STUDIES OF POLYPHENYLALANINE SYNTHESIS WITH RETICULOCYTE RIBOSOMES*

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The stimulation of amino acid incorporation in cell-free systems from bacteria by synthetic polynucleotides has been used to study the genetic code (Nirenberg and Matthaei, 1961; Lengyel et al, 1961). Arnstein et al (1962) and more recently Maxwell (1962) and Weinstein and Schechter (1962) have reported similar studies using cell-free systems from animal tissues. In this communication, reticulocyte ribosomes are shown to incorporate C^{14}-phenylalanine at similar levels to bacterial systems when stimulated by poly U, under conditions where hemoglobin synthesis is greatly reduced. The results indicate that different sites are involved in the two types of peptide bond synthesis.

The addition of poly U to the regular complete system (Allen and Schweet, 1962) containing reticulocyte ribosomes resulted in a 20-fold stimulation of C^{14}-phenylalanine incorporation. The stimulation of incorporation by poly U was inhibited by RN-ase and Puromycin, and depended on the presence of ATP, transfer RNA and ribosome The incorporation of C^{14}-phenylalanine under optimal conditions ranged from 10 to 20 mμmoles per mg. of ribosomal protein in different ribosome preparations. These amounts are similar to those obtained in bacterial systems (reviewed by Schweet and Bishop, 1962) and 10 to 40 times greater than previously reported for animal systems. They are also similar to the amount of hemoglobin synthesized based on C^{14}-leucine incorporation (Fig. 1). In different ribosome preparations, the polyphenylalanine

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synthesis ranged from about 80 to 180% of the hemoglobin synthesis in the same preparation.

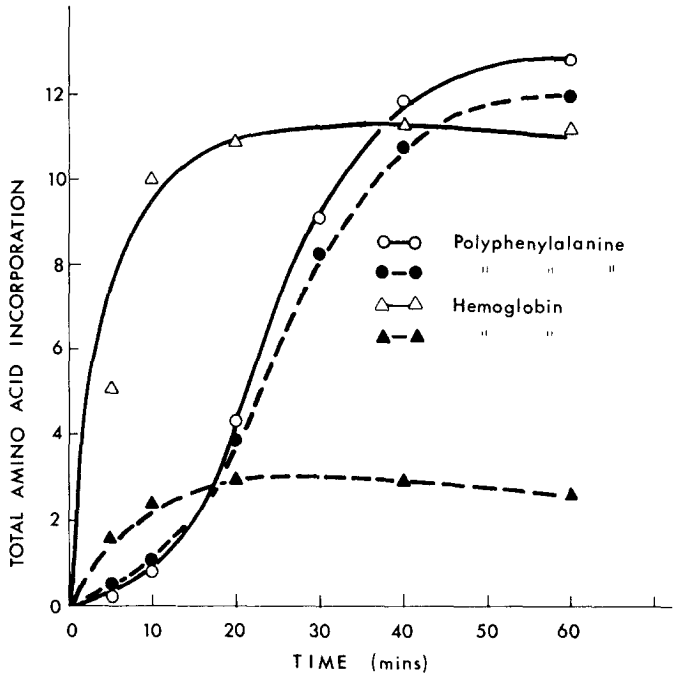

Fig. 1. Time course of polyphenylalanine and hemoglobin synthesis. Polyphenylalanine synthesis in regular ribosomes (○) and pre-incubated ribosomes (●). Hemoglobin synthesis in regular ribosomes (△) and pre-incubated ribosomes (▲). Pre-incubated ribosomes were prepared by incubation of unwashed ribosomes in the complete system (Allen and Schweet, 1962) with C^{12}-amino acids for 30 minutes. The ribosomes were then diluted and centrifuged as described for washed ribosomes. C^{14}-leucine incorporation x 7, or C^{14}-phenylalanine incorporation x 17.5 in the absence of poly U, was used to calculate hemoglobin synthesis. Data are given as mumoles per mg ribosomal protein. See Table I for assay conditions.

C^{14}-leucine incorporation was not influenced by the presence of poly U, confirming the report by Arnstein et al (1962). Thus, polyphenylalanine synthesis takes place in addition to the normal hemoglobin synthesis. In the presence of poly U, the distribution of C^{14}-leucine in ribosomes and soluble protein was also unaffected. During hemoglobin synthesis in the cell-free system, the ribosomes contain incomplete chains, some of which are completely "new" chains, that is they contain N-terminal C^{14}-valine (Morris et al, 1962). If poly U were to occupy a hemoglobin-synthesizing site after the release of a completed chain, since these "new" globin chains could not form, the C^{14}-leucine incorporated into the ribosome

should be decreased, compared to the incorporation into soluble protein. This was not the case, suggesting that the 2 types of protein synthesis involve separate sites.

Support for this view was obtained from several types of experiments where hemoglobin and polyphenylalanine synthesis were dissociated. When ribosomes were pre-incubated in the complete system with C^{12}-amino acids, polyphenylalanine synthesis was unimpaired, but further hemoglobin synthesis was greatly reduced (Fig. 1). This result differs from that of Arnstein et al (1962), but different conditions were used. It should be noted that the pronounced time lag in polyphenylalan synthesis was similar in regular and pre-incubated ribosomes. Thus, this lag does arise from the need for hemoglobin synthesis to be completed before poly U can ente a particular ribosome. Incubation of ribosomes in potassium chloride in the absenc of magnesium ion also decreased hemoglobin synthesis without affecting polyphenylalanine synthesis (Table I). No protein synthesis occurs during this "shock" and it is likely that this treatment involves loss of the hemoglobin messenger RNA from the ribosome, although this remains to be proven. If polyphenylalanine synthesis depended solely on the presence of "empty" hemoglobin sites, it might be expected that these ribosomes would show more polyphenylalanine synthesis or a faster initial rate. This was not the case.

The best evidence, however, for the existence of separate sites is the differential inhibition of the 2 types of synthesis by Puromycin. Both polyphenylalanine and hemoglobin synthesis show a similar sensitivity to inhibition by Puromycin added directly to the incorporation system containing poly U. However, since Puromycin acts by replacing partially completed chains on the ribosome (Morris et al, 1962), it seemed likely that by pre-treating with Puromycin in the absence of poly U and then re-isolating these ribosomes, only hemoglobin sites would be inhibited. Using ribosomes pre-treated in this way, polyphenylalanine synthesis was unimpaired and hemoglobin synthesis was inhibited by 65% (Table I). Since the Puromycin inhibition must result from inhibitor attachment to the ribosomes, different sites for polyphenylalanine and hemoglobin synthesis appear to be present. It is likely, but not yet demonstrated, that the different sites are on different

ribosomes. Finally, it should be noted that the combination of pre-incubation and "shock" (Table I) produced ribosomes with a background incorporation of C^{14}-phenyl alanine of 0.085 mμmoles per mg which was stimulated 100-fold by the addition of poly U. This type of stimulation would be sufficient for study of "coding" problems with synthetic polynucleotides.

TABLE I

DISSOCIATION OF HEMOGLOBIN AND POLYPHENYLALANINE SYNTHESIS

Treatment	Polyphenylalanine Synthesis	Hemoglobin Synthesis
1. None	15.6	12.6
Shocked in KCl	16.0	3.6
2. None	9.4	10.4
Pre-treated with Puromycin	9.8	3.6
Pre-incubated and Shocked	8.6	1.5

The values are given as mμmoles of total amino acid incorporation per mg of ribosomal protein added. Each tube contained 1.5 mg ribosomal protein and other constituents of the complete system (Allen and Schweet, 1962), plus 300 μg of poly U. Incubation time was 60 minutes. Shocked ribosomes were prepared by incubating ribosomes (1.5 mg protein) for 10 minutes at 37° with 100 μmoles KCl; 50 μmoles Tris chloride, pH 7.5; and 20 μmoles GSH in a final volume of 0.75 ml. The control (None) was incubated at 4° under the same conditions. Pre-treatment with Puromycin was done by incubation of ribosomes in the complete system with 6×10^{-4}M inhibitor for 5 minutes at 37°, in the absence of poly U. The mixture was diluted with an equal volume of medium B (Allen and Schweet, 1962). The ribosomes were removed by centrifugation and the pellet rinsed to remove residual free Puromycin, homogenized and assayed as usual.

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