major activity for the synthesis of III resides in the microsomal fraction which is separated from the mitochondria during their purification.

Acknowledgment

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Stimulation of Protein Synthesis *in Vitro* by Partially Degraded Ribosomal Ribonucleic Acid and Transfer Ribonucleic Acid*

John J. Holland, Clayton A. Buck,† and B. J. McCarthy

ABSTRACT: An *in vitro* protein-synthesizing system from *Escherichia coli* is capable of using purified ribosomal ribonucleic acid (RNA) as a template for amino acid incorporation under certain conditions. This template activity is optimal when neomycin is added and when the secondary structure of ribosomal RNA is destroyed by heating to cause a number of phosphodiester bond cleavages in each molecule. After more extensive heating to destroy its secondary structure, soluble RNA also acts as a template for amino acid incorporation.

Nirenberg and Matthaei (1961) showed in their original study of cell-free protein synthesis that ribonucleic acid (RNA) extracted from washed *Escherichia coli* ribosomes was capable of slight stimulation of protein synthesis. However, the specific activity of ribosomal RNA was less than 5% of that of tobacco mosaic virus (TMV) RNA. Furthermore, sucrose gradient sedimentation studies indicated that only a fraction of the RNA from ribosomes was active and that the soluble RNA fraction was inactive as a template. Subsequently, other investigators have used this system to measure messenger RNA (m-RNA) activity in total RNA extracted from bacterial and mammalian cells. This assay is made on the assumption that ribosomal and transfer RNA (t-RNA) contribute little to the template activity.

It is shown below that both ribosomal and soluble

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TABLE I: Stimulation of ^{14}C-Arginine Incorporation by *E. coli* Ribosomal RNA in the Presence of Neomycin.[a]

Additions	Cpm/mg of Protein
None	440
10 μg of neomycin	959
500 μg of unheated ribosomal RNA	854
500 μg of unheated ribosomal RNA and 10 μg of neomycin	17,070
500 μg of heated ribosomal RNA	4,860
500 μg of heated ribosomal RNA and 10 μg of neomycin	38,600

[a] The reaction mixture (0.3 ml) contained per milliliter: 50 μmoles of NH_4Cl, 12 μmoles of $Mg(CH_3COO)_2$, 6 μmoles of mercaptoethanol, 20 μmoles of Tris pH 7.8, 0.1 μmole of GTP, 1 μmole of ATP, 5 μmoles of sodium salt of phosphoenolpyruvate, 20 μg of pyruvate kinase, 3.3 mg of 30S protein, 10 mμmoles each of all cold amino acids, 1.0 μcurie of ^{14}C-arginine. The 30S protein was added to the mixtures last and incubation was at 37° for 60 min. "Heated" RNA was heated at 100° for 20 min prior to addition.

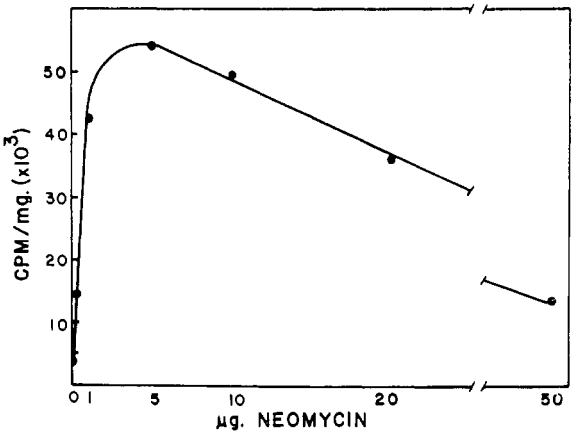

FIGURE 1: Effect of varying concentrations of neomycin in promoting template activity of *E. coli* ribosomal RNA. Reaction conditions as in Table I except that varying amounts of neomycin are added to the 0.3-ml reaction mixture as indicated, and 500 μg of *E. coli* ribosomal RNA was employed in each reaction mixture. The data are expressed as counts per minute per mg of protein.

RNA (s-RNA) can be converted to template RNA by the addition of neomycin and the disruption of secondary structure by heating. We have also recently found that neomycin promotes direct template activity of denatured deoxyribonucleic acid (DNA) from a variety of sources (McCarthy and Holland, 1965; Holland and McCarthy, 1964). It appears that streptomycinoid antibiotics can effect template activity in all three types of polynucleotide which perform other functions in the living cell.

Materials and Methods

The cell-free system was essentially that of Nirenberg (1964), modified by dissociation of ribosomal subunits through dialysis to a low magnesium concentration. *E. coli* strain K12 was grown at 37° in Difco brain–heart infusion broth and harvested while still in the log phase of growth. Washed cells were suspended in two or three volumes of a solution containing 0.01 M Tris–HCl, pH 7.9, 0.01 M magnesium acetate, 0.06 M KCl, and 0.006 M β-mercaptoethanol. The frozen cells were disrupted in a French pressure cell at 10,000–13,000 psi. The supernatant solution, after removal of cell wall debris by centrifugation at 30,000*g* for 15 min (30S), was dialyzed against the above buffer modified by reduction of the magnesium acetate concentration to 10^{-4} M for 24 hr at 4°. The dialyzed 30S fraction was placed in sealed tubes in small aliquots and stored frozen at −70° or below until used.

Ribosomes were obtained by centrifugation at 105,000*g* for 3 hr after disruption of *E. coli* or mammalian cells in the standard buffer. Ribosomal RNA was obtained by phenol extraction of a solution of ribosomes in 0.1 M KCl–10^{-3} M EDTA, pH 7.0. The RNA was repeatedly precipitated with ethanol and dissolved in 0.1 M KCl. The final precipitate was dried in an air stream, dissolved in distilled water, and stored frozen until used. Ribosomal subunits were obtained by dialysis of intact ribosomes against the above standard buffer, modified by reduction of the magnesium acetate concentration to 10^{-4} M for 24 hr at 4° followed by centrifugation at 105,000*g* for 4 hr. Where indicated, 50S and 30S ribosomal subunits were partially purified by differential centrifugation at 105,000*g* as described by Tissières *et al.* (1960).

Soluble (transfer) RNA was obtained from *E. coli* by two methods. In one the s-RNA was extracted across cell walls as described by Von Ehrenstein and Lipmann (1961). A paste of packed *E. coli* cells was suspended in a solution of 0.01 M magnesium acetate buffered to pH 7.0 with 0.001 M Tris–HCl and shaken for 1 hr at room temperature with an equal volume of water-saturated phenol. The RNA was then repeatedly precipitated with ethanol. In the other method cells were disrupted in a French pressure cell at 10,000–13,000 psi in standard buffer, centrifuged at 30,000*g* for 10 min to remove cell wall debris, then at 105,000*g* for 3 hr to sediment ribosomes, and the final supernatant solution was extracted with phenol. Since s-RNA, prepared by both of these methods, was contaminated with small amounts of ribosomal RNA and its breakdown products, it was further purified by sucrose gradient sedimentation. Contaminating DNA was degraded with deoxyribonuclease and the enzyme was removed by phenol extrac-

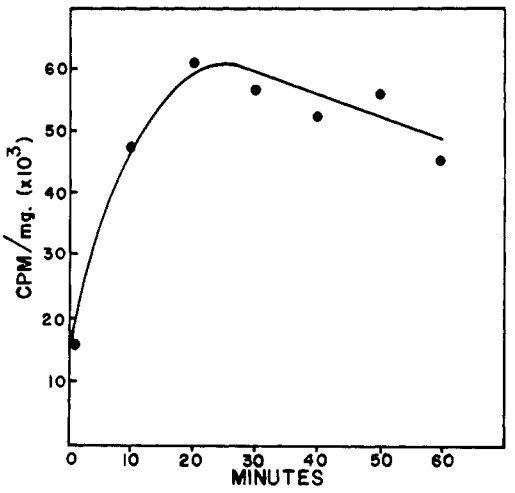

FIGURE 2: Effect of heating on template activity of *E. coli* ribosomal RNA. Reaction conditions as in Table I, except that each reaction mixture contained *E. coli* ribosomal RNA which had been heated for varying periods at 100° in 10^{-3} M EDTA, pH 7.0. The data are expressed as counts per minute per mg of protein. Neomycin (10 μg) was present in each case. All reaction mixtures were incubated 1 hr at 37°.

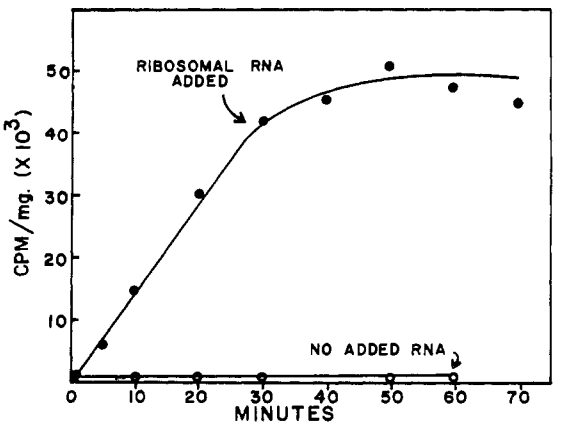

FIGURE 3: Kinetics of ^{14}C-arginine incorporation in the presence or absence of ribosomal RNA from *E. coli*. Reaction conditions as in Table I except that 600 μg of *E. coli* ribosomal RNA was boiled for 10 min and 10 μg of neomycin was employed.

tion followed by repeated ethanol precipitation. Only RNA sedimenting in the 4S peak was employed for testing of template activity of s-RNA.

The reaction mixture described in Table I was modified from that of Nirenberg (1964) by substituting NH_4Cl for KCl (Conway, 1964), by omitting uridine (UTP) and cytidine triphosphates (CTP) and increasing the level of guanosine triphosphate (GTP), and by employment of neomycin where indicated. After incubation at 37° the reaction was stopped by addition of 1 drop of 1 N NaOH. The mixture was diluted to 3 ml with 0.15 M NaCl solution and 3 ml of 10% trichloroacetic acid (TCA) was added, followed by heating for 15 min at 90–100° (Siekevitz, 1952). The protein precipitate was collected twice by centrifugation with intervening dispersal in NaOH, and the final precipitate was collected on a nitrocellulose membrane filter, dried, and counted in a thin-window gas-flow counter or a Packard scintillation counter.

Unlabeled amino acids, ATP, GTP, phosphoenolpyruvate, pyruvate kinase, and pancreatic ribonuclease were obtained from Calbiochem. Deoxyribonuclease I was a crystalline enzyme electrophoretically purified to remove traces of ribonuclease obtained from Worthington Biochemical Co. Puromycin was a generous gift from Lederle Laboratories, chloramphenicol was obtained from Parke, Davis and Co., and neomycin was a product of E. R. Squibb and Sons. RNA from the bacteriophage f2 was a generous gift of Mrs. Virginia Merriam of the Department of Biochemistry, University of Washington. The following ^{14}C amino acids were obtained from New England Nuclear Corp.: L-alanine (110 mcuries/mmole), L-arginine (222 mcuries/mmole), L-aspartic acid (148 mcuries/mmole), L-isoleucine (222 mcuries/mmole), L-lysine (228 mcuries/mmole), L-phenylalanine (333 mcuries/mmole), L-proline (185 mcuries/mmole), L-threonine (148 mcuries/mmole), L-tyrosine (375 mcuries/mmole), and L-valine (185 mcuries/mmole).

Results

Template Activity of Ribosomal RNA. Table I shows that untreated ribosomal RNA gave slight stimulation of ^{14}C-arginine incorporation in agreement with the report of Nirenberg and Matthaei (1961). It can also be seen that heating of the ribosomal RNA at 100° to disrupt its secondary structure gave an RNA product which was somewhat more effective. Addition of 10 μg of neomycin to the reaction mixture made native ribosomal RNA an efficient template but heated RNA in the presence of neomycin was even more effective.

Figure 1 is a dose-response curve showing that neomycin was most effective in promoting template activity of ribosomal RNA when in the range 1–10 μg/reaction mixture. Figure 2 shows the template activity in the presence of neomycin of ribosomal RNA which had been heated at 100° for varying periods. Heating for about 20 min degraded ribosomal RNA to a form which was maximally active as a template, but further heat degradation led to slow loss of activity.

The kinetics of amino acid incorporation with an *E. coli* ribosomal RNA template in this system is shown in Figure 3. The rate of incorporation was constant for 20–30 min, then decreased. Figure 4 is a dose-response curve showing that amino acid incorporation is linearly proportional to the amount of added *E. coli* or mouse cell ribosomal RNA up to 100–200 μg.

Table II shows that the incorporation of many dif-

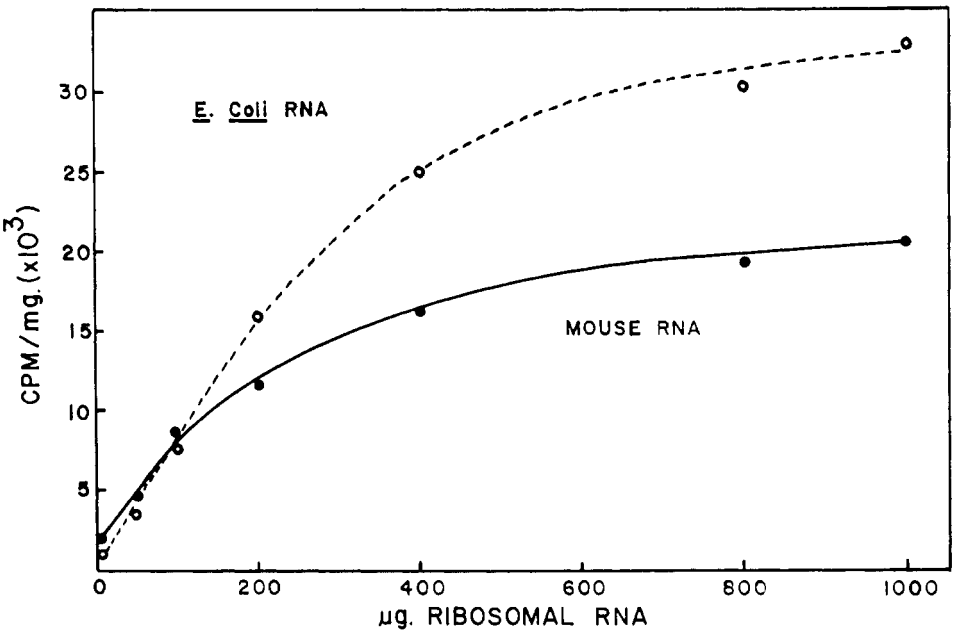

FIGURE 4: Effect of varying amounts of *E. coli* and mouse ribosomal RNA on ^{14}C-lysine incorporation. Reaction conditions as in Table I. Neomycin (10 µg) was present in each reaction mixture and incubation was for 60 min at 37°. Each ribosomal RNA sampled was boiled for 10 min.

ferent amino acids is stimulated by heated *E. coli* ribosomal RNA in the presence of neomycin. It is notable that certain amino acids such as arginine, lysine, and threonine were incorporated considerably better than others such as phenylalanine, valine, and isoleucine. This may result from the abundance of coding triplets in the nucleotide sequence of ribosomal RNA, although it could equally well be a manifestation of misreading of code words induced by the neomycin (Davies *et al.*, 1965).

Energy Requirement and Susceptibility to Inhibitors. Omission of adenosine triphosphate (ATP), GTP, and phosphoenolpyruvate prevents amino acid incorporation in this system (Table III). Furthermore, incorporation is very sensitive to ribonuclease but resistant to deoxyribonuclease. Both puromycin and chloramphenicol block incorporation, as previously shown for cases of stimulation by virus RNA and synthetic polyribonucleotides (Nirenberg and Matthaei, 1961) and single-stranded DNA (Holland and McCarthy, 1964).

Correspondence between Template Activity and Ribosomal RNA during Purification. Figure 5 indicates that it is probably ribosomal RNA itself and not a contaminating "m-RNA" which is active here. Intact *E. coli* ribosomes were dissociated to their 30S and 50S subunits by dialysis against ribosome buffer containing 10^{-4} M magnesium acetate. The dissociated subunits were then passed through a column of Sephadex G-200 in the same buffer. The RNA in each fraction removed from the column was extracted with phenol and tested for template activity after washing by repeated ethanol precipitation and heating. The template activity appears in a region corresponding to the optical density profile of the ribosomal RNA (Figure 5).

Any ribosome-bound m-RNA would have been released from the 30S subunit attachment site during dialysis in 10^{-4} M Mg^{2+} buffer and would have been at least degraded so that it would not elute in the void volume of the column along with the bulk ribosomal RNA.

Table IV provides further evidence that ribosomal RNA itself is active; 30S and 50S subunits were partially purified by differential centrifugation and their RNA was extracted, heated, and tested for stimulatory ability. RNA from both subunits showed about the same specific activity. The same proved true when 30S and 50S subunits were separated by sucrose gradient centrifugation.

Template Activity of Heat-Degraded t-RNA. Like ribosomal RNA, s-RNA is a major component of living cells, much more abundant than m-RNA, yet inactive as a template. However, similar heat degradation and exposure to neomycin will convert s-RNA to an effective template material. It can be seen in Table V that native (unheated) s-RNA was relatively ineffective as a template and it sometimes inhibited amino acid incorporation at higher concentrations in this system. However, s-RNA became very active in stimulating amino acid incorporation after heating for 90 min at 100°. Prolonged heating was required to cause significant cleavage and activity of the small s-RNA molecules as shown by Figure 6. In contrast, Figure 7 shows that similar heating of f2 phage RNA had no such potentiating effect on its template activity. Figure 8

TABLE II: Stimulation of Incorporation of Different ^{14}C Amino Acids by *E. coli* Ribosomal RNA in the Presence of Neomycin.[a]

^{14}C Amino Acid	μg of RNA	Cpm/mg	μμmoles Incorporated/ mg of Protein
Valine	None	603	21
	500	7,310	254
Isoleucine	None	1,250	40
	500	9,780	310
Tyrosine	None	985	25
	500	16,100	412
Alanine	None	683	32
	500	13,500	639
Proline	None	775	27
	500	20,000	690
Aspartic acid	None	371	15
	600	9,940	388
Threonine	None	850	33
	600	19,700	742
Lysine	None	1,025	33
	800	68,100	2,130
Arginine	None	1,572	49
	500	27,500	870
Phenylalanine	None	2,600	70
	500	9,760	260

[a] Reaction conditions as in Table I. Neomycin (10 μg) was present in all reaction mixtures with 1 μcurie of the various ^{14}C amino acids. The ribosomal RNA was boiled for 10 min.

TABLE III: Effect of Inhibitors or Omission of Energy Requirement on Amino Acid Incorporation Stimulated by *E. coli* Ribosomal RNA.[a]

Additions or Omissions	Cpm/mg of Protein
None	38,500
RNA omitted	3,680
ATP, GTP, PEP omitted	419
100 μg of deoxyribonuclease added	30,900
1 μg of ribonuclease added	442
100 μg of puromycin added	283
100 μg of chloramphenicol added	597

[a] Reaction mixture as in Table I. Neomycin (10 μg) and 1 μcurie of ^{14}C-arginine were employed in each incubation mixture. Reaction mixtures also contained 400 μg of ribosomal RNA previously boiled for 10 min, unless otherwise stated.

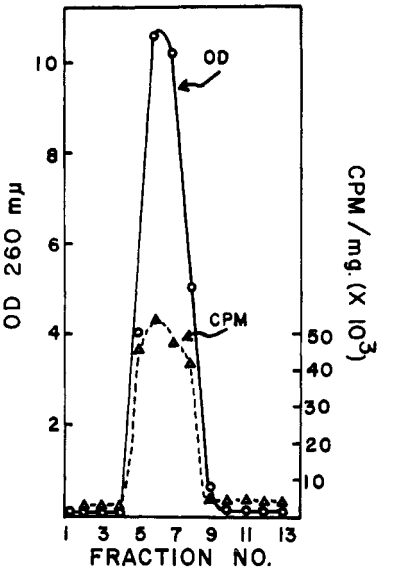

FIGURE 5: Correlation between the Sephadex G-200 elution profile of *E. coli* ribosomal subunits and the template activity of RNA extracted from each eluted fraction. See text for description.

TABLE IV: Template Activity of the RNA from the Two Subunits of *E. coli* Ribosomes.[a]

Additions	Cpm/mg of Protein
None	1,920
500 μg of RNA from 30S subunits[b]	38,600
500 μg of RNA from 50S subunits[b]	38,800
None	1,450
100 μg of RNA from 50S subunits[c]	15,000
300 μg of RNA from 30S subunits[c]	37,000

[a] Reaction mixtures as in Table I with 10 μg of neomycin and 1 μcurie of ^{14}C-arginine present in each case. In each case the ribosomal RNA was boiled for 10 min prior to use. [b] Ribosomal subunit RNA was obtained by phenol extraction of partially purified 30S or 50S subunits. [c] Ribosomal subunit RNA was obtained from 30S and 50S subunits separated by sucrose gradient centrifugation [2.5 hr at 105,000*g* in 10–30% sucrose, 0.01 M Tris buffer pH 7.8, 0.06 M KCl, 10^{-4} M $Mg(CH_3COO)_2$, 10^{-3} M β-mercaptoethanol].

shows that the template activity of s-RNA preparations sediments in a sucrose gradient in the 4S region in general agreement with the profile of optical density at 260 mμ. Parallel experiments documented the amino acid accepting activity of s-RNA prepared in this fashion.

TABLE V: Template Activity of 4S t-RNA from *E. coli*.[a]

Additions	Cpm/mg of Protein
None	2,710
100 μg of unheated s-RNA	2,750
100 μg of heated s-RNA	20,400
300 μg of unheated s-RNA	4,350
300 μg of heated s-RNA	40,200

[a]Reaction conditions as in Table I except that 10 μg of neomycin and 1 μcurie of ^{14}C-arginine were present in each case. s-RNA was the RNA from the 4S peak in a sucrose gradient. "Heated" RNA was heated for 90 min at 100° before use.

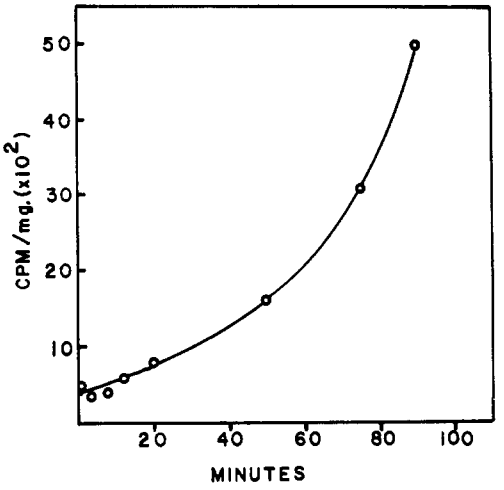

FIGURE 6: Effect of heating *E. coli* s-RNA on its template activity. *E. coli* s-RNA was heated for varying periods at 100°, then tested for template activity in the presence of 10 μg of neomycin as in Table I. s-RNA (100 μg) was used in each reaction mixture with 0.2 μcurie of ^{14}C-lysine.

Susceptibility of the Product to Proteolytic Enzymes. The amino acid labeled product directed by ribosomal RNA exhibits the susceptibility to proteolytic enzymes expected of a polypeptide. Table VI shows that both crystalline trypsin and pronase rendered the product of ribosomal RNA stimulation soluble in 5% trichloroacetic acid.

Discussion

The above results provide evidence that both t-RNA and ribosomal RNA can act as templates for amino acid incorporation in a cell-free system after disruption of their secondary structure and in the presence of

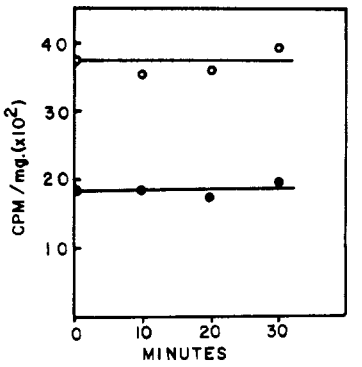

FIGURE 7: Effect of heating f2 phage RNA on its template activity. After heating at 100° for varying periods, 60-μg samples of f2 phage RNA were tested for template activity. Each reaction mixture contained 0.2 μcurie ^{14}C-valine. ○ = in the presence of 10 μg of neomycin, ● = no neomycin added.

neomycin. These findings should be considered together with the report of template activity of single-stranded DNA also effected by aminoglycoside antibiotics (Holland and McCarthy, 1964; McCarthy and Holland, 1965). The possibility, therefore, arises of a barrier to translation which is common to all these three classes of polynucleotides, which perform other functions in the living cell. Whatever this natural impediment, it is apparent that it may be surmounted in a cell-free system by the addition of streptomycinoid antibiotic molecules.

A clue to a possible explanation is provided by recent experiments concerned with ribosome biosynthesis. RNA extracted from incomplete ribosomal particles produced in the presence of chloramphenicol has template activity in an *E. coli* amino acid incorporating system (Otaka *et al.*, 1964). The same is true of the RNA extracted from precursor ribosome particles accumulated during methionine starvation or a methionine-requiring mutant of a relaxed RC^{rel} strain of *E. coli* (Nakada, 1965). In both of these cases the RNA is nonmethylated or poorly methylated (Gordon and Boman, 1964; Gordon *et al.*, 1964). These and other findings have led to the suggestion that newly formed ribosomal RNA does have template activity, perhaps for the specification of the amino acid sequence of ribosomal protein which is terminated by methylation after completion of the ribosomal particles (Nakada, 1965). Since s-RNA and DNA do not normally possess template activity and are also methylated, it may be the addition of methyl groups which prevents template activity in a polynucleotide. There is no reason to suppose that s-RNA and DNA ever act as templates for protein synthesis *in vivo*, so that an efficient mechanism to prevent ribosome attachment is to be expected. It should also be remembered that virus RNA and m-RNA, which are naturally occurring templates, are free of methyl groups (Srinivasan and Borek, 1964). The higher degree of order promoted by the insertion of

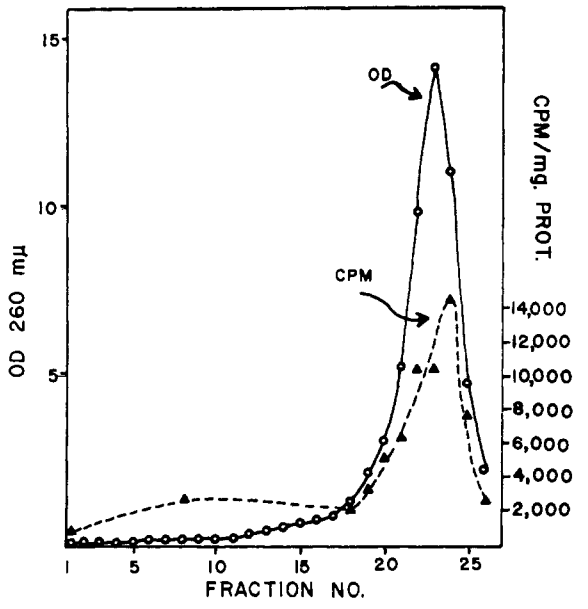

FIGURE 8: Sucrose gradient sedimentation of *E. coli* s-RNA and its template activity after heating. *E. coli* s-RNA was placed on a 5–20% sucrose gradient containing 0.1 M KCl, 2×10^{-3} M EDTA, and 2×10^{-2} M Tris-HCl, pH 7.5, and centrifuged at 105,000*g* for 300 min in the Spinco SW 39 rotor. Fractions were collected, their optical density at 260 mμ was measured, and the RNA in each fraction was recovered by ethanol precipitation with a small amount of glycogen carrier. The RNA was dissolved in water, heated at 100° for 90 min, and tested for template activity as in Table I.

TABLE VI: Sensitivity to Proteolytic Enzymes of the Product of Ribosomal RNA Template.

Product	Acid-Insoluble Cpm[a]
Control untreated product	16,490
Product after trypsin digestion	1,263
Product after pronase digestion	1,130

[a] Counts per minute precipitable by 5% trichloroacetic acid. The product of a reaction mixture (as in Table I) was passed through a column of Sephadex G-25 in standard buffer to remove free ^{14}C-arginine. The front peak (in the void volume) containing radiolabeled product was divided into three 1-ml aliquots, each of which was incubated for 30 min at 37° alone or with 1 mg of either enzyme as indicated in standard buffer. Remaining acid-insoluble material was precipitated three times with 5% TCA and the final precipitate was spread evenly on a filter and counted.

methyl groups may itself be sufficient to prevent template activity although it is possible that more specific effects are involved. The conversion of polynucleotides to active templates by neomycin and other aminoglycoside antibiotics may then be a result of the deformation of their secondary structure to allow interactions with ribosomes. Whatever the detailed mechanism of action and whether methylated bases are important, it seems likely that the special activity of these antibiotics in relaxing requirements for translation is a function of their affinity for both ribosomes and free nucleic acids.

Consistent with this concept of ordered structure as a barrier to translation is the finding that heating to produce chain scission is also effective in promoting template activity. If it is assumed that the kinetics of chain scission of ribosomal RNA and s-RNA are the same as with viral RNA (Gordon *et al.*, 1963), then one or a few breaks are sufficient to obtain good activity. In addition to destruction of helical regions such breakage could also allow reading by providing more terminal attachment sites or causing a reading frame shift. It should also be noted that neomycin is not effective unless the RNA has been degraded. This is not the case when single-stranded DNA is used as a template (McCarthy and Holland, 1965), probably because these molecules exist as random coils. Similarly, heat treatment of a natural viral m-RNA does not increase its specific activity in the presence or absence of neomycin.

Preliminary experiments have shown that proteins of considerable size may be synthesized with ribosomal RNA as template. Further experiments may show whether or not they are recognizable as ribosomal proteins. Certainly misreading of the code is to be expected by analogy with experiments concerned with antibiotic effects on RNA code words (Davies *et al.*, 1964; So *et al.*, 1964; Davies *et al.*, 1965). It has already been shown, however, that ribosomes are the sites sensitive to this effect of streptomycinoid antibiotics (Spotts and Stanier, 1961; Cox *et al.*, 1964; Speyer *et al.*, 1962), and misreading may be eliminated by the use of antibiotic-resistant ribosomes (Davies *et al.*, 1964).

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Comparison of Experimental Binding Data and Theoretical Models in Proteins Containing Subunits*

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ABSTRACT: Models for subunit interactions are examined by means of interpreting ligand saturation curves. Equations are derived to indicate the effect of variables such as the strength of binding of the ligand, the geometrical arrangement of subunits, the strength of interaction between subunits, the energy of the conformation change, and the effect of nonidentical subunits. Rapid methods, *i.e.*, equations and nomograms, are developed to fit theoretical curves to experimental data with a minimum of parameters. Applying these procedures to the binding of oxygen by hemoglobin as an illustrative example, it is seen that a number of simple models can represent the published data accurately. In general it appears that unique mechanisms cannot be established from ligand saturation curves by themselves, but the mathematical analysis of the curves indicates possible sources of additional information to make such distinctions possible.

For many years it has been known that the binding of oxygen to hemoglobin follows a sigmoid curve which differs appreciably from the typical Michaelis–Menten equation covering the same concentration range. An empirical equation designed by Hill (1910), $Y = kp^n/(1 + kp^n)$, gave a reasonable approximation to the data with $n \approx 2.6$. Adair (1925) obtained a closer fit using a four-constant equation in which the constants related the successive affinity constants of oxygen to the four heme groups in hemoglobin. Adair's equation did not provide any theoretical explanation for the changing affinity constants, but it was capable of fitting the data quite accurately. Pauling (1935) made the first attempt to relate the change in these constants to the geometry of the protein by assuming a single affinity constant and an interaction term which depended on the geometry of the four subunits. Excellent recent reviews on linked functions and binding of hemoglobin have been presented by Wyman (1964) and Rossi-Fanelli *et al.* (1964).

The recent emphasis on the properties of proteins has highlighted the importance of the hemoglobin problem in several ways. In the first place, protein conformational changes provide perhaps the best explanation for the "interaction" between heme groups during the binding of oxygen. This hypothesis is supported by the elegant studies of Perutz and co-workers (1964) who found that the hemes lie far from each other in the hemoglobin molecule and that a conformational change apparently occurs when oxygen is absorbed to hemoglobin. In the second place, the widespread observation of conformational effects in enzyme systems in general and in regulatory systems in particular together with the observation that most of these enzymes are composed of subunits indicates that the hemoglobin interactions may not be an isolated phenomenon but rather one manifestation of a general situation (Grisolia, 1964; Umbarger, 1964; Gerhart and Pardee, 1962; Monod *et al.*, 1963; Koshland, 1963).

Monod *et al.* (1965) have recently proposed an interesting new model to explain the hemoglobin satura-

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