Isolation and Characterization of an Endonuclease from *Escherichia coli* Specific for Ribonucleic Acid in Ribonucleic Acid-Deoxyribonucleic Acid Hybrid Structures*

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**SUMMARY**

A ribonuclease which degrades RNA specifically in RNA-DNA hybrid structures has been purified 1500-fold from *Escherichia coli* B and 200-fold from *E. coli* strain D110 pol A, endo 1+, thy-). For maximal activity, the enzyme requires Mg$_2^+$ and the presence of a sulfhydryl reagent. The enzyme is capable of digesting more than 95% of the RNA in RNA-DNA hybrids to acid-soluble products. The products are oligonucleotides with 5'-phosphate and 3'-hydroxyl termini. The enzyme acts as an endonuclease and does not require free 3' or 5' termini for activity. However, it cannot cleave the phosphodiester bond covalently linking ribonucleotides to DNA. With circular single stranded DNA templates deoxynucleotide incorporation catalyzed by DNA polymerase II is dependent on RNA synthesis. This reaction can be stimulated by RNase H.

During these studies an additional ribonuclease H activity was detected in *E. coli* B and purified 160-fold. Unlike RNase H, this activity is N-ethylmaleimide-resistant and sensitive to antiserum prepared against DNA polymerase I and attacks [3'-H, 5'-32P]poly(A) - poly(dT) in the 5' → 3' direction.

A ribonuclease activity which specifically degrades RNA in RNA-DNA hybrid structures was first described by Hausen and Stein (1) in calf thymus. This enzyme, called RNase H, catalyzed the hydrolysis of [3H]poly(U) - poly(dA) but not [3H]poly(U) - poly(A) or ribosomal RNA. In addition to homopolymeric RNA-DNA hybrids, RNA-DNA hybrids generated by DNA-dependent RNA polymerase were degraded before, but not after heat denaturation. RNase H activity appears to be ubiquitous; this activity has been detected in human KB cells (2), and in purified preparations of oncornaviral RNA dependent DNA polymerase (3-5). Recently, an enzyme capable of degrading RNA in RNA-DNA hybrids was isolated from *Escherichia coli* which was reported active at neutral pH in the presence of Mn$_2^+$ (6). In addition, RNase III preparations showed activity on RNA-DNA hybrids (7, 8).

The present interest in RNase H of *E. coli* stems from the observation of Brutlag et al. (9) that DNA replication of M13 phage is dependent, in vivo, on RNA synthesis beyond what is used as messenger RNA. Furthermore, Wickner et al. (10) have shown that RNA, presumably in RNA-M13-DNA hybrid structures, provides 3'-hydroxyl primer strands for DNA synthesis, as shown by the covalent attachment of DNA to its 5' end to RNA. The presence of a 3'-hydroxyl priming end in a duplex structure is required by all known DNA polymerases, Pol I (11), Pol II (12), and Pol III (13). Thus, if RNA priming is required for normal DNA replication then a mechanism must be available to remove the RNA and allow joining of synthesized DNA pieces by DNA ligase. We have found two enzymes capable of performing this role in *E. coli*. One is DNA polymerase I, which degrades RNA-DNA hybrids in the 5' → 3' direction, while the other, an N-ethylmaleimide-sensitive endonuclease with RNase H specificity, is found in both wild type and pol A1 strains of *E. coli*. The purpose of this communication is to describe the isolation and properties of these activities.

**EXPERIMENTAL PROCEDURE**

**Materials**

Synthetic radioactive homopolymers were obtained from Schwarz-Mann BioResearch. The specific activities of these polymers were [3H]poly(A), 51 mCi per mmole; [3H]poly(I), 19 mCi per mmole; [3H]poly(C), 8 mCi per mmole; [3H]poly(U), 8.6 mCi per mmole. Unlabeled deoxyhomopolymers were from General Biochemicals while labeled nucleoside triphosphates were from New England Nuclear Corp.

Highly purified RNA polymerase of *E. coli* was obtained from Mrs. Sue Wickner and Dr. Lyn Yarbrough of this department. Dr. Yarbrough also carried out the purification steps from *E. coli* B described below up to the 5m agarose step, when RNA polymerase was separated from RNase H and purified further. DNA
polymerase I (Jovin, Fraction VII) (14) was purified by Dr. Barry Ginsberg of this department and DNA polymerase II was purified as described by Wiedner et al. (15). E. coli unwinding protein was purified by the method of Sigal et al. (16). Purified preparations of E. coli unwinding protein were assayed by their ability to render single stranded DNA resistant to the action of nuclease SI (17). Reaction mixtures (0.1 ml) contained 1 µM denatured T7 [3H]DNA (30 cpm per pmole), 20 mM Tris- HCl buffer, pH 7.5, and varying amounts of unwinding protein. After 10 min at 38°, 0.025 ml of a solution containing 0.8 mM potassium acetate buffer, pH 4.8, 0.8 mM NaCl, 2 mM ZnCl₂ was added, followed by 0.56 unit of nuclease SI (1 unit is equivalent to 1 µmole of acid-soluble nucleotide formed in 30 min at 38°). The mixture was incubated for 30 min at 38°, and acid-insoluble radioactivity measured. One unit of unwinding protein activity is defined as the ability to protect 1 nmole of denatured DNA from digestion by nuclease SI during the 30-min incubation.

**Bacterial Strains**—A thymine-requiring derivative of strain HfrH and phage fd were obtained from Dr. Borries Kemper of this department. E. coli D110 (pol A₃, endo 1, thy⁻) was a gift of Dr. Robb Moses, Baylor College of Medicine. For purification of RNase H, E. coli D110 was grown to an optical density (measured at 650 nm) of 1.0 in nutrient broth supplemented with vitamin B₁ (10 µg per ml) and thymine (10 µg per ml), harvested by centrifugation, and frozen. Large quantities of E. coli B were purchased from General Biochemicals.

**Methods**

**Assay of RNase H Activity**—The assay for RNase H activity was carried out as follows: reaction mixtures (0.05 ml) containing 24 µM [3H]poly(A) (32 cpm per pmole, 400 nucleotides in length), 24 µM poly(dT), 40 mM Tris- HCl, pH 7.7, 4 mM MgCl₂, 1 mM dithioerythritol, 30 µg per ml of bovine serum albumin, 0.002 ml of 100% glycerol, and varying amounts of RNase H were incubated for 20 min at 30°. The reaction was stopped by the addition of 0.1 ml of cold 0.1 M sodium pyrophosphate, 0.05 M of denatured salmon sperm DNA (1 mg per ml), 0.1 M of albumin (10 mg per ml), and 0.3 M of 10% trichloroacetic acid. The acid-insoluble material was removed by centrifugation for 2 min at 40,000 rpm in an International refrigerated centrifuge and the supernatant fluid was counted in 10 ml of scintillation fluid (naphthalene, 95 g; 2,5-diphenyloxazole 7 g; 1,4-bis[2-(5-phenylxazoyl)]benzene, 50 mg; and dioxane, to 1 liter) in a scintillation counter. One unit of activity is defined as the amount of enzyme producing 1 nmole of acid-soluble material in 20 min at 30°. During purification of RNase H from E. coli strain D110, the activity on [3H]poly(I)·poly(dC) hybrids was followed. The assay was as described above, except that 3.8 µM [3H]poly(I)·poly(dC) (10 cpm per pmole) was substituted for the [3H]poly(A) and poly(dT). The decrease in acid-precipitable radioactivity was determined. The radioactive material banding at the hybrid density of 1.787 g per cc was collected and dialyzed overnight against 0.01 M Tris-HCl, pH 7.7.

**Preparation of fd DNA·RNA hybrids with E. coli DNA polymerase—**RNA synthesis was carried out in reaction mixtures (0.52 ml) containing 46 µM fd DNA, 100 units of E. coli RNA polymerase, 40 mM Tris-HCl, pH 7.7, 8.1 mM MgCl₂, 1.9 mM dithioerythritol, 14 µg per ml of bovine serum albumin, 100 mM KCl, 115 µM each of ATP, CTP, GTP, and 54 µM [3H]UTP (2570 cpm per pmole). After incubation for 60 min at 37°, the reaction was stopped by the addition of 5 µMol of EDTA. The [3H]RNA·DNA hybrid product was isolated by equilibrium density gradient centrifugation in CsCl as follows: 700 µMoles of Tris-HCl, pH 7.7, 150 µMoles of KCl, 2 µMoles of EDTA, and 10.25 g of solid CsCl were added to the mixture and water was added to a final volume of 10 ml. The density of the solution was 1.756 g per cc as measured by refractive index. The mixture was centrifuged at 37,000 rpm for 40 hours at 20° in a Spinco No. 40 rotor in polycyilomer tubes. Fractions were collected from a hole pierced in the bottom of the tube, refractive indices measured, and the amount of acid-insoluble radioactivity determined. The radioactive material banding at the hybrid density of 1.787 g per cc was collected and dialyzed overnight against 0.01 M Tris-HCl, pH 7.7.

**Separation on DEAE-cellulose of Products Generated from Poly(A)·[3H]Poly(A)·poly(dT)**—It was treated with excess E. coli RNase H under the assay conditions described above until all of the radioactive material was acid-soluble. The reaction was stopped by the addition of 0.05 M of denatured salmon sperm DNA (1 mg per ml) and 0.3 M of 5% trichloroacetic acid. After centrifugation for 2 min at 4000 rpm, the supernatant containing acid-soluble material was collected, trichloroacetic acid was removed by ether extraction, and the residue was evaporated to dryness in vacuo. Unlabeled poly(A)·poly(dT) was also treated with RNase H in a parallel incubation mixture as described above and added as carrier. The combined degradation products were dissolved in 1 ml of a solution containing 0.02 M Tris-HCl, pH 7.5, and 8 M urea (Buffer A) and applied to a DEAE-cellulose column (0.5 x 110 cm) equilibrated with Buffer A at room temperature. The adsorbed oligonucleotides were eluted with a 300-ml linear NaCl gradient (0 to 0.5 M) in Buffer A at a flow rate of 15 ml per hour. Fractions of 2 ml were collected, and an aliquot of each was counted in 10 ml of Bray's scintillation fluid (20). Absorbance at 260 nm was measured in the continuous flow cell of a Gilford spectrophotometer during elution. The oligonucleotide peaks were pooled separately and the fractions containing (pApA)₄ dialyzed overnight against water. The chromotographic separation was kindly performed by Dr. L. Schulman of this department and all operations were carried out with siliconized glassware to prevent loss of radioactive nucleotides by adsorption.

**RESULTS**

**Preparation of RNase H from E. coli B**—All operations were carried out at 4°. Two hundred grams of E. coli B were ground with 600 g of glass beads in a Waring Blender to a thick paste in 500 ml of a solution containing 5 M NaCl, 0.05 M Tris HCl, pH 8, 0.1 M EDTA, and 0.1 M dithiothreitol (Buffer B). The mixture was separated from glass beads by centrifugation at 5,000 x g for 20 min (crude extract). Nucleic acid was removed by polyethylene glycol-dextran phase partition as described by Albertsson (21) and Alberts (22) as follows. Polyethylene glycol 6000 (Carbowax, Union Carbide), 6.7 g/100 ml of supernatant, and dextran T-500 (Pharmacia), 1.6 g/100 ml of

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supernatant, were added and stirred in the cold until dissolved. Phase separation was effected by centrifugation at 10,000 \times g for 20 min and the polyethylene glycol phase dialyzed overnight against Buffer B without NaCl. The resulting polyethylene glycol extract (1500 ml) was passed through a 300-ml DNA cellulose column (which contained 0.5 mg of denatured calf thymus DNA per ml of packed volume) and washed with 0.15 M NaCl in Buffer B until the eluate had an absorbance at 280 nm of less than 0.08. RNase H activity was eluted with 200 ml of 1.2 M NaCl in Buffer B, along with RNA polymerase, DNA polymerase, and other DNA-binding proteins (22). This fraction was concentrated by precipitation with 1.5 volumes of a saturated ammonium sulfate solution (saturated at room temperature and adjusted to pH 8 with NaOH before use). The pellet was suspended in 8 ml of 0.02 M Tris-HCl, pH 8, 0.05 M NaCl, 0.1 mM dithiothreitol, and 0.1 mM EDTA in 5% glycerol (Buffer C), and dialyzed 2 hours against 500 ml of Buffer C (which was changed three times during the dialysis). The dialysate was passed through a 250-ml 5% agarose column (60 x 2.5 cm) equilibrated in Buffer C, and fractions containing RNase II activity (eluted after 1 column volume) were pooled and dialyzed overnight against 1 liter of a solution containing 0.01 M Tris-HCl, pH 8, 0.01 M 2-mercaptoethanol, 0.1 mM EDTA, and 30% glycerol (Buffer D) (5% agarose fraction). RNA polymerase was eluted in earlier fractions (0.58 column volume) and was used for other studies.

The dialysate 5% agarose fraction (salt concentration by conductivity measurement was 0.02 M NaCl) was applied to a column of DEAE-cellulose (2.1 x 15 cm) equilibrated with Buffer D in 10% glycerol. The column was eluted with a 400-ml linear gradient of NH4Cl (0 to 0.6 M in Buffer D in 10% glycerol); 6-ml fractions were collected and aliquots assayed for RNase II as described above. Two major peaks of RNase H activity were detected, eluting at 0.07 and 0.14 M NH4Cl, respectively (Fig. 1). A third minor peak of activity was detected in the wash fraction and was not further studied. The major peaks were pooled separately and dialyzed against a solution containing 0.02 M potassium phosphate, pH 6.5, 0.01 M 2-mercaptoethanol, 0.1 mM EDTA, and 30% glycerol (Buffer E). The peak eluting at 0.07 M (Peak A) contained 50% of the total RNase H activity applied to the column while the peak eluting at 0.14 M (Peak B) represented 20% of the original activity. These peaks of RNase H activity could be further distinguished by their sensitivity to N-ethylmaleimide. Peak A was inhibited more than 90% by 20 mM N-ethylmaleimide while Peak B was resistant to this concentration of the reagent. No further purification of Peak A was achieved by chromatography on phosphocellulose.

The material in Peak B (4.2 mg of protein) was applied to a column of phosphocellulose (10 x 2.1 cm) previously equilibrated with a solution containing Buffer E in 10% glycerol and the enzyme was eluted with a linear gradient of 200 ml of potassium phosphate, pH 6.5 (0.03 to 0.9 M) in Buffer E. The N-ethylmaleimide-resistant RNase H activity was eluted with 0.13 M potassium phosphate (Fig. 2). DNA polymerase I activity was also eluted at 0.13 M potassium phosphate and the ratio of RNase H activity to polymerase activity was constant (1.5 to 2.7) over the peak of activity (Fig. 2, inset).

A summary of the purification of RNase H from E. coli B is shown in Table I A. The RNase H activity contained in Peak A was purified approximately 2,000-fold over crude extracts with a yield of about 4%. The enzyme activity was stored in a solution containing 0.05 M Tris-HCl, pH 7.7, 0.05 M NaCl, 0.01 M 2-mercaptoethanol, 0.1 mM EDTA, and 50% glycerol at -20° and was stable for more than 2 months. The enzyme was free of detectable DNase activity toward [H]poly[d(A-T)] and did not alter the sedimentation properties of fd [3C]DNA when examined in neutral sucrose gradients. Some RNase H preparations after DEAE-cellulose or phosphocellulose chromatography contained a contaminating RNase activity which attacked poly(U) and poly(C) but not poly(I) or poly(A). This activity required Mg2+, was stable after heating to 65° for 5 min, and was separated from RNase H by chromatography on Sephadex G-100 (Fig. 3). This pyrimidine-specific RNase eluted near the V0 of the Sephadex column, well resolved from the RNase H activity,
TABLE I

Purification of RNase H from Escherichia coli

RNase H was purified as described under "Results." The initial steps of the procedure described for the isolation of RNase H from E. coli B were also used for the purification of RNA polymerase. Proteins were measured by the procedure of Bücher (23).

A. Isolation from E. coli B

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>13,600</td>
<td>430,000</td>
<td>32 (100%)</td>
<td></td>
</tr>
<tr>
<td>Polyethylene glycol extract</td>
<td>4,550</td>
<td>355,000</td>
<td>78 (82%)</td>
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<tr>
<td>5m agarose</td>
<td>39,000</td>
<td>3,600</td>
<td>150 (16%)</td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose Peak A</td>
<td>0.2</td>
<td>14,000</td>
<td>70 (80%)</td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose Peak B</td>
<td>4.2</td>
<td>6,400</td>
<td>1,500 (1,500)</td>
<td></td>
</tr>
<tr>
<td>Phosphocellulose of Peak A</td>
<td>0.2</td>
<td>15,700</td>
<td>79 (80%)</td>
<td></td>
</tr>
<tr>
<td>Phosphocellulose of Peak B</td>
<td>0.6</td>
<td>2,900</td>
<td>4,800 (16%)</td>
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</tbody>
</table>

B. Isolation from E. coli strain D110

<table>
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<th>Fraction</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
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<td>Crude extract</td>
<td>430</td>
<td>10,000</td>
<td>44 (100%)</td>
<td></td>
</tr>
<tr>
<td>Polyethylene glycol extract</td>
<td>78</td>
<td>10,400</td>
<td>133 (100%)</td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>2.5</td>
<td>1,720</td>
<td>680 (100%)</td>
<td></td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>0.09</td>
<td>750</td>
<td>8,300 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

*The results obtained with these fractions are unreliable as a measure of RNase H activity because this activity includes RNases capable of acting on poly(A) in the absence of poly(dt).

Fig. 3. Separation of RNase H from a pyrimidine-specific RNase. The phosphocellulose fraction of RNase H isolated from Escherichia coli B (750 units) was chromatographed on a Sephadex G-100 column (25 X 1.1 cm) equilibrated with 0.01 M potassium phosphate, pH 6.5, 0.01 M 2-mercaptoethanol, 0.1 mM EDTA, and 20% glycerol. Fractions were assayed as described under "Experimental Procedure" except that 20 μM [3H]poly(U) was substituted for [3H]poly(A)-poly(dt) for the RNase assay. RNase H activity, O--O: pyrimidine-specific RNase, O--O. The recovery of RNase H activity applied to the column was 40%.

This enzyme has properties different from known RNases of E. coli and remains to be further characterized.

The RNase H activity contained in Peak B was purified 150-fold with a yield of 0.7% (Table I). The activity in this fraction chromatographed coincidently with DNA polymerase I activity which was measured with "activated" calf thymus DNA (15). The RNase H and DNA polymerase activities in Peak B were both resistant to 0.02 M N-ethylmaleimide and completely inhibited by rabbit antiserum prepared against purified DNA polymerase I (Fraction VII) described by Jovin et al. (14). We conclude that Peak B is DNA polymerase I in keeping with the reported ability of DNA polymerase I to attack RNA in hybrid structures (5, 24).

Purification of RNase H from E. coli strain D110—Eight grams of E. coli D110 (pol A1, endo-1) were ground for 10 min with 10 g of alumina A-301 and extracted with 24 ml of 0.05 M Tris-HCl, pH 7.5, 1 mM dithiothreitol, and 0.1 mM EDTA. The suspension was centrifuged at 10,000 X g for 10 min and the precipitate discarded. The supernatant was centrifuged at 30,000 X g for 20 min and the pellet discarded. To the supernatant fluid (crude extract, 19 ml) was added 5.1 g of NaCl, 6.1 ml of 30% polyethylene glycol, and 2.1 ml of 20% Dextran T500. This mixture was stirred for 30 min and then separated by centrifugation at 16,000 X g for 10 min. The polyethylene glycol phase (26.5 ml) was dialyzed against a solution (1 liter) of Buffer D in 50% glycerol which was changed four times over a 4-hour period (conductivity measurements indicated less than 0.03 M NaCl). The polyethylene glycol extract (78 mg of protein) was applied to a DEAE-cellulose column (2 x 26 cm) previously equilibrated with Buffer D in 10% glycerol. The enzyme was eluted with a 300-ml linear gradient of 0 to 0.6 M NaCl and the RNase II activity eluted between 0.05 to 0.06 M NaCl.

Fractions containing RNase H activity were pooled and dialyzed overnight against 0.01 M potassium phosphate, pH 6.5, 0.01 M 2-mercaptoethanol, 0.1 mM EDTA (Buffer F) in 50% glycerol (conductivity measurement indicated 0.02 M NH₄Cl). The DEAE-cellulose fraction (25 mg of protein) was applied to a phosphocellulose column (2 x 22 cm), previously equilibrated with Buffer F in 10% glycerol, and eluted with a 240-ml linear gradient of 0 to 0.6 M NH₄Cl. RNase H activity eluted at 0.29 M NH₄Cl and was dialyzed overnight against a solution containing 0.02 M Tris-HCl, pH 7.5, 0.01 M 2-mercaptoethanol, 0.1 mM EDTA in 50% glycerol. This phosphocellulose fraction was stored at -10°C and was stable for at least 3 months.

The purification of RNase H (Peak A) from E. coli strain D110 is summarized in Table II. The amount of RNase H activity isolated was comparable to that obtained from E. coli B. This purification procedure was carried out without the DNA cellulose step and the final enzyme preparation is of lower purity compared to those preparations isolated with the DNA cellulose step included. The enzyme preparation described in Table II was free of detectable DNase and known RNases. However it contained the pyrimidine-specific RNase which was separated by Sephadex chromatography as described above. After DEAE-cellulose chromatography a second peak of activity (corresponding to 9% of the RNase H activity applied to the column) was observed eluting at 0.12 M NH₄Cl in spite of the pol A mutation in strain D110. This activity may correspond to the DNA polymerase I fragment which contains the 5' → 3' exonuclease activity present in pol A strains.1 No differences in properties were noted between RNase H activity isolated from E. coli B or strain D110.

Requirements of E. coli RNAse H—The requirements for RNase H activity measured by acid solubilization of poly(A) are sum-

1 Dr. J. R. Lehman of Stanford University has determined that Escherichia coli pol A1 mutants contain normal levels of the 5' → 3' exonuclease activity.
mized in Table II. Magnesium is required with optimal activity observed between 2 to 4 mm Mg²⁺. The requirement for Mg²⁺ could be only partially replaced by Mn²⁺. The enzyme required sulfhydryl reagents for maximal activity, was inhibited by N-ethylmaleimide and possessed a broad pH optimum between pH 7.5 to 9.1. Half-maximal activity was observed at pH 6.9. The enzyme activity was relatively insensitive to salt and retained 50% of its activity in the presence of 0.3 M NaCl. With [³²P]RNA-fd DNA hybrids as substrate, the enzyme retained 50% activity in the presence of 0.4 M NaCl.

Under the assay conditions employed, the reaction was linear for more than 60 min and up to 60% degradation of added poly(A). After this point, the rate decreased markedly, although a final yield of 92% degradation was achieved. While equal amounts of poly(A) and poly(dT) were used in the standard assay, catalytic amounts of poly(dT) can be used; under the latter condition, poly(A) degradation can exceed the amount of added poly(dT) by as much as 20-fold. In these experiments, the ratio of poly(dT) to poly(A) varied from 0.01 to 5.0. During the course of these reactions no evidence of degradation of poly(dT) was detected. The addition of excess pancreatic DNase I at any time completely inhibited the further degradation of RNA. Under the assay conditions employed (see “Experimental Procedure”) RNase H activity was proportional to enzyme concentration between 0.05 to approximately 0.5 unit (Fig. 4).

Studies on Extent of RNA Degradation. The extent of degradation of poly(A) in the presence of poly(dT) is shown in Fig. 5. In the presence of 6-fold excess RNase H, 92% of the poly(A) was degraded in 5 min. The addition of more enzyme at 15 min had no further effect. In contrast, the addition of an equal amount of poly(A) resulted again in the degradation of 92% of this material, indicating that only the supply of RNA-DNA hybrids limited the reaction under the conditions employed.

RNase H also degrades RNA in RNA-DNA hybrids formed with E. coli RNA polymerase and fd DNA (25). The RNA polymerase product, after isolation by isopycnic banding in CsCl, was almost quantitatively (95%) susceptible to RNase H digestion. The RNA resistant to RNase H action (5%) was converted to acid-soluble material by pancreatic RNase (2 μg per ml) in 0.25 M NaCl.

Nature of Products Formed by RNase H Action on Poly(A).-The products of complete digestion of [³²P]poly(A)-poly(dT) by RNase H were analyzed by chromatography on DEAE-cellulose (Fig. 6). The radioactivity eluting from the column was resolved into oligonucleotides of various chain lengths as follows: AMP 4%, (pA)₂ 16%, (pA)₃ 31%, (pA)₄ 27%, (pA)₅ 19%, (pA)₆ 2.9%. The radioactivity in the (pA)₅ peak was pooled, dialyzed against water, lyophilized, and further analyzed as follows. Treatment of this material with bacterial alkaline phosphatase resulted in a decrease of the negative charge and co-migration with standard (Ap)dA on electrophoreses at pH 3.5. Snake venom phosphodiesterase (26) completely digested the pentanucleotide product (not treated with alkaline phosphatase) to AMP at pH 8.6. These results indicated the oligonucleotide product contained 3'-hydroxyl and 5'-phosphate termini and possessed the structure (pA)₅.

Specificity of Action of RNase II. The specificity of RNase H for various synthetic homopolymers was examined (Table III). Each labeled ribohomopolymer was tested alone or after annealing to its complementary unlabeled ribo- or deoxyribohomopolymer for sensitivity to RNase II. All ribohomopolymers

![Fig. 4. Dependency of RNase H activity on enzyme concentration.](http://www.jbc.org/)

![Fig. 5. Extent of degradation of poly(A) in hybrid structure by RNase H.](http://www.jbc.org/)
were cleaved in the presence of their complementary deoxyribopolymers. Little activity was detected with polynucleotides alone or polymers annealed to their complementary ribopolymers.

The rate of degradation of different homopolymeric RNA-DNA hybrids by RNase H varied as much as 10-fold. In general, the rate of hydrolysis (in decreasing order) was the following: poly(I)·poly(dC), poly(C)·poly(dG), poly(A)·poly(dT), poly(U)·poly(dA).

The concentration of polynucleotides in each reaction mixture was 75% of that applied to the column.

In parallel reaction with 30 nmoles of unlabeled poly(A) and poly(dT), 19 nmoles were incubated as described under "Experimental Procedure" for 30 min with 18 units of RNase H. The products of a control reaction containing 20 mM Tris.HCl, pH 8.0, 10 mM MgCl₂, 6 mM dithiothreitol, 8 µg/ml of bovine serum albumin, 50 µg/ml of rifampicin, 0.1 mM each of dATP, dCTP, and dGTP, 52 µM [α-32P]dTTP (1000 cpm per pmole), 44 µM fd DNA-RNA hybrid (in which 50% of DNA was covered with hybrid RNA), 0.28 unit of DNA polymerase II, 0.1 unit of Escherichia coli unwinding protein, and 0.2 µM each of dATP, dCTP, and dGTP, was studied in vitro.

The rate of degradation of RNA in fd DNA-RNA hybrid structures was in the same range of activity noted with synthetic polymers. It is interesting to note that RNase H activity associated with RNA-dependent DNA polymerase of avian myeloblastosis virus does not attack poly(U)·poly(dA) but does attack all other RNA-DNA hybrids examined (3-5).

**RNA-Primed DNA Synthesis on fd DNA**—Since the filamentous phages fd and M13 are known to form an RNA-DNA hybrid during SS to RF replication (10), the effect of RNase H on DNA polymerization with this intermediate was studied in order to clarify the role of this enzyme in DNA replication. In vitro DNA synthesis was assayed under conditions (see Fig. 7) in which the number of priming ends was limiting and the rate of deoxynucleotide incorporation was dependent on the concentration of RNA-DNA hybrids. DNA synthesis catalyzed by DNA polymerase II was maximally stimulated 2-fold by E. coli unwinding protein, as first reported by Sigal et al. (16). The addition of RNase H to the reaction containing polymerase and unwinding protein increased the rate another 2.5-fold (Fig. 7). The addition of RNase H in the absence of unwinding protein increased the rate 2-fold. Since RNase H is an endonuclease, it could stimulate DNA polymerization by creating new 3'-hydroxyl priming ends or by degrading RNA hybrids which interfere with the progression of DNA polymerase II along the template DNA.

**DISCUSSION**

The present studies indicate that E. coli contains an N-ethylmaleimide-sensitive enzyme which completely degrades RNA present in an RNA-DNA hybrid structure producing 5'-phosphate and 3'-hydroxyl-terminated oligonucleotide products. This activity can be readily differentiated from other well-characterized RNases of E. coli. Unlike RNase I (27) it requires...
Mg\(^{2+}\) and degrades single stranded RNA poorly, if at all. RNase II (28) is an exonuclease which acts processively and attacks only single stranded RNA, while RNase III (7, 8) degrades duplex RNA as well as RNA in hybrid structures. RNase IV (29) appears to be an enzyme which introduces a limited number of specific breaks in large molecular weight RNA. The characteristics of each of these systems is clearly different from those observed with RNase H.

The mechanism of action of *E. coli* RNase H has been studied (4) and it was shown to act by endonucleolytic attack. Poly(A) was labeled at the 5' end with \([\gamma\text{-}^32\text{P}]\)ATP and polynucleotide kinase, and extended at the 3' end with [\(\text{Hi}\)]ADP and polynucleotide phosphorylase. The label at both ends was rendered acid-soluble at an equal rate in the presence of poly(dT). In addition, poly(A) substrates which are circular or which are blocked at one or both ends with covalently bound cell lulose or poly(C) or both were all susceptible to RNase I attack in the presence of poly(dT). With such modified poly(A) substrates lacking ends, the enzyme must act endonucleolytically. The same substrates were tested with purified RNase II of avian myeloblastosis virus, a processive exonuclease (4). In contrast to results with *E. coli* RNase H, all poly(A) substrates without ends were resistant to attack by the viral enzyme.

A second RNase H activity detected in extracts of *E. coli*, resistant to N-ethylmaleimide, was separated from the RNase H described above by chromatography on DEAE-cellulose. When this enzyme was tested on the doubly labeled poly(A) substrate described above it released the \(\gamma\text{-}^32\text{P}\)-labeled 5' end preferentially, indicating a 5' → 3' exonuclease. The activity copurified with DNA polymerase I activity and was inactivated by antiserum prepared against DNA polymerase I (Jovin et al., 14), Fraction VII, suggesting that this activity is associated with the polymerase. Similar results have been obtained by Baltimore and Shendur (5) as well as by Scheckman et al. (24).

A number of laboratories (30) have shown that single stranded circular DNA (fr, M13, or \(\epsilon\chi\chi\)147) can be activated as an effective template for *in vitro* DNA synthesis by the action of RNA polymerase. The de novo initiated RNA chains in hybrid structure provide 3'-hydroxyl termini which act as primers for deoxynucleotide incorporation. With such structures, the extent of deoxynucleotide incorporation should depend upon the amount of DNA template in single stranded structure not hydrogen-bonded to RNA. Under conditions in which 30 to 50% of the 32P DNA existed as RNA-DNA hybrid structure, DNA polymerase I catalyzed deoxynucleotide incorporation was stimulated by the addition of RNase H (Fig. 7). These results, coupled with the recent demonstration of the role of RNase H in DNA synthesis in *E. coli* (9, 31), suggest that RNase H may play a role in DNA replication. Since RNase H of *E. coli* is an endonuclease, it should excise RNA regions in RNA-DNA duplex structures, the DNA repair machinery could then fill in regions of the duplex previously occupied by RNA.

With this model in mind, we examined the extent of removal of RNA from RNA-DNA hybrids in which the RNA ends were free or covalently linked to DNA at the 5' or 3' end. With fr DNA-RNA hybrids more than 95% of the RNA was rendered acid-soluble by RNase II of *E. coli*. Hybrid RNA covalently attached at its 5' end to DNA was prepared by joining poly(A) to \([5\text{-}^32\text{P}]\)poly(dA) with DNA ligase in the presence of NAD\(^{+}\) and poly(dT).2 The ultimate AMP residue covalently linked to poly(dA) was resistant to RNase H treatment, as shown by the failure of RNase H treatment to convert the joining \([5\text{-}^32\text{P}]\)phosphodiester bond (sensitive to alkaline hydrolysis) to alkali-resistant \([5\text{-}^32\text{P}]\)poly(dA). Thus, the enzyme is unable to remove a ribonucleotide covalently attached to the 5' end of DNA even though it is in hybrid structure.

In *E. coli* there exists a second mechanism by which ribonucleotides can be covalently linked to DNA, namely chain extension catalyzed by RNA polymerase (32). Using this enzyme, a single \([\alpha\text{-}^32\text{P}]\)UMP residue was added to the 3'-hydroxyl end of poly(dA-T). This ribonucleotide covalently bound to the 3' end of DNA was also resistant to RNase H action.

Two other systems are capable of attacking ends containing ribonucleotides in hybrid structures. One is exonuclease III which acts in a 5' → 3' direction (33) while the other is the 5' → 3' exonuclease activity of DNA polymerase I. Thus the combined action of RNase H and these two enzymes (or possibly other activities with similar properties) should remove all ribonucleotides in RNA-DNA duplex structures.

The role of RNA polymerase as initiator in supporting deoxynucleotide incorporation as described above is clear. However, the origin of RNA in circular duplex DNA (34) most likely arises by chain extension by RNA polymerase. The results with DNA ligase suggest that if the RNA segment was synthesized by chain initiation and was used as a primer for DNA synthesis, then the resulting 3' DNA end contiguous with RNA could not be covalently joined. Thus initiation by RNA polymerase would preclude joining by DNA ligase.

**Note Added in Proof**—Purified RNase H preparations (*E. coli* B, phosphocellulose fraction) contained detectable endonuclease activity on duplex DNA measured by the cellulose nitrate filter binding assay (35) as follows. Reaction mixtures (0.05 ml) containing \([\gamma\text{-}^32\text{P}]\)colicin E1 DNA (542 cpm per n mole, 2 n moles), 10 mM MgCl\(_2\), 20 mM Tris-HCl (pH 8), and enzyme were incubated for 15 min at 38\(^\circ\)C. Reactions were stopped with 1 ml of a solution containing 1 M KCl, 1 mM Tris-HCl (pH 7.5), and 1 mM EDTA and heated at 100\(^\circ\)C for 10 min, rapidly cooled in ice, and passed through nitrocellulose filters. Intact circular duplex colicin E1 DNA passes through the filter while nicked denatured colicin E1 DNA is retained. Caution in the case of this assay is necessary because dithioerythritol introduces nicks into the colicin E1 DNA; similar results were obtained by Dr. L. Grossman (California Institute of Technology) with PM2 and mitochondrial DNA; 20% of the circles bound to the cellulose nitrate in the presence of 0.6 mM dithioerythritol. The DNA endonuclease activity was separated from RNase H activity (5800 units in 9 ml) by chromatography on a 382-ml Sephadex G-100 column (3.3 × 45 cm) equilibrated with Buffer D. RNase H eluted between 50 to 66% column volume with a recovery of 76% while DNA endonuclease activity eluted between 66 to 77% column volume. The Sephadex G-100-treated RNase H preparation was unstable and lost 50% of its activity in 1 to 2 weeks. The enzyme was stabilized by adsorption of RNase H activity in this fraction to a 10-ml phosphocellulose column equilibrated with Buffer D, followed by elution with 30 ml of 0.4 M NH\(_4\)Cl in Buffer D (recovery of activity 100%); fractions containing

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2 Unpublished results obtained by Dr. K. Nath of this department. *E. coli* coli DNA ligase (as well as T4 DNA ligase) did not link the 3' end of poly(dA) to \([5\text{-}^32\text{P}]\)poly(dA) in the presence of poly(dT). On the other hand, these enzymes do covalently link poly(A) to the 3' end of \([5\text{-}^32\text{P}]\)poly(dA) in the presence of poly(dT).
RNase H activity were pooled and dialyzed against 2 liters of 65% glycerol in Buffer D for 3 hours at 4°C.

REFERENCES

3. LEIS, J., BERKOWER, I., AND HURWITZ, J. (1972) Biochemistry 11, 1732
4. ANDO, T. (1966) J Biochem., Tokyo, Japan, 61, 158
Isolation and Characterization of an Endonuclease from *Escherichia coli* Specific for Ribonucleic Acid in Ribonucleic Acid-Deoxyribonucleic Acid Hybrid Structures

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