In this manner a hydrophobic environment analogous to that found in native BSA is possibly generated.

Studies of macromolecule–small molecule interactions on synthetic polymers by Klotz and coworkers (Klotz *et al.*, 1969; Klotz and Harris, 1971) have shown that linear polymers have a much lower binding ability for hydrophobic anions (methyl orange) than highly cross-linked or branched polymers. These authors attribute the difference in behavior to the difference in conformation, the "open and extended" conformation of the linear polymers being less favorable than the "compact locally concentrated" conformation of the other polymers or of native BSA. In view of our results we may add that some secondary interactions promoted by the proximity of nonpolar groups may be essential in binding processes involving hydrophobic interactions.

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# *Lactobacillus plantarum* Exoribonuclease. Effect of Urea Treatment on the Processive Mode of Degradation*

Enore C. Gardonio and David M. Logan†

ABSTRACT: The effect of urea on *Lactobacillus plantarum* exoribonuclease (a processive nuclease) has been tested. Although dilute urea has no effect on enzyme activity, treatment with 1.67 M urea leads to a biphasic, logarithmic loss of activity. This loss of activity is accompanied by decreases in both the $K_m$ and $V_{max}$ over an order of magnitude but by no change in the ionic requirements of the enzyme. More importantly the residual enzyme activity regardless of the prior urea treatment is always strictly processive with both synthetic polynucleotides and yeast RNA. Thus the factors conferring processivity are not affected by urea treatment.

Recently at least three bacterial enzymes have been shown to degrade single-stranded RNA processively. That is they degrade individual RNA strands essentially to completion before starting to degrade another RNA strand. These are polynucleotide phosphorylase from *Escherichia coli* (Thang *et al.*, 1967) and from *Micrococcus luteus* (Klee and Singer, 1968), ribonuclease II from *E. coli* (Nossal and Singer, 1968), and exoribonuclease from *Lactobacillus plantarum* (Logan and Singer, 1968). It is not known what chemical or structural features of those enzymes lead to processive degradation rather than the more common random degradation.

We have studied the effect of the general denaturing agent urea on the *L. plantarum* enzyme to see if processivity is lost under conditions leading to local denaturation of the protein. However under our experimental conditions which lead to extensive loss of activity and changes in the enzyme's kinetic parameters, the residual activity is always expressed as strictly processive degradation.

## Materials and Methods

The growth of *L. plantarum* and the purification and assay of the *L. plantarum* exoribonuclease have been described previously (Logan and Singer, 1968; Gardonio and Logan, 1971). Tris·HCl buffer (pH 8.6) was used instead of 2-amino-2-methyl-1,3-propanediol buffer (pH 8.6). A fraction III enzyme preparation was used in all the experiments reported here except the gel electrophoresis experiments which were performed with fraction V enzyme.

[$^3$H]- and [$^{14}$C]poly(A) were prepared in our laboratory by standard techniques from [$^3$H]- and [$^{14}$C]ADP (Amersham–Searle and Schwarz BioResearch) and polynucleotide phosphorylase kindly supplied by Dr. M. F. Singer or purchased

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TABLE I: Effect of Diluted Urea on Enzyme Activity.[a]

| Expt | Urea Concn (M) | Enzyme Act. (mμmole/min) |
|---|---|---|
| 1 | 0 | 0.89 |
| | 0.125 | 1.01 |
| 2 | 0 | 0.55 |
| | 0.10 | 0.53 |

[a] Activity was measured under standard assay conditions with different amounts of fraction III enzyme. Urea was added where indicated from a concentrated stock solution. The activity reported is the initial rate of degradation.

from Miles Chemical Co. Nessler's solution was prepared by method 1 of Dawson *et al.* (1969). Reagent grade chemicals were obtained from Fisher Scientific Inc., and British Drug House, Inc. Bio-Gel P-2 was purchased from Bio-Rad Laboratories.

The mode of degradation was assessed in three ways. As previously reported (Logan and Singer, 1968) the degradation of terminally labeled polymers and the chromatography of degradation products on Sephadex G-100 can be shown to differentiate between random and processive degradation. In addition we have used thin-layer chromatography on *P*EI-cellulose[1] to reduce the possibility of trace amounts of oligonucleotide remaining undetected. Thin-layer chromatography was performed on precoated PEI-cellulose sheets (Baker Chemical Co.). Aliquots (5 μl) from reaction mixtures were spotted directly on the sheets after varying incubation periods and the spots were freed of salts and urea by an ascending wash with water. The sheets were then dried and the chromatographs were developed with 1.6 M LiCl. They were visualized under uv light. A processive enzyme under these conditions gives rise to only two spots: undegraded polymer at the origin and 5′-AMP. In contrast, with a random enzyme the intermediate portion of the chromatogram shows spots corresponding to oligonucleotides of different chain lengths.

Urea concentration was assayed by the method of Bernt and Bergmeyer (1965).

Gel electrophoresis was performed in 7.5% acrylamide gels by the technique of Clarke (1964). Urea of the indicated concentration was added to the electrolyte solution.

## Results

*Effect of Urea on Enzyme Assay.* Table I shows the results of two experiments in which urea was added to the assay mixture to assess the effect on the measured activity. The final urea concentrations were adjusted to be at least the final concentration which would arise from dilution of the enzyme after urea treatment. Urea at these dilute concentrations has no demonstrable effect on the measured activity.

*Enzyme Inactivation by Urea.* EFFECT OF TEMPERATURE. Figure 1 shows the effect of different temperatures on the inactivation of the enzyme in 1.67 M urea. The inactivation is biphasic when plotted on a semilogarithmic plot of residual activity *vs.* duration of treatment. The general reaction consists of rapid initial inactivation followed by a second slower

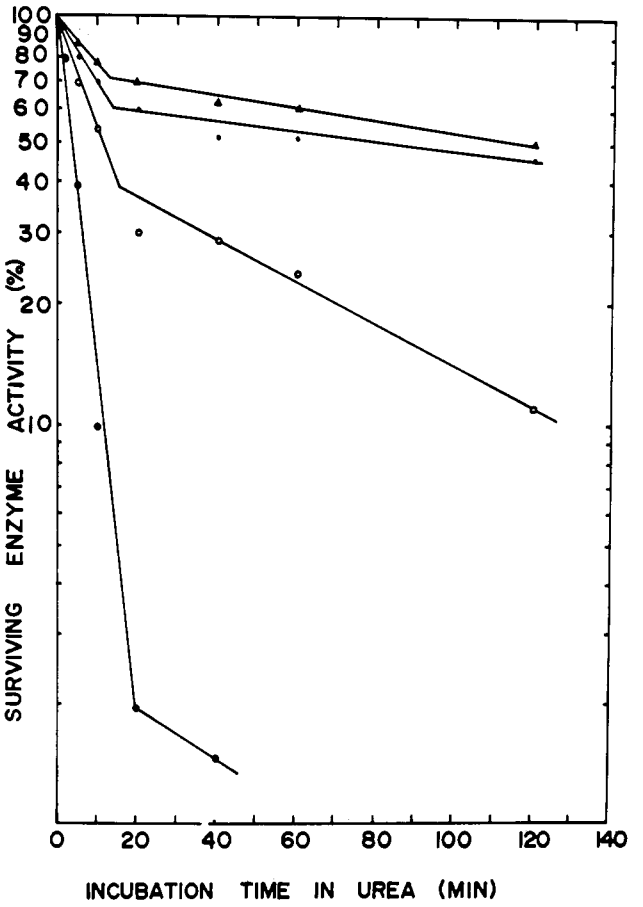


FIGURE 1: The effect of temperature on enzyme inactivation in 1.67 M urea. Inactivation and assays of residual activity were performed as described in the text. Temperatures tested were 15° (▲), 23° (•), 30° (○), and 37° (●).

inactivation phase. The rate of the initial inactivation is accelerated by increasing temperature in the range 15–37°: initial rate 37°/initial rate 15° = 3.9/1. On the other hand, the rate of the slower inactivation process is much less affected by increasing temperature. However the relative amounts of fast and slow inactivation components are very sensitive to temperature. At 30° about 40–45% of the inactivation occurs at the slow rate (based on extrapolation of the data for slow inactivation to the ordinate) while at 37° only 2–3% of the inactivation occurs at the slow rate.

EFFECT ON ENZYME KINETICS. The loss of activity by the enzyme during urea treatment is accompanied by changes in the enzymes' kinetic parameters. Figure 2 shows Lineweaver–Burk plots of data obtained using enzyme fractions treated with 1.67 M urea at 37° for differing durations. Under these conditions there is a continuous decrease in both the $K_m$ and $V_{max}$ during the course of the enzyme inactivation (Table II). Under these inactivation conditions there is no evidence of substrate inhibition at high substrate concentrations. On the other hand when the inactivation conditions are adjusted to give a larger slowly inactivating component (a lower inactivation temperature) the residual activity shows a significant inhibition at high substrate concentrations. An example of this is shown in Figure 3.

In this case double-reciprocal plots of data obtained using treated enzyme show definite substrate inhibition at higher substrate concentrations. The enzyme fractions used in each case were obtained by different treatments but in both cases

[1] Abbreviation used is: PEI-cellulose, polyethylenimine cellulose.

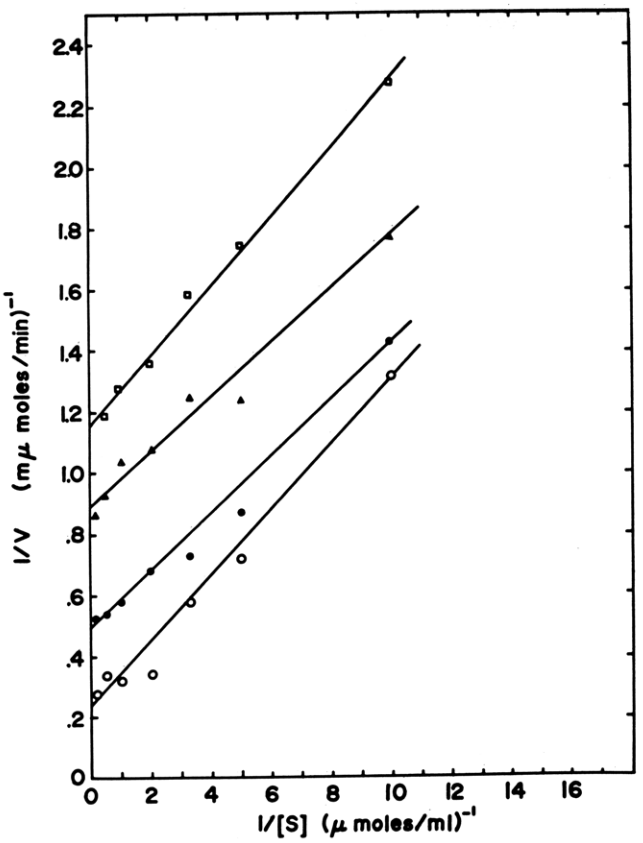


FIGURE 2: Lineweaver–Burk plots obtained using partially inactivated enzyme. Enzyme was partially inactivated by incubation at 37° in 1.67 M urea for different durations. After different periods the enzymes were assayed for residual activity and the effect of substrate concentration on enzyme activity. Data are shown for enzymes having 100% (○), 80% (●), 37% (▲), and 9% (□) surviving activity.

the treatments were long enough to ensure that the slow phase of inactivation had been reached.

*Gel Analysis of Treated Enzymes.* Enzyme fractions (fraction V) were inactivated as described above at 37 and 23° in 1.67 M urea. At 37° the inactivation is essentially linear

TABLE II: Kinetic Constants for Urea-Treated Enzyme.[a]

| Residual Enzyme Act. (%) | $K_m$ ($\times 10^{-3}$ M) | $V_{max}$ (mμmoles/min) |
|---|---|---|
| 100 | 0.54 | 4.90 |
| 94 | 0.44 | 3.70 |
| 80 | 0.20 | 2.08 |
| 61 | 0.13 | 1.45 |
| 37 | 0.098 | 1.15 |
| 9 | 0.084 | 0.87 |

[a] Fraction III enzyme was incubated with 1.67 M urea for varying durations. The enzyme solution was then diluted and assayed for residual activity and for the effect of polymer concentration on activity. The latter data were plotted on double-reciprocal plots (Figure 3) from which the $K_m$ and $V_{max}$ were calculated. $K_m$ is given in polymer mononucleotide concentration and $V_{max}$ in millimicromoles per minute of mononucleotide produced in a standard assay.

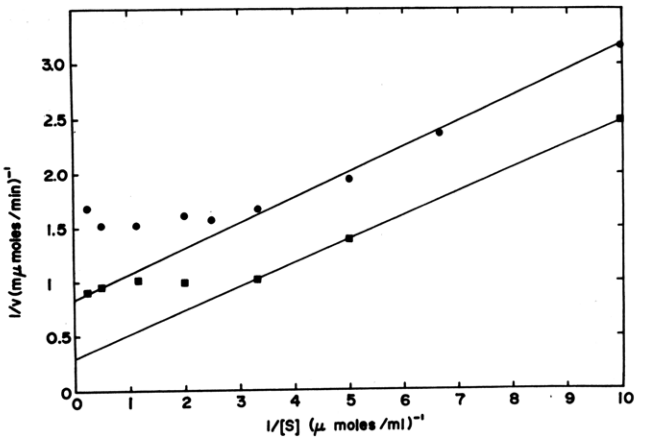


FIGURE 3: Lineweaver–Burk plots of enzyme inactivated under conditions leading to substrate inhibition at high substrate concentrations. Data shown are for enzyme inactivated at 30° for 60 min in 1.67 M urea (●) and at 23° for 60 min in 1.67 M urea (■).

whereas the 23° inactivation curve shows a large slowly inactivating component. At various intervals during the inactivation samples were removed and analyzed by gel electrophoresis. The patterns obtained are shown in Figure 4. In all cases urea treatment produces only one band. At 37° the $R_F$ of this band is essentially unchanged throughout the experiment. The enzyme fractions incubated at 23° how-

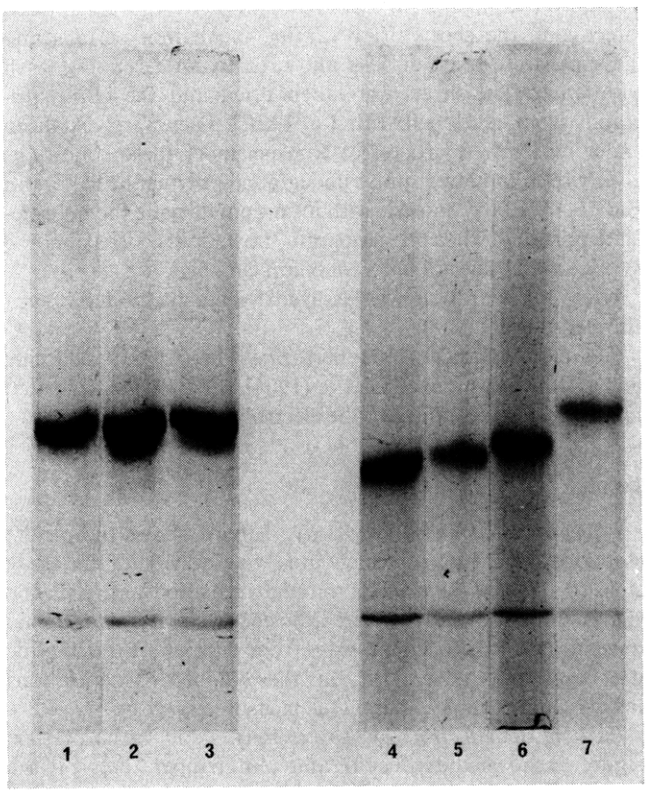


FIGURE 4: Disc gel electropherograms of exoribonuclease treated with urea. Gels 1, 2, and 3 were obtained with enzyme treated at 37° in 1.67 M urea for 0 min (1), 15 min (2), and 30 min (3). Gels 4, 5, 6, and 7 were obtained with enzyme treated at 23° in 1.67 M urea for 0 min (4), 15 min (5), 30 min (6), and 90 min (7). The faint leading band on each gel is an oligopeptide marker. The input to each gel was approximately 20 μg of fraction V enzyme.

TABLE III: Ion Requirements of Untreated and Urea-Treated Enzymes.[a]

| | Act. (cpm) |
|---|---|
| A. Untreated Enzyme | |
| Complete buffer | 898 (100) |
| − $NH_4Cl$ | 164 (18) |
| + 0.01 M EDTA | 7 (0.8) |
| B. Treated enzyme | |
| Complete buffer | 492 (100) |
| − $NH_4Cl$ | 84 (17) |
| + 0.01 M EDTA | 6 (1.2) |

[a] Fraction III enzyme was incubated with 1.67 M urea until 55% activity remained. Control and treated enzyme were then assayed in standard assays modified as indicated. Activity is expressed as counts per minute of [$^{14}$C]AMP released from [$^{14}$C]poly(A) ($4.7 \times 10^4$ cpm/μmole of polymer nucleotide). The numbers in parentheses are the activity (per cent) of that measured in the complete assay system.

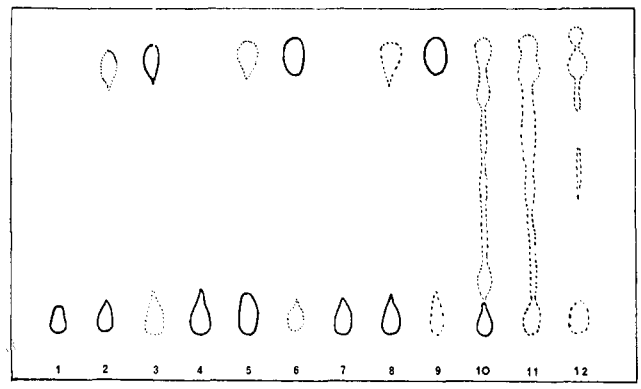


FIGURE 5: Tracing of thin-layer chromatographs of poly(A) and its degradation products. The degradations were carried out by: 1–3 non-urea-treated enzyme, 4–6 enzyme treated at 37° in 1.67 M urea to 50% survival of activity, 7–9 enzyme treated at 37° in 1.67 M urea to 7% survival of activity, 10–12 snake venom phosphodiesterase. Fractions 1, 4, 7, and 10 are zero time, fractions 2, 5, 8, and 11 were incubated to give 30–40% degradation in each case, and fractions 3, 6, 9, and 12 were incubated to give >90% degradation of the poly(A) in each case. The dotted outlines indicate low intensity of uv absorbance.

ever are significantly retarded on the gels when inactivated to the slow portion of their inactivation curves.

*Mode of Enzyme Action Following Urea Treatment.* At several stages of different urea treatments the modified enzyme was assessed for two parameters, first, surviving activity (Figure 1) and, second, the mode of polymer degradation. The latter was tested in two ways. Firstly, untreated and treated enzymes were used to degrade terminally labeled polymers. In all cases the data for the urea-treated enzymes were the same as that previously reported for untreated enzyme (Logan and Singer, 1968) indicating processive degradation. This technique will not however indicate the production of a small number of oligonucleotides nor the accumulation of a relatively stable terminal oligonucleotide such as that found with *E. coli* RNase II (Nossal and Singer, 1968). We have tested for these possibilities by thin-layer chromatography. A processive enzyme produces only mononucleotides and undegraded polymer whereas a random enzyme produces oligonucleotides of various lengths. Figure 5 shows the separation of the degradation products of poly(A) degraded to different extents with untreated enzyme, two different urea treated enzymes, and snake venom phosphodiesterase. The latter enzyme which acts randomly produces a distinct 5′-AMP spot and in addition there is a streak of variable intensity from the origin almost to the 5′-AMP spot. This indicates the production of oligonucleotides of different lengths. In contrast both of the urea-treated enzymes show strictly processive degradation (no oligonucleotides) even when the enzyme is treated so that only 7% activity remains.

The following substrates have been tested with urea-treated enzyme: poly(A), poly(U), poly(C), (pA)nU, (pA)nC, (pA)nG, (pU)nG, and high molecular weight yeast RNA. Under all conditions of temperature, urea concentration, and extent of degradation tested, the enzyme shows no trace of random degradation.

*Ion Requirements of Urea-Treated Enzyme.* Untreated enzyme requires $Mg^{2+}$ and $NH_4^+$ ions for maximum activity. Urea-treated enzyme was tested to see if these requirements remained or are modified. Table III shows that with enzyme, treated at room temperature with 1.67 M urea until 45% of the initial activity was lost, there is no change in the requirement for these ions.

*Enzyme Reactivation.* The removal of urea from a treated enzyme solution does not result in any recovery of enzyme activity. This was tested first by extensive dialysis of an enzyme solution containing 1.67 M urea. The enzyme was treated at 30° for 75 min at which time 23% of the original activity remained. The enzyme solution was then dialyzed against water (1250 × volume) and assayed for activity after different durations of dialysis. After 6-hr sampling there was no increase in activity and in fact a small diminution of activity was found after 3 hr beyond that explainable by dilution of the dialysate.

Urea was also removed from the enzyme solution by chromatography on a Bio Gel P-2 column. In this case enzyme was incubated at 36° for 30 min in 1.67 M urea. The reaction mixture was then chilled to 4° and an aliquot was removed to measure residual activity. The remaining solution was added to a P-2 column (2.5 × 15 cm) and the column was run with 0.02 M Tris·HCl (pH 7.8)–20% glycerol. All eluted fractions containing enzyme activity were pooled and assayed for total activity recovered. The recovery of input activity to the column was 48% which is less than the recovery from a control column testing untreated enzyme. In an identical control column the recovered activity of untreated enzyme was 52%. Thus there is no increase in activity due to the removal of urea. The urea concentration in the pooled fraction from the column was less than $0.3 \times 10^{-3}$ M as assayed by the procedure of Bernt and Bergmeyer (1965).

## Discussion

Extensive treatment of the processive enzyme *L. plantarum* exoribonuclease with urea leads to a loss of activity by the enzyme and a reduction in both the $V_{max}$ and $K_m$. However, there is no concomitant loss of the ability of the residual enzyme to act in a strictly processive manner regardless of the substrate tested. Thus processive degradation appears to depend on factors other than simply the secondary and tertiary structure of the enzyme. The effect of primary structure modifications is currently being studied.

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# *Lactobacillus plantarum* Exoribonuclease. Further Purification and Characterization*

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ABSTRACT: *Lactobacillus plantarum* exoribonuclease, a processive nuclease, has been purified to essential homogeneity. This has been achieved by elution of the enzyme from phosphocellulose columns with RNA and KCl. The purified enzyme gives rise to a single band on disc gel electrophoresis. The molecular weight of the enzyme measured by gel chromatography is $9.0 \times 10^4$. The enzyme apparently degrades RNA in the 3′ to 5′ direction.

Partial purification of the processive nuclease *Lactobacillus plantarum* exoribonuclease has already been published (Logan and Singer, 1968). Further studies on the processive mode of degradation and physical chemistry of the enzyme (Gardonio and Logan, 1971) have however required a more highly purified enzyme. In this paper we report on the further purification of the enzyme to essential homogeneity. Substantial purification has been achieved by the technique of substrate elution of the enzyme from phosphocellulose columns using yeast RNA as substrate. In addition the molecular weight and direction of degradation of the enzyme have been determined.

## Materials and Methods

The methods of maintaining, growing, and harvesting *Lactobacillus plantarum* (ATCC8014) were described previously (Logan and Singer, 1968). Enzyme activity was also assayed as previously described with the exception that 0.25 M Tris (Fisher) was used in place of 0.25 M 2-amino-2-methyl-1,3-propanediol·HCl buffer.

For fraction IV a different initial assay was used.

The RNA which elutes with the enzyme from the phosphocellulose column was used as the enzyme's initial substrate in reaction mixtures supplemented with appropriate amounts of $NH_4Cl$ and $MgCl_2$. Several incubation periods were used (0, 0.5, 1, 3, and 5 min) in each assay to determine at which times linear breakdown occurred. The reaction was stopped by addition of 0.1 ml of carrier RNA solution (Singer and Tolbert, 1965) and the addition of 0.4 ml of cold 95% ethanol. The reaction tubes were chilled to 0° and after 10-min incubation they were then centrifuged at 3500*g* for 10 min. The supernatant (300 μl) was diluted to 1 ml with water and the absorbance at 260 mμ was recorded. The column fractions showing activity were then incubated at 37° in the presence of 1.5 mM $MgCl_2$ and 0.4 M $NH_4Cl$ until the accompanying RNA was degraded. (This degradation often required several hours.) The reaction mixture was then dialysed against water until free of RNA nucleotides and salts and an aliquot of the remaining enzyme was then assayed by the standard assay procedure.

Enzyme fractions I, II, and III were prepared as previously described with the exception of the initial cell lysis step. Frozen cells were thawed and mixed into a thick slurry by the addition of cold 0.02 M Tris·HCl (pH 7.8) (approximately one-fifth volume). This slurry was then frozen in a pressure cell and the cells were broken in a modified Hughes press. The lysate was suspended in twice its volume of the above buffer and the purification was carried out as described by Logan and Singer (1968).

$(pA)_3$* poly(A) in which the 5′-terminal trinucleotide is labeled with $^3H$ was prepared by Dr. N. G. Nossal and was generously supplied by Dr. M. F. Singer. Degradation of total polymer material and terminally labeled material was measured as described by Nossal and Singer (1968).

Protein was determined by the method of Lowry *et al.* (1951).

Polyacrylamide disc gel electrophoresis was performed by the method of Clarke (1964) as modified by Tobe and Loughton (1969). All samples were run on 7.5% polyacrylamide gels with a constant current of 3 mA/gel tube.

Sephadex G-100 (Pharmacia), DEAE (DE-32, Whatman), and phosphocellulose (P-11, Whatman) were prepared according to the manufacturer's instructions.

Molecular weight was determined from the position of elution of the enzyme from a Sephadex G-100 column (2.5 × 87 cm) as described by Andrews (1964; Andrews and Folley,

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