Partial Purification and Properties of a Nuclease from Chicken Pancreas*

(Received for publication, June 9, 1965)

JAMES ELEY‡ and JAY S. ROTH§

From the Institute of Cellular Biology, University of Connecticut, Storrs, Connecticut 06268

SUMMARY

A nuclease has been isolated from an acid homogenate of chicken pancreas and purified over 300-fold. The purified preparation was free of phosphomonoesterase and phosphodiesterase activity, but rapidly hydrolyzed ribonucleic acid and deoxyribonucleic acid. The enzyme which by several criteria was a single protein required Mg++ or Mn++ for activity; addition of excess ethylenediaminetetraacetate completely inhibited all of the nuclease action. The pH optimum for RNA was around 9.5 and for DNA around 8.8. The enzyme was found to be completely inactivated by heating for 10 min at 70°, but on heating at 80° from one-quarter to one-third of the activity was restored. There appeared to be no free sulfhydryl groups essential for enzyme activity. Iodoacetate at the proper concentration completely inhibited ribonuclease activity, but had little or no effect on deoxyribonuclease activity.

A large number of nuclease have been isolated from a variety of different sources in recent years. These enzymes have proved to be quite useful for several purposes and have been employed as probes of deoxyribonuclease acid conformation to determine sequential arrangements of nucleotides in deoxyribonucleic acid and ribonuclease acid, to remove specific polynucleotides, and to study possible evolutionary relationships among proteins with similar catalytic activity.

Our interest in the chicken pancreas nuclease arose from the chance observation that ribonuclease activity in chicken pancreas was absolutely dependent on the presence of magnesium ion, a property that had not been described before for a ribonuclease. Further study of the chicken pancreatic nuclease showed that it is a true nuclease capable of hydrolyzing RNA and DNA but not synthetic diester substrates. The enzyme appears to be the only one in chicken pancreas capable of hydrolyzing polynucleotide material and although similar nucleases have been described in the literature, none had come from an organism as high in the evolutionary scale as the chicken. In addition, the chicken nuclease has several interesting properties which may be unique and have not been, to our knowledge, observed with any other enzyme. These are complete inactivation by heating at 70° followed by partial reactivation at 80° and complete abolishment of RNase activity by iodoacetate without significant effect on DNase activity.

MATERIALS AND METHODS

Enzyme Assays

Ribonuclease-Assay for RNase was patterned after the method of Roth (2). The routine assay contained, in the order of mixing, 50 mM carbonate-bicarbonate buffer, pH 10, 4 mM MgCl₂, water, and enzyme to give a total volume of 2.0 ml. At zero time 1 ml of 1% RNA was added. After incubating the tubes in a water bath at 37° for 30 min, the reaction was stopped by adding 3.0 ml of precipitating agent and mixing thoroughly. The precipitating agent was a 1 N HCl solution in 76% ethanol containing 0.5% lanthanum chloride. The tubes were allowed to stand for 10 min and were then filtered through 9-cm circles of Whatman No. 42 filter paper with the use of watch glasses to cover the funnels. A 1.0-ml aliquot of the filtrate was diluted 50-fold with water and the absorbance at 260 μ determined. Any deviations from these conditions are noted in the text.

Deoxyribonuclease—The assay contained, in the order of mixing, 50 mM Tris-HCl buffer, pH 8.5, 4 mM MgCl₂, water, and enzyme source to give a total volume of 2.0 ml. At zero time, 1 ml of 0.1% DNA, that had been heated in a boiling water bath for 30 min and then cooled in ice water, was added to give a total volume of 3.0 ml and the tubes were incubated in a water bath at 37° for 30 min. The reaction was stopped by adding 3.0 ml of ice-cold 10% perchloric acid. The tubes were immersed in ice for 10 min and then centrifuged at 2000 × g for 30 min at 4° in an International PR2 refrigerated centrifuge. The supernatant fluid was poured off and its absorbance read at 260 μ. Deoxyribonuclease activity was also measured in some cases by the method of Dische as described by Ashwell (3).

* Supported by Grant P-303 from the American Cancer Society, Grant CB-430 from the National Science Foundation, and Grant CA-57821 from the National Cancer Institute, National Institutes of Health.
‡ Present address, Department of Pathology, University of Colorado Medical School, Denver. This paper was abstracted from a thesis presented in partial fulfillment of the requirements for the Ph.D. degree, University of Connecticut, June 1965.
§ Recipient of Career Award RC-31-63 from the National Cancer Institute, National Institutes of Health.

Note—Curtis, Burdon, and Smellie (1) have reported on a nuclease purified from rat liver mitochondria with properties quite similar to chicken nuclease.
Phosphodiesterase Activity—The method of De Garille and Laskowski was utilized with some modifications (4). A 0.1% solution of calcium bis(p-nitrophenyl) phosphate was prepared and stored in the frozen state. The assay contained 0.5 mg of substrate, 2.7 mM MgCl₂, 33 mM Tris-HCl buffer (pH 8.5), enzyme, and enough water to give a volume of 3.0 ml. The enzyme was added at zero time and the tubes incubated in a water bath at 37° for periods of 30 min to 4 hours. The hydrolysis of the ester was followed by measuring the increase in absorbance at 440 μm. A substrate blank was run by omitting enzyme and in some cases an enzyme blank was also run by omitting substrate. Phosphodiesterase activity was also measured with the 5'- and 3'-p-nitrophenyl esters of thymidylic acid. The method of measurement of the hydrolysis of the 3'-ester was patterned after that of Ralph, Smith, and Khorana (5). The assay contained 0.9 mg of substrate, 80 mM buffer, enzyme source, and enough water to give a total volume of 0.5 ml. The buffers used were Tris-HCl (pH 8.5) and acetate (pH 5.2). In some assays 2 mM MgCl₂ was also added. The tubes were incubated in a water bath at 37° for 4 hours, after which the color was developed by adding 1 ml of 0.1 N NaOH. The amount of ester hydrolysis was determined by measurement of the absorbance at 440 μm. A substrate blank was run at the same time.

The method of measurement of hydrolysis of the 5'-ester was similar to that of Razzell and Khorana (6). The assay contained 0.5 mg of substrate, 2.7 mM MgCl₂, 33 mM Tris-HCl buffer (pH 8.5), enzyme, and enough water to give a volume of 3.0 ml. The tubes were incubated in a water bath at 37° for various times and the absorbance at 440 μm was read after addition of 1.0 ml of 0.1 N sodium hydroxide. A substrate blank was run simultaneously.

Nucleotidase—Measurement of 3'- and 5'-nucleotidase activities was carried out by the methods of Heppel and Hilmoe (7) and Shuster and Kaplan (8), respectively. Protein was measured by the method of Lowry et al. (9) and phosphate by the method of Fiske and SubbaRow as described by Leloir and Cardini (10).

Chemicals and Ion Exchange Agents

DEAE- and carboxymethyl cellulose were obtained from Schleicher and Schuell, DEAE-Sephadex A-50 and CM-Sephadex were obtained from Pharmacia. Calcium phosphate gel and calcium bis(p-nitrophenyl) phosphate were purchased from Sigma. Salmon sperm DNA and 5'-p-nitrophenyl thymidylicate were obtained from Calbiochem. Crystalline bovine pancreatic RNase and DNase were products of Worthington. Schwartz yeast RNA, a product of Schwartz BioResearch, lot No. 6080, was utilized for all of the assays unless otherwise indicated. A 1% solution of this RNA made to pH 7.0 with NaOH was stored frozen and thawed as needed. High molecular weight yeast RNA was prepared by the method of Crestfield, Smith, and Allen (11). Schwartz RNA, free of contaminating metal ions, was prepared by Dr. Robert Wojnar (12). All of the salts were reagent grade. Potassium and sodium phosphate were used interchangeably for buffers unless indicated otherwise. Buffers were either made fresh or stored at 4°. The disodium salt of EDTA was adjusted to pH 7.0 with NaOH.

RESULTS

Preparation and Purification of Enzyme

Chicken pancreases were obtained fresh from a chicken processing plant. The pancreases were immersed in ice water, and extraneous tissue was removed after chilling. If not used immediately they were stored frozen. There was no diminution of enzyme activity in tissue that had been stored at −17° for up to 6 months.

All of the steps in the purification procedure were carried out in a cold room at 4°. Frozen or fresh tissue, 10 g, was homogenized in 100 ml of 0.1 N H₂SO₄ in a TenBroeck glass homogenizer. Homogenization was as rapid as possible and the homogenate was assayed immediately for nuclease activity since the enzyme was rapidly inactivated in the acid medium.

The homogenate was immediately centrifuged at 78,000 × g for 15 min. The precipitate which contained little nuclease activity was discarded and the cloudy supernatant fraction was immediately brought to pH 7.5 with 6 M NaOH. The neutralized supernatant fraction was left at 4° for 15 min and then centrifuged at 78,000 × g for 1 hour.

Ammonium Sulfate Fractionation—The clear supernatant fluid was brought to 55% saturation with ammonium sulfate by adding 0.351 g of salt per ml of fluid with constant stirring. After standing for 15 min, the white precipitate was removed by centrifugation at 35,000 × g for 15 min. The precipitate contained little nuclease activity. The supernatant fraction from the 55% precipitate was brought to 90% saturation with ammonium sulfate by adding 0.264 g of salt per ml of fluid with constant stirring. This was allowed to stand for at least 30 min (standing for up to 24 hours does not affect the results) and then was centrifuged at 35,000 × g for 30 min. The super- natant fraction was discarded and the precipitate was carefully drained and dissolved in 25 ml of 0.05 M phosphate buffer, pH 7.5. The solution was next dialyzed against 4 liters of 0.05 M phosphate buffer, pH 7.5, for 16 to 24 hours. Dialysis for as long as 36 hours caused no significant decrease in enzyme activity although dialysis in 0.025 m buffer for as long as 24 hours caused considerable inactivation.

Fractionation on DEAE-Sephadex A-50—The clear, colorless dialyze was placed on a DEAE-Sephadex A-50 column that had been equilibrated with 0.05 M phosphate buffer, pH 7.5. The column, 1.8 × 40 cm, was filled to a height of about 28 cm and required about 6 g of dry Sephadex. After all of the protein solution had run onto the column, two 2- to 3-ml aliquots of buffer were added to wash down any remaining protein. Elution was first carried out with 50 to 100 ml of the 0.05 M buffer and then with a linear gradient consisting of 200 ml of the 0.05 M buffer in the mixer and 200 ml of 0.2 M KH₂PO₄ in the reservoir. In some cases smaller volumes were used for elution. The column was allowed to run freely; elution usually took 15 to 22 hours. The eluate was collected in 5- to 7-ml portions by means of a fraction collector. An ultraviolet attachment recorded the absorbance at 280 nm of the eluate. The tubes were assayed for nuclease activity and those tubes containing the activity peak were combined.

Dialysis and Calcium Phosphate Gel Fractionation—The DEAE-Sephadex eluate was dialyzed against 4 liters of 0.02 M or 0.05 M phosphate buffer, pH 7.5, for 18 to 24 hours, and the dialyze was thoroughly mixed with calcium phosphate gel (1 g of gel per mg of protein) and allowed to stand in
TABLE I
Nuclease purification

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Amount</th>
<th>Total protein</th>
<th>Total units</th>
<th>RNase</th>
<th>DNase</th>
<th>RNase:DNase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>1350</td>
<td>1020</td>
<td>1060</td>
<td>0.75</td>
<td>0.77</td>
<td>0.61-1.20</td>
</tr>
<tr>
<td>Neutral supernatant</td>
<td>222</td>
<td>823</td>
<td>1002</td>
<td>3.7</td>
<td>4.5</td>
<td>0.78-1.34</td>
</tr>
<tr>
<td>45% precipitate</td>
<td>138</td>
<td>87</td>
<td></td>
<td>0.6</td>
<td></td>
<td></td>
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<tr>
<td>90% precipitate</td>
<td>113</td>
<td>947</td>
<td>768</td>
<td>16</td>
<td>13</td>
<td>0.72-1.14</td>
</tr>
<tr>
<td>90% supernatant</td>
<td>25</td>
<td>343</td>
<td>418</td>
<td>14</td>
<td>17</td>
<td>0.91-1.21</td>
</tr>
<tr>
<td>Dialysate</td>
<td>27</td>
<td>386</td>
<td>391</td>
<td>26</td>
<td>26</td>
<td>0.93-1.25</td>
</tr>
<tr>
<td>DEAE-Sephadex eluate</td>
<td>156</td>
<td>434</td>
<td>359</td>
<td>140</td>
<td>115</td>
<td>0.65-1.19</td>
</tr>
<tr>
<td>Dialyzed DEAE-Sephadex eluate</td>
<td>168</td>
<td>342</td>
<td>225</td>
<td>105</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Gel eluate, 0.2 M</td>
<td>10</td>
<td>0.7</td>
<td>244</td>
<td>184</td>
<td>350</td>
<td>0.75-0.91</td>
</tr>
<tr>
<td>Wash 1</td>
<td>10</td>
<td>0.25</td>
<td>60</td>
<td>68</td>
<td>275</td>
<td>0.85-1.00</td>
</tr>
<tr>
<td>Wash 2</td>
<td>10</td>
<td></td>
<td>68</td>
<td>68</td>
<td>275</td>
<td></td>
</tr>
</tbody>
</table>

* A unit of enzyme activity is defined as the amount of enzyme that gives an increase of 1.0 in the absorbance at 260 μm in the RNase assay.

* The ratios of the specific activity of RNase to DNase for each of the ratios of the specific activities of RNase to DNase are given. The mean is in parentheses.

water for 10 min. The gel was collected in 50-ml centrifuge tubes by centrifuging at 2000 × g for 5 min in the International PR2 refrigerated centrifuge. The collected gel was washed once with 5 to 10 ml of 0.05 M phosphate buffer, pH 7.0, and then extracted once with 5 ml of 0.1 M phosphate buffer and twice with 10 ml of 0.2 M buffer, both pH 7.0. The latter extracts were combined although the major portion of the enzyme appears in the first 0.2 M wash. The purified enzyme could be stored at 4 °C or after lyophilization in a freezer. Little activity is lost after storage for 2 months.

Variations in Purification Procedure—Homogenization in water instead of acid extracted more protein but gave much less pure final product than did homogenization in acid. The use of a second fractionation on DEAE-Sephadex instead of the phosphate gel was tried but gave less purification and more dilution. The enzyme was not readily eluted from a carboxymethyl cellulose or IRC-50 column.

Table I gives the results of a typical nuclease purification from chicken pancreas homogenized in 0.1 N H₂SO₄. Throughout the procedure the specific activities for RNase and DNase maintained a nearly constant ratio with the exception of two fractions, the 90% ammonium sulfate supernatant fraction and the calcium phosphate gel eluate. All of the other fractions have given constant RNase to DNase activity ratios in all of the purifications.

The average purification was about 350-fold and ranged from 300 to 600. Recoveries in each step were generally good, usually 80 to 90% of the nuclease activity processed. The one exception was the 90% precipitate fraction; nuclease recovery here was never greater than 60% of the activity present in the 50% supernatant fraction. If the 90% saturated ammonium sulfate solution was left standing for as long as 24 hours before centrifugation, there was no increase in the activity precipitated.

The unpurified RNase activity was never fully accounted for in the 90% supernatant fraction. In the typical results given in Table I, 40% of the RNase activity was not precipitated in the 90% step and only 15% was found in the supernatant fraction; thus about 25% of the activity was lost. In contrast, the DNase activity (shown also in Table I) was fully recovered in the 90% precipitate plus 90% supernatant fraction. Assays carried out to test the effect of ammonium sulfate on the RNase and DNase activities of the nuclease indicated that this salt had no effect under the conditions utilized.

Fig. 1 gives the elution pattern obtained from a DEAE-Sephadex A-50 column. The nuclease activity usually appeared as two partially separated peaks (occasionally as a single peak). The RNase and DNase activities always coincided. Protein came out in three major fractions, one ahead of the nuclease activity and the remaining two coincident with the two enzyme activity peaks. Since two nuclease peaks occurred, it was thought that two enzymes might be present. To test for this possibility, each tube of the eluate was assayed both with and without MgCl₂; in all cases, MgCl₂ was necessary for activity. It appears likely that the two peaks represent two different forms of the same enzyme, either a slight chemical modification brought about by the acid treatment or perhaps a conformational change which causes a difference in behavior on the cellulose column.

In the last column of Table I are listed the deviations and averages of the ratios of the specific activities of RNase to DNase for each of the fractions. The ratios are quite constant throughout the purification procedure. The only significant deviation from a ratio of 1 was in the 90% supernatant fraction which reflects the poor recovery of RNase in this fraction previously cited.

Contaminating Enzymes in Purified Enzyme Preparation—Several tests were carried out on purified enzyme fractions for the presence of contaminating enzymes. A DEAE-Sephadex eluate (0.38 unit) with a specific activity of 98 or a calcium phosphate gel eluate with a specific activity of 190 (1.5 units) gave no hydrolysis of calcium bis-p-nitrophenyl phosphate after 4 or 8 hours of incubation, respectively. The gel eluate also did not hydrolyze thymidine 5'-p-nitrophenyl phosphate in 8 hours of incubation. Thymidine 3'-p-nitrophenyl phosphate was not hydrolyzed in 4 hours by 0.9 unit of a gel eluate with specific activity of 210. From these results it appeared that the purified preparations were free of phosphodiesterase activity. Nucleotidase activity was assayed at pH 7.8 and 8.7. No release of
Fig. 1. Elution pattern of protein, RNase activity, and DNase activity from a DEAE-Sephadex A-50 column. The column, 2 X 21 cm, contained 4 g (dry weight) of absorbent. At tube 1, 28 ml of a dialysate containing 28 mg of protein were added. Elution by a linear gradient was started at tube 12. The reservoir contained 125 ml of 0.3 M NaH₂PO₄ and the mixer contained 125 ml of 0.05 M phosphate buffer, pH 7.5. The eluate was collected in fractions of about 5 ml. Every other tube was assayed for RNase and DNase activity and the absorbance at 230 nm of each tube was read.

Fig. 2. Heat inactivation of purified enzyme at 70 ° and 80 °. The enzyme source was a gel eluate with a specific activity of 190. Aliquots of 0.4 ml were heated at 80 ° for the times indicated, cooled, diluted to 0.8 ml with water, and assayed. For procedures, see Fig. 3 and text. Control samples that were not heated, but were otherwise treated in the same manner as the heated samples, gave absorbance values of 0.237 for the RNase assay and 0.148 for the DNase assay.

Properties of Nuclease

Heat Stability—As indicated in Fig. 2, the partly purified enzyme is completely inactivated by heating for 10 min at 70 °. On heating, however, at 75-80 °, considerable activity was retained; on the other hand, an aliquot heated in boiling water for 10 min retained little or no activity. In the experiment illustrated in Fig. 2, aliquots of purified enzyme with a specific activity of 190 were heated at either 70 ° or 80 ° for the times indicated. After 10 min of heating at 80 °, 64% of the initial RNase and 56% of the initial DNase activity remained; percentages are based on the activity of the unheated control. The activities slowly decreased with increase in the time of heating, but even after 60 min, 22% of the RNase and 24% of the DNase activity still remained. No nuclease activity was detected in any of the samples heated at 70 °.

The results of these two experiments support the proposal that one enzyme is responsible for both the RNase and DNase activities. From the behavior of the enzyme to heating, it is clear that there is complete inactivation at 70 ° followed by a partial reactivation at 80 °. This is an unusual phenomenon and may indicate partial rearrangement of the enzyme molecule into a different conformation which has, nevertheless, some enzyme activity.

pH Optima—The nuclease activities at various pH values are given in Fig. 3. Each assay was performed in triplicate. Two of the samples were mixed in test tubes in the usual way (see “Materials and Methods”) and the third was mixed in a 5-ml beaker and also incubated in a water bath at 37 °. The pH in the beakers was read immediately after mixing and then again after 27 min of heating in the water bath. The initial pH readings are plotted in the graph since the change in pH during the assay was never greater than 0.08 and was usually about 0.0 pH unit.

The pH curve for RNase activity as depicted in Fig. 3 shows no activity below pH 7.5 and a sharp peak of activity at pH 9.7 to 9.9. Another experiment (not illustrated), in which the region from pH 8.6 to 10.4 was investigated more thoroughly, gave a pH optimum of 9.3 to 9.8 with little activity at pH 10.3.

The relationship between DNase activity and pH is also given in Fig. 3. This experiment was conducted in the same manner as cited above for RNase. The results indicated that activity extended over a wide range from pH 5 to 10 with a maximum around pH 8.8. In this case, heat-denatured DNA was used as substrate, but a similar experiment with native DNA gave nearly identical results. Heat-denatured DNA is hydrolyzed 2 to 4 times faster than native DNA.
For these experiments acetate, phosphate, Tris, glycine, and carbonate buffers were used with an overlap of pH between each set; except for the acetate-phosphate range, all of the buffers were 0.1 M. Glycine buffers gave significantly lower activities (90 to 93%) for the RNase assay than did carbonate-bicarbonate buffers of the same pH.

Effect of Metal Ions on Nuclease Activities—The procedure for these experiments and the results are given in Table II. In all of the cases the percentage values are based on the activity of a control which contained Mg++ as the only metal ion. The results in Table II indicate that Ni++, Zn++, Pb++, Hg++, and Cu++ inhibited both RNase and DNase activities. In other experiments not cited in this table, it was found that Hg++ could inhibit RNase activity completely when RNA prepared according to the directions of Crestfield, Smith, and Allen (11) was the substrate; RNase activity was not restored when excess Mg++ was added. Assays were also carried out for each of the salts listed in Table II in the absence of MgCl₂. The only other metal ion which activated the enzyme was Mn++. The manganese-activated enzyme retained both nuclease activities. These results indicate that metal ion effects on RNase and DNase activities were qualitatively the same but quantitatively different. Based on the assumption that one enzyme is responsible for both activities, these differences may arise from two distinct hydrolytic mechanisms for the two substrates. Another possibility is that the metal ions interact to different degrees with the two substrates.

Effect of Sulfhydryl Inhibitors on Nuclease Activities—Table III presents the effects of p-chloromercuribenzenesulfonic acid, sodium arsenite, and iodoacetate on the RNase and DNase activities of the enzyme. Solutions of p-chloromercuribenzenesulfonic acid and iodoacetate were adjusted with NaOH to pH 8.5 for the DNase assay and to pH 9.5 for the RNase assay. The assay tubes were incubated in a water bath at 37°C for 15 min prior to adding the substrate. Blanks to which no enzyme was added were run for each sample. The amount of enzyme used per assay had no detectable absorption at 260 μμ. Enzyme controls contained no p-chloromercuribenzenesulfonic acid or iodoacetate.

Another experiment identical with that above was run with RNA prepared according to the method of Crestfield, Smith, and Allen (11). Neither RNase nor DNase activities were much affected by sodium arsenite or p-chloromercuribenzenesulfonic acid but iodoacetate completely inhibited RNase activity while it did not affect DNase activity to an appreciable extent. A similar experiment with a p-chloromercuribenzenesulfonic acid concentration twice as great as used here gave identical results for RNase activity and gave 64% of the control value for the DNase assay. The experiments were done with heated DNA. A blank space indicates that the sample was not run.

Table II

<table>
<thead>
<tr>
<th>Additions to assay</th>
<th>RNA³</th>
<th>Heated DNA⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Schwarz</td>
<td>Crestfield, Smith, and Allen²</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>33</td>
<td>44</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>82</td>
<td>98</td>
</tr>
<tr>
<td>ZnCl₂</td>
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<td>40</td>
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<tr>
<td>HgCl₂</td>
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</tr>
<tr>
<td>FeSO₄</td>
<td>93</td>
<td>96</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>89</td>
<td>89</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>72</td>
<td>37</td>
</tr>
<tr>
<td>Fe(NO₃)₃</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>110</td>
<td>95</td>
</tr>
<tr>
<td>PbCl₂</td>
<td>75</td>
<td>25</td>
</tr>
</tbody>
</table>

- The concentrations of the additions were 1.7 X 10⁻³ M except for PbCl₂ which was 7.0 X 10⁻⁴ M.
- The assay procedures as described in "Materials and Methods" were followed. The components were mixed in the following order: water, nucleic acid, MgCl₂, metal salt, buffer, and at zero time the enzyme was added. The substrates are indicated in each column. Carbonate buffer was used for all of the RNase assays except for those containing PbCl₂. In these assays Tris-HCl was used. The latter buffer was used for all of the DNase assays. All of the assays were done in 0.05M MgCl₂. Those carried out with RNA prepared according to Crestfield, Smith, and Allen (11) had all of the components decreased by one-half.
- The percentage values are based on the activity of controls, which contained 4 μM MgCl₂ as the only metal salt, taken as 100%.
- The assay for the control contained excess of enzyme per tube and the DNase assays contained 0.24 unit of enzyme per tube. Each value represents the average of two assays. The experiments were done with heated DNA. A blank space indicates that the sample was not run.

³ RNA prepared according to the method of Crestfield, Smith, and Allen (11).
The enzyme source was a gel eluate with a specific activity of 190. Each assay contained 0.1 unit of enzyme.

<table>
<thead>
<tr>
<th>Reagent in assay</th>
<th>Concentration</th>
<th>RNase activity</th>
<th>DNase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unheated enzyme</td>
<td>Heated enzyme</td>
</tr>
<tr>
<td>p-Chloromercuribenzene sulfonic acid</td>
<td>$4.3 \times 10^{-4}$</td>
<td>98</td>
<td>86</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>$7.7 \times 10^{-4}$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NaAsO$_2^-$</td>
<td>$1.7 \times 10^{-3}$</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* The assay procedures are described in "Materials and Methods." All of the components, except the substrates, were mixed and incubated in a water bath at 37° for 15 min. At zero time the substrates were added. Controls were run without any p-chloromercuribenzene sulfonic acid, iodoacetate, or NaAsO$_2^-$. Each figure represents the average of two assays for RNase and three assays for DNase. Results from three different experiments are given for the DNase activity. Two other similar experiments were also performed for RNase activity; the results were the same as those listed.

DISCUSSION

The chicken pancreas enzyme under investigation has been classified as a nuclease because of the following facts. (a) It hydrolyzes both RNA and DNA. (b) It does not hydrolyze any of the three synthetic substrates, calcium bis(p-nitrophenoxy)phosphate, thymidine 3′- or 5′-p-nitrophenoxyphosphate.

The evidence for assignment of both RNase and DNase activities to one enzyme may be summarized as follows. (a) During purification of over 300-fold, the ratio of the specific activities (RNase to DNase) remains nearly constant. (b) Both activities show the same unusual behavior of inactivation followed by reactivation on heating. (c) Both activities require Mg$^{++}$ or Mn$^{++}$ and can be completely inhibited by EDTA. (d) Hydrolysis of both RNA and DNA gives products terminated in a 5′-phosphate and 3′-hydroxyl. (e) Both activities have an alkaline pH optimum. (f) No physical separation of the two activities was obtained by any of a variety of methods tried.

Despite this ample evidence that only one enzyme is involved, there are two facts not in accord with this conclusion. (a) Iodoacetate eliminated the ability of the nuclease to hydrolyze RNA, but it had little or no effect on its ability to hydrolyze DNA. (b) Various metals had the same qualitative effect on both activities, but the quantitative effect was different in some cases.

The latter fact can be rather easily dismissed since the binding of metal cations by the two different substrates is probably quite different. The effect of iodoacetate is not so easily explained. Here it is unlikely that the compound acted on the substrate and altered the RNA in such a manner that the enzyme could no longer degrade it. If such were the case, the presence of similar reactive groups in both RNA and DNA would require that the hydrolysis of each substrate be affected to about the same extent. This was certainly not the case. It seems much more likely that the effect of iodoacetate was on the enzyme itself which leads to two possibilities. (a) Separate enzymes were responsible for the ability to hydrolyze RNA and DNA, and (b) one enzyme was responsible for both hydrolytic activities, but iodoacetate affected a site absolutely necessary for RNase activity but not involved in DNase activity. The latter possibility is an intriguing one in view of the fact that p-iodoacetate at pH 5.5 reacts with bovine pancreatic RNase combining specifically with a histidine group and inactivating the enzyme (13). This suggests the possibility that the chicken nuclease may have at least one active site similar to that of bovine RNase.

At a stage of evolution as high as the chicken, the presence of one enzyme that hydrolyzes both RNA and DNA and which may have two totally or partially separate active sites is of considerable interest. It is significant that it appears that all of the RNase and DNase activity that is present in the pancreas fractions obtained during purification can be attributed to this enzyme. Thus it is possible that there is combined in this single protein, functions that are performed by separate enzymes in higher organisms, e.g. bovine pancreatic RNase and DNase. It is interesting to note that sometime after this paper was written, Rahman (14) reported that an RNase occurs in rat liver which has a pH optimum of 9.5, is strongly inhibited by EDTA, and is activated by Mg$^{++}$. It was not determined whether this enzyme can degrade DNA. Interestingly enough, also this enzyme was strongly inhibited by monovalent cations (14).

Since p-chloromercuribenzenesulfonic acid and sodium arsenite did not seriously affect the enzymatic activities, it can be tentatively concluded that free sulfhydryl groups are not required for enzyme activity. However, further studies are necessary to establish this point firmly and to determine whether there are —SH groups in the protein.

The reactivation of the enzyme at temperatures greater than 70° is another interesting aspect of this protein. We are not aware of any other enzyme that shows this phenomenon and thus it is presented as the strongest evidence that one enzyme hydrolyzes both RNA and DNA. The most obvious explanation is that during heating the conformation of the molecule changes so that around 70° most or all of the enzyme is in an inactive form. Upon heating at a higher temperature, 80°, enough energy is available for the molecule to assume an active form, presumably differing in conformation from the original form. The only evidence to suggest that a new conformation may be obtained is the fact that the DNase activity of the heat-inactivated enzyme was inhibited more by iodoacetate than was the unheated enzyme. The number of experiments performed was too few to attribute much significance to this last observation, but it does present a possible tool for investigation of this question. Further purification and studies on the macromolecular properties of the enzyme and on its amino acid composition and sequence are in progress.

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James Eley and Jay S. Roth


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