A comparative study of acid deoxyribonucleases extracted from different tissues and species[1]

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Dedicated to Dr. W. H. Cook on the occasion of his sixty-fifth birthday

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The chromatographic and enzymic properties, the sedimentation coefficients, and the mechanism of action on native deoxyribonucleic acid (DNA) shown by acid deoxyribonuclease (DNase) preparations obtained from different animal sources were compared. The results obtained strongly indicate that the enzymatic activity is associated with protein molecules endowed with very similar physical, chemical, and enzymic properties. The enzyme levels in the tissues may vary by as much as three orders of magnitude. The highest acid DNase levels are found in lymphatic and tumoral tissues, the lowest ones in cells like sperms and erythrocytes that do not reproduce themselves.

Acid deoxyribonuclease (DNase) has been intensively investigated in this laboratory during the past few years. The most interesting properties shown by acid DNase from hog spleen are the following: its ability to cleave simultaneously and at the same level both strands of native DNA (1–3); its competitive inhibition by some natural and synthetic polyribonucleotides (4, 5); its ability to hydrolyze a synthetic substrate, bis(*p*-nitrophenyl)phosphate (6). The physical and chemical properties of the enzyme have been investigated in some detail (7) and it has been shown that each enzyme molecule is formed by two subunits (8). More recently, the specificity of acid DNase has been greatly clarified (9). A review of the work carried out in Strasbourg has been published elsewhere (10), and the reader is referred to it for further details.

It would be obviously very interesting to know the biological role played by acid DNase in the cell. As a first step toward this goal, we have tried to establish whether the acid DNase activities found by many authors in different tissues and cells are carried by similar protein molecules, a point which is by no means clear (see Laskowski (11) for a recent review of the literature). Ideally, this problem could be solved by comparing the chemical, physical and enzymic properties of highly purified enzyme preparations obtained from different sources. This being an impossibly long and difficult task, we compared the following properties of partially purified acid DNase preparations obtained from different sources: (*a*) sedimentation coefficients; (*b*) mechanism of action on native DNA; (*c*) chromatographic behavior on DEAE-cellulose, hydroxyapatite, and Sephadex G-75 columns; (*d*) pH–activity curves and the effects of SO_4^{2-}, Mg^{2+}, and EDTA ions.

Materials and Methods

Materials

The following biological materials were used as sources of acid DNases: (*a*) epithelial tissues: hog liver, hog kidney, hog pancreas; (*b*) lymphatic tissues: hog spleen, calf spleen, calf thymus; (*c*) tumor tissues: a fibroblastic sarcoma from AKR mice and a mammary epithelioma from C_3H mice; (*d*) non-multiplying cells: chicken erythrocytes, hog erythrocytes, mackerel spermatozoa; (*e*) biological fluids: hog serum, bull seminal plasma, human urine pooled from several healthy individuals; (*f*) an invertebrate, the clam *Mercenaria mercenaria*. For several materials two different preparations were investigated.

Most materials were used as soon as possible after collection; alternatively, materials were frozen and stored at −15 °C for periods of time up to a few weeks. In the case of hog pancreas, a commercial organ powder (VioBin, Monticello, Ill.) was used; in the case of *M. mercenaria*, a powder extract prepared by Dr. A. P. Russell was used.

Methods

Enzyme Extraction

All operations were carried out at 0–4 °C. Calf thymus, calf spleen, sarcomas and epitheliomas were extracted according to Bernardi (14), and hog liver and kidney according to Bernardi *et al.* (15). Chicken erythrocytes were washed, lysed, and extracted according to Bernardi

[1]This is paper VI in the series "Studies on acid deoxyribonuclease." For the previous paper in the series see reference 9. Part of this work has been presented in a thesis (12) and in a preliminary communication (13).

TABLE I

Activities of some acid DNase preparations*

Enzyme source	Amount of material (g or ml)	Total extracted activity (units)	Extracted activity (units per g or ml)	Specific activity after chromatographic purification
Hog spleen	1000 g	3000	3	350
Calf thymus	230 g	1270	5.5	66
Mouse epithelioma	30 g	135	4.5	20
Mouse sarcoma	48 g	195	4.0	15
Chicken erythrocytes	2000 ml	103	0.05	3
Hog erythrocytes	1800 ml	1.2	0.0006	
Mackerel sperms	159 g	1	0.006	0.002
Bull seminal serum	25 ml	2	0.08	0.5
Human urine	5400 ml	7.5	0.0014	1
M. mercenaria (powder)	0.176 g	43		20

*Very different values were obtained for the specific activities of different enzyme preparations, owing to the large differences in enzyme levels in different materials, amounts of material treated, and purification procedures used. Activities were determined after ammonium sulfate fractionation, except in the cases of mouse sarcoma and epithelioma and *M. mercenaria*, where they were determined in the extracts.

and Sadron (2). Hog erythrocytes were washed five times, following the procedure used for chicken erythrocytes; they were lysed after it was verified on smears stained according to May–Grunwald–Giemsa that there were no platelets and white blood cells. Hog blood serum and bull seminal plasma were obtained by centrifugation. Mackerel semen was dispersed in 0.15 *M* NaCl and centrifuged; sperms were washed four times with the same solvent; the suspension was adjusted to pH 2.5 with 0.2 *N* H_2SO_4; and after 15 h in this solvent, it was homogenized in a Potter–Elvehjem homogenizer. The hog pancreas powder was dispersed in distilled water, dialyzed against distilled water, and clarified by centrifugation. The *M. mercenaria* extract was dispersed in 0.15 *M* NaCl and clarified by centrifugation.

Enzyme Fractionation

Enzyme extracts were fractionated, except where otherwise stated, using the following two steps (6, 15): (*a*) acidification to pH 2.5 by dropwise addition of 0.1 *N* H_2SO_4, (*b*) fractionation between 0.4 and 0.8 saturation with $(NH_4)_2SO_4$. This treatment was used in the case of enzyme extracts from hog erythrocytes, hog serum, and mackerel sperms (in which last case an acidic treatment had already been used to lyse the cells (see above)). Extracts from calf thymus and calf spleen were fractionated according to Bernardi (14), and those from hog liver, kidney, and spleen were fractionated according to Bernardi *et al.* (15). Extracts from chicken erythrocytes, hog pancreas, and urine were fractionated only with $(NH_4)_2SO_4$. Extracts from sarcoma, epithelioma, and *M. mercenaria* were used as such.

Enzyme Assay

Acid DNase activity was assayed by determining acid-soluble oligonucleotides formed by DNA degradation as described elsewhere (6, 15, 16). Activity units and specific activity have been already defined (6, 15, 16).

Sucrose-gradient centrifugations were performed according to Martin and Ames (17), using experimental conditions described elsewhere (7), with beef heart cytochrome *c* (type V; Sigma, St Louis, Mo.) as a reference protein.

Initial degradation of native DNA by acid DNase preparations was investigated by viscometry, to determine the mechanism of action of the enzymes, using the experimental conditions of Bernardi and Cordonnier (18). Kinetic data were treated according to Schumaker *et al.* (19).

Column chromatography of enzyme preparations was performed as already described (6). In most cases, two successive chromatograms were run, the first on DEAE-cellulose, the second on hydroxyapatite columns using the experimental conditions of Bernardi and Griffé (6). DEAE-cellulose was equilibrated with 0.005 *M* phosphate buffer, pH 8.0. Under this condition, the column does not adsorb hog spleen acid DNase. A pH and molarity gradient (limiting buffers, 0.005 *M* phosphate, pH 8.0, and 0.15 *M* acetate, pH 5.0, respectively) was then applied to elute the proteins which had been retained by the column. Hydroxyapatite was equilibrated with 0.05 *M* phosphate buffer, pH 6.8, and elution was done using a linear molarity gradient of phosphate buffer, pH 6.8 (0.05–0.5 *M*). Under these conditions, hog spleen acid DNase is eluted at a molarity of 0.25.

Results

Table I summarizes the values for total extracted activity obtained from 10 of the preparations studied. The specific activities obtained after chromatographic purification (see below) are also presented in Table I. These values are reported since they give at least an order of magnitude of the enzyme levels present in different biological materials.

Sedimentation Coefficients

These were determined on all enzyme preparations except that from hog pancreas; the second hog liver enzyme fraction from DEAE-cellulose was also investigated. In all cases the sedimen-

tation coefficients were found to be equal to 3.3 ± 0.1. Figure 1 shows two examples of sucrose density-gradient centrifugations.

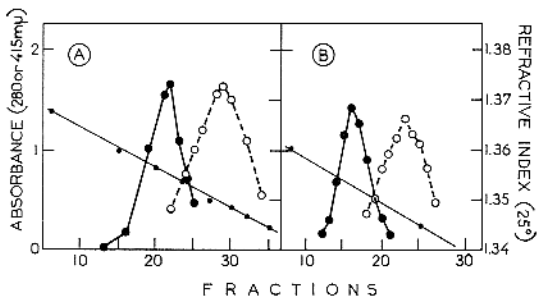

FIG. 1. Sucrose-gradient centrifugation of acid DNase preparations from mouse epithelioma (*A*) and hog erythrocytes (*B*). 36 and 30 fractions were collected in the two cases, respectively. (●), Acid DNase activity; (○), absorbance at 415 mμ of cytochrome *c*. The scale on the right indicates the refractive index of fractions at 25°. A linear molarity gradient was obtained by using 5% and 20% sucrose solutions in 0.15 *M* acetate buffer – 0.01 *M* EDTA, pH 5.0. Centrifugation was carried out at 4° and 38 000 r.p.m. for 16 h in a SW-39 rotor using a Spinco model L preparative ultracentrifuge.

Mechanism of Action on Native DNA

The kinetics of the initial degradation of native DNA from calf thymus were investigated by viscometry on the purified enzyme preparations from mouse epithelioma, chicken erythrocytes, human urine, hog erythrocytes, and *M. mercenaria*. In all cases, the initial degradation took place according to a "single-hit" kinetics, as already found for hog spleen acid DNase (1–3). The results obtained are shown in Table II. Examples of degradation kinetics are shown in Fig. 2.

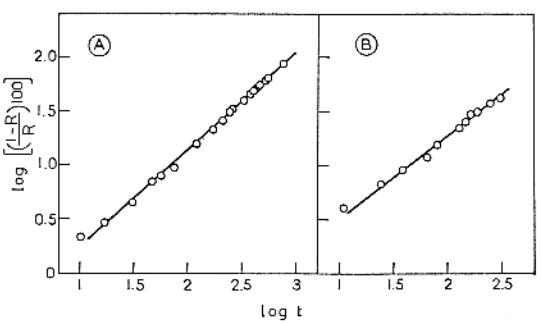

FIG. 2. Initial degradation of calf thymus DNA by acid DNase preparations from hog spleen (*A*) and mouse epithelioma (*B*) as followed by viscometry. *R* is defined in a footnote to Table II, and *t* is digestion time. For other experimental details see Materials and Methods. The slope of the straight line is equal to *n*, the apparent number of strands.

TABLE II

Mechanism of degradation of native DNA by acid DNase preparations

Enzyme source	Method	n^a
Hog spleen	Light scattering	1 ± 0.1^b
Calf spleen	"	1.1^b
Calf thymus	"	1.0^b
Pancreatic DNase[c]	"	$1.7–2.0^b$
Hog spleen	Viscometry	0.9^d
Mouse epithelioma	"	0.9
Chicken erythrocytes	"	0.9
Hog erythrocytes	"	0.9
M. mercenaria	"	0.9
Human urine	"	0.9
Pancreatic DNase[c]	"	1.5

[a] *n* is the apparent number of strands, such as obtained from the slope of log $(1 - R)/R$ versus log *t* (see Fig. 2). *R* is equal to M_t/M_0 or to η_t/η_0, where M_t, η_t, M_0, and η_0 are the weight-average molecular weights or the specific viscosities (at an average shear gradient equal to 45 sec^{-1}) at digestion times *t* and 0, respectively.

[b] Data of Bernardi and Sadron (2); see this reference for the experimental conditions used.

[c] Data obtained with pancreatic DNase are shown to provide a comparison.

[d] Data of Bernardi and Cordonnier (17); see this reference for the experimental conditions used.

Chromatographic Properties

The acid DNase activities present in the enzyme preparations obtained from calf thymus, mouse sarcoma and epithelioma, human urine, and *M. mercenaria* were not adsorbed by DEAE-cellulose columns equilibrated with 0.005 *M* phosphate buffer, pH 8.0. Upon the successive chromatography of the active fraction on hydroxyapatite columns, DNase activities were eluted by 0.25 *M* phosphate buffer, pH 6.8. This chromatographic behavior is exactly that shown by acid DNase from hog spleen (6). As an example, the results obtained with the preparation from mouse epithelioma are shown in Fig. 3. The active fractions obtained from hydroxyapatite columns were then loaded on Sephadex G-75 columns. It was found that, in all cases, the elution volume of the DNase activity was the same as that of hog spleen acid DNase. Extracts from hog erythrocytes and mackerel sperms were chromatographed on hydroxyapatite only; in both cases, elution took place at about 0.25 *M* phosphate buffer, pH 6.8.

A different chromatographic behavior was observed for the enzymatic activities present in bull seminal plasma, hog serum and liver, and chicken erythrocytes. The chromatographic behavior of the last two preparations was studied in some detail, and will be described here. The acid DNase activity of chicken erythrocytes was adsorbed and eluted from the DEAE-cellulose column during the pH–molarity gradient

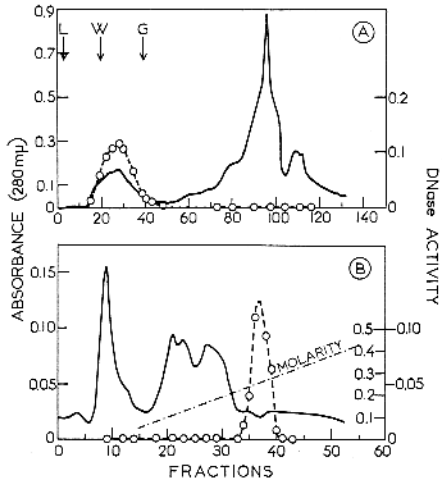

FIG. 3. *A*. Chromatography of a mouse mammary epithelioma extract obtained from 24 g of tissue on a 2 × 25 cm DEAE-cellulose column. 10-ml fractions were collected. *B*. Chromatography of fractions 16–40 from the previous experiment on a 1 × 10 cm hydroxyapatite column. 4.5-ml fractions were collected. The absorbance at 280 mμ is indicated by a continuous line (left scale) and the acid DNase activity per milliliter by a broken line (right outer scale). Effluent molarity is indicated by the right inner scale. Arrows marked L, W, and G show the fractions corresponding to the loading of the product on the column, the washing of the column by the equilibration buffer, and the start of the gradient, respectively. See Materials and Methods for the solvents used for elution.

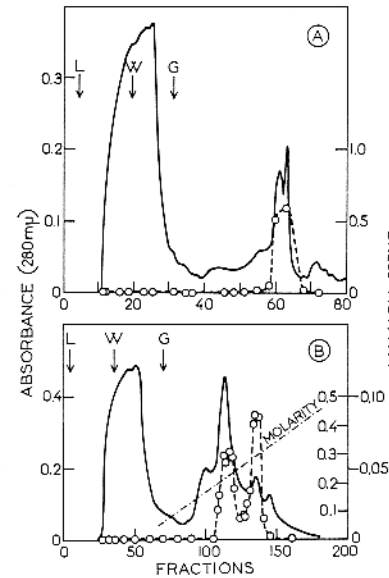

FIG. 4. *A*. Chromatography of a chicken erythrocyte extract obtained from 2000 ml of blood on a 2 × 20 cm DEAE-cellulose column. 10-ml fractions were collected. *B*. Chromatography of a chicken erythrocyte extract obtained from 800 ml of blood on a 3 × 24 cm hydroxyapatite column. 7-ml fractions were collected. Other indications as in Fig. 3.

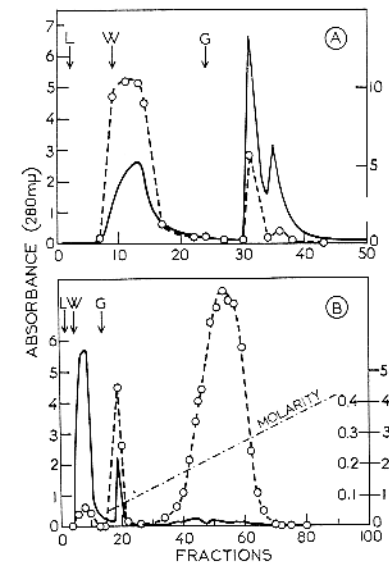

FIG. 5. *A*. Chromatography of a hog liver extract on a 1.3 × 17 cm DEAE-cellulose column. 4-ml fractions were collected. *B*. Chromatography of a hog liver extract on a 1 × 13 cm hydroxyapatite column. 4-ml fractions were collected. Other indications as in Fig. 3.

(Fig. 4*A*). When unfractionated material was chromatographed on a hydroxyapatite column, the DNase activity was eluted between 0.20 and 0.30 *M* phosphate buffer, pH 6.8, in two peaks (Fig. 4*B*); the same behavior was found upon rechromatography. The elution volume of the activity from Sephadex G-75 columns was the same as that shown by the other enzyme preparations mentioned above. The acid DNase preparation of hog liver showed two activity peaks upon chromatography on DEAE-cellulose; a major component was not retained, whereas a minor one was eluted by the pH–molarity gradient (Fig. 5*A*). The unfractionated preparation also showed two activity peaks on hydroxyapatite; the first one was eluted at 0.05 *M*, the second one at 0.25 *M* phosphate buffer (Fig. 5*B*). When the two active fractions from DEAE-cellulose were chromatographed on hydroxyapatite, the first fraction was eluted at about 0.25 *M* phosphate buffer whereas the second one was eluted at about 0.05 *M* (Figs. 6*A*, 6*B*). This finding shows that the first acid DNase peak obtained from DEAE-cellulose corresponds to the second activity peak from hydroxyapatite,

whereas the second, minor, component from DEAE-cellulose can be identified with the activity eluting at 0.05 *M* phosphate from hydroxyapatite.

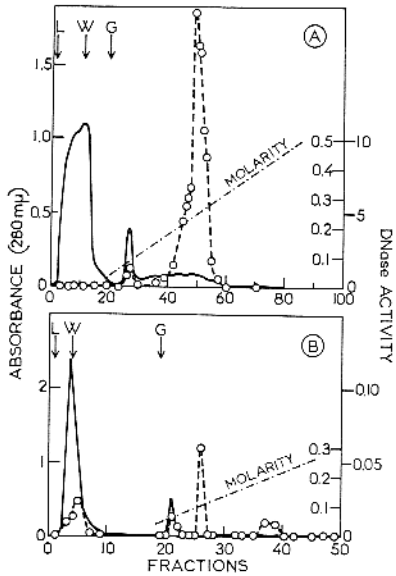

FIG. 6. *A*. Chromatography of fractions 7–18 from the DEAE-cellulose column chromatography shown in Fig. 5*A*, on a 1 × 13 hydroxyapatite column. 4-ml fractions were collected. *B*. Chromatography of fractions 31–32 from the DEAE-cellulose column chromatography shown in Fig. 5*A*, on a 1 × 6 cm hydroxyapatite column. 3-ml fractions were collected. Other indications as in Fig. 3.

The bull seminal plasma preparation also showed two activity peaks on DEAE-cellulose, the first one being eluted by the equilibration buffer, the second one by the pH–molarity gradient. Further chromatographic investigations on these components did not give satisfactory results because of their low activities.

pH–Activity Curves: Effect of Mg^{2+}, SO_4^{2-}, and EDTA

These were investigated using four enzyme preparations showing different specific activities; highly purified hog spleen acid DNase (6, 15) was used as a reference. The enzyme preparations used were those obtained from calf thymus, mouse epithelioma, mouse sarcoma, and chicken erythrocytes; their specific activities were 66, 20, 15, and 3, respectively (Table 1), as compared with 350 for the hog spleen enzyme.

Some of the results obtained are shown in Figs. 7–9. At ionic strength $\mu = 0.15$ and in the presence of 0.01 *M* EDTA, the pH optimum was found to be close to 4.9. EDTA is an activator, whereas Mg^{2+} and SO_4^{2-} are inhibitors. With

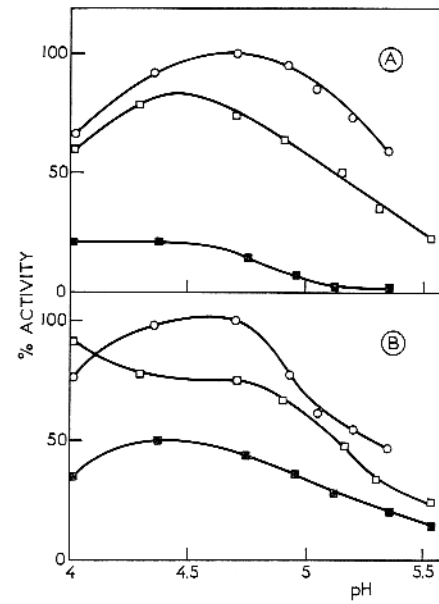

FIG. 7. Activity of chromatographically homogeneous hog spleen acid DNase (*A*) (data of ref. 6) and of calf thymus acid DNase (*B*). Ionic strength was equal to 0.15. Solvents were acetate buffer – 0.01 *M* EDTA (○), acetate buffer – 0.01 *M* $MgCl_2$ (□), acetate buffer – 0.01 *M* Na_2SO_4 (■).

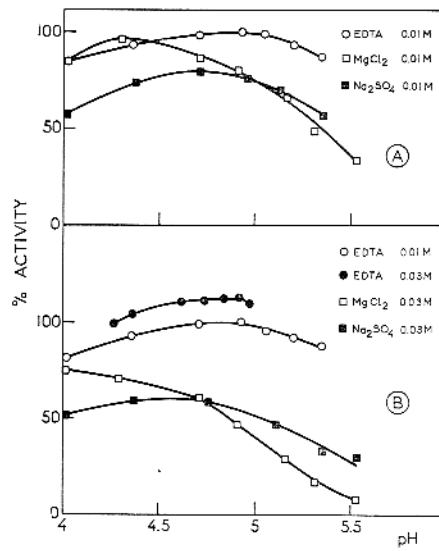

FIG. 8. Acid DNase activity of the mouse epithelioma enzyme preparation in the presence of two concentrations of EDTA, Mg^{2+}, and SO_4^{2-}. Solvents were acetate buffers added with EDTA, Mg^{2+}, or SO_4^{2-}, as indicated in the figure.

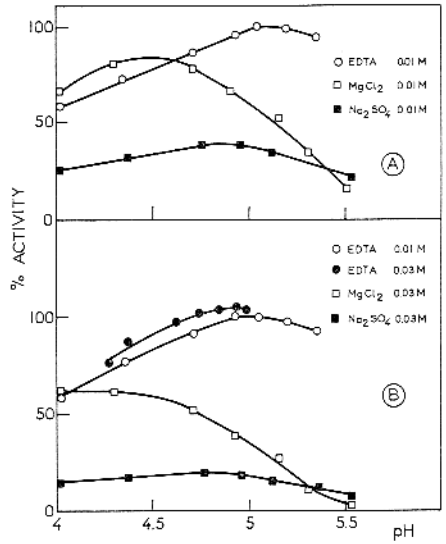

FIG. 9. Acid DNase activity of the chicken erythrocyte enzyme preparation in the presence of two concentrations of EDTA, Mg^{2+}, and SO_4^{2-}. Solvents were acetate buffers added with EDTA, Mg^{2+}, or SO_4^{2-}, as indicated in the figure.

low specific-activity preparations, these inhibitory effects become evident only with higher concentrations of Mg^{2+} and SO_4^{2-}. At least to some extent, this phenomenon seems to be due to the presence of other proteins in the impure enzyme preparation used. In fact, if one investigates the effect of these ions on hog spleen acid DNase at different levels of purity, it is evident that inhibitory effects increase with the purity of the enzyme, particularly for Mg^{2+} inhibition (Table III).

TABLE III

Inhibition of hog spleen acid DNase activity by sulfate and magnesium

Specific activity	% Activity* in the presence of:	
	0.01 M Mg^{2+}	0.01 M SO_4^{2-}
3.8	45	9
45	29	3.3
150	15	4
250	5.7	2.7

*Activities were measured in 0.15 M acetate buffer, pH 5.0, with the additions indicated. The activities measured in 0.15 M acetate buffer – 0.01 M EDTA, pH 5.0, were taken as equal to 100%.

Discussion

It is very difficult to know, on the basis of the present results, the acid DNase levels of different cells. The data presented in Table I can, however, help in providing at least a gross estimate. Clearly, tissues with a high proliferating activity, like lymphatic and tumoral tissues, have the highest acid DNase levels. In contrast, cells which do not reproduce themselves, like sperms and erythrocytes, are extremely poor in acid DNase, the enzyme level being three orders of magnitude lower. This relationship between levels of acid DNase activity and capacity for proliferation or regeneration of a given tissue was first observed by Allfrey and Mirsky (20) in 1952. It is interesting to point out that the enzyme level of nucleated chicken erythrocytes is so low that these authors were unable to detect it. The acid DNase level of anucleated mammalian erythrocytes is even lower, and one might wonder whether the enzyme did not come from a trace contamination of erythrocytes by other blood cells. The precautions taken in preparing the cells and the finding of very small amounts of DNA in human erythrocytes (21) lead one to believe that an acid DNase activity is really present in anucleated erythrocytes. The presence of acid DNase activity in biological fluids opens the problem of the origin of the enzyme; further work needs to be done to understand from which cells the enzyme is derived and by which mechanism it is released.

Two main lines of evidence gathered in the present work support the conclusion that acid DNase activities present in different cells and species are associated with very similar protein molecules. First, the sedimentation coefficients of the acid DNase preparations investigated so far have been found to be the same within experimental error. Second, the results of the kinetic experiments indicate that all enzyme preparations show the typical "single-hit" kinetics of degradation of native DNA already known for spleen acid DNase. This kinetics has been shown to be due to the simultaneous breakage of both DNA strands at the same level (1–3).

The chromatographic and enzymic properties shown by the enzyme preparations are in general agreement with the conclusion mentioned above, since in most cases they were very similar to those of spleen acid DNase, which was taken as a reference because it was better known. Some exceptions were found, however, and certainly they deserve to be investigated in more detail. At

the present time the different chromatographic behavior of some enzyme preparations may be explained as due to differences in the primary structures which would be expected to exist in enzymes derived from different animal species, and/or the fact that the presence of accompanying proteins, which differ so much both in quantity and in quality from one preparation to the other, may interfere with the "normal" chromatographic behavior of the enzyme. Similar explanations may hold for the slight differences in the pH–activity curves or in the effects of EDTA, Mg^{2+}, and SO_4^{2-}. The variable effects of EDTA, Mg^{2+}, and SO_4^{2-} observed with enzyme preparations of different purity (see Table III) may explain some of the contradictory results obtained with crude enzyme preparations by previous authors (see refs. 11 and 22 for reviews of the literature).

In conclusion, it is likely that, in spite of variations in the chromatographic and enzymic properties shown by some enzyme preparations, acid DNase activity is carried by very similar protein molecules, possibly present in all cells of eukariotic organisms.

Acknowledgments

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