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RESEARCH ARTICLES

Unusual Resistance of Peptidyl Transferase to Protein Extraction Procedures

Harry F. Noller, Vernita Hoffarth, Ludwika Zimniak

Peptidyl transferase, the ribosomal activity responsible for catalysis of peptide bond formation, is resistant to vigorous procedures that are conventionally employed to remove proteins from protein–nucleic acid complexes. When the "fragment reaction" was used as a model assay for peptide bond formation, *Escherichia coli* ribosomes or 50*S* subunits retained 20 to 40 percent activity after extensive treatment with proteinase K and SDS, but lost activity after extraction with phenol or exposure to EDTA. Ribosomes from the thermophilic eubacterium *Thermus aquaticus* remained more than 80 percent active after treatment with proteinase K and SDS, which was followed by vigorous extraction with phenol. This activity is attributable to peptidyl transferase, as judged by specific inhibition by the peptidyl transferase–specific antibiotics chloramphenicol and carbomycin. In contrast, activity is abolished by treatment with ribonuclease T1. These findings support the possibility that 23*S* ribosomal RNA participates in the peptidyl transferase function.

There is much evidence to support the view that ribosomal RNA (rRNA) participates directly in protein synthesis (*1*, *2*), and it has even been argued that the fundamental mechanism underlying translation may be RNA-based (*3*, *4*). Indeed, demonstration of the ability of RNA to perform enzymatic catalysis in other biological contexts (*5*, *6*) has drawn increased attention to the functional potential of rRNA. However, apart from the well-established role of the 3′ terminus of 16*S* rRNA in mRNA selection, direct proof of this has been elusive. For example, efforts to carry out steps of protein synthesis with protein-free preparations of rRNA have not been successful [but see (*7*)], possibly because billions of years of co-evolution of ribosomal proteins and rRNA have led to a requirement for ribosomal proteins to achieve proper folding and function of the rRNA (*8*–*10*).

Localization of peptidyl transferase to the large ribosomal subunit. In our efforts to study the biological activity of rRNA, we have chosen as a model system the peptidyl transferase reaction, which is the source of the catalysis of peptide bond formation, and is also the single catalytic activity that has unambiguously been shown to be an integral part of the ribosome structure (*11*). In spite of many attempts by several laboratories, peptidyl transferase activity has never been detected in RNA-free preparations of ribosomal proteins. An important attraction of peptidyl transferase is that it can be monitored with a simplified assay known as the "fragment reaction" (Fig. 1), which measures the transfer of *N*-formyl-methionine from a short fragment of tRNA to the amino group of puromycin to form a model peptide bond (*12*). The fragment reaction requires only the large ribosomal subunit, appropriate ionic conditions, and 33 percent methanol or ethanol, in addition to the f-Met-oligonucleotide and puromycin substrates. Thus, there is no requirement for the small ribosomal subunit, mRNA, protein factors, guanosine triphosphate (GTP), or even complete tRNA molecules. The authenticity of the model reaction is supported by the stereochemical specificity of the substrates and highly specific inhibition of the reaction by antibiotics that are known peptidyl transferase inhibitors (*13*).

Earlier studies showed that this system can be simplified even further by stepwise removal of ribosomal proteins from the 50S subunit with high concentrations of salt (*14*–*16*). In one study, removal of approximately half of the proteins from the 50S subunit resulted in loss of peptidyl transferase functions; full activity was restored by reconstitution of the resulting core particles with purified protein L16 (*16*). These same preparations of purified L16 showed no detectable peptidyl transferase activity, however. Another study provided evidence for an L16-dependent, conformational change in similar protein-deficient 50S core particles (*17*). The temperature dependence of the kinetics of this process corresponds to an activation energy of about 30 kcal/mol, suggesting the occurrence of a fairly substantial structural rearrangement. These experiments indicate that protein L16 plays an important role in proper assembly of the core particle. Reconstitution experiments, in which individual components were omitted, showed that proteins L2, L3, L4, L15, L16, and L18, as well as 23S rRNA were essential for reconstitution of peptidyl transferase activity (*18*); of this group, L18 could also be excluded on the basis of other studies (*19*). This list most likely represents an overestimate of the number of proteins actually needed for ca-

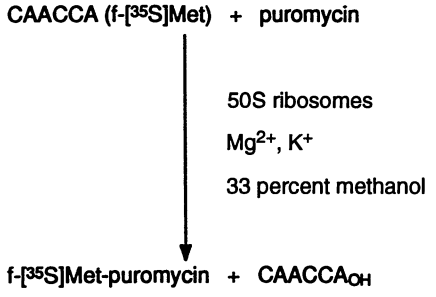

Fig. 1. The "fragment reaction." Peptidyl transferase activity is measured by formation of f-[^{35}S]Met-puromycin from reaction of the CAACCA(f-[^{35}S]Met) oligonucleotide fragment, derived from the 3′ end of f-[^{35}S]Met-tRNA by RNase T1, with puromycin, in the presence of 33 percent methanol (*12*). The oligonucleotide fragment and puromycin serve as peptidyl-tRNA and aminoacyl-tRNA analogues, respectively.

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talysis, since some of them may be important only for assembly, and others may be needed for binding the full-length tRNA used in these experiments.

In vitro assembly experiments have shown that correct folding of rRNA depends on sequential, cooperative interactions with ribosomal proteins (*8*, *9*); the above-mentioned L16-dependent event is one such example. Many features of the higher order structure of rRNA are lost during conventional RNA extraction procedures (*20*). Thus, the requirement for certain ribosomal proteins could be explained by their role in stabilizing the active conformation of 23*S* rRNA. Our strategy, therefore, was to attempt to remove all of the 50*S* proteins without disturbing crucial elements of the RNA conformation. This includes avoidance of denaturing conditions, chelating agents, or extremes of pH.

Resistance of peptidyl transferase to protein extraction procedures. Experiments with *Escherichia coli* 70*S* ribosomes or 50*S* subunits showed that peptidyl transferase activity survives treatment with 0.5 percent SDS and proteinase K at 1 mg/ml for 1 hour at 37°C, as shown by the formation of *N*-formyl-[^{35}S]methionyl-puromycin (Fig. 2). However, phenol extraction (Fig. 2) or exposure to 5 mM EDTA abolished the activity.

One possible explanation for the loss of activity on phenol extraction is that, in the mesophilic bacterium *E. coli*, some crucial higher order RNA structural feature might be unstable in the absence of r-proteins. We therefore subjected ribosomes from the thermophilic eubacterium *Thermus aquaticus* to a similar extraction because its rRNA structure might be inherently more robust than that of *E. coli*. Indeed, we found that the peptidyl transferase activity of *T. aquaticus* 50*S* subunits was resistant not only to treatment with proteinase K and SDS at 37°C (Fig. 2), but also at 40°, 50°, and 60°C (Fig. 3). Surprisingly, loss of activity was seen only when subunits were incubated at 60°C in the absence of SDS and proteinase K; this result may be due to low levels of endogenous ribonuclease in the subunit preparations. As in the case of *E. coli* ribosomes, activity was abolished by treatment with EDTA. In contrast to the behavior of *E. coli* ribosomes, *T. aquaticus* peptidyl transferase was resistant to vigorous extraction with phenol, even in combination with SDS–proteinase K digestion (Fig. 2). Peptidyl transferase activity also withstood extraction with two other detergents known for their ability to disrupt protein-RNA interactions, triisopropylnaphthalene sulfonate (TNS) and *p*-aminosalicylate (PAS) (*21*). When *T. aquaticus* 50*S* subunits were extracted with TNS, either alone or in combination with PAS and phenol, they retained significant activity (Table 1). The lower activity observed after PAS treatment may be caused by sequestration of magnesium ions.

An important question is whether the activity that survives the extraction procedures is actually due to peptidyl transferase.

Table 1. Peptidyl transferase activity of *T. aquaticus* 50*S* subunits following treatments with various detergents (*21*) and in combination with phenol. *Thermus aquaticus* 50*S* subunits (300 μg) were treated, as indicated, with 1 percent triisopropylnaphthalene sulfonic acid, sodium salt (TNS), 5 percent *p*-aminosalicylic acid (PAS), or 0.5 percent SDS plus proteinase K at 1 mg/ml for 2 hours at 37°C in 50 μl of buffer A. After addition of 200 μl of buffer A and 250 μl of neutralized, water-saturated phenol, the mixture was vortexed for 40 minutes at 4°C. A second phenol extraction was carried out for 10 minutes, the aqueous phase was extracted three times with ether, and the RNA-containing material was recovered by precipitation with three volumes of ethanol. The precipitate was redissolved in 50 μl of buffer A and heated for 10 minutes at 42°C, and 1.5 A_{260} units assayed for peptidyl transferase activity (*32*). The results shown are the net ethyl acetate extractable ^{35}S (counts per minute) after subtracting 182 cpm background from a control reaction lacking ribosomes; the background is the result of an unidentified side product that is resolved from the f-Met-puromycin product as a fast-moving spot on paper electrophoresis (Figs. 2 and 4).

Treatment	Peptidyl transferase activity, ^{35}S	
	cpm	percent
Phenol only	1515	96
1 percent TNS	1469	93
1 percent TNS, 5 percent PAS	896	57
1 percent TNS, phenol	1212	77
1 percent TNS, 5 percent PAS, phenol	359	23
0.5 percent SDS, proteinase K, phenol	1375	87

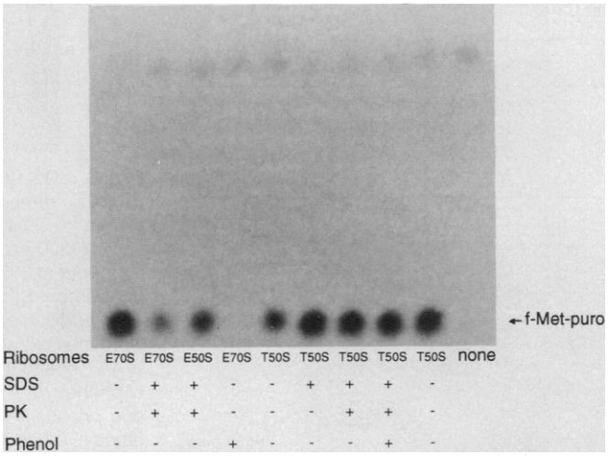

Fig. 2. Peptidyl transferase activity of *E. coli* and *T. aquaticus* ribosomes after protein extraction. Ribosomes and subunits, prepared as described (*20*, *31*), were suspended in buffer A (5 mM $MgCl_2$, 150 mM NH_4Cl, 25 mM tris-HCl, pH 7.5), with 375 μg of *E. coli* 70*S* ribosomes, 250 μg of *E. coli* 50*S* subunits, or 150 μg of *T. aquaticus* 50*S* subunits in a volume of 500 μl for the following treatments. SDS (with or without proteinase K digestion), 0.5 percent SDS, 1 hour at 37°C; PK, proteinase K at 1 mg/ml; phenol, addition of 200 μl of buffer A, 250 μl of neutralized, water-saturated phenol, followed by vortexing for 45 minutes at 4°C. The phenol phase was removed, the aqueous phase was extracted four times with ether, precipitated with three volumes of ethanol, and resuspended in 50 μl of buffer A. Samples were heated for 10 minutes at 42°C and assayed for peptidyl transferase activity (*32*). An autoradiograph of the products after high-voltage paper electrophoresis is shown. The position of the f-[^{35}S]Met-puromycin product is shown. The cathode is at the top. E70S, E50S, *E. coli* 70*S* ribosomes, or 50*S* subunits, respectively; T50*S*, *T. aquaticus* 50*S* subunits.

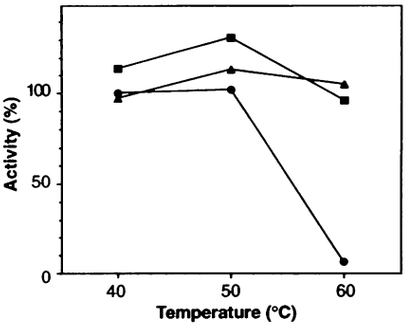

Fig. 3. Peptidyl transferase activity after treatment of *T. aquaticus* 50*S* subunits with SDS and proteinase K at increasing temperatures. *T. aquaticus* subunits (120 μg) were treated with proteinase K at 1 mg/ml with (■) or without (▲) 0.5 percent SDS, for 1 hour at the indicated temperature. Extracted material was recovered by precipitation with ethanol, redissolved in buffer A, and assayed (*32*). Control subunits (●) were treated identically except that proteinase K and SDS were omitted. Full (100 percent) activity is defined as that of control subunits incubated for 1 hour at 40°C and corresponds to 1295 cpm.

Authentic eubacterial peptidyl transferase is known to be inhibited specifically by the antibiotics chloramphenicol and carbomycin (*13*). Formation of f-Met-puromycin by *T. aquaticus* 50S subunits is indeed inhibited by these two antibiotics (Fig. 4), with carbomycin showing significantly stronger inhibition than chloramphenicol. No inhibition was observed with erythromycin, a 50S subunit-specific antibiotic that is known not to affect peptidyl transferase, or with anisomycin, which inhibits peptidyl transferase in eukaryotic and archaebacterial ribosomes, but not in those of eubacteria. Similar inhibition by these drugs was observed in *Thermus* 50S subunits treated with SDS, proteinase K, and phenol (Fig. 4), evidence that the extraction-resistant activity is indeed peptidyl transferase.

In contrast to its resistance to extensive digestion with proteinase K, peptidyl transferase activity is highly sensitive to treatment with ribonuclease (RNase). Digestion was effected by RNase T1, which cleaves specifically at guanine residues, so that cleavage of the oligonucleotide substrate (which lacks guanines) could be excluded. Brief digestion of *T. aquaticus* 50S subunits with RNase T1 caused significant loss of activity, and digestion of the extracted material abolished peptidyl transferase activity (Fig. 4). This result is in agreement with earlier studies on *E. coli* ribosomes, in which peptidyl transferase was inactivated by treatment with RNase T1 (*22*) or with the guanine-specific reagent kethoxal (*23*).

Protein composition of extracted particles. Treatment of *T. aquaticus* 50S subunits with SDS and proteinase K, followed by two successive phenol extractions, typically yielded material that was more than 80 percent active, compared to untreated subunits. When ribosomes were labeled with [^{35}S]methionine, the amount of protein remaining in the extracted material was typically about 5 percent of that of untreated subunits. The extraction-resistant polypeptides were removed by acetic acid treatment (*24*) for analysis by SDS gel electrophoresis (peptidyl transferase activity is irreversibly lost when exposed to acetic acid). A typical result is shown in Fig. 5, which shows the polypeptide material that survives one, two, or three successive phenol extractions after SDS and proteinase K treatment of *T. aquaticus* ribosomes. Coomassie blue staining (Fig. 5A) or ^{35}S autoradiography (Fig. 5B) show that most of the remaining polypeptide material runs with the SDS front (<10 kD). In addition, three or four slower migrating polypeptides are seen, and their intensity diminishes with successive extraction steps. The decreasing intensity of these bands was not correlated with loss of activity in this experiment; indeed, full peptidyl transferase activity persisted after three successive extractions, when 95 percent of the [^{35}S]methionine-labeled material had been removed (Table 2). It has so far proved difficult to remove the remaining extraction-resistant polypeptide material reproducibly, without resorting to treatments that are disruptive to RNA structure. With certain subunit preparations, more than 99 percent of the ^{35}S-labeled protein was removed under conditions where high activity is normally preserved although this is not typically the case.

The unusual resistance of *T. aquaticus* peptidyl transferase to protease digestion, ionic detergents, and phenol extraction, in contrast to its sensitivity to ribonuclease, points to the importance of 23S rRNA for this ribosome-catalyzed reaction. However,

Table 2. Extraction of protein from [^{35}S]methionine-labeled ribosomes. *Thermus aquaticus* ribosomes labeled in vivo with [^{35}S]methionine (*31*) were treated as described in Fig. 5. Samples were removed for quantitation of ^{35}S and for the peptidyl transferase assay (*32*). The peptidyl transferase activity represents ethyl acetate extractable ^{35}S (cpm) after subtraction of 183 cpm background (see legend to Table 1).

Treatment	[^{35}S]Protein remaining		Peptidyl transferase activity	
	cpm	percent	cpm	percent
Control; no treatment	16392	100	1488	100
SDS, proteinase K, 1× phenol	1894	12	1308	88
SDS, proteinase K, 2× phenol	1156	7	1522	102
SDS, proteinase K, 3× phenol	860	5	1638	110

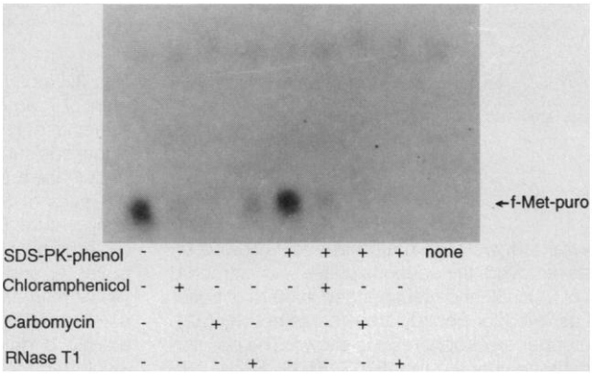

Fig. 4. Sensitivity of *T. aquaticus* subunits or subunits treated with SDS and proteinase K and then extracted with phenol to peptidyl transferase inhibitors or ribonuclease. *Thermus aquaticus* 50S subunits, either untreated or extracted as in Fig. 2, were assayed in the presence of 0.2 mM chloramphenicol, 0.2 mM carbomycin, or after treatment for 10 minutes at 37°C with 5 μg of ribonuclease T1 per 225 μg of 50S subunits or the molar equivalent of extracted subunits.

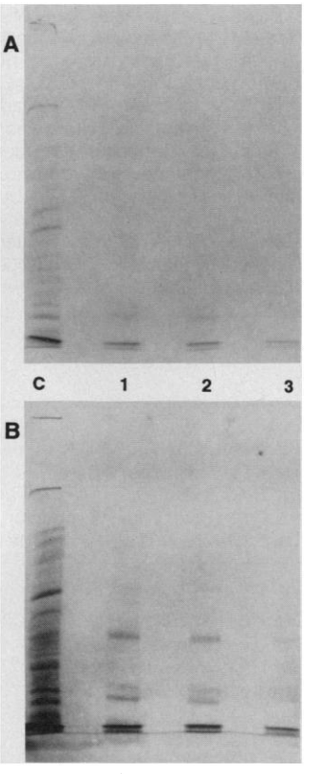

Fig. 5. SDS gel electrophoresis of ribosomal proteins remaining after extraction of ribosomes. ^{35}S-labeled *T. aquaticus* 70S ribosomes (300 μg; 230,000 cpm) were incubated for 1 hour at 37°C in 50 μl of buffer A containing 0.5 percent SDS and proteinase K at 1 mg/ml. After addition of 200 μl of buffer A, the mixtures were then extracted by vortexing for 40 minutes at 4°C, one to three times with an equal volume of phenol. After four ether extractions, the aqueous phases were precipitated with three volumes of ethanol, redissolved in 100 μl of buffer A, of which 60 μl was extracted with 66 percent acetic acid as described (*24*), and analyzed by SDS gel electrophoresis (*33*). The gel was stained with Coomassie blue (**A**) and autoradiographed (**B**). The lanes show proteins associated with (C) untreated ribosomes, and those remaining after one, two, or three successive phenol extractions.

the presence of significant amounts of polypeptide material, even after vigorous extraction, prevents us from concluding that peptide bond formation is catalyzed solely by RNA. The approximately 5 percent of the ribosomal protein remaining could correspond to stoichiometric amounts of as many as two or three intact ribosomal proteins (depending upon molecular masses). If any proteins are, in fact, required for peptidyl transferase function, they must be few in number. Preliminary indications from gel electrophoresis (Fig. 5) suggest that much of the remaining protein is in the form of peptide fragments. Further characterization is required to assess whether any specific protein or its fragments might be present in stoichiometric amounts following extraction. Another important issue is the fraction of active particles in the starting ribosome population; if only a small fraction were active, the remaining protein could belong to an extraction-resistant subpopulation of active particles. While we have no direct measure of the fraction of ribosomes that is active in the peptidyl transferase reaction, there is good evidence that a high proportion of the ribosomes in these preparations is active in other ribosomal functions. The preparations of *E. coli* ribosomes used are virtually fully active in binding tRNA, as judged by complete or nearly complete protection by tRNA of bases in 16S and 23S rRNA from chemical probes (*25, 26*). Similarly complete protection of bases in 23S rRNA was observed when the CAACCA(f-Met) peptidyl transferase substrate was bound to *E. coli* 50S subunits in the presence of sparsomycin (*27*). Since activity of *T. aquaticus* ribosomes and subunits in the fragment reaction is similar to that of their *E. coli* counterparts, similar arguments are applicable to these ribosomes as well.

Implication of specific sites in 23S rRNA in the peptidyl transferase function. Our findings are precedented by an abundance of circumstantial evidence implicating 23S rRNA in the peptidyl transferase function (*1*). Affinity labeling studies, in which tRNAs with various chemically reactive groups attached to their aminoacyl ends have been reacted with ribosomes, show that highly conserved regions of 23S rRNA, most notably the central loop of domain V, are in close proximity to the site of peptide bond formation (*28*). Chloramphenicol and carbomycin, two antibiotics that are known specifically to inhibit the peptidyl transferase reaction, protect certain bases in the central loop of domain V from chemical probes (*29*). Point mutations conferring resistance to chloramphenicol and anisomycin, another peptidyl transferase inhibitor, have also been found in this part of 23S rRNA, and some of them occur at positions that are identical to those that are protected by chloramphenicol and carbomycin (*30*). Chemical footprinting studies have shown that tRNA interacts, either directly or indirectly, with bases in this same region of 23S rRNA, several of which are again identical with the sites of protection by these antibiotics or of mutations conferring resistance to them (*26*). Furthermore, it has been shown by stepwise deletion experiments that the part of the tRNA structure responsible for these interactions is the aminoacyl-CCA 3′ end (*26*). Indeed, N-blocked aminoacylated oligonucleotides, such as the CAACCA-(f-Met) fragment used in our experiments, bound in the presence of sparsomycin and ethanol, protect virtually all of the same bases in 23S rRNA that are protected by normal binding of the intact tRNA (*27*). Taken together, these results provide convincing evidence for interaction between the CCA end of tRNA and a region of 23S rRNA in and around the central loop of domain V. Finally, the absolute conservation of many bases in this region of 23S rRNA in all known large subunit rRNA sequences, which include eubacterial, archaebacterial, eukaryotic, plastid, and mitochondrial sequences, is in keeping with important functional, as opposed to structural, constraints. Direct proof for the hypothesis that peptide bond formation is catalyzed solely by rRNA will require demonstration of activity with completely protein-free preparations, such as in vitro transcripts of 23S rRNA.

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31. *Thermus aquaticus* (strain provided by D. Gelfand, Cetus Corp.) was grown to saturation at 70°C overnight in a shaking water bath in the following medium: 1× Castenholz salts (*34*), 5× Nitsch's trace elements (*34*), yeast extract at 4 g/liter, bactopeptone at 8 g/liter, and NaCl at 2 g/liter. Ribosomes were prepared from frozen *T. aquaticus* cells as described for *E. coli* ribosomes (*20*), except that buffers contained 25 mM $MgCl_2$ for preparation of 70*S* ribosomes and 2 mM $MgCl_2$ for 50*S* subunits. Ribosomes and subunits were stored at −70°C in 25 mM $MgCl_2$, 100 mM NH_4Cl, 6 mM 2-mercaptoethanol, 0.5 mM EDTA, 20 mM tris-HCl, pH 7.5. For preparation of [^{35}S]methionine-labeled ribosomes, the same procedure was followed, except that cells were grown in media containing 1× Castenholz salts, 10× Nitsch's trace elements, bactopeptone at 0.2 g/liter, and [^{35}S]methionine (10 μCi/ml; 1000 mCi/mmol specific activity).
32. Peptidyl transferase activity was assayed by the fragment reaction (*12*), typically in a total volume of 50 μl, containing 25 μl of ribosomes in buffer A, CAACCA(f-[^{35}S]Met) at 20,000 cpm, prepared as described (*12*), 2 mM neutralized puromycin, 10 μl of buffer B (2 M potassium acetate, 100 mM $MgCl_2$, 250 mM tris-HCl, pH 7.5). The reaction was initiated by addition of 25 μl of methanol, held at 4°C for 10 minutes, and terminated by addition of 50 μl of 0.3 M sodium acetate, pH 5.5, saturated with $MgSO_4$. The mixture was extracted with 1 ml of ethyl acetate, and 0.5 ml of the extract was dried down, subjected to high-voltage paper electrophoresis in pyridine acetate, pH 3.5 at 3000 V for 2 hours, and autoradiographed. Under these conditions, the initial rate of peptide bond formation was measured, and the assay was linear with respect to ribosome concentration within the amounts used in these experiments.
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