Proc. Nat. Acad. Sci. USA
Vol. 69, No. 10, pp. 3009–3013, October 1972

Purification and Properties of Bacteriophage T4-Induced RNA Ligase*

(poly(A)/circle formation/RNA modification/RNA synthesis)

ROBERT SILBER†, V. G. MALATHI, AND JERARD HURWITZ

Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, New York 10461; and
† Department of Medicine, NYU School of Medicine, New York, N.Y. 10016

Communicated by Severo Ochoa, August 7, 1972

ABSTRACT **An enzyme, purified 300-fold from *Escherichia coli* infected with bacteriophage T4, catalyzes the conversion of 5′-termini of polyribonucleotides to internal phosphodiester bonds. The reaction requires ATP and Mg^{++}. For every 5′-^{32}P terminus rendered resistant to alkaline phosphatase, an equal amount of AMP and PPi are formed. Various polyribonucleotides are substrates in the reaction; to date, the best substrate is [5′-^{32}P]polyriboadenylate. With the latter substrate, no evidence of intermolecular reaction was obtained. However, the 5′-^{32}P termini of poly(A) rendered resistant to alkaline phosphatase are also resistant to attack by RNase II, polynucleotide phosphorylase, and low concentrations of venom phosphodiesterase. Since the product formed with poly(A) lacks 3′-hydroxyl ends, as measured with these exonucleases, the enzyme appears to convert linear molecules of polyriboadenylate to a circular form by the intramolecular covalent linkage of the 5′-phosphate end to the 3′-hydroxyl terminus.**

DNA ligase from bacteriophage T4 and *Escherichia coli* catalyzes the esterification of single-stranded breaks within a DNA duplex (1–4). The T4 enzyme also catalyzes the joining of ribo-oligoadenylates when hydrogen bonded to polydeoxythymidylate, and of deoxy-oligothymidylates when hydrogen bonded to poly(A), but has not been shown to mediate the joining of polyribonucleotides in the absence of complementary DNA chains (5). During studies on DNA ligase, we noted that crude fractions of T4-infected *E. coli*, in addition to sealing single-stranded breaks in DNA, reacted with various 5′-^{32}P-labeled polyribonucleotides to render the 5′-phosphate terminus resistant to the action of bacterial alkaline phosphatase. We describe the partial purification and some properties of an enzyme that catalyzes the formation of an internal phosphodiester bond at the 5′-phosphate terminus of polyribonucleotides. Evidence is presented that this enzyme is distinct from DNA ligase, and functions as an RNA ligase.

MATERIALS AND METHODS

Materials. Unlabeled nucleotides were purchased from Schwarz-Mann. [γ-^{32}P]ATP was supplied by Amersham or New England Nuclear Corp. Polyadenylic [poly(A)], polyinosinic [poly(I)], polyuridylic [poly(U)], polycytidylic [poly(C)], and polydeoxyadenylic [poly(dA)] acids were obtained from Miles Chemical Corp. The following enzymes were supplied by Worthington Biochemical Corp.: bacterial alkaline phosphatase, pancreatic RNase and DNase, micrococcal nuclease, and venom phosphodiesterase. Ribonuclease II was a gift from Dr. Randolph Holmes and polynucleotide phosphorylase was a gift from Dr. Maxine Singer.

Preparation of Substrates. The 5′-hydroxyl-terminated polyribonucleotides were prepared by partial digestion (30% conversion to acid-soluble material) of long-chain polyribonucleotides with micrococcal nuclease, followed by treatment with alkaline phosphatase to remove 3′-PO_4 groups. The 5′ terminus was labeled with ^{32}P with polynucleotide kinase, which was free of detectable RNase activity. Polynucleotide kinase was purified from T4-infected *E. coli* D-110 by a modification (6) of the method of Richardson (7). To improve the removal of RNase activity remaining in the DEAE-cellulose eluate (fraction IV), we substituted a 0.05–0.3 M KCl gradient for the step-wise elution used by others during phosphocellulose chromatography. The eluate from the phosphocellulose column was free of detectable RNase, as judged by the acid solubilization of [^{3}H]poly(A) or [^{3}H]poly(U). It was also free of endonuclease, as judged by a lack of effect on the sedimentation velocity of these substrates in a sucrose gradient.

Preparation of [^{32}P]Poly(A). The reaction mixture (2.2 ml) contained the following: 1000 A_{260} units of poly(A), 50 μmol of glycine–NaOH buffer (pH 9.2), 5 μmol of $CaCl_2$, and 5 μg of micrococcal nuclease. After incubation at 38° for 10 min, 150 μg of bacterial alkaline phosphatase was added and the incubation was continued at 80° for 20 min. An additional 150 μg of this phosphatase was added, and the mixture was incubated at 80° for another 20 min. The reaction was terminated by the addition of 3 ml of aqueous distilled phenol. The phenol layer was extracted twice with 2-ml portions of 0.05 M NaCl. The aqueous layers were combined, extracted four times with ether, and dialyzed for 15 hr against 1 liter of 0.2 M NaCl, followed by an overnight dialysis against 0.1 M NaCl. The average chain length of the product was measured with polynucleotide kinase; it ranged from 30 to 75 nucleotides. Similar conditions were used for poly(I) (340 A_{260} units), except for the addition of 10 μg of micrococcal nuclease. With poly(C) and poly(U), the concentration of micrococcal nuclease was lowered to 2.5 μg.

^{32}P Labeling of 5′-OH Terminated Polynucleotide. The 5′-hydroxyl terminated polynucleotides were labeled with ^{32}P as follows: the reaction mixture contained 0.1 M Tris·HCl (pH 7.5), 1 mM dithiothreitol, 10 mM $MgCl_2$, 30 μg/ml bovine-serum albumin, 30 A_{260} units/ml 5′-hydroxy-terminated ribonucleotide, 0.2 mM [γ-^{32}P]ATP, and 150 units/ml polynucleotide kinase. The mixture was incubated at 38° for 15 min, then

* This is paper I in a series, "Reaction at Ends of RNA Chains."

TABLE 1. *Purification of RNA ligase*

Fraction	Total units	Specific activity (units/mg protein)
Crude extract	8320	3.6
Ammonium sulfate I*	2640	9.1
DEAE-cellulose	1540	49
Ammonium sulfate II	1485	66
Sephadex G-75	1281	183
DEAE-Sephadex	660	1150

* Assay of RNA ligase activity in streptomycin supernatant fractions preceding this step were unreliable. For this reason data on the streptomycin fraction are not given.

an additional 50 units of polynucleotide kinase was added. Quantitative phosphorylation usually occurred after a total of 30 min of incubation. The reaction was stopped with phenol treatment as described above, and the excess [γ-^{32}P]ATP was removed by dialysis or filtration through a column of Sephadex G-25.

RNA Ligase Assay was routinely performed as follows: a reaction mixture (0.1 ml) containing 7 nmol of [5′-^{32}P]-poly(A) (0.2 nmol of 5′-^{32}P termini), 5 μmol of Tris·HCl (pH 7.5), 1.0 μmol of $MgCl_2$, 10 nmol of ATP, 5 μg of albumin, 0.13 μmol of dithiothreitol, and enzyme were incubated for 30 min at 38°. The ligase reaction was terminated by heating at 100° for 2 min, after which 0.1 ml H_2O and 1.5 units of alkaline phosphatase were added, and the mixture was incubated for 35 min at 80°. The phosphatase reaction was terminated by the addition of 5 ml of 5% Cl_3CCOOH and 0.1 ml of 0.1 M sodium pyrophosphate; acid-insoluble material was collected on GF/C filter paper and its radioactivity was determined. One unit of enzyme activity is defined as 1 nmol of 5′-^{32}P terminus rendered resistant to phosphatase in 30 min.

Purification of RNA Ligase. E. coli D-110 (30 g), infected with T4 *amt*B5 for 60 min at 37°, were collected and suspended in 75 ml of a solution containing 20 mM Tris·HCl (pH 7.5)–0.5 mM 2-mercaptoethanol–0.1 mM EDTA (buffer A). The cells were disrupted by sonication for 10 min with a Branson sonifier. The suspension was centrifuged at 105,000 × *g* for 30 min; the supernatant fluid (75 ml, crude extract) was treated with 30 ml of 5% streptomycin sulfate, and the mixture was centrifuged at 10,000 × *g* for 15 min. The supernatant fluid (100 ml) was treated with 29.1 g of solid ammonium sulfate (50% saturation); after 30 min at 0°, the pellet was collected, dissolved in 10 ml of buffer A (ammonium sulfate I), and dialyzed against 500 ml of solution containing 0.1 M KCl–0.02 M Tris·HCl (pH 7.5)–1 mM 2-mercaptoethanol–0.1 mM EDTA (buffer B) for about 12 hr. The dialyzed fraction was applied to a DEAE-cellulose (DE-52) column (3 × 12 cm) previously equilibrated with buffer B. The loaded column was washed successively with 75 ml of buffer B, 150 ml of buffer B containing 0.25 M KCl, and 100 ml of buffer B containing 0.5 M KCl. The enzyme activity eluted in two peaks, the first appearing with 0.25 M KCl and the second appearing with 0.5 M KCl. RNase activity was determined in individual fractions. Those enzyme fractions from both peaks, which were relatively low in ribonuclease, were pooled and precipitated with solid ammonium sulfate (70% saturation) and dissolved in 2.5 ml of buffer B (AS II).

The AS II fraction was applied to a column of Sephadex G-75 (60 cm × 1.5 cm) previously equilibrated with buffer B. The column was washed with buffer B and 1.2-ml fractions were collected. The enzyme was included in the gel and appeared in fractions 19–25 after about half of the RNase activity had eluted. Since the first third of the fractions containing enzyme usually contained most of the RNase activity, they were discarded. The remainder of the fractions was pooled and dialyzed against buffer B, whose pH had been lowered to 6.5 with 0.05 M cacodylate buffer, pH 6.5 (Buffer B–cacodylate). The enzyme was then applied to a 10 × 1-cm column of DEAE-Sephadex that had been washed with Buffer B–cacodylate. The column was washed with 20 ml of this buffer and eluted with a 0.1–0.5 M gradient of KCl in 50 ml Buffer B–cacodylate. RNase activity still present in the Sephadex fraction eluted at 0.37 M KCl, while RNA ligase appeared at about 0.44 M KCl. The last third of the fractions containing RNA ligase was usually free of ribonuclease activity.

This procedure resulted in a 300-fold purification, with a recovery of 8% (Table 1). A recovery of 30% is obtained if the activity in the RNase-rich fractions, which were intentionally discarded, is included in the calculation. The ammonium sulfate II fraction is stable for at least 2 weeks stored at 0°. The DEAE-Sephadex eluate lost about 30% of its activity in 24 hr. The remaining activity was stable for 5 days at 0°, but then declined so that after 12 days all activity

TABLE 2. *Requirements of RNA ligase system*

Expt.	Additions	Activity resistant to alkaline phosphatase (pmol/30 min)
A	1. tRNA + ATP + Mg^{++}	5.91
	2. Omit ATP or Mg^{++}	<0.1
	3. (1) + RNase (5 μg)	<0.1
	4. (1) + DNase (5 μg)	5.70
B	1. Poly(A) + ATP + Mg^{++}	47.2
	2. Omit ATP or Mg^{++}	<0.1
	3. (1) + RNase (5 μg)	39.4
	4. (1) + DNase (5 μg)	46.4
	5. (1) + Micrococcal nuclease (1 μg) + Ca^{++}	2.1
	6. (1) + 0.1 mM *p*-hydroxybenzoate	<0.1
	7. (1) + 1 mM Pi	50.0
	8. (1) + 0.1 mM PPi	<0.1

All reaction mixtures (0.1 ml) contained the following: 5 μmol of Tris·HCl pH 7.5, 0.5 μmol of $MgCl_2$, 5 nmol of dithiothreitol, 5 nmol of ATP, 3 μg of bovine-serum albumin. The substrate in Exp. A was [5′-^{32}P]tRNA (*E. coli*, 18 nmol, containing 0.38 nmol of 5′-^{32}P termini, 425 cpm/pmol) and in Exp. B, poly(A) (14 nmol, containing 0.44 nmol of ^{32}P, 280 cpm/pmol). In Exp. B, where Pi and PPi were used, the reaction was terminated by the addition of trichloroacetic acid, and the precipitate was washed with 1% Cl_3CCOOH. The precipitate was neutralized with dilute NH_4OH, adjusted to pH 8.0 with Tris·HCl, and incubated with 1 unit of alkaline phosphatase for 35 min at 80°.

was lost. We have been unsuccessful in stabilizing the enzyme preparation at this stage of purity. RNA ligase activity was separated from DNA ligase by DEAE-cellulose; all detectable DNA ligase activity eluted before the RNA ligase. The DEAE-Sephadex fraction was free of poly(A) polymerase, RNA polymerase, polynucleotide phosphorylase, and ribonucleases III and H. It still contained pyrophosphatase activity.

RESULTS

Effect of Phage Infection. The formation of an alkaline phosphatase-resistant product catalyzed by crude extracts of bacteriophage-infected *E. coli* D110 was examined as a function of time after phage infection. After infection by T2, T4, and T6 detectable increases in activity were evident by 3 min; the presence of chloramphenicol prevented this increase. Infection with T4 *am*H39X, a mutant that fails to induce an active DNA ligase (8), induced RNA ligase activity similar to the level observed with ordinary T4 infection, while the DNA ligase activity observed after infection with the mutant was less than 5% that noted after T4 infection. Infection with the following phages did not result in elevation of RNA ligase activity: T1, T3, T5, T7, and RNA-phage Qβ. The induction of phage λ did not result in a detectable increase in activity. Current studies in mouse cell line JLSV-9 indicate the presence of an activity that converts [5′-^{32}P]poly(A) to an alkaline phosphatase-resistant form.

Requirements of Purified RNA Ligase. The enzyme possesses a broad pH optimum between 7.5 and 8.2 in Tris·HCl buffer. The conversion of 5′-phosphate ends in t-RNA or poly(A) to a form resistant to alkaline phosphatase is dependent on the addition of ATP and Mg^{++} (Table 2). The K_m for ATP was 0.2 μM and that for Mg was 400 μM. Mg^{++} concentrations above 10 mM had a marked inhibitory effect on the reaction. The ATP requirement was not satisfied by GTP, CTP, or UTP, nor was there any effect of AMP or ADP on the reaction. When ATP was replaced by dATP, the reaction proceeded at 20% the rate observed with ATP. Complete loss of activity was noted with tRNA in the presence of RNase, but the reaction was insensitive to DNase. The activity with poly(A), as expected, is resistant to DNase, almost completely resistant to RNase, and sensitive to micrococcal nuclease. No activity was detected in the presence of *p*-hydroxymercuribenzoate (1 mM) or PPi, while Pi had no effect on the reaction. A molecular weight of 52,000 ± 5,000 was determined for RNA ligase by gel filtration (9) with Sephadex G-100.

TABLE 3. *Comparison of various [5′-^{32}P]polyribonucleotides*

Additions	5′-^{32}P-terminus added (pmol)	Alkaline phosphatase resistant (pmol/30 min)
tRNA (34.7)	167	3.9
Poly(A) (1.4)	50	7.8
Poly(I) (21.5)	185	2.1
Poly(C) (30)	270	0.1
Poly(U) (23)	185	0.07
Poly(dA) (7)	70	<0.01

Reaction mixtures (0.1 ml) contained 5 μmol of Tris·HCl (pH 7.5), 0.5 μmol of Mg^{++}, 5 nmol of ATP, 5 nmol of dithiothreitol, 0.6 μg of protein (Sephadex fraction), and polynucleotides as indicated. Numbers in parenthesis indicate the nmol of nucleotide added.

TABLE 4. *Characterization of poly(A) and poly(I) products*

Material hydrolyzed	% Product recovered as pAp	Ap	pIp	Ip
1. [5′-^{32}P]poly(A) (1.54 × 10^4 cpm)	>95	<5	<5	<5
2. Poly(A); alkaline phosphatase resistant (8.3 × 10^3 cpm)	4	93	<2	<2
3. [5′-^{32}P]Poly(I) (1.12 × 10^4 cpm)	<5	<5	<95	>5
4. Poly(I); alkaline phosphatase resistant (5.1 × 10^3 cpm)	<3	<3	3	92

Reaction products were prepared as described in Table 2. The substrate, poly(A) or poly(I), and their respective ligase products were hydrolyzed in 0.5 M KOH at 38° for 18 hr. The KOH was cautiously neutralized with Dowex 50 H^+, and aliquots were spotted on Whatman 3 MM paper and electrophoresed for 2.5 hr at 5000 V. Markers corresponding to the indicated nucleotides were included. The paper was cut into 1-cm strips and counted in toluene–PPO–POPOP in a Packard scintillation counter. Numbers in parentheses indicate the amount of ^{32}P used in each electropherogram.

Activity with Other Polynucleotides and Effect of Chain Length. All 5′-^{32}P-terminated polyribonucleotides tested were active. The polypurines (A and I) were most effective (Table 3), while polypyrimidines (C and U), in general, are less effective than the polypurines; [5′-^{32}P]poly(C) and poly(U) were less than 25 and 10% as effective as poly(A), respectively, while poly(dA) was completely inactive as a substrate. In general, activity with the polypyrimidines as substrates diminished with storage of the enzyme. The length of the polyribonucleotide substrate influences the reaction velocity; maximal rates were obtained with poly(A) of an average chain length between 34–40. This rate was about twice that observed with substrates with an average chain length of 70–100 nucleotides, and almost 10 times that observed with poly(A) of average chain length 300. The reaction velocity was also dependent on the concentration of poly(A). The rate was directly proportional to the substrate concentration from 3 to 14 nmol of nucleotide. Since the reaction proceeds best with poly(A), this polymer was used for most of the studies on product characterization.

Stoichiometry of the Reaction. The products formed from ATP in reaction mixtures with poly(A) are AMP and PPi, in amounts equivalent to ends rendered resistant to alkaline phosphatase (10). Purified enzyme preparations also catalyze the release of PPi from ATP in the absence of added poly(A). The nature of this activity is unknown. When [^{3}H]ATP was used in the reaction, the incorporation of label into acid-soluble product was 5% (or less) of the termini rendered alkaline phosphatase resistant.

Evidence of Internal Phosphodiester Bond Formation. The alkaline phosphatase-resistant product is converted to an acid-soluble form after hydrolysis with 0.5 M KOH for 15 hr. The ^{32}P in the hydrolysate is Norit adsorbable, but rendered nonadsorbable to charcoal after treatment with alkaline phosphatase. When alkaline phosphatase-resistant products prepared from reactions with [5′-^{32}P]poly(A) were isolated

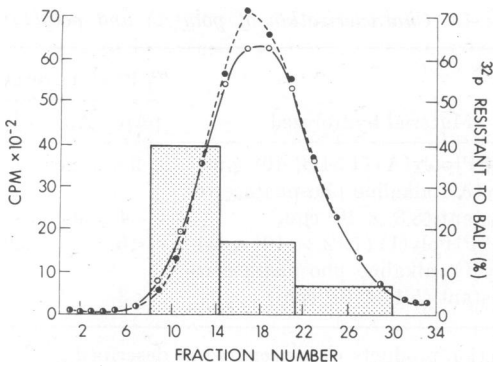

FIG. 1. Sedimentation pattern of [5′-^{32}P]poly(A) and [5′-^{32}P]poly(A) RNA ligase product. The reaction mixture (0.1 ml) contained 4 nmol of [^{32}P]poly(A), 0.5 μmol of $MgCl_2$, 10 nmol of ATP, 5 μmol of Tris·HCl (pH 7.5), and 3.2 units of enzyme. After 90 min at 30°, about 70% of the acid-insoluble ^{32}P termini were resistant to alkaline phosphatase. Reactions were terminated by the addition of 0.04 ml of a solution containing 5.5 M HCHO, 1 M sodium phosphate buffer (pH 7.0), and 10% sodium dodecyl sulfate, and the mixture was heated at 65° for 10 min. The entire solution was added to 5.2 ml of a 5–25% sodium dodecyl sulfate–sodium phosphate–HCHO sucrose gradient, which was centrifuged at 50,000 rpm for 36 hr at 20° in an SW65 rotor. Fractions were collected and aliquots counted in Bray's scintillation fluid. Enzyme-treated sample (●——●); Control (○——○). Phosphatase-resistant material (*shaded area*).

and hydrolyzed with 0.5 M KOH (Table 4), the ^{32}P was recovered in 2′(3′)-AMP; alkaline hydrolysis of the starting substrate, [5′-^{32}P]poly(A), yielded labeled 2′(3′),5′-ADP. These results indicate that the 5′-^{32}P terminus has been converted to an internal phosphodiester bond during the enzymatic reaction. When similar experiments were done with [5′-^{32}P]poly(I) as substrate, the radioactivity was recovered as 2′(3′)-IMP; the recovery of the radioactivity in this experiment as 2′(3′)-IMP and not as 2′(3′)-AMP indicates that addition of AMP residues at the 5′-phosphate terminus does not occur.

Properties of RNA Ligase Product. The sedimentation properties of the poly(A) product formed with RNA ligase containing ^{32}P resistant to alkaline phosphatase was compared with [5′-^{32}P]poly(A) from a reaction mixture containing heat-inactivated enzyme (Fig. 1). No difference was noted between the sedimentation of the two preparations. However, a marked skewing in distribution of the alkaline phosphatase-resistant product was observed; significantly more phosphatase-resistant material was found in the lower than in the upper part of the gradient. Since the poly(A) preparation was not fully homogeneous for a given chain length, the preponderance of alkaline phosphatase-resistant material at the lower part of the gradient may reflect more efficient use of the longer poly(A) chains as substrate for RNA ligase. This finding could also represent a slight increase in sedimentation velocity of the product as compared to the substrate, but recentrifugation of material isolated from the bottom of the gradient failed to support this hypothesis. When reaction mixtures were subjected to polyacrylamide gel electrophoresis, no difference in migration was found between ligase-treated and control samples. Gel filtration through Sephadex G-50 also revealed a similar pattern for enzyme-treated and control mixtures. The ultracentrifugation, electrophoresis, and gel filtration observations effectively rule out an intermolecular reaction between [5′-^{32}P]poly(A) chains, and suggest the generation of circular molecules by an intramolecular reaction between the 5′-phosphate and 3′-hydroxyl ends of the same oligomer.

Evidence for Formation of Circles. The intramolecular reaction postulated to explain the above results should lead to the disappearance of 3′-hydroxyl ends. This possibility was examined by the use of exonucleases that require a 3′-terminus for hydrolysis. Three enzymes were used for this purpose: venom phosphodiesterase, RNase II, and polynucleotide phosphorylase. The RNA ligase product was insusceptible to degradation by venom phosphodiesterase under conditions carefully adjusted with respect to time of incubation and enzyme concentration (10). Incubation for longer time or with higher concentration of this enzyme led to degradation of the product, presumably due to endonuclease contamination of the enzyme preparation. There was excellent agree-

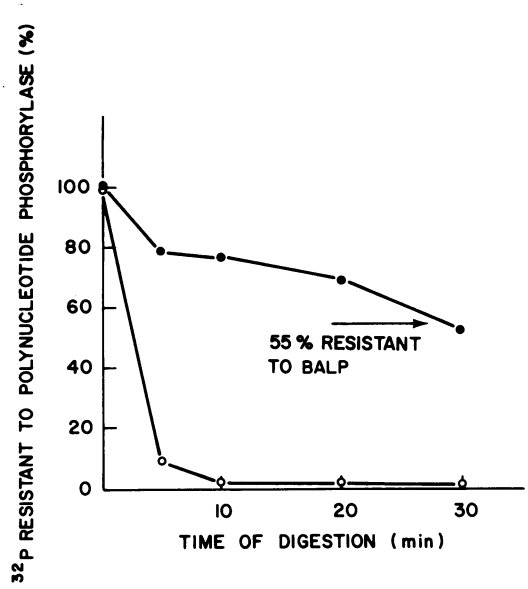

FIG. 2. Effect of RNA ligase on the susceptibility of poly(A) to digestion by polynucleotide phosphorylase. Preparations of poly(A) were obtained from fractions 2–8 (out of 20) of a sucrose gradient containing poly(A) of average chain length 40 after treatment with RNA ligase in a standard reaction medium of total volume 0.3 ml; a control reaction mixture was prepared that lacked enzyme. The conditions used were as described for Fig. 1, except that detergent and formaldehyde were omitted from the gradient and the material was centrifuged for 36 hr at 48,000 rpm in an SW 50.1 rotor. Sucrose was removed by dialysis against demineralized water and the poly(A) was concentrated. For treatment with polynucleotide phosphorylase, the reaction mixture (0.1 ml) contained: 10 nmol of Tris·HCl (pH 8.3), 0.5 nmol of $MgCl_2$, 0.1 nmol of EDTA, 1 μmol of K_2HPO_4, 20 μg of BSA, [5′-^{32}P]poly(A) (2–10 pmol of ends), and 0.014 unit of polynucleotide phosphorylase. The reaction was terminated at the indicated times by the addition of 5 ml of 5% Cl_3CCOOH and 0.1 ml of 0.1 M sodium pyrophosphate. The mixture was collected on GF/C filters and counted. The curve represented by the symbols (●——●) indicates the resistance to polynucleotide phosphorylase of poly(A) subjected to enzyme treatment; the curve (○——○) indicates resistance to polynucleotide phosphorylase of poly(A) not exposed to RNA ligase.

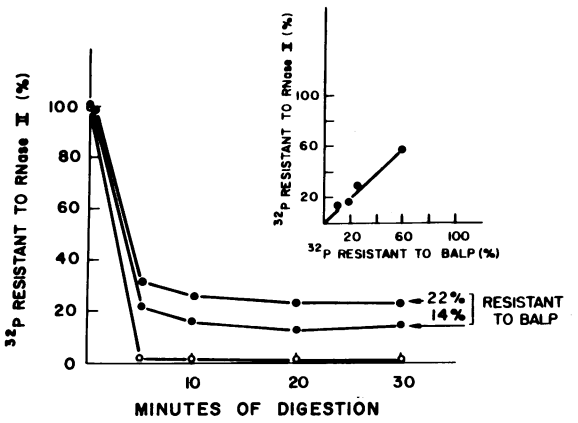

FIG. 3. Effect of RNA ligase on the susceptibility of poly(A) to digestion by RNase II. Preparations of poly(A) were obtained as described in Fig. 2. For treatment with RNase II the reaction mixture (0.1 ml) contained: 10 μmol of Tris·HCl, 10 μmol of KCl, 1.5 μmol of $MgCl_2$, 100 μg of bovine-serum albumin, and [5′-^{32}P]poly(A) 2–10 pmol of ends as in Fig. 2. Resistance to RNase II of enzyme-treated material (●——●) and control (○——○) obtained in two experiments using the same RNA ligase preparation are shown. The relationship between resistance to alkaline phosphatase and RNase II obtained with the product formed with four different preparations of RNA ligase is shown in the *insert*.

ment between the percentage of total ^{32}P not susceptible to venom phosphodiesterase and the amount resistant to alkaline phosphatase action. Similar observations were made with polynucleotide phosphorylase and RNase II (Figs. 2 and 3). In each case resistance of the product to exonuclease degradation was observed and good correlation was noted with alkaline phosphatase resistance. Sequential treatment with either RNase II or polynucleotide phosphorylase, followed by alkaline phosphatase, revealed that prior treatment with either exonuclease had increased the alkaline phosphatase resistance of the acid-insoluble material to 100%. This finding can be interpreted as reflecting degradation of noncircular molecules by the exonuclease to an acid-soluble form, with sparing of the alkaline phosphatase-resistant material; it provides evidence that the alkaline phosphatase resistance is present on the same population of molecules as the exonuclease resistance. After treatment with 7–28 milliunits of micrococcal nuclease (amounts that did not produce acid-soluble products) poly(A) product, isolated as described in Fig. 1 and pretreated with alkaline phosphatase, became sensitive to degradation by RNase II. The effect of the micrococcal endonuclease, which makes ends available for the action to the exonucleolytic action of RNase II, further suggests that the RNA ligase product formed with poly(A) is circular.

DISCUSSION

The data reported here indicate that the RNA ligase action in *E. coli* infected with T-even phages is not due to DNA ligase. The evidence supporting this conclusion can be summarized as follows: (*i*) only T-even phages induce RNA ligase, while T1, T3, and T7 are capable of inducing DNA ligase; (*ii*) highly purified DNA ligase from *E. coli* or phage-infected *E. coli* does not substitute for the RNA ligase reaction with poly(A) or tRNA; (*iii*) the purification of both activities has yielded preparations of RNA ligase free of DNA ligase and DNA ligase free of RNA ligase; (*iv*) infection with the T4 mutant T4 *amH*39X induced the RNA, but not the DNA, ligase activity.

The evidence presented above indicates that the reaction with [5′-^{32}P]poly(A) generates a circular product. Relatively short chains of poly(A) are required for optimal activity. It may be that intramolecular stacking of purine polymers offers conditions where the 3′-hydroxyl and 5′-phosphate termini are properly aligned for joining. In contrast to the action of DNA ligase, duplex structures are not required and may actually be inhibitory in the RNA ligase reaction (10); the reason for this is unclear.

The function of the RNA ligase *in vivo* is not known, but several possible roles can be suggested. The formation of a circular form of RNA has been described for the replicating form of encephalomyocarditis virus (11). Host cell repair of double-stranded RNA has also been reported for this virus (12). The possibility of a role in repair of RNA, or in the prevention of exonuclease degradation of RNA by formation of a circular polyribonucleotide, are possible biologic functions that warrant further study. The availability of an enzyme potentially capable of joining RNA molecules raises the possibility of recombination in RNA genetic systems.

Work of R. S. was done during the tenure of Guggenheim Fellowship. V. G. M. was a Trainee, United States Public Health Service Grant, AM 5282. This research was supported by the National Institutes of Health, National Science Foundation, and the American Cancer Society.

1. Gellert, M. (1967) *Proc. Nat. Acad. Sci. USA* **57**, 148–155.
2. Weiss, B. & Richardson, C. C. (1967) *Proc. Nat. Acad. Sci. USA* **57**, 1021–1028.
3. Olivera, B. M. & Lehman, I. R. (1967) *Proc. Nat. Acad. Sci. USA* **57**, 1426–1433.
4. Gefter, M. L., Becker, A. & Hurwitz, J. (1968) *Proc. Nat. Acad. Sci. USA* **58**, 240–247.
5. Kleppe, K., van de Sande, J. H. & Khorana, H. G. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 68–73.
6. Hanggi, U. J., Streeck, R. E., Voigt, H. P. & Zachau, H. G. (1967) *Biochim. Biophys. Acta* **217**, 278–293.
7. Richardson, C. C. (1965) *Proc. Nat. Acad. Sci. USA* **54**, 158–165.
8. Fareed, G. C. & Richardson, C. C. (1967) *Proc. Nat. Acad. Sci. USA* **58**, 665–672.
9. Andrews, P. (1964) *Biochem. J.* **91**, 222–233.
10. Leis, J., Silber, R., Malathi, V. G. & Hurwitz, J. (1972) in *Mechanisms and Prospects of Genetic Exchange*, in press.
11. Agol, V. I., Drygin, Y. F., Romanova, L. I. & Bogdanov, A. A. (1970) *FEBS Lett.* **8**, 13–16.
12. Zavadova, Z. (1971) *Nature* **233**, 123.