T_1 RIBONUCLEASE INHIBITION OF POLYURIDYLIC ACID-STIMULATED POLY-PHENYLALANINE SYNTHESIS

David W. Allen and Paul C. Zamecnik

The John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston

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Polyuridylic acid (poly U) has been found to stimulate phenylalanine (phe) incorporation in the rabbit reticulocyte cell-free system (Arnstein *et al.*, 1962; Weinstein and Schechter, 1962; Arlinghaus and Schweet, 1962). Thus it is possible to study both hemoglobin and polyphenylalanine (poly phe) cell-free synthesis under comparable conditions. Hemoglobin synthesis occurs on a multiple ribosomal structure, the polysome, containing approximately 5 ribosomes (Warner *et al.*, 1963; Gierer, 1963). Poly U has been found to combine preferentially with unaggregated reticulocyte ribosomes and to produce polysomes active in poly phe synthesis (Gierer, 1963). Small amounts of pancreatic RNase which produce negligible gross hydrolysis of RNA depolymerize the polysome and inhibit protein synthesis (Arnstein, 1961). The RNase is considered to hydrolyze the RNA linking the ribosomes in the polysome, presumably the messenger RNA (Warner *et al.*, 1963; Gierer, 1963).

To study this phenomenon further a cell-free rabbit reticulocyte system was employed in which the addition of poly U produced a 5-fold increase in C^{14}-phe incorporation. Serial dilutions of both pancreatic RNase (Worthington 3 times crystallized) and RNase T_1 (T_1) were used to inhibit C^{14}-phe incorporation both with and without poly U. Poly U is digested by pan-

creatic RNase but not T_1, whereas a mixed polynucleotide such as hemoglobin messenger RNA should be hydrolyzed by both enzymes.

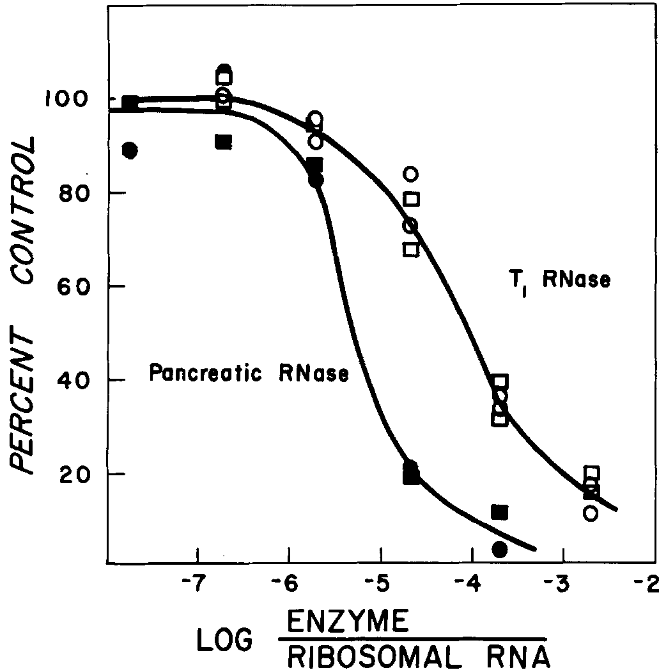

Fig. 1. The results of several experiments compare the inhibition of phe incorporation with or without poly U by either pancreatic RNase or T_1. Rabbit reticulocyte ribosomes were prepared by the method of Schweet et al. (1958) and stored as the pellet at -15°C under 0.25 M sucrose. The ribosomes were suspended in 0.01 M magnesium acetate, 0.06 M KCl, and 0.01 M tris HCl buffer, pH 7.8. A complete cell-free system was used similar to that previously described (Allen and Zamecnik, 1962), except for the alteration in the medium noted above, reduction of the final volume to 0.25 ml, and the addition of 0.25 mg/ml yeast soluble RNA, an amount which yielded maximal incorporation under these conditions. To alternate tubes 20 micrograms of poly U were added. Serial dilutions of pancreatic RNase, or T_1 were added as well as 0.08 micromoles of C^{14} DL-phe (1.82×10^6 cpm/micromole), the tubes incubated 45 minutes at 37°C, and the protein precipitated and counted as previously described (Allen and Zamecnik, 1962). Results are expressed by plotting the percent of the proper uninhibited control (e.g. 1223 cpm/mg with poly U, or 257 cpm/mg without poly U) against the logarithm of the ratio of the weight of the enzyme to the weight of the ribosomal RNA. Aliquots inhibited by T_1 are represented as O with poly U, and □ without poly U. Aliquots inhibited by pancreatic RNase are represented by ● with poly U and ■ without poly U.

Fig. 1 shows that with each enzyme over a large range of dilution there is nearly the same percentage inhibition of both hemoglobin synthesis and poly phe synthesis. C^{14}-phe incorporation without poly U (indicated by squares) is taken as a measure of hemoglobin synthesis and C^{14}-phe incorporation with poly U (indicated by circles) is taken as a measure of poly phe synthesis. The inhibition of polyphenylalanine synthesis would not be predicted if the messenger RNA is considered the only site of action of T_1, since the poly U messenger RNA should be immune to T_1 action.

This finding is not due to an enzymatic impurity in the T_1 which acts on the poly U. The T_1 used in these experiments was prepared from takadiastase by Dr. Peter Bergquist using a modification of the method of Rushizky and Sober (1962) and has been shown to be specific for the phosphodiester bond involving guanosine 3' phosphate. As a further test, poly U was treated with either pancreatic RNase or by T_1 at concentrations of the enzymes that inhibited protein synthesis 95%. Whereas pancreatic RNase completely hydrolyzed poly U, T_1 produced no change in poly U detectable by chromatography, nor in its activity in stimulating phe incorporation.

Since T_1 inhibits poly phe synthesis without hydrolyzing the poly U messenger, the effect of T_1 on the poly U induced polysome was studied by sucrose gradient analysis, following the technique of Warner _et al._ (1963) (Fig. 2). The polysomes were produced and labeled by a 15 minute incubation at 37°C of ribosomes with poly U and C^{14}-phe. The effect of a further incubation for 15 minutes at 37°C with T_1 is compared with a similarly incubated control tube minus the enzyme. Fig. 2 shows that T_1 has largely depolymerized the poly U induced

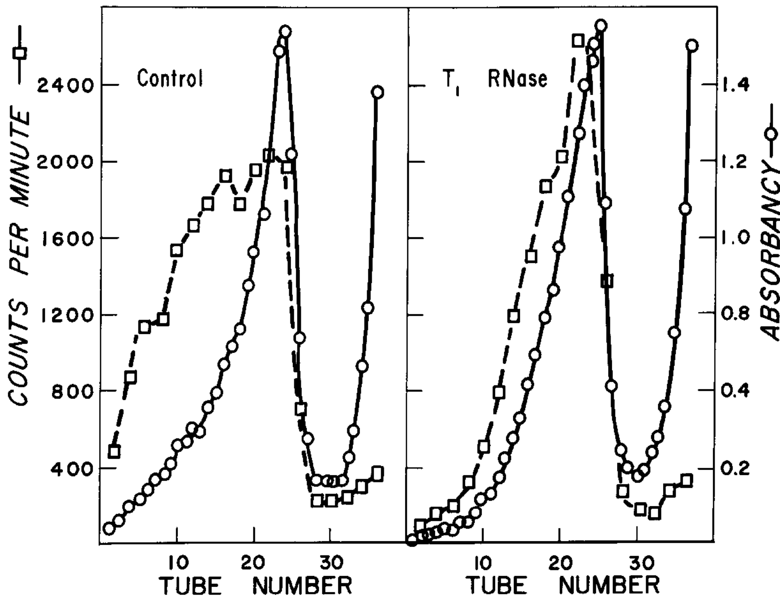

Fig. 2. Depolymerization of poly U induced polysomes by T_1 as studied by the sucrose gradient technique. Two samples of 8 mg of ribosomes containing 3.6 mg of RNA were incubated with the complete cell-free system as described in Fig. 1, 0.2 mg poly U, 0.4 micromoles DL phe (1.82 x 10^6 cpm/micromole) in a final volume of 1.2 ml for 15 minutes at 37°C. Then 8 micrograms of T_1 were added to one tube and both samples incubated 15 minutes longer. One ml of the incubation mixture was layered on 30 ml of a linear 5-20% sucrose gradient in 0.0015 M $MgCl_2$, 0.01 M tris HCl, pH 7.8, and 0.01 M KCl and centrifuged 25,000 rpm at 5°C for 3 hours in the Spinco SW25 swinging bucket centrifuge. Ten drop fractions were collected from the bottom of the tube and the absorbancy at 260 mμ (- O -) and radioactivity (--□ --) determined, following the technique of Warner et al. (1963).

polysomes in tubes 2-18, resulting in a more slowly sedimenting component which nearly coincides with the major 260 mμ absorbing component around tube 25 (the 80 s monomer). The C^{14}-poly phe remains attached to the disaggregated ribosomes. Other experiments demonstrated that pancreatic RNase similarly depolymerized the polysomes. Incubation alone produced depolymerization, but at a slower rate. For this reason, the T_1 treated polysomes are compared with an incubated control. An unincubated control shows the majority of radioactive labeling in the heavier polysome

fractions (around tube 14) and only a small percent of the counts in the 80 s fraction.

Thus T_1 depolymerizes an aggregate of ribosomes produced by addition of poly U but does not act on the poly U. T_1 then may be concluded to act on a ribosomal RNA required for formation of the polysome. Consistent with this conclusion, ribosomes treated with T_1, then washed, show inhibited poly phe synthesis compared to untreated but similarly washed controls. Evidence that T_1 acts on RNA at or near the ribosomal binding site for poly U is demonstrated by the protective action of preincubation of ribosomes with poly U (Table I).

TABLE I

EFFECT OF PREINCUBATION WITH POLY U ON T_1 INHIBITION OF C^{14}VAL AND C^{14}PHE INCORPORATION

No.	cpm/mg C^{14}phe		cpm/mg C^{14}val	
	Control	T_1	Control	T_1
1. Preincubated with poly U	1174	523	164	13
2. Poly U added after preincubation	1237	309	192	15
3. No poly U added	192	28	270	16

All samples contained 1.6 mg of ribosomes (0.8 mg ribosomal RNA) and the complete cell-free system described in Fig. 1, and were preincubated for 10 minutes at 37°C prior to addition of the radioactive label, one third of the samples (No. 1) containing in addition at this time 20 micrograms of poly U per tube. After chilling the tubes to 0°C the same amount of poly U was added to a second third of the tubes (No. 2) and the final third received no poly U (No. 3). Where stated, the tubes received 1.6 micrograms of T_1 RNase (T_1) or no enzyme (control). Either 0.08 micromoles of DL C^{14}phe (1.82×10^6 cpm/micromole) or 0.04 micromoles L C^{14}val (4.8×10^6 cpm/micromole) was used as a radioactive label. The samples were incubated for 45 minutes at 37°C, then the protein precipitated and the radioactivity determined as previously described. The results are averages of duplicate tubes which agreed within 10%.

Preincubation of the system with poly U partially protects C^{14}phe incorporation against subsequent T_1 action (523 cpm/mgm _vs_ 309 cpm/mg), whereas T_1 inhibition of C^{14}valine (C^{14}val) incorporation (measuring hemoglobin synthesis) is not similarly spared by preincubation with poly U (13 cpm/mgm _vs_ 15 cpm/mg). The protective effect of preincubation of the system with poly U against T_1 inhibition of C^{14}phe incorporation is quite reproducible. In 6 replications of the experiment shown in Table I (including this experiment), the average percentage of the control tube without preincubation with poly U ($\pm$ standard deviation) was $26\pm3\%$ and with preincubation with poly U was $46\pm5\%$.

T_1 inhibition of poly phe synthesis provides evidence that a ribosomal RNA other than messenger RNA may be essential for protein synthesis and polysome formation. Three possible functions of this T_1 sensitive ribosomal RNA are suggested: (1) this RNA rather than poly U holds the polysome together; (2) the RNA in question is the binding site for the soluble RNA to which the growing polypeptide chain is attached and which is hydrogen bonded to the messenger; or (3) it is the ribosomal binding site of the poly U messenger holding the ribosomes together.

The first possibility seems remote since there is no evidence for an RNA other than messenger RNA holding the polysome together and it makes the polymerization of ribosomes by the addition of artificial messenger (Gierer, 1963) an unnecessarily complicated concept. The second possible site of T_1 action, the ribosomal binding site of the soluble RNA linked to nascent polypeptide and messenger, is made unlikely by the observation that the nascent polypeptide remains with the single ribosome following T_1 depolymerization of the polysome (Fig. 2). The third possibility that T_1 acts on the ribosomal binding site for poly U is favored by its simplicity, and the protective effect of preincu-

bation with poly U (Table I). This provides evidence for a ribosomal binding site for poly U which, considering its T_1 sensitivity, may be a single-stranded RNA containing one or more internal guanylic acid residues.

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