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Purification and Properties of Ribonuclease H of Calf Thymus

(S-adenosylmethionine/Mg, Co, Mn/poly(dT)·poly(rA)/phage f1 DNA)

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ABSTRACT **Ribonuclease H of calf thymus has been purified better than 3000-fold to yield an almost homogeneous preparation. The enzyme, which comprises about 0.03% of the total protein in the initial extract, is a slightly acidic protein (pI = 4.95) of molecular weight of about 64,000, possibly composed of subunits. The enzyme requires a metal ion for activation; the conditions for activation by Mg, Co, and Mn are described. It is inhibited by S-adenosylmethionine. The substrates cleaved were poly(dT)·poly(rA) and the DNA–RNA hybrid made from phage f1 DNA; ribosomal RNA was not attacked.**

Ribonuclease H (1), widely distributed in cellular systems, is an enzyme specific for the cleavage of the RNA moiety of a DNA–RNA hybrid. It is not impossible that enzymes having this specificity play an important role in the biosynthesis of DNA, since a RNA primer (2–5) may well form the initial tract of a growing DNA chain before its repair and ligation. We report here the almost complete purification of ribonuclease H of calf thymus; this amounted to a better than 3000-fold enrichment over the initial extract.

MATERIALS AND METHODS

Materials. Unlabeled deoxyribo- and ribonucleoside triphosphates came from Schwarz/Mann and Sigma; the latter also supplied *S*-adenosylmethionine. Radioactive precursors were furnished by New England Nuclear Corp.; Sephadex G-50, G-100, and CM-50 by Pharmacia; microgranular DEAE-cellulose DE-52 by Whatman; polyethylene glycol 6000 by J.T. Baker Chem. Co.

Enzymes. RNA polymerase (EC 2.7.7.6) was isolated from *Escherichia coli* by a modification of a procedure in the literature (6). Terminal deoxynucleotidyl transferase was isolated from calf thymus (7).

Substrates. The hybrid poly(dT)·poly[^{14}C](rA) was formed by hybridization of equimolar quantities of poly(dT) and poly[^{14}C](rA). Poly(dT), synthesized enzymically (7), served as template for the polymerization of [^{14}C]ATP by RNA polymerase. The incubation mixture contained in a total volume of 4.0 ml of 50 mM Tris·HCl buffer (pH 8.0) and 0.13 M NaCl: 1.6 μmol of poly(dT), 15 μmol of [^{14}C]ATP (400 cpm/nmol), 15 μmol of $MnCl_2$, 15 μmol of dithiothreitol, and 120 μg of RNA polymerase. The reaction was started by the addition of ATP. After incubation for 48 hr at 35° under argon, when 87% of the ATP used had been polymerized, the reaction was stopped by the addition of 300 μmol of EDTA. Enough poly(dT) was added to make it equal in proportion to the poly(rA) formed, the NaCl concentration was adjusted to 0.4 M, and the mixture was kept at 72° for 30 min; this was followed by slow cooling (3 hr) to 25°. The hybrid poly(dT)·poly(rA) was freed of ATP and EDTA by filtration through a column of Sephadex G-50 that had been equilibrated with 0.4 M NaCl–0.01 M Tris·HCl (pH 7.5). The solution was stored over $CHCl_3$ at 4°.

The preparation of the hybrid of phage f1 DNA with a DNA/RNA ratio of 1 has been described (5). ^{3}H-labeled 16S RNA from *Bacillus subtilis* (80,000 cpm/μg) was kindly given us by Dr. L. Margulies.

Analytical Procedures. All procedures used here have been listed in the corresponding section of a previous paper (8).

Assay of Ribonuclease H Activity and Definition of Unit. As this enzyme appears to be an endonuclease (9), the proportion of DNA–RNA hybrid that is cleaved depends upon the ratio of hybrid to enzyme. We obtained valid assays when the enzyme concentration was so chosen as to permit the cleavage of 75–90% of the hybrid used within 15 min. Each enzyme sample to be tested in the course of purification was diluted to varying degrees with a 0.5% solution of bovine-serum albumin in 0.6 M Tris·HCl buffer (pH 8.0), and portions of 10 μl of each dilution were used for the assay. Each assay sample (total volume 120 μl) consisted of 0.05 M Tris·HCl (pH 8.0), 0.35 M NaCl, 0.02 M $CoCl_2$, 10 nmol of the hybrid poly(dT)·poly[^{14}C](rA), 50 μg of bovine-serum albumin, and enzyme. Incubation was for 15 min at 35°. The reaction was stopped with 10% trichloroacetic acid, and the mixture was filtered 10 min later through a membrane filter (Schleicher & Schuell, B-6) which was counted in a scintillation counter.

The ribonuclease H unit is defined as the quantity of enzyme catalyzing the conversion of 1 nmol of hybridized poly(rA) to an acid-soluble form in 15 min at 35°, under conditions when 75–90% of the hybrid used is so attacked.

Assay of Ribonuclease H for Ribonuclease Activity of the Pancreatic Type. The assay mixtures contained, in a total of 0.24 ml: 0.05 M Tris·HCl (pH 8.0); 100 μg of ribonuclease H (60,000 units), corresponding to Step VIII described below; 1.5 μg of ^{3}H-labeled 16S RNA (125,000 cpm); and either 20 mM $CoCl_2$ and 0.35 M NaCl, or 2.0 mM $MnCl_2$, 0.2 M ammonium sulfate, and 0.01 M thioglycol, or 25.0 mM $MgCl_2$ and 0.05 M NaCl. In control experiments the enzyme was omitted. Incubation was for 1 hr at 35°. The reaction was stopped with 10% trichloroacetic acid, and the mixture was chilled for 10 min in ice and filtered through a membrane filter whose radioactivity was measured.

TABLE 1. *Isolation of ribonuclease H from calf thymus*

Step	Procedure	Volume (ml)	Total protein (mg)	Total activity (10^6 units)	Specific activity	Recovery (%)
I	Crude extract	11,400	106,000	26.80	253	100
II	Precipitation with polyethylene glycol	800	22,000	22.79	1,036	85
III	Fractionation with ammonium sulfate	350	7,670	16.70	2,177	62.3
IV	Removal of nucleic acid	67	3,760	14.70	3,910	54.9
V	Fractionation on CM-Sephadex	14	460	11.00	23,913	41.0
VI	First fractionation on DEAE-cellulose	3.1	62.5	8.05	128,800	30.0
VII	Fractionation on Sephadex G-100	32.8	21.8	6.04	277,064	22.5
VIII	Second fractionation on DEAE-cellulose	2.2	5.6	4.27	762,500	15.9

In this preparation, 4.5 kg of calf thymus were processed. See *text* for definition of unit and experimental details. The specific activity is expressed as units/mg of protein.

PREPARATION OF RIBONUCLEASE H

Introductory Remarks. The starting material was commercially available frozen calf thymus. In different tissue samples the specific activity of the enzyme in the crude extract varied between 160 and 270 units/mg of protein, a variation not reflected in the final yield. The entire enzyme activity can be extracted from the organ with 0.1 M phosphate buffer of pH 6.5, but these extracts are not suitable for further purification, although they are useful for the evaluation of other extraction procedures. It proved best to extract the enzyme from the thymus tissue with 0.01 M aqueous thioglycol in a proportion of 3 parts of fluid to 1 part of tissue (v/w).* Polyethylene glycol, used previously for the fractionation of plasma proteins (10), is of great value for the first fractionation step, since otherwise fractionation with $(NH_4)_2SO_4$ cannot be applied successfully.

The pH values and saturation levels of ammonium sulfate stated hereafter apply to a temperature of 25°. The following sequence of purification steps took place at 4°, unless noted otherwise. It is summarized in Table 1.

Buffers. The buffers used in the isolation of the enzyme are designated by letters. *Buffer A:* 0.05 M Tris·HCl (pH 7.8)–0.01 M thioglycol; *buffer B:* same as A, but with 0.05 M KCl; *buffer C:* same as A, but with 0.08 M KCl; *buffer D:* same as A, but with 0.1 M KCl; *buffer E:* same as A, but with 0.15 M KCl; *buffer F:* 0.05 M Tris·HCl (pH 7.5)–0.3 M KCl–0.01 M thioglycol; *buffer G:* 0.1 M Tris·HCl (pH 7.8)–0.01 M thioglycol; *buffer H:* same as G, but with 0.08 M KCl; *buffer I:* 0.2 M sodium acetate (pH 5.4)–0.01 M thioglycol; *buffer J:* same as I, but with 0.05 M KCl; *buffer K:* same as I, but with 0.13 M KCl.

Step I: Crude Extract. The still half-frozen tissue is first cut into slices, 2- to 3-cm thick, which are freed of fat before being sliced further to small cubes which are permitted to thaw. About 4.5 kg were processed in 1 day in 4 portions. The tissue was mixed with 3 parts of 0.01 M aqueous thioglycol and comminuted for 1 min in a low-speed blendor. The mixture was centrifuged (13,000 × *g*, 90 min), the supernatant fluid was poured through a large Büchner funnel equipped with several layers of glass wool in order to remove lipids, and the filtrate was stored overnight at 4°.

Step II: Precipitation with Polyethylene Glycol. The extract is diluted with 0.01 M thioglycol solution to bring the protein concentration to 9–10 mg/ml, and 1 M phosphate buffer of about pH 5.9 (9 parts KH_2PO_4 and 1 part K_2HPO_4) is added to produce a molarity of 0.1 M. The subsequent operation requires vigorous agitation with a motor stirrer, as the solution becomes increasingly viscous. Powdered polyethylene glycol (80 g/liter) is added slowly, with the avoidance of lumping. When all is dissolved, slow stirring is continued for 1 hr and the mixture is centrifuged (13,000 × *g*, 30 min). The sediment, retaining about 10% of the activity, is dis-

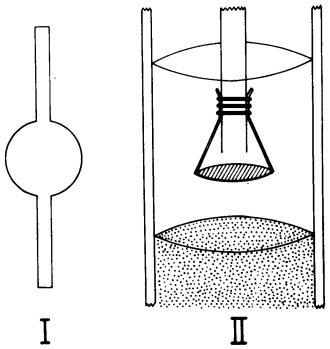

FIG. 1. Arrangement for delivery of solutions to surface of gel bed. A piece of Mylar or another flexible material is cut into the shape indicated in *I*, with the diameter of the disc thrice that of the delivery tube; the device is fastened to the latter, as shown in *II*. The delivery tube is placed about 0.5 cm above the surface of the adsorbent.

* When aqueous thioglycol was applied to thymus in the proportions 2/1, 3/1, 4/1 (v/w), the percentages of enzyme extracted were 83, 65, and 20% of the total activity, respectively. The remaining activity could be extracted from the sediments with phosphate buffer. The 3/1 proportion was chosen because these extracts retained their entire activity for 3 days at 4°, whereas all others lost 20% of their activity in 24 hr.

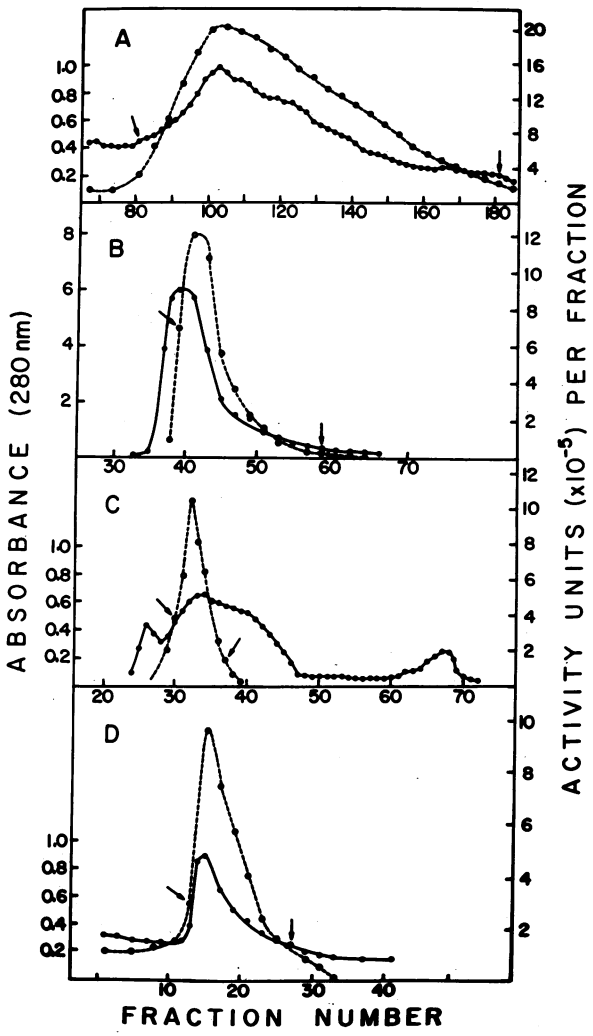

FIG. 2. Final steps of purification of ribonuclease H of calf thymus. The eluate fractions between the *two arrows* were used in the subsequent step. The steps are numbered as in Table 1. In all profiles, the enzyme activity is indicated by *open circles*, the protein concentration by *closed circles*. (*A*) Step V, fractionation on CM-50 at pH 5.4; 8-ml fractions; 78.5% of the activity applied after Step IV was recovered and 96% of this was used in the next step. (*B*) Step VI, first fractionation on DEAE-cellulose at pH 7.8; 3.3-ml fractions; 90% of the activity was recovered and 87% of this was used. (*C*) Step VII, fractionation on Sephadex G-100 at pH 7.5; 2-ml fractions; 87% of the activity was recovered and 86% of this was used; (*D*) Step VIII, second fractionation on DEAE-cellulose at pH 7.8; 2-ml fractions; 95% of the activity was recovered and 83% of this was used for isolation.

carded and the solution is brought to 0.2 M by the addition of the 1 M phosphate buffer. The bulk of the enzyme now is precipitated by the addition, with vigorous stirring, of 136 g/liter of polyethylene glycol, and slow stirring is continued for 90 min. The mixture, including the foam, is centrifuged (13,000 × *g*, 30 min) and the cups are kept inclined for 15 min, in order to drain the supernatant away from the rubbery sediment. The liquid then is drawn off and the cup walls are rinsed with cold water without touching the sediments. The sediments are cut into small pieces and dissolved in 700 ml of 0.1 M phosphate buffer (pH 6.8) containing 0.01 M thioglycol.† At this step the enzyme can be stored if several preparations are to be worked up simultaneously. With a protein concentration of 30–35 mg/ml and under argon it can be kept for more than 2 weeks at 4° without loss of activity.

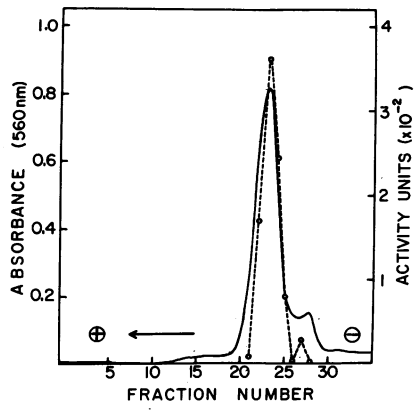

FIG. 3. Polyacrylamide gel electrophoresis of ribonuclease H after Step VIII. The electrophoresis was performed at 4 mA per gel and pH 8.9 in 7% acrylamide at 5°. The gel then was halved; one half was cut into fractions and assayed for enzyme activity, the other half was stained with Coomassie Brilliant Blue and destained, both according to a recent paper (11), and scanned in a Gilford spectrophotometer.

Step III: Fractionation with Ammonium Sulfate. The enzyme solution resulting from the preceding step is diluted with 0.1 M phosphate–0.01 M thioglycol (pH 6.8) to a protein concentration of 15 mg/ml, and solid $(NH_4)_2SO_4$ (243 g/liter) is added slowly with gentle stirring to achieve 40% saturation. The mixture is stirred for 1 hr and centrifuged (16,000 × *g*, 1 hr).‡ The addition to the supernatant liquid of 76.6 g/liter of $(NH_4)_2SO_4$ produces 52.5% saturation. The mixture, after being stirred for 1 hr, is centrifuged (16,000 × *g*, 1 hr), and the sediment is taken up in 300 ml of Buffer A.

Step IV: Removal of Nucleic Acid. The solution resulting from the preceding step is diluted with 1.5 liter of Buffer A and applied to a DEAE-cellulose column (4 × 20 cm), previously equilibrated with 500 ml of the buffer, at a flow rate of 300 ml/hr. In this experiment and in all subsequent steps the columns were equipped with the device shown in Fig. 1. After the collection of the effluent, which contains the bulk of the enzyme, the column is washed with Buffer E until the absorbance of the eluates at 280 nm drops below 0.5. From the combined effluents the enzyme is again precipitated by the addition of 351 g/liter of $(NH_4)_2SO_4$ and stirring is continued for 2 hr. After centrifugation (16,000 × *g*, 1 hr) the sediment is dissolved in 50 ml of Buffer I.

Step V: Fractionation on CM-Sephadex. The enzyme solution is freed of ammonium sulfate by being passed through a

† Since phosphate interferes with Co^{2+} in the assay mixture, a small portion of the sediment was dissolved in Buffer A for the assay of enzymic activity.

‡ Residual polyethylene glycol may form a top layer after centrifugation. In this case, the supernatant fluid is poured carefully through cheesecloth fastened to the top of the centrifuge cup.

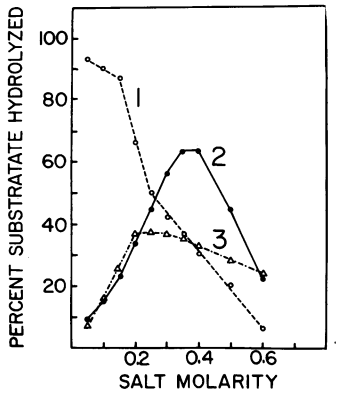

FIG. 4. Activation of ribonuclease H by metal ions as a function of salt molarity. Incubation for 15 min at 35°. All assay mixtures (total volume, 0.12 ml) contained 0.05 M Tris·HCl (pH 8.0); 10 nmol poly(dT)·poly(rA); 50 μg of bovine-serum albumin. *Curve 1:* 25 mM $MgCl_2$; 7 ng of enzyme; indicated molarities of NaCl. *Curve 2:* 20 mM $CoCl_2$; 10 ng of enzyme; indicated molarities of NaCl. *Curve 3:* 2 mM $MnCl_2$; 14 ng of enzyme; 0.01 M thioglycol; indicated molarities of ammonium sulfate. This activation is inhibited by KCl or NaCl. All values shown on *Curves 1* and *3* have been normalized by computation to an enzyme concentration of 10 ng per assay.

Sephadex-G50 column (4 × 30 cm) previously equilibrated with Buffer J. The effluent is diluted to 1 liter with the same buffer. In the meantime a batch of Sephadex CM-50 has been equilibrated with Buffer J and filled into a column (8 × 16 cm) that is again equilibrated with the buffer. The diluted enzyme solution is applied, at a flow rate of 400 ml/hr, to this column, which is used within 4 hr after its preparation (8).§ The column is washed with 1–2 liters of the same buffer until the absorbance of the eluates at 280 nm equals, or is below, 0.2. The enzyme then is eluted with Buffer K at a flow rate of 400 ml/hr, fractions of 8 ml being collected. A typical elution diagram is shown in Fig. 2*A*. All fractions showing at least a 50% enrichment over the preceding step are combined, and the enzyme is concentrated by precipitation with 430 g/liter of $(NH_4)_2SO_4$ and collected as in Step III. The suspension of the sediment in 10 ml of Buffer A is dialyzed overnight against 4 liters of the same buffer, during which time the enzyme goes into solution.

Step VI: First Fractionation on DEAE-Cellulose. The enzyme solution, diluted with 120 ml of Buffer A, is applied to a column (2.5 × 23 cm) of DEAE-cellulose, equilibrated previously with Buffer A, at a flow rate of 100 ml/hr. The column is washed with Buffer G (70 ml/hr) until the absorbance of the eluates at 280 nm does not surpass 0.2. Elution of the enzyme then is performed with Buffer H with the collection of fractions of 3.3 ml (Fig. 2*B*). Eluate fractions showing at least a 1.5-fold enrichment are combined, precipitation with 430 g/liter of $(NH_4)_2SO_4$ is again performed, and the precipitate is collected (25,000 × *g*, 1 hr) after stirring for 1 hr. It is suspended in a glass–Teflon grinder in 25 ml of $(NH_4)_2SO_4$ (277 g/liter of Buffer A); the suspension is stirred for 1 hr and centrifuged (25,000 × *g*, 1 hr). From the supernatant solution the enzyme is precipitated by the addition of more $(NH_4)_2SO_4$ (134 g/liter) to achieve a saturation of 65%. Stirring is continued for 2 hr and followed by centrifugation (25,000 × *g*, 1 hr). The sediment is dissolved in 2 ml of Buffer F and the solution is adjusted with the same buffer to a protein concentration of 20 mg/ml.

§ A precipitate that forms during the dilution of the enzyme solution collects at the top of the column. It does not interfere with washing and elution. Before the latter operation, however, the top portion of the adsorbent is gently stirred up and permitted to settle in order to prevent channel formation.

TABLE 2. *Inhibition of ribonuclease H by S-adenosylmethionine*

S-Adenosylmethionine (mM)	Percent inhibition with specified substrates and metal ions					
	poly(dT)·poly(rA)			Phage f1 DNA–RNA hybrid		
	Co	Mn	Mg	Co	Mn	Mg
1.5	70	65	83	75	71	85
3	95	85	100	93	89	100
6	100	100	100	100	100	100

Uniform conditions: incubation for 15 min at 35°; total volume, 0.12 ml; 0.05 M Tris·HCl (pH 8.0); 50 μg of bovine-serum albumin; 10 nmol of substrate; indicated amounts of *S*-adenosylmethionine. Variations: (*1*) 20 mM $CoCl_2$, 0.35 M NaCl, 0.01 μg of enzyme; (*2*) 2 mM $MnCl_2$, 0.2 M ammonium sulfate, 0.014 μg of enzyme; (*3*) 25 mM $MgCl_2$, 0.05 M NaCl, 7 ng of enzyme.

Step VII: Fractionation on Sephadex G-100. This adsorbent is packed into a column (1.5 × 85 cm) 2 days before use and equilibrated with Buffer F at a flow rate of 20 ml/hr. The enzyme solution is applied to the column in two equal portions of 1.55 ml. After the elution of the first portion with the same buffer (20 ml/hr; 2-ml fractions) the second portion is placed on the column and eluted in the same manner. The elution profile of one of the portions is shown in Fig. 2*C*. Again, all fractions enriched at least 1.5 times are combined.

Step VIII: Second Fractionation on DEAE-Cellulose. The column (1.5 × 8 cm) is equilibrated with Buffer A. The enzyme solution is placed on the column in 2-ml portions, each being diluted with 3 volumes of aqueous 0.01 M thioglycol directly before application, and a flow rate of no less than 50 ml/hr is maintained. The column is washed with Buffer B until the absorbance of effluents has dropped to 0.05. Washing is continued with two bed volumes of Buffer C (10 ml/hr) and the enzyme then is eluted with Buffer D, as shown in Fig. 2*D*¶. To the combined eluates $(NH_4)_2SO_4$ (430 g/liter) is added slowly, so as to reduce foaming; stirring is continued for 2 hr, followed by centrifugation (20,000 × *g*, 30 min). The centrifuge cups are drained completely, and the sediment is suspended in 5 ml of $(NH_4)_2SO_4$ solution (295 g/liter of Buffer A); the suspension is stirred for 1 hr and centrifuged (16,000 × *g*, 30 min). The sediment retains 10–15% of the activity. It is taken up in the storage buffer, consisting of 0.05 M Tris·HCl (pH 7.5), 0.4 M NaCl, 0.03 M $MgCl_2$,

¶ It is possible to elute the enzyme with more of Buffer C, but the eluates are extremely dilute and the enzyme loses activity rapidly. For this reason, care should be taken to use no more than the specified volume of this buffer for washing. Even the eluates with Buffer D are fairly dilute so that in the first and last fractions protein could not be determined accurately. For this reason, all fractions containing at least 2% of the total activity applied to the column were taken.

0.5 mM dithiothreitol, and 50% glycerol, and kept at −20°. The bulk of the enzyme is precipitated from the supernatant fluid by addition of $(NH_4)_2SO_4$ (117 g/liter); the mixture is stirred for 2 hr and centrifuged (16,000 × *g*, 1 hr). The sediment is also taken up in the storage buffer and stored at −20°. Under these conditions, the enzyme is stable.

PRELIMINARY REVIEW OF PROPERTIES AND CONCLUDING REMARKS

Ribonuclease H is a slightly acidic protein with an isoelectric point of 4.95. Its molecular weight, estimated from gel filtration, is 64,400; from sucrose density gradient centrifugation, the value is 63,000, when a partial specific volume of 0.725 is assumed. Electrophoresis in the presence of sodium dodecyl sulfate showed three main bands corresponding to molecular weights of 33,500, 27,500, and 17,000. Whether the enzyme consists of several units or is a dimer of the heaviest of these components remains to be decided. The gel electropherogram of the purified enzyme itself (Step VIII in Table 1) is shown in Fig. 3. The active protein corresponds to about 88% of the total. The nature of the slower shoulder requires further study; since the enzyme is quite unstable, especially in dilute solutions, this may be an inactivation product. It is noteworthy that the 280/260 absorbance ratio of the enzyme is 1.57; it is possible that it contains a nucleotide component as constituent or as contaminant.

The final preparation of ribonuclease H is entirely devoid of ribonuclease activity of the pancreatic type[‖]. When tested with the DNA–RNA hybrid of phage f1 DNA its enzymic activity [as % of that displayed with poly(dT)·poly(rA) as substrate] was: 66.5% in the presence of Mg ions, 61.7% in the presence of Co ions, and 54.3% with Mn ions.

The enzyme requires a metal ion for activity; the optimum concentrations are 25 mM Mg^{2+}, 20 mM Co^{2+}, and 2 mM Mg^{2+}. Activation curves in the presence of different salt molarities are shown in Fig. 4. Mg^{2+} ions at a low salt molarity are the most active; Co^{2+} shows 70%, Mn^{2+} 40% of the Mg^{2+} activity. For routine assays during the purification of the enzyme Co ions were chosen, as they act at a high NaCl molarity at which many other interfering enzymes are inactive.

[‖] In the assay procedure described before, the difference of the counts of labeled RNA recovered in the absence and the presence of ribonuclease H was ±400 cpm or ±0.3% of the radioactivity present. The quantity of ribonuclease H used would have sufficed to split 72 mg of poly(rA) in hybrid form.

The enzyme is completely inactivated by *p*-chloromercuribenzoate and by 1.0 mM Hg^{2+} salts; 0.5 mM dithiothreitol has no effect when activation is by Mg^{2+}, but shows an 80% inhibition, reversible by dilution, when activation is performed with Co or Mn ions. More striking is the observation that the enzymic activity is inhibited by *S*-adenosylmethionine (Table 2). The latter cannot be replaced by adenosine triphosphate or its deoxy analogue, or by adenosine 3′,5′-monophosphate.

We are continuing the study of ribonuclease H with respect both to simplifying and refining the isolation procedure and to exploring its usefulness as a diagnostic tool for the investigation of processes of cellular growth. It is believed that the final purification step described here has rendered the preparation free of other interfering enzymes. To indicate some of the directions further work may take, it could be mentioned that this enzyme, as has been suggested recently (12), may be operative in removing the RNA primer portion of a newly made DNA chain. This enzyme could also be of value for probing homopolymer stretches in a DNA or RNA which, only after hybridization with suitable ribo- or deoxyribo-oligonucleotides, should become substrates susceptible of being cleaved by ribonuclease H.

The work has been supported by research grants from the National Institutes of Health, U.S. Public Health Service, and the American Cancer Society. This paper is dedicated to Prof. Edgar Lederer of the University of Paris on his 65th birthday.

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