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*SYNTHETIC POLYNUCLEOTIDES AND THE AMINO ACID CODE**

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Communicated October 25, 1961

The problem of coding in protein biosynthesis, i.e., of how a certain sequence of four different nucleotides in an RNA[1] chain can specify a given sequence of 20 different amino acids in a polypeptide chain, has been considered by several investigators in the last decade. Until now these studies have been either theoretical[2, 3] or statistical[4–6] in nature.

Increased knowledge of the mechanism of protein biosynthesis, the concept of messenger RNA as the actual template,[7] and especially the observation of Brenner *et al.*[8] (cf. also Gros *et al.*[9]) that after infection of *Escherichia coli* with T_2 phage the newly formed messenger RNA is added to preexisting ribosomes (on which most of the protein synthesis of the infected cell occurs) suggested the use of synthetic polyribonucleotides, with known nucleotide sequences, as messengers and a possible method of experimental approach to the nucleotide code. The simplest possible sequence is that in homopolynucleotides which, if active, should prescribe the formation of homopolypeptide chains. These homopolynucleotides as well as copolymers containing two or more different nucleotide species in predetermined ratios can be synthesized by polynucleotide phosphorylase.[10]

We wish to report that various kinds of synthetic polynucleotides are active as messengers in an *E. coli* system and indeed determine the incorporation of different amino acids into an acid-insoluble product. That is the case for phenylalanine, serine, and tyrosine with polymers containing uridylic acid, uridylic acid and cytidylic acid, and uridylic acid and adenylic acid respectively.

Recently Nirenberg and Matthaei[11] succeeded in obtaining a poly U-dependent synthesis of polyphenylalanine in a system of *E. coli* supernatant and ribosomes.

Preparations.—*E. coli* ribosomes were prepared from log phase cells grown on enriched media (0.5 per cent glucose, 0.5 per cent trypticase, 0.5 per cent yeast extract). The cells were washed twice with ice-cold 0.2 *M* KCl and once with 0.02 *M* Tris-HCl buffer, pH 7.9, containing 0.01 *M* $MgCl_2$ and 0.005 *M* mercaptoethylamine. They were then resuspended in 2 volumes of the same medium and disrupted for 3 min at maximum power in a 10 kc Raytheon oscillator at 5°. The suspension was centrifuged twice for 30 min at 3–4° and 30,000 *g* to remove debris and intact cells. The extract was centrifuged at low temperature for 2 hr in the preparative Spinco centrifuge at 105,000 *g*. The clear supernatant fluid was collected, the sediment containing the ribosomes was rinsed with Tris-HCl buffer, pH 7.9, containing 0.01 *M* $MgCl_2$, 0.02 *M* KCl, 0.2 *M* sucrose, and 0.5 per cent Lubrol (Imperial Chemical Industries) and suspended in this medium, with use of a glass homogenizer, to give a concentration of 20 mg of protein/ml. This suspension was layered over a solution containing 0.034 *M* Tris-HCl buffer, pH 7.9, 0.01 *M* $MgCl_2$, 0.6 *M* KCl, and 0.3 *M* sucrose, and again centrifuged for 3 hr at 105,000 *g*. The particles were then suspended in 0.1 *M* Tris-HCl buffer, pH 7.9, containing 0.01 *M* $MgCl_2$ and 0.02 *M* KCl, to give a concentration of 10 mg of protein/ml, frozen in dry ice, and kept in a freezer at −18°. The ribosomes are stable under these conditions. They can also be kept after lyophilization. Rat liver ribosomes were prepared by the method of Rendi and Hultin.[12] Rat liver supernatant was prepared by the method of Zamecnik and Keller.[13] For some experiments the precipitate obtained by bringing the *E. coli* supernatant to pH 5.0 was dissolved in 0.05 *M* Tris-HCl buffer, pH 7.9, containing 0.005 *M* mercaptoethylamine, and used in place of the supernatant. This treatment aimed at reducing the level of endogenous amino acids in the system. Transfer RNA (S-RNA) was prepared from *E. coli* supernatant as described by Hoagland *et al.*[14]

Polyribonucleotides were prepared with *Azotobacter vinelandii* polynucleotide phosphorylase (specific activity, 62) as previously described.[10] After precipitation with ethanol, the polymers were dissolved in 0.04 *M* NaCl–0.005 *M* sodium citrate and the solution was extracted with phenol according to Gierer and Schramm.[15] The aqueous solution was dialyzed against distilled water for 5 hr at 3° and the polymer recovered by lyophilization. Poly UC was prepared from a mixture of UDP and CDP in molar ratio 5:1, poly UA from a mixture of UDP and ADP in a molar ratio 5:1, and poly CU from UDP and CDP in molar ratio 1:5. The preparation of polythio U has been previously described.[16] 5-Fluorouridine 5′-diphosphate, used in the preparation of polyfluoro U,[7] was a synthetic product kindly supplied by Dr. Charles Heidelberger, University of Wisconsin, Madison, Wisconsin. The sedimentation coefficients of the polynucleotides used in this work are listed in Table 1. Nucleoside diphosphates were obtained from the Schwarz Laboratories, Mount Vernon, N. Y., and the Pabst Laboratories, Milwaukee, Wisconsin. C^{14}-

labeled amino acids were obtained from the California Biochemical Corporation under allocation from the Atomic Energy Commission.

Methods.—In order to decrease the "blank" incorporation of labeled amino acids in the absence of added polynucleotides, *E. coli* ribosomes and supernatant were preincubated for 15 min at 37° in a mixture containing in 1.0 ml the following components (in μmoles unless otherwise specified): Tris-HCl buffer, pH 7.9, 37; Lubrol, 1.8 mg; KCl, 55; $MgCl_2$, 13; mercaptoethylamine, 11; ATP, 0.9; GTP, 0.22; creatine phosphate, 12; creatine kinase, 44 μg; each of 20 nonlabeled amino acids, 0.09; ribosomes with 4 mg of protein; and supernatant with 4 mg of protein. For amino acid incorporation each sample contained 0.1 ml of the above preincubated mixture and 0.15 ml of a basic reaction mixture of the following composition (in μmoles unless otherwise specified): Tris-HCl buffer, pH 7.9, 10.5; Lubrol, 0.5 mg; KCl, 15.3; $MgCl_2$, 2.5; mercaptoethylamine, 3.1; ATP, 0.25; GTP, 0.06; creatine phosphate, 3.3; and creatine kinase, 12.5 μg. Other additions, as noted in the tables, included C^{14}-labeled amino acid (specific radioactivity 1 to 10 μc/μmole), 0.025 μc; transfer RNA, 0.5–1.0 mg; and polynucleotide, 0.04 μmole as mononucleotide. The latter amount was found to be optimal for poly U; higher concentrations were inhibitory. After incubation for 1 hr at 37° the reaction was stopped by the addition of 5 ml of 10 per cent trichloroacetic acid and the samples were worked up according to the procedure of Zamecnik *et al.*[18] The radioactivity of the acid-insoluble residue was measured with a windowless gas-flow counter. Amino acid incorporation is expressed throughout in mμmoles/mg ribosomal protein.

TABLE 1

SEDIMENTATION COEFFICIENTS OF SYNTHETIC POLYNUCLEOTIDES

Polynucleotide	$S_{20,w}$
Poly A	17.7
Poly U (sample 1)	10.3
Poly U (sample 2)	4.5
Poly C	8.6
Poly UC	9.5
Poly UA	9.1
Poly thio U	19.0
Poly fluoro U	9.4

In the experiment in which rat liver and *E. coli* ribosomes were compared, the preincubation described above was omitted and no transfer RNA was added. Protein was determined by the method of Lowry *et al.*[19]

Results.—Poly U-dependent incorporation of phenylalanine in E. coli system: Investigation of the incorporation of different C^{14}-labeled amino acids into an acid-insoluble product by an *E. coli* supernatant plus ribosomes system, with and without the addition of poly U, showed that out of 19 amino acids (C^{14}-labeled asparagine was not available to us) tested individually only the incorporation of phenylalanine was markedly stimulated by poly U. A small stimulation of the incorporation of leucine and isoleucine was also observed. The effect of poly U on the incorporation of phenylalanine is shown in Table 2. It may also be seen that addition of *E. coli* transfer RNA brought about a further pronounced increase of phenylalanine incorporation. Since poly U did not affect the loading of transfer RNA with phenylalanine, catalyzed by the amino acid activating enzymes in the supernatant, it appears that poly U affects the transfer of activated phenylalanine residues from the specific phenylalanine-transfer RNA to the ribosomes; this indicates that poly U acts as messenger RNA in this system. As shown by Nirenberg and Matthaei,[11] polyphenylalanine, which is exceedingly insoluble, is formed under these conditions. With the highest concentration of transfer RNA used (Table 2) one mole of phenylalanine was incorporated for every 3.25 uridylic acid

TABLE 2

EFFECT OF *E. coli* TRANSFER RNA ON THE POLY U-DEPENDENT INCORPORATION OF PHENYLALANINE*

Additions to basal system		
Poly U	Transfer RNA (mg/ml)	Phenylalanine incorporation†
−	0	0.1
−	9	0.3
+	0	3.6
+	2	15.2
+	6	23.5
+	9	24.6

* Incubation 30 min at 37°.
† mμmoles/mg of ribosomal protein.

residues in the poly U added. The incorporation of phenylalanine was inhibited by puromycin and, to a lesser extent, by chloramphenicol.

Experiments with other homopolymers: The effect of poly A, poly thio U, and poly fluoro U was investigated in experiments similar to those of Table 2 but *without the addition of transfer RNA*. Poly A did not stimulate the incorporation of any of 19 amino acids tested. Addition of poly A to a system containing poly U completely inhibited the effect of the latter. This is undoubtedly due to formation of the double-stranded, helical poly A + U complex. Poly thio U (which appears to be multi-stranded[20, 21]) had no effect,[22] and poly fluoro U (which like poly U is single-stranded[20]) had but a small effect on phenylalanine incorporation. Poly C had a small but consistent effect on the incorporation of proline (cf. footnote 11) but had no influence on that of any other amino acid. It may be of interest that a short-chain poly U (poly U sample 2, Table 1) was about 40 per cent as effective as an equimolar amount of the longer-chain poly U used throughout this work (poly U sample 1, Table 1) in promoting phenylalanine incorporation.

Experiments with rat liver ribosomes: Poly U had no effect on the incorporation of phenylalanine by a system of rat liver supernatant plus ribosomes (Table 3).

TABLE 3

POLY U-DEPENDENT INCORPORATION OF PHENYLALANINE BY *E. coli* AND RAT LIVER RIBOSOMES

		Phenylalanine incorporation*		
Supernatant	Ribosomes	(*a*) No poly U	(*b*) With poly U	Ratio *b/a*
Liver	Liver	0.40	0.46	1.2
E. coli	*E. coli*	0.07	3.29	47.0
Liver	*E. coli*	0.06	0.17	2.8
E. coli	Liver	0.16	1.81	11.3

* mμmoles/mg of ribosomal protein.

However, no transfer RNA was added in these experiments and, considering the pronounced effect of transfer RNA on the *E. coli* system, this negative result cannot be considered conclusive. With the unsupplemented rat liver system there was no effect on either poly A, poly U, or poly C on the incorporation of any of 19 amino acids tried singly. However, as seen in Table 3, poly U had a fairly marked effect on the incorporation of phenylalanine by a system of *E. coli* supernatant plus rat liver ribosomes. This was not true for the opposite combination, i.e., liver supernatant plus *E. coli* ribosomes. Thus, the ineffectiveness of poly U with the rat liver system is related to the soluble components of this system. It is too early to speculate on the significance of this observation, but the finding that

rat liver ribosomes can be substituted for *E. coli* ribosomes for poly U-dependent incorporation of phenylalanine is not without interest.

Effect of synthetic homo- and copolymers on amino acid incorporation in E. coli system: The results of these experiments are summarized in Table 4. The in-

TABLE 4

AMINO ACID INCORPORATION IN *E. coli* SYSTEM WITH VARIOUS POLYNUCLEOTIDES*

	Polynucleotide					
Amino acid	None	Poly U	Poly C	Poly UC	Poly UA	Poly CU
Phenylalanine	0.03	**13**		7	3	0.02
Serine	0.02	0.02	0.01	1.6	0.01	
Tyrosine	0.02			0.02	**0.75**	
Leucine	0.02	0.3		**1.5**	0.46	0.03
Isoleucine	0.01	0.09		0.32	**0.62**	0.007
Proline	0.02	0.02	0.06	**0.6**	0.03	0.14

* mμmoles/mg of ribosomal protein. 19 amino acids were tested individually in all cases, but the ones giving negative results have been omitted from the table. All values (except those for poly CU) are averages of at least two separate experiments.

corporation of phenylalanine was stimulated to a decreasing extent by poly U, poly UC, and poly UA. Most significant is the fact that some of the copolymers stimulated the incorporation of amino acids other than phenylalanine. Thus, whereas poly UC, and only this polymer, stimulated the incorporation of serine, only poly UA stimulated the incorporation of tyrosine. Less clearcut results were obtained with leucine and isoleucine, the incorporation of both of which was significantly stimulated by poly U, poly UC, and poly UA. Highest incorporation of leucine was promoted by poly UC whereas poly UA brought about the highest incorporation of isoleucine. Ambiguous results were also obtained with proline, the incorporation of which was stimulated by poly C, poly CU, and poly UC in order of increasing effectiveness.

Discussion.—In contrast to poly UC (U:C = 5:1) poly CU (U:C = 1:5) was almost ineffective in promoting amino acid incorporation (Table 4). The reason for this pronounced discrepancy may be sought in the fact that, contrary to natural messenger RNA (or to poly U), the synthetic copolymers do not provide an unbroken sequence of code units. Hence, as far as amino acid incorporation is concerned, there must be many gaps along their chains. This situation might result in the formation of short-chain, acid-soluble polypeptides which would escape detection by the procedure used here. Copolymers such as poly UC and poly UA containing relatively long unbroken U sequences together with some other code units might give rise to short, but acid-insoluble, polyphenylalanine chains with occasional serine, leucine, tyrosine, or isoleucine residues. Viewed in this way, a high proportion of U in a synthetic copolymer would provide a convenient "handle" for incorporation of amino acids other than phenylalanine into short, acid-insoluble polyphenylalanine chains.

If poly U codes for polyphenylalanine, a short sequence of three or more U residues would be the code letter for phenylalanine. If for the sake of simplicity we assume a triplet code, the ratio of UUU to UUC (or UCU, or CUU) triplets in a random copolymer of the composition of poly UC would be the same as the U:C ratio of the polymer, i.e., 5:1. The ratio of UUU to UCC (or CUC, or CCU) triplets would be 25:1. From Table 4 the ratio of phenylalanine to serine incorporation with poly UC was 4.4:1. On the basis of this result the triplet code letter

for serine would be either UUC, UCU, or CUU. The ratio of phenylalanine to tyrosine incorporated with poly UA (U:A = 5:1) was 4.0. This would make either UUA, UAU, or AUU as the likely triplet code letter for tyrosine.

The overlapping results obtained with leucine and isoleucine are difficult to explain, but, if maximal stimulation is taken as the meaningful result, the triplet code letters for these two amino acids would contain 2U and 1C and 2U and 1A, respectively. Difficulties are also encountered with proline (Table 4). Using the same criterion as for leucine and isoleucine, one would tentatively assign a triplet code letter containing 1U and 2C to this amino acid. On the other hand, the greater effectiveness of poly UC as compared to poly C might be due to the provision of an insoluble polyphenylalanine "handle" for the proline residues. Thus it is possible that CCC is really the triplet code letter for proline. It is of interest in this connection that, in a nitrous acid mutant of tobacco mosaic virus described by Tsugita and Fraenkel-Conrat,[23] a proline residue was replaced by leucine. As the nitrous acid effect is due to deamination of C to U, replacement of C by U would be in line with the code letters suggested for these amino acids. Our results would also be compatible with the fact that proline:phenylalanine ratio of wild cucumber and tobacco mosaic virus protein varies in the same direction as the C:U ratio of their respective nucleic acids.[24]

Experiments now in progress with other homo- and copolymers prepared with polynucleotide phosphorylase and deductions from amino acid replacement data as used by Woese[5] may be expected to be of further help in deciphering the nucleotide code.

Summary. Polyribonucleotides synthesized with polynucleotide phosphorylase stimulated the incorporation of certain amino acids into an acid-insoluble product by a system of consisting of *E. coli* high-speed supernatant and ribosomes. Addition of *E. coli* transfer RNA brought about a further pronounced increase of the incorporation of phenylalanine in the presence of poly U. This indicates that the polymers affect the transfer of activated amino acid residues from transfer RNA to ribosomes and act as messenger or template RNA in this system. In one experiment with poly U and phenylalanine, without addition of transfer RNA, rat liver ribosomes could be substituted for their *E. coli* counterparts.

While poly U promoted phenylalanine incorporation, poly UC promoted the incorporation of phenylalanine and serine, and poly UA stimulated the incorporation of phenylalanine and tyrosine. These and other results reported in this paper would appear to open up an experimental approach to the study of the coding problem in protein biosynthesis.

We are indebted to Carlos Basilio for the preparation of poly CU, to R. C. Warner and Yo-Yun Chen for the sedimentation coefficients of the synthetic polynucleotides, and Albert Lenny and Horace Lozina for skillful technical assistance.

Note added in proof.—The results of further experiments to be reported in these PROCEEDINGS, with use of other copolymers (UG, UAC, where G stands for guanylic acid residues), extend to eleven the list of amino acids incorporated into an acid-insoluble product by the *E. coli* system with different polymers. It now includes cysteine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine.

* Aided by grants from the National Institute of Arthritis and Metabolic Diseases (Grant A-1845) of the U.S. Public Health Service, and the Rockefeller Foundation.

[1] Abbreviations: RNA, ribonucleic acid; the capital letters A, U, and C are used for the nucleotides adenylic, uridylic, and cytidylic acid, respectively, or their corresponding residues in polynucleotide chains; ADP, UDP, and CDP, the 5'-diphosphates of adenosine, uridine, and cytidine; ATP and GTP, the 5'-triphosphates of adenosine and guanosine; Tris, tris(hydroxymethyl)aminomethane.

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THE PREPARATION OF CRYSTALLINE TRANSALDOLASE FROM CANDIDA UTILIS

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Communicated October 23, 1961

Transaldolase was first isolated from brewer's yeast[1] and shown to catalyze the reversible reaction:

$$\text{Sedoheptulose 7-phosphate} + \text{D-glyceraldehyde 3-phosphate} \rightleftharpoons \text{D-erythrose 4-phosphate} + \text{D-fructose 6-phosphate} \quad (1)$$

In the course of the reaction a three-carbon unit equivalant to dihydroxyacetone is transferred from the donor, which may be either sedoheptulose 7-phosphate or D-fructose 6-phosphate, to the acceptor, either D-glyceraldehyde 3-phosphate or