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Purification and Enzymatic Characterization of Three EndoDNase Isoenzymes from *Physarum polycephalum*

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Three alkaline DNases, A, B, and C, with preference for the digestion of double-stranded DNA (dsDNA) were partially purified from microplasmodia of *Physarum polycephalum*. They were very similar but differed in their isoelectric points. These were pH 5.8 for DNase A, 7.1 for DNase B, and 9.1 for DNase C. All three enzymes consisted of a single polypeptide chain with a molecular weight of 16,000 to 17,000, which readily formed high molecular weight complexes with low enzyme activity. These complexes could be reversibly dissociated by urea, and DNase activity was quantitatively reactivated. The DNases hydrolyzed the substrate DNA by an endonucleolytic mechanism which gave 5′-phosphorylated products. Divalent cations, $MnCl_2$ or $MgCl_2$, were essential for enzyme activity at the optimum pH of approximately 8.5 and at low ionic strength. The optimal conditions of pH, buffer, divalent cations and ionic strength and the extent of inhibition by salt, phosphate ions or urea differed slightly but significantly between the different isoenzymes.

Several DNA-degrading enzyme activities are found in crude extracts of microplasmodia of the acellular slime mold *Physarum polycephalum* (*1*) and they show growth-, cell cycle-, and differentiation-dependent variations in their activities (*2*). Alkaline DNases are generally thought to be involved in highly regulated processes, *e.g.* DNA replication in nuclei and mitochondria (*3–9*).

This study describes the purification of several alkaline DNase isoenzymes, originally detected in crude microplasmodial extracts and collectively named "alkaline DNase activity at pH 7.6" (*1*).

Abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

MATERIALS AND METHODS

Physarum polycephalum microplasmodia (M3c) originated from a gift of R. Braun (Bern). They were cultured and collected at near-stationary growth phase, as described elsewhere (*10*).

Purification of DNase Activity—All procedures were performed at 4°C, except when otherwise noted. The microplasmodia from 6 liters of culture were homogenized by sonication in buffer A (10 mM Tris·HCl, 10 mM KCl, pH 7.0). The sonicate (Fraction I) was centrifuged as described elsewhere (*10*), and the supernatant (Fraction II) was chromatographed over 1.0 kg of DEAE-cellulose (DE-52, Whatman), as described previously (*10*) and as specified below. The column

(9.0×35 cm) was washed at a flow rate of 7 ml/min with 2 liters of buffer A and was eluted with 5 liters of a linear gradient from 10 to 410 mM KCl in 10 mM Tris·HCl, pH 7.0, and with 1.5 liters of buffer A containing 0.4 M KCl. Fractions of 25.5 ml were collected. Enzyme activities were measured as described below and fractions with activity on native DNA were pooled. Urea was added to this slightly turbid fraction to 5 M, after which the fraction clarified and was called Fraction III.

Fraction III was loaded on a column of hydroxylapatite (6.5×10 cm) (Biorad, DNA-grade), equilibrated with 1 mM potassium phosphate buffer, pH 6.8, in 5 M urea. The column was eluted at a flow rate of 2.5 ml/min with 0.7 liter of 1 mM potassium phosphate buffer, pH 6.8, in 5 M urea and with 0.5 liter of a linear gradient from 1 mM to 500 mM potassium phosphate (pH 6.8) in 5 M urea. Fractions of 20 ml were collected. Activity on native DNA was only found in the flow-through fractions. These were pooled and called Fraction IV.

Fraction IV was loaded on a column of DEAE-cellulose (2.6×31.5 cm) (DE-52, Whatman), equilibrated with buffer B (10 mM Tris·HCl, 10 mM KCl, 5 M urea, pH 7.0). The column was eluted with 0.3 liter of buffer B and with 1.2 liters of a linear gradient from 0 to 0.4 M KCl in buffer B. Fractions of 27 ml were collected. The fractions with activity on native DNA were pooled, concentrated 12-fold against dry polyethylene glycol 6000 (Merck) and dialyzed against 3 changes of 5 liters of 5 M urea in 1 mM Tris·HCl, pH 7.0. The dialyzed concentrate was called Fraction V.

Fraction V was focused for 67 h at 500 V and 4°C in a 110 ml LKB isoelectric focusing column with Ampholines, pH 3.5 to 10 (1%, w/v, LKB), in a linear gradient from 5 to 45% (w/v) sucrose containing 5 M urea and with the cathode at the top. Fractions of 1.18 ml were collected at a flow rate of 0.4 ml/min, the pH of the fractions was measured at 4°C and the activities on native and denatured DNA were measured as described below. Fractions with activity on native DNA were collected in such a way that each peak of enzymatic activity was pooled separately. The pooled activities from the acid, the neutral, and the alkaline pH regions of the column were called A, B, and C, respectively. They were each concentrated to 3 ml against dry polyethylene glycol 6000 and chromatographed on a column of Sephadex G-100 (2.6×68 cm) (Pharmacia), equilibrated with buffer B. The flow rate was 0.22 ml/min. Fractions of 4.4 ml were collected. The fractions with activity on native DNA from gel filtrations A, B, and C were pooled and called Fraction VIA, Fraction VIB, and Fraction VIC, respectively.

Assays of Enzyme Activity—DNase activity was measured essentially according to the nuclease assay described by Waterborg and Kuyper (*10*). The incubation mixture (0.7 ml) contained 0.1 ml of enzyme solution in buffer B, 0.1 ml of native or heat-denatured herring sperm DNA (Boehringer) and 35 mM Tris·HCl, pH 8.5, and 1 mM $MnCl_2$. The incubation at 30°C was stopped in ice, the mixture was acidified with $HClO_4$, and the absorbance at 260 nm of the clarified supernatant (1.25 ml) was measured, correcting for blank values.

One Unit of DNase activity was defined as the amount of enzyme which produced, under the given assay conditions with native DNA during incubation for one hour at 30°C, an increase in the absorbance at 260 nm of 1.0 in the acid supernatant.

The activities of the zinc-metallo endonuclease on DNA and RNA and of acid and alkaline phosphodiesterases and phosphatases on bis-*p*-nitrophenylphosphate (Merck) and *p*-nitrophenylphosphate (Merck) were measured as described elsewhere (*10*).

Molecular Weight Determinations—The molecular weights of the DNases were determined by chromatography in buffer A and buffer B on Sephadex G-100 (Pharmacia) and by polyacrylamide-SDS gel electrophoresis according to Rosenthal and Lacks (*11*) on linear 6 to 18 percent (w/v) polyacrylamide gradient gels with 0.01 mg/ml native salmon sperm DNA (Sigma) and with protein standards of known molecular weights. The gels were incubated in the dark at 30°C in 35 mM Tris·HCl, pH 8, 1 mM $MnCl_2$, 0.02% (w/v) NaN_3, 0.001 mg/ml ethidium bromide (Serva) and with or without 5 M urea.

The protein content of fractions was determined according to Lowry *et al.* (*12*) and the conductivity was measured with a CDM3 conductivity meter (Radiometer).

Stability—The temperature stability of the DNases was determined by incubation of Fraction VIA, VIB, and VIC in buffer B for 2, 5, 10, 20, and 45 min at the given temperatures. The remaining enzyme activity was measured as described above.

Mode of DNase Hydrolysis—The fragmentation of DNA was studied during a series of digestions with the assay system described above, except that the DNA concentration was raised 5-fold, that 1 mM $MnCl_2$ or 3 mM $MgCl_2$ was used and that all three DNases were tested. The incubation at 30°C was stopped in ice. Fractions of 1.0 ml containing 20 OD units at 260 nm were loaded on a column of Sephadex G-200 (Pharmacia) (2.6×20 cm), equilibrated with buffer A. The flow rate was 0.25 ml/min and fractions of 1.0 ml were collected. The absorbance of the fractions was measured at 260 nm.

The location of the phosphate groups in the polynucleotide products after limited hydrolysis of DNA was determined as described elsewhere (*13*, *14*). DNA was digested at 3 mM $MgCl_2$ to 2.0 percent acid solubility, as described above. The reaction was stopped by heating (10 min 100°C) in order to inactivate the DNase and to denature the DNA. Digested DNA (0.18 mg in 0.05 ml) was incubated at 37°C for 2.5 h with 320 mUnits of *E. coli* alkaline phosphatase (Miles) in 0.002 ml of distilled water and the incubation was stopped by heating (10 min, 100°C). Snake venom phosphodiesterase (Worthington, 25 mUnits in 0.025 ml of distilled water) or calf spleen phosphodiesterase (Worthington, 410 mUnits in 0.025 ml of 20 mM EDTA, 0.04% (w/v) Tween 20, and 200 mM Tris·HCl, pH 7) was added and the production of acid-soluble material during the incubation at 37°C was followed for 5 h by measuring the absorbance at 260 nm in the acid supernatant after the addition of 0.025 ml of bovine serum albumin (Sigma, 50 mg/ml in distilled water) and 0.10 ml of 2.5 N $HClO_4$. Appropriate blanks were treated in parallel.

RESULTS

Nuclease activity on native, double-stranded DNA (dsDNA) was observed in crude homogenates from microplasmodia of *Physarum polycephalum*. This activity was partly due to the action of a zinc-metallo endonuclease (*10*) and several phosphodiesterases. However, part of the activity could be attributed to a DNase activity with preference for dsDNA. This enzyme was detected as a discrete activity only after at least partial removal of contaminating enzymes by DEAE-

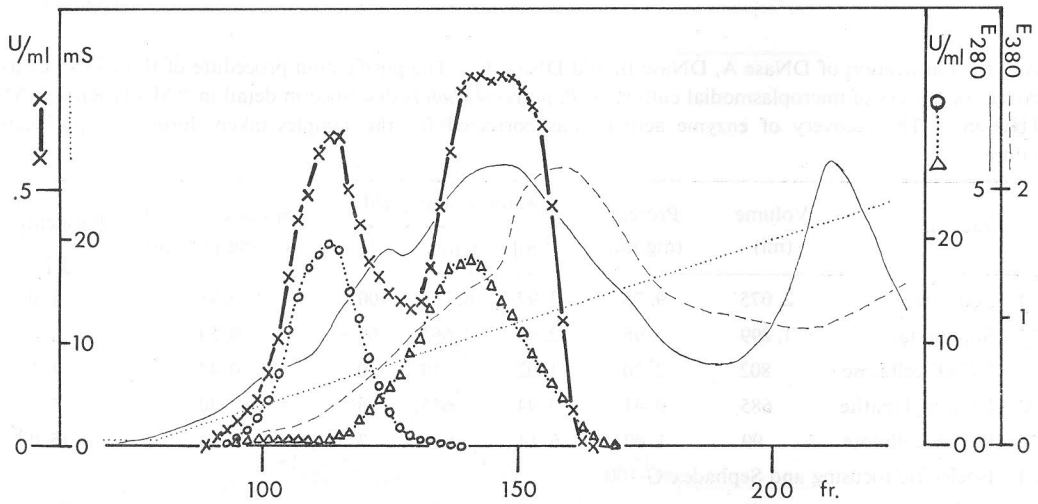

Fig. 1. DEAE-cellulose chromatography. Chromatography of Fraction II on DEAE-cellulose was performed and DNase activity on dsDNA (×) and on ssDNA (○), phosphodiesterase activity (△), absorbance at 280 nm (——) and at 380 nm (– – –) and the conductivity (·····) of the fractions were determined as described in "MATERIALS AND METHODS."

cellulose chromatography (Fig. 1). The overestimation of the DNase with preference for dsDNA in Fraction I and II was approx. 5-fold (Table I).

The DNase activity after DEAE-cellulose chromatography was highly unstable. Loss of activity was found during storage at −20°C, during dialysis in buffer A, during ammonium sulfate fractionation, during gel filtration and during rechromatography over DEAE-cellulose. Loss of preference for dsDNA was also observed and this seemed to be correlated to the loss of activity. When more than 90 percent of the activity on dsDNA had disappeared, the activity for dsDNA relative to that for ssDNA had dropped from 2 to as low as 0.1. The loss of activity was also accompanied by an increase in the molecular weight of the enzyme activity. Initially, gel filtration on Sephadex G-100 showed that almost all the activity had an apparent molecular weight of approx. 16,000 to 17,000. During inactivation, this activity almost completely disappeared and low activity with higher molecular weights of up to 100,000 and more and without preference for dsDNA appeared (results not shown). Apparently a complex of high molecular weight was formed, in which the DNase enzyme had low activity.

The high molecular weight complex could not be dissociated by 3 M KCl, as tested by gel filtration. It was also insensitive to bovine pancreatic DNase 1, which showed that DNA was not part of the complex (*3*). However, the addition of 5 or 8 M urea could reactivate the DNase to its original level of enzyme activity, irrespective of whether 10 percent or more than 95 percent of the activity had been lost. The complex dissociated and the DNase again appeared as a homogeneous peak of activity on Sephadex G-100 at MW 17,000. The reactivation also restored the preference for dsDNA. This reactivation and dissociation by urea could also be demonstrated on polyacrylamide-SDS-DNA gels (Fig. 2). The molecular weight of 16,000 of the DNase, which was active after protein dissociation by SDS, showed that the enzyme consists of a single polypeptide chain.

The DNase activity was stable in the presence of 5 M urea during storage, dialysis, or chromatography. The removal of urea, whether from Fraction III or from more purified preparations, again resulted in inactivation and the formation of high molecular weight forms. Dialysis overnight of Fraction III against buffer A at 4°C inactivated approx. one-third of the activity. Complete reactivation was obtained within 12 h at 4°C after the addition of urea to 5 M. 2 M urea was ineffective for reactivation.

In the presence of 5 M urea, no DNase activity was bound to DEAE-cellulose and hydroxylapatite and all contamination by phosphodiesterases and phosphatases could be removed, because these activities were retained. The low level of the

TABLE I. Purification of DNase A, DNase B, and DNase C. The purification procedure of three DNases from the extract of 6 liters of microplasmodial culture of *P. polycephalum* is described in detail in "MATERIALS AND METHODS." The recovery of enzyme activity was corrected for the samples taken during the purification procedure.

Fraction		Volume (ml)	Protein (mg/ml)	Activity and yield			Specific activity (U/mg protein)	Purification
				U/ml	Units	%		
I	Sonicate	2,075	9.75	3.97	8,232	100	0.41	1.0
II	Supernatant	1,899	4.98	2.98	5,662	69	0.59	1.5
III	DEAE-cellulose	802	2.20	1.02	819	10	0.47	1.1
IV	Hydroxylapatite	685	0.41	0.94	645	8	2.30	5.7
V	DEAE-cellulose	99	1.00	6.14	606	7	6.14	15.0
VI	Isoelectric focusing and Sephadex G-100							
	DNase A	71	0.047	0.51	36	0.4	10.9	27
	DNase B	107	0.059	3.13	336	4.1	52.8	130
	DNase C	96	0.042	0.29	27	0.3	6.8	17

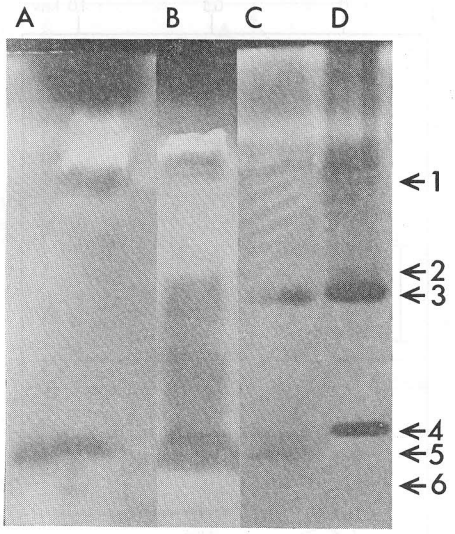

Fig. 2. Polyacrylamide-SDS-DNA gel electrophoresis. The 6 to 18% gradient gel (*11*) was loaded with: A, microplasmodia lysed in 1% SDS; B, DNase preparation Fraction III, except that this preparation was dialyzed against buffer A; C, DNase preparation Fraction III, dialyzed against buffer B and with some DNase 1 added as an internal marker for enzyme activity, (5) DNase activity; D, Nuclease molecular weight markers, bovine pancreatic DNase 1 (31,000) and micrococcal nuclease (16,800), in this gel which was stained with ethidium bromide. Arrows point to the positions of protein molecular weight markers in a parallel gel stained with Coomassie Blue: (1) bovine serum albumin (68,000), (2) ovalbumin (43,000), (3) bovine pancreatic DNase 1 (31,000), (4) micrococcal nuclease (16,800), and (6) lysozyme (13,400).

final contaminant, zinc-metallo endonuclease, in Fraction V was separated from the DNase activities during isoelectric focusing because it focused at pH 4.6 (Fig. 3). During this purification step three DNase activities were found reproducibly at pH values of 5.8 ± 0.1, 7.1 ± 0.1, and 9.1 ± 0.2 (Fig. 3). Gel filtration removed from these activities the slightly inhibitory Ampholines, which caused an inhibition of 25 percent at 0.3% (w/v), and showed that all three activities had identical molecular weights (Fig. 4). The three preparations, Fraction VIA, VIB, and VIC, were specific for DNA and did not contain activity on RNA or on phosphodiesterase and phosphatase substrates. These preparations, called DNase A, DNase B, and DNase C, respectively, were used for all further experiments.

The stability of the three DNase activities was identical. They retained more than 75 percent activity when stored in buffer B at −20°C for 6 months. Repeated freezing and thawing caused some loss of activity. Fifty percent inactivation was found during incubations in buffer B at 40, 45, and 50°C for 16.0 ± 1.0, 4.9 ± 0.3, and 1.25 ± 0.25 min, respectively. No loss of activity was found during DNase assays for up to 50 h at 30°C at the reduced concentration of 0.7 M urea in the incubation mixture.

For all three DNases, deviations from linearity were observed during the digestion of DNA to

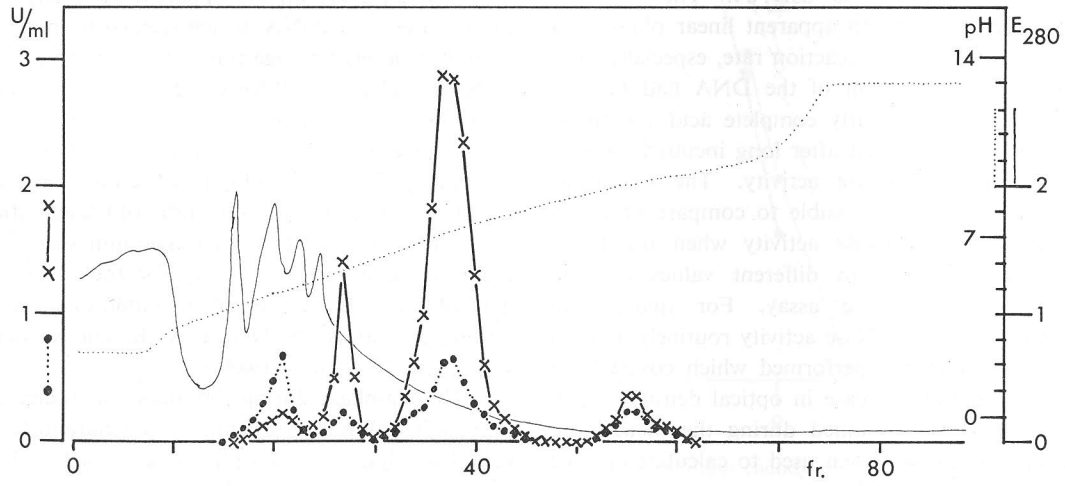

Fig. 3. Isoelectric focusing. Fraction V was focused and the activities on dsDNA (×) and ssDNA (●), pH (·····) and the absorbance at 280 nm (——) of the fractions were determined as described in " MATERIALS AND METHODS."

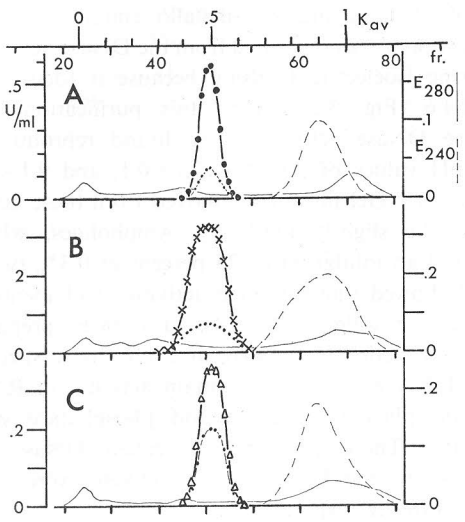

Fig. 4. Sephadex G-100 gel filtration. A. Gel filtration of DNase A (acid isoelectric point), prepared by isoelectric focusing and concentration. Determination of activities on dsDNA (●) and ssDNA (·····), and absorbances at 280 nm (——) and 240 nm (– – –) as described in " MATERIALS AND METHODS." The K_{av} values of the column were determined as described elsewhere (*15*). B. Gel filtration of DNase B (neutral isoelectric point), prepared and determined as for Fig. 4A. C. Gel filtration of DNase C (alkaline isoelectric point), prepared and determined as for Fig. 4A.

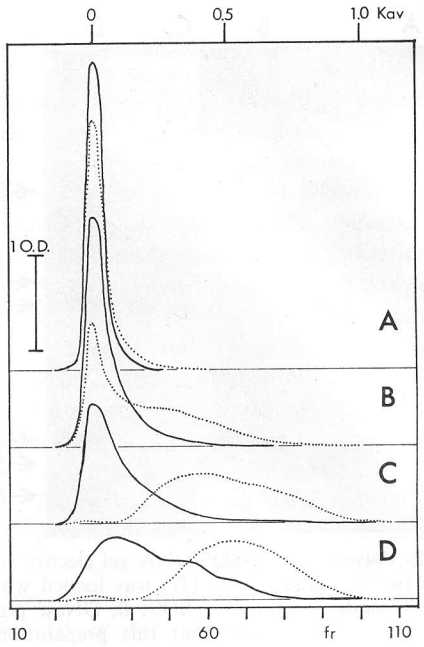

Fig. 5. Sephadex G-200 analysis of dsDNA digested with DNase B. dsDNA was digested with DNase B to 0% (A), 2.0% (B), 13.2% (C), and 36% (D) acid solubility and 20 OD were chromatographed on Sephadex G-200 with (·····) or without (——) denaturation for 10 min at 100°C, as described in " MATERIALS AND METHODS." The K_{av} values of the column were determined as described elsewhere (*15*). The bar indicates of 1 OD unit at 260 nm.

acid-soluble products. A clear lag-phase curve was found for digestions with up to 20 percent acid solubility of the dsDNA. The digestion proceeded through an apparent linear phase to a slowing-down of the reaction rate, especially after more than 50 percent of the DNA had become acid-soluble, but nearly complete acid solubilization could be reached after long incubations with high levels of DNase activity. The non-linear kinetics made it impossible to compare or determine levels of DNase activity when the DNA had been digested to different values of acid solubility during the assay. For quantitative determination of DNase activity routinely a series of incubations was performed which covered the range where an increase in optical density at 260 nm of 1.0 was obtained during the incubation, and this result was then used to calculate enzyme activity (Units per ml).

A difference was noted in the form of the time curves for the digestion of dsDNA and ssDNA. This had an effect on the apparent preference of the DNases for dsDNA, which seemed to increase at higher levels of digestion. For example, the preference of DNase B for dsDNA increased from 2, when five percent of the dsDNA had become acid-soluble, to more than 4 at thirty percent acid solubility. To avoid ambiguity, the preference of the DNases was defined as the ratio of the activities on dsDNA and ssDNA after digestion such that the increase in optical density for the assay on dsDNA was 1.0. Under these standardized conditions the ratios for DNase A, B, and C were 4.0, 2.5, and 1.6, respectively.

The lag-phase during the digestions indicated that the DNA was fragmented by an endonucleolytic hydrolysis of phosphodiester bonds. The analysis of digested DNA on Sephadex G-200 confirmed this conclusion (Fig. 5). The molecular weight of the DNA decreased sharply without the

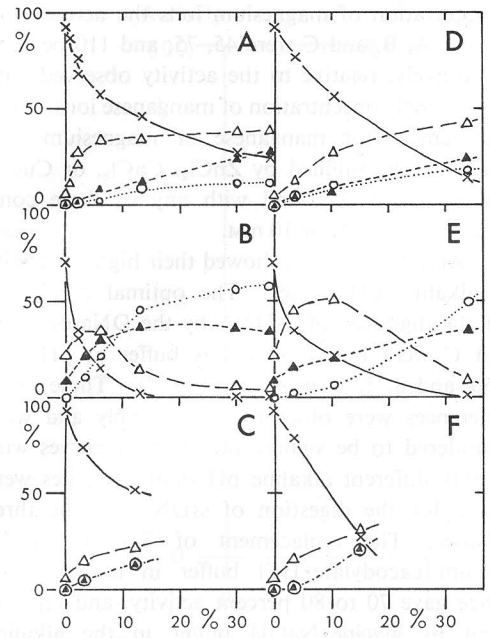

Fig. 6.

production of mononucleotides and with only small amounts of small, acid-soluble oligonucleotide fragments. The analysis also showed that under conditions when almost no change in the molecular weight had occurred in the dsDNA, the strands in the DNA were already very strongly fragmented. Apparently, the major part of the hydrolysis events consisted of unrelated cleavages within the two single strands of the double-stranded

Fig. 6. Kinetic analysis of DNA digested with DNase B. Digestions of dsDNA and ssDNA with DNase B were carried out and analyzed on Sephadex G-200 as already described (Fig. 5). The distribution of various length classes of DNA is plotted against the percentage acid solubility. Optical density up to $K_{av}=0.07$ (×), from K_{av} 0.07 to 0.29 (△), from K_{av} 0.29 to 0.50 (▲), and from K_{av} 0.50 to 1.0 (○). A. Digest of dsDNA at 1 mM $MnCl_2$, analysis without denaturation. B. Digest of dsDNA at 1 mM $MnCl_2$, analysis after denaturation. C. Digest of ssDNA at 1 mM $MnCl_2$. D. Digest of dsDNA at 3 mM $MgCl_2$, analysis without denaturation. E. Digest of dsDNA at 3 mM $MgCl_2$, analysis after denaturation. F. Digest of ssDNA at 3 mM $MgCl_2$.

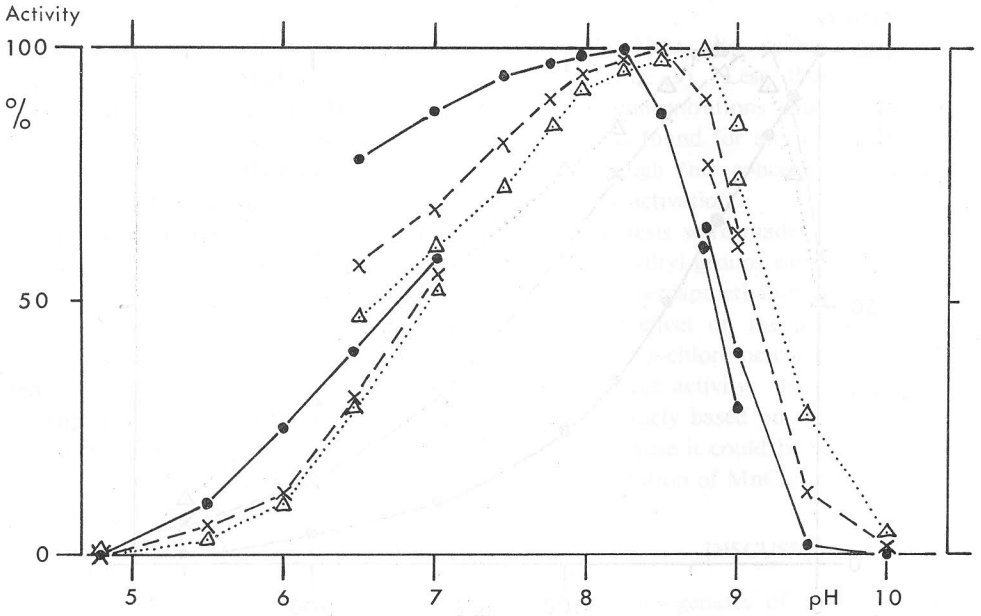

Fig. 7. Optimum pH for DNase activity. The effects of 35 mM sodium cacodylate·HCl buffer (pH 4.8–7.0), 35 mM Tris·HCl buffer (pH 6.5–9.0), and 35 mM glycine·NaOH buffer (pH 8.8–10.0) on the digestion of dsDNA with DNase A (●), DNase B (×), and DNase C (△) at 1 mM $MnCl_2$ are plotted as the activity relative to the highest value found.

DNA. Manganese and magnesium ions showed a difference in the relative frequency of single-stranded and double-stranded breaks (Fig. 6). The two ions also affected the endonucleolytic digestion patterns of ssDNA in different ways (Fig. 6).

Snake venom phosphodiesterase readily digested the polynucleotide products to complete acid solubility. The rate of this digestion was independent of the removal of phosphate termini by preincubation with phosphatase. With spleen phosphodiesterase fast digestion was only found after phosphatase treatment. This proved that 3′-hydroxyl and 5′-phosphate termini were produced.

DNase activity was absolutely dependent on activation by the divalent cations Mn^{2+} or Mg^{2+}. The absence of these ions or the addition of EDTA or EGTA in excess over the concentration of manganese or magnesium ions completely abolished the activity. The requirements for optimal activity differed for the three DNases. Optimal concentrations of $MnCl_2$ were 2.5, 1.2, and 0.8 mM and those of $MgCl_2$ were 7.5, 3.5, and 2.0 mM for DNase A, B, and C, respectively. At the optimal concentration of magnesium ions the activities of DNase A, B, and C were 45, 75, and 110 percent, respectively, relative to the activity observed with the optimal concentration of manganese ions. The requirement for manganese or magnesium ions could not be fulfilled by $ZnCl_2$, $CaCl_2$, or $CuCl_2$. No activity was found with any of these compounds at 1, 2, 5, or 10 mM.

All three DNases showed their highest activity at alkaline pH values. The optimal conditions for the digestion of dsDNA by the DNases A, B, and C were found with Tris buffer at pH 8.25, 8.50, and 8.75, respectively (Fig. 7). These slight differences were observed reproducibly and were considered to be significant. Similar curves with slightly different alkaline pH optimal values were found for the digestion of ssDNA by the three DNases. The replacement of Tris buffer by sodium cacodylate·HCl buffer in the acid pH range gave 70 to 80 percent activity, and replacement by glycine·NaOH buffer in the alkaline pH range gave 120 to 80 percent activity (Fig. 7).

All three DNases were strongly affected by ionic strength. The optimal concentrations for the Tris buffer, when dsDNA was digested at

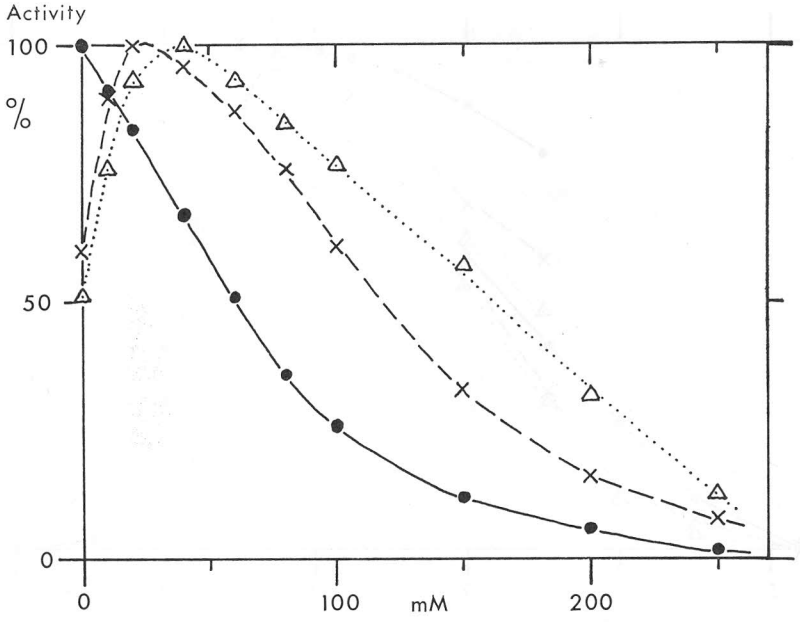

Fig. 8. Optimum Tris concentration for DNase activity. The effect of Tris·HCl buffer, pH 8.5, on the digestion of dsDNA with DNase A (●), DNase B (×), and DNase C (△) at 1 mM $MnCl_2$ is plotted as the activity relative to the highest value found. Distilled water·NaOH, pH approx. 8.5, was used as "0 mM."

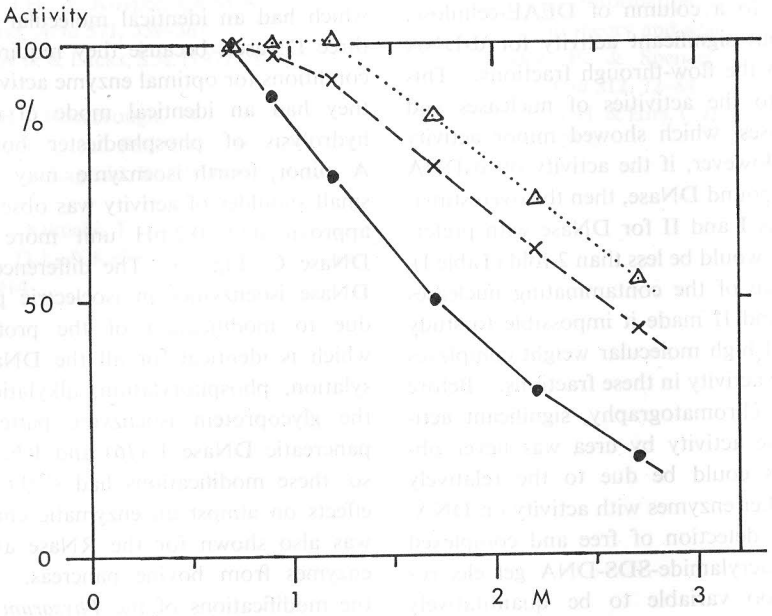

Fig. 9. Inhibition of DNase activity by urea. The effect of urea on the digestion of dsDNA with DNase A (●), DNase B (×), and DNase C (△) at 35 mM Tris·HCl, pH 8.5, and 1 mM $MnCl_2$ is plotted as the activity relative to the activity without the extra addition of urea. Under the starting conditions 0.7 M urea was present from the enzyme preparations added to the assay in buffer B.

1 mM $MnCl_2$, were 0, 25, and 40 mM for DNase A, B, and C, respectively. Higher concentrations caused severe inhibition (Fig. 8). However, the optimal buffer concentration was dependent on the choice of substrate, *i.e.* dsDNA or ssDNA, and activating ion, *i.e.* Mn^{2+} or Mg^{2+}.

DNase A was always more strongly affected by the addition of inhibitory substances than DNase B, and DNase C was always less affected. This was found for the inhibition by salts such as Tris, phosphate, KCl, NaCl, ammonium sulfate, and urea. The phosphate ions caused such a strong, competitive inhibition of DNase activity —fifty percent inhibition of DNase A, B, and C was caused by 0.4, 0.7, and 1.0 mM potassium phosphate—that phosphate buffer could not be used as a replacement for Tris buffer. The inhibition by KCl, NaCl, or ammonium sulfate was exemplified by the inhibition caused by Tris (Fig. 8) or by KCl, which inhibited the activities of DNase A, B, and C by fifty percent at 35, 45, and 60 mM, respectively, while at 200 mM KCl less than ten percent of the activity of all three DNases remained. Urea also caused inhibition of the DNases (Fig. 9). Less than full activity was found at concentrations exceeding 0.5 M and no activity was found for any of the enzymes at 5 M urea, although this concentration was necessary for enzyme activation.

When tests were made to determine whether or not sulfhydryl groups were essential for enzyme activity, 2-mercaptoethanol at 1, 2, and 5 mM showed no effect on the activity of any of the DNases, but *p*-chloromercuribenzoate did strongly inhibit enzyme activity. However, this effect was probably largely based on the binding of divalent cations, because it could be reduced by increasing the concentration of $MnCl_2$ or $MgCl_2$.

DISCUSSION

In crude homogenates of microplasmodia of *P. polycephalum* no DNA-degrading activity with preference for dsDNA was detected (*1*). After partial removal of nucleases not specific for DNA, DNase activity with preference for dsDNA was

detected bound to a column of DEAE-cellulose, although low but significant activity for dsDNA was observed in the flow-through fractions. This could be due to the activities of nucleases and phosphodiesterases, which showed minor activity for dsDNA. However, if the activity on dsDNA were due to the bound DNase, then the overestimation in Fractions I and II for DNase with preference for dsDNA would be less than 2-fold (Table I).

The presence of the contaminating nucleases in Fractions I and II made it impossible to study the formation of high molecular weight complexes with low DNase activity in these fractions. Before DEAE-cellulose chromatography, significant activation of DNase activity by urea was never observed, but this could be due to the relatively high levels of other enzymes with activity on DNA. The method of detection of free and complexed DNase by polyacrylamide-SDS-DNA gel electrophoresis was too variable to be quantitatively useful, although the method was reproducible in a qualitative way. To prevent losses of DNase activity the procedure from collecting microplasmodia to ion exchange chromatography was executed as quickly as possible. The purification procedure also avoided the occurrence of high protein concentrations, such as those found during ammonium sulfate fractionation, because complexation and inactivation appeared to be much faster under these conditions.

The analysis of the fragmentation of DNA showed a clear endonucleolytic mode of action. The cleavage of phosphodiester bonds, which gave 5′-phosphorylated products, proceeded for the major part by the formation of single-strand breaks in the dsDNA. The analysis of dsDNA after limited digestion showed that it was heavily nicked under conditions where the native molecular weight was nearly unchanged. However, some double-strand cleavage did occur, but the method of analysis could not discriminate between single-hit kinetics, *i.e.* simultaneous scission of both strands, and double-hit kinetics, *i.e.* double-strand breaks originating from closely spaced but unrelated single-strand nicks (*16*).

DNase A, B, and C were separated by isoelectric focusing, and were considered to be isoenzymes because they behaved identically during purification, except during isoelectric focusing, because they contained a single polypeptide chain which had an identical molecular weight for the three DNases, because they required very similar conditions for optimal enzyme activity and because they had an identical mode of endonucleolytic hydrolysis of phosphodiester bonds in DNA. A minor, fourth isoenzyme may be present: a small shoulder of activity was observed repeatedly approximately 0.2 pH unit more alkaline than DNase C (Fig. 3). The differences between the DNase isoenzymes in isoelectric point could be due to modification of the protein backbone, which is identical for all the DNases, by glycosylation, phosphorylation, alkylation, *etc.*, as in the glycoprotein isoenzyme patterns of bovine pancreatic DNase 1 (*16*) and RNase A (*17*). If so, these modifications had slight but significant effects on almost all enzymatic characteristics, as was also shown for the RNase and DNase isoenzymes from bovine pancreas. The nature of the modifications of the *Physarum* DNases could not be determined because the final preparations were not pure enough. Higher purity material might be obtained from the growth medium of *Physarum*, into which DNase activity with preference for dsDNA is secreted. A further study of the process of secretion might indicate the function *in vivo* of the DNase isoenzymes from *P. polycephalum*.

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