Purification and Properties of an Acid Deoxyribonuclease from the Snail *Helix aspersa* Müll.†

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ABSTRACT: An acid deoxyribonuclease is isolated from the hepatopancreas of common snail *Helix aspersa* Müll. It is specific for DNA. The activity of the enzyme was purified 1100-fold by an eight-step procedure including dichloromethane treatment, streptomycine and ammonium sulfate precipitation, chromatography on DEAE-cellulose, Sephadex G-100, DEAE-Sephadex, and P-cellulose. The preparation is homogeneous as judged by electrophoresis on polyacrylamide gel. The enzyme activity is obtained free of contaminating acid or alcaline phosphomonoesterases, exonuclease, and unspecific phosphodiesterase. The enzyme does not require divalent cation for activity and has a pH optimum of 5.6. Ionic strength is critical for maximum activity. The isoelectric point of this protein is 5.9; $s_{20,w}$ is 3 S, its molecular weight is 30,000 as determined by gel filtration, $K_m = 1.3 \times 10^{-5}$ M, and the apparent energy of activation is 11,300 cal/mole. Fifty per cent of the enzymatic activity is lost in 10 M urea after 3 min. Heating the enzyme at 60° for 9 min reduces enzymatic activity to half. The enzyme is not inhibited by polyribonucleotides at its optimum pH but they are inhibitors at lower pH's. This acid *Helix* endodeoxyribonuclease is different by all criteria tested from splenic acid endonuclease of beef.

Sequencing of DNA which has not yet been achieved requires endodeoxyribonucleases specific for particular sequences of DNA. Several restriction enzymes could now be used for this purpose; but, being very specific (for example, 6 base pairs recognized by the *Hemophilus influenzae* enzyme) (Kelly and Smith, 1970) they yield very long pieces of DNA, still too long for sequence analysis.

Consequently, there is an urgent need for endonucleases which could discriminate among base sequences so as to degrade DNA in small oligonucleotides enriched in nonhydrolyzable sequences. The present and accompanying papers describe an endonuclease extracted from the hepatopancreas of the common snail (*Helix aspersa* Müll.) which partially fulfills these conditions. Moreover, this enzyme displays a pH optimum which warranted a comparison of its main properties with those of the acid DNases studied by Cordonnier and Bernardi (1968), among which several could be of great significance: they are found in all higher organisms thus far investigated (Cordonnier and Bernardi, 1968), suggesting an important function, still unknown, common to all eukaryotic cells; moreover, they obey an allosteric mechanism of control of their activity (Bernardi, 1968; Lesca and Paoletti, 1969). They differ from all the other known nucleases found in bacteria and virus since they are active in an acid pH range and do not require divalent cations.

A careful investigation of the properties of the snail enzyme, which has been purified to homogeneity and is devoid of any nucleolytic enzyme contamination, reveals that besides sharing common properties with other acid DNases, it differs from them in several ways.

Furthermore the physical and chemical properties have been investigated in details in order to correlate this DNase with the others already described.

Materials and Methods

Nucleic Acids. [*methyl*-^{3}H]DNA is extracted according to Laval *et al.* (1970) from *Escherichia coli* Hfr Uvil 1858 grown on M 63 medium, containing 5 mCi per liter of [*methyl*-^{3}H]-thymine. Its specific activity is 10^7 cpm/μmole of DNA (all concentrations are expressed in nucleotides of DNA). RNA content is less than 3% as estimated with the orcinol method (Defrance and De Lesdain, 1962); protein as determined by the Lowry *et al.* (1951) procedure represents less than 1% of the DNA. Upon centrifugation on sucrose gradient, it sediments as a sharp single peak at 20 S.

Total rat liver RNA is prepared according to Hiatt (1962) and contains the three well-defined fractions (28, 17, and 4 S) found after centrifugation in the analytical Spinco Model E. RNA concentrations are determined by spectrophotometry at 260 nm, assuming that a 1-μg/ml solution has an absorbance of 0.030 (Hiatt, 1962). Yeast RNA is a gift from the Choay Laboratories, Paris. Poly(A), poly(U), poly(C), and poly(I) are purchased from Miles Research laboratories.

Enzymes and Chemicals. Splenic endonuclease of beef is prepared according to the procedure described by Bernardi and Griffe (1964) for hog spleen endonuclease. Electrophoretically pure chymotrypsin and pancreatic RNase (fraction D of Taborsky, 1959) are a generous gift from Dr. E. Sach. Crystalline horse heart cytochrome *c* (type V) and soya bean trypsin inhibitor are obtained from the Sigma Chemical Co. Bovine serum albumin fraction V is an Armour product.

DEAE-cellulose (Cellex D 0.86 mequiv/g) is purchased from Bio-Rad Laboratories, Richmond, Calif.

DEAE-Sephadex A-50 (3.5 ± 0.5 mequiv/g) and Sephadex G-100 (Pharmacia, Uppsala, Sweden) are treated before use according to the manufacturer's instructions. Cellulose phosphate, Whatmann Chromedia PI (7.4 mequiv/g), is obtained from W. and R. Balston Ltd. and treated according to Paul and Lehman (1966) before use. Bis(*p*-nitrophenyl)phosphoric

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acid (sodium salt) is purchased from the British Drug House, England, and sodium *p*-nitrophenylphosphate from Merck-Darmstadt, Germany. Streptomycine sulfate is a gift from Dr. Dupuis, Rhône-Poulenc, France. Ammonium sulfate (enzyme grade) and sucrose are purchased from Mann Research Laboratories. All the reagents used are analytical grade, purchased either from Prolabo, Paris, France, or from Merck, Darmstadt, Germany.

Assay Methods. Helix DNase ACTIVITY. General Method. This assay measures the conversion of native DNA labeled [*methyl*-^{3}H]thymine to acid-soluble oligonucleotides. The incubation mixture (0.5 ml) contains 50 μmoles of Na acetate buffer (pH 5.6)–15.8 nmoles of DNA–enzyme. All the determinations are performed at 37° unless otherwise stated. Dilutions of enzyme are made in 0.05 M acetate buffer (pH 5.6) containing 0.05% bovine serum albumin. After 10 min at 37°, 0.5 ml of cold 0.5 N perchloric acid is added, then, 2 min later, 0.2 ml of solution of serum albumin (1 mg/ml) is added. After 5 min at 0°, the precipitate is removed by centrifugation for 10 min at 12,000*g*, and 0.1 ml of supernatant is poured into a counting vial. Finally, 3 ml of ethanol and 10 ml of scintillator solution (4 g of 2,5-diphenyloxazole–100 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene in 1 l. of toluene) are added. Radioactivity is determined in a Packard Tri-Carb liquid scintillator spectrometer. The blank reaction in which the solution for dilution of the enzyme is substituted to the enzyme always yields less than 0.3% of the acid-precipitable material as acid-soluble counts. The amount of acid-soluble oligonucleotides released is proportional to the amount of enzyme added between 5 and 75% of substrate hydrolysis at 37°.

A unit of enzyme activity is defined as the amount of protein liberating 1 nmole of acid-soluble oligonucleotide in 1 min at 37° in the conditions described above. One unit introduces 69 endonucleolytic breaks per minute per 1×10^6 of DNA molecular weight as described in the following paper (Laval and Paoletti, 1972).

An alternative assay used to determine the ionic requirements of this enzyme measures the collapsing of the secondary structure of the DNA through the fluorescence of the complex DNA–ethidium bromide (Le Pecq and Paoletti, 1966). The incubation mixture (15 ml) contains 1.2 mmoles of thymus DNA–1.5 mmoles of acetate buffer (pH 5.6); after preincubation for 5 min at 37° with occasional stirring to allow equilibration of temperature, 1 ml of enzyme dilution is added. Every 5 min, 3-ml aliquots are transfered into 1 ml of 0.0375 M Tris solution containing 0.0825 M sodium citrate and 50 μg/ml of ethidium bromide. Fluorescence intensity is determined at room temperature with a spectrophotometer Zeiss PM QII equipped with the fluorescence attachment ZFM4 and activity calculated from the slope of the curve IF[1] *vs.* time (see Table I).

PHOSPHOMONOESTERASE (PMase) ACTIVITIES. Phosphomonoesterase activities are assayed by measuring the liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate according to Chersi *et al.* (1966) either in 0.1 M acetate buffer (pH 4.3) or in 0.1 M glycine buffer (pH 9.1)–0.01 M $MgCl_2$.

PHOSPHODIESTERASE ACTIVITY. Phosphodiesterase activity is assayed on bis(*p*-nitrophenyl) phosphate in 0.1 M acetate buffer (pH 5.6) according to Privat de Garilhe and Laskowsky (1955).

RIBONUCLEASE (RNase) ACTIVITY. RNase activity is assayed in 0.1 M acetate buffer (pH 5.0) according to Schneider and Hogeboom (1952).

In order to detect trace contamination of DNase by RNase S values of the 3 fractions of rat liver RNA are measured in the analytical ultracentrifuge after having been incubated with the purified enzyme at a concentration twice that used for the inhibition experiments by polyribonucleotides.

Sedimentation Analysis of Proteins. Sucrose gradient centrifugation is carried out according to Martin and Ames (1961) in a Spinco LHT preparative ultracentrifuge at 4°. A linear molarity gradient (4.3 ml) is obtained from 5 and 20% sucrose solutions in 0.05 M acetate buffer (pH 5.6) with a gradient maker device and a polystaltic pump (Buchler, Fort Lee, N. Y.). Beef heart cytochrome *c*, which absorbs selectively at 415 nm, is added as reference protein to the enzyme solution to be layered on the top of the sucrose gradient. After centrifugation, the bottom of the tube is punctured, and 32 ± 1 fractions are then analyzed for endonuclease activity and cytochrome *c* content. Linearity of the sucrose gradient is checked with a Zeiss refractometer.

Isoelectric Point Determination. The pH gradients are generated with carrier ampholytes (Ampholine) in the LKB 8100 electrofocusing column of 110 ml, purchased from LKB Produkter AB, Stockholm, Sweden, and used as described by the manufacturer. The voltage depends on the pH range; values will be given for each experiment. Water at 4° is circulated through the mantle during the experiment. Fractions of 1.6 ml are collected at flow rate of 1.6 ml/min. pH measurements are performed with a Tacusel pH meter at 0°.

Acrylamide Gel Electrophoresis. Preparations of *Helix* endonuclease of different specific activities are examined by acrylamide gel electrophoresis at 4°, as described by Ornstein (1964) and Davis (1964); 7.5% pore gels are used in Tris-glycine (pH 8.3). Voltage is 160 V and current 3 mA/tube for about 120 min at 4°. Bromophenol marked the advancing front which is always ahead of the protein bands. Gels are

TABLE I: Effect of Organic and Inorganic Salts on Endonuclease Activity.[a]

Tested Compound	Concentration Giving 50% Inhibition (μmole/ml)
$CaCl_2$	16
$MnCl_2$	7.5
$Mg(CH_3O)_2$	6
$CoCl_2$	3.75
NaF	2.6
LiCl	80
KCl	86
NaCl	80
C_sCl	70
EDTA	55
NH_4Cl	80
$(NH_4)_2SO_4$	30
KH_2PO_4–K_2HPO_4	28

[a] Activities have been determined in 0.1 M acetate buffer (pH 5.6) by the ethidium bromide test described in Methods. The table lists the concentration of the salt needed to give 50% inhibition of endonuclease activity.

[1] Abbreviation used is: IF, fluorescence intensity.

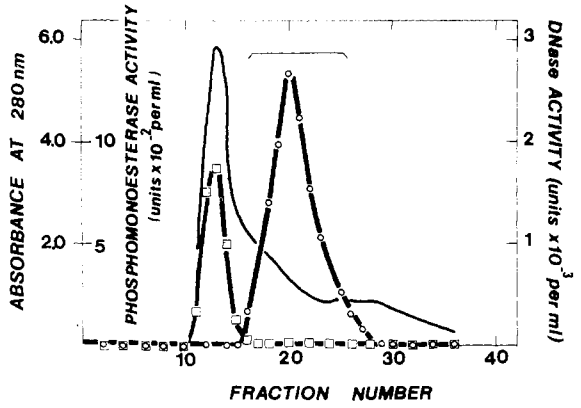

FIGURE 1: Chromatography of fraction V on Sephadex G-100: (——) absorbance at 280 nm (left scale); (O——O) endonuclease activity (right outer scale); (□——□) alkaline phosphomonoesterase activity (left inner scale). Fractions between the brackets are processed further. (For details, see text.)

stained with Coomassie Blue and destained electrophoretically. They are photographed, and then the film is scanned with the Joyce Loebel microdensitometer.

Protein Determination. Protein concentration is determined by the method of Lowry *et al.* (1951) and in effluent solutions from chromatography columns, by the absorbance at 280 nm.

Miscellaneous. Dialysis tubing is boiled in 10% sodium carbonate containing 0.001 M EDTA for 10 min and washed with cold, distilled water; this cycle is repeated; the bags are kept at 4° in a 50% solution of ethanol. Molarity gradients are checked with a Zeiss refractometer.

Results

Purification and Criteria of Purity of Helix Endonuclease. PREPARATION OF EXTRACTS. The snails, *Helix aspersa* Müll., are obtained from L. Payan 84-Mazan, France; it is advisable to prepare the enzyme from animals just collected at the end of the summer in order to obtain extract of a maximum specific activity.

Snails (4 kg) are washed four times in 10 l. of 0.15 M NaCl in order to remove a heavy viscous slime and five times in 10 l. of distilled water.

The shells of the snails are broken and the hepatopancreas excised; these organs are collected in a cooled receptacle. All the subsequent operations are performed at 4°. The hepatopancrease juice is discarded from the gland. Hepatopancreas (378 g) are collected, mixed with 378 ml of chilled 0.01 M acetate buffer (pH 5.6), and homogenized for 4 min with the Ultra-Turrax at full speed. The temperature of the mixture is not allowed to rise above 10°. The resulting homogenate is centrifuged at 17,000*g* for 15 min in a Servall RC2 centrifuge. The supernatant (615 ml) is collected. The extraction procedure is repeated twice on the pellets, the first time with 260 ml of buffer and the second time with 176 ml. The supernatants are pooled (fraction I: 1045 ml, 534 units/ml, 30.4 mg of protein per ml) (see Table II).

DICHLOROMETHANE TREATMENT. This step is included in the purification procedure in order to remove lipid contaminants (Sih *et al.*, 1963) which otherwise would interfere with chromatographic purification.

Fraction I is divided into 300-ml portions to which are added 60 ml of precooled CH_2Cl_2 at −20°; the two layers are vigorously shaken for 30 sec. The resulting emulsion is immediately centrifuged for 15 min at 17,000*g*, and the aqueous layer is recovered. Each portion of fraction I is treated in the same way. The resulting solution is fraction II (970 ml, 413 units/ml, 21.2 mg of protein per ml); it is brown and limpid.

TABLE II: Purification Procedure.

Step		Volume (ml)	Total Protein (mg)	Total Units (× 10^{-4})	Specific Activity
I	Extract	1045	31,780	55.8	17.7
II	Dichloromethane treatment	970	20,560	40	21.7
III	Streptomycine supernatant	1090	12,420	29.6	24.3
IV	Ammonium sulfate dialysate	133.5	4,270	23.6	55
V	DEAE-cellulose and ammonium sulfate precipitation	9.5	528	16	310
VI	Sephadex G-100 chromatography	116	92.8	12.4	1,400
VII	DEAE-Sephadex chromatography	61.5	32.8	9.4	2,880
VIII	Phosphocellulose chromatography	29	0.84	1.6	19,200

STREPTOMYCIN PRECIPITATION. To fraction II is added 175 ml of a chilled, freshly prepared 5% (w/v) solution of streptomycin sulfate over a period of 125 min with continuous stirring. The resulting suspension is stirred for an additional 10 min, then centrifuged for 10 min at 17,000*g*. The supernatant fluid (fraction III: 1090 ml, 272 units/ml, 11.4 mg of protein per ml) is immediately subjected to ammonium sulfate fractionation.

AMMONIUM SULFATE FRACTIONATION. Ammonium sulfate (308 g) is added with continuous stirring to fraction III over a 1-hr period. The suspension is allowed to stand for 15 min and is centrifuged. To the supernatant is added, under continuous stirring, 319 g of ammonium sulfate over a 90-min period. The precipitate is allowed to form for 1 hr, then centrifuged. The resulting precipitate is dissolved in 50 ml of water and titrated to pH 7.75 (at 4°) by addition of 0.5 M Tris and dialyzed for 14 hr against 4 changes of 2 l. of 0.01 M Tris buffer (pH 7.75) (fraction IV: 133.5 ml, 1770 units/ml, and 32 mg of protein per ml; pH 7.5 at 25°).

In fraction IV phosphodiesterase, basic and acid phosphomonoesterases, and RNase activities are present in the dark brown solution.

DEAE-CELLULOSE FRACTIONATION. A column of DEAE-cellulose (4.9 cm^2 × 19.5 cm) is equilibrated with 0.01 M Tris buffer (pH 7.5), then washed with 2 l. of the same buffer. Half the volume of fraction IV (*i.e.*, 66.5 ml) is poured on the column at a rate of 60 ml/hr and then followed by 100 ml of 0.01 M Tris buffer (pH 7.5). A linear gradient from 0 to 0.2 M NaCl in 0.01 M Tris buffer (pH 7.5) is applied. The flow rate is 85 ml/hr, and 10-ml fractions are collected. Approximately, 85% of the activity is eluted in a sharp peak between 2.1 and

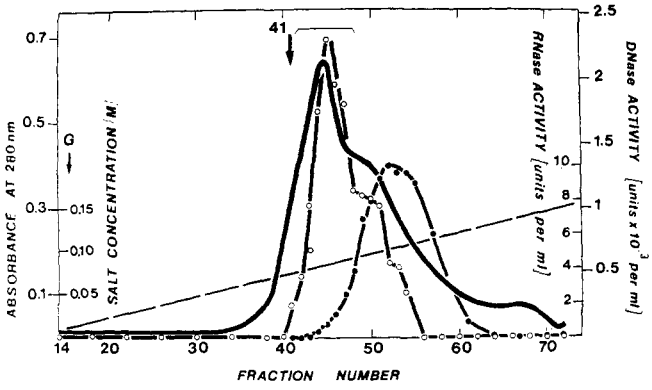

FIGURE 2: Chromatography of fraction VI on DEAE-Sephadex A-50: (——) absorbance at 280 nm (left outer scale); (●——●) RNase activity (units per ml) (right minor scale); (○——○) DNase activity (units per ml) (left outer scale); and the broken line is the molarity (left inner scale). (For details, see text.) Arrow marked G corresponds to the starting of the gradient.

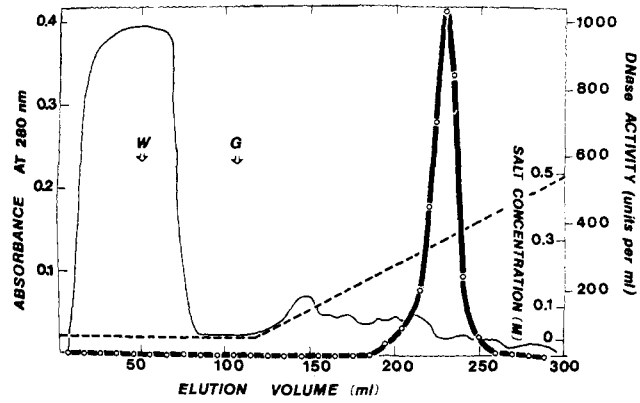

FIGURE 3: Chromatography of fraction VII on P-cellulose. Arrows marked W and G show the fraction corresponding to the washing of the column by the equilibration buffer and to the start of the gradient, respectively. (——) Absorbance at 280 nm; (○——○) endonuclease activity (right outer scale); (- - -) molarity of NaCl in the effluent (left inner scale).

3.6 resin bed volumes of effluent. The peaks of endonuclease and alcaline phosphomonoesterase activities coincide. RNase and acid phosphomonoesterase activity peaks are eluted between 2.8 and 4.2 resin bed volumes for the former and 3.6 and 5.1 for the latter. Acid phosphodiesterase activity is eluted as a broad peak between 2.4 and 4.0 resin bed volume. A second column is run with the second half of fraction IV.

Endonuclease fractions from the two columns with a specific activity greater than 200 are pooled (136 ml) and concentrated for the next step by adding 88 g of ammonium sulfate under continuous stirring. The precipitate is allowed to collect for 3 hr, recovered by centrifugation, then dissolved in a minimum amount of 0.01 M Tris buffer (pH 7.5) (fraction V: 9.5 ml, 16,850 units/ml, 55.6 mg of protein per ml). This fraction contains 50% of the activity applied to the column and can be stored at 4° for 2 weeks without any loss of activity. This step leads to an almost complete elimination of the acid phosphomonoesterase and acid phosphodiesterase and to the removal of more than 80% of the RNase.

GEL FILTRATION ON SEPHADEX G-100. A column of Sephadex G-100 (4.9 $cm^2 \times 39.5$ cm) is prepared and washed with 2 l. of 0.01 M Tris buffer (pH 7.5). The same buffer is used for elution of the protein solution. An aliquot of 3.2 ml of fraction V is loaded on the column and eluted at 40 ml/hr rate. Fractions of 5 ml are collected. The elution volume of the midpoint of the alkaline phosphatase activity peak is 62 ml and that of deoxyribonuclease activity 98 ml, resulting in a complete separation of the two activities (Figure 1). The peak fractions which contain nuclease without phosphomonoesterase activity are pooled. The column is washed and used again for the two other aliquots. All the peaks containing nuclease activity are pooled. This pool is designated fraction VI (116 ml: 1120 units/ml, 0.7 mg of protein per ml); 78% of the proteins applied to the column is recovered, and a fivefold purification of the material is achieved.

DEAE-SEPHADEX CHROMATOGRAPHY. A column of DEAE-Sephadex (4.9 $cm^2 \times 30$ cm) is equilibrated with 0.01 M Tris buffer (pH 7.5). Fraction VI (116 ml) is applied to the resin, then washed with 20 ml of 0.01 M Tris buffer (pH 7.5). A linear gradient (0.01–0.18 M NaCl) in 0.01 M Tris buffer (pH 7.5, total volume 500 ml) is applied. Fractions (9 ml) are collected. DNase activity appears as a main peak showing a shoulder probably due to the shrinking of the resin (Figure 2). RNase activity is almost completely separated from DNase. The peak fractions under brackets which contain less than 4 units per ml of RNase are pooled and adjusted to pH 5.8 by the slow addition of 0.1 N acetic acid (fraction VII: 61.5 ml, 1530 units/ml, 0.53 mg of protein per ml). When DNase completely devoid of any traces of RNase, as checked by analytical centrifugation of RNA (see Material and Methods), is needed, fraction 41 is used (see below, inhibition by polyribonucleotides).

PHOSPHOCELLULOSE CHROMATOGRAPHY. Phosphocellulose is equilibrated with 0.02 M acetate buffer (pH 5.8). A column (1.95 $cm^2 \times 12$ cm) is prepared and washed with 500 ml of the same buffer. Fraction VII (61.5 ml) is applied to the resin in 35 min, then washed with 45 ml of 0.02 M acetate buffer (pH 5.8). A linear gradient (0–0.8 M NaCl) in 0.02 M acetate buffer (pH 5.8) (total volume 200 ml) is applied at a flow rate of 40 ml/hr, and 5-ml fractions are collected (Figure 3). The *Helix* DNase is eluted at 0.3 M. Fraction VIII (29 ml, 550 units/ml, and 30 μg per ml of proteins) is used, unless otherwise stated, in the studies to be reported here and in the following papers (Laval and Paoletti, 1972; Laval *et al.*, 1972[2]).

CRITERIA OF PURITY. Fraction VIII containing *Helix* DNase proves to be homogeneous as judged by polyacrylamide gel electrophoresis (Figure 4). Phosphomonoesterases are assayed as described in Material and Methods. Upon 6-hr incubation of 70 units of enzyme, protected by bovine serum albumin at either pH 4.3 or at pH 9.1 in the presence of Mg^{2+}, there is no liberation of *p*-nitrophenol. A similar assay run on bis(*p*-nitrophenyl) phosphate rules out the presence of unspecific phosphodiesterase. Exonuclease activity is also absent, as no mononucleotides are detected by chromatography on DEAE-cellulose in 7 M urea, according to the method of Tomlinson and Tenner (1963), of an extensive digest of DNA by the *Helix* endonuclease (for details, see accompanying paper). The P-cellulose fraction VIII contains a slight ribonuclease contaminant; 55 units of DNase of this fraction liberate 10^{-3} nmole of P-RNA per min. It should be recalled that the first part of the DEAE-Sephadex fraction (Figure 2, tube number 41) is devoid of any RNase contami-

[2] Unpublished data.

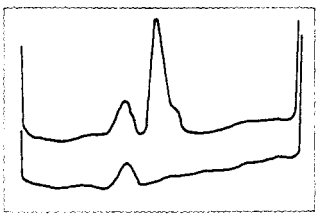

FIGURE 4: Microdensitometer tracings of polyacrylamide gel electrophoretograms of *Helix* DNase. Lower: fraction VIII (4 μg); upper: fraction VIII (4 μg) and pancreatic DNase (5 μg) have been run simultaneously in order to compare the two proteins. Electrophoretic conditions are described in Materials and Methods. The origin is on the left.

nation, as checked on the ultracentrifugation assay of RNA described in Materials and Methods.

STABILITY. Upon conservation at 4° half of the activity is lost in about 2 months, but no loss of activity occurs upon conservation in 40% glycerol at −20° for 5 months (less than 5%).

Physical and Chemical Properties of the Enzyme Protein. MOLECULAR WEIGHT. Molecular weight of *Helix* endonuclease is estimated by Sephadex G-100 gel filtration according to the procedure described by Andrews (1964). The column is successively calibrated with cytochrome *c*, soya bean trypsin inhibitor, chymotrypsin, and albumin by measuring the elution volume (V_e) of each protein in a separate run. The void volume (V_0) is the elution volume for a high molecular weight substance completely excluded from the gel particles (Dextran Blue).

In 0.05 M Tris-HCl buffer–0.1 M KCl (pH 7.5), the enzymatic activity is eluted as a single sharp symetric peak and a molecular weight of 30,000 is calculated (Figure 5) assuming that the protein is a globular one. After zone centrifugation in a sucrose density gradient according to Martin and Ames (1961), a sedimentation coefficient of 3 ± 0.1 S is determined using an $s_{20,w}$ of 1.7 S for cytochrome *c* (Ehrenberg, 1957)

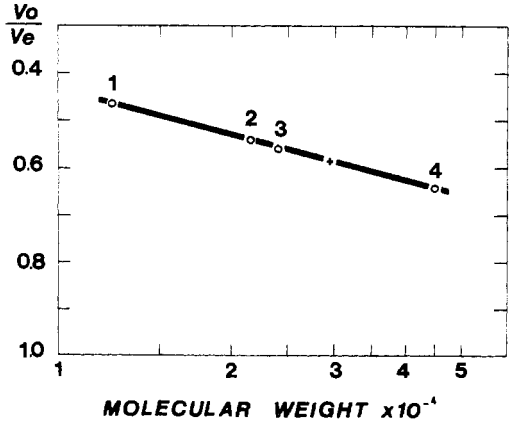

FIGURE 5: Molecular weight of endonuclease by gel filtration on Sephadex G-100. Calibration plot of V_0/V_e *vs.* log molecular weight of standard proteins. The ratio V_0/V_e of the proteins used as standard are plotted against logarithm of the molecular weight. V_0 void volume; V_e, elution volume; 1, cytochrome *c*; 2, soya bean trypsin inhibitor; 3, chymotrypsin; 4, ovalbumin; +, 100 μl of enzyme of the P-cellulose step is layered on the column (1 cm × 95); the enzyme activity, measured as described in Materials and Methods (General Method), is eluted as a single sharp symmetric peak. Flow rate is 12 ml/hr.

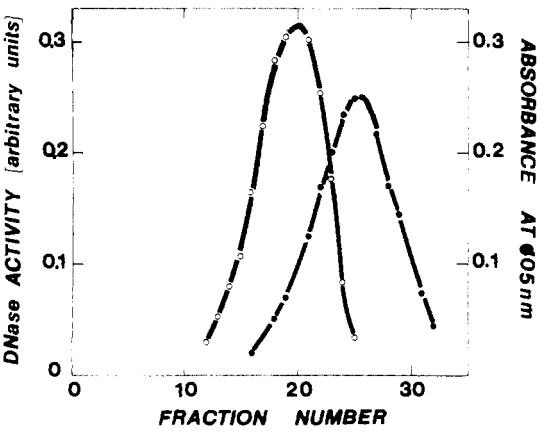

FIGURE 6: Sucrose gradient centrifugation of *Helix* DNase. A linear molarity gradient of 5–20% sucrose in 0.05 M acetate (pH 5.6) was obtained as described in Materials and Methods; 14 units of DNase (fraction VIII) and 250 μg of cytochrome *c* are centrifuged for 16 hr at 5° at 39 krpm using a SW 39 rotor and a Spinco Model LHT ultracentrifuge. A total of 32 fractions is collected in 1 ml of 0.1 M acetate buffer containing 0.01% of bovine serum albumine, except for fractions 10, 20, 26, and 30, for which refractive index is determined prior to the addition of the buffer. (○——○) DNase activity measured at 45° (arbitrary units) (left scale); (●——●) cytochrome *c* (OD 405 nm) right scale.

(Figure 6). This value is in good agreement with the estimation of molecular weight by gel filtration.

UREA AND HEAT INACTIVATION. The rate of inactivation of *Helix* endonuclease at 37° in 10 M urea is expressed as the logarithm of the percentage of the remaining activity after incubation in 10 M urea for increasing time (Figure 7). This concentration of urea was selected after experiments which established that the enzyme is still active in the presence of

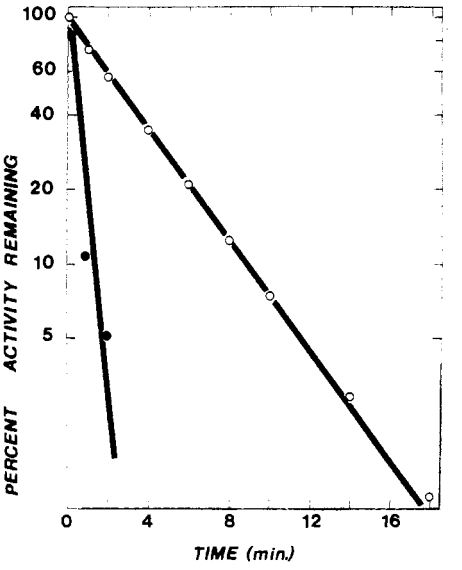

FIGURE 7: Rate of inactivation of *Helix* and splenic endonuclease by 10 M urea. At 37°, *Helix* enzyme fraction VII (0.2 ml) dialyzed against 0.1 M acetate buffer (pH 5.6) is added to 0.4 ml of 15 M urea in 0.1 M acetate (pH 5.6); 50-μl aliquots are withdrawn and diluted one-tenth with chilled 0.1 M acetate buffer containing 0.05 M bovine serum albumin, and assayed as described under Methods. Remaining activity is determined from a plot of activity *vs.* incubation time. Splenic enzyme was treated under similar conditions. (○——○) *Helix* endonuclease; (●——●) spleen endonuclease.

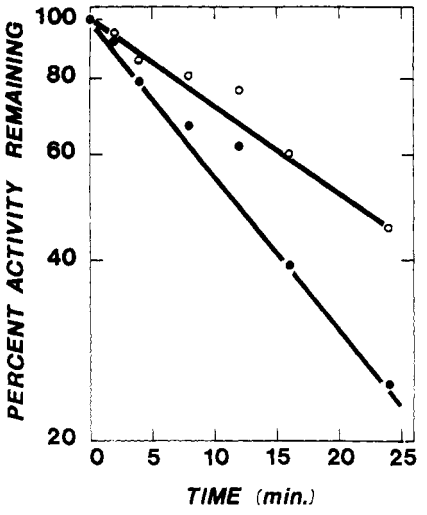

FIGURE 8: Heat inactivation of *Helix* DNase. *Helix* enzyme fraction VII is dialyzed against 0.1 M acetate buffer (pH 5.6) and heated at 60°. After different periods of time, aliquots are withdrawn, and diluted one-tenth in chilled 0.1 M acetate buffer (pH 5.6) containing 0.05% bovine serum albumin. Activity is assayed as described under Methods. Splenic enzyme is treated under similar conditions. (○——○) *Helix* endonuclease; (●——●) spleen endonuclease.

8.0 M urea. *Helix* DNase is more resistant to urea denaturation than splenic enzyme. Half-lives of the two enzymes being respectively 3 min and 15 sec under the same experimental conditions.

The percentage of the remaining activities after incubation of *Helix* and splenic endonucleases at 60° is shown in Figure 8. The rate of thermal inactivation is not very different for the two enzymes.

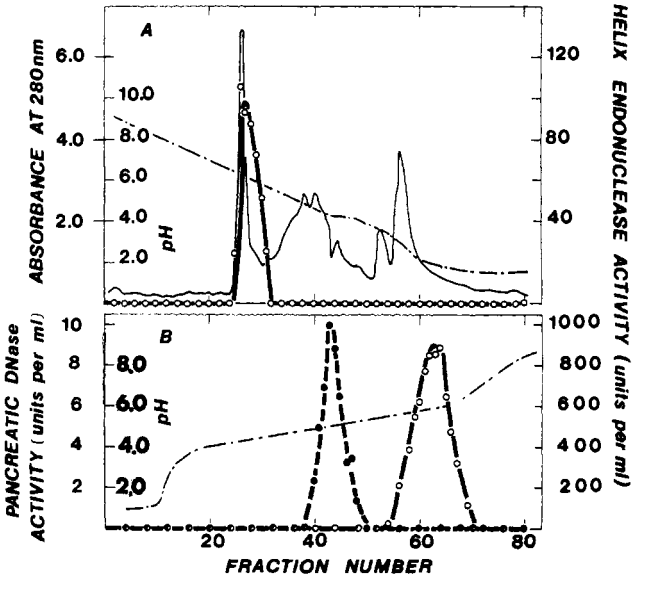

FIGURE 9: Electrofocusing of *Helix* endonuclease. A. Extract treated by dichloromethane. Fraction II is prepared for this experiment as described above (3.4 ml, 1390 units, 56 mg of proteins). Electrofocusing is done with Ampholine (pH range 3.10); voltage is 500 V for 45 min, then gradually increased to 700 V for 65 hr. Anode at the top. Recovery of DNase activity is 61%. (——) A_{280}; (○——○) *Helix* DNase activity B. Purified *Helix* DNase and reference protein (pancreatic DNase). 8000 units of *Helix* DNase (fraction VIII) and 30 μg of pancreatic DNase are submitted to electrofocalization in Ampholine (pH range 4–6); voltage of 400 V, 3 mA, rising to 500 V, 2 mA in the first 2 hr. Anode at the bottom. Recovery of *Helix* DNase is 68%. (○——○) *Helix* DNase; (●——●) pancreatic DNase.

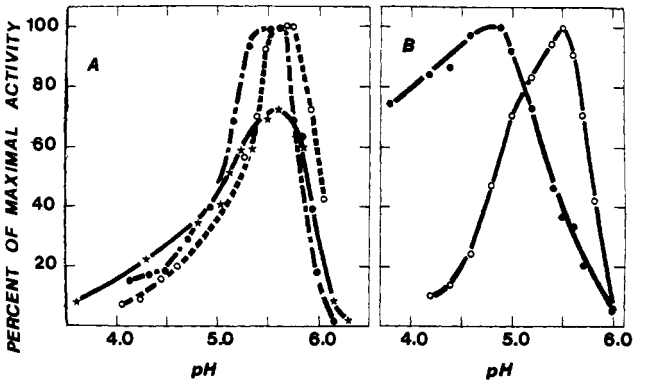

FIGURE 10: Activities of *Helix* and splenic endonucleases as a function of pH and solvant. A. *Helix* endonuclease. Citrate buffer: 0.05 M (★). Sodium acetate buffer: [acetate] 0.1 M (●) and 0.05 M (○). B. Comparison of *Helix* and spleen endonuclease activities. Ionic strength acetate buffer [Na] 0.1 M.

ELECTROFOCUSING EXPERIMENTS. Figure 9b shows that the isoelectric point of *Helix* DNase is 5.9 ± 0.1. The pancreatic DNase used as a marker displays an isoelectric point of 4.95, a result in agreement with several published values obtained from other techniques (Kunitz, 1949; Polson, 1957). Another electrofocusing experiment is performed on a roughly purified extract (fraction II) in order to ascertain that the extract does not contain another acid DNase which could be lost during the purification procedure. Figure 9a shows that this is not the case, as in a large pH range (3–10) only one single symetric peak of endonuclease activity can be characterized with an isoelectric point of 5.9 ± 0.1.

Catalytical Properties of the Enzyme. ENVIRONMENTAL CONDITIONS. pH. The *Helix* endonuclease displays optimal activity between 5.3 and 5.6, whereas beef splenic acid DNase shows a broad peak between 3.8 and 5.0 under identical ionic

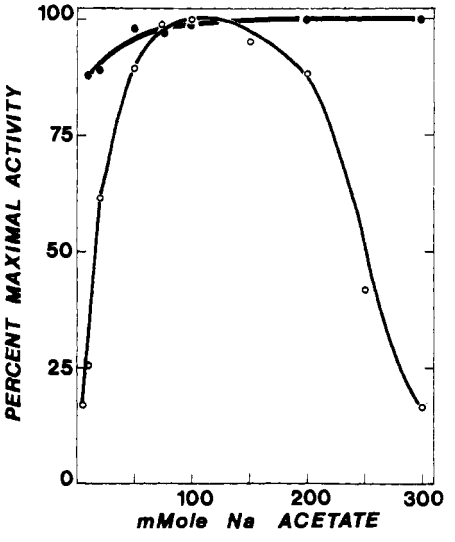

FIGURE 11: Activity of *Helix* endonuclease as a function of ionic strength. Data obtained with the splenic acid endonuclease are presented for comparison. Determination according to the general method. Solvent is acetate buffer at pH 5.6 for the *Helix* enzyme (○——○) and pH 4.8 (0.01 M EDTA) for the splenic enzyme (●——●).

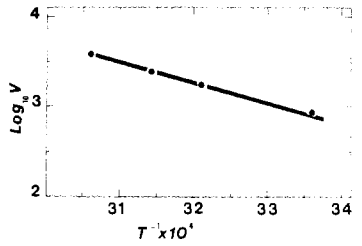

FIGURE 12: Activity of *Helix* endonuclease *vs.* temperature. The initial velocity of enzyme is determined at 25°, 37°, 45°, and 54° with the general method.

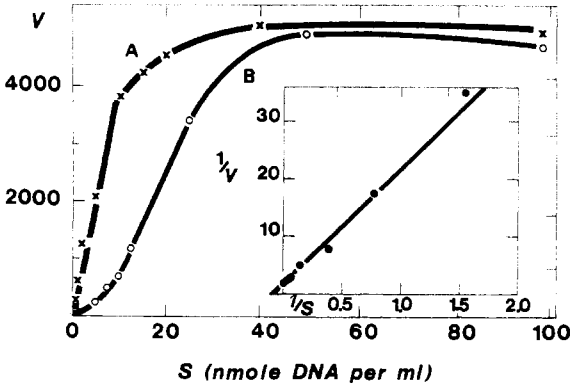

FIGURE 13: *Helix* endonuclease activity as a function of substrate concentration. A. Control solvent is 0.1 M acetate buffer (pH 5.6); temperature 37°; activities are determined with the general method (×——×) Insert: Lineweaver–Burk plot of graph A. B. In the presence of the acid DNase inhibitor. Inhibitor: (1.4 U of inhibitor per ml) (○——○).

strength. With the *Helix* enzyme similar results are obtained either with acetate or citrate buffer (Figure 10).

Ionic Strength. Influence of ionic strength is critical. The protein exhibits a low catalytic activity at low and high ionic strength and is nearly fully active only in a limited range of molarity (about 0.025–0.200 M). This property is at variance with the splenic DNase (Figure 11).

Effect of Different Ions. Several ions are tested within a wide range of concentration usually from 0.5 μmole per ml to 20 μmoles per ml. They are all inhibitory to different extents and no divalent cations are necessary for maximum activity. Dithiothreitol (0.01 M final concentration) has no effect on the enzyme activity. Mg^{2+} and SO_4^{2-} both exhibit inhibitory effects, as described also for the splenic enzyme (Cordonnier and Bernardi, 1968).

Temperature. The apparent energy of activation is 11.3 kcal. The enzyme is still fully active at 54°; it undergoes a progressive denaturation at 60° as previously described (Figure 12).

Inhibitory Effect of RNA and Synthetic Polyribonucleotides. Total rat liver RNA, poly(A), poly(I), poly(C), poly(U), and the complex poly(rA:rU) at concentrations between 1 and 20 μg per ml show no inhibition, if assay is performed in 0.1 M acetate buffer (pH 5.6). However, at pH 5.0, the *Helix* endonuclease can be inhibited of about 50% in the presence of 2 μg of total RNA in the incubation mixture; it should be recalled that, at pH 5.0, the activity of the enzyme is already diminished of 40% as compared to the activity at pH 5.6.

KINETICS. The K_m of *Helix* endonuclease is 1.3×10^{-5} M in 0.1 M acetate (pH 5.6) (Figure 13).

Lesca and Paoletti (1969) isolated from the liver of mouse a protein inhibitor of acid DNase; this inhibitor was shown to change the kinetics of acid DNase from a Michaelian one (without inhibitor) to a sigmoidal one (with inhibitor). We therefore investigated the kinetics of *Helix* endonuclease in the presence and in the absence of this inhibitor. For each substrate concentration the initial enzyme velocity is measured. Results are shown in Figure 13B. The kinetics in the absence of inhibitor obey Michaelis law, whereas in its presence the plot of *V vs.* (S) results in a sigmoidal-shaped curve, the inhibition being less pronounced for high substrate concentrations.

Discussion

An acid endonuclease from the hepatopancreas of a common snail *Helix aspersa* Müll. has been purified 1100-fold and obtained as a homogeneous preparation as judged by gel electrophoresis of one single species of protein of molecular weight 30,000, devoid of detectable contaminating activities except for traces of RNase; the ratio of DNase/RNase activity being of the order of 5×10^4. This low level of contamination is not critical for most experiments.

Moreover, there are two ways to get rid of this contamination if needed. (a) Select a portion of the DEAE-Sephadex peak which does not contain any RNase before further processing the preparation; such a procedure results in a loss of DNase; (b) perform a complementary purification step in the electrofocusing apparatus (unpublished experiment). It should be noted that the separation of DNase from RNase by DEAE-Sephadex chromatography as described in this paper also has been efficient in our hands for beef acid spleen DNase and pancreatic DNase (unpublished results).

Our purification procedure has been elaborated in order to avoid extreme conditions of pH which were described by Kunitz (1949) for pancreatic DNase and by Bernardi and Griffe (1964) for acid splenic DNase. These latter authors emphasize the risk of a partial hydrolysis of glycosidic linkages by using low pH. Although the evidence of such linkages in *Helix* endonuclease is not established, they have been found in pancreatic DNase (Catley *et al.*, 1969) as well as in spleen DNase (Bernardi *et al.*, 1965); pH 2.5 precipitation followed by ammonium sulfate fractionation has nevertheless been attempted successfully. The recovery of the enzyme is low partly because the central parts of the chromatographic peaks displaying the highest specific activities or less contamination by other enzymes are purposedly selected. The decrease of activity from fraction I to fraction VIII is not due to the disappearance of other endonuclease(s) present in crude extracts because the electrofocusing of fraction II reveals only the endonuclease presently described.

It should be noted that the enzyme displays some affinity for anionic (phosphocellulose) as well as for cationic (DEAE-Sephadex and DEAE-cellulose) chromatographic exchange resins; this holds also for other enzymes such as *E. coli* RNA polymerase (Burgess, 1969). The K_m of the *Helix* endonuclease is of the same order of magnitude as the ones measured for the splenic DNase (1.2×10^{-5} M) (Jacquemin-Sablon *et al.*, 1964) and for *E. coli* endonuclease I (2.5×10^{-5} M) (Lehman *et al.*, 1962), but well below that of the pancreatic DNase (about 5×10^{-3} M) (Le Talaer *et al.*, 1962).

No data can ascertain the monomeric or oligomeric structure of this enzyme. Consequently, any discussion on the

inhibitory effect of the spleen DNase inhibitor on *Helix* endonuclease is unwarranted; it should be recalled that the model of action at the molecular level of this inhibitor predicts a dimeric structure of the enzyme for explaining the non-Michaelian kinetics (Lesca and Paoletti, 1969).

Other noticeable characteristics of this enzyme are: narrowness of the pH range of activity (50% of maximum activity at pH 5.0 and 5.9), reduction of activity at both low and high ionic strength, and resistance to the denaturation by urea.

Acid DNase activities present in different species are thought to be associated with proteins very similar with respect to their chromatographic and enzymatic properties and sedimentation coefficients (Coordonnier and Bernardi, 1968).

In spite of sharing some of those common properties (molecular weight, chromatography on hydroxylapatite (unpublished data), and reduced activity toward denatured DNA (Laval *et al.*, 1966; Laval and Paoletti, 1972)) *Helix* endonuclease is definitely an enzyme different from the former ones, especially from the hog splenic acid DNase, which is the most investigated in this series, according to the following criteria: (a) isoelectric point, 5.9 for *Helix* enzyme and 10.2 for the hog spleen enzyme (Bernardi, 1968), although different techniques have been used; (b) pH dependency; (c) ionic strength dependency; (d) chromatographic behavior, in our hands, the *Helix* DNase is recovered in the breakthrough peak on CM-Sephadex (0.05 M phosphate buffer, pH 6.8), whereas the spleen enzyme is retained in the same conditions, and (e) the specificity toward the base sequences which shows striking differences (Laval *et al.*, 1972).[2] Moreover, the hepatopancreas of *Helix* does not contain any endonuclease which could be similar to the splenic enzyme or to the endonuclease which is present in another gasteropode, the clam *Mercenaria* (Cordonnier and Bernardi, 1968). Studies on the four endonucleases from another mollusc, *Octopus vulgaris*, are too preliminary to decide to which group these enzymes belong (Antonoglou and Georgatsos, 1968).

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