ABSTRACT

The nuclease described by Carell, E.F., Egan, J.M. and Pratt, E.A. [Arch. Biochem. Biophys. (1970) 138, 26-31] has been purified 1000-fold from <i>Euglena gracilis</i> strain Z. The enzyme catalyzes the hydrolysis of both polyribonucleotides and polydeoxyribonucleotides. The relative rates of hydrolysis of synthetic and natural polynucleotides was found to be: poly (U) 100, poly (dT) 33, denatured calf-thymus DNA 33, yeast tRNA 9, E. coli total RNA 6, poly (da-dT) 5, poly (A) < 1, poly (C) < .05, and poly (G) < .05. The enzyme attacks polynucleotides in an endonucleolytic fashion, yielding products terminated with a 3'-phosphate. Poly (U) appears to be hydrolyzed completely to 3'-UMP; both RNA and DNA appear to have some phosphodiester bonds resistant to enzyme catalyzed hydrolysis. Because of its mode of action and its inducibility by light, we propose the name endonuclease L for this enzyme.

In 1970, Carrell, Egan and Pratt reported the presence of a deoxyribonuclease in crude extracts of <i>Euglena gracilis</i> that preferred denatured to native DNA as substrate, had a pH optimum of 9.4, and was activated by calcium ions. The enzymatic activity was low in dark adapted cells and increased several fold during the light-induction of chloroplast development. This light-dependent increase in enzymatic activity was abolished by puromycin suggesting that a light-induced synthesis of enzyme was occurring. In this report we show that the <i>Euglena</i> enzyme catalyzes the hydrolysis of both polyribonucleotides as well as polydeoxyribonucleotides in an endonucleolytic fashion. Because of these properties and its inducibility by light we feel the name <i>Euglena</i> endonuclease L is more appropriate than "<i>Euglena</i> alkaline deoxyribonuclease" as suggested.

MATERIALS AND METHODS

<i>Cells</i> -- <i>Euglena gracilis</i> strain Z was obtained from the algae collection, Indiana University (#753), <i>Escherichia coli</i> Hfr X-839 OR11 (thy- pyr-) was obtained from Roy Curtiss III through the courtesy of James N. Adams (Department of Microbiology, The University of South Dakota). Bacteriophage PM2 and its host, <i>Pseudomonas</i> BAL-31, were obtained from R.B. Setlow.
Polynucleotides -- E. coli [³H]DNA was extracted and purified by the method of Miura³ from E. coli Hfr X-839 OR11 (thy-pyr" ) grown in the presence of 0.25 μCi/ml of [methyl-³H]-thymidine. E. coli [³H]RNA was isolated by phenol extraction of cells grown in nutrient broth containing 1.6 μCi/ml of [5-³H]-uridine by the method of Bolton⁴. The isolated RNA was approximately equally labeled in the uracil and cytosine moieties. The radioactive RNA was diluted with non-radioactive RNA isolated in the same way to achieve a specific activity of 1000 cpm/nmole. Poly (A), poly (C), poly (G), poly (U), poly (dT) and poly (dA-dT) were purchased from P-L Biochemicals.

PM2 DNA labeled with [³H] thymidine was isolated by a slight modification of published procedures⁵. The isolated DNA had a specific activity of 6000 cpm/nmole.

Determination of DNA, RNA, and Protein -- The concentration of DNA was determined by the method of Burton⁶ using calf thymus DNA (Worthington Biochemical Corporation) as standard. The concentration of calf thymus DNA was based on phosphate content determined according to the method of Ames and Dubin⁷. RNA concentrations were estimated from the absorbance at 260 nm, assuming that an absorbance of 1.00 corresponds to 150 nmole/ml. All concentrations of nucleic acids are expressed as nucleotide equivalents. Protein was determined by the method of Lowry et al.⁸ or the spectrophotometric method of Warburg and Christian⁹.

Enzymatic Assays -- DNase and RNase activities were assayed either by the production of acid-soluble fragments or the hyperchromicity using a Gilford recording spectrophotometer. For the production of acid soluble fragments from DNA the assay mixture (0.3 ml) contained 0.1 M sodium glycinate (pH 9.4), 25 mM CaCl₂, 0.1 mM EDTA, 300 nmoles of heat denatured calf thymus DNA (Sigma, Type V) and enzyme appropriately diluted in a solution composed of 0.01 M Tris (pH 9.1) -0.2 mg/ml bovine serum albumin 0.1 mM EDTA. Incubations of 15 to 60 min at 37° were stopped by the addition of 0.5 ml of 3.5% HClO₄. After 5 min at 0° the solution was centrifuged at 12,000 x g for 10 min and 0.5 ml of the supernatant solution was diluted to 1.0 ml with H₂O and the absorbance at 260 nm measured. A unit of DNase activity was defined as the amount of enzyme producing one nmole of acid-soluble material (nucleotide equivalents) in 15 min assuming a millimolar extinction coefficient of 10.0. For the experiment using various detergents to solubilize endonuclease L (Table I), 5 nmoles of denatured (³H)DNA (5500 cpm/nmole) was substituted for the calf thymus DNA because of the large amount of 260 nm absorbing material in the controls.

The production of acid-soluble fragments from RNA was measured exactly the
Crude enzyme was prepared as described under purification. An ammonium sulfate fraction was prepared by adding 262 g per liter of crude extract, centrifuging for 10 min at 15,000 x g, and suspending the pellet in .05 M Tris, pH 7.4 to give a volume equal to 0.3 that of the crude. Five ml aliquots of the ammonium sulfate fraction were made 1% with respect to detergent, incubated 15 min at 37° with periodic stirring, followed by centrifugation for 10 min at 15,000 x g. The resulting pellets were re-extracted with 6 ml of 1% detergent - .05 M Tris, pH 7.4, the two supernatant solutions combined and assayed using radioactive DNA (see Methods).

<table>
<thead>
<tr>
<th>Detergent Used</th>
<th>Protein mg/ml</th>
<th>Total Units</th>
<th>Units/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>4.1</td>
<td>111</td>
<td>3.6</td>
</tr>
<tr>
<td>2. Triton X-100</td>
<td>8.9</td>
<td>617</td>
<td>5.1</td>
</tr>
<tr>
<td>3. Sarkosyl</td>
<td>13.9</td>
<td>2625 (1075)*</td>
<td>14.8</td>
</tr>
<tr>
<td>4. Sodium deoxycholate</td>
<td>3.4</td>
<td>1780 (1700)*</td>
<td>40.5</td>
</tr>
<tr>
<td>5. Brij 58</td>
<td>7.6</td>
<td>542</td>
<td>5.3</td>
</tr>
<tr>
<td>6. Lubrol PX</td>
<td>7.1</td>
<td>456</td>
<td>4.8</td>
</tr>
</tbody>
</table>

*Assayed after dialysis for 20 hrs versus .01 M Tris, pH 7.4 followed by a 10 min centrifugation at 15,000 x g to remove insoluble material formed during dialysis.

The measurement of hyperchromicity upon hydrolysis of DNA, the assay mixture (1.0 ml) contained 0.1 M sodium glycinate (pH 9.4), 24 mM CaCl₂, 0.1 mM EDTA, 150 nmoles of heat denatured calf thymus DNA, 40 µg bovine serum albumin and enzyme. A full scale of 0.1 absorbance was utilized and the increase in absorbance measured over a period of about 10 to 15 min at 30°.

The measurement of hyperchromicity upon hydrolysis of RNA was carried out as for the DNA except 165 nmoles of E. coli RNA was the substrate and the CaCl₂ concentration was 15 mM.
The assay conditions for the nicking of PM2 double stranded circular DNA was the same as for the production of acid soluble fragments except 5 nmoles of PM2 DNA was added and the reaction was terminated with .02 ml 0.5 M EDTA, pH 7. The amount of intact double stranded circles was determined by layering 0.1 ml of the assay mixture on top of 5 to 20% alkaline sucrose gradients. The 5 and 20% alkaline sucrose contained 0.5 M NaCl, 1 mM EDTA, and 0.2 N NaOH. Centrifugation was for 60 min at 45,000 rpm at 20°. About 25 fractions were collected on paper strips and processed as described.

*Papex Chromatography -- System 1:* ascending chromatography with 65% isopropanol: 16.7% concentrated HCl : 18.3% H2O (v/v) as solvent. Whatman No. 1 paper was used. This system is capable of differentiating 5'-UMP, 3'-UMP, and 2', 3'-cyclic UMP

*System 2:* descending chromatography in 76% ethanol; Whatman No. 1 paper that had been dipped in a 0.1 saturated solution of (NH4)2SO4 (pH 7.0) and dried was used. This system is capable of separating all four ribonucleosides or their monophosphates.

*System 3:* descending chromatography with sec-butanol; n-propanol; 1M ammonium acetate (pH 4.8), 5 : 3 : 3 (v/v) as solvent. Whatman No. 1 paper was used. This system can differentiate 3'-dTMP from 5'-dTMP.

**RESULTS**

*Solubilization with Detergents -- Preliminary experiments demonstrated* that the bulk of the enzymatic activity was bound in particulate form. Thus, after centrifugation of a crude extract for 1 hr at 105,000 x g more than 70% of the activity was still associated with the pellet. After purification by ammonium sulfate fractionation, most of the activity could still be recovered in the pellet after centrifuging 10 min at 15,000 x g. High concentrations of salt (1 M KCl) were unsuccessful in releasing activity from the pellet (data not shown). However, a significant fraction of the activity could be released from the particulate fraction by extraction with detergents. Table I summarizes the relative efficiency of several detergents in solubilizing the enzyme. Sarkosyl and sodium deoxycholate were most efficient at extracting the enzyme. However, upon subsequent dialysis more than half of the activity was lost from the sarkosyl extract whereas very little of the activity was lost from the deoxycholate extract. Of equal importance was the fact that deoxycholate extracted less protein resulting in a much higher specific activity than any other detergent. Hence, deoxycholate was the detergent used in the subsequent purification.

*Purification -- Euglena gracilis* strain Z was grown in heterotrophic
media (Difco) and harvested in log phase (125 g wet weight from 33 l of media). The cells were washed twice in distilled water, once with 0.05 M Tris-HCl (pH 7.4), then stored at -20° until used. The frozen cells were suspended in .05 M Tris-HCl (pH 7.4) (4 times the wet weight of the cells), and broken by sonication of 80 ml batches for 2.5 min with a Blackstone sonifier. The sonified cells were centrifuged for 10 min at 15,000 x g and the precipitate was discarded. The supernatant solution is the crude extract.

Streptomycin sulfate fractionation. To 560 ml of crude extract were slowly (over a period of 30 min) added 1/20 volume of 10% streptomycin sulfate. After all the streptomycin was added, the suspension was stirred for 15 min at 0°, then centrifuged for 10 min at 15,000 x g. The precipitate was discarded.

First (NH₄)₂SO₄ fractionation. To the streptomycin supernatant solution was added 205 g of (NH₄)₂SO₄ per liter of solution. After stirring for 30 min at 0°, the precipitate was removed by centrifugation (10 min at 15,000 x g) and resuspended in 0.01 M Tris-HCl (pH 7.4) to give a volume equal to about 0.1 that of the crude extract.

Second (NH₄)₂SO₄ fractionation. To the first (NH₄)₂SO₄ fraction was added 205 g/l of (NH₄)₂SO₄. The subsequent treatment was exactly the same as the first (NH₄)₂SO₄ fraction. Although this step does not increase the specific activity of the enzyme it does usually aid in the removal of a calcium-independent RNase.

Deoxycholate extraction. Sufficient 20% sodium deoxycholate (pH 7.4) was added to the second ammonium sulfate fraction to give a final concentration of 1%. The solution was incubated for 15 min at 37° with periodic stirring, cooled to 0° and centrifuged for 10 min at 15,000 x g. The pellet was re-extracted twice with 1% sodium deoxycholate -.01 M Tris-HCl (pH 7.4) using one-half the volume of the original ammonium sulfate fraction. The combined supernatant solutions are the deoxycholate extract.

Phosphocellulose chromatography. The deoxycholate extract was dialyzed for 48 hrs against 3 ten liter changes of 5 mM Tris-HCl (pH 7.4). The length of time of dialysis is important for the successful adsorption of the enzyme on to the phosphocellulose. Rapid dialysis using a hollow fiber device was unsuccessful because the enzyme did not stick to the phosphocellulose even though conductivity measurements indicated an efficient dialysis had occurred. A possible explanation is that deoxycholate is bound by the enzyme and only slowly released upon prolonged dialysis.

The dialyzed extract was charged onto a 2.8 x 20 cm phosphocelulose column that had been equilibrated with 0.01 M Tris-HCl (pH 7.4). The column
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was washed successively with 125 ml portions of 0.01 M Tris-HCl, 0.01 M Tris-HCl - 0.1 M KCl, and 0.01 M Tris-HCl - 0.2 M KCl (all at pH 7.4). The enzyme was eluted with 0.01 M Tris-HCl - 0.5 M KCl (pH 7.4). The active fractions were pooled and concentrated to about 10 ml in an Amicon Ultrafiltration cell (UM-2 membrane). The enzyme was stored at -20° and showed no significant loss of activity after several months even with repeated freezing and thawing. Table II summarizes the purification. Units are based on the hydrolysis of DNA. An approximate 1000-fold purification was achieved with an 18% yield.

Properties of Enzyme -- Hydrolysis of both RNA and DNA. The purified enzyme hydrolyzes either DNA or RNA, both of which are dependent upon the presence of calcium ions. Table II shows that the ratio of the DNase to the RNase activity remains quite constant over the last four steps of purification. The crude extract contains a large amount of RNase activity even in the presence of EDTA, hence meaningful measurements of the calcium-dependent RNase were not possible.

Divalent cation requirement. Both the DNase and RNase activities of the purified enzyme require calcium for activity, with the optimal concentration at 20-30 mM for DNase and 10-15 mM for RNase (Fig. 1). The RNase activity is more sensitive to inhibition at high calcium concentrations than is the DNase activity.

Table II. Purification of Euglena gracilis Endonuclease L from 125 g of Cells

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>DNase</th>
<th>RNase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml</td>
<td>Units</td>
<td>units/mg</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>10.0</td>
<td>5.48 x 10^5</td>
<td>98</td>
<td>100</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>1st (NH₄)_2SO₄ extract</td>
<td>15.5</td>
<td>3.4 x 10^5</td>
<td>384</td>
<td>62</td>
<td>7.15</td>
<td></td>
</tr>
<tr>
<td>2nd (NH₄)_2SO₄ extract</td>
<td>14.7</td>
<td>3.8 x 10^5</td>
<td>452</td>
<td>69</td>
<td>6.16</td>
<td></td>
</tr>
<tr>
<td>Deoxycholate extract</td>
<td>2.4</td>
<td>1.2 x 10^5</td>
<td>440</td>
<td>22</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>Cellulose phosphate chromatography</td>
<td>.019</td>
<td>9.8 x 10^4</td>
<td>97684</td>
<td>18</td>
<td>7.4</td>
<td></td>
</tr>
</tbody>
</table>

Activity and specific activity are based on DNase activity.

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Figure 1. Effect of CaCl₂ concentration on DNase and RNase activities were determined by the hyperchromicity assay as described in Materials and Methods.

activity. The same requirement for calcium is observed for the DNase activity as the DNA concentration is varied over a 10-fold range. The following divalent cations failed to substitute for calcium in the DNase assay when tested as concentrations of 0.1, 1.0, and 10.0 mM: MgCl₂, MnCl₂, CuCl₂, CoCl₂, NiCl₂.

Optimal pH. The optimal pH for both DNase and RNase is 9.4 to 9.5 in sodium glycinate buffer (Fig. 2). There is no significant difference in activity between Tris-HCl and sodium glycinate buffers at the same pH.

Effect of ionic strength. Figure 3 shows that in the presence of 24 mM CaCl₂ the DNase activity is not inhibited at KCl concentrations of 0.1 M or below. About 75% and 50% of the activity remains at 0.2 and 0.3 M KCl respectively. The response to NaCl is identical. Since the optimal calcium concentration is so high the question arose whether CaCl₂ may be showing two effects in the stimulation of the hydrolysis of DNA; one as an activator and secondly an ionic strength effect. To test this possibility, the effect of
Figure 2. Effect of pH on DNase and RNase activities of endonuclease L. Both the DNase and RNase activities were determined by the hyperchromicity assay as described in Materials and Methods. The pH values were directly determined in the incubation mixture at 30°. X--X Tris-HCl; •--• sodium glycinate.

Figure 3. Effect of KCl concentration on the DNase activity of endonuclease L. The DNase was measured by the hyperchromicity assay as described in Materials and Methods.
KCl on the enzyme-catalyzed hydrolysis of DNA at a suboptimal concentration of CaCl₂ (3 mM) was determined. Figure 3 shows that KCl does not show any significant stimulation under these conditions. Hence, we conclude that Ca²⁺ is acting only as an activator in the reaction and does not have a non-specific ionic strength activating effect.

Apparent Km values for DNA and RNA. Figure 4 shows Lineweaver-Burk plots using \( \text{E. coli} \) RNA and denatured calf thymus DNA as substrates. The apparent Km's are \( 2.4 \times 10^{-6} \) M and \( 7.4 \times 10^{-5} \) M for DNA and RNA respectively. It should be noted that four times the amount of enzyme was used for the RNase assays as for the DNase assays; hence, the Vmax values are not as close as one might conclude from the Lineweaver-Burk plot.

Competition of RNA hydrolysis by DNA. Table III shows that denatured calf thymus DNA inhibits the hydrolysis of \( \text{E. coli} \) RNA, consistent with the

![Figure 4](image)

Figure 4. Reciprocal plots for the determination of apparent Km values for calf thymus DNA and \( \text{E. coli} \) RNA as substrates for endonuclease L. Both the DNase and RNase activities were determined by the hyperchromicity assay as described in Materials and Methods. 72 units were used for the DNase assay whereas 288 units were used for the RNase assays. The open circles refer to DNA as substrate, the X to RNA as substrate.
nucleotide that a single protein is responsible for the hydrolysis of both DNA and RNA.

Light-induction of both DNase and RNase activities. As a further test of the hypothesis that a single protein is responsible for the hydrolysis of both RNA and DNA, we measured the light induction of both activities. Since crude extracts contain a calcium-independent ribonuclease activity, we had to purify the enzyme through the first ammonium sulfate fraction in order to obtain meaningful assays of the calcium-dependent RNase. Figure 5 shows that both the DNase and calcium-dependent RNase activities are very low in dark-adapted cells and increase in a parallel fashion upon exposure to light.

Substrate specificity. Table IV shows that of the four homopolyribonucleotides, only polyuridylic acid is hydrolyzed to any significant extent as judged by the production of acid soluble fragments. Indeed, a polyuridylic acid is hydrolyzed three times faster than denatured calf thymus DNA and eleven and sixteen times faster than yeast tRNA and E. coli total RNA, respectively. No hydrolysis of poly U occurs in the absence of Ca\(^{2+}\). Although Km values for the synthetic polynucleotides are not known, the concentrations used in the assay are at least 10 times greater than the apparent Km values found for E. coli RNA and denatured calf thymus DNA.

The products of polyuridylic acid hydrolysis were investigated by gel filtration and paper chromatography. Figure 6, panel A illustrated the resolution of a dinucleotide, pTpT, from uridylic acid by gel filtration on Bio-gel P-2. We assumed that the dinucleotide pUpU would elute in a similar fashion to pTpT. Figure 6, panel B shows an analysis of the products of polyuridylic
Figure 5. Light-induction of calcium-dependent DNase and RNase activities in dark-grown Euglena gracilis. Euglena gracilis strain Z was grown on heterotrophic medium (Difco) in the dark in a gyratory environmental chamber (Psycrotherm-New Brunswick Scientific Co., Inc.) to a cell density of 2.2 x 10^8 cells/ml. One liter batches of cells were harvested at various times after exposure to light and cell extracts were prepared and fractionated through the first ammonium sulfate fraction as described under purification. DNase and RNase activities were measured by the production of acid-soluble material as described under Materials and Methods except that denatured E. coli [3H] DNA (434 cpm/nmole) was substituted for the calf-thymus denatured DNA. Specific activity is defined as moles nucleic acid made acid-soluble in 15 min at 37° per mg protein. DNA activity; X RNase activity. The bars refer to the variation in the assays.

Table IV. Hydrolysis of Polynucleotides

<table>
<thead>
<tr>
<th>Polynucleotide</th>
<th>Amount in Assay</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (U)</td>
<td>342</td>
<td>100</td>
</tr>
<tr>
<td>Poly (dT)</td>
<td>112</td>
<td>33</td>
</tr>
<tr>
<td>Denatured Calf Thymus DNA</td>
<td>300</td>
<td>33</td>
</tr>
<tr>
<td>Yeast tRNA</td>
<td>300</td>
<td>9</td>
</tr>
<tr>
<td>E. coli RNA (total)</td>
<td>50</td>
<td>6</td>
</tr>
<tr>
<td>Poly (dA-dT)</td>
<td>210</td>
<td>5</td>
</tr>
<tr>
<td>Poly (A)</td>
<td>226</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Poly (G)</td>
<td>221</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>Poly (C)</td>
<td>313</td>
<td>&lt; .05</td>
</tr>
</tbody>
</table>

The incubation mixture (.3 ml) contained 0.1 M sodium glycinate (pH 9.6) 0.1 mM EDTA, 15 mM CaCl_2 (25 mM in the case of denatured calf-thymus DNA) 40 μg of bovine serum albumin, and enzyme. Incubation was for 30 min at 37° and was terminated by the addition of 0.1 ml of bovine serum albumin (10 mg/ml) plus 0.5 ml of 3.5% HCl. After 5 min at 0° the solution was centrifuged at 12,000 x g for 10 min and the absorbance at 260 nm of the supernatant solution determined. Activity (nmole polynucleotide made acid soluble) is expressed relative to poly (U) which is set at 100.
Figure 6. Gel Filtration of the products of poly (U) hydrolysis catalyzed by endonuclease L. A 1 x 60 cm column of Bio-gel P-2 was equilibrated with .05 M NH₄HCO₃ (pH 8.0). Panel A indicates the elution profile of pTpT and 2' (3')-UMP. Panel B shows the elution profile of poly (U) hydrolyzed to various extents by endonuclease L. The conditions of hydrolysis were the same as for the determination of acid-soluble fragments from RNA except that the CaCl₂ concentration was 15 mM, 342 nmoles of poly (U) was the substrate, and the reaction was terminated with 0.05 ml of 0.1 M EDTA (pH 7.0). → no endonuclease L; △ 144 units of endonuclease L and incubation for 15 min; ← 144 units of endonuclease L and incubation for 30 min; O 288 units of endonuclease L and incubation for 30 min.
Figure 7. Gel filtration of the products of [3H] RNA hydrolysis catalyzed by endonuclease L. The same column as described in Fig. 6 was used. The incubation medium was the same as for the determination of acid-soluble fragments from RNA except that the CaCl₂ concentration was 15 mM, 20 nmoles of [3H] RNA was the substrate, and the reaction was terminated with 0.05 ml of 0.1 M EDTA (pH 7.0). 0.5 umole of 2'-(3')-UMP was added as a marker in each case. All the absorbance, indicated by X, is due to the marker as the amount of RNA added did not contribute significantly to the absorbance. • no endonuclease L; A- - A 288 units of endonuclease L incubated for 30 min; O 288 units of endonuclease L incubated for 60 min; O--O 288 units of endonuclease L incubated for 90 min.
acid hydrolysis with increasing extent of hydrolysis. After 15 min of incubation with 144 units of endonuclease L (which converts 100% of polyuridylic acid to acid soluble fragments) most of the material resides in dinucleotide and larger fragments. After further digestion, all of the polyuridylic acid is converted to material that elutes in the 3'-UMP region. To confirm that the material was really uridine monophosphate, we subjected the material in the UMP peak region from the most completely hydrolyzed poly (U) to ascending paper chromatography using system #1 which is capable of resolving 5'-UMP, 3'-UMP, and 2', 3'-cyclic UMP. The single product migrated identically to 3'-UMP. Hence, endonuclease L is a 3'-phosphate former.

Figure 7 shows the analysis of the products of hydrolysis of E. coli [3H]-RNA (labeled equally in cytosine and uracil residues) by Bio-gel P-2 chromatography. Incubation for 30 min with 288 units of endonuclease L converts more than 90% of the RNA to acid-soluble fragments. But as shown in panel A, very little free 3'-UMP is released. Panel B shows the presence of resistant or very slowly hydrolyzed fragments even after 60 and 90 min incubation. Paper chromatography of the peak eluting between 26 to 37 ml in system 2 revealed small amounts of 3'-UMP and 3'-CMP and large amounts of unidentified material. Judging from the elution from the Bio-gel column, the unidentified material is most probably a mixture of different di- and trinucleotides.

The products of an exhaustive hydrolysis of thymine labeled E. coli DNA were examined by paper chromatography in solvent 3 with the results shown in Figure 8. A considerable amount of thymidylic acid is released which migrates...
with the 3'-dTMP marker. Thus, hydrolysis of DNA by endonuclease L results in products terminated with 3'-phosphate as was the case when RNA was the substrate. A considerable amount of thymine containing oligonucleotides are still resistant to hydrolysis by endonuclease L.

The best criterion for determining whether an enzyme is an endonuclease or an exonuclease is its ability to catalyze the hydrolysis of circular nucleic acids. Figure 9 shows that PM2 covalently closed circular DNA is nicked by endonuclease L. No significant reaction occurs in the absence of calcium as shown in panel B of Figure 9. Thus, our conclusion that this enzyme should be classified as an endonuclease.

Figure 9. Sedimentation profile of PM2 DNA in alkaline sucrose gradients. Panel A, no enzyme; panel B 75 units of endonuclease L but no calcium; panel C 75 units of enzyme with calcium. Sedimentation was from right to left. Intact double stranded circles appear in fractions 6 to 9.
DISCUSSION

The evidence that a single protein is responsible for the hydrolysis of both RNA and DNA is the following:

1. A constant ratio of activities over the last four steps of purification.
2. Similar requirements for high calcium ion for optimal activity.
3. Similar pH optima.
4. RNA hydrolysis is inhibited by denatured DNA.
5. Both RNase and DNase activities are induced by light in a similar fashion.
6. The products of both RNA and DNA hydrolysis are terminated by 3'-phosphates.

_Euglena_ endonuclease L has similar enzymological properties to _Chlamydomonas_ endonuclease A. Both enzymes are similar in that they hydrolyze both RNA and DNA, prefer single-stranded substrates, liberate products terminated by 3'-phosphates, have a very alkaline pH optima, and are activated by calcium ions. Some minor differences are apparent. Copper can substitute for calcium in the case of _Chlamydomonas_ endonuclease A but not the _Euglena_ enzyme. Also, the _Chlamydomonas_ enzyme is more sensitive to inhibition by low salt, especially sodium salts, than is the _Euglena_ enzyme. Finally, the _Euglena_ enzyme appears to be tightly bound to particulate matter and can be released only by extraction with detergents. Such was not the case with _Chlamydomonas_ endonuclease A. The overall similarities in catalytic properties lead to the temptation to speculate that perhaps _Euglena_ endonuclease L and _Chlamydomonas_ endonuclease A are prototypes of nucleases associated with chloroplast metabolism. However, much more work with diverse photosynthetic species is necessary to lend much credence to this hypothesis.

It should be pointed out that the _in vitro_ catalytic properties of these two algal enzymes, in particular the one from _Euglena_, is quite similar to the well-studied enzyme from _Staphylococcus aureus_.

The most intriguing result is the finding that of the four homopolyribonucleotides only poly (U) is hydrolyzed to any significant extent and it is hydrolyzed all the way to 3'-UMP. Does this specificity represent a specificity for phosphodiester bonds involving uridylic acid (and thymidylic acid in DNA) or does this represent a discrimination against the secondary structures of poly (C), poly (A), and poly (G)? Clearly, secondary structure is an important factor in determining the rate of hydrolysis as native DNA is converted to acid soluble fragments only about 3-5% the rate of heat denatured.
DNA. On the other hand, poly (dA-dT) which has much secondary structure is hydrolyzed at least 5 times faster than poly A and 100 times faster than poly C or poly G. This question of specificity can only be answered by examining the identity of the bases adjacent to the phosphodiester bond hydrolyzed in DNA and RNA.

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