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Studies on a Calf Thymus Ribonuclease Specific for Ribonucleic Acid–Deoxyribonucleic Acid Hybrids†

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ABSTRACT: Ribonuclease H, an enzyme that specifically hydrolyzes the RNA moiety of RNA–DNA hybrids, has been extensively purified from calf thymus. The enzyme is apparently extranuclear in origin. It appears to have a molecular weight of 74,000, and only one major subunit. It requires covalent cations for activity, and prefers Mn^{2+} over Mg^{2+}. The enzyme acts on poly(rAdT) as an endonuclease to produce mono- and oligoribonucleotides, terminated by a 5′-phosphate. No activity could be demonstrated on natural double- or single-stranded DNA, or on single-stranded RNA. The homopolymer hybrids tested either served as substrate for the enzyme or acted as an inhibitor. No single-stranded homopolymer was a substrate or an inhibitor, except poly(rA) which was an inhibitor. Double-stranded viral RNA and several of the homopolymer double-stranded ribonucleic acids are inhibitors of the reaction. The pattern of inhibition of calf thymus ribonuclease H by a number of different rifampicin derivatives has been investigated.

A ribonuclease (ribonuclease H) has been described in extracts of calf thymus that appears to hydrolyze specifically the RNA portion of RNA–DNA hybrids (Stein and Hausen, 1969; Hausen and Stein, 1970). The DNA portion of the hybrid remains intact. Single- and double-stranded DNA and double-stranded RNA have minimal substrate activity. Ribosomal RNA has less than 2% of the substrate activity of the enzymatically synthesized hybrids.

A similar enzyme activity has been described in preparations of avian myeloblastosis virus[1] (AMV) by Molling *et al.* (1971), in mouse KB cells (Keller and Crouch, 1972), and chick embryo extracts (Crouch, 1973[2]).

Such an enzyme is of interest to those concerned with the structure of nucleic acids from several points of view. The enzyme's presence and specificity, if confirmed, raise the question of the possible significance of the hybrids that are its physiologic substrate. Moreover, a ribonuclease specific for the RNA moiety of a RNA–DNA hybrid could be of great value as a tool for the characterization and identification of hybrid nucleic acids. Further information is, however, required regarding the enzyme's specificity and mechanism of action. The purpose of the work reported here has been to further purify and characterize the calf thymus ribonuclease H.

The enzyme from calf thymus has been purified more than 200-fold and its specificity for RNA–DNA hybrids has been confirmed. No activity was detected using double- or single-stranded DNA, or single-stranded RNA. Similar findings were observed with a number of homopolymer materials. The hybrid homopolymer combinations tested were found to be either substrates or inhibitors of the enzyme. Some preliminary characterization of the protein has been accomplished, and data related to size, subunit structure, and mode of activity are presented.

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[1] Abbreviation used is: AMV, avian myeloblastosis virus.

[2] Crouch, R. (1973), private communication.

TABLE I: Purification Summary.[a]

Step	Vol (ml)	Protein (mg)	Sp Act. (units/mg)	Yield (%)
I, 9000 rpm supernatant	1900	6080	0.0298	100
II, 25–55% ammonium sulfate	400	3760	0.0452	93
III, Protamine sulfate	550	2420	0.0588	79
IV, DEAE	220	88	0.288	14
V, Hydroxylapatite	47	4.7	0.974	2.5
VI, Sephadex G-200	4	0.4	5.48	1

[a] Procedures used in purification of ribonuclease H are as described under Methods. One unit of activity is 1 μmol of poly(rA) phosphate rendered Cl_3CCOOH soluble per min, at 37°.

Materials and Methods

Materials. Calf thymus was obtained from a commercial slaughterhouse, sliced with scissors, and frozen at −70° until use. Material stored for up to 6 months showed no appreciable loss in specific activity. Ultrapure ammonium sulfate was purchased from Mann, protamine sulfate from Eli Lilly, microgranular DEAE-cellulose from Whatman, and Sephadex G-200 (superfine) from Pharmacia. Hydroxylapatite was prepared by Mr. D. L. Rogerson at the National Institutes of Health, according to the method of Tiselius *et al.* (1956).

Native Nucleic Acids. DNA from bacteriophage λ was prepared from *E. coli* M 5107 (temperature-sensitive repressor) by the method of Nash and Merril (1969), employing equilibrium centrifugation in CsCl. The material was then taken through 3–5 phenol extractions, 10–12 ethanol fractionations, and exhaustive dialysis. Single-stranded DNA was prepared by heat denaturation (100°, 10 min), followed by rapid cooling in 0.02 M Tris (pH 7.8). MSII RNA was prepared by the method of Strauss and Sinsheimer (1963). The identity of each of these materials was confirmed spectrophotometrically (Cary 15) and by measurement of the bouyant density in Cs_2SO_4 or $CsCl_2$. Reovirus RNA was the kind gift of Dr. W. K. Joklik.

Homopolymers. Homopolymers were purchased from Miles or P-L Biochemicals. Tritium-labeled polymers were purchased from New England Nuclear or Schwarz. The absorption spectrum of each polymer was determined in 0.01 M Tris (pH 7.8). Concentrations were calculated from published spectral data (see notation in Table II). An extinction coefficient for poly(dG) was calculated by comparison of the absorption spectrum of poly(dG) at pH 7.8 with that of an identical sample in 0.1 M KOH. Moreover, we also compared the absorption spectrum of poly(dG) in 0.1 M KOH to that of dGMP in the same solvent. The two spectra were virtually identical, thus indicating that the spectrophotometric characteristics of polymerized dGMP are not significantly different from that of the monomeric species in this solvent. The extinction coefficient calculated for poly(dG) on this basis (E_p = 9.0 at 260 mμ at pH 7.8) agrees well with the published value of 8.99 (Bollum, 1966).

Homopolymers were combined to form double-stranded ribonucleotides, or ribonucleotide–deoxyribonucleotide hybrids, by mixing equimolar amounts of the substituent material in 0.01 M Tris (pH 7.8)–0.1 M NaCl, at room temperature for 30 min. The spectrum of each complex was compared with and found to be similar to published patterns (Table II); concentrations of each complex (except "poly(rCdG)") were calculated using the published E_p vlaues. The values calculated from the constituent concentrations and the measured concentrations assuming the cited E_p values for the hybrid complexes were in agreement within 5%. Full characterization of "poly(rCdG)" has not been presented in the literature. Therefore, the concentration of "poly(rCdG)" was calculated from the constituent concentrations. The nature of the "complex" formed upon mixing poly(rC) with poly(dG) is not therefore known. For this reason, "poly(rCdG)" will be written within quotation marks in this paper. Full characterization of this complex falls outside the scope of this work. All polynucleotide concentrations are expressed as total nucleotide phosphate.

Assay. The hybrid ribonuclease assay was similar to that previously described (Hausen and Stein, 1970).

Radioactivity contained in the poly(rA) portion of rAdT hybrids was rendered acid soluble by the action of the enzyme. The reaction mixture contained, in 0.5 ml, 0.03 M Tris (pH 7.8), 0.1 M $(NH_4)_2SO_4$, 1 mM $MnCl_2$, and 10 μM [^{3}H]rAdT (12,800 cpm/nmol). The reaction was terminated by addition of 0.5 ml of 10% Cl_3CCOOH and 100 μg of tRNA as carrier, allowed to stand in ice for 15 min, and centrifuged at 2000 rpm for 5 min. A 0.5-ml aliquot of the supernatant fluid was removed for scintillation counting. Under these conditions, the assay is linear with protein concentration and with time. One unit of enzyme activity is equal to 1 μmol of poly(rA) phosphate rendered acid-soluble/min, at 37°. The assay is readily inhibited by poly(rA); therefore, in order to avoid the presence of free poly(rA) in the rAdT used for the assay, a small excess of poly(dT) must be added.

All the experiments reported here were done with enzyme carried through the complete purification procedure (fraction 6).

Protein. Protein concentrations were measured by the method of Lowry *et al.* (1951).

Results

Purification of Ribonuclease H. A purification protocol is summarized in Table I.

Homogenization. Frozen calf thymus (200 g) was pulverized with a hammer and then homogenized at low speed for 3 min in 2000 ml of 0.05 M Tris (pH 7.8), 0.25 M sucrose, 0.025 M KCl, 0.003 M $MgCl_2$, and 0.0015 M $CaCl_2$, in a 1-gal Waring Blendor at 4°. The homogenate was filtered through two and then four thicknesses of cheesecloth, and centrifuged at 9000 rpm (Sorvall RC2-B) for 20 min. The supernatant fluid was filtered through glass wool to remove floating fat (fraction 1).

This procedure is reported to remove the nuclei from calf thymus homogenates (Goldberg and Moon, 1970). As determined microscopically after staining with hematoxylin and eosin very few nuclei remain in the supernatant solution after centrifugation. More than 95% of the diphenylamine reacting material (Burton, 1956) is removed from the solution, whereas more than 90% of the enzyme activity remains in the supernatant solution.

Ammonium Sulfate Fraction. Solid ammonium sulfate was added to 25% saturation (17.5 g/100 ml). The solution was stirred for 30 min and centrifuged for 20 min at 9000 rpm, and the precipitate was discarded. Solid ammonium sulfate was added to the supernatant fluid to 55% saturation (21

g/100 ml added). This was stirred for 30 min and centrifuged at 9000 rpm for 20 min and the supernatnat fluid was discarded. The precipitate was resuspended in about 400 ml of 0.02 M Tris (pH 7.8)–0.05 M KCl, and insoluble material was removed by centrifugation at 13,000 rpm for 20 min (fraction 2).

Protamine Sulfate Fraction. The protein concentration of fraction 2 was adjusted to 10 mg/ml with the same buffer and 1 ml of a 10-mg/ml solution of protamine sulfate was added for each 20 ml of enzyme solution. The mixture was stirred for 30 min and centrifuged at 13,000 rpm for 20 min, and the precipitate was discarded. The supernatant fluid is fraction 3.

DEAE Chromatography. Fraction 3 was dialyzed against 10 vol of 0.02 M Tris (pH 7.8)–0.05 M KCl, twice for 2 hr each, and applied to a 200-ml DEAE column previously equilibrated against the same buffer. The column was washed with the same buffer until the absorbance at 280 mμ was less than 0.05, and then a KCl gradient of 0.05–0.25 M in six column volumes was applied. The enzyme begins to appear in the eluate at about 0.07 M KCl. The fractions containing the enzyme were pooled (fraction 4).

The dialysis step prior to application of fraction 3 enzyme to the DEAE column is described as taking a total of 4 hr. It has been found that if the dialysis was prolonged, progressively larger amounts of enzyme were lost as an insoluble precipitate. Four hours of dialysis was found to be sufficient to permit all of the enzyme to bind to the column, but, as noted, this binding was such that enzymatic activity was eluted rather promptly upon application of the salt gradient. The inability of the enzyme to tolerate low ionic strength in the early stages of the purification procedure made the achievement of satisfactory conditions for other types of ion exchange chromatography difficult.

Hydroxylapatite Chromatography. Fraction 4 was applied directly to a 20-ml hydroxylapatite column previously equilibrated with 0.02 M KPO_4 (pH 6.8). After application, the column was washed with 0.06 M KPO_4 at the same pH, and then the enzyme was eluted with a KPO_4 gradient: 0.06–0.25 M in 20 column volumes. The enzyme was in the first protein peak eluted by the gradient (fraction 5).

Sephadex Chromatography. The material in fraction 5 was precipitated with solid ammonium sulfate (60% saturation), stirred for 45 min, and centrifuged at 15,000 rpm for 20 min. The precipitate was dissolved in 1–2 ml of 0.05 M Tris (pH 7.8) and applied to a 200-ml Sephadex G-200 (superfine) column previously equilibrated with the same buffer. The 1-ml fractions containing enzyme activity were pooled in accordance with the yield and purity requirements of a given experiment. The experiments described here were done with the 4–5 ml from the activity peak (fraction 6).

Purity of the Enzyme Preparation. The enzyme was eluted from the Sephadex column as a discrete peak. While it was not homogeneous the degree of contamination of the peak tubes was relatively small. Electrophoresis on sodium dodecyl sulfate–acrylamide gels revealed the presence of only one major component, moving with an R_F of 0.4. Moreover, the band in the acrylamide gel pattern which moves with an R_F of 0.4 is the only one which became progressively enriched over the last steps in the purification procedure. It has not been possible to elute any of the bands from acrylamide gels and to demonstrate enzyme activity, regardless of whether the gels were polymerized with ammonium persulfate or riboflavine.

Molecular Weight and Subunit Structure. The subunit molecular weight of calf thymus ribonuclease H was determined by acrylamide gel electrophoresis in sodium dodecyl sulfate by the method of Neville (1971). Using standards of known molecular weight, a logarithmic relationship was obtained between molecular weight and migration; calf thymus ribonuclease H gave an average mol wt of 74,000. This value is not significantly different from the value of 76,000 that was obtained by sedimentation equilibrium analysis (Spinco Model E) of the native enzyme at two speeds in 0.01 M Tris (pH 7.8). These data indicate that the enzyme has a single subunit. It is noteworthy that the value reported by Keller and Crouch (1972) for the molecular weight of ribonuclease H from KB cells is very close to that reported here for calf thymus ribonuclease H.

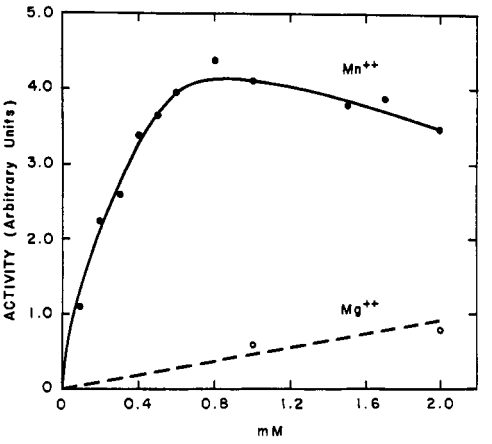

FIGURE 1: Activity measured in the standard reaction mixture, in the presence of variable divalent cation.

Properties of the Enzyme. Temperature and pH dependence of the purified enzyme were essentially as reported by Hausen and Stein (1970). Similarly, the necessity for divalent cation and the ability of Mg^{2+} to substitute only partially for Mn^{2+} were confirmed (Figure 1). Assays with increasing Mg^{2+} concentration showed a peak value of about 75% of the peak value using Mn^{2+}, and this at 15 mM Mg^{2+}. For the purified enzyme the optimal concentration for Mn^{2+} in the reaction mixture was 1 mM, rather than the value of 2 mM previously reported (Hausen and Stein, 1970).

Substrate Specificity. As shown in Table II, a large number of compounds have been tested for their ability to act as a substrate for ribonuclease H. The enzyme was used in all assays at a final concentration of about 0.7–1.0 μg/ml. All potential substrates were tested at several concentrations up to 15 μM, and most were tested up to 20–30 μM. (Radioactive poly(rA) was tested as a substrate up to 45 μM. No detectable radioactive acid-soluble products were found indicating that poly(A) is not a substrate.) As shown in Table II, only poly(rAdT) was found to serve as a substrate for the enzyme with K_m = 1.9 μM.

Inhibition of Ribonuclease H Activity. The ability of the compounds listed in Table II to serve as inhibitors of the hydrolysis of poly(rAdT) was examined. In each case where inhibition could not be demonstrated, the materials in question were tested to at least 15 μM. Where inhibition was observed, the data presented in Table II represent the measurement of the initial rates of the reaction using increasing amounts of poly(rAdT) as substrate, at two or three different concentrations of the inhibitor. Data were calculated as described by Dixon and Webb (1964).

Poly(rA) acts as a noncompetitive inhibitor of the reaction,

TABLE II: Substrates and Inhibitors of Ribonuclease H.[a]

Material	K_m (μM)	Mode of Inhibition	K_i (μM)
Substrates			
rAdT[b]	1.9		
Inhibitors			
rIdC[c]		Competitive	1.9
rCdG (see Methods)		"Uncompetitive"	
Reovirus RNA	Not done	Competitive	7.3
rArU[b]		Noncompetitive	2.5
rIrC[c]		None	
Ms II RNA		None	
rA[b]		Noncompetitive	2.0
rC[c]		None	
rI[c]		None	
ss-λ-DNA[d]		None	
ds-λ-DNA[d]		None	

[a] Materials were prepared as described under Methods. The K_m value is the mean of six determinations. K_i values are the mean of two or three determinations, by procedures described under Methods. References for extinction coefficients of the materials listed are the following. [b] Riley *et al.*, 1966. [c] Chamberlin and Patterson, 1955. [d] Chamberlin and Berg, 1964.

as shown in Figure 2. The K_i was estimated to be 2.0 μM. Measurements using poly(rArU) gave data similar to that shown in the figure; the K_i was calculated to be 2.5 μM. Reovirus RNA, representing double-stranded RNA, gave a competitive pattern of inhibition, similar to that shown in Figure 3. The K_i was calculated to be 7.3 μM.

Poly(rIdC) was found to be a competitive inhibitor (Figure 3); the K_i was calculated to be 1.9 μM. The data obtained for inhibition by poly(rCrG) are illustrated in Figure 4. This pattern is apparently consistent with that of an "uncompetitive" inhibitor.

MsII RNA, used in these studies as a model for single-stranded RNA, was tested as a potential inhibitor up to a concentration of 75 μM, with no apparent effect on the reaction.

In review of the results of Molling *et al.* (1971), it was of

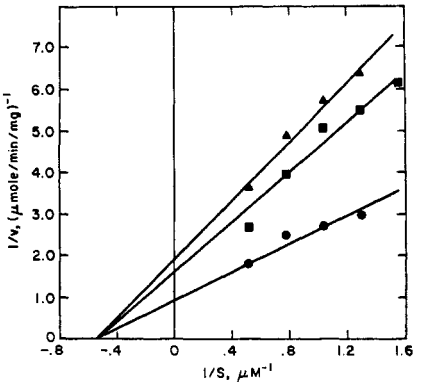

FIGURE 2: Initial reaction rate of 1.0-μg fraction 6 enzyme with variable concentrations of poly(rAdT), in the presence of different concentrations of poly(rA): (▲) 2.6 μM poly(rA); (■) 1.3 μM poly(rA); (●) zero poly(rA).

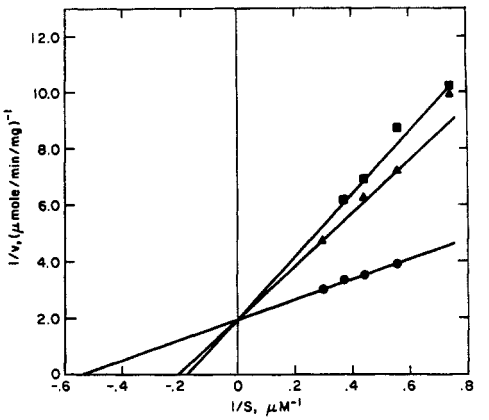

FIGURE 3: Initial reaction rate of 1.0-μg fraction 6 enzyme with variable concentrations of poly(rAdT), in the presence of different concentrations of poly(rIdC): (■) 2.16 μM poly(rIdC); (▲) 1.9 μM poly(rIdC); (●) zero poly(rIdC).

interest to examine the effect of rifampicin derivatives on ribonuclease H. Table III summarizes the results of experiments in which four different rifampicin derivatives were tested each at four different concentrations for their effect on ribonuclease H activity. The results are expressed as per cent residual activity, measured as the initial rates in the presence (or absence) of the stated amount of inhibitor.

It can be seen that the degree of inhibition varies considerably between the four rifampicin derivatives. Moreover, the degree of inhibition of ribonuclease H does not correlate in any way with the degree of inhibition of viral reverse transcriptase or of mammalian DNA polymerase. This is in contrast to the results of Molling *et al.* (1971) that, however, were based on the examination of the effect of a single rifampicin derivative on ribonuclease H from a different source (AMV). Calculated on a molar basis it can be estimated roughly that 0.2 mM PR/19 is required to inhibit 0.003 μM ribonuclease H, assayed on rAdT at 1–2 μM concentration. Thus, the mechanism of inhibition of ribonuclease H must be different from the stoichiometric binding to the enzyme that is the basis for the inhibition of *E. coli* RNA polymerase by rifampicin.

The mode of action of the enzyme was further explored by thin-layer electrophoresis analysis of the reaction products

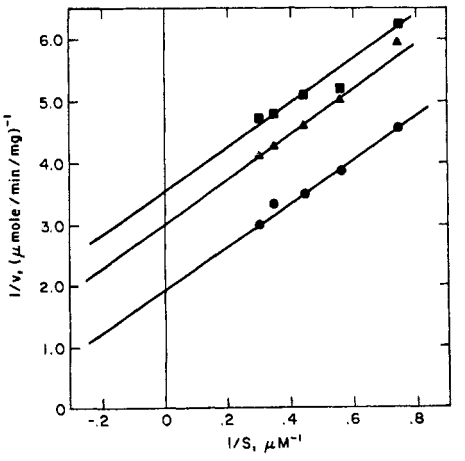

FIGURE 4: Initial reaction rate of 1.0-μg fraction 6 enzyme with variable concentrations of poly(rAdT), in the presence of different concentrations of poly(rCdG): (■) 2.6 μM poly(rCdG); (▲) 1.94 μM poly(rCdG); (●) zero poly(rCdG).

TABLE III

Inhibitor[a]		Inhibitor Concn (μg/ml) Needed for 50% Inhibition[b] of		% Residual RNase H Act.[c] for Inhibitor (μg/ml)			
Manufacturer's Code	N.C.I. No.[d]	Rauscher Leukemia Virus Reverse Transcriptase	Human Lymphocytes DNA Polymerase	50	100	200	400
AF/ABDF CIS	139-209	45	12	95	92	78	32
AF/013	143-483	20	15	100	100	100	
PR/19	143-465	<50	>250		91	51	15
AF/AP	143-416	>50	>1000	100	100	100	69

[a] The rifampicin derivatives were kindly supplied by Professor P. Sensi of Lepetit S.p.A., Milan, Italy. The chemical names of the compounds are as follows: AF/ABDP CIS, 3-(4-benzyl-2,6-dimethylpiperazinoiminomethyl)rifamycin SV (cis) (2,6-dimethyl-4-benzyl-4-demethylrifampicin); AF/013, 3-(formylrifamycin SV)-*n*-octyl oxime; PR/19, 3′-acetyl-1′-benzyl-2′-methylpyrrolo[3,2-*c*]-4-desoxyrifamycin SV; AF/AP, 3-piperazinoiminomethylrifamycin SV (*N*-demethylrifampicin). [b] The data on inhibitor concentration were derived from extensive studies of Yang *et al.* (1972) on rifamycin antibiotics. [c] The amount of ribonuclease H used was 0.23 μg/ml. [d] The number used for identification of these compounds by the chemotherapy program of the National Cancer Institute.

on polyethylenimine plates. This study was carried out in cooperation with Dr. Robert Crouch. Using poly(rAdT) hybrids labeled in the α position with ^{32}P, the standard reaction mixture was allowed to run to "completion" and the products were electrophoresed as described by Crouch (1972).[3] Similar to the results of Keller and Crouch (1972) with ribonuclease H from KB cells, a series of oligonucleotides containing one to nine bases was found with an average chain length of 5.6. In order to gain information on the mode of action of the enzyme, a reaction mixture similar to the one described above was first treated with ribonuclease H, and then digested with KOH, and the reaction products were chromatographed in two dimensions (Randerath and Randerath, 1967). Similar to the findings of Keller and Crouch (1972), 3′,5′-deoxyadenosine diphosphate (pAp) was found as a major product and the proportion of ^{32}P appearing in pAp (33%) is as expected from the average length of the oligonucleotides generated from rAdT by ribonuclease H.

Enzymatic Action. The ability of phosphate to stimulate the activity of the enzyme on poly(rAdT) was examined; there was no stimulation of the enzymatic activity when 6 mM KPO_4 was added to the reaction mixture. When 1.2 mM KPO_4 was substituted for Tris in the standard reaction mixture, no activity was detected using poly(rA) as the substrate.

Discussion

Purification Procedure. The purification procedure as described is relatively straightforward. While it does not result in a homogeneous enzyme preparation, it does represent a 150-fold increase in specific activity over that reported previously for calf thymus ribonuclease H.

The data concerning the initial homogenization and centrifugation procedure, by which method Goldberg and Moon (1970) were able to isolate intact nuclei containing RNA polymerase from the precipitate, suggest that ribonuclease H is extranuclear. The possibility that the enzyme is associated with other cellular organelles is not excluded.

[3] Crouch, R. (1972), manuscript in preparation.

Properties of the Enzyme. The temperature and pH dependence found during these studies were essentially the same as described by Hausen and Stein (1970). Also, the enzyme was found to require divalent cation for activity, and to clearly prefer Mn^{2+} over Mg^{2+} (Figure 2). This result differs from that reported by Keller and Crouch (1972) for the ribonuclease H isolated from KB cells, and from that reported by Molling *et al.* (1971), for AMV, where the enzyme prefers Mg^{2+} to Mn^{2+}. This suggests that the enzymes from these tissues are different, but in view of the similarity of the reaction products, it is not certain that this difference is functionally significant.

As shown in Figure 2, purified ribonuclease H has an optimal Mn^{2+} requirement of 0.8–1.2 mM, as opposed to a value of 2.0 mM reported by Hausen and Stein (1970). This difference might be explained by nonspecific binding of Mn^{2+} by other proteins in the less pure enzyme preparation. Enzymatic digestion of poly(rAdT) produces a series of oligonucleotides of from one to nine bases, a behavior consistent with an endonuclease. This is similar to the findings of Keller and Crouch (1972) with the enzyme from KB cells. The enzyme appears to attack the 3′ oxygen–phosphate bond, leaving the oligonucleotides with a terminal 5′-phosphate; again similar results are being reported by Keller and Crouch (1972).

Substrate Specificity. In the various studies of ribonuclease H cited (Hausen and Stein, 1970; Keller and Crouch, 1972; Molling *et al.*, 1971), different criteria for what constitutes a "hybrid" have been employed. Particularly as the function of the enzyme is not known, the question of which criteria have biological relevance cannot be answered, and the selection of standards by which substrate materials are classified is necessarily arbitrary. We have limited our work to natural nucleic acids and artificial homopolymers that conform to published spectral data and that band sharply in cesium gradients with buoyant densities in accord with published values. The bacteriophage and virus nucleic acids used satisfy these criteria. Using *E. coli* RNA polymerase and a variety of natural DNA templates, we have not been able to produce "natural" hybrid molecules that gave a single band at the expected density on Cs_2SO_4 gradients.

Artificial homopolymers have been used extensively in these studies to minimize questions of structure of the various molecules employed as much as possible, and in the interest of reproducibility. However, the complexities of homopolymer structures are illustrated by the work of Riley *et al.* (1966) and that cited in the review by Felsenfeld and Miles (1967). Freely acknowledged is the unresolved question of the relation of these materials and their structures to whatever the enzyme's biological substrate might be.

Among the materials listed in Table II, only poly(rAdT) was found to serve as a substract for ribonuclease H. None of the natural materials listed, tested as models for double- and single-stranded DNA, and for single-stranded RNA, served as substrates. This we interpret as corroborating the data presented by Hausen and Stein (1970) for the calf thymus enzyme and the findings with the enzymes derived from other tissues (Keller and Crouch, 1972; Molling *et al.*, 1971).

With a view to the possibility that the structure of the homopolymer hybrids might not exactly mimic that of the physiologically relevant substrates, mixing studies were performed to ascertain the capacity of these materials to bind to the enzyme under the reaction conditions utilized. Poly(rIrC) and "poly(rCdG)" were both inhibitors of activity on poly(rAdT) (Table II).

Keller and Crouch (1972) have shown that for the enzyme derived from KB cells, activity can be demonstrated on hybrids made with *E. coli* RNA polymerase on ϕX-174 DNA templates. Hybrids constructed with [α-^{32}P]GTP or -UTP will, upon hydrolysis, yield 3′,5′-NDP spots corresponding to all four bases when the reaction products are digested with base and electrophoresed on polyethylenimine plates. This suggests that under these conditions the enzyme does not exhibit significant base specificity, and therefore one can conclude that adenosine need not be adjacent to the site of attack by the enzyme. In light of this finding, the inhibition of enzyme activity by poly(rIdC) and "poly(rCdG)" is not easily explained. More work will be required to elucidate the special features of poly(rIdC) and "poly(rCdG)" that render these materials unsuitable as substrates but capable to be recognized by the enzyme. Both reovirus RNA, a natural double-stranded RNA, and poly(rArU) inhibit the activity of the enzyme. Poly(rIrC), on the other hand, is not inhibitory. The significance of the different patterns of inhibition observed is not clear.

Poly(rA) is an inhibitor of the reaction. The relation of its structure in solution to that of double-stranded RNA or RNA–DNA hybrids is not clear. Different conditions might explain why this inhibition was not observed by Hausen and Stein (1970).

Classification of the Enzyme. As noted above, the enzyme does not hydrolyze poly(rA), even when phosphate is substituted for Tris in the reaction medium, and there is no stimulation of the activity of the enzyme on poly(rAdT) by phosphate. Thus, ribonuclease H is not polynucleotide phosphorylase "acting in reverse" (Singer, 1966).

The molecular weight of the enzyme makes it unlikely that it is one of the ribonucleases already described. The work of Keller and Crouch (1972), and that reported here, indicate that ribonuclease H is an endonuclease, and its reaction products are mono- and oligonucleotides terminated by a 5′-phosphate. Of the vertebrate ribonucleases listed in Barnard's recent review (1969), only the sheep kidney nuclease (Kasai and Grunberg-Manago, 1967) has a specificity similar to the enzyme described here since it hydrolyzes tRNA into 5′-phosphate oligonucleotides without formation of mononucleotides. Bacterial ribonuclease III (Robertson *et al.*, 1968) works on double-stranded ribonucleic acids, but its reaction products are mononucleotide 3′-monophosphates. Thus, it would seem that ribonuclease H is a new enzyme.

Function of the Enzyme. The function of a ribonuclease that is specific for RNA–DNA hybrids is not clear. Keller (1972) has proposed a role for covalently bound RNA in the initiation of DNA replication, and has suggested that ribonuclease H might be relevant in this regard. No specific information concerning this hypothesis is presented here, although the evidence that the enzyme is extranuclear disputes this idea.

Molling *et al.* (1971) have reported that ribonuclease H activity is present in extracts of AMV and that the activity remains associated with reverse transcriptase activity after sucrose velocity gradient contrifugation and DEAE-Sephadex chromatography. The patterns of inhibition by different rifampicin derivatives are clearly not the same for calf thymus ribonuclease H and mammalian reverse transcriptase; thus, it would appear that the ribonuclease H found in AMV extracts and calf thymus, although similar in their specificity, are different in other respects. An enzyme that degrades the RNA portion of an RNA–DNA hybrid, while presumably leaving the DNA portion intact, would be in accord with the model of RNA tumor virus replication that includes transfer of base sequence information from single-stranded RNA to double-stranded DNA by way of an RNA–DNA hybrid intermediate. However, the participation of a ribonuclease H-like enzyme in this process is still only hypothetical. Ribonuclease H has now been obtained from calf thymus, KB and chick embryo cells, *E. coli*, and various RNA tumor virus preparations contaminated to some unknown degree with material from the cells in which they grew. The biological role of this enzyme remains an interesting and unanswered question.

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Characterization and Poly(adenylic acid) Content of Ehrlich Ascites Cell Ribonucleic Acids Fractionated on Unmodified Cellulose Columns†

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ABSTRACT: Conditions are described whereby synthetic poly(A) as well as denatured DNA, heterogeneous nuclear RNA (hnRNA), and heterogeneous cytoplasmic RNA (hcRNA) from Ehrlich ascites cells can be selectively adsorbed to unmodified cellulose columns. In this manner, these species can be isolated essentially free from other types of RNA and double-stranded DNA (ds-DNA). Evidence is presented which indicates that the selective binding of these hRNAs to cellulose is the result of a hydrophobic interaction between poly(A) moieties in the RNAs and the polyaromatic lignins present as minor constituents in all plant celluloses. Hybridization of nuclear and cytoplasmic RNAs to [^{3}H]poly(U) before and after cellulose chromatography revealed that over 98% of the poly(A)-containing RNAs bound to the cellulose. The poly(A) moiety was found to represent approximately 4.2% of the nucleotides in bound hcRNA and 2.0% of the bound hnRNA. The specificity of the cellulose to bind poly(A)-containing RNAs was examined by prehybridizing nuclear and cytoplasmic RNAs to excess poly(U), thereby preventing poly(A) sequences from binding. The amount of bound hcRNA was reduced 90% and the bound hnRNA 80% by this procedure, indicating that most of the RNAs which normally bind to the cellulose do so *via* their poly(A) moieties. In addition, mengovirus RNA was found to contain a poly(A) region approximately 50 nucleotides long, compared with regions 150–200 nucleotides long in hRNA; the viral RNA did not adsorb effectively to the cellulose, suggesting that the length of the poly(A) moiety is important in the binding of RNAs to cellulose. The binding ability of hRNAs synthesized in the presence of 0.04 μg/ml of actinomycin D also was examined. Approximately 50% of the hcRNAs and 40% of the hnRNAs did not bind and, therefore, did not contain significant amounts of poly(A). Finally, the poly(A) content of various sedimentation classes of cellulose-bound hRNAs was determined by hybridization to [^{3}H]poly(U). Poly(A) was detected in all sedimentation classes of hnRNA and hcRNA and there was a relative decrease in the proportion of poly(A) in the hRNA molecules with increasing sedimentation rate.

The functions and interrelationships between the heterogeneous RNAs (hRNAs)[1] in animal cells are not well understood. However, progress toward achieving such an understanding has been aided by the recent development of several procedures for the isolation of hRNA (Edmonds and Caramela, 1969; Kates, 1970; Lee *et al.*, 1971; Sullivan and Roberts, 1971; Sheldon *et al.*, 1972; Kitos *et al.*, 1972). These procedures have facilitated investigations concerning synthesis, processing, transport, and utilization of hRNA, as well as providing the means of isolating hRNA for studies on hybridization and *in vitro* protein synthesis.

One of these methods for isolating hRNA involves chromatography on unmodified cellulose columns (Sullivan and Roberts, 1971; Kitos *et al.*, 1972; Schutz *et al.*, 1972). In this report we show that the basis for the RNA fractionation is a selective hydrophobic bonding of hRNA to polyaromatic lignins, minor constituents of the cellulose. Also, we present evidence that over 98% of the cells' poly(A)-containing RNAs bind to the cellulose, that the single-stranded poly(A) moiety is required for binding, and that hRNA which does not contain poly(A) does not adsorb to the cellulose. Finally, the bound and unbound hcRNA and hnRNAs are characterized with respect to their sedimentation profiles, nucleotide compositions and poly(A) contents.

Materials and Methods

The poly(A), poly(I), poly(C), and [^{3}H]poly(U) (78.1 μCi/mmol of P, >50,000 molecular weight) used in these

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[1] Abbreviations used are: hRNA, heterogeneous RNA; hnRNA, heterogeneous nuclear RNA; hcRNA, heterogeneous cytoplasmic RNA; rpRNA, ribosomal precursor RNA.