metabolism, suggests once more the interesting possibility that the substances studied by Shapiro may have their origin in algal metabolism (*3*). Allen has reported that for pure cultures of six species of *Chlamydomonas*, 10 to 45 percent of the oxidizable organic matter formed by the cells is excreted into the medium (*7*).

An extension of this analysis to other groups of algae and a rigorous study and comparison of their excretion products with the organic material found in natural waters may clarify the nature, origin, and role of these substances (*8*).

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28 September 1962

Fate of a Synthetic Polynucleotide Directing Cell-Free Protein Synthesis
I. Characteristics of Degradation

Abstract. Tritiated-polyuridylic acid was degraded rapidly in extracts of *Escherichia coli* and degradation was not dependent on concomitant polyphenylalanine synthesis. Since a large portion of the polymer was degraded before appreciable polyphenylalanine was synthesized, the catalytic activity of the undegraded polyuridylic acid in directing protein synthesis was suggested. The predominant breakdown products were 5′-mononucleotide phosphates. Possible enzymatic routes of polyuridylic acid breakdown are discussed.

Polyuridylic acid (*1*) has been found to direct the incorporation of phenylalanine into polyphenylalanine in a cell-free *Escherichia coli* protein synthesizing system (*2*). The ease of preparation of this synthetic polynucleotide from isotopically labeled UDP and its uniform base composition have facilitated the study of the fate of this compound which is believed to represent a simple model of "messenger RNA." This and the accompanying report (*3*) describe several aspects of the fate of poly U in extracts of *E. coli* which are actively synthesizing polyphenylalanine.

Experimental procedure. An extract of *E. coli*, "pre-incubated S-30" (*2*), was separated into ribosomal and supernatant fractions by centrifugation at 105,000*g* for 3 hours. The upper two-thirds of the supernatant solution was saved. The lower one-third was gently swirled for 5 seconds over the ribosomal pellet and decanted, and the ribosomes were resuspended in one-third the original volume of 0.1*M* tris-HCl, *p*H 7.8, 0.01*M* magnesium acetate, 0.05*M* KCl, and 0.006*M* β-mercaptoethanol. By systematically varying the proportions of ribosomal and supernatant solutions in the presence of a constant amount of poly U [80 mμmole (pU) per milliliter of reaction mixture] the optimal proportion of these solutions for maximal polyphenylalanine synthesis was determined and used in all the experiments. Protein was determined by a modification of the method of Lowry *et al.* (*4*).

To prepare H^3-poly U, UDP (*5*) was tritiated by the Wilzbach procedure (*5*) and subjected to paper electrophoresis for 2 hours at 1000 v in 0.05*M* ammonium formate buffer *p*H 3.5 followed by radioautography. There was only one radioactive spot, and this migrated with authentic UDP. The H^3-UDP, without purification, was converted by polynucleotide phosphorylase which had been obtained from *Micrococcus lysodeikticus* and purified through the $(NH_4)_2SO_4$ step (*6*), to H^3-poly U which was then deproteinized (*7*). The resulting polymer had a specific radioactivity of approximately 3×10^5 count/min μmole (pU) when counted under optimal conditions in a liquid scintillation counter. The average sedimentation constant of this material ($s_{20,w}$) was 8.2 as determined in 0.01*M* sodium cacodylate, *p*H 6.9, and 0.1*M* NaCl in the Beckman model E analytical ultracentrifuge with ultraviolet optics. The approximate weight-average molecular weight of the H^3-poly U, estimated from the relationship between $s_{20,w}$ and molecular weight of poly U described by Fresco (*7a*) was found to be 5×10^5. The degree of heterogeneity of the polymer is shown in Fig. 1. The theoretical distribution of $s_{20,w}$ for a homogeneous poly U with an average $s_{20,w}$ of 8.2 was calculated (*8*) and fell within a very narrow range. The diffusion coefficient for poly U was given to us by J. Fresco. The width of the observed distribution indicated that the polymer was very heterogeneous.

Bray's solution (*9*) was used for counting H^3-poly U and its degradation products, in a Packard Tri-Carb liquid scintillation counter. Appropriate corrections were made for quenching produced by extraneous materials.

After paper chromatography of H^3-poly U and its degradation products, the paper was cut into 1- by 1½-inch pieces which were placed vertically into counting vials. The contents of the paper were eluted by

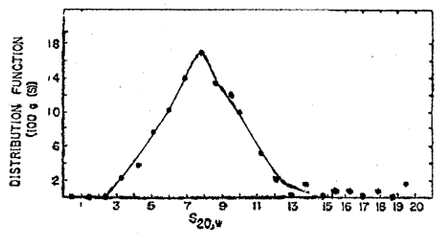

Fig. 1. Heterogeneity of H^3-poly U. A solution containing 30 μg/ml H^3-poly U in 0.1*M* NaCl, 0.01*M* sodium cacodylate, *p*H 6.9, was centrifuged in the Beckman model E analytical ultracentrifuge equipped with ultraviolet optics. Photographs were taken at 8-minute intervals. Calculation of the sedimentation coefficients were made, as described by Schumaker and Schachtman (*22*), using a photograph obtained after 44.8 minutes of centrifugation at 56,100 rev/min.

wetting its upper edge with 1 ml of H_2O, whereupon 16 ml of Bray's solution was added. The vial was stored at 0°C in the dark for 24 hours, and then counted. Although addition of H_2O to wet and elute the paper caused quenching, fewer counts were obtained if this step was omitted, possibly due to trapping of the H^3-material in the interstices of the paper. In control experiments with the H_2O technique, about 80 percent of counts that were placed on paper strips could be counted. Therefore, this correction was made when determining the radioactivity of chromatographic strips.

C^{14}-phenylalanine (uniformly labeled, specific activity 45 μc/μmole) (*5*) was diluted with C^{12}-phenylalanine as needed. The composition of the reaction mixture used for determination of C^{14}-phenylalanine incorporation into protein (*2*) is given in the legend for Fig. 2. The protein precipitates were counted in a proportional gas flow counter with a Micromil window. The presence of tritium in the poly

Table 1. Identification of H^3-products formed from poly U. The concentration of H^3-poly U was 50 mμmole (pU) per millimeter of reaction mixture.

Probable degradative enzyme	Mononucleotide	Percentage of total H^3-poly U added after incubation time	
		3 min	30 min
Polynucleotide phosphorylase (*13*)	UDP	20	13
	UTP	24	11
	5′-UMP	14	48
"Ribosomal ribonuclease" (*19*)	2′-, 3′-cyclic UMP	6	5
	2′- and 3′-UMP	0	1
Total*		65	80

*Refers to the total percentage of H^3-poly U recovered as mononucleotide at the indicated times. The values include 1 percent and 2 percent uridine found, respectively, after 3 and 30 minutes of incubation.

U did not interfere with the determination.

Since poly U is not precipitated by trichloroacetic acid or perchloric acid (*10*), except in the presence of extracts of *E. coli*, the usual assays for measuring degradation of RNA by observing a decrease in acid precipitable material could not be used. Since poly U was precipitated by mixtures of ethanol and magnesium acetate, the following precipitation procedure was used: H^3-poly U in 0.2 ml of the reaction mixture described in Fig. 2 was added to 2 volumes of absolute ethanol and 1 volume of 0.1*M* magnesium acetate. The mixture was kept at 4°C for 30 minutes and centrifuged at 2000*g* for 30 minutes at 0°C; the supernatant was decanted. The precipitate was resuspended in 5 ml of 0.05*M* magnesium acetate in 50 percent ethanol, recentrifuged, suspended in 10 ml of Bray's solution, and counted. About 80 percent of H^3-poly U could be recovered in the final precipitate even when as little as 0.5 mμmole of (pU) in poly U was used. H^3-poly U degraded with crystalline pancreatic ribonuclease was not precipitated by this technique. Addition of the H^3-poly U precipitate to Bray's solution produced slight quenching, and a quenching correction therefore was applied to all samples.

To identify the products of degradation of H^3-poly U, the reactions were stopped after 3 and 30 minutes of incubation by addition of perchloric acid (final concentration 3 percent). The mixtures were kept at 4°C for 30 minutes and then centrifuged at 10,000*g* for 30 minutes at 4°C. The supernatant solution was decanted, the *p*H was adjusted to approximately 7 with KOH, and the resultant precipitate of potassium perchlorate was removed by centrifugation as before. The degree of recovery of H^3-material was determined by counting triplicate aliquots of the neutralized supernatants and of the starting material. Aliquots of the recovered material then were chromatographed and gave identical results with samples concentrated by lyophilization.

To rule out the possibility that a nuclease which might have escaped perchloric acid precipitation was reactivated after neutralization, a sample of H^3-poly U was mixed with neutralized supernatant solution, kept at 4°C for 3 hours, and then chromatographed. This treatment produced no degradation of the polymer.

Mononucleotides and oligonucleotides were separated from poly U by descending chromatography in *n*-propanol, NH_4OH, and H_2O (55:10:35) (solvent A) (*11*). Polynucleotides remain at the origin in this system. Oligonucleotides migrate, but more slowly than mononucleotides. Polynucleotides and oligonucleotides are not hydrolyzed by the concentration of NH_4OH used (*11*). The uridine mononucleotides were separated by descending cochromatography with authentic uridine mononucleotide markers on Whatman No. 3 paper at 25°C in solvent A and in ethanol and ammonium acetate, *p*H 7.4 (70:30), (solvent B) (*12*). The 2′-, 3′-cyclic UMP and uridine were separated from noncyclic uridine mononucleotides by chromatography for 16 hours in both of these solvents. UTP, UDP, 5′-UMP and 2′- and 3′-UMP (mixture) were separated after 40 hours in solvent B. Separation of 2′- and 3′-UMP from uridine 5′-phosphates also was achieved with solvent A on dried paper which had previously been saturated with 0.2*M* sodium borate, *p*H 9.2. The positions of the nucleotides were determined by visual inspection upon illumination with an ultraviolet lamp.

Results. The incorporation of C^{14}-phenylalanine into protein (Fig. 2) was proportional to the amount of H^3-poly U added in the range of 0 to 70 mμmole (pU) in poly U per milliliter of reaction mixture. Subsequent experiments were usually performed with poly U concentrations corresponding to the linear portion of the concentration curve. In the experiment shown, one C^{14}-phenylalanine residue was incorporated for each 4 (pU) added within this range. Although the same preparations of *Escherichia coli* extract and H^3-poly U were used in all experiments reported, the ratio of C^{14}-phenylalanine incorporated to H^3-poly U added decreased from 1:3 to 1:7 during the course of this investigation.

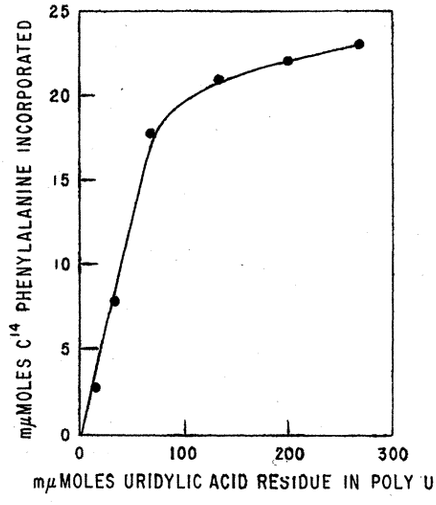

Fig. 2. Relation between concentration of H^3-poly U and polyphenylalanine synthesized. Each reaction mixture contained the following in 1 ml: 100 μmole tris hydrochloride, *p*H 7.8; 10 μmole magnesium acetate; 50 μmole KCl; 6 μmole β-mercaptoethanol; 1 μmole ATP; 5 μmole potassium phosphenolpyruvate; 0.12 μmole GTP; 0.12 μmole C^{14}-phenylalanine containing 300,000 count/min; 20 μg PEP, crystalline; *E. coli* 105,000*g* supernatant solution and ribosomal solution containing 1.8 mg and 0.8 mg protein, respectively. The indicated number of millimicromoles of (pU) in H^3-poly U was added to 1 ml reaction mixtures. The mixtures were incubated for 70 minutes at 35°C, and reactions were terminated by addition of 5 ml of cold 10 percent trichloroacetic acid. The precipitates were washed and counted by a modification of the method of Siekevitz (*23*). The slight incorporation of C^{14}-phenylalanine which was found in control tubes was subtracted from the amount incorporated in tubes containing poly U.

The data of Fig. 3 demonstrate that incorporation of phenylalanine into protein proceeded at an almost linear rate for approximately 20 minutes. In contrast, after 3 minutes of incubation more than 80 percent of the H^3-poly U added had become soluble in the mixture of alcohol and magnesium acetate, a fact that suggested rapid degradation to smaller soluble products. Degradation then proceeded more slowly and, after 30 minutes of incubation, there was an increase in precipitable material.

The composition of the degradation products of H^3-poly U after 3 minutes and 30 minutes of incubation is shown in Fig. 4. After 3 minutes of incubation, 81 percent of the added H^3-material was recovered for chromatography; 65 percent had been degraded to mononucleotides, and a small amount to oligonucleotides. After 30 minutes of incubation 88 percent of the H^3-material present in the reaction mixture was recovered, of which 80 percent was in mononucleotides. The remainder of the H^3-material either remained at the origin or was precipitated by the perchloric acid treatment.

Specific mononucleotides were identified by additional chromatography on paper; the composition of the mononucleotide fraction is shown in Table 1. Ninety percent of the mononucleotides had phosphates on the 5′-position, and only a small amount of 2′-, 3′-cyclic UMP was found. The major change between 3 and 30 minutes was the increase of 5′-UMP in the latter sample.

Since polynucleotide phosphorylase (*13*) is present in *E. coli* and since the degradative product of its action is a nucleoside diphosphate, the conversion of a nucleoside diphosphate (UDP) to other mononucleotides was studied in the protein synthesizing system. To a 1.0 ml reaction mixture, 200 mμmole of H^3-UDP was added and the mixture was incubated for 30 minutes at 35°C. After incubation, 20 percent was recovered as 5′-UDP, 65 percent as 5′-UMP and 15 percent as 5′-UTP.

It may be seen in Table 2 that rapid degradation of H^3-poly U occurred in the absence of ATP, GTP, and the ATP-generating system even though C^{14}-phenylalanine incorporation into protein did not occur in such re-

Table 2. Effect of C^{14} phenylalanine incorporation into protein upon H^3-poly U degradation. The complete reaction mixture was prepared as described in Fig. 2 with C^{12}- instead of C^{14}-phenylalanine. GTP, ATP, potassium phosphoenolpyruvate, phosphoenolpyruvate kinase, and phenylalanine were omitted from the other reaction mixture; 33 mμmole of (pU) in H^3-poly U were added to each reaction mixture. The mixtures were incubated at 35°C. Similar reaction mixtures were assayed for C^{14}-phenylalanine incorporation into protein. In 1.0 ml of the complete reaction mixture 4.5 mμmole of C^{14}-phenylalanine were incorporated whereas only 0.03 mμmole was incorporated in the system depleted of GTP, ATP, PEP, and PEP kinase.

Incubation time (min)	Precipitable H^3-polyU [mμmole (pU) per ml reaction mixture]	
	Complete	No GTP, ATP, PEP, PEP kinase, phenylalanine
0	27	28
1	6	9
8	5	5
16	4	2

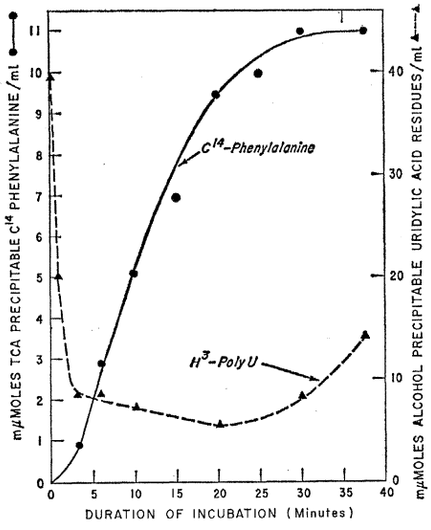

Fig. 3. Comparison between the rate of polyphenylalanine synthesis and the rate of H^3-poly U degradation. Each reaction mixture contained the components described in Fig. 2 including 50 mμmole of (pU) in H^3-poly U per milliliter. In appropriate tubes, 0.12 μmole of C^{14}-phenylalanine (10^6 count/min) were added per milliliter of reaction mixture. The reaction mixture used for assay of precipitable H^3-poly U contained C^{12}- rather than C^{14}-phenylalanine. The two reaction mixtures, each in a total volume of 2 ml, were incubated for 40 minutes, and 0.2-ml aliquots were removed at the indicated time intervals and were transferred to either 2 ml of 10 percent trichloroacetic acid at 4°C for assay of phenylalanine incorporation into protein or into 0.2 ml of magnesium acetate + 0.4 ml of absolute ethanol at 4°C for analysis of precipitable H^3-poly U.

action mixtures. H^3-poly U was degraded also, although not as rapidly, by incubating with triply washed ribosomes, suspended only in 0.1*M* tris HCl, *p*H 7.8, 0.01*M* magnesium acetate, and 0.05*M* KCl. The major product of degradation by washed ribosomes was 5′-UMP; and only 10 percent of 2′-, 3′-cyclic UMP was found. Rapid degradation by the supernatant from centrifugation at 105,000*g* in the absence of ribosomes ATP, GTP, PEP, PEP kinase, and phenylalanine was also observed. Since H^3-poly U was rapidly degraded in the absence of materials required for protein synthesis, such degradation is not dependent on concomitant polyphenylalanine formation.

Because of the marked contrast between the prolonged duration of incorporation of C^{14}-phenylalanine into protein and the rapid initial degradation of poly U, the possible resynthesis of active polynucleotide from mononucleotides by the extract was explored. Addition of a mixture of uridine mononucleotides did not stimulate the incorporation of C^{14}-phenylalanine into protein (Table 3). Since a small amount of intact oligonucleotide or polynucleotide might be required to "prime" the incorporation of the mononucleotides into active polymer, the effect of addition of mononucleotides to a system containing a small amount of poly U also was tested. Again mononucleotides did not stimulate C^{14}-phenylalanine incorporation (Table 3). It would appear then, that the prolonged course of protein synthesis is not related to resynthesis of active polymer from mononucleotides.

Discussion. "Messenger RNA" is believed to be rapidly degraded by *E. coli* cells (*14*); however, the enzymic route of such degradation and its possible relationship to the process of protein synthesis are unknown. Since it has been shown that poly U directs C^{14}-phenylalanine incorporation into protein in a cell-free extract of *E. coli*, the characteristics of degradation of this synthetic "messenger RNA" were studied in this system.

When limiting amounts of poly U were added to reaction mixtures, about 80 percent was converted rapidly to mononucleotides and oligonucleotides. This rapid degradation preceded or did not require significant C^{14}-phenylalanine incorporation and thus appeared to be unrelated to protein synthesis. A similar lack of dependence of degradation of "messenger RNA" on con-

Table 3. Effect of uridine mononucleotides upon C^{14}-phenylalanine incorporation into protein. Composition of the reaction mixtures are those given for Fig. 2, except that H^1- rather than H^3-poly U was added where indicated. Incorporation of C^{14}-phenylalanine into protein was proportional to H^3-poly U added in the range of 0 to 21 mμmole of (pU) in poly U per 0.35 ml of reaction mixtures. The total volume of each reaction mixture was 0.35 ml. Samples were incubated for 60 minutes at 35°C. "Mononucleotides+" refers to an equimolar solution of UTP, UDP, 5′-UMP, and 2′- and 3′- UMP (mixed).

Addition	C^{14}-phenylalanine (count/min)
None	60
Mononucleotides+, 6 mμmole each	74
Mononucleotides+, 30 mμmole each	56
Poly U, 3 mμmole*	485
Poly U, 3 mμmole* + mononucleotides+, 6 mμmole each	503
Poly U, 3 mμmole* + UDP, 15 mμmole	436

*Millimicromoles of (pU) in poly U.

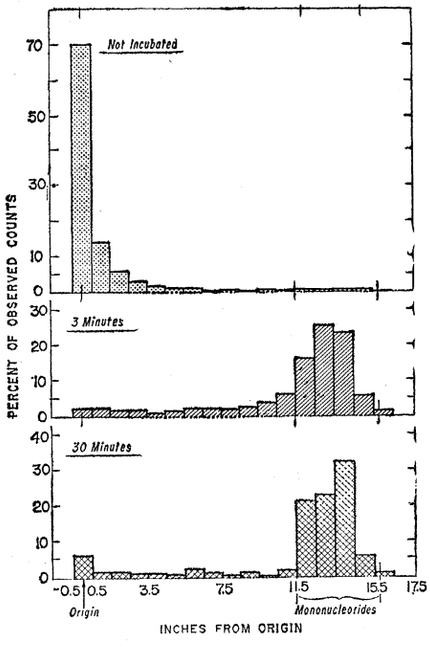

Fig. 4. Chromatography of degradation products of H^3-poly U. Reaction mixtures (5.0 ml) were prepared as described in Fig. 2, except that C^{12}-phenylalanine was used; 50 mμmole of (pU) in H^3-poly U were added per milliliter of reaction mixture. Incubation and precipitation procedures are described in the text. Samples containing approximately 2000 counts/min were chromatographed for 40 hours in solvent A. The mononucleotide peak corresponds to the positions of UTP, UDP, 5′-UMP, and 2′- and 3′-UMP. The trailing of radioactivity may represent oligonucleotides. Since uridine and 2′-, 3′-cyclic UMP migrate the length of the chromatogram and are eluted during 40 hours, the time of chromatography was reduced to 16 hours when identifying these compounds. The extract of *E. coli* used for this experiment incorporated 1 mμmole of C^{14}-phenylalanine into protein for each 4.5 mμmole of (pU) in H^3-poly U.

comitant protein synthesis has recently been demonstrated by Tissières and Watson (*15*).

It is not yet known whether poly U acts catalytically or stoichiometrically, that is, whether one molecule of poly U directs the synthesis of many or only one molecule of polyphenylalanine. Since more than 80 percent of the poly U added to reaction mixtures was degraded before significant polyphenylalanine synthesis had occurred, only a small fraction of the poly U added may actually function in directing protein synthesis. In making estimates of the number of phenylalanine residues directed into protein by each uridylic acid residue in poly U, the amount of poly U which had been degraded before significant protein synthesis occurred should therefore be taken into consideration. From the data of Fig. 3 it can be estimated that one uridylic acid residue in the poly U which survives rapid degradation acts to direct one phenylalanine into protein. If the suggestion that the coding ratio is three nucleotides per amino acid (*16*) can be applied to poly U, it might be concluded that one poly U molecule directs the synthesis of more than one polyphenylalanine molecule and thus behaves catalytically. Recent experiments with intact *E. coli* (*17*) have suggested that "messenger RNA" may act catalytically in vivo.

To help clarify the enzymic routes of H^3-poly U breakdown, the H^3-products formed during incubation were identified. The action of the two well-characterized ribonucleases from *E. coli*, polynucleotide phosphorylase (*13*) and ribosomal ribonuclease (*18, 19*), can be distinguished by the nature of the products formed. Polynucleotide phosphorylase catalyzes a reversible phosphorolysis of RNA in the presence of inorganic phosphate and produces nucleoside-5′-diphosphates. However, during incubation of our reaction mixture, UDP was partially converted to UTP presumably by the action of phosphoenolpyruvate kinase (*20*) and to 5′-UMP. Thus the formation of H^3-5′-UTP, H^3-5′-UDP, and H^3-5′UMP may represent degradation of H^3-poly U by polynucleotide phosphorylase. In contrast, ribosomal ribonuclease catalyzes the formation of nucleoside-2′-,3′-cyclic phosphates which are more slowly converted by the same enzyme to nucleoside-3′-phosphates (*19*). This enzyme is further characterized by its existence in a latent ribosome-bound form in the presence of 0.01*M* magnesium (*18, 19*), the concentration used in our investigation. Another ribosomal ribonuclease from *E. coli* has been reported by Wade and Lovett (*21*) but has not been purified or extensively characterized. It catalyzes the hydrolysis of RNA to nucleotide-5′-monophosphates and is active even in the presence of 0.01*M* magnesium.

Since most of the H^3-poly U which has been degraded in reaction mixtures can be recovered as 5′-mononucleotides, such degradation possibly may be ascribed to catalysis by polynucleotide phosphorylase or by the ribonuclease of Wade and Lovett, or by both. Relatively little degradation of poly U by ribosomal ribonuclease was found after either short or long incubations. Whether the enzymes involved in poly U degradation are also responsible for the in vivo degradation of "messenger RNA" remains to be determined.

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1. Abbreviations used: GTP, adenosine and guanosine-5′-triphosphate, respectively; PEP, potassium phosphoenolpyruvate; PEP kinase, phosphoenolpyruvate kinase, crystalline; poly U, polyuridylic acid; (pU), uridylic acid residues in poly U; tris HCl, tris (hydroxymethyl) aminomethane hydrochloride; UTP, UDP, and 5′ UMP, uridine-5′-tri-, di-, and monophosphate, respectively; 2′- and 3′-UMP, a mixture of uridine 2′-monophosphate and uridine 3′-monophosphate; 2′-, 3′-cyclic UMP, uridine-2′, 3′-cyclic monophosphate.
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Fate of a Synthetic Polynucleotide Directing Cell-Free Protein Synthesis
II. Association with Ribosomes

Abstract. Tritiated-poly U, when added to cell-free extracts of *Escherichia coli*, became associated with ribosomes. This association occurred at 3°C and did not require high-energy phosphate compounds. Small amounts of tritiated polyuridylic acid produced polydisperse ribosomal aggregates with sedimentation constants of approximately 100 to 130. C^{14}-phenylalanine initially was incorporated into protein only on these particles, which suggests that they are the sites of polyphenylalanine synthesis.

Rapidly synthesized fractions of RNA have been demonstrated in *Escherichia coli* (*1–3*) and have been considered to function in protein synthesis as "messenger RNA." Recently, Risebrough, Tissières, and Watson have reported that such RNA fractions associate with ribosomal particles, particularly with 100 S ribosomes (*2*). Since poly U (*4*) functions in cell-free extracts of *E. coli* as template RNA, some characteristics of its association with ribosomes have been studied.

Experimental procedure. The same preparations of extracts of *E. coli* and H^3-poly U discussed in the accompanying report (*5*) were used in this study. The rate of polyphenylalanine synthesis and the relationship between the amount of poly U added to a reaction mixture and the amount of phenylalanine incorporated into protein remained within the ranges cited (*5*). The sedimentation constants of the components of the ribosomal solution are shown in Fig. 1. The sedimentation constant of the major ribosomal component was approximately 70 S. Few 100 S ribosomes were found. For some of the experiments a preparation of washed 70 S ribosomes was prepared by repeated centrifugation of ribosomes in 0.1*M* tris HCl, *p*H 7.8, 0.01*M* magnesium acetate, and 0.05*M* KCl (*6*). The final ribosomal pellet suspended in this medium contained 3 mg of ribosomal protein per milliliter; it was stored in aliquots at −20°C.

Sucrose density gradient centrifugation experiments were performed as described