INABILITY TO DETECT RNASE V IN ESCHERICHIA COLI AND COMPARISON OF OTHER RIBONUCLEASES BEFORE AND AFTER INFECTION WITH COLIPHAGE T7

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SUMMARY: No marked changes in the activities of polynucleotide phosphorylase or RNases I, II, and III were observed in Escherichia coli after infection with phage T7. We were unable to detect activity with the reported properties of RNase V in extracts of E. coli.

In E. coli infected with phage T7 the half-life of mRNA at 30° is prolonged from approximately 2 or 3 min to 20 or 30 min (17). In such infected cells both phage- and host-specific mRNA appear to be stabilized (16). The experiments reported here were undertaken to see if infection by T7 is associated with changes in activities of the known ribonucleases of E. coli.

Ribonuclease Activities in T7-Infected Cells. Enzyme activities were measured in crude extracts of uninfected and T7-infected E. coli strains B and N464. PNPase[1/] and RNases I, II, and III were specifically assayed by taking advantage of their differing substrate specificities and metal ion requirements. RNase IV was not investigated because a suitable quantitative assay was not available (2). As described in detail below, no activity with the properties reported for RNase V (4-9) was detected in our extracts.

No marked change in the activity of any of the enzymes tested was observed after infection with phage T7 (Table I). E. coli N464 is a multiple mutant of E. coli K12[2/]. Phage T7 infects strain N464 efficiently, and our data confirm the low levels of PNPase and RNase I as well as the thermolability

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1/ Abbreviations used: Polynucleotide phosphorylase (PNPase), β,γ-methylene GTP (GOPOPCP), aurintricarboxylic acid (ATA).

2/ We are grateful to Dr. David Schlessinger for making this strain available.

TABLE I. Nuclease activities in uninfected and phage T7-infected E. coli

E. coli strain	T7 infected	Fraction	PNPase	RNase I	RNase II	RNase III
			μmoles nucleotide per hr per mg protein			
B	No	S30	1.17	4.6	15.4	0.49
B	Yes	S30	1.05	3.5	13.6	0.48
N464	No	S30	0.13	0.46	7.4	0.50
		Heated S30	0.11	0	0.14	0.048
N464	Yes	S30	0.13	0.15	5.6	0.66
		Heated S30	0.11	0	0.13	0.097

E. coli B (15), E. coli N464 (4), and coliphage T7 (15) were grown in tryptone broth at 30° as described by Studier (15). Exponentially growing 250 ml cultures at 1.2 - 1.4 x 10^8 cells/ml were infected with approximately 15 T7 phages per cell, giving 99% killing of each host in less than 2 min. At 10 min after adding phage the infected and control cells were chilled rapidly, collected by centrifugation at 10,000 x g, washed twice in buffer (0.01 M Tris-HCl, pH 7.8, 0.014 M magnesium acetate, 0.06 M KCl), suspended in 2.5 ml aliquots of buffer made 0.006 M in 2-mercaptoethanol, sonicated as described by Singer and Tolbert (13), and centrifuged 20 min at 20,000 x g. The supernatants were treated in the cold with 2 μg/ml of DNase (Worthington, one time crystallized, RNase free) and centrifuged 30 min at 30,000 x g to yield S30 extracts. Samples of the extracts from E. coli N464 were rapidly heated and maintained at 50° for 10 min. Enzyme assays were performed at 37° with several appropriate concentrations of S30 extracts, and the average specific enzymatic activities were expressed as μmoles nucleotide/hr/mg protein. The concentrations of protein in the S30 extracts were 1.4 to 3.1 mg/ml. The following assays were used: RNase I, assay A of Neu and Heppel (11) with yeast tRNA as substrate; RNase II, Singer and Tolbert (13); RNase III, Robertson et al. (12) with poly I • ^{3}H-poly C substrate at 50 nmoles nucleotide/ml; PNPase, Chou and Singer (1); and protein, Lowry et al. (10). Radioactive samples were counted in triton-toluene scintillator (3) using internal standards to determine specific radioactivity of the substrates.

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of RNase II in this strain (4). RNase III was also partially inactivated at 50°.

Experiments with RNase V. RNase V was described (4) as a nucleolytic activity responsible for degradation of mRNA and detectable in extracts of E. coli N464 after heating to inactivate RNase II. The reported properties

TABLE II. Nuclease activity with RNase V assay system: Cofactors and inhibitors

RNase V Assay Conditions	Activity (μmoles/hr/ml of extract)	% Inhibition
Complete	0.139	0
-GTP	0.152	0
-GSH	0.130	6
-tRNA	0.111	20
-GTP, GSH, tRNA	0.152	0
+GOPOPCP, 0.95 mM	0.102	27
+Fusidic acid, 1 mM	0.093	33
+ATA, 0.09 mM	0.097	30

E. coli N464[3/] was grown at 30° in medium containing 8 g nutrient broth plus 8 g technical grade casamino acids per liter, rapidly chilled, harvested by centrifugation early in log phase (about 0.3 - 0.4 g cells/liter), washed twice with TM2 buffer (0.01 M Tris-HCl, pH 7.5, 0.01 M Mg^{2+}), and stored frozen as a pellet. Thawed cells were disrupted by grinding with 2 g alumina/g cells, and extracted with 1.5 ml TM2 buffer/g cells plus DNase at 2 μg/ml. Crude extract (S10) was obtained by two successive centrifugations at 10,000 x g for 15 min followed by overnight dialysis of the supernatant against 100 volumes of TM2 buffer. S10 (31.4 mg protein/ml) was heated 10 min at 50°C. RNase V assays (4) were carried out at 37° in 0.1 ml or 0.05 ml reaction mixtures for 30 min unless otherwise noted. RNase V assay mixtures contained the following: 40 mM Tris-Cl, pH 7.6; 0.5 mM GTP; 10 mM GSH; 20 mM magnesium acetate; 50 mM NH_4Cl; E. coli tRNA, 100 μg/ml; ^{14}C-poly U (Schwarz, 0.44 mCi/mmole), 60 nmoles nucleotide/ml; and 200 μl/ml of S10.

of RNase V include a requirement for ribosomes, supernatant factors, and several other components of the protein synthetic system, as well as an exonucleolytic mechanism of degradation in the 5'→3' direction (5). Table II shows typical results obtained in this laboratory when assays for RNase V were carried out with heated, unfractionated extracts of E. coli N464[3/]. There is no requirement for GTP, GSH, or added tRNA. There is partial inhibition by GOPOPCP, fusidic acid, and ATA, although the inhibition is less striking than that reported for RNase V (4, 7). When the crude extract

3/ We are indebted to Dr. David Schlessinger for the detailed protocol for preparation of crude extracts and cell fractions. Extracts referred to as S30 (4, 5) were in fact S10 and are so designated herein.

is separated into supernatant and ribosomal fractions, activity in the RNase V system is found only in the heated ribosomes (Fig. 1A, B). The nuclease in the S100 fraction is completely inactivated by heating and does not stimulate the activity of the ribosomes (Fig. 1B). Activity was also measured in these extracts using salt and buffer conditions optimal for RNase II. With the same high concentrations of S10, S100, and ribosomes and with the same substrate as prescribed for the standard RNase V assay (4), the apparent RNase II activity is somewhat lower than the RNase V activity (Fig. 1A, B, C). The RNase II detected after heating is associated with the ribosomes and is not stimulated by S100 (Fig. 1C). RNase II is known to bind to ribosomes (13, 14). The data presented here suggest that the ribosome-bound RNase II of N464 is more stable to heat than is

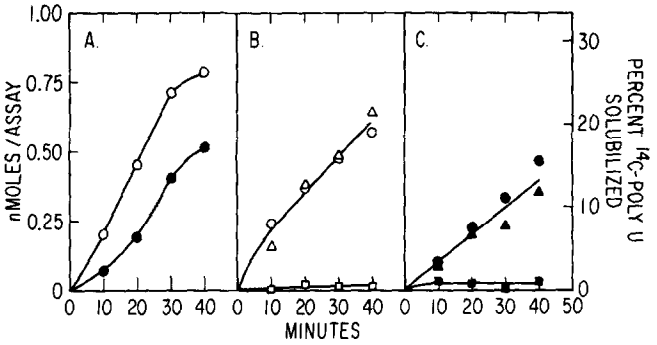

Figure 1. Ribonuclease activity of heated extracts from E. coli N464 in RNase V and RNase II assays[3]. Methods and RNase V assay were as described for Table II. RNase II assays were as described previously (13) (0.1 M Tris-Cl, pH 7.5, 0.1 M KCl, 1.5 mM $MgCl_2$) except that ^{14}C-poly U (60 nmoles nucleotide per ml) was substrate. The upper 2/3 of supernatant fluid after centrifugation of S10 at 109,000 x g for 2 hr is S100 (10.8 mg protein per ml). The ribosomal pellet was washed twice with TM2 by similar centrifugation and suspended in TM2 at 20 mg ribosomes per ml. S100 and ribosomes in this experiment were heated 5 min at 50° (essentially identical results were obtained with other preparations heated 5 or 10 min). Concentrations in assays were 200 µl of S10 or S100 per ml and 2.0 mg ribosomes per ml (4, 5). Results are expressed per 50 µl aliquot of the reaction mixture with--drawn at each indicated time. The amount of substrate hydrolyzed is indicated on the left hand ordinate, and the percent hydrolysis is on the right hand ordinate.
A. Nuclease activity in heated S10 extracts: RNase V conditions (-o-), RNase II conditions (-•-).
B. RNase V activity of ribosomes (-o-), S100 (-□-), S100 + ribosomes (-Δ-).
C. RNase II activity of ribosomes (-•-), S100 (-■-), S100 + ribosomes (-▲-).

the soluble RNase II[4/].

The specific activity of the nuclease in a typical heated S10 extract assayed with standard RNase V conditions (Table II) was 4.4 nmoles per hour per mg protein, in agreement with published results (4, 5). The activity in independent preparations varied up to two fold. RNase V activity thus represents less than 0.5% of the RNase II activity in unheated S10 extracts from E. coli N464. We have found, however, that the high concentrations of heated extract used in RNase V assays (Table II, Fig. 1, and Ref. #4, 5) are in fact strongly inhibitory to the residual activity observed with either the RNase V or the RNase II assay system (Fig. 2A). At low concentrations of extract the residual RNase II activity approached 50 nmoles per hour per mg protein, which is 10-fold greater than that observed with standard RNase V conditions and is consistent with the expected RNase II activity (the higher values in Table I reflect the use of poly A substrate at saturating concentrations). At the lowest enzyme concentrations in Fig. 2A, the residual RNase II activity is proportional to enzyme concentration and is slightly greater than the apparent RNase V activity.

Highly purified RNase II (13) is also strongly inhibited by high concentrations of heated S10 from N464 (Fig. 2B). Furthermore, in the presence of this inhibitory extract, the Mg^{2+} optimum of purified RNase II is shifted to concentrations above that found for the purified enzyme itself (Fig. 2C) (13, 14) and approaches the Mg^{2+} optimum reported for RNase V (4). In our experiments the activity of the heated N464 extracts in standard RNase V assays was also optimal at high Mg^{2+} levels (Fig. 2C).

The explanation for the discrepancies between our results and those of Schlessinger and co-workers is not clear. We have been unable to detect

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4/ A similar conclusion has been reached by A. Bothwell and D. Apirion, as reported in the accompanying paper. We are grateful to these workers for communicating their results prior to publication.

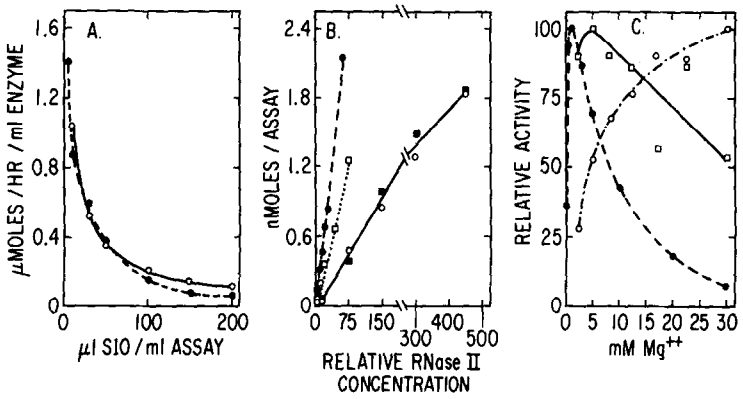

Figure 2. Enzyme preparations and methods as described for Table II and Figure 1.
A. High concentrations of heated S10 from E. coli N464 inhibit the nuclease activity in the extract. At each concentration of S10 the reaction was shown to proceed linearly with time, and less than 20% of the ^{14}C-poly U was hydrolyzed. RNase V conditions (-o-). RNase II conditions (--•--).
B. Inhibition of purified RNase II by heated S10 from E. coli N464. The RNase II used was purified in this laboratory by Drs. J. J. Gastles and G. P. Tolbert from E. coli MRE 600 and had a specific activity of 1210 units/mg protein (13). Hydrolysis of ^{14}C-poly U by purified RNase II was measured at 37° in reactions incubated for 10 min under the following conditions: RNase II assay (--•--), RNase V assay (--□--) omitting S10, RNase II assay plus 200 μl S10/ml (-■-), and RNase V assay with 200 μl S10/ml (-o-).
C. Magnesium optima for hydrolysis of ^{14}C-poly U by purified RNase II (--•--), by heated S10 from E. coli N464 under RNase V conditions (-•-o-•-), and by purified RNase II under conditions of the RNase V assay in presence of 200 μl/ml of heated S10 (-□-). Appropriate blanks have been subtracted, and for each set of assays the maximum observed activity has been normalized to a value of 100.

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any activity with the reported properties of RNase V. We have not investigated the direction of hydrolysis by the residual nuclease because the interpretation of such experiments when performed with crude enzyme preparations is very difficult. The activity we measure under the conditions described by Schlessinger and co-workers for RNase V appears most likely to be residual RNase II associated with ribosomes[4].

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