MOLECULAR PHARMACOLOGY, 9, 229–236

Increased Binding of Transfer Ribonucleic Acid Species to Ribosomes under Conditions Interfering with Their Aminoacylation

KENNETH BALKOW[1] AND MARCO RABINOVITZ

Laboratory of Physiology, National Cancer Institute, Bethesda, Maryland 20014

(Received November 2, 1972)

SUMMARY

BALKOW, KENNETH AND RABINOVITZ, MARCO: Increased binding of transfer ribonucleic acid species to ribosomes under conditions interfering with their aminoacylation *Mol. Pharmacol.* **9**, 229–236 (1973).

The α-amino alcohols, histidinol and valinol, inhibited protein synthesis in the rabbit reticulocyte cell-free system by interfering with activation of histidine and valine, respectively. The lower rate of translation resulted in an increase in the polyribosome component of such systems. Histidinol promoted an accumulation of $tRNA^{His}$ associated with ribosomes, and a similar result was found with valinol. The data indicate that deacylated tRNA is bound to ribosomes in a protein-synthesizing system when the acylated form is unavailable.

INTRODUCTION

The generally accepted mechanism of protein biosynthesis (1, 2) requires that aminoacyl-tRNA molecules bind to messenger codons sequentially, and that the amino acid residue be incorporated into growing peptide chains by peptidyltransferase, a ribosome-associated activity. In agreement with this mechanism, a soluble protein (elongation factor I) which is active in the binding of aminoacyl-tRNA to the ribosome has been isolated from many cell types. An intermediate in this process is a ternary complex between elongation factor, aminoacyl-tRNA, and GTP (3–9). Evidence has accumulated that elongation factor I recognizes only the charged form of tRNA (3, 6, 10).

The α-amino alcohols, histidinol and valinol, are analogues of histidine and valine, respectively. They cannot attach to tRNA or be incorporated into protein, and are shown here to be effective inhibitors of aminoacyl-tRNA formation and protein synthesis in reticulocyte cell-free systems. The consequent decrease in the rate of translation causes the polyribosome component of lysates to increase. Unexpectedly, we found that histidinol also increased the amount of $tRNA^{His}$ associated with ribosomes. Analogous results were found with valinol. Thus it appears that the deacylated forms of tRNA are not necessarily excluded from binding to ribosomes synthesizing polypeptide chains. The increase in tRNA binding and polysome content, as well as the inhibition of protein synthesis, could be completely overcome or reversed by the addition of an excess of the corresponding natural amino acid. The significance of these results to the mechanism of transfer RNA

[1] Fellow of the Damon Runyon Memorial Fund for Cancer Research.

binding to ribosomes during protein synthesis is discussed.

MATERIALS AND METHODS

Reagents. L-Histidinol was purchased from Calbiochem, and DL-valinol from the Research Organic/Inorganic Chemical Corporation. The following uniformly labeled [^{14}C]L-amino acids were purchased from New England Nuclear Corporation: valine (210 Ci/mole), histidine (252 Ci/mole), and lysine (260 Ci/mole).

Preparation of rabbit reticulocytes and cell-free extracts. The procedure for preparation of rabbit reticulocyte lysates and supernatant fractions has been previously described (11).

Aminoacyl-tRNA synthetases were isolated from a pH 5 precipitate of reticulocyte supernatant with the use of DEAE-cellulose as described by Muench and Berg (12). The enzyme preparation was stored at a concentration of 8 mg/ml at −80°, and contained 1 mM potassium phosphate buffer (pH 6.8), 10% glycerol, and 20 mM β-mercaptoethanol.

Transfer RNA was prepared from a pH 5 precipitate of reticulocyte supernatant with the use of the phenol extraction procedure described below for ribosomal RNA. It was dissolved in water at a concentration of 2 mg/ml and stored at −20°. A solution containing 1 mg of RNA per milliliter was assumed to have an absorbance of 24 at 260 nm.

Protein synthesis in lysate cell-free system. Optimal conditions for protein synthesis in the reticulocyte cell-free system have been previously described (11). The final hemin concentration used in all experiments was 35 μM, and incubations were performed at 34°. In the experiments described in Figs. 1–3 the concentration of leucine added was 30 μM, and the specific activity of [^{14}C]leucine was 50 Ci/mole. In all other experiments the leucine concentration was 300 μM. An aliquot (25 μl) of the synthesizing system was used to determine the radioactivity incorporated into protein. It was added to 6 ml of cold 10 mM NaCl containing 24 μmoles of [^{12}C]L-leucine. Two milliliters of 20% trichloracetic acid were added to this mixture while it was being vigorously agitated. After 15 min at 0°, 20 min at 85°, and another 15 min at 0°, the suspension was filtered on to a Millipore filter (0.45-μ pore size). It was washed with 5% trichloracetic acid, dried, and counted in a gas flow counter with a Micromil window at an efficiency of 34%.

Preparation of ribosomes and ribosomal RNA. In those experiments in which ribosomal RNA was to be isolated from the incubated lysate, the standard incubation volume was scaled up at least 40 times (final volume, 4.4 ml) and all amino acids were present in the nonradioactive form. After incubation, the tubes were cooled to 0°, diluted with 2.3 volumes of 10 mM Tris-HCl (pH 7.4)–30 mM KCl–2 mM magnesium acetate, and the sample was layered over a cushion of 35% (w/v) sucrose in the same buffer. The material was centrifuged for 4 hr at 254,000 × *g* (average) in the 60Ti rotor of a Beckman L2-65B ultracentrifuge. The ribosome pellet was rinsed with distilled water and suspended in 2.5 ml of buffer A [10 mM Tris-HCl (pH 7.4)–100 mM KCl–2 mM magnesium acetate]. The suspension was shaken for 30 min at room temperature with 0.1 volume of 5.5% sodium dodecyl sulfate and an equal volume of phenol saturated with buffer A. The aqueous layer was separated by centrifugation, and the phenol layer was re-extracted with 2.5 ml of buffer A. The pooled aqueous layers were precipitated with 0.1 volume of 1 M ammonium acetate, pH 5, and 3 volumes of ethanol. The precipitated RNA was recovered by centrifugation, washed once with ethanol, and dissolved in 2.0 ml of 1.8 M Tris-HCl, pH 8. After incubation at 37° for 90 min to deacylate aminoacyl-tRNA (13), the pH was adjusted to 5 with 1 M ammonium acetate and 1 M acetic acid and the tRNA was reprecipitated with 3 volumes of ethanol. The final precipitate was washed once with ethanol and dissolved in water at a concentration of 1.5 mg/ml. This crude RNA preparation, which contains ribosome-bound tRNA, was assayed for amino acid acceptor capacity as described below.

Aminoacylation of tRNA. The amino acid acceptor capacity of RNA preparations was determined in an incubation mixture similar to that described by Pawelek *et al.* (14).

The final concentrations of added components were as follows: ATP, 8 mM; magnesium acetate, 12 mM; Tris-HCl, pH 7.4, 50 mM; β-mercaptoethanol, 3 mM; unpurified ribosomal RNA, 0.75 mg/ml; aminoacyl-tRNA synthetases, 0.4 mg/ml; and [^{14}C]-amino acid, 0.5 μCi/ml, 2 μM. The final volume of each incubation mixture was 0.2 ml, and tubes were incubated at 37° for 10 min. In some experiments supernatant tRNA, 0.05 mg/ml, replaced the unpurified ribosomal RNA preparation. It has been reported that 1.67 is the optimal Mg^{2+}:ATP ratio for enzymes charging either lysine, histidine, or valine in this system (15). This is close to the value of 1.5 used in these studies.

Reactions were terminated by the addition of 3 ml of cold 5% trichloracetic acid containing 2 mg/ml of valine, lysine, and histidine. The precipitated RNA was collected on Millipore filters, washed with cold 5% trichloracetic acid, and dried in a vacuum oven. The filters were placed in toluene scintillator, and the RNA-associated radioactivity was counted in a Packard liquid scintillation spectrometer, with an efficiency of 85% for ^{14}C.

Periodate oxidation of ribosome-bound tRNA. Preparations of ribosomal RNA were chromatographed on DEAE-cellulose to remove high molecular weight RNA. Approximately 5 mg of RNA were adsorbed on a column (10 cm × 4 mm) of Whatman DE-52 that had been equilibrated with 0.1 M NaCl and 50 mM ammonium acetate (pH 5). The column was washed with the same buffer, and the low molecular weight RNA (a mixture of transfer RNA and ribosomal 5 S RNA) was eluted with 1 M NaCl and 50 mM ammonium acetate (pH 5). RNA was recovered from the column fractions by precipitation with ethanol. A portion was dissolved in 1.8 M Tris-HCl, pH 8 (approximately 1 mg/ml), and incubated at 37° for 90 min to deacylate charged tRNA. The RNA was reprecipitated at pH 5 by the addition of 3 volumes of ethanol and used for aminoacylation studies as described above. A second portion was dissolved in water at a concentration of 1 mg/ml and subjected to mild oxidation by periodate as described by Mosteller *et al.* (16). A 0.1-ml aliquot was incubated with 0.05 ml of 2.2 mM sodium metaperiodate and 0.05 ml of 0.13 M ammonium acetate (pH 5) for 10 min at 20°; 0.067 ml of 0.01 M glucose was added, and the incubation was continued for a further 10 min. The tRNA was precipitated by the addition of 0.1 volume of 1 M ammonium acetate (pH 5) and 3 volumes of ethanol, and then subjected to deacylation in 1.8 M Tris-HCl (pH 8) as described for the untreated preparation. The oxidized tRNA was reisolated by ethanol precipitation and used for aminoacylation studies as described above.

Sucrose gradient analysis of ribosome-polyribosome component of lysates. The standard lysate incubation mixture was diluted with 1.2 volumes of 10 mM Tris-HCl (pH 7.5)–30 mM KCl–2 mM magnesium acetate, and a 0.4-ml sample was layered over a linear 15–30% (w/w) sucrose gradient in the same buffer. The gradients were centrifuged for 75 min at 40,000 rpm in the SW 41 rotor of a Beckman L2-65B ultracentrifuge and analyzed as previously described (17).

RESULTS

Effect of amino acid analogues on protein synthesis. The effect of 1 mM L-histidinol or 10 mM DL-valinol on the time course of protein synthesis in the reticulocyte cell-free system is shown in Fig. 1. Both analogues decreased the rate of protein synthesis by approximately 95%. The experiments described later on the binding of tRNA to ribosomes utilized these same concentrations of amino acid analogues. However, neither histidinol nor valinol inhibited protein synthesis in the presence of an excess of the corresponding natural amino acid (Figs. 2 and 3). This suggested that they both inhibit protein synthesis by interfering specifically with the activation of a single amino acid. Further support for this view came from the experiments described in the next section.

Effect of amino acid analogues on aminoacyl-tRNA formation. Crude aminoacyl-tRNA synthetases were used to investigate the effects of amino acid analogues on charging of reticulocyte tRNA (see MATERIALS AND METHODS). Histidinol (1 mM) and

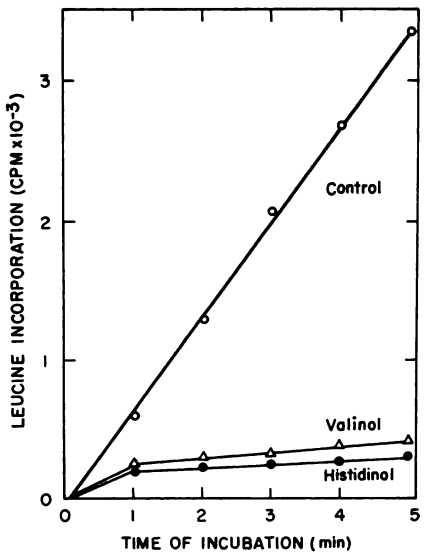

FIG. 1. *Effect of histidinol and valinol on kinetics of protein synthesis*

The reticulocyte cell-free system was incubated in the presence of a complete amino acid mixture (○——○), in the presence of L-histidinol (1 mM) and an amino acid mixture lacking histidine (●——●), or in the presence of DL-valinol (10 mM) and an amino acid mixture lacking valine (△——△). Aliquots (25 μl) were removed at the indicated times, and the leucine incorporation was determined as described in MATERIALS AND METHODS.

valinol (10 mM) blocked formation of the corresponding aminoacyl-tRNA by over 95% in a 10-min incubation period (Figs. 4 and 5). Neither analogue affected the aminoacylation of $tRNA^{Lys}$.

Polyribosome profiles of lysates incubated with amino acid analogues. During the first 5 min of incubation of the lysate system with a complete amino acid mixture the polyribosome profile underwent little change, except for some degradation of the largest polyribosomes (Fig. 6A and B). In the presence of histidinol (1 mM) or valinol (10 mM) there was considerable conversion of monomeric ribosomes and ribosomal subunits to polyribosomes (Fig. 6C and 6D). This is attributed to the decreased rate of translation, enabling messenger RNA to become almost saturated with ribosomes. Similar observations were reported by Lodish (18), who utilized antibiotics to restrict the rate of ribosome movement. In preparations containing some lysates, a proportion of certain polyribosome components sedimented more rapidly after incubation with the amino acid analogues. This is particularly evident in the dimer and trimer regions of the gradients of Fig. 6C and 6D, where split peaks can clearly be seen. Similar species were found by Hoerz and McCarty (19, 20) in lysates during the onset of inhibition of chain initiation by sodium fluoride, and were shown to consist of polyribosomes with an extra attached 40 S ribosomal subunit. This suggested to these authors that fluoride inhibited the attachment of the 60 S subunit to the initiation complex. Since there is no reason to assume that the α-amino alcohols cause direct blockade of this process, the access of the 60 S ribosomal subunit must be hindered by some secondary, pos-

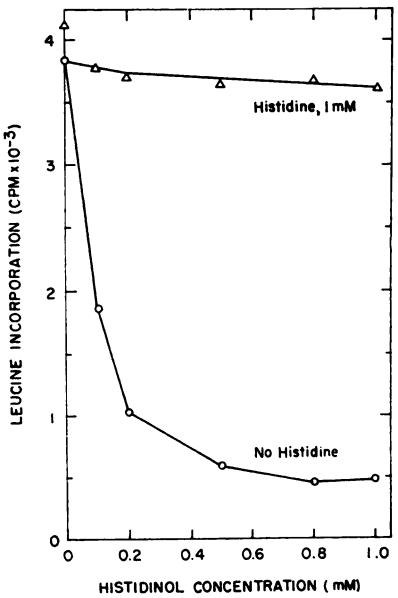

FIG. 2. *Effect of histidine on inhibition of protein synthesis by histidinol*

Samples of the reticulocyte cell-free system were incubated with different concentrations of histidinol and an amino acid mixture lacking histidine. After 5 min of incubation at 34°, 25-μl samples were removed to determine leucine incorporation (see MATERIALS AND METHODS). Another set of tubes was incubated under the same conditions, but with the addition of 1 mM L-histidine.

sibly steric, effect. This phenomenon is currently under further investigation.

Effect of amino acid analogues on association of tRNA with ribosomes. After incubation of the reticulocyte cell-free system with 1 mM histidinol or 10 mM valinol, the amount of histidine- or valine-accepting tRNA bound to ribosomes was found to increase (Table 1). A much more marked increase was noted with histidinol than with valinol. The amount of bound lysine-accepting tRNA was also found to increase, presumably because of the increased proportion of polyribosomes present in the lysate. The increase of $tRNA^{His}$ and $tRNA^{Val}$ under conditions in which their aminoacylation is almost completely blocked suggests that the deacylated forms of these tRNA species are capable of binding to ribosomes. No increase of $tRNA^{Val}$ binding to ribosomes occurred in the presence of 5 mM NaF (Table 2).

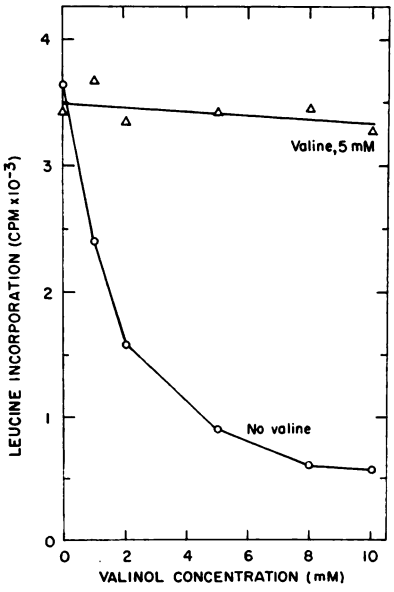

FIG. 3. *Effect of valine on inhibition of protein synthesis by valinol*

Samples of the reticulocyte cell-free system were incubated with different concentrations of valinol and an amino acid mixture lacking valine. After 5 min of incubation at 34°, 25-μl samples were removed to determine leucine incorporation (see MATERIALS AND METHODS). Another set of tubes was incubated under the same conditions, but with the addition of 5 mM L-valine.

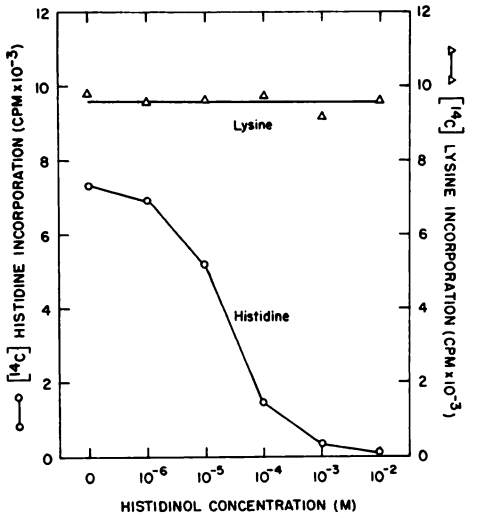

FIG. 4. *Effect of histidinol on charging of tRNA*

tRNA was extracted from reticulocyte supernatant fraction and used as substrate for aminoacylation as described in MATERIALS AND METHODS. Each incubation mixture contained 0.05 mg/ml of tRNA and different concentrations of histidinol as indicated.

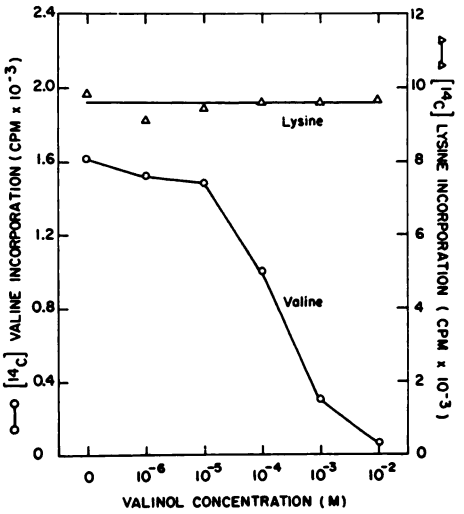

FIG. 5. *Effect of valinol on charging of tRNA*

Experimental conditions were the same as described in Fig. 4.

Since fluoride causes polyribosome disaggregation in the reticulocyte cell-free system (19), this observation suggests that the

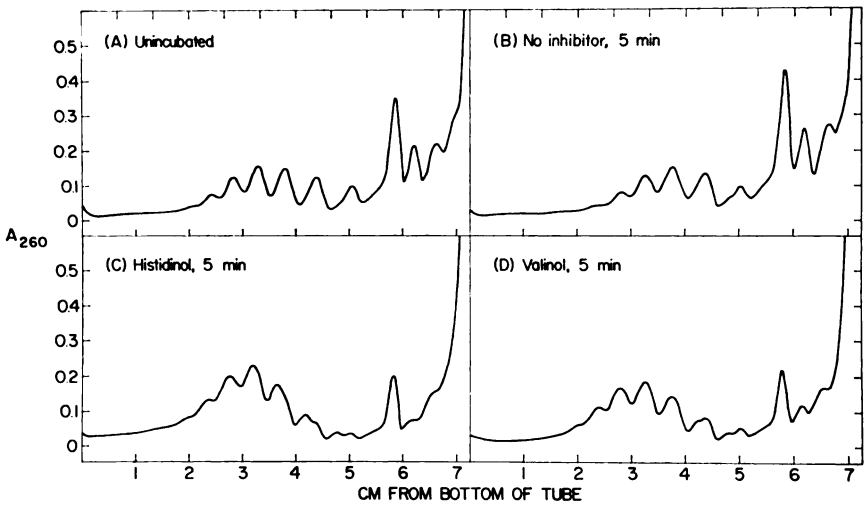

FIG. 6. *Ribosome aggregation in the presence of amino acid analogues*

The reticulocyte cell-free system was incubated for 5 min in the presence and absence of amino acid analogues, with the amino acid mixtures described in the legend to Fig. 1. Samples were analyzed on sucrose gradients as described in MATERIALS AND METHODS. The direction of sedimentation is from right to left. A. Unincubated lysate. B. No inhibitor. C. L-Histidinol, 1 mM. D. DL-Valinol, 10 mM.

accumulated tRNA is bound to the polyribosomes. The observed decrease of ribosome-bound $tRNA^{Val}$ after fluoride treatment is in agreement with the results of Culp *et al.* (21).

We attempted to verify that the tRNA which accumulated on ribosomes in the presence of histidinol was in the deacylated form by subjecting it to mild oxidation with periodate. This procedure destroys the acceptor capacity of deacylated tRNA, but charged tRNA is protected against periodate attack (22). As expected, the ribosome-bound tRNA retained only 5% of its acceptor capacity for histidine after such treatment (Table 3). Although this value was significantly lower than that obtained for $tRNA^{His}$ from control ribosomes, it should be noted that a large proportion of the $tRNA^{His}$ and $tRNA^{Lys}$ in each preparation was periodate-sensitive. However, it is difficult to obtain quantitative results with this technique, since we do not know the extent to which tRNA becomes deacylated during the isolation procedure. It is also to be expected that ribosomes contain some deacylated tRNA which has already participated in peptide bond formation and is awaiting release. That such tRNA may constitute an appreciable portion of ribosome-bound tRNA has been demonstrated in a rat liver system by Wettstein and Noll (23).

DISCUSSION

The binding of deacylated tRNA to ribosomes has been studied directly by a number of workers (24–29). Levin and Nirenberg (24) reported that $tRNA^{Phe}$ and Phe-$tRNA^{Phe}$ were bound with approximately equal affinities to ribosomes containing a poly U template. It was further suggested (25) that deacylated tRNA might function as an inhibitor of protein synthesis under conditions of low amino acid supply. However, since these studies were carried out in the absence of components required for protein synthesis and at very high magnesium ion concentrations (20 mM), it is possible that deacylated tRNA might be excluded from binding to ribosomes under physiological conditions. Indeed, binding of aminoacyl-tRNA to ribosomes catalyzed by elongation factor I and GTP is not inhibited by deacylated tRNA (6, 30). Moreover, elongation factor I can form a ternary complex with GTP and aminoacyl-tRNA but not with uncharged tRNA (3, 6, 10).

In the present experiments charging of

tRNA with valine or histidine in the reticulocyte cell-free protein-synthesizing system was prevented by the addition of the corresponding α-amino alcohol. Consequently there was severe inhibition of protein synthesis and an increase in the proportion of polyribosomes in the lysate. However, increased amounts of valine- or histidine-accepting tRNA were recovered from ribosomes after treatment with the appropriate analogue, suggesting that charging of tRNA is not an absolute prerequisite for ribosome binding. Addition of an excess of the corresponding natural amino acid caused the amount of ribosome-bound valine or histidine tRNA to return to its original level. A further decrease in the level of $tRNA^{Val}$ was observed in the presence of NaF, which inhibits chain initiation and causes disaggregation of polyribosomes in this system. This suggested that the accumulated $tRNA^{Val}$ was bound to the polyribosome

TABLE 1

Effect of valinol and histidinol on binding of tRNA to ribisomes

The protein-synthesizing system was incubated with either an amino acid mixture lacking valine (A) or an amino acid mixture lacking histidine (B), with the additional components described below. The ribosome fraction was isolated by centrifugation, and RNA was extracted and assayed for amino acid acceptor capacity as described in MATERIALS AND METHODS. Assays were performed in duplicate, and blank values (no added RNA) of 0.5 for valine, 0.7 for lysine, and 2.7 for histidine have been subtracted.

	Acceptor capacity	
	Valine	Lysine
	pmoles amino acid/mg total RNA	
A. Valine omitted		
No inhibitor, 5 min	9.3	10.9
10 mM valinol, 5 min	17.7	18.0
10 mM valinol, 5 min; then 10 mM valine, 5 min	9.6	11.9
B. Histidine omitted	Histidine	Lysine
No inhibitor, 5 min	13.7	10.8
1 mM histidinol, 5 min	60.2	15.5
1 mM histidinol, 5 min; then 2 mM histidine, 5 min	14.7	11.5

TABLE 2

Effect of sodium fluoride on valinol-induced accumulation of $tRNA^{Val}$

The reticulocyte cell-free system was incubated for 5 min with an amino acid mixture lacking valine, with the additional components described below. The ribosome fraction was isolated by centrifugation, and RNA was extracted and assayed for amino acid acceptor capacity as described in MATERIALS AND METHODS. Assays were performed in duplicate, and blank values (no added RNA) of 0.5 for valine and 1.0 for lysine have been subtracted.

System	Acceptor capacity	
	Valine	Lysine
	pmoles amino acid/mg total RNA	
No inhibitor	11.8	13.3
5 mM valinol	20.1	20.6
5 mM NaF	7.7	4.8
10 mM valinol + 5 mM NaF	5.8	4.2

TABLE 3

Sensitivity of ribosome-bound tRNA to periodate oxidation

The protein-synthesizing system was incubated for 5 min with an amino acid mixture lacking histidine and with the addition of either 1 mM L-histidinol (A) or 1 mM L-histidine (B). The ribosome fraction was obtained by centrifugation, and bound tRNA was isolated as described in MATERIALS AND METHODS. The procedure for periodate oxidation is also described in MATERIALS AND METHODS. Assays for amino acid acceptor capacity were performed in duplicate, and contained 0.05–0.10 mg/ml of RNA. Blank values of 37 for histidine and 16 for lysine have been subtracted.

System	Acceptor capacity	
	Histidine	Lysine
	pmoles amino acid/mg total RNA	
A. 1 mM L-histidinol added		
Periodate-treated	117	102
Untreated	2260	510
Activity remaining (%)	5	20
B. 1 mM L-histidine added		
Periodate-treated	62	124
Untreated	322	284
Activity remaining (%)	19	44

fraction. Moreover, the parallel changes in polyribosome content and binding of $tRNA^{Val}$ or $tRNA^{His}$ indicate that binding is codon-specific. These results suggest either that elongation factor I is not absolutely specific for the charged form of tRNA or that uncharged tRNA binds to ribosomes by a pathway which does not involve interaction with elongation factor I. The design of the present experiments does not yet enable us to decide between these possibilities.

An additional possibility is that the deacylated tRNA accumulates in a complex of aminoacyl-tRNA synthetases. Such complexes, which are of high molecular weight and contaminate crude ribosome preparations, have recently been described in mammalian cells (31–34). However, since accumulation of tRNA is not observed in the presence of either NaF or the natural amino acid, it cannot simply be the result of contamination with a free synthetase complex.

REFERENCES

1. P. Lengyel and D. Söll, *Bacteriol. Rev.* **33**, 264–301 (1969).
2. F. Lipmann and J. Lucas-Lenard, *Annu. Rev. Biochem.* **40**, 409–448 (1971).
3. J. M. Ravel, R. L. Shorey, C. W. Garner, R. C. Dawkins, and W. Shive, *Cold Spring Harbor Symp. Quant. Biol.* **34**, 321–330 (1969).
4. J. Gordon, *Proc. Nat. Acad. Sci. U. S. A.* **58**, 1574–1578 (1967).
5. H. Weissbach, N. Brot, D. Miller, M. Rosman, and R. Ertel, *Cold Spring Harbor Symp. Quant. Biol.* **34**, 419–431 (1969).
6. A. Skoultchi, Y. Ono, J. Waterson, and P. Lengyel, *Cold Spring Harbor Symp. Quant. Biol.* **34**, 437–454 (1969).
7. J. Lucas-Lenard, P. Tao, and A. Haenni, *Cold Spring Harbor Symp. Quant. Biol.* **34**, 455–462 (1969).
8. F. Ibuki and K. Moldave, *J. Biol. Chem.* **243**, 791–798 (1968).
9. J. Hradec, *Biochem. J.* **126**, 933–943 (1972).
10. F. Ibuki and K. Moldave, *J. Biol. Chem.* **243**, 44–50 (1968).
11. C. R. Maxwell, C. S. Kamper, and M. Rabinovitz, *J. Mol. Biol.* **58**, 317–327 (1971).
12. K. H. Muench and P. Berg, "Procedures in Nucleic Acid Research" pp. 375–383, Harper and Row, New York, (1966).
13. P. Sarin and P. C. Zamecnik, *Biochim. Biophys. Acta* **91**, 653–655 (1964).
14. J. Pawelek, W. Godchaux, III, J. Grasso, A. I. Skoultchi, J. Eisenstadt, and P. Lengyel, *Biochim. Biophys. Acta* **232**, 289–305 (1971).
15. D. W. E. Smith and A. L. McNamara, *Science* **171**, 577–579 (1971).
16. R. D. Mosteller, W. J. Culp, and B. Hardesty, *J. Biol. Chem.* **243**, 6343–6352 (1968).
17. S. Mizuno, J. M. Fisher, and M. Rabinovitz, *Biochim. Biophys. Acta* **272**, 638–650 (1972).
18. H. F. Lodish, *J. Biol. Chem.* **246**, 7131–7138 (1971).
19. W. Hoerz and K. S. McCarty, *Proc. Nat. Acad. Sci. U. S. A.* **63**, 1206–1213 (1969).
20. W. Hoerz and K. S. McCarty, *Biochim. Biophys. Acta* **228**, 526–535 (1971).
21. W. Culp, J. Morrisey, and B. Hardesty, *Biochem. Biophys. Res. Commun.* **40**, 777–785 (1970).
22. J. Preiss, P. Berg, E. Ofengand, F. Bergmann, and M. Dieckmann, *Proc. Nat. Acad. Sci. U. S. A.* **45**, 319–328 (1959).
23. F. O. Wettstein and H. Noll, *J. Mol. Biol.* **11**, 35–53 (1965).
24. J. G. Levin and M. Nirenberg, *J. Mol. Biol.* **34**, 467–480 (1968).
25. J. G. Levin, *J. Biol. Chem.* **245**, 3195–3202 (1970).
26. A. Kaji and H. Kaji, *Biochem. Biophys. Res. Commun.* **13**, 186–192 (1963).
27. H. Kaji and A. Kaji, *Proc. Nat. Acad. Sci. U. S. A.* **52**, 1541–1547 (1964).
28. N. W. Seeds, J. A. Retsema, and T. W. Conway, *J. Mol. Biol.* **27**, 421–430 (1967).
29. J. Hradec, *Biochem. J.* **126**, 923–931 (1972).
30. J. M. Ravel, R. L. Shorey, S. Froehner, and W. Shive. *Arch. Biochem. Biophys.* **125**, 514–526 (1968).
31. A. K. Bandyopadhyay and M. P. Deutscher, *J. Mol. Biol.* **60**, 113–122 (1971).
32. W. K. Roberts and W. H. Coleman, *Biochem. Biophys. Res. Commun.* **46**, 206–214 (1972).
33. C. J. G. M. Vennegoor, A. L. H. Stols, and H. Bloemendal, *J. Mol. Biol.* **65**, 375–378 (1972).
34. J. D. Irvin and B. Hardesty, *Biochemistry* **11**, 1915–1920 (1972).