *et al.* (1967a) (Figure 8). The denaturable Leu-tRNA elutes earlier than the undenatured Leu-tRNA because of its slightly greater molecular size (Adams *et al.*, 1967). Further identification of the two peaks was obtained by assays for the ability to be enzymatically deacylated. As shown by Lindahl *et al.* (1967b) the denatured Leu-tRNA cannot be recognized by its synthetase for the enzymatic deacylation reaction unless it is first renatured. In complete confirmation of this work it was found that while the native Leu-tRNA species (peak 2 of Figure 8) was readily deacylated in an AMP- and PP-dependent reaction, the denatured Leu-tRNA (peak 1 of Figure 8) was completely resistant to AMP- and PP-dependent deacylation. (A small amount of chemical deacylation was observed with all samples.) If, however, the denatured Leu-tRNA was first renatured, then deacylation proceeded as readily as with the native Leu-tRNA.

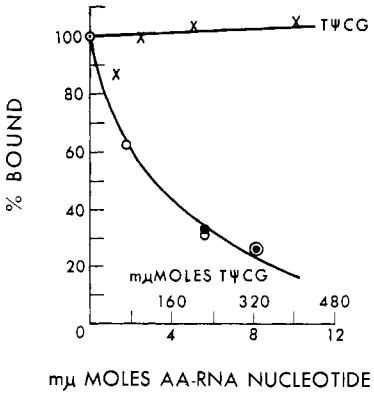

FIGURE 7: Ability of TpψpCpGp to mimic the action of AA-tRNA. Unmodified AA-tRNA, ○; TpψpCpGp, ×; unmodified AA-tRNA plus 200 mμmoles of TpψpCpGp, ●. Assays and analysis as in Figure 5A. The concentration of TpψpCpGp is expressed as millimicromoles of tetranucleotide.

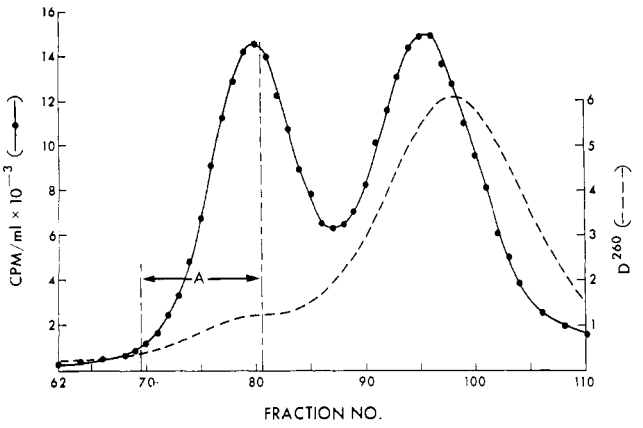

FIGURE 8: Partial purification of the denaturable RNA^{Leu} from baker's yeast by Sephadex G-100 chromatography. [^{14}C]Leu-tRNA, prepared as described in Methods, was passed over a 1.2 × 78 cm column of Sephadex G-75 equilibrated in 20 mM cacodylate–2 mM EDTA (pH 7.0) to remove residual ATP. The sample was denatured by heating at 60° for 2 min in 10 mM cacodylate–1 mM EDTA (pH 7.0). After cooling to 0°, the sample (3.0 ml) was applied to a 2.5 × 95 cm column of Sephadex G-100 equilibrated at 4° in 150 mM KCl–10 mM cacodylate buffer (pH 7.0), 5 mM $MgCl_2$, and 0.5 mM EDTA (Lindahl *et al.*, 1967a). The column was eluted with this buffer at 0.5 ml/min and 4.0-ml fractions were collected. Recovery of ^{14}C was 109%. The tubes indicated as fraction A were pooled to obtain the denatured Leu-tRNA species and recovered by ethanol precipitation. [^{14}C]Leucine, —●—; OD_{260}, — — —.

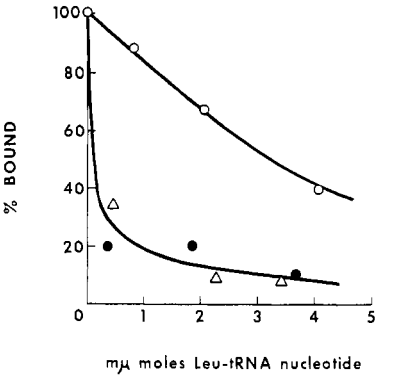

FIGURE 9: Ability of denatured and renatured Leu-tRNA of yeast to react with E–GTP complex of wheat embryo. Denatured Leu-tRNA ○; renatured Leu-tRNA, ●; native Leu-tRNA, △. Denatured Leu-tRNA prepared by Sephadex G-100 chromatography (Figure 8) was divided into two portions and dissolved in (a) 10 mM cacodylate buffer–1 mM EDTA (pH 7.0) and (b) 10 mM cacodylate–1 mM EDTA–10 mM $MgCl_2$ (pH 7.0). The two solutions were heated at 60° for 2 min to (a) denature and (b) renature the Leu-tRNA (Lindahl *et al.*, 1967b). After cooling to 0° the samples were tested for reactivity with the E–GTP complex. Formation of complex and reaction with AA-tRNA was carried out as described in Methods. Native Leu-tRNA was obtained by pooling fractions 91–102 from the column shown in Figure 8. It was recovered by ethanol precipitation and dissolved in water. Leu-tRNA nucleotide was calculated on the basis of precipitable [^{14}C]leucine present after the renaturation or denaturation treatment. A chain length of 85 (Adams *et al.*, 1967) was used.

The denatured and renatured Leu-tRNAs were then tested for their ability to react with the E–GTP complex (Figure 9). It is clear that the renatured and native Leu-tRNAs are very similar in reactivity, while the denatured Leu-tRNA is much less reactive. The small amount of activity observed for the denatured species may be intrinsic or may be due to the occurrence of a small amount (*ca.* 10%) of renaturation during the work-up or final assay.

Because of a lack of material, competition of denatured Leu-tRNA with native Leu-tRNA was not examined.

TABLE V: Effect of Tetracycline on Ability of AA-tRNA to React with E–GTP Complex.[a]

AA-tRNA Nucleotide Added (mμmoles)	% GTP Bound		
	Control	+Ethanol	+Tetracycline and Ethanol
0	100	100	100
4.8	55	57	51
8.0	45	44	23

[a] Formation of E–GTP complex and reaction with AA-tRNA was carried out as described in Methods except that the reaction mixtures contained 10% ethanol or 7.4×10^{-4} M tetracycline were indicated; 100% bound corresponds to 2120, 2397, and 2170 cpm for columns 2, 3, and 4, respectively.

Discussion

The general mechanism of protein synthesis operative in wheat embryos is undoubtedly very similar to the widely studied process in bacteria and mammals. However, since some differences have been established such as the incompatibility between the transfer enzymes and ribosomes of wheat and *E. coli* (Allende and Bravo, 1966), the presence of a GTP binding protein in wheat extracts with similar properties to the one described in *E. coli* (Allende *et al.*, 1967; Gordon, 1967, 1968) is of some interest. The wheat transfer enzymes have not been studied or fractionated to any extent. It is impossible therefore to discuss their correlation with the three *E. coli* transfer enzymes that have been identified (Lucas-Lenard and Lipmann, 1966). The results shown in Figures 3 and 4 and Table II, however, demonstrate that at least two factors are necessary for polypeptide synthesis in this system. One of them, responsible for GTP binding, could be rapidly inactivated by heating at 42° and was adsorbed on nitrocellulose membranes except when aminoacyl-tRNA was present. Under the conditions used, this factor was not retained by DEAE-cellulose resin. The second factor was not destroyed by mild heating and could be separated from the GTP binding protein by DEAE-cellulose chromatography. This latter factor must have been present in the nitrocellulose membrane filtrates (see Figure 4) that were assayed after the addition of aminoacyl-tRNA.

With the use of this enzyme system from wheat, it has been possible to demonstrate extensive specificity in the recognition of tRNA and AA-tRNA by the E–GTP complex. Reaction can take place only when the amino acid is attached and bears a free amino group. Chemical blockage of the amino function

by acetylation, removal by nitrous acid treatment, or removal of the entire amino acid all make the tRNA inactive. The failure of unacylated tRNA to make the protein–GTP complex filterable has been observed previously in *E. coli* (Ertel *et al.*, 1968b; Gordon, 1968; Ravel *et al.*, 1967), and *B. stearothermophilus* (Skoultchi *et al.*, 1968). However, in these experiments, the tRNA had not previously been incubated in a charging reaction, leaving open the possibility of an effect unrelated to the presence of amino acid on the tRNA. In the present experiments, two methods were used to make deacylated tRNA from AA-tRNA and its lack of activity was confirmed. In addition, the failure to compete with AA-tRNA was demonstrated. The inability of *N*-acetyl-AA-tRNA to make the E–GTP complex filterable has also been observed by others (Ertel *et al.*, 1968b; Ravel *et al.*, 1967) with *E. coli* extracts.

On the other hand, there does not appear to be much, if any species specificity in this reaction. *E. coli* and yeast AA-tRNA were used in these experiments with protein complexes from wheat embryo and efficient reaction occurred as illustrated by the calculated dissociation constant of 8×10^{-8} M. Moreover, AA-tRNA from wheat embryo behaved similarly, although deacylated tRNA from wheat was completely inactive. Lack of species specificity was also observed in the experiments of Skoultchi *et al.* (1968).

Failure of tetracycline to affect the formation of protein–GTP–AA-tRNA complexes indicates that this antibiotic prevents binding to the A site on the ribosome (Gottesman, 1967; Sarkar and Thach, 1968) by interference at the ribosomal level. Indeed this result might have been anticipated since tetracycline blocks nonenzymatic binding to ribosomes (Suarez and Nathans, 1965) as well as enzymatic binding (Lucas-Lenard and Haenni, 1968). The recent report of a specific binding of tetracycline to the 30S subunit (Connamacher and Mandel, 1968) is also consistent with this view.

Similarly the failure of the tetranucleotide TpψpCpGp to affect the interaction of AA-tRNA with E–GTP indicates that its site of action must also be subsequent to the formation of the E–GTP–AA-tRNA complex.

The need for a free amino group on the amino acid of AA-tRNA could be interpreted in at least three ways. (1) A free amino group is all that is essential for correct substrate recognition by the protein–GTP complex; (2) the attachment of an unacylated amino acid alters the conformational properties of the tRNA (Sarin and Zamecnik, 1965) so that it can be recognized by the protein–GTP complex, but the amino group is not recognized directly by the protein; or (3) proper substrate specificity requires a correct tRNA conformation as well as a free amino group on the amino acid.

The experiment with denatured Leu-tRNA (Figure 9) appears to rule out the first explanation, but does not allow a distinction between the second and third alternatives. In agreement with these findings, a recent paper by Ono *et al.* (1968) reports that Met-tRNA^{Met} but not Met-tRNA^{fMet} reacts with the E–GTP complex from *B. stearothermophilus*. Thus Met-tRNA^{fMet} appears to be another example of an amino acid esterified to a "denatured" tRNA which is unable to react.

The specificity requirements for E–GTP–AA-tRNA formation appear to be the way unwanted N-acylated AA-tRNAs are kept from binding to the A site. Although nonenzymatic binding to the A site of both *N*-acetyl- (Suarez and Nathans, 1965; de Groot *et al.*, 1967) and deamino-AA-tRNA (F. Chapeville, personal communication) has been observed, as shown here, enzymatic binding would not be possible for these analogs. One might have expected that the A site itself would have evolved the desired selectivity so that additional factors would not be necessary. However, the apparent lack of inherent specificity may not be accidental in view of the observations of Thach and coworkers (Sarkar and Thach, 1968; Hershey and Thach, 1967) which suggest that F factor dependent binding of fMet-tRNA to ribosomes also occurs initially at the A site. Nevertheless it should be noted that an alternative model has been suggested to explain their findings (Bretscher, 1968).

If the A site must maintain the capacity to bind either N-acylated or free AA-tRNA, one of the functions of T_u and T_s may be to select only free AA-tRNA for chain-elogation reactions. Specifically, fMet-tRNA and Met-tRNA^{fMet} would be excluded by this mechanism from T factor dependent binding in response to internal AUG codons. It should be noted, however, that in the presence of a mRNA containing AUG codons, but not AUG trinucleotide, fMet-tRNA is apparently prevented from binding to 70S ribosomes even at 18–30 mM Mg (Nomura and Lowry, 1967; Salas *et al.*, 1967) suggesting that additional exclusion mechanisms may operate.

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