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Covalent Attachment of a Peptidyl-Transfer RNA Analog to the 50S Subunit of *Escherichia coli* Ribosomes

(protein synthesis/acrylamide gel/α-bromoamide/affinity label)

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ABSTRACT The peptidyl-tRNA analog, *N*-bromoacetyl-Phenylalanyl-tRNA^{Phe} has been prepared. Its binding to the 70S ribosome of *E. coli* is totally dependent upon polyuridylic acid. The analog becomes covalently attached to the 50S particle. It is associated with only one protein fraction after polyacrylamide-gel separation of total 50S proteins. The analog also reacts with 23S ribosomal RNA or a protein that remains tightly bound to this RNA after treatment with LiCl–urea and sodium dodecyl sulfate. The analog can function as a peptidyl-tRNA for at least one peptide transfer, but it then inhibits further chain elongation. This result strongly suggests that this analog becomes covalently bound at the P-site of the ribosome.

70S Ribosomes of *Escherichia coli* contain more than 50 different components. The exact structural and functional role of any of these components in protein synthesis is not known with clarity. While a great deal of progress has been made in characterizing the location and function of 30S proteins (1), much less is known about the proteins of the 50S particle. At least one central step of protein synthesis, the formation of peptide bonds, is localized exclusively on the 50S particle (2, 3). The critical component(s) for this function, peptidyl transfer, have not been unambiguously identified. In this work, we report an effort to identify a 50S ribosomal protein that is located adjacent to the peptide group of ribosome-bound peptidyl-tRNA. Such a protein would be a likely candidate to be peptidyl transferase.

The approach used in this work is an extension of the idea of affinity labeling (4). In studies of individual enzymes, substrate analogs bearing a chemically reactive group have been used to probe the location of residues at or near an active site. In the present work, the protein-reactive group is attached to a tRNA rather than to a small molecule. Initially, we are interested in determining to which of the ribosomal components this reagent can couple. The identification of the particular groups within a protein or RNA molecule that have been labeled is reserved for future studies. It should be evident that affinity labeling in this manner can provide a critical bridge between the relatively imprecise kind of structural information available about a large particle like the ribosome and the high-resolution structural data accessible for single proteins. On the other hand, for a structure as complex as the ribosome, it is not clear how specifically defined a given binding site will be.

METHODS AND RESULTS

Ribosomes

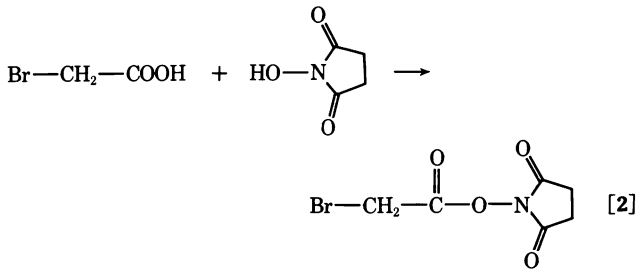
70S ribosomes were prepared from mid-logarithmic-phase *E. coli* Q13 by the method of Traub *et al.* (5). The following buffers were used: A [10 mM Tris (pH 7.4)–50 mM NH_4Cl–6 mM 2-mercaptoethanol–10 mM $MgCl_2$]; B (same as A, except 0.25 mM $MgCl_2$); C (same as A, except 1.0 μM $MgCl_2$ and no 2-mercaptoethanol). 30S and 50S ribosomal subunits were prepared from 70S ribosomes by dialysis against buffer B for 48 hr at 4°, and were separated on 10–30%, 38-ml sucrose gradients in an SW 27 rotor at 25,000 rpm for 16 hr. Unfolded ribosomes were prepared by dialysis of 70S particles against buffer C for 48 hr at 4°. Analytical sucrose gradients of 5 ml were centrifuged in an SW 50.1 rotor at 45,000 rpm for 1–4 hr.

Preparation of the peptidyl-tRNA analog

Previous studies have shown that *N*-acetyl-Phe-tRNA^{Phe} behaves like a peptidyl-tRNA in the presence of the synthetic mRNA, poly(U) (6, 7). As an affinity label, we have synthesized *N*-bromoacetyl-Phe-tRNA^{Phe} (BrAcPhe-tRNA) as shown below. Labeled Phe-tRNA^{Phe} was prepared from unfractionated *E. coli* B tRNA and [^{14}C]Phe (450 Ci/mol) with a crude supernatant fraction from *E. coli* Q13 (8, 9).

$$\text{ATP} + \text{tRNA}^{Phe} + \text{Phe} \rightarrow \text{Phe-tRNA}^{Phe} + \text{AMP} + \text{PP}_i \quad [1]$$

N-hydroxysuccinimide was condensed with α-bromoacetic acid in ethyl acetate containing one equivalent of dicyclohexylcarbodiimide, a procedure analogous to that of de Groot *et al.* (10).



[2]

The product of this reaction was dissolved in dimethyl-

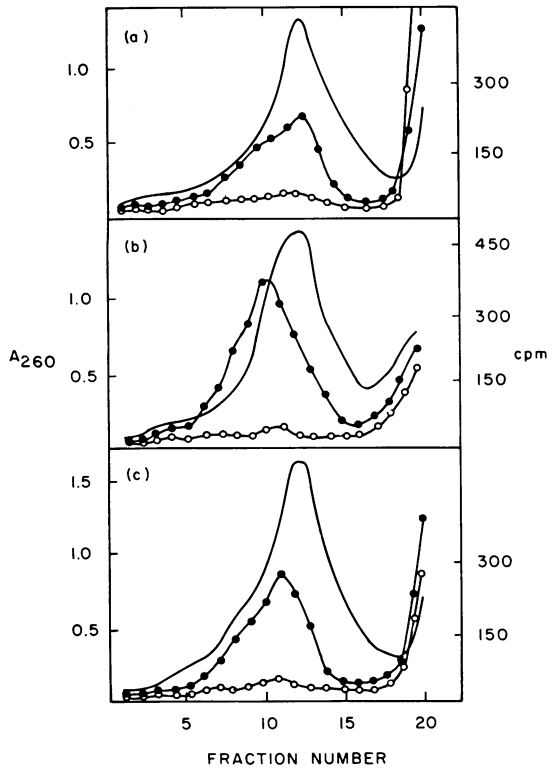


FIG. 1. Binding of Phe-tRNA and derivatives to 70S ribosomes. —— A_{260}; ●——● cpm [^{14}C]Phe with 0.1 A_{260} of poly(U) per 2 A_{260} of ribosomes; O——O cpm [^{14}C]Phe without poly(U). All samples were run on 5–25% sucrose gradients for 1 hr after a 30-min incubation at 37° in buffer A. In addition to ribosomes, samples contained (*a*) Phe-tRNA; (*b*) *N*-acetyl-Phe-tRNA; (*c*) BrAcPhe-tRNA, each at equal concentrations.

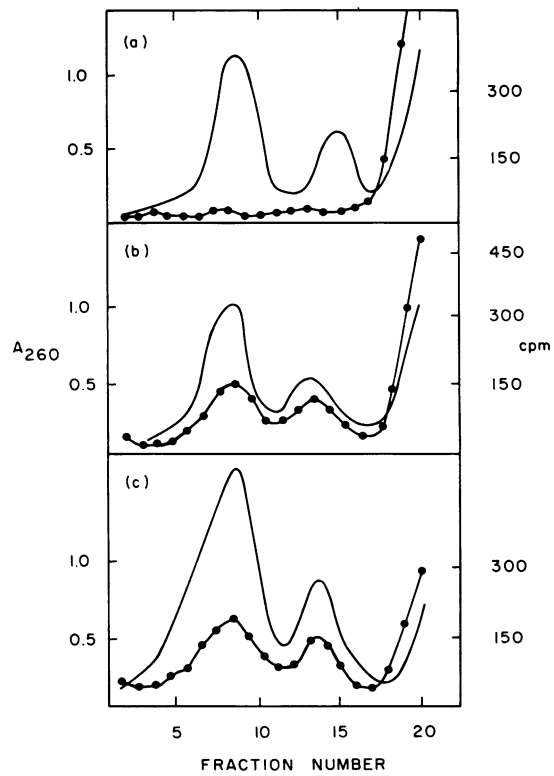
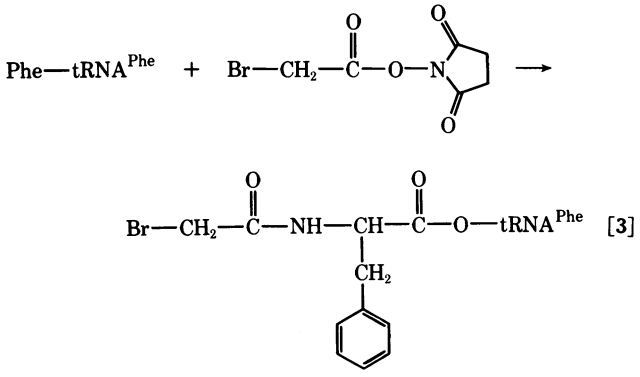


FIG. 2. Retention of Phe-tRNA and derivatives on 30S and 50S ribosomes after dissociation of 70S particles by dialysis against buffer B. —— A_{260}; ●——● cpm [^{14}C]Phe, on 10–30% sucrose gradients for 2 hr. These samples were prepared from the samples containing poly(U) shown in Fig. 1. Samples contained (*a*) Phe-tRNA; (*b*) *N*-acetyl-Phe-tRNA; (*c*) BrAcPhe-tRNA.

sulfoxide and added in a 2:1 weight ratio to a solution of Phe-tRNA in 50 mM phosphate buffer (pH 6.8). Incubation at 37° for 3 hr yielded BrAcPhe-tRNA.



[3]

N-acetyl-Phe-tRNA^{Phe} was made in an exactly comparable way. BrAcPhe-tRNA differs from the *N*-acetyl derivative simply by replacement of one hydrogen by a bromine. This alteration should not cause any serious steric perturbations, as the bromine is located at the position normally occupied by an amino-acid side chain in a natural peptidyl-tRNA. In practice, the product of reaction [3] typically contained 65–70% affinity label; the remainder was unreacted Phe-tRNA^{Phe}, as determined by Cu^{2+} hydrolysis (11). The α-bromoamide moiety of the affinity label can be expected to react with free amino groups, lysine, cysteine, or histidine residues of proteins (12), as well as with certain RNA bases (13).

BrAcPhe-tRNA binding to ribosomes and subunits

Initial binding of the affinity label to 70S ribosomes from *E. coli* parallels that of *N*-acetyl-Phe-tRNA^{Phe}. In 10 mM Mg^{2+} the binding is totally directed by poly(U). Though the extent of BrAcPhe-tRNA binding is only half that of *N*-acetyl-Phe-tRNA^{Phe}, it is equal to that of Phe-tRNA^{Phe}. These results are shown in Fig. 1.

Ribosomes from each sample shown in Fig. 1 were separated into 50S and 30S subparticles by dialysis against buffer B. Sucrose gradient analysis of the dialyzed samples showed that both in the case of BrAcPhe-tRNA and *N*-acetyl-Phe-tRNA^{Phe}, radioactivity remained with both subunits. In contrast, samples that contained Phe-tRNA^{Phe} lost essentially all of their bound tRNA when the 70S ribosomes were separated into subparticles. These results are shown in Fig. 2.

To obtain some preliminary evidence as to whether the BrAcPhe-tRNA was covalently attached, tRNA-containing ribosomes were dialyzed against 1 μM Mg^{2+}. This dialysis produces unfolded subparticles, and results in a series of more-slowly sedimenting peaks on a sucrose gradient (see Fig. 3 *a*, *b*). No *N*-acetyl-Phe-tRNA^{Phe} remains attached to unfolded ribosomal subparticles. In contrast, ribosomes

reacted with BrAcPhe-tRNA retain about 90% of their initial bound tRNA when the ribosomes are unfolded. The fact that unfolding of the ribosome is necessary to release *N*-acetyl-Phe-tRNA^{Phe} is consistent with earlier work of Gilbert (14, 15), who showed that unfolding was also necessary to release bound peptidyl-tRNA.

Two controls were performed to assure that the BrAcPhe-tRNA reaction had occurred with a specific ribosome site (or sites), and was not some kind of random or nonfunctionally significant binding. All binding observed is poly(U) dependent. If BrAcPhe-tRNA is added in the presence of poly(U), but the 30-min incubation is omitted, no tRNA remains bound to subsequently unfolded ribosomes. This result is shown in Fig. 3*b*. The ability of *E. coli* 70S ribosomes reacted with BrAcPhe-tRNA to perform poly(U)-directed polyphenylalanine synthesis after addition of GTP and a ribosomal supernatant to the sample is strongly inhibited (Table 1). The success of the controls, and the parallelism of binding of BrAcPhe-tRNA and *N*-acetyl-Phe-tRNA^{Phe} provide circumstantial evidence that the affinity label is binding to the P-site of the ribosomes. If this were so, aminoacyl-tRNA should still be able to bind to the A-site of BrAcPhe-tRNA-treated 70S ribosomes. In addition, since the formation of a peptide bond requires no additional factors (2), it might be possible to observe one step of the peptidyl transferase reaction—formation of a peptide bond between the bromoacetyl-Phe moiety of BrAcPhe-tRNA and the Phe-tRNA^{Phe}. To examine this point, nonradioactive BrAcPhe-tRNA or *N*-acetyl-Phe-tRNA^{Phe} and radioactive Phe-tRNA^{Phe} were added to ribosomes simultaneously, and the mixture was incubated for 30 min with poly(U). The ribosomes were then checked for the presence of covalently attached radioactive amino acids by sucrose gradient centrifugation of unfolded particles as described above. Radioactive phenylalanine is found attached only to the BrAcPhe-tRNA sample. These results are shown in Fig. 3*c* and *d*. It is apparent that ribosomes labeled with BrAcPhe-tRNA can still perform at least one cycle of peptide transfer. This reaction produces what is probably *N*-acetyl-Phe-[^{14}C]Phe-tRNA^{Phe} covalently attached to ribosomes. It is hard to imagine any other plausible explanation for our observations. Whether the initial tRNA^{Phe} is subsequently released from the P-site is not known. These results suggest that BrAcPhe-tRNA is bound to the P-site. To provide stronger proof, we will have to either identify the reaction product or show that the reaction is blocked by antibiotics known to affect peptide transfer. These experiments have not yet been attempted.

TABLE 1. *Poly(U)-directed synthesis of polyphenylalanine*

First incubation	Phenylalanine incorporation (cpm)	
	−Poly(U)	+Poly(U)
N-Acetyl-Phe-tRNA^{Phe}	68	1400
BrAcPhe-tRNA	50	400*

Polyphenylalanine synthesis was performed in 0.1-ml reaction mixtures, as described by Nirenberg and Matthaei (16). Samples were first incubated for 30 min in the absence of tRNA, [^{14}C]-phenylalanine, and supernatant. Then, these ingredients were added and protein synthesis took place for 25 min.

* Part of this incorporation may be due to the 30–35% contamination of the BrAcPhe-tRNA preparation by [^{14}C]Phe-tRNA.

BrAcPhe-tRNA attachment to proteins and nucleic acids

Results presented thus far suggest that BrAcPhe-tRNA binds to the P-site, and becomes covalently attached to ribosomes. To provide more convincing evidence and to narrow down the possible sites of attachment, 50S particles containing BrAcPhe-tRNA and *N*-acetyl-Phe-tRNA^{Phe} were separated into an RNA fraction and a total 50S protein fraction by treatment with LiCl–urea (17). Similar separations were done for 30S particles. From the four resulting protein samples, only total 50S protein from ribosomes reacted with BrAcPhe-tRNA contains substantial amounts of radioactivity (Table 2). Thus, these experiments prove that BrAcPhe-tRNA becomes covalently attached to one or more of the 50S ribosomal proteins.

Of the four ribosomal RNA samples, again only the rRNA reacted with BrAcPhe-tRNA from 50S particles was radio-

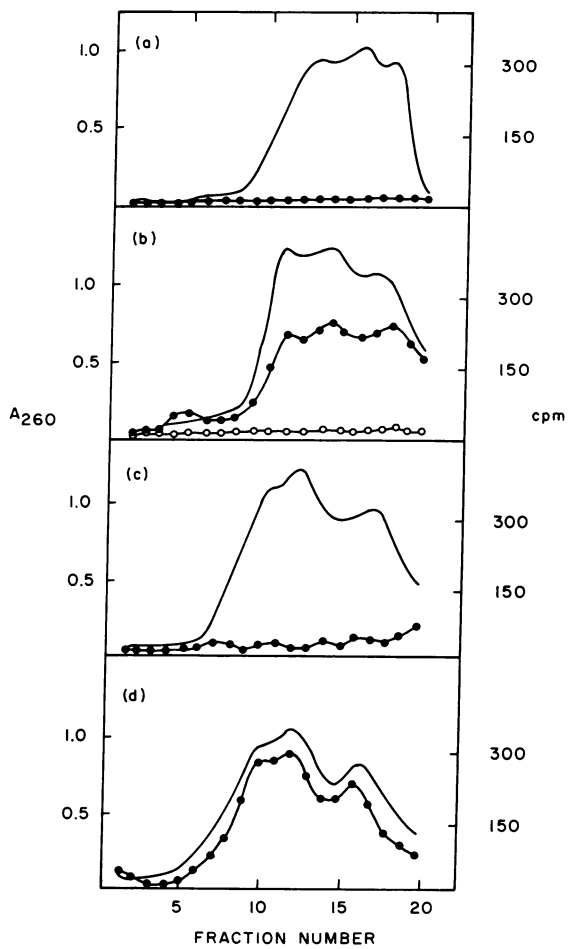
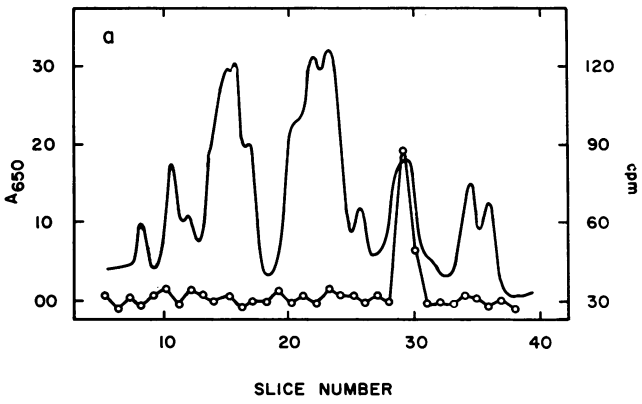


FIG. 3. Retention of radioactive phenylalanine and derivatives on unfolded 70S ribosomes. Ribosomes were unfolded by dialysis against buffer C. —— A_{260}, ●——● [^{14}C]Phe. All samples were 10–30% sucrose gradients for 3.5 hr. (*a*) Ribosomes containing *N*-acetyl-Phe-tRNA from Fig. 1. (*b*) Ribosomes containing BrAcPhe-tRNA from Fig. 1. O——O [^{14}C]Phe in ribosomes from an unincubated sample. (*c*) Ribosomes incubated in buffer A containing poly(U), [^{14}C]Phe-tRNA^{Phe}, and [^{12}C]*N*-acetyl-Phe-tRNA^{Phe}, and then unfolded. (*d*) Same as *c*, except that [^{12}C]-BrAcPhe-tRNA was used instead of *N*-acetyl-Phe-tRNA^{Phe}.



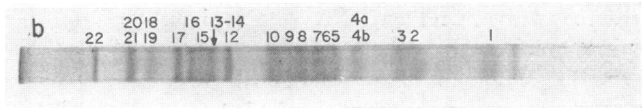


FIG. 4. Urea gels of 50S proteins from ribosomes reacted with BrAcPhe-tRNA. Gels contained 7.5% acrylamide–2% bisacrylamide–8 M urea, and were run at pH 4.5. Gels were stained with Coomassie Brilliant Blue (19) and scanned at 650 nm in a Gilford Spectrophotometer with a linear transport attachment. Gels to be analyzed for radioactivity were not stained, but were cut into 2-mm slices, which were then (each) dissolved in 0.4 ml of 30% H_2O_2 and counted in a Packard Tri-Carb 3375. (*a*)——A_{650} and O——O ^{14}C cpm, for simultaneous gels. (*b*) Photograph of 50S gel. Bands are numbered according to Traut *et al.* (18).

active. It contained almost as much radioactivity as did the 50S protein fraction. A sucrose gradient study of sodium dodecyl sulfate-treated ribosomes labeled with BrAcPhe-tRNA showed that the radioactivity cosediments with 23S rRNA (results not shown here).

The results described above prove that BrAcPhe-tRNA reacts covalently with the 50S subunit. An attempt was made to identify which of the 50S proteins was covalently labeled. 50S subparticles were dialyzed against 4 M urea and were separated by acrylamide-gel electrophoresis. In early experiments, samples were treated with pancreatic ribonuclease to remove any tRNA that might remain attached to the labeled protein and change its electrophoretic mobility. However, identical results were obtained when RNase treatment was omitted. It can be seen from Fig. 4 that there is only a single peak of radioactivity in these gels. This peak has been observed in gels run on five different samples of BrAcPhe-tRNA-modified ribosome. This peak overlaps the peak in the gel scan corresponding to the 50S protein bands *2* and *3*. Traut (18) has shown that band *3* contains the single protein, 3 VI, and that band *2* contains two proteins, 2 IX and 2 XI.

DISCUSSION

We have shown that one peak on acrylamide gels prepared from 50S ribosomal proteins corresponds to the site of covalent attachment of the peptidyl-tRNA analog, BrAcPhe-tRNA. In addition, a considerable amount of BrAcPhe-tRNA is found attached to the 23S rRNA after removal of proteins with LiCl–urea and treatment with sodium dodecyl sulfate. Several experiments suggest that BrAcPhe-tRNA is bound to the P-site. The most compelling argument is the fact that simultaneous binding of unlabeled BrAcPhe-tRNA and radioactive Phe-tRNA^{Phe} leads to covalent attachment of radioactive amino acids to ribosomes. From this result, it seems likely that the bromoacetyl moiety of BrAcPhe-tRNA has covalently reacted with the 50S particle at or near the peptidyl-transferase site. However, these detailed findings raise more questions about the nature of the site attached to BrAcPhe-tRNA than they answer. It is worth outlining some of the alternatives. One possibility is that the pattern of BrAcPhe-tRNA reactivity is really quite heterogeneous. Several different rRNA sites may have been attacked; more than one of the three proteins present in the radioactive gel peak may have been labeled. In this case, one would have to conclude that either the 3′-end of tRNA bound in the P-site has considerable conformational flexibility, or that some BrAcPhe-tRNA binds to the A-site and reacts there.

TABLE 2. *Extent of covalent attachment of BrAcPhe-tRNA to ribosomal proteins and nucleic acid*

First incubation		Total proteins (cpm)	rRNA (cpm)
N-acetyl-Phe-tRNA^{phe}	30S	35	35
	50S	32	30
BrAcPhe-tRNA	30S	32	37
	50S	520	480

50S and 30S particles were isolated from 70S samples as described in Fig. 1. Proteins and nucleic acid were separated by the LiCl–urea method (17). Proteins were dialyzed against 4 M urea, and any remaining tRNA was digested with pancreatic ribonuclease, which is active under these conditions. Both ribosomal RNA and protein were then precipitated with trichloroacetic acid and counted in a liquid scintillation counter in a toluene-based solvent.

It seems more likely, however, that the reactivity of BrAcPhe-tRNA has been fairly selective. Only one of the three proteins present in the radioactive gel band, 3 VI, remains in a 2 M LiCl-core particle (18). This protein is resistant to trypsin digestion (20). From these results, it seems unlikely that protein 3 VI is exposed enough to react with BrAcPhe-tRNA. In contrast, Chang and Flaks have shown (20) that one of the other two proteins, either 2 IX or 2 XI, is extremely labile to trypsin digestion in the 50S particles. These studies indicate some likelihood that this protein may be among those involved in the ability of ribosomes to carry out peptidyl transfer. Earlier work from Staehelin's laboratory (21) showed that one or both of these proteins appear in a lightly staining band on an acrylamide gel of β–γ split proteins. This set of proteins seems to be involved either structurally or functionally in peptidyl-transferase activity. However, neither Staehelin nor Chang and Flaks concluded that proteins 2 IX or 2 XI are definitely involved in peptidyl transfer. Although the situation is clearly complex, a diverse set of experimental results now suggests that proteins 2 IX and 2 XI merit close scrutiny as proteins at or near the peptidyl-transferase site.

If the BrAcPhe-tRNA reaction is really highly specific, how can the presence of radioactivity in the 23S rRNA fraction be explained? There are two possibilities. Nomura (22) has shown that there are two 50S proteins that are very tightly bound to 23S rRNA. These may not be removed by

treatment with detergent. The radioactivity we find in the RNA could be associated with one of these proteins. Or, it could be associated with rRNA itself, since it is known that an α-bromoamide is capable of alkylating some of the nucleic acid bases (13). Thus, the heterogeneity of labeling we observe may result from more than one mode of BrAcPhe-tRNA binding to ribosomes, or it may result from proteins and rRNA being closely packed near the 3'-end of binding site of BrAcPhe-tRNA. But, there is another intriguing possibility. Traut *et al.* have shown that one of the two proteins (2 IX or 2 XI) is present only to the extent of 0.6 mol/mol of ribosomes (18). Thus, the heterogeneous labeling by BrAcPhe-tRNA may arise from a fundamental heterogeneity of 50S particles.

One last unexplained observation is the fact that the tRNA moiety of BrAcPhe-tRNA is lost prior to acrylamide gel electrophoresis of the proteins separated by LiCl–urea. This loss could be due to the instability of the ester bond between the phenylalanine and the tRNA, to the presence of ribonuclease contamination, or, possibly, to a ribosome-catalyzed cleavage.

While much work clearly remains to be done, it seems likely that the use of BrAcPhe-tRNA and other reactive tRNA analogs should ultimately enable a much more detailed picture of the mechanism of protein synthesis to be developed.

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