THE BIOLOGIC ACTIVITY OF ENZYMICALLY-ALTERED E. COLI TRANSFER RNA

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Transfer RNA (s-RNA) retains the ability to both accept and transfer amino acids after heating and after exposure to 6 M urea (Takanami, et al., 1961). Preiss, et al., (1959) have shown that acceptor activity is destroyed by limited digestion with venom diesterase, whereas comparable digestion with spleen diesterase had less effect. Data are presented in this paper which show that removal of the terminal 5'-phosphate from s-RNA does not diminish these biologic activities. On the other hand, most of the transfer, as well as the acceptor, activity is lost after partial digestion by spleen phosphodiesterase.

MATERIALS AND METHODS

Escherichia coli transfer RNA was prepared by the method of Ofengand, et al., (1961); C^{14}-amino acid-transfer RNA, washed ribosomes, and transfer enzyme were prepared as described by Nathans and Lipmann (1961). Spleen phosphodiesterase was prepared as previously described (Hilmoe, 1960), and E. coli alkaline phosphatase with a specific activity of 1400 [1/] was fractionated by a modification of the procedure of Garen and Levinthal (1960). Uniformly labeled C^{14}-mixed amino acids, prepared from an algal protein hydrolysate (0.8 mc/mg), and uniformly labeled C^{14}-phenylalanine (1.3 mc per mg) were purchased from the New England Nuclear Corporation. The sodium salt of phosphoenol pyruvate was obtained from Calbiochem and pyruvate kinase from Sigma Chemical Company. Polyuridylic acid was a gift from Dr. Leon A. Heppel. The method of Ames and Dubin (1960) was used for the inorganic phosphate determinations. Radioactivity was counted in a thin window gas flow counter.

Enzyme for use in the amino acid acceptor assay was prepared by dialyzing against 0.01 M Tris, pH 7.5, the 30,000 x g supernatant solution obtained from a glass bead extract of frozen E. coli cells (Preiss, et al., 1961).

[1/] Specific activity is expressed as micromoles of AMP hydrolyzed per hour per milligram of enzyme at 37°.

The assay included, in a volume of 0.2 ml: 20 μmoles phosphate buffer, pH 7.0; 0.3 μmoles ATP; 1 μmole $MgCl_2$; 0.2 μmoles mercaptoethanol; 0.5 mg s-RNA; 0.3 μg C^{14}-mixed amino acids; and 25 μl extract. After 20 minutes at 37°, the reaction was stopped by adding 2 ml of cold 10% trichloroacetic acid. The precipitate was collected on a Millipore filter disc, washed with 10-15 ml 10% trichloroacetic acid, dried, and counted. Enzyme blanks without added s-RNA were subtracted. Phosphate was employed both as a buffer and to suppress E. coli alkaline phosphatase, for this enzyme is not easily destroyed after use (Heppel, et al., 1961) and it hydrolyzes ATP (Heppel, et al., 1961). Nevertheless, assays with phosphatase present usually showed about 5 per cent inhibition.

RESULTS AND COMMENTS

Incubation of s-RNA with E. coli alkaline phosphatase at 60° led to complete removal of terminal phosphate in 3 hours (Fig. 1). Continued incubation for 3 hours resulted in no further release of inorganic phosphate, nor was there any rise above the control in ultraviolet absorbing material (260 mμ) soluble in either 0.13% uranium acetate in 1.3% perchloric acid or in 0.24 M NaCl in 64% ethanol. Increasing the enzyme three-fold in a similar incubation for 10 hours also failed to give evidence of endonucleolytic attack. The ability of the RNA to accept mixed amino acids (Fig. 1) and phenylalanine (not shown) decreased gradually, but at a rate no greater than that of heated controls without phosphatase.

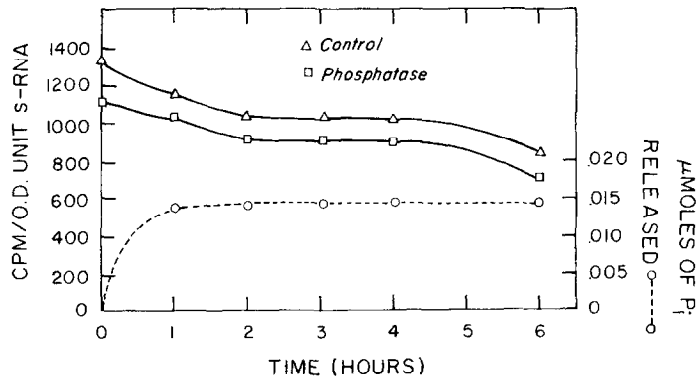

Fig. 1. Amino acid acceptor activity of E. coli s-RNA after treatment with E. coli alkaline phosphatase. Duplicate 0.1 ml mixtures containing 0.5 mg s-RNA, 10 μmoles Tris, pH 8.3, and 3.5 μg alkaline phosphatase (where indicated) were incubated at 60° for the times designated. P_i determinations were carried out on the supernatant fluid from one set following precipitation of the RNA with 0.4 ml of 0.17% uranium acetate in 1.7% perchloric acid. The RNA in the remaining tubes was precipitated with 0.4 ml 80% ethanol containing 0.3 M NaCl; each precipitate was then dissolved in 0.25 ml H_2O and 0.1 ml aliquots were used in the amino acid acceptor assay described in the text.

Dephosphorylated s-RNA was examined for its ability to transfer amino acids to ribosomes. Mixed C^{14}-amino acids or C^{14}-phenylalanine were first attached to s-RNA which had previously been exposed to alkaline phosphatase. Thereupon the s-RNA was incubated with purified transfer enzyme and washed ribosomes in the presence of a GTP regenerating system. The results for phenylalanine transfer in the presence of polyuridylic acid appear in Fig. 2. Essentially similar results were obtained with the mixed amino acids without added "messenger" RNA. Preparations of dephosphorylated s-RNA were as effective as normal s-RNA in the amino acid transfer system.

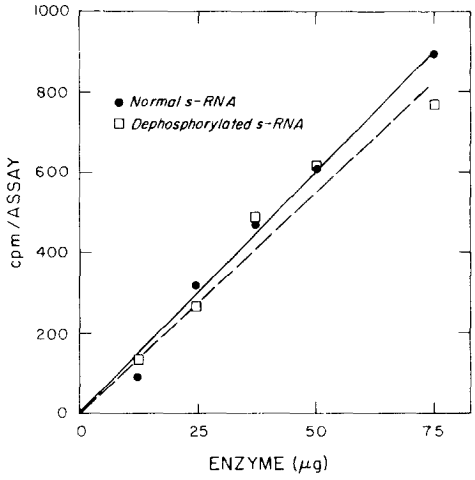

Fig. 2. Transfer of C^{14}-phenylalanine from s-RNA to ribosomes. Incubations (0.5 ml) contained 0.05 M Tris, pH 7.4, 0.013 M $MgCl_2$, 0.03 M KCl, 0.01 M GSH, 0.0006 M GTP, 0.01 M phosphoenol pyruvate, 30 μg pyruvate kinase, 5 μg poly U, 0.5 mg ribosomes, enzyme, and 0.05 mg of normal C^{14}-phenylalanine s-RNA (1,740 cpm), or 0.075 mg of phosphatase-treated C^{14}-phenylalanine s-RNA (1590 cpm). Incubations (37°, 5 min.) were terminated by adding 5 ml of 10% TCA. Precipitates were heated (90°, 20 min.), filtered and washed on Millipore filters. Values have been corrected for non-enzymatic ribosomal amino acid incorporation. This assay is that described by Nathans and Lipmann (1961).

These results indicate that the removal of the terminal phosphate from transfer RNA in no way affects its biologic activity as judged by its ability to accept and transfer amino acids. The gradual decrease with time in acceptor activity of the heated control s-RNA varies with the preparation of RNA. This is undoubtedly due to nucleases in the s-RNA; phenol extraction markedly decreases this loss in activity. We have utilized this ability of s-RNA to accept amino acids after exposure to phosphomonoesterase as an assay during further purification to remove contaminating nuclease activity from commercial E. coli alkaline phosphatase.

The effect of spleen phosphodiesterase was now examined. Acceptor activity of both normal and dephosphorylated s-RNA was lost relatively rapidly upon incubation with this enzyme (Table I). Transfer activity also decreased sharply after short digestion. In such experiments the amino acids were attached prior to incubation with the phosphodiesterase. The data for phenyl-

TABLE I. Effect of spleen phosphodiesterase upon amino acid acceptor activity of E. coli s-RNA before and after phosphatase treatment

Spleen diesterase (ml)	s-RNA No Phosphatase		s-RNA + Phosphatase	
	Solubilization (per cent)	Incorporation (cpm)	Solubilization (per cent)	Incorporation (cpm)
0	-	1067	-	787
.005	0.26	1007	0.22	780
.015	0.49	762	1.21	666
.030	1.47	651	2.57	641
.050	2.51	575	6.24	453
.065	3.54	464	8.70	358

E. coli s-RNA (3.5 absorbancy units, 260 mμ, 0.01 N NaOH) was incubated in a volume of 0.1 ml containing 10 μmoles Tris-HCl buffer, pH 8.3, and 3.5 μg E. coli alkaline phosphatase for 2 hours at 60°. Control tubes were treated similarly except that no phosphatase was added. s-RNA was recovered by precipitation with 3 volumes of 80% ethanol containing 0.3 M NaCl and digested with spleen diesterase (6.8 units/ml) by incubating at 37° for 30 minutes in a volume of 0.1 ml containing 5 μmoles succinate buffer, pH 6.5, and the enzyme. The amino acid acceptor activity was measured on the product recovered by precipitation with ethanol-NaCl and solubilization was determined from the absorbancy at 260 mμ of the supernatant fluid (corrected for hyperchromicity).

alanine transfer are presented in Figure 3. The same results were obtained when these experiments were done using mixed amino acid-s-RNA. A difficulty, also observed by others, is the instability of the amino acid-s-RNA complex. Thus, under the conditions necessary for spleen digestion (pH 6.5, 37°, 30 minutes) 30 and 46 per cent of the radioactive amino acids were released from the normal and dephosphorylated s-RNA, respectively; this was exactly the same in the presence or absence of spleen enzyme. In view of the inhibitory effect in

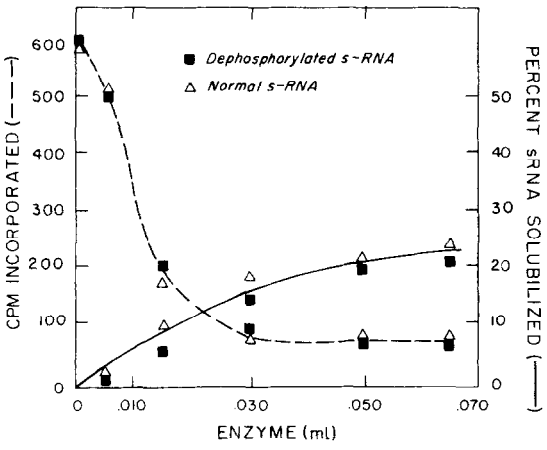

Fig. 3. Effect of spleen phosphodiesterase digestion of C^{14}-phenylalanine-RNA on transfer of phenylalanine to ribosomes. The incubations with spleen enzyme, precipitation of the s-RNA, and calculation of the per cent solubilization are as described under Table I except that the tubes containing normal s-RNA (0.15 mg containing 5,200 cpm C^{14}-phenylalanine) were incubated for 60 minutes, and tubes containing phosphatase-treated s-RNA (0.15 mg containing 3,180 cpm C^{14}-phenylalanine) were incubated for 30 minutes. Transfer was then measured as in Fig. 2.

oligonucleotides of a 5'-terminal phosphate (Heppel, et al., 1956), we were surprised to find only a two-fold difference in rates of attack upon normal and dephosphorylated s-RNA. It is possible that the mechanism of action is not entirely stepwise, especially on large molecules, and by virtue of endonuclease attack the inhibition due to the 5'-phosphate group is overcome. Another possibility is that preparations of spleen phosphodiesterase still contain traces of nuclease activity. Because of these uncertainties we feel that the data pertaining to the spleen enzyme must be interpreted with caution.

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