Purification and Properties of a 3′-Phosphoryl Former Endodeoxyribonuclease from Eggs of *Asterias forbesi*†

Clementina Carestia, Sofia Gauthier, Aldo Granieri, and Eduardo Scarano*,‡

ABSTRACT: A DNA endonuclease has been purified from eggs of *Asterias forbesi* by a simple four-step-purification procedure. The purified enzyme is at least 96% pure and is free of phosphatase, phosphodiesterase, and RNase. It has a pH optimum of 6.5 and does not require divalent cations. The enzyme produces 3′-phosphoryl and 5′-hydroxyl end groups. The products of exhaustive hydrolysis can be grouped in two fractions. The first fraction, 40%, contains a small amount of mononucleotides and di-, tri-, tetra-, penta-, and hexanucleotides. The second fraction, 60%, contains oligonucleotides larger than hexanucleotides.

Endodeoxyribonucleases are interesting enzymes because of the possible role in several biological phenomena, e.g., genetic recombination. Moreover, DNases are useful tools for the study of the primary structure of nucleic acids. Bernardi et al. (1973) have developed a method to establish the frequency in DNA of short sequences produced by hydrolysis with specific DNases.

To our knowledge no DNase has been purified and characterized from eggs, neither of invertebrates nor of vertebrates.

To investigate the possible role of endonucleases in meiosis and fertilization, we searched for endonuclease activity in unfertilized eggs of *Asterias forbesi*. This animal possesses the characteristic of carrying out meiosis after fertilization. Our interest was also to search for an enzyme that could hydrolyze DNA in relatively large fragments.

In the present work is reported the characterization and purification from unfertilized eggs of *A. forbesi* of an endodeoxyribonuclease that produces 3′-phosphoryl and 5′-hydroxyl end-groups at nicks.

Materials and Methods

Biological Materials. Asterias forbesi, Echinodermata phylum, subphylum *Asterozoa,* class Stelleroidea, were obtained from Marine Biological Laboratory of Woods Hole, Mass. Ovaries were collected and suspended in filtered sea water (1 g of ovaries/10 mL of water). The eggs were obtained by adding 1-methyladenine at the final concentration of 1 μg/mL. Ovaries were discarded by filtration on gauze and eggs sedimented by centrifugation for 10 min in an IEC International Centrifuge at 1000*g* and then washed once with filtered sea water.

Nucleic Acids. DNA from salmon sperm, sodium salt type III, Worthington Biochemical Co., was dissolved in 0.015 M NaCl-0.0015 M sodium citrate; DNA from other sources was prepared according to Marmur (1961). DNA was estimated by the diphenylamine colorimetric assay according to Burton (1956) and by determination of A_{260}. Yeast RNA type IX, Worthington Biochemical Co., was dissolved in 0.015 M NaCl-0.0015 M sodium citrate. DNA from salmon sperm was denatured by heating at 100 °C for 10 min followed by fast cooling.

Assays. DNase Assay. The enzymatic activity was tested in a standard reaction mixture containing 4.1 mg of salmon sperm DNA and 600 μmol of Tris[1]-HCl (pH 7.4) buffer, in a final volume of 6 mL. The activity was determined by the decrease in viscosity of the reaction mixture, by the formation of acid-soluble oligonucleotides, and by the increase of the absorbancy at 260 nm of the incubation mixture. The viscosity decrease of the reaction mixture was measured with a Cannon Fenske viscosimeter at 37 °C. The viscosity of the incubation mixture at 37 °C was 3 cP. No changes in viscosity were observed when the reaction mixture without enzyme was allowed to remain at the incubation temperature for 2–3 h. The decrease in viscosity was linear up to 3 min. The formation of acid-soluble oligonucleotides was determined spectrophotometrically. To 100 μL of the reaction mixture 1 mL of cold perchloric acid was added at 4 °C. After 30 min at 4 °C, the samples were centrifuged for 10 min at 20 000*g* in a SS34 rotor in a Sorvall centrifuge to remove the precipitate. The absorbancy at 260 nm of the supernatant was measured against a blank obtained from the reaction mixture without enzyme. The increase of the $A_{260\,nm}$ of the reaction mixture was determined as a function of time after the addition of the enzyme.

Under the standard assay conditions, *0.1 unit of the enzyme was defined as the amount of the enzyme which catalyzes a viscosity decrease of 0.375 cP in 3 min at 37 °C.* The decrease in viscosity was linear with the amount of the enzyme in the range of 0.075–0.30 unit. The increase of $A_{260\,nm}$ at 60 min and the production of acid-soluble oligonucleotides at 30 min were linear with the amount of the enzyme in the same range.

Phosphatase Assay. Phosphatase activity was measured as described by Garen and Levinthal (1971).

RNase Activity. RNase activity was measured according to Kunitz (1946).

Phosphodiesterase Activity. Phosphodiesterase activity was measured according to Koerner and Sinsheimer (1957).

Protein Determination. Protein concentration was estimated by the procedure of Lowry et al. (1969) and by the microbiuret procedure according to Goa (1953).

† From the International Institute of Genetics and Biophysics, Naples, and the Institute of Molecular Biology, II Faculty of Medicine, University of Naples, Italy. *Received December 13, 1976.*

‡ The present work was started by E. Scarano, Lillie Fellow for 1970, at the Marine Biological Laboratory, Woods Hole, Mass.

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; CM, carboxymethyl.

TABLE I: Purification Procedure.

Step	Vol (mL)	Total protein (mg)	Total unit	Spec. act. (unit/mg)
Acetone powder	1.4	36.95	4.48	0.12
Ultracentrifugation	1.0	16.00	2.90	0.18
CM-Sephadex	3.0	0.45	2.98	6.60
Ammonium sulfate precip.	3.0	0.03	2.78	92.70

Sedimentation of DNA on Sucrose Gradients. Linear sucrose gradients (5–20%) in 10 mM Tris-HCl buffer (pH 7.6)–1 mM EDTA were run at 23 000 rpm for 18 h at 4 °C in a Spinco L_2 ultracentrifuge using the SW 25.1 rotor. The alkaline gradients were prepared by dissolving the sucrose in 0.1 M NaOH–0.001 M EDTA. The gradients were collected in 1.1-mL fractions by puncturing the tubes at the bottom. The A_{260} and A_{280} were determined in a PMQII Zeiss spectrophotometer. Residual hyperchromicity of DNA of the neutral gradients was measured after heat denaturation of the samples dialyzed against 0.015 M NaCl–0.005 M sodium citrate.

Determination of the Products of the Reaction. The analysis of the product of the reaction was carried out according to Tomlison and Tener (1963). The samples were loaded on a column of DEAE-cellulose (0.8 × 12 cm) in chloride form and fractionation was carried out with a linear gradient of LiCl in 7 M urea and 0.01 M lithium acetate (pH 5.1). [^{3}H]dCMP was added to the sample as mononucleotide marker. The A_{271} was determined in a PMQII Zeiss spectrophotometer. Fifty microliters of each fraction was poured in a glass vial, and 10 mL of Insta-Gel (Packard) was added. The radioactivity was determined in a 3385 Packard liquid scintillator spectrometer.

Electrophoresis on Polyacrylamide Gels. The purity of different preparations of *A. forbesi* endodeoxyribonuclease was checked by 7.5% polyacrylamide gels electrophoresis, according to Davis (1964). Gels were run at 4 °C, 160 V, 5 mA/gel, for 2 h in the following buffers: 0.02 M Tris–glycine (pH 8.0); 0.02 M citric acid–sodium citrate (pH 5.6 and 6.6). Bromophenol blue was used as marker of the front. Gels were stained with Coomassie blue and scanned with a Gilford densitometer.

Identification of End Groups of Oligonucleotides. The standard reaction mixture was incubated until the digestion product was 100% acid soluble (0.14 unit of enzyme was added at 0 time and at 6 h). The reaction was stopped by heating at 100 °C for 10 min and the mixture was divided in 1.5-mL fractions. One fraction was incubated with spleen phosphodiesterase (Worthington Biochemical Co.) according to Bernardi and Bernardi (1968); another was incubated with venom phosphodiesterase (from *Crotalus adamanteus* from Worthington Biochemical Co.) according to Felix et al. (1960). The samples were lyophilized and chromatographed on Whatman 3 MM paper using 2-propanol–HCl–H_2O (65:16.7:18.3 v/v) as solvent. To test the reliability of the experimental conditions, control experiments were carried out with oligonucleotides obtained by hydrolysis with DNase I or with DNase II.

Results

The results of the purification procedure are shown in Table I. All operations were carried out at 4 °C.

Crude Enzyme. One volume of unfertilized eggs was added to 10 volumes of acetone at −18 °C in a Waring Blendor and blenderized for 1 min. The suspension was rapidly filtered and

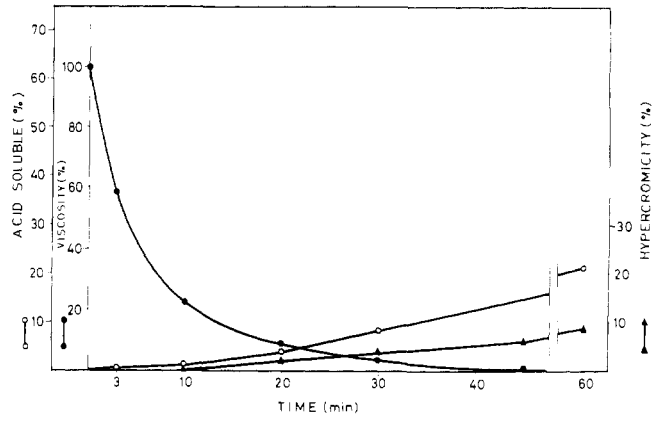

FIGURE 1: Time kinetics of the enzymatic hydrolysis. Salmon sperm DNA was incubated under the conditions of the standard assay; 0.140 unit of enzyme was added at 0 time. (●) Viscosity decrease; (○) acid-soluble oligonucleotides; (▲) hyperchromicity.

the precipitate was washed until the filtered acetone was completely clear and colorless. The acetone powder was dried under vacuum and stored at −30 °C. The weight of the acetone powder was one-tenth that of the eggs. The acetone powder was suspended at a concentration of 5% in 0.1 M K_2HPO_4–1 mM mercaptoethanol (pH 8.0), homogenized for 10 min in a Kontes Glass Duall Potter (v = 1725–1300 rpm), and extracted under magnetic stirring for 15 min at 4 °C. The suspension was centrifuged for 15 min at 4340g in a Sorvall RC2 centrifuge and the precipitate was discarded.

Ultracentrifugation. The supernatant was centrifuged for 60 min at 127 000g in the 40 rotor in a L_250 Spinco ultracentrifuge. The precipitate was discarded.

CM-Sephadex Fractionation. One-milliliter aliquots of the supernatant were mixed to 3 mL of 0.05 M Veronal buffer (pH 8.0); CM-Sephadex C-50 (equilibrated in the same buffer) was added in the proportion of 1 mg/3 mg of proteins. The suspension was stirred at 4 °C for 30 min, and the CM-Sephadex was recovered by centrifugation. The enzyme was eluted by stirring the CM-Sephadex with 3 mL of 1 M KCl in 0.1 M phosphate buffer, pH 6.5, for 30 min. The resin was centrifuged down and the supernatant saved for the next step.

Ammonium Sulfate Fractionation. Finally powdered ammonium sulfate was added slowly to the enzyme solution at a saturation of 40%. After 20 min under mechanical stirring, the solution was centrifuged for 18 min at 20 000g in the SS34 rotor in a Sorvall RC2 centrifuge and the precipitate discarded. The supernatant was brought to 60% saturation of ammonium sulfate and, after 20 min of mechanical stirring, the precipitate was collected by centrifugation and dissolved in 0.1 M phosphate buffer (pH 6.5) at a concentration of 10 μg of protein per mL.

Stability. The acetone powder was maintained for 36 months at −30 °C without any loss of activity. The other enzyme fractions were maintained for 12 months at −30 °C without any loss of activity.

Criteria of Purity. The purified enzyme electrophorized at different pHs on 7.5% polyacrylamide gels gave one protein band. Under our conditions, 2–3 μg of protein can be revealed; 90 μg of protein was loaded on each gel.

Phosphatase, phosphodiesterase, and RNase activities were assayed as described under Materials and Methods. Enzyme, 0.235 unit, was tested in each assay. None of these activities was observed in the purified enzyme preparation.

Characteristics of the Enzymatic Reaction. In Figure 1 is

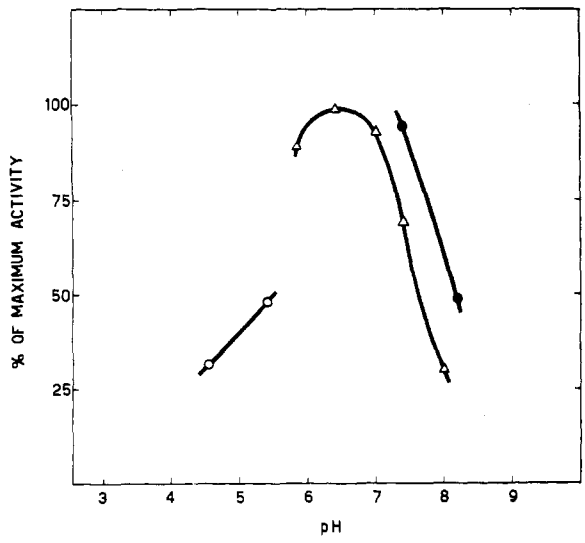

FIGURE 2: pH-activity curve. Ordinate: % of maximum activity. Abscissa: pH. Three buffers were used at 0.1 M concentration as follows: (●) Tris-HCl; (△) Tris-maleate; (○) sodium acetate-acetic acid. The activity was measured viscosimetrically.

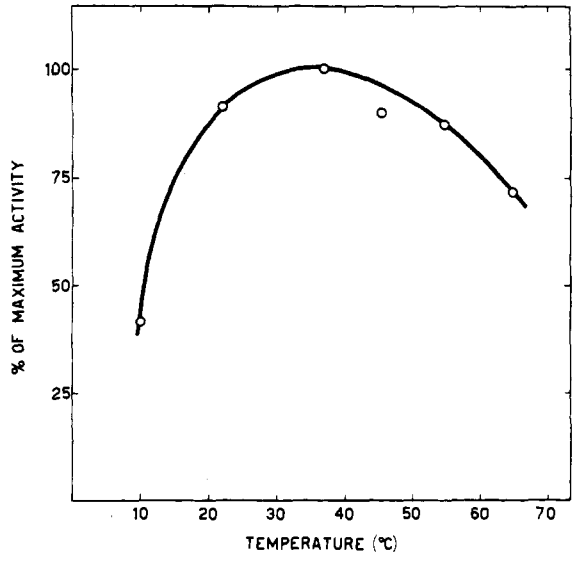

FIGURE 3: Temperature-activity curve. Ordinate: % of maximum activity. Abscissa: temperature in degrees centigrade. The activity was measured viscosimetrically.

depicted the decrease of viscosity, the hyperchromicity, and the production of acid-soluble oligonucleotides. The decrease of viscosity starts immediately upon addition of the enzyme, whereas the production of acid-soluble oligonucleotides and the hyperchromicity occur only after a certain period of time.

pH-Activity Curve. Figure 2 shows the pH-activity curve; the enzymatic activity was determined between pH 4 and 5.4 in 0.1 M acetate buffer, between 5.4 and 7.9 in 0.1 M Tris-maleate buffer, and between 7.4 and 8.2 in 0.1 M Tris-HCl. The optimum was 6.5. The same pH-activity curve was obtained by measuring the formation of acid-soluble oligonucleotides.

Temperature-Activity Curve. Figure 3 shows that the optimal temperature is at 37 °C.

Effects of NaCl. The enzymatic activity is sensitive to NaCl. At 10 mM Tris-maleate (pH 6.5), the activity is increased three times at 0.15 M NaCl.

Bivalent Cations. Mg^{2+} and Ca^{2+} tested up to 1.5 mM and Co^{2+} and Mn^{2+} tested up to 1 mM failed to give any effect on the enzymatic activity. The enzyme is active even in the presence of 5 mM EDTA.

Enzymatic Activity on Denatured DNA. The enzymatic activity on denatured DNA, measured by the production of acid-soluble oligonucleotides, is one-tenth of that on native DNA.

Characterization on Sucrose Gradient of the Products. Salmon sperm DNA was hydrolyzed under the condition of the standard assay for 3, 10, and 30 min and was run on neutral and alkaline gradients, as described under Materials and Methods. A decrease of the sedimentation rate, measured both under neutral and alkaline conditions, occurs in all samples (Figure 4). The decrease is already present at 3 min of digestion. The residual hyperchromicity of the DNA of the reaction mixture and of the neutral gradient fractions was measured. It was 26.7% at 0 time, 26% at 3 min, and 25% at 10 min.

Characterization of the Product of Exhaustive Digestion. Salmon sperm DNA was digested for 30 h under the standard assay conditions. Enzyme (0.140 unit) was added at 0, 3, and 6 h. Streptomycin sulfate, which does not inhibit the enzyme, was added in order to avoid bacterial growth. The final product, 100% acid soluble, was fractionated on DEAE-cellulose column, as described under Materials and Methods. One hundred percent of the oligonucleotides was recovered by elution with 0.03-0.3 M LiCl gradient (Figure 5). Peaks of di-, tri-, tetra-, penta-, and hexanucleotides are separated. A small fraction of mononucleotides is also eluted. The mean value, calculated from five experiments, of each fraction is 2% mononucleotides, 8.4% dinucleotides, 8.8% trinucleotides, 6.2% tetranucleotides, 6.4% pentanucleotides, 8.5% hexanucleotides, and 60% oligonucleotides containing more than six nucleotides.

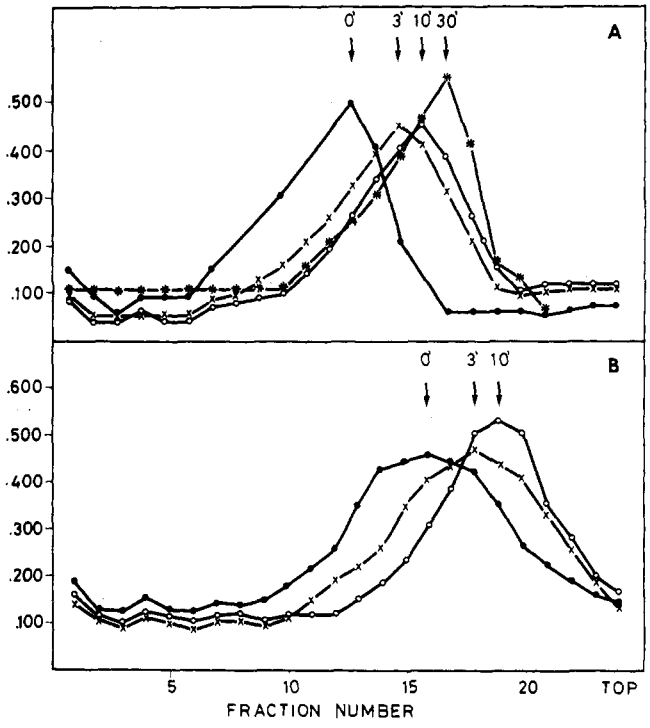

FIGURE 4: Sedimentation on neutral (A) and alkaline (B) sucrose gradient of salmon sperm DNA hydrolyzed for 3, 10, and 30 min. The assay conditions were the standard ones; the sucrose gradients were prepared as described under Materials and Methods.

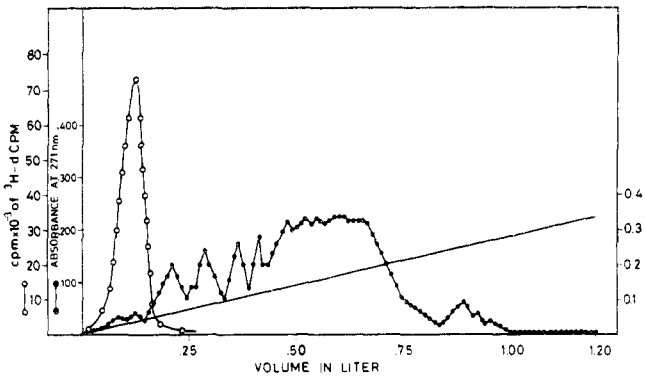

FIGURE 5: Chromatography on DEAE-cellulose (chloride form) column of extensive digestion products. Fractions of 10 mL were collected and the absorbancy was measured at 271 nm; tracer amounts of [^{3}H]dCMP were added to the digestion product in order to have a mononucleotides marker. (○) [^{3}H]dCMP cpm; (●) absorbance at 271 nm.

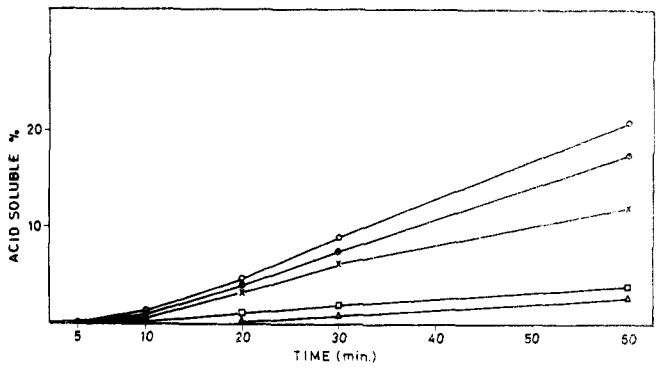

FIGURE 7: Hydrolysis of different DNAs, measured as production of acid-soluble oligonucleotides, as described under Materials and Methods. (○) Salmon sperm DNA; (●) BHK cell DNA; (X) calf thymus DNA; (□) *E. coli* DNA; (△) sea urchin embryos DNA.

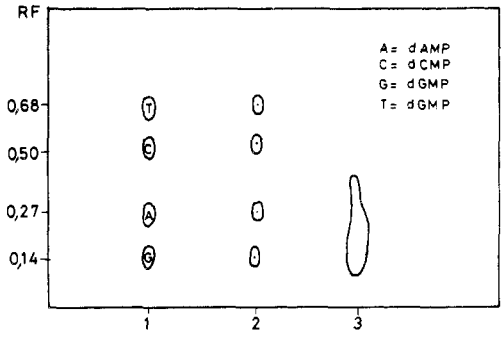

FIGURE 6: Chromatography on Whatman 3 MM paper of the four deoxyribonucleotides (1); of the spleen phosphodiesterase digest (2); of the venom phosphodiesterase digest (3). The digestions were performed on the product of *Asterias forbesi* DNase hydrolysis as described under Materials and Methods.

End-Group Analysis of the Products. The products of the reaction were analyzed as described under Materials and Methods in order to identify the end groups of the oligonucleotides produced. Only spleen phosphodiesterase hydrolyzed the products into the four deoxyribonucleotides. Digestion with venom phosphodiesterase did not give any hydrolysis of the products of the enzyme reaction (Figure 6). These results show that the enzyme produces oligonucleotides with 3′-phosphoryl and 5′-hydroxyl end groups.

Different DNAs as Substrates. The enzymatic activity was assayed with DNAs having different (A + T)/(C + G) ratios: sea urchin embryos DNA (*Paracentrotus lividus*), 1.85; BHK cell DNA, 1.33; salmon sperm DNA, 1.43; calf thymus DNA, 1.30; *E. coli* DNA, 1.00. All the tested DNAs were treated as described by Marmur (1961). The assay conditions were the standard ones; the activity was measured as production of acid-soluble oligonucleotides (Figure 7). The enzymatic action appears to be correlated with the DNA composition. The velocity of the enzymatic reaction is higher for DNAs with higher A + T content, except for the sea urchin embryos DNA, which is hydrolyzed at a much slower rate.

Discussion

The enzyme purified from eggs of *A. forbesi* can be classified as an endodeoxyribonuclease because of its specificity toward DNA and the kinetics of the hydrolysis. Mononucleotides appear in a very small amount only after an exhaustive hydrolysis.

The endodeoxyribonuclease from *A. forbesi* has been purified about 800 times over the acetone powder extract by a simple and fast procedure. As judged by electrophoresis on polyacrylamide gel, the maximum amount of possible protein contamination is 3%.

The purified enzyme in a first phase degrades the substrate by producing pieces of double-strand DNA. This is demonstrated: (1) by the very rapid decrease of viscosity; (2) by the kinetics of the changes of sedimentation rate of products on both neutral and alkaline sucrose gradient; (3) by the high residual hyperchromicity of the products (more than 90%) at 3′ and 10′; (4) by the absence during this phase of acid-soluble oligonucleotides among the products. Only after the initial rapid decrease of the viscosity the production of acid-soluble oligonucleotides begins and continues until all the DNA is converted into acid-soluble products. The present data do not permit to distinguish whether the enzyme hydrolyzes the substrate in the first phase by a purely diplotomic mechanism or by a mixed diplotomic and haplotomic mechanism (Bernardi, 1968).

A mechanism of action, involving double breaks in native DNA, was first described by Oth et al. (1958) and has been extensively studied by Bernardi and Sadron (1964) and by Bernardi (1968) for DNase II or acid DNase. Recently this mechanism of action has also been found by Sabeur et al. (1974) for a DNase purified from testes of crab *Cancer pagurus*, which resembles DNase I for pH optimum, divalent cation requirements, and inhibition by ionic strength.

The enzyme from eggs of *A. forbesi* appears to hydrolyze, at a faster rate, DNAs with high A + T content, except for the sea urchin embryos DNA. Salmon sperm DNA treated by the same procedure used to purify DNA from sea urchin embryos is not altered in its substrate properties. The sea urchin embryos DNA might have a lower affinity for the egg DNase than expected from its base composition. Higher affinity of some DNases for DNA with higher A + T content has already been described by Sabeur et al. (1974) for a neutral DNase. Parisi and De Petrocellis (1972) have observed an alkaline DNase activity in sea urchin embryos extracts. The sea urchin embryo DNase preferentially cleaves DNA with high A + T content.

The endodeoxyribonuclease from *A. forbesi* cleaves the internucleotide linkage producing 3′-phosphoryl end groups since the products are not hydrolyzed by venom phosphodiesterase and 5′-hydroxyl end groups since spleen phosphodiesterase hydrolyzes the product to mononucleotides. Endo-

deoxyribonucleases that produce at nicks 3′-phosphoryl and 5′-hydroxyl end groups are the endodeoxyribonucleases of calf thymus (Fredericq and Oth, 1958), of venom of *Bothrops atrox* (Georgatsos and Laskowsky, 1961), of salmon testis (Yamamoto, 1971), and of hog spleen (Bernardi and Griffé, 1964). These enzymes have pH optima between 3.8 and 6, do not require divalent cations, and are activated by high ionic strength. The endodeoxyribonuclease from *A. forbesi* resembles these enzymes but differs essentially for its pH optimum that is near neutrality. In invertebrates other DNases have been described, which show some differences in respect to "typical" DNases II. Laval and Paoletti (1972) have found in the hepatopancreas of the snail *Aspersa müll* a DNase II with pH optimum of 5.6. Georgatsos and Antonoglou (1964) described three endodeoxyribonucleolytic activities: two of them are activated by Ca^{2+} and have pH optima respectively of 6 and 8; the third one does not require cations and has an optimum of 7. Georgatsos (1964) purified also a DNase with a pH optimum of 8, producing 3′-phosphoryl end groups.

The endodeoxyribonuclease from *A. forbesi* is the first DNase purified from eggs.

The biological role of the egg endodeoxyribonuclease might be the inactivation of DNA of the polar bodies since the enzyme produces strand breaks which cannot be repaired by polynucleotide ligases that require adjacent 3′-hydroxyl and 5′-phosphoryl end groups at nicks. In addition, neither of the two end groups produced by the enzyme can be elongated by DNA polymerases. It might be significant that all 3′-phosphomonoester former endo-DNases, which have been described, are endocellular enzymes. As already pointed out by Scarano (1969), it might be of interest to study DNA endonucleases 3′-phosphomonoester formers in embryonic cells of *Ascaris* and of *Sciara*, and in erythroblasts, i.e., in cells, where DNA inactivation and elimination occur.

References

Bernardi, A., and Bernardi, G. (1968), *Biochim. Biophys. Acta 155,* 360.

Bernardi, G. (1968), *Adv. Enzymol. 31,* 1.

Bernardi, G., Ehrlich, S. D., and Thiery, J. P. (1973), *Nature (London), New Biol. 246,* 36.

Bernardi, G., and Griffé, M. (1964), *Biochemistry 3,* 1419.

Bernardi, G., and Sandron, C. (1964), *Biochemistry 3,* 1411.

Burton, K. (1956), *Biochem. J. 62,* 315.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Felix, F., Potter, J. L., and Laskowski, M. (1960), *J. Biol. Chem. 235,* 1150.

Fredericq, E., and Oth, A. (1958), *Biochim. Biophys. Acta 29,* 281.

Garen, A., and Levinthal, C. (1971), *Biochim. Biophys. Acta 38,* 470.

Georgatsos, J. G. (1964), *Biochim. Biophys. Acta 95,* 544.

Georgatsos, J. G., and Antonoglou, O. (1964), *Enzymology 27,* 141.

Georgatsos, J. G., and Laskowski, M. Sr. (1962), *Biochemistry 1,* 288.

Goa, J. (1953), *Scand. J. Clin. Lab. Invest. 5,* 218.

Koerner, J. F., and Sinsheimer, R. L. (1957), *J. Biol. Chem. 228,* 1049.

Kunitz, M. (1946), *J. Biol. Chem. 164,* 563.

Laval, J., and Paoletti, C. (1972), *Biochemistry 11,* 3596.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1969), *J. Biol. Chem. 42,* 24.

Marmur, J. (1961), *J. Mol. Biol. 3,* 208.

Oth, A., Fredericq, E., and Hacha, R. (1958), *Biochim. Biophys. Acta 29,* 287.

Parisi, E., and De Petrocellis, B. (1972), *Biochem. Biophys. Res. Commun. 49,* 706.

Sabeur, G., Sicard, P. J., and Aubel-Sandron, G. (1974), *Biochemistry 13,* 3203.

Scarano, E. (1969), *Ann. Embryol. Morphogen., Suppl. 1,* 51.

Tomlison, R. V., and Tener, G. M. (1963), *Biochemistry 2,* 697.

Yamamoto, M. (1971), *Biochim. Biophys. Acta 228,* 95.