template RNA. Other explanations, however, are fully plausible, and it is not possible at this state to rule out alternative interpretations. In the following paper, further experiments on amino acid incorporation using the system described here are presented. It will be shown that in addition to the usual requirements, the system is stimulated by template RNA.

Summary.—Cell-free *E. coli* extracts have been obtained which actively incorporate amino acids into protein. Methods were devised whereby these extracts could be dialyzed and stored for long periods of time at $-15°$ without undue loss of activity. The characteristics of amino acid incorporation by such stored extracts were strongly suggestive of *de novo* protein synthesis, for incorporation required both ribosomes and 105,000 $\times$ *g* supernatant fractions, ATP and an ATP-generating system, was stimulated by a mixture of other L-amino acids, and was markedly inhibited by puromycin, chloramphenicol, and RNAase. The initial rate of amino acid incorporation was not inhibited by DNAase; subsequent incorporation was greatly inhibited. The possible relationship of the DNAase inhibition of amino acid incorporation into protein to the synthesis of "messenger" RNA was briefly discussed.

* Supported by a NATO Postdoctoral Research Fellowship.

[1] Tissières, A., D. Schlessinger, and F. Gros, these PROCEEDINGS, **46**, 1450 (1960).
[2] Matthaei, J. H., and M. W. Nirenberg, *Fed. Proc.*, **20**, 391 (1961).
[3] Kameyama, T., and G. D. Novelli, *Biochem. Biophys. Res. Comm.*, **2**, 393 (1960).
[4] Kirsch, J. F., P. Siekevitz, and G. E. Palade, *J. Biol. Chem.*, **235**, 1419 (1960).
[5] Mora, P. T., E. Merler, and P. Maury, *J. Am. Chem. Soc.*, **81**, 5449 (1959).
[6] Anson, M. L., *J. Gen. Physiol.*, **22**, 79 (1938).
[7] Sevag, M. G., D. B. Lackmann, and J. Smolens, *J. Biol. Chem.*, **124**, 425 (1938).
[8] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *ibid.*, **193**, 265 (1951).
[9] Allfrey, V. G., and A. E. Mirsky, these PROCEEDINGS, **44**, 981 (1958).
[10] Hurwitz, J., A. Bresler, and R. Diringer, *Biochem. Biophys. Res. Comm.*, **3**, 15 (1960).
[11] Stevens, A., *ibid.*, **3**, 92 (1960).
[12] Weiss, S. B., and T. Nakamoto, *J. Biol. Chem.*, **236**, PC18 (1961).
[13] Siekevitz, P., *ibid.*, **195**, 549 (1952).

THE DEPENDENCE OF CELL- FREE PROTEIN SYNTHESIS IN E. COLI UPON NATURALLY OCCURRING OR SYNTHETIC POLYRIBONUCLEOTIDES

BY MARSHALL W. NIRENBERG AND J. HEINRICH MATTHAEI*

NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

Communicated by Joseph E. Smadel, August 3, 1961

A stable cell-free system has been obtained from *E. coli* which incorporates C^{14}-valine into protein at a rapid rate. It was shown that this apparent protein synthesis was energy-dependent, was stimulated by a mixture of L-amino acids, and was markedly inhibited by RNAase, puromycin, and chloramphenicol.[1] The present communication describes a novel characteristic of the system, that is, a requirement for template RNA, needed for amino acid incorporation even in the

presence of soluble RNA and ribosomes. It will also be shown that the amino acid incorporation stimulated by the addition of template RNA has many properties expected of *de novo* protein synthesis. Naturally occurring RNA as well as a synthetic polynucleotide were active in this system. The synthetic polynucleotide appears to contain the code for the synthesis of a "protein" containing only one amino acid. Part of these data have been presented in preliminary reports.[2, 3]

Methods and Materials.—The preparation of enzyme extracts was modified in certain respects from the procedure previously presented.[1] *E. coli* W3100 cells harvested in early log phase were washed and were disrupted by grinding with alumina (twice the weight of washed cells) at 5° for 5 min as described previously.[1] The alumina was extracted with an equivalent weight of buffer containing 0.01 *M* Tris(hydroxymethyl)aminomethane, pH 7.8, 0.01 *M* magnesium acetate, 0.06 *M* KCl, 0.006 *M* mercaptoethanol (standard buffer). Alumina and intact cells were removed by centrifugation at 20,000 × *g* for 20 min. The supernatant fluid was decanted, and 3 μg DNAase per ml (Worthington Biochemical Co.) were added, rapidly reducing the viscosity of the suspension, which was then centrifuged again at 20,000 × *g* for 20 min. The supernatant fluid was aspirated and was centrifuged at 30,000 × *g* for 30 min to clear the extract of remaining debris. The liquid layer was aspirated (S-30) and was centrifuged at 105,000 × *g* for 2 hr to sediment the ribosomes. The supernatant solution (S-100) was aspirated, and the solution just above the pellet was decanted and discarded. The ribosomes were washed by resuspension in the standard buffer and centrifugation again at 105,000 × *g* for 2 hr. Supernatant fluid was discarded and the ribosomes were suspended in standard buffer (W-Rib). Fractions S-30, S-100, and W-Rib were dialyzed against 60 volumes of standard buffer overnight at 5° and were divided into aliquots for storage at −15°.

In some cases, fresh S-30 was incubated for 40 min at 35°. The reaction mixture components in μmoles per ml were as follows: 80 Tris, pH 7.8; 8 magnesium acetate; 50 KCl; 9 mercaptoethanol; 0.075 each of 20 amino acids;[1] 2.5 ATP, K salt; 2.5 PEP, K salt; 15 μg PEP kinase (Boehringen & Sons, Mannheim, Germany). After incubation, the reaction mixture was dialyzed at 5° for 10 hr against 60 volumes of standard buffer, changed once during the course of dialysis. The incubated S-30 fraction was stored in aliquots at −15° until needed (Incubated-S-30).

RNA fractions were prepared by phenol extraction using freshly distilled phenol. Ribosomal RNA was prepared from fresh, washed ribosomes obtained by the method given above. In later RNA preparations, a 0.2% solution of sodium dodecyl sulfate recrystallized by the method of Crestfield *et al.*[4] was added to the suspension of ribosomes before phenol treatment. The suspension was shaken at room temperature for 5 min. Higher yields of RNA appeared to be obtained when the sodium dodecyl sulfate step was used; however, good RNA preparations were also obtained when this step was omitted. An equal volume of H_2O-saturated phenol was added to ribosomes suspended in standard buffer after treatment with sodium dodecyl sulfate, and the suspension was shaken vigorously at room temperature for 8–10 min. The aqueous phase was aspirated from the phenol phase after centrifugation at 1,450 × *g* for 15 min. The aqueous layer was extracted two more times in the same manner, using $^1/_2$ volume of H_2O-saturated phenol in each case. The final aqueous phase was chilled to 5° and NaCl was added to a final concentration of 0.1%. Two volumes of ethyl alcohol at −20° were added with stirring to precipitate the RNA. The suspension was centrifuged at 20,000 × *g* for 15 min and the supernatant solution was decanted and discarded. The RNA pellet was dissolved in minimal concentrations of standard buffer (minus mercaptoethanol) by gentle homogenization in a glass Potter-Elvehjem homogenizer (usually the volume of buffer used was about $^1/_3$ the volume of the original ribosome suspension). The opalescent solution of RNA was dialyzed for 18 hr against 100 volumes of standard buffer (minus mercaptoethanol) at 5°. The dialyzing buffer was changed once. After dialysis, the RNA solution was centrifuged at 20,000 × *g* for 15 min and the pellet was discarded. The RNA solution, which contained less than 1% protein, was divided into aliquots and was stored at −15° until needed.

Soluble RNA was prepared from 105,000 × *g* supernatant solution by the phenol extraction method described above. Soluble RNA was also stored at −15°. Alkali-degraded RNA was prepared by incubating RNA samples with 0.3 *M* KOH at 35° for 18 hr. The solutions then were neutralized and dialyzed against standard buffer (minus mercaptoethanol). RNAase-digested samples of RNA were prepared by incubating RNA with 2 μg per ml of crystalline

RNAase (Worthington Biochemical Company) at 35° for 60 min. RNAase was destroyed by four phenol extractions performed as given above. After the last phenol extraction, the samples were dialyzed against standard buffer minus mercaptoethanol. RNA samples were treated with trypsin by incubation with 20 μg per ml of twice recrystallized trypsin (Worthington Biochemical Company) at 35° for 60 min. The solution was treated four times with phenol and was dialyzed in the same manner.

The radioactive amino acids used, their source, and their respective specific activities are as follows: U-C^{14}-glycine, U-C^{14}-L-isoleucine, U-C^{14}-L-tyrosine, U-C^{14}-L-leucine, U-C^{14}-L-proline, L-histidine-2(ring)-C^{14}, U-C^{14}-L-phenylalanine, U-C^{14}-L-threonine, L-methionine (methyl-C^{14}), U-C^{14}-L-arginine, and U-C^{14}-L-lysine obtained from Nuclear-Chicago Corporation, 5.8, 6.2, 5.95, 6.25, 10.5, 3.96, 10.3, 3.9, 6.5, 5.8, 8.3 mC/mM, respectively; C^{14}-L-aspartic acid, C^{14}-Lglutamic acid, C^{14}-L-alanine, obtained from Volk, 1.04, 1.18, 0.75 m C/mM, respectively; D-L-tryptophan-3 C^{14}, obtained from New England Nuclear Corporation, 2.5 mC/mM; S^{35}-L-cystine obtained from the Abbott Laboratories, 2.4 mC/mM; U-C^{14}-L-serine obtained from the Nuclear-Chicago Corporation, 0.2 mC/mM. Other materials and methods used in this study are described in the accompanying paper.[1] All assays were performed in duplicate.

Results.—Stimulation by ribosomal RNA: In the previous paper,[1] it was shown that DNAase markedly decreased amino acid incorporation in this system after 20 min. For the purpose of this investigation, 30,000 × *g* supernatant fluid fractions previously incubated with DNAase and other components of the reaction mixtures (Incubated-S-30 fractions) were used for many of the experiments.

Figure 1 shows that incorporation of C^{14}-L-valine into protein by Incubated-S-30 fraction was stimulated by the addition of purified *E. coli* soluble RNA. Maximal stimulation was obtained with approximately 1 mg soluble RNA. In some experiments, increasing the concentration 5-fold did not further stimulate the system. Soluble RNA was added to all reaction mixtures unless otherwise specified.

Figure 2 demonstrates that *E. coli* ribosomal RNA preparations markedly stimu-

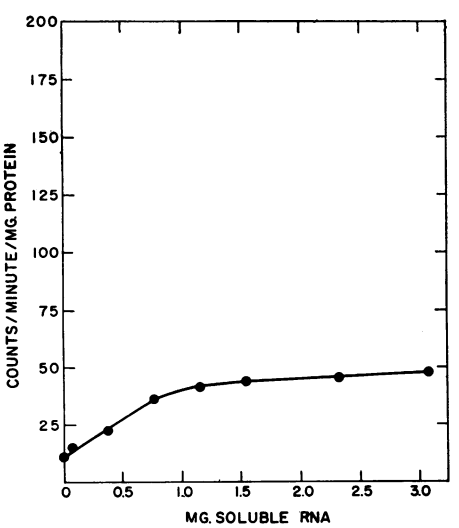


FIG 1.—Stimulation of amino acid incorporation into protein by *E. coli* soluble RNA. Composition of reaction mixtures is specified in Table 1. Samples were incubated at 35° for 20 min. Reaction mixtures contained 4.4 mg. of Incubated-S-30 protein.

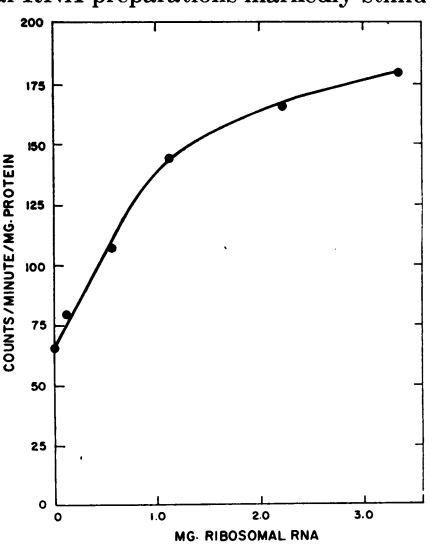


FIG. 2.—Stimulation of amino acid incorporation into protein by *E. coli* ribosomal RNA in the presence of soluble RNA. Composition of reaction mixtures is specified in Table 1. Samples were incubated at 35° for 20 min. Reaction mixtures contained 4.4 mg of Incubated-S-30 protein and 1.0 mg *E. coli* soluble RNA.

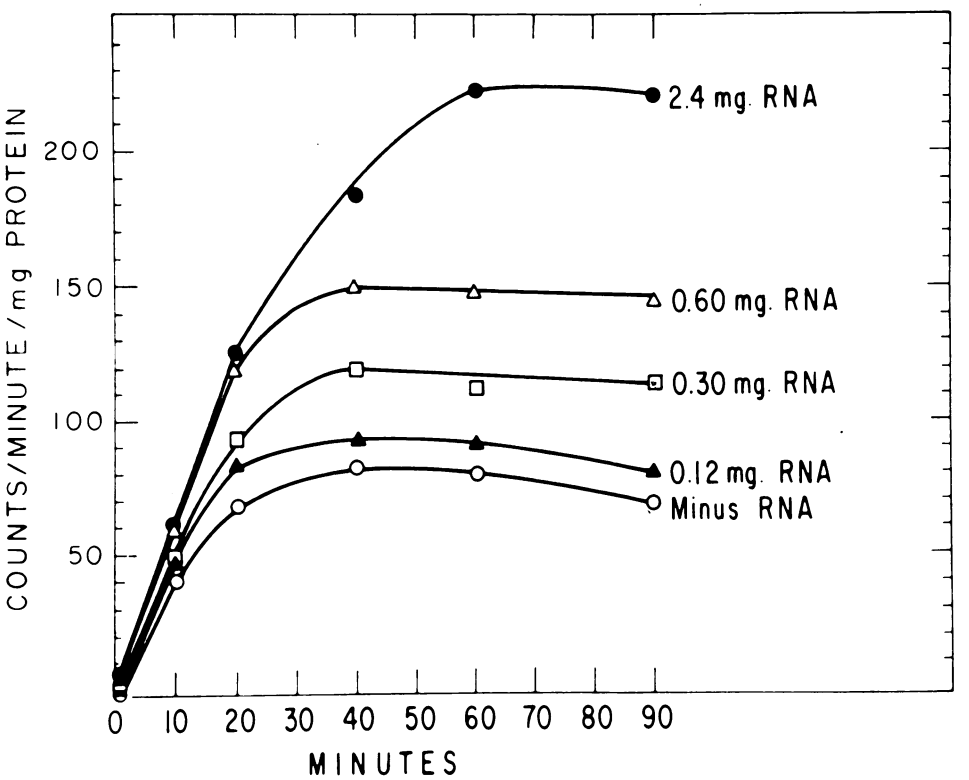


FIG. 3.—Dependence of C^{14}-L-valine incorporation into protein upon ribosomal RNA. The composition of the reaction mixtures and the incubation conditions are presented in Table 1. Reaction mixtures contained 0.98 mg of *E. coli* soluble RNA and 4.4 mg of Incubated-S-30-protein.

lated incorporation of C^{14}-valine into protein even though maximally stimulating concentrations of soluble RNA were present in the reaction mixtures. A linear relationship between the concentration of ribosomal RNA and C^{14}-valine incorporation into protein was obtained when low concentrations of ribosomal RNA were used. Increasing the soluble RNA concentration up to 3-fold did not replace the effect observed when ribosomal RNA was added.

The effect of ribosomal RNA in stimulating incorporation of C^{14}-valine into protein is presented in more detail in Figure 3. In the absence of ribosomal RNA, incorporation of C^{14}-valine into protein by the incubated-S-30 fraction was quite low when compared with S-30 (not incubated before storage at $-15°$) and stopped almost completely after 30 min. At low concentrations of ribosomal RNA, maximum amino acid incorporation into protein was proportional to the amount of ribosomal RNA added, suggesting stoichiometric rather than catalytic action of ribosomal RNA. Total incorporation of C^{14}-valine into protein was increased more than 3-fold by ribosomal RNA in this experiment even in the presence of maximally stimulating concentrations of soluble RNA. Ribosomal RNA may be added at any time during the course of the reaction, and, after further incubation, an increase in incorporation of C^{14}-valine into protein will result.

Characteristics of amino acid incorporation stimulated by ribosomal RNA: In Table 1 are presented the characteristics of C^{14}-L-valine incorporation into protein

TABLE 1

CHARACTERISTICS OF C^{14}-L-VALINE INCORPORATION INTO PROTEIN

Experiment no.	Addition	Counts/min/mg protein
1	− Ribosomal RNA	42
	+ " "	204
	+ " " + 0.15 μmole Chloramphenicol	58
	+ " " + 0.20 μmole Puromycin	7
	+ " " deproteinized at zero time	8
2	− Ribosomal RNA	35
	+ " "	101
	+ " " − ATP, PEP, PEP kinase	7
	+ " " + 10 μg RNAase	6
	+ " " + 10 μg DNAase	110
	+ Boiled Ribosomal RNA	127
	+ Ribosomal RNA, deproteinized at zero time	8
3	− Ribosomal RNA	34
	− " " − 20 L amino acids	21
	+ " "	99
	+ " " − 20-L-amino acids	52

The reaction mixtures contained the following in μmole/ml: 100 Tris(hydroxymethyl) aminomethane, pH 7.8; 10 magnesium acetate; 50 KCl; 6.0 mercaptoethanol; 1.0 ATP; 5.0 phosphoenolpyruvate, K salt; 20 μg phosphoenolpyruvate kinase, crystalline; 0.05 each of 20 L-amino acids minus valine; 0.03 each of GTP, CTP, and UTP; 0.015 C^{14}-L-valine (~70,000 counts); 3.1 mg. *E. coli* ribosomal RNA where indicated, and 1.0 mg *E. coli* soluble RNA; 3.2, 3.2, and 1.4 mg of incubated-S-30 protein were present in Experiments 1, 2, and 3, respectively. In addition 4.4 mg protein of W-Rib were added in Experiment 3. Total volume was 1.0 ml. Samples were incubated at 35° for 20 min, were deproteinized with 10 per cent trichloroacetic acid, and the precipitates were washed and counted by the method of Siekevitz.[22]

stimulated by the addition of ribosomal RNA. Amino acid incorporation was strongly inhibited by 0.15 μmoles of chloramphenicol and 0.20 μmoles/ml reaction mixture of puromycin. Furthermore, the incorporation was completely dependent upon the addition of ATP and an ATP-generating system and was totally inhibited by 10 μg/ml RNAase. Equivalent amounts of DNAase had no effect upon the incorporation stimulated by the addition of ribosomal RNA. Placing a ribosomal RNA preparation in a boiling water bath for 10 min did not destroy its C^{14}-valine incorporation activity; instead, a slight increase in activity was consistently observed. However, when these RNA preparations were placed in a boiling water bath, a copious, white precipitate resulted. Upon cooling the suspension in an ice bath, the precipitate immediately dissolved.

The data of Table 1 also demonstrate that the incorporation of amino acids into protein in the presence of ribosomal RNA was further stimulated by the addition of a mixture of 20 L-amino acids, suggesting cell-free protein synthesis.

C- and N-terminal analyses of the ribosomal RNA-dependent product of the reaction were performed with carboxypeptidase and 1-fluoro-2,4-dinitrobenzene respectively (Dr. Frank Tietze kindly performed these analyses). Four per cent of the radioactivity was released from the C-terminal end and 1% was associated with the N-terminal end. The remainder of the C^{14}-label was internal. Similar results were obtained when reactions were performed using S-30 enzyme fractions which had not been treated with DNAase. Protein precipitates isolated from reaction mixtures after incubation were completely hydrolyzed with HCl, and the C^{14}-label incorporated into protein was demonstrated to be valine by paper chromatography.

Many of the experiments presented in this paper were performed with enzyme fractions prepared with DNAase added to reduce their viscosity. Ribosomal

RNA also stimulated C^{14}-valine incorporation when enzyme extracts prepared in the absence of DNAase were used.

To be effective in stimulating amino acid incorporation into protein, the ribosomal RNA *required the presence of washed ribosomes.* The data of Table 2 show

TABLE 2

THE INEFFECTIVENESS OF RIBOSOMAL RNA IN STIMULATING C^{14}-L-VALINE INCORPORATION INTO PROTEIN IN THE PRESENCE OF RIBOSOMES OR 105,000 × *g* SUPERNATANT SOLUTIONS ALONE

Additions	Counts/min
Complete	51
" + 2.1 mg Ribosomal RNA	202
" − Ribosomes	17
" − Ribosomes + 2.1 mg Ribosomal RNA	20
" − Supernatant solution	36
" − Supernatant solution + 2.1 mg Ribosomal RNA	45
" Deproteinized at zero time	25

The components of the reaction mixtures and the incubation conditions are presented in Table 1. 0.86 and 3.3 mg protein were present in the ribosome (W-Rib) and 105,000 × *g* supernatant (S-100) fractions, respectively.

that both ribosomes and 105,000 × *g* supernatant solution were necessary for ribosomal RNA-dependent amino acid incorporation. No incorporation of amino acids into protein occurred when the 105,000 × *g* supernatant solution alone was added to ribosomal RNA preparations, demonstrating that ribosomal RNA preparations were not contaminated with intact ribosomes. This conclusion also was substantiated by showing that the activities of ribosomal RNA preparations were not destroyed by boiling, although the activities of the ribosomes were destroyed by such treatment.

The effect of ribosomal RNA upon the incorporation of seven different amino acids is presented in Table 3. The addition of ribosomal RNA increased the incorporation of every amino acid tested.

The effect shown by ribosomal RNA was not observed when other polyanions were used, such as polyadenylic acid, highly polymerized salmon sperm DNA, or a high-molecular-weight polymer of glucose carboxylic acid (Table 4). Pretreatment of ribosomal RNA with trypsin did not affect its biological activity. However, treatment of the ribosomal RNA with either RNAase or alkali resulted in a complete loss of stimulating activity. The active principle, therefore, appears to be RNA.

The sedimentation characteristics of the ribosomal RNA preparations were examined in the Spinco Model E ultracentrifuge (Fig. 4*A*). Particles having the characteristics of S-30, S-50, or S-70 ribosomes were not observed in these preparations. The S_{20}^{W} of the first peak was 23, that of the second peak 16, and that of the third, small peak, 4. Pretreatment with trypsin did not affect the S_{20}^{W} values of the peaks appreciably (Fig. 4*C*); however, treatment with RNAase completely destroyed the peaks (Fig. 4*B*), confirming the ancillary evidence which had suggested that the major component was high-molecular-weight RNA.

Preliminary attempts at fractionation of the ribosomal RNA were performed by means of density-gradient centrifugation employing a linear sucrose gradient. The results of o[illegible]uch experiment are presented in Figure 5. Amino acid incorporation acti[illegible]he RNA did not follow absorbancy at 260 mμ; instead, the activity se[illegible]ncentrated around fraction No. 5, which was approximately one-third[illegible] from the bottom of the tube. These results again

TABLE 3

SPECIFICITY OF AMINO ACID INCORPORATION STIMULATED BY RIBOSOMAL RNA

C^{14}-Amino Acid	Addition	Counts/min/mg protein
C^{14}-L-Valine	Complete	25
"	" + Ribosomal RNA	137
C^{14}-L-Threonine	"	31
"	" + Ribosomal RNA	121
C^{14}-L-Methionine	"	121
"	" + Ribosomal RNA	177
C^{14}-L-Arginine	"	49
"	" + Ribosomal RNA	224
C^{14}-L-Phenylalanine	"	77
"	" + Ribosomal RNA	147
C^{14}-L-Lysine	"	36
"	" + Ribosomal RNA	175
C^{14}-L-Leucine	"	134
"	" + Ribosomal RNA	272
"	" Deproteinized at zero time	6

The composition of the reaction mixtures are presented in Table 1. The mixture of 20 L-amino acids included all amino acids except the C^{14}-amino acid added to one reaction mixture. Reaction mixtures contained 4.4 mg Incubated-S-30 protein. Samples were incubated at 35° for 60 min. 2.1 mg ribosomal RNA were added where indicated.

TABLE 4

RIBOSOMAL RNA CONTROL EXPERIMENTS DESCRIBED IN TEXT

Experiment No.	Addition	Counts/min/mg protein
1	Complete	54
	" + 2.4 mg Ribosomal RNA	144
	" + 2.0 mg Polyadenylic acid	10
	" + 2.0 mg Salmon sperm DNA	41
	" + 2.0 mg Polyglucose carboxylic acid	49
	" + 2.4 mg Ribosomal RNA, deproteinized at zero time	7
2	Complete	39
	" + 2.0 mg Ribosomal RNA*	150
	" + 2.1 mg Ribosomal RNA preincubated with trypsin*	166
	" + 2.0 mg Ribosomal RNA preincubated with RNAase*,†	47
	" Deproteinized at zero time	8
3	Complete	20
	" + 1.2 mg Ribosomal RNA	82
	" + 1.2 mg Alkali degraded ribosomal RNA†	21
	" Deproteinized at zero time	7

The composition of the reaction mixtures and the incubation conditions are given in Table 1. 4.4, 3.2, and 4.4 mg Incubated-S-30 protein were present in Experiments 1, 2, and 3, respectively. 2.4, 0.98, and 0 mg *E. coli* soluble RNA were present in Experiments 1, 2, and 3, respectively.

* Ribosomal RNA preparations were deproteinized by phenol extraction after enzymatic digestion as specified under *Methods and Materials.*

† mg Ribosomal RNA refers to RNA concentration before digestion.

demonstrate that the activity was not associated with a soluble RNA fraction, present in maximum concentration in fraction No. 11, near the top of the tube. In addition, all amino acid incorporation analyses were performed in the presence of added soluble RNA, and the addition of more soluble RNA would not stimulate C^{14}-L-valine incorporation into protein.

Effects of RNA obtained from different species: The data of Table 5 demonstrate that RNA from different sources stimulates C^{14}-valine incorporation into protein. Yeast ribosomal RNA prepared by the method of Crestfield *et al.*[4] was considerably more effective in stimulating incorporation than equivalent amounts of *E. coli* ribosomal RNA. Yeast ribosomal RNA prepared by this method has little or no amino acid acceptor activity and has a molecular weight [redacted] 26,000.[7] Tobacco mosaic virus RNA prepared by phenol extraction and [redacted]ecular weight of

TABLE 5

STIMULATION OF AMINO ACID INCORPORATION BY RNA FRACTIONS PREPARED FROM DIFFERENT SPECIES

Additions	Counts/min/mg protein
None	42
+ 0.5 mg *E. coli* ribosomal RNA	75
+ 0.5 mg Yeast ribosomal RNA	430
+ 0.5 mg Tobacco mosaic virus RNA	872
+ 0.5 mg Ehrlich ascites tumor microsomal RNA	65

The components of the reaction mixtures and the incubation conditions are presented in Table 1. Reaction samples contained 1.9 mg Incubated-S-30 protein.

approximately 1,700,000† stimulated amino acid incorporation strongly. Marked stimulation due to tobacco mosaic virus RNA was observed also with *E. coli* enzyme extracts which had not been treated with DNAase. More complete details of this work will be presented in a later publication.

Stimulation of amino acid incorporation by synthetic polynucleotides: The data of Figure 6 show that the addition of 10 μg of polyuridylic acid‡ per ml of reaction mixture resulted in a remarkable stimulation of C^{14}-L-phenylalanine incorporation. Phenylalanine incorporation was almost completely dependent upon the addition of polyuridylic acid, and incorporation proceeded, after a slight lag period, at a linear rate for approximately 30 min.

The data of Table 6 demonstrate that no other polynucleotide tested could replace polyuridylic acid. The absolute specificity of polyuridylic acid was con-

TABLE 6

POLYNUCLEOTIDE SPECIFICITY FOR PHENYLALANINE INCORPORATION

Experiment no.	Additions	Counts/min/mg protein
1	None	44
	+ 10 μg Polyuridylic acid	39,800
	+ 10 μg Polyadenylic acid	50
	+ 10 μg Polycytidylic acid	38
	+ 10 μg Polyinosinic acid	57
	+ 10 μg Polyadenylic-uridylic acid (2/1 ratio)	53
	+ 10 μg Polyuridylic acid + 20 μg polyadenylic acid	60
	Deproteinized at zero time	17
2	None	75
	+ 10 μg UMP	81
	+ 10 μg UDP	77
	+ 10 μg UTP	72
	Deproteinized at zero time	6

Components of the reaction mixtures are presented in Table 1. Reaction mixtures contained 2.3 mg Incubated-S-30 protein. 0.02 μmoles U-C^{14}-L-phenylalanine (~125,000 counts/minute) was added to each reaction mixture. Samples were incubated at 35° for 60 min.

firmed by demonstrating that randomly mixed polymers of adenylic and uridylic acid‡ (Poly A-U, 2/1 ratio and 4/1 ratio) were inactive in this system. A solution of polyuridylic acid and polyadenylic acid (which forms triple-stranded helices) had no activity whatsoever, suggesting that single-strandedness is a necessary requisite for activity. Experiment 2 in Table 6 demonstrates that UMP, UDP, or UTP were unable to stimulate phenylalanine incorporation.

The data of Table 7 demonstrate that both ribosomes and 100,000 × *g* supernatant solution, as well as ATP and an ATP-generating system, were required for the polyuridylic acid–dependent incorporation of phenylalanine. Incorporation was inhibited by puromycin, chloramphenicol, and RNAase. The incorpora-

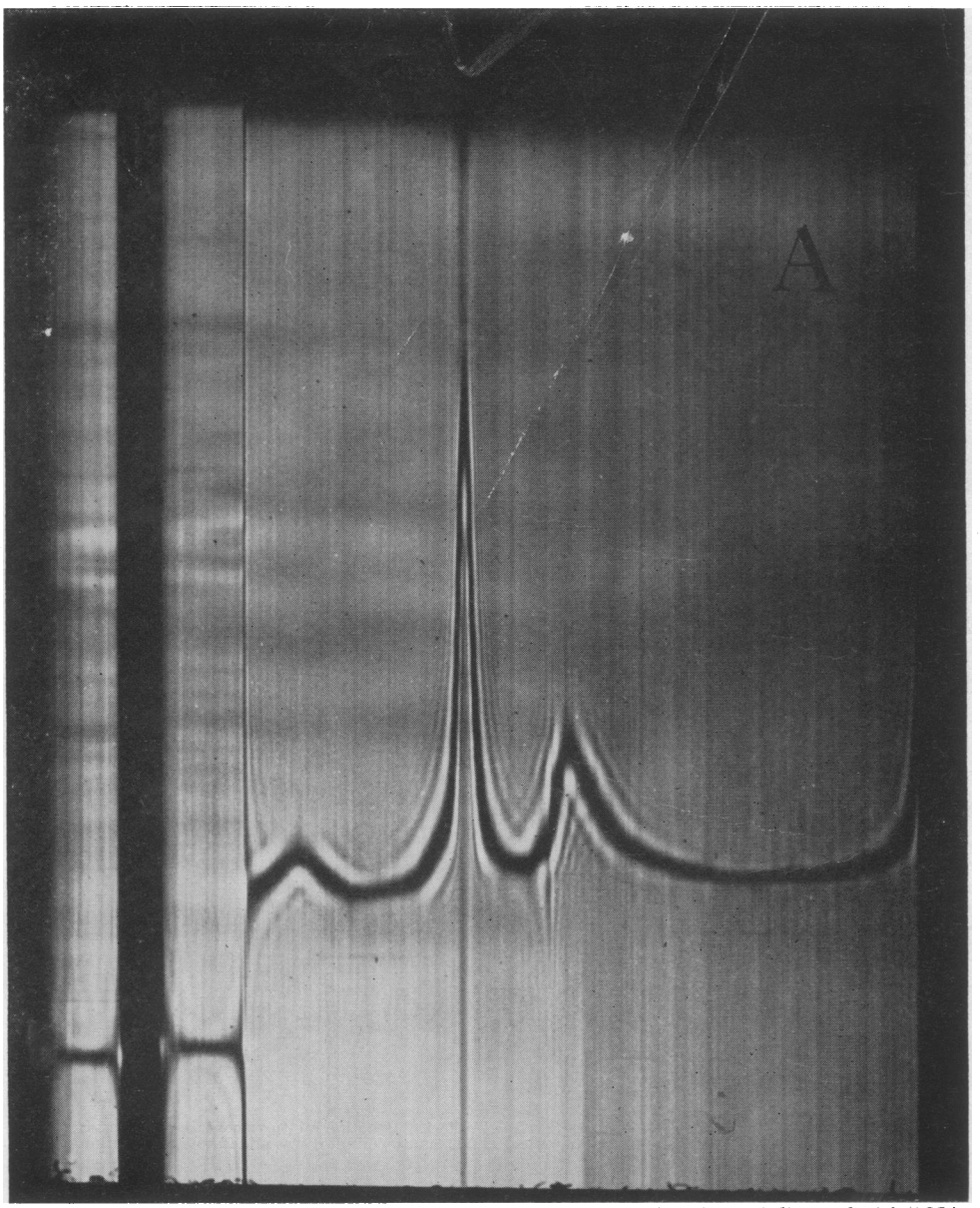
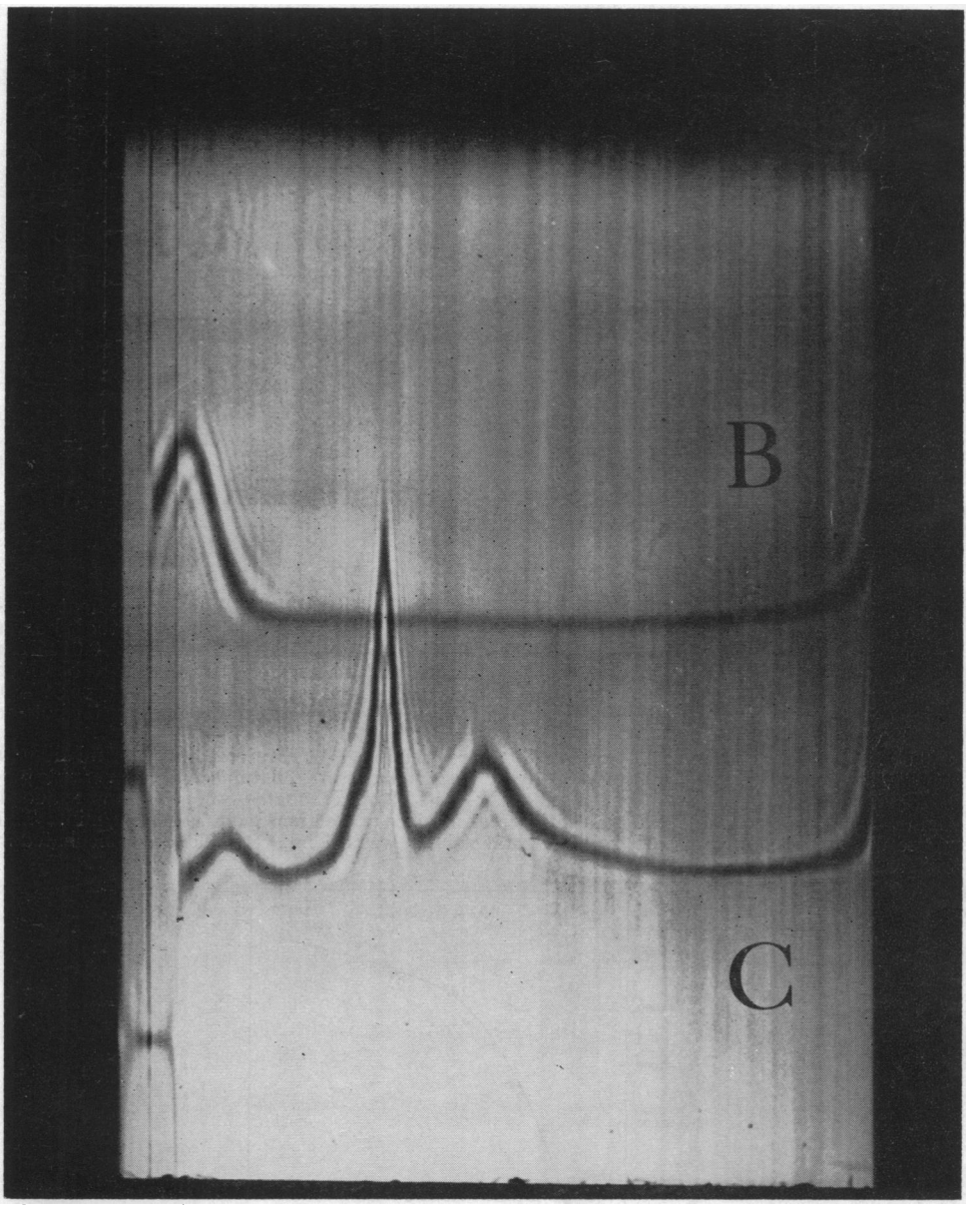


FIG. 4.—*E. coli* ribosomal RNA preparations. (*A*) Untreated (above). (*B*) digested with RNAase; (*C*) digested with trypsin. Preparation and digestion of samples presented under *Methods and Materials*. 9.8 and 10.5 mg/ml RNA were present in *A* and *C*. 11.5 mg/ml RNA was present in *B*
(*Continued on facing page*)

tion was not inhibited by addition of DNAase. Omitting a mixture of 19 L-amino acids did not inhibit phenylalanine incorporation, suggesting that polyuridylic acid stimulated the incorporation of L-phenylalanine alone. This conclusion was substantiated by the data presented in Table 8. Polyuridylic acid had little effect in stimulating the incorporation of 17 other radioactive amino acids. Each labeled amino acid was tested individually, and these data, corroborating the results given in Table 8, will be presented in a subsequent publication.



(*Fig. 4—continued*)

before digestion. Photographs were taken in a model E Spinco ultracentrifuge equipped with schlieren optics.

The product of the reaction was partially characterized and the results are presented in Table 9. The physical characteristics of the product of the reaction resembled those of authentic poly-L-phenylalanine, for, unlike many other polypeptides and proteins, both the product of the reaction and the polymer were resistant to hydrolysis by 6*N* HCl at 100° for 8 hr but were completely hydrolyzed by 12*N* HCl at 120–130° for 48 hr.

Poly-L-phenylalanine is insoluble in most solvents[25] but is soluble in 33 per cent

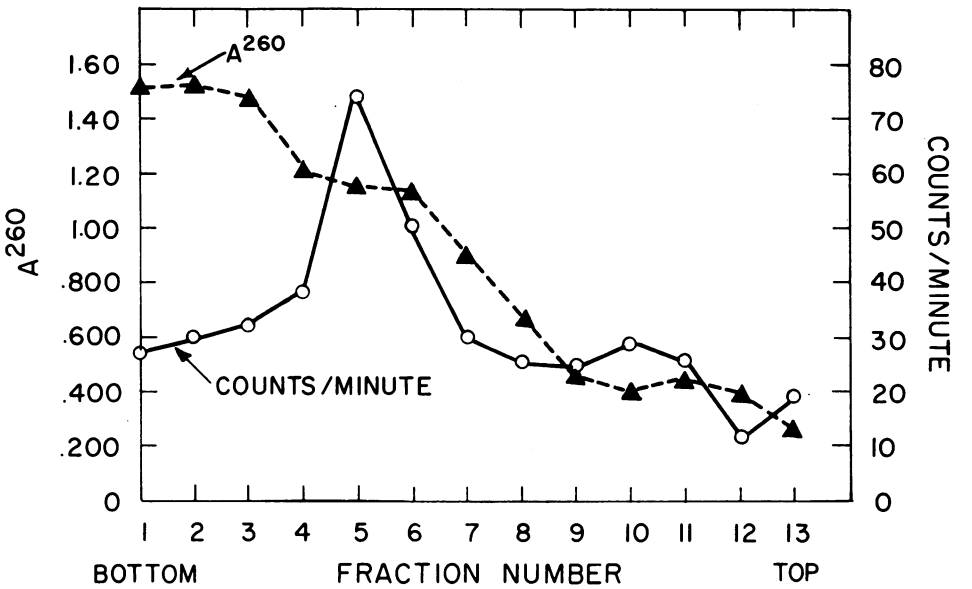


FIG. 5.—Sucrose density-gradient centrifugation of ribosomal RNA. A linear gradient of sucrose concentration ranging from 20 per cent at the bottom to 5 per cent at the top of the tube was prepared.[23] The sucrose solutions (4.4 ml total volume) contained 0.01 *M* Tris, pH 7.8, 0.01 *M* Mg acetate and 0.06 *M* KCl. 0.4 ml of ribosomal RNA (4.6 mg) was layered on top of each tube which was centrifuged at 38,000 × *g* for 4.5 hours at 3° in a swinging bucket rotor, Spinco type SW-39, using a Spinco Model L ultracentrifuge. 0.30 ml fractions were collected after piercing the bottom of the tube.[24]

0.025 ml aliquots diluted to 0.3 ml with H_2O were used for A^{260} measurements. 0.25 ml aliquots were used for amino acid incorporation assays. Reaction mixtures contained the components presented in Table 1. 0.7 mg of *E. coli* soluble RNA and 2.2 mg Incubated-S-30 protein were added. Control assays plus 0.25 ml 12.5 per cent sucrose in place of fractions gave 79 counts/min. This figure was subtracted from each value. Total volume was 0.7 ml. Samples were incubated at 35° for 20 min.

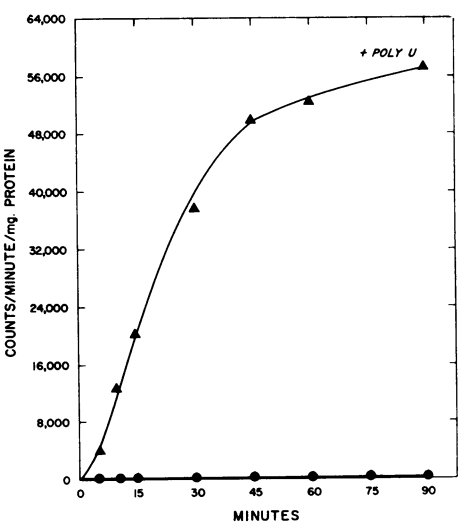


FIG. 6.—Stimulation of U-C^{14}-L-phenylalanine incorporation by polyuridylic acid. ● without polyuridylic acid; ▲ 10 μg polyuridylic acid added. The components of the reaction mixtures and the incubation conditions are given in Table 1. 0.024 μmole U-C^{14}-L-phenylalanine (~500,000 counts/min) and 2.3 mg Incubated-S-30 protein were added/ml of reaction mixture.

TABLE 7

CHARACTERISTICS OF POLYURIDYLIC ACID–DEPENDENT PHENYLALANINE INCORPORATION

Additions	Counts/min/mg protein
Minus polyuridylic acid	70
None	29,500
Minus 100,000 × *g* supernatant solution	106
Minus ribosomes	52
Minus ATP, PEP, and PEP kinase	83
+ 0.02 μmoles puromycin	7,100
+ 0.31 μmoles chloramphenicol	12,550
+ 6 μg RNAase	120
+ 6 μg DNAase	27,600
Minus amino acid mixture	31,700
Deproteinized at zero time	30

The components of the reaction mixtures are presented in Table 1. 10 μg of polyuridylic acid were added to all samples except the specified one. 2.3 mg of Incubated-S-30 protein were added to each reaction mixture except those in which ribosomes alone and 100,000 × *g* supernatant solution alone were tested. 0.7 mg W-Rib protein and 1.3 mg S-100 protein were used respectively. 0.02 μmoles U-C^{14}-L-phenylalanine, Sp. Act. = 10.3 mC/mM (~125,000 counts/minute) were added to each reaction mixture. Samples were incubated at 35° for 60 min.

TABLE 8

SPECIFICITY OF AMINO ACID INCORPORATION STIMULATED BY POLYURIDYLIC ACID

Experiment no.	C^{14}-amino acids present	Additions	Counts/min/mg protein
1	Phenylalanine	Deproteinized at zero time	25
		None	68
		+ 10 μg polyuridylic acid	38,300
2	Glycine, alanine, serine, aspartic acid, glutamic acid	Deproteinized at zero time	17
		None	20
		+ 10 μg polyuridylic acid	33
3	Leucine, isoleucine, threonine, methionine, arginine, histidine, lysine, tyrosine, tryptophan, proline, valine	Deproteinized at zero time	73
		None	276
		+ 10 μg polyuridylic acid	899
4	S^{35}-cysteine	Deproteinized at zero time	6
		None	95
		+ 10 μg polyuridylic acid	113

Components of the reaction mixtures are presented in Table 1. The unlabeled amino acid mixture was omitted. 0.015 μM of each labeled amino acid was used. The specific activities of the labeled amino acids are present in the *Methods and Materials* section. 2.3 mg of protein of preincubated S-30 enzyme fraction were added to each reaction mixture. All samples were incubated at 35° for 30 min.

TABLE 9

COMPARISON OF CHARACTERISTICS OF PRODUCT OF REACTION AND POLY-L-PHENYLALANINE

Treatment	Product of reaction	Poly-L-phenylalanine
6 *N* HCl for 8 hours at 100°	Partially hydrolyzed	Partially hydrolyzed
12 *N* HCl for 48 hours at 120–130°	Completely hydrolyzed	Completely hydrolyzed
Extraction with 33% HBr in glacial acetic acid	Soluble	Soluble
Extraction* with the following solvents: H_2O, benzene, nitrobenzene, chloroform, N,N-dimethylformamide, ethanol, petroleum ether, concentrated phosphoric acid, glacial acetic acid, dioxane, phenol, acetone, ethyl acetate, pyridine, acetophenone, formic acid	Insoluble	Insoluble

* The product was said to be insoluble if <0.002 gm of product was soluble in 100 ml of solvent at 24°. Extractions were performed by adding 0.5 mg of authentic poly-L-phenylalanine and the C^{14}-product of a reaction mixture (1800 counts/min) to 5.0 ml of solvent. The suspensions were vigorously shaken for 30 min at 24° and were centrifuged. The precipitates were plated and their radioactivity was determined.

HBr in glacial acetic acid.§ The product of the reaction had the same apparent solubility as authentic poly-L-phenylalanine. The product of the reaction was purified by means of its unusual solubility behavior. Reaction mixtures were deproteinized after incubation, and precipitated proteins were washed in the usual

manner according to the method of Siekevitz.[22] Dried protein pellets containing added carrier poly-L-phenylalanine were then extracted with 33 per cent HBr in glacial acetic acid, and the large amount of insoluble material was discarded. Polyphenylalanine was then precipitated from solution by the addition of H_2O and was washed several times with H_2O. Seventy per cent of the total amount of C^{14}-L-phenylalanine incorporated into protein due to the addition of polyuridylic acid could be recovered by this procedure. Complete hydrolysis of the purified reaction product with 12*N* HCl followed by paper electrophoresis** demonstrated that the reaction product contained C^{14}-phenylalanine. No other radioactive spots were found.

Discussion.—In this investigation, we have demonstrated that template RNA is a requirement for cell-free amino acid incorporation. Addition of soluble RNA could not replace template RNA in this system. In addition, the density-gradient centrifugation experiments showed that the active fractions in the ribosomal RNA preparations sedimented much faster than soluble RNA. It should be noted that ribosomal RNA is qualitatively different from soluble RNA, since bases such as pseudouracil, methylated guanines, etc., found in soluble RNA, are not present in ribosomal RNA.[5]

The bulk of the RNA in our ribosomal RNA fractions may be inactive as templates, for tobacco mosaic virus RNA was 20 times as active in stimulating amino acid incorporation as equivalent amounts of *E. coli* ribosomal RNA. In addition, preliminary fractionation of ribosomal RNA indicated that only a portion of the total RNA was active.

It should be emphasized that ribosomal RNA could not substitute for ribosomes, indicating that ribosomes were not assembled from the added RNA *in toto*. The function of ribosomal RNA remains an enigma, although at least part of the total RNA is thought to serve as templates for protein synthesis and has been termed "messenger" RNA.[12–14] Alternatively, a part of the RNA may be essential for the synthesis of active ribosomes from smaller ribosomal particles.[15–21]

Ribosomal RNA may be an aggregate of subunits which can dissociate after proper treatment.[6–8] Phenol extraction of *E. coli* ribosomes yields two types of RNA molecules with S_w^{20} of 23 and 16 (Fig. 4), equivalent to molecular weights of 1,000,000 and 560,000, respectively.[9, 10] These RNA species can be degraded by boiling to products having sedimentation coefficients of 13.1, 8.8, and 4.4, corresponding to molecular weights of 288,000, 144,000, and 29,000. Although the sedimentation distributions of the latter preparations suggest a high degree of homogeneity among the molecules of each class, these observations do not eliminate the possibility that the subunits are linked to one another *via* covalent bonds.[8] Preliminary evidence indicates that the subunits may be active in our system, since the supernatant solution obtained after boiling *E. coli* ribosomal RNA for 10 min and centrifugation at 105,000 $\times$ *g* for 60 min was active. Examination of boiled ribosomal RNA with the Spinco Model E ultracentrifuge showed a dispersed peak with a sedimentation coefficient of 4–8. This may be the same material found in the sucrose density-gradient experiment (using non-boiled RNA preparations), where a small peak of activity somewhat heavier than soluble RNA was usually noted (Fig. 5).

In our system, at low concentrations of ribosomal RNA, amino acid incorporation

into protein was proportional to the amount of ribosomal RNA added, suggesting a stoichiometric rather than a catalytic action of ribosomal RNA. In contrast, soluble RNA has been shown to act in a catalytic fashion.[11]

The results indicate that polyuridylic acid contains the information for the synthesis of a protein having many of the characteristics of poly-L-phenylalanine. This synthesis was very similar to the cell-free protein synthesis obtained when naturally-occurring template RNA was added, i.e., both ribosomes and 100,000 $\times$ g supernatant solutions were required, and the incorporation was inhibited by puromycin or chloramphenicol. One or more uridylic acid residues therefore appear to be the code for phenylalanine. Whether the code is of the singlet, triplet, etc., type has not yet been determined. Polyuridylic acid seemingly functions as a synthetic template or messenger RNA, and this stable, cell-free *E. coli* system may well synthesize any protein corresponding to meaningful information contained in added RNA.

Summary.—A stable, cell-free system has been obtained from *E. coli* in which the amount of incorporation of amino acids into protein was dependent upon the addition of heat-stable template RNA preparations. Soluble RNA could not replace template RNA fractions. In addition, the amino acid incorporation required both ribosomes and 105,000 $\times$ g supernatant solution. The correlation between the amount of incorporation and the amount of added RNA suggested stoichiometric rather than catalytic activity of the template RNA. The template RNA–dependent amino acid incorporation also required ATP and an ATP-generating system, was stimulated by a complete mixture of L-amino acids, and was markedly inhibited by puromycin, chloramphenicol, and RNAase. Addition of a synthetic polynucleotide, polyuridylic acid, specifically resulted in the incorporation of L-phenylalanine into a protein resembling poly-L-phenylalanine. Polyuridylic acid appears to function as a synthetic template or messenger RNA. The implications of these findings are briefly discussed.

Note added in proof.—The ratio between uridylic acid units of the polymer required and molecules of L-phenylalanine incorporated, in recent experiments, has approached the value of 1:1. Direct evidence for the number of uridylic acid residues forming the code for phenylalanine as well as for the eventual stoichiometric action of the template is not yet established. As polyuridylic acid codes the incorporation of L-phenylalanine, polycytidylic acid‡ specifically mediates the incorporation of L-proline into a TCA-precipitable product. Complete data on these findings will be included in a subsequent publication.

* Supported by a NATO Postdoctoral Research Fellowship.

† Dr. Frankel-Conrat, personal communication.

‡ We thank Drs. Leon A. Heppel and Maxine F. Singer for samples of these polyribonucleotides, and Dr. George Rushizky for TMV-RNA.

§ We thank Dr. Michael Sela for this information.

** We thank Drs. William Dreyer and Elwood Bynum for performing the high-voltage electrophoretic analyses.

[1] Matthaei, J. H., and M. W. Nirenberg, these PROCEEDINGS, **47**, 1580 (1961).

[2] Matthaei, J. H., and M. W. Nirenberg, *Biochem. & Biophys. Res. Comm.*, **4**, 404 (1961).

[3] Matthaei, J. H., and M. W. Nirenberg, *Fed. Proc.*, **20**, 391 (1961).

[4] Crestfield, A. M., K. C. Smith, and F. W. Allen, *J. Biol. Chem.*, **216**, 185 (1955).

[5] Davis, F. F., A. F. Carlucci, and I. F. Roubein, *ibid.*, **234**, 1525 (1959).

[6] Hall, B. D., and P. Doty, *J. Mol. Biol.*, **1**, 111 (1959).

[7] Osawa, S., *Biochim. Biophys. Acta*, **43**, 110 (1960).

[8] Aronson, A. I., and B. J. McCarthy, *Biophys. J.*, **1**, 215 (1961).

[9] Kurland, C. G., *J. Mol. Biol.*, **2**, 83 (1960).
[10] Littauer, U. Z., H. Eisenberg, *Biochim. Biophys. Acta*, **32**, 320 (1959).
[11] Hoagland, M. B., and L. T. Comly, these PROCEEDINGS, **46**, 1554 (1960).
[12] Volkin, E., L. Astrachan, and J. L. Countryman, *Virology*, **6**, 545 (1958).
[13] Nomura, M., B. D. Hall, and S. Spiegelman, *J. Mol. Biol.*, **2**, 306 (1960).
[14] Hall, B. D., and S Spiegelman, these PROCEEDINGS, **47**, 137 (1961).
[15] Bolton, E. T., B. H. Hoyen, and D. B. Ritter, in *Microsomal Particles and Protein Synthesis*, ed. R. B. Roberts (New York: Pergamon Press, 1958), p. 18.
[16] Tissières, A., J. D. Watson, D. Schlessinger, and B. R. Hollingworth, *J. Mol. Biol.*, **1**, 221 (1959).
[17] Tissières, A., D. Schlessinger, and F. Gros, these PROCEEDINGS, **46**, 1450 (1960).
[18] McCarthy, B. J., and A. I. Aronson, *Biophys. J.*, **1**, 227 (1961).
[19] Hershey, A. D., *J. Gen. Physiol.*, **38**, 145 (1954).
[20] Siminovitch, L., and A. F. Graham, *Canad. J. Microbiol.*, **2**, 585 (1956).
[21] Davern, C. I., and M. Meselson, *J. Mol. Biol.*, **2**, 153 (1960).
[22] Siekevitz, P., *J. Biol. Chem.*, **195**, 549 (1952).
[23] Britten, R. J., and R. B. Roberts, *Science*, **131**, 32 (1960).
[24] Martin, R., and B. Ames, *J. Biol. Chem.*, **236**, 1372 (1961).
[25] Bamford, C. H., A. Elliott, and W. E. Hanby, *Synthetic Polypeptides* (New York: Academic Press, 1956), p. 322.

MUTAGENS AND INFECTIOUS NUCLEIC ACIDS

BY JOHN H. NORTHROP* AND FILIPPO CAVALLERO†

THE UNIVERSITY OF CALIFORNIA AT BERKELEY AND THE ROCKEFELLER INSTITUTE

Communicated August 14, 1961

Cultures of bacteria usually contain a few cells which differ from the rest in some hereditary character. The difference may be colony type, resistance to antibiotics, virus production, or other properties.

The cells which are resistant to antibiotics may be proved by Lederberg's[1] replica technique to be mutants of the sensitive cells.[2] These resistant cells produce a nucleic acid (transforming principle) which is able to transfer the hereditary character to another cell, and at the same time cause more of itself to be formed.[3, 4] This nucleic acid, therefore, appears as a result of the mutation (or perhaps its appearance is the mutation).

The virus-producing cells also contain a nucleic acid[5] (the virus) which can transfer genetic information to another cell and also cause more of itself to be formed, exactly like the transforming principle. The viral nucleic acid, however, is generally assumed to be formed from a hypothetical "pro-virus,"[13] which had infected the culture at some time in the past.

There are, therefore, two entirely different hypotheses for the origin of transforming-principle nucleic acid and viral nucleic acid, although both have the same chemical and biological properties. This appears improbable and, in any event, violates the principle of economy of hypotheses.

One of the writers suggested,[14, 15] therefore, that the virus-producing cell also is a mutant, like the antibiotic-resistant cell. The fact that the number of such cells is generally increased by mutagenic agents[13] was cited as evidence.