STIMULATION BY RNA POLYMERASE OF AMINO ACID INCORPORATION INTO PROTEINS BY EXTRACTS OF ESCHERICHIA COLI†

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It has been observed by Tissieres, Schlessinger and Gros (1960) that incorporation of amino acids into protein by extracts of E. coli was inhibited by DNase. An explanation for this result has recently been provided by the demonstration that amino acid incorporation is stimulated by RNA formed by the action of RNA polymerase with viral DNA as primer (Wood and Berg, 1962; Ning, Stevens and Loper, 1962; Eisenstadt, Kameyama and Novelli, 1962).

In this communication we present evidence that 1) viral DNA can be replaced by bacterial DNA, 2) inhibition of RNA synthesis with actinomycin D results in commensurate inhibition of protein synthesis and that 3) RNA containing base analogues is usually incapable of stimulating amino acid incorporation.

The requirements for incorporation of C^{14} leucine into protein are shown in Table I, part A. For maximum incorporation both RNA

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Table I

Effect of DNA and RNA Polymerase on Amino Acid Incorporation

	Reaction Mixture	C^{14} leucine Incorporated
		mμmoles
A.	1. Complete (with T2 DNA)	.16
	2. Omit RNA polymerase	.02
	3. Omit T2 DNA	.05
	4. Complete + DNase (10 μg)	.03
	5. Complete + RNase (10 μg)	<.01
B.	1. Complete (with Pneumococcal DNA)	.16
	2. Complete (with Slime mold DNA)	.09
	3. Omit RNA polymerase	.01
C.	1. Complete (with T2 DNA)	.13
	2. Omit T2 DNA and RNA polymerase	.02
	3. Complete + actinomycin D (2.5 mμmoles)	.04
	4. Complete + actinomycin D (10.0 mμmoles)	.02
	5. Omit T2 DNA and RNA polymerase plus poly UG (7.2 mμmoles)	.10
	6. Omit T2 DNA and RNA polymerase plus poly UG (18 mμmoles)	.15
	7. Omit T2 DNA and RNA polymerase plus poly UG (36 mμmoles)	.22
	8. As 6 plus actinomycin D (2.5 mμmoles)	.15
	9. As 6 plus actinomycin D (10 mμmoles)	.17

The complete reaction mixture (0.30 ml) contained: Tris buffer, pH 7.8, 5.0 μmoles; magnesium acetate, 2.0 μmoles; $MnCl_2$, 0.5 μmoles; KCl, 12 μmoles; β-mercaptoethanol, 3 μmoles; 17 unlabeled amino acids, (alanine, arginine, aspartic, cysteine, glutamic, glycine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, valine), 20 mμmoles (each); C^{14} leucine, 40 mμmoles, (specific activity 10,000 cpm per mμmole); ATP, 500 mμmoles; GTP, 100 mμmoles; CTP, 50 mμmoles; UTP, 50 mμmoles, T2 DNA equivalent to 60 mμmoles of nucleotide, or 22 mμmoles of pneumococcal DNA or 14.8 mμmoles of slime mold DNA where indicated; RNA polymerase, 3.4 units; ribosomal fraction, 250 μg protein, and soluble fraction I, 100 μg protein. The ribosomes

and soluble fraction were prepared as described by Wood and Berg (1962). Actinomycin and poly UG were added as indicated. The latter was a gift from Dr. L.A. Heppel of the National Institutes of Health and had a G:U ratio of 0.85 as determined spectrophotometrically and by base analysis. The latter measurement was made by Dr. L.A. Heppel.

After 60 minutes at 38° the reaction was terminated by the addition of 2.5 ml of 7% perchloric acid and the precipitate suspended in 2 ml of 10% TCA, heated for 10 minutes at 100° and cooled. 1.5 mg of serum albumin were added, the suspension centrifuged, and the precipitate washed twice with 3 ml of 1% perchloric acid. The final precipitate was dissolved in 1.5 ml of 0.2 N NH_4OH decanted onto planchets, dried, and counted in a windowless gas flow counter. No correction was made for self-absorption.

In a parallel experiment, with the complete system except that non-radioactive leucine and α-P^{32}-labeled UTP were used, 2.5 and 10 mμmoles of actinomycin D inhibited UMP incorporation 88% and 94% respectively. Ribonucleotide incorporation was measured as described by Furth, Hurwitz, and Anders (1962).

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polymerase and DNA must be present. Addition of DNase reduces incorporation of labeled amino acid to the level obtained when RNA polymerase and DNA are omitted.

Wood and Berg (1962) and Ning, Stevens and Loper (1962) reported that in this reaction, DNA isolated from the T phages markedly stimulated amino acid incorporation, while DNA's obtained from other sources were considerably less active. In agreement with these results we found DNA from Micrococcus lysodeikticus, E. coli, calf thymus and Bacillus subtilis to be inactive or poorly active in our test system. However, we have found that DNA from Diplococcus pneumoniae[1] and from Dictyostelium discoideum (a cellular slime mold)[2] does

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1/ Pneumococcal DNA, active in transformation, was generously supplied by Miss A. Evans.

2/ Slime mold DNA, which was generously supplied by Dr. Julius Marmur, Brandeis University, has the unusually high (A+T/G+C) value of 4.0.

stimulate amino acid incorporation (Table I, part B). It is interesting to note that these two species of DNA resemble bacteriophage DNA in having a high content of A + T ($>$ 60%) as compared with the ineffective species.

Other experiments support the conclusion that stimulation by RNA polymerase of amino acid incorporation is a result of the RNA formed, rather than of some unsuspected activity of the enzyme preparations employed. As shown in Table I, part C, actinomycin D, a potent inhibitor of RNA polymerase (Reich et al., 1961; Goldberg and Rabinowitz, 1962; Hurwitz et al., 1962) inhibits amino acid incorporation at the same low concentrations which inhibit polynucleotide synthesis. Polynucleotide added to the system stimulated protein synthesis even in the presence of actinomycin, as demonstrated by use of limiting amounts of poly UG. The inhibition of protein synthesis by actinomycin in vivo (Kirk, 1960; Levinthal et al., 1962; Hurwitz et al., 1962) must therefore result from the effect of this substance on RNA synthesis rather than from subsequent action on messenger RNA in polypeptide synthesis.

It was of interest to examine the role in protein synthesis of hydrogen bonding between the bases of RNA synthesized by RNA polymerase and those of ribosomal and soluble RNA. In these experiments, UTP was replaced by each of the 4 analogues that replace UTP in the RNA polymerase reaction (Kahan and Hurwitz, 1962). Fluoro-UTP, although the least effective analogue as substrate for the RNA polymerase reaction (measured by incorporation of α-P^{32} labeled GTP), was the only analogue that supported amino acid incorporation approximating that observed with UTP. The stimulation of amino

acid incorporation was reduced when UTP was replaced by ψ-UTP, riboTTP or Br-UTP (Table 2). These same results were obtained whether leucine, tyrosine, lysine, isoleucine and valine incorporation was measured.

Table 2

Effect of Replacement of UTP by Analogues of UTP on RNA Polymerase Stimulation of Amino Acid Incorporation

Reaction Mixture	Incorporation of C^{14} leucine	Incorporation of GMP^{32}
	mμmoles	
1. Complete	.21	5.8
2. Omit UTP	.07	2.2
3. Omit UTP add Bromo-UTP	.06	4.4
4. Omit UTP add Fluoro-UTP	.16	3.2
5. Omit UTP add Pseudo-UTP	.07	7.1
6. Omit UTP add Ribo-TTP	.07	5.1
7. Omit DNA	.04	4.8

Amino acid incorporation was carried out as described in Table I with T2 DNA. Where indicated, UTP was replaced by analogues, (prepared as described by Kahan and Hurwitz, 1962) in equivalent amounts.

The effects of synthetic polynucleotides containing base analogues on the incorporation of amino acids is variable. Among heteropolymers, hypoxanthine is effective in place of guanine, but xanthine is not (Basilio et al., 1962). With homopolymers, neither polyfluorouridylic (Lengyel et al., 1962) nor polyribothymidylic

acids (Haschemeyer and Rich, 1962) replaced polyribouridylate. However, polynucleotides containing 60% UMP and 40% F-UMP were as effective as polyuridylic acid in stimulating polyphenylalanine production[3]. The latter observation prompted us to investigate the extent of analogue incorporation in place of UMP in RNA synthesized in our test system because endogenous UTP is probably present in the crude extracts employed. RNA prepared in these extracts was labeled with α-P^{32}-GTP in the presence of either Br-UTP or F-UTP. The product was isolated and hydrolyzed with alkali, or RNase and spleen phosphodiesterase, to the nucleoside 2' and 3' phosphate. By electrophoresis at pH 3.5, UMP was isolated together with its analogues and this mixture separated into its components by electrophoresis in 0.1 M ammonium carbonate, pH 9.2. Both RNA preparations possessed 4-5 times as much label in each analogue as in UMP.

These results suggest that factors in addition to hydrogen bonding ability, perhaps the size of substitutions at C5 of the pyrimidine ring, render F-UMP intrinsically more suitable in protein synthesis than Br-UMP. However, the possibility that UMP is preferentially concentrated in reaction mixtures containing FUTP, to the exclusion of analogue, in a fraction of the total RNA which supports protein synthesis cannot be excluded. It will be necessary to synthesize polynucleotides with RNA polymerase in purified reaction mixtures and combine these with crude extracts, in order to determine whether protein synthesis can be supported by a polymer in which all of the UMP is replaced by F-UMP.

3/ Personal communication from Dr. Joseph F. Speyer.

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