

thymine dimer leaving a 3'-phosphoryl terminus. Nucleotides are not released during this incision step. The subsequent thymine dimer excision is carried out by the second enzyme, an exonuclease also purified extensively (1500-fold) by Grossman *et al.* This enzyme, whose activity is dependent upon added magnesium ion, acts on unirradiated denatured DNA releasing 5'-mononucleotides by an exonucleolytic mechanism starting at either the 3' or 5' terminus. Native DNA is resistant to the action of the exonuclease; however, the enzyme will attack irradiated native DNA that has been pretreated with the endonuclease in the 5' → 3' direction liberating an average of 6 nucleotides per endonucleolytic break. The digestion products consist of mono-, di-, and trinucleotides; and the thymine dimers are contained in the trinucleotide fragments. Purified preparations of the exonuclease are devoid of DNA polymerase activity, suggesting that the UV exonuclease is not a 5' → 3' exonuclease component of the *M. luteus* DNA polymerase.

It would appear from these studies that the excision of thymine, and more generally pyrimidine dimers may be a two-step process. The initial single-stranded incision is probably dependent upon the presence of a distorted area in the DNA duplex resulting from the formation of a thymine dimer. Once the initial break has been introduced, a short single-stranded region containing the photoproduct results which is then susceptible to the action of the exonuclease.

Grossman *et al.* have isolated a mutant of *M. luteus* by nitrosoguanidine mutagenesis which is abnormally sensitive to UV and X-irradiation and also shows a reduced capacity to support the replication of UV-irradiated phages (the *hcr⁻* phenotype). Extracts of this mutant have a correspondingly low level of UV endonuclease activity (76). Takagi *et al.* have transformed the mutant with DNA derived from UV-resistant cells and found that the UV-resistant transformants displayed the same level of sensitivity to UV irradiation as the wild type; however, the UV endonuclease activity in the extract remained at the same low level observed in the original mutant strain (75). All attempts to isolate a revertant have thus far been unsuccessful, suggesting that the mutant bacterium may harbor a double mutation. Thus, while it appears that the UV endonuclease may be involved in the repair of UV damage *in vivo*, this point has not been firmly established.

11

Spleen Acid Deoxyribonuclease

GIORGIO BERNARDI

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I. Introduction

Acid deoxyribonuclease (DNase) is an enzyme which splits the phosphodiester bonds of native DNA by both a diplotomic and a haplotomic mechanism (see Section III,C) leaving the terminal phosphate in a 3' position. The enzyme is very widely distributed in animal cells and appears to be localized in the lysosomes. The best known acid DNase is that from hog spleen; this explains why most of the data presented here refer to this enzyme. It should be stressed, however, that the properties of acid DNases obtained from the tissues of other vertebrates appear to be extremely similar to those of the hog spleen enzyme;

therefore, the results obtained with hog spleen acid DNase may be of a more general validity.

The present article will review the progress made in our knowledge of acid DNase during the past 10 years, therefore after the second edition of "The Enzymes" (1). No attempt has been made to cover exhaustively the literature on this subject since several reviews dealing with acid DNase have been published in the meantime (2-6).

The DNase activity having a pH optimum comprised between 4.5 and 5.5, first observed in animal tissues in the late 1940's, was referred to by the early investigators as acid DNase. Later the name DNase II, intended to mean the second type of animal DNase was suggested for the acid DNase activity (7) to contrast it with pancreatic DNase, which was called DNase I. Subsequently, DNases have been classified as 5'-monoester formers and 3'-monoester formers (1), a division which is not identical with that of DNases I and II, even if frequently it is assumed to be so. More recently, it has been suggested to abandon the distinction between DNase I and DNase II altogether (5). Here the original terminology will be used since it is the least inconsistent among those proposed so far.

II. Physical and Chemical Properties

A. ISOLATION

Methods leading to homogeneous acid DNase preparations from hog spleen have been described (8-11). The following is a very brief outline of the method of Bernardi *et al.* (10) as presently used in the author's laboratory. Hog spleens are trimmed, ground, and homogenized with 0.05 M H₂SO₄; the homogenate is acidified to pH 2.5 with 0.1 M H₂SO₄ and centrifuged; the supernatant so obtained is fractionated be-

1. M. Laskowski, "The Enzymes," 2nd ed., Vol. 5, p. 123, 1961.
2. M. Privat de Garilhe, "Les nucléases." Hermann, Paris, 1964.
3. I. R. Lehman, *Ann. Rev. Biochem.* **36**, 645 (1967).
4. W. E. Razzell, *Experientia* **23**, 321 (1967).
5. M. Laskowski, *Advan. Enzymol.* **29**, 165 (1967).
6. G. Bernardi, *Advan. Enzymol.* **31**, 1 (1968).
7. L. Cunningham and M. Laskowski, *BBA* **11**, 590 (1953).
8. G. Bernardi, M. Griffé, and E. Appella, *Nature* **198**, 186 (1963).
9. G. Bernardi, and M. Griffé, *Biochemistry* **3**, 1419 (1964).
10. G. Bernardi, A. Bernardi, and A. Chersi, *BBA* **129**, 1 (1966).
11. G. Bernardi, *Procedures Nucleic Acid Res.* p. 102 (1966).

tween 40 and 80% saturation of (NH₄)₂SO₄; the final precipitate is dialyzed against 0.05 M phosphate buffer, pH 6.8, and clarified by centrifugation. The crude enzyme solution so obtained is purified using three chromatographic steps involving DEAE-Sephadex, hydroxyapatite, and CM-Sephadex, respectively. The enzyme is eluted from the last column in two activity peaks, the first component representing less than 20% of the total activity. The two components, called A and B, respectively, are rechromatographed separately on CM-Sephadex columns, dialyzed against 0.001 M acetate buffer, pH 5.0, concentrated by freeze-drying to about 1% concentration, then frozen and stored at -15°.

Hog spleen acid DNase, as obtained by the above procedure, is completely free of contaminating phosphatase, exonuclease, and adenosine deaminase activities. The enzyme has a weak intrinsic hydrolytic activity on bis(*p*-nitrophenyl) phosphate and the *p*-nitrophenyl derivatives of deoxyribonucleoside 3'-phosphates (see Section III,D,3).

B. PHYSICAL AND CHEMICAL PROPERTIES

The physical properties and the amino acid analysis of hog-spleen acid DNase B (main component) are given in Tables I and II, respectively.

The high ammonia level of the acid hydrolyzate and the high amide level seen in Pronase digests suggest that a very large percentage of the dicarboxylic acids may be present in the protein as the corresponding amides. Glucosamine (12) and mannose (R. G. Winzler, personal

TABLE I
PHYSICAL PROPERTIES OF HOG SPLEEN ACID DEOXYRIBONUCLEASE^a

$s_{20,w}$ (Svedbergs) (analytical centrifugation)	3.4
(sucrose gradient centrifugation)	3.3 ± 0.1
$D_{20,w}$ (10 ⁻⁷ cm ² /sec) ^b	7.8
\bar{V} (ml/g) ^c	0.72
Molecular weight (from s and D)	3.8 × 10 ⁴
(from sedimentation equilibrium) ^d	4.1 × 10 ⁴
f/f_0	1.34
$E_{280}^{1\%,1\text{ cm}}$	12.1

^a From Bernardi *et al.* (12).

^b This value was obtained at concentrations of 0.5% and about 0.1%.

^c Value calculated from amino acid composition.

^d Townend and Bernardi (13).

12. G. Bernardi, E. Appella, and R. Zito, *Biochemistry* **4**, 1725 (1965).

13. R. Townend and G. Bernardi, *ABB*, submitted for publication.

TABLE II
AMINO ACID ANALYSIS OF HOG SPLEEN DEOXYRIBONUCLEASE^a

Amino acid	Grams of amino acid residues per 100 g of protein ^b in hydrolysis time of			Corrected values ^c	Moles of amino acid/mole of protein MW 38,000	Nearest integral No. of residues/mole of protein ^d
	22 hr	48 hr	72 hr			
Lys	6.94	6.80	6.95	6.89	20.44	20
His	2.13	2.17	2.31	2.20	6.08	6
(NH ₃)	(23.05)	(23.65)	(24.06)	(22.5)	(49.4)	(49)
Arg	5.04	5.55	5.37	5.46	13.30	13
Asp	10.19	10.14	9.36	9.89	32.68	33
Thr	5.44	5.20	4.80	5.63	21.16	21
Ser	8.30	8.10	7.18	8.46	36.93	37
Glu	11.03	11.09	10.66	10.93	32.19	32
Pro	7.09	7.14	6.51	6.91	27.05	27
Gly	3.88	3.97	3.87	3.90	25.99	26
Ala	4.84	4.72	4.33	4.63	24.77	25
½-Cys	1.77	1.79	1.79	1.79	6.65 ^e	8
Val	2.62	3.33	3.35	3.35	12.84	13
Met	1.63	1.65	1.30	1.53	4.45	4
Ileu	2.08	2.49	2.52	2.52	8.47	8
Leu	10.47	11.13	10.49	10.67	35.87	36
Tyr	5.23	5.17	4.83	5.28	12.31	12
Phe	6.30	6.56	6.07	6.31	16.30	16
Try	6.30				6.3 ^f	6
Glucosamine	3.07	3.32	3.06	3.15	7.45	(8)
Total	98.05	100.32	94.75	99.50		343
N recovery (%)	98.9	98.7	97.4			

^a From Bernardi *et al.* (12).

^b Total N is 17.2%; total S is 1.0%.

^c In calculating the corrected values, the criteria given by Tristram and Smith (14) have been followed.

^d The selection of the integral numbers of residues has been done also taking into account results from other analyses.

^e After performic acid oxidation, cysteic acid 8.2 residues.

^f From N-bromosuccinimide titration.

communication, 1967) are present in the protein; in the tryptic digests, the glucosamine and neutral sugar residues are found on a single peptide spot. No free sulfhydryl groups can be detected in acid DNase, both native and denatured (G. Bernardi, unpublished observations); therefore, the eight half-cystine residues must form four disulfide bridges.

Optical rotatory dispersion, circular dichroism, and infrared spectra of

acid DNase have shown that the enzyme contains little α -helix; anti-parallel pleated sheet β -structure is probably present in the molecule (13a).

C. A AND B COMPONENTS

A comparison of the properties of the two acid DNase components A and B showed no differences in the sedimentation velocities, elution volumes from Sephadex G-100 columns, ultraviolet spectra, orcinol reaction, and enzymological properties (DNase and phosphodiesterase activities). Two differences (besides their different behavior on CM-Sephadex columns, which indicates that component A is likely to be slightly less basic than component B) have been found so far between the two components: (1) the level of both glucosamine and mannose are definitely lower in component A compared to component B (R. G. Winzler, personal communication, 1967); (2) one particular single tryptic peptide spot of component A is resolved into two spots in the otherwise identical peptide map of component B. Since the amount of component A may be reduced to zero by avoiding acidification, the latter finding may tentatively be explained (10) by assuming that the difference between A and B results from the deamidation of an asparagine (or a glutamine) residue adjacent to a lysine (or an arginine) by the acid treatment involved during the preparation of the enzyme, leading to a peptide bond resistant to trypsin. Alternatively, it may be thought that during acidification a peptide bond has been split by cathepsins present in the homogenate. The loss of sugars might also be due to acid hydrolysis or to an enzyme attack. It is possible that the two chromatographic components of spleen acid DNase seen by Koszalka *et al.* (15) on Amberlite IRC-50 had an origin similar to components A and B, since 5 N H₂SO₄ was used by these authors to adjust the tissue extract to pH 4.0.

D. DIMERIC STRUCTURE

Some chemical and physical results seem to suggest that acid DNase may have a dimeric structure (6, 16). When the enzyme is reduced, carboxymethylated, digested with crystalline trypsin (treated with 1,1-

13a. S. N. Timasheff and G. Bernardi, *ABB* 141, 53 (1970).

14. G. R. Tristram and R. H. Smith, *Advan. Protein Chem.* 18, 227 (1967).

15. T. R. Koszalka, R. Falkenheim, and K. I. Altman, *BBA* 33, 647 (1957).

16. G. Bernardi, *JMB* 13, 603 (1965).

tosylamido-2-phenethyl-chloromethyl ketone to inactivate contaminating chymotrypsin), and mapped, 17–19 peptides are found as opposed to 32–34 arginine + lysine residues present in each enzyme molecule of MW = 38,000. Arginine, tryptophan, and histidine peptides are found in half, or less than half, the number of the respective amino acids in the supposedly dimeric protein.

Other results also suggesting a dimeric structure come from sedimentation studies. At a 1% concentration, the sedimentation coefficient of the enzyme, which is 2.8 S in acetate or phosphate buffers, drops to 1.75 and 2.1 S in 6 M guanidine at pH 5.6 and 8.6, respectively; in the presence of β -mercaptoethanol, the sedimentation coefficient is still lower, as expected: 1.5 S in 6 M guanidine and only 0.8 S in 8 M urea; in 4 M urea–0.05 M β -mercaptoethanol, pH 5.0, two boundaries, having sedimentation coefficients equal to 2.6 and 0.8 S, respectively, can be seen.

Cooperative binding of the synthetic substrate bis(*p*-nitrophenyl) phosphate and of a protein inhibitor (see Sections III,D,2 and 3) might also be considered as an indirect indication of a dimeric structure.

Recent equilibrium sedimentation studies (13) show, however, that the molecular weight of acid DNase in 6 M guanidine (with or without 0.1 M β -mercaptoethanol) is close to 40,000. Clearly, further investigations on acid DNase are needed for a better understanding of the relationships between structure and mechanism of action of this enzyme.

III. Catalytic Properties

A. GENERAL FEATURES OF DNA DEGRADATION

Three different phases can be distinguished in the degradation of native DNA by acid DNase as follows (see Fig. 1):

(1) The initial phase, in which the macromolecular and biological properties of DNA are dramatically modified, whereas no change can be detected in its spectral properties and no acid-soluble fragments are formed. In terms of molecular weight this phase extends from the initial molecular weight to about 10^5 daltons [weight average molecular weight, M_w , of double-stranded fragments (17, 18)].

(2) The middle phase, which is characterized by a hyperchromic shift and the formation of acid-soluble oligonucleotides; monoesterified phosphate can be detected. In this phase, the increase of ultraviolet absorption and of acid-soluble oligonucleotides is linear with the reciprocal

17. G. Bernardi, *Nature* **206**, 779 (1965).

18. G. Bernardi, *BBA* **174**, 423 (1969).

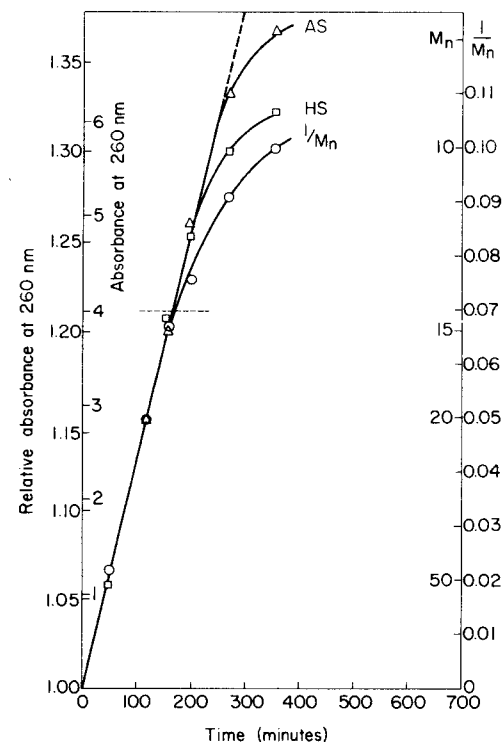


Fig. 1. Hyperchromic shifts (\square ; left-hand outer scale), acid solubility (\triangle ; left-hand inner scale; values corrected for dilution), reciprocal average size (\circ ; right-hand outer scale) of oligonucleotides from calf thymus DNA during acid DNase digestion. The DNA sample ($A_{260} = 8.0$) was digested at 23° in 0.05 M ammonium acetate–0.01 M EDTA, pH 5.5. The horizontal broken line indicates the limit between the middle phase and the terminal phase of the digestion. In this experiment the initial lag time of both hyperchromic shift and acid solubility is not apparent because of the high enzyme concentration used (19).

number average molecular weight of oligonucleotides, $1/M_n$, that is the relative number of terminal nucleotides. Under the experimental conditions of Fig. 1, the average chain length in this phase is comprised between 100 and 14 nucleotides (19). It is important to stress that the limits of the middle phase may be shifted by changes in the experimental conditions of the digestion (temperature, pH, and ionic strength of the solvent).

(3) The terminal phase, which shows an increasingly slower, further increase in the hyperchromic shift and acid-soluble oligonucleotide formation. Both phenomena, however, are no more linear with number of

19. C. Soave, J. P. Thierry, S. D. Ehrlich, and G. Bernardi, in preparation.

end groups liberated. Under the experimental conditions of Fig. 1 this phase extends from a chain length of 14 to a chain length of 6.

B. METHODS OF INVESTIGATION AND ACTIVITY UNITS

The degradation of native DNA by acid DNase may be investigated, by physical, biological, and chemical methods.

It should be pointed out that none of these methods can be used to follow the entire course of the enzymic degradation and also that all methods, with the only exception of the terminal phosphate determination, are indirect methods. The routine use of indirect assay procedures (most commonly the formation of acid-soluble oligonucleotides, the hyperchromic shift, or the viscosity drop) has made it very difficult to define a satisfactory activity unit. Since a linear relationship exists between the formation of terminal phosphate groups and both hyperchromic shift and the liberation of acid-soluble oligonucleotides (Fig. 1), the indirect methods can be standardized against the direct method. It is possible, therefore, to define acid DNase activity according to the recommendations of the Commission on Enzymes of the International Union of Biochemistry: One unit of DNase is defined as the amount of enzyme which catalyzes the formation of 1 μ mole of terminal phosphate per minute at 25° under optimal conditions. One such unit is equivalent to about 325 units defined (9–11) as the amount of enzyme catalyzing the liberation of oligonucleotides having a corrected A_{260} equal to 1, at 37°, in 0.15 M acetate buffer, 0.01 M ethylenediaminetetraacetate (EDTA), pH 5.0, the DNA concentration being 400 μ g/ml.

C. MECHANISM OF THE INITIAL DEGRADATION OF NATIVE DNA

Acid DNase initially degrades native, double-stranded DNA according to two mechanisms (see Fig. 2): (1) a diplotomic mechanism (6, 20)—

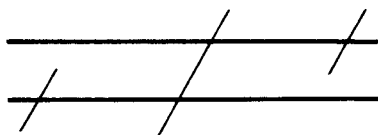


FIG. 2. Scheme of the mixed haplotomic and diplotomic mechanism of degradation of native DNA by acid DNase.

20. The introduction of this terminology (6) is justified by the fact that a mechanism of DNA degradation involving the simultaneous breakage of both strands at the same level shows a "single hit" kinetics whereas that caused by single breaks shows a "double hit" kinetics; this is, of course, a source of ambiguity.

from the Greek *diplós*, double, and *tomé*, break—by which both strands are simultaneously split at the same level, and (2) a haplotomic mechanism (6)—from the Greek *haplós*, single—causing scissions on one or another of the two strands. Both mechanisms are operational from the very beginning of the digestion.

The diplotomic mechanism, first suggested on the basis of qualitative evidence (21), has been rigorously established by kinetic work (22–25) and later confirmed using different experimental approaches (26, 27). This mechanism is responsible for the absence of a time lag in the molecular weight decrease and for the linearity of plots of $1/M_w$ vs. digestion time, which characterize the acid DNase digestion. A diplotomic degradation of native DNA has subsequently been demonstrated to take place also with other DNases, e.g., *E. coli* endonuclease I (28, 29) and *D. pneumoniae* DNase (30). In spite of the fact that kinetically these enzymes degrade native DNA like acid DNase, it is conceivable that their mechanism of action at the molecular level is different. Among other differences, the two bacterial enzymes have molecular weights close to half the molecular weight of acid DNase and form 3'-OH-ended oligonucleotides.

The haplotomic mechanism is similar to that already known to occur with pancreatic DNase (31, 32) and contributes to the molecular weight decrease only after a time lag, during which single breaks accumulate on the DNA strands. The ratio of total bonds broken to bonds broken by the diplotomic mechanism has been estimated, in different ways, to lie at least initially between 1.5 and 3 (33).

As can be expected, the initial degradation of DNA by acid DNase not only causes a drastic change in the macromolecular properties of the substrate, but also strongly affects its biological activity. Young and Sinsheimer (26) have been able to show that close to one diplotomic

21. A. Oth, E. Fredericq, and R. Hacha, *BBA* **29**, 287 (1958).
22. G. Bernardi and C. Sadron, *Nature* **191**, 809 (1961).
23. G. Bernardi and C. Sadron, *A. Baselli Conference on Nucleic Acids and Their Role in Biol., Milan, 1964* p. 62.
24. G. Bernardi and C. Sadron, *Biochemistry* **3**, 1411 (1964).
25. L. A. MacHattie, G. Bernardi, and C. A. Thomas, Jr., *Science* **141**, 59 (1963).
26. E. T. Young, II and R. L. Sinsheimer, *JBC* **240**, (1965).
27. E. Melgar and D. A. Goldthwait, *JBC* **243**, 4401 (1968).
28. G. Bernardi and C. Cordonnier, *JMB* **11**, 141 (1965).
29. F. W. Studier, *JBM* **11**, 373 (1965).
30. H. Kopecka and G. Bernardi, *6th Meeting Fed. European Biochem. Soc., Madrid, 1969* (abstracts).
31. C. A. Thomas, *JACS* **78**, 1861 (1956).
32. V. N. Schumaker, E. G. Richards, and H. K. Schachman, *JACS* **78**, 4320 (1960).
33. G. Bernardi and M. L. Bach, *JMB* **37**, 87 (1968).

break per λ -DNA molecule is sufficient to destroy its infectivity, whereas, on the average, four phosphodiester bonds can be hydrolyzed by pancreatic DNase in a λ -DNA molecule before its infectivity is lost. Bernardi and Bach (33) have found no feature in the inactivation of transforming *H. influenzae* DNA that could specifically be related to the diplotomic mechanism of action, in agreement with the fact that transformation occurs by integration of single-stranded and not of double-stranded DNA into the host genome. An interesting finding was that at comparable levels of bond breakage acid DNase is much more inactivating than pancreatic DNase, *E. coli*, endonuclease I, or sonication; for example, a Poisson average of one inactivating event per cathomycin marker (37% survival) requires more than 50 breaks per molecule of 12×10^6 daltons by pancreatic DNase but less than 2-4 breaks by acid DNase.

D. GENERAL CATALYTIC PROPERTIES

These have generally been investigated by hyperchromic shift or acid solubility assays and therefore bear on the middle phase of the DNA degradation.

1. Effect of Substrate Concentration, pH, and Ions

When acid DNase activity is assayed by the acid-solubility method the optimal DNA concentration is 0.4 mg/ml (9) and higher substrate concentrations appear to be inhibitory (16, 21, 34). It has been shown, however, that this inhibition is because increasing substrate concentration decreases the efficiency of acid-soluble oligonucleotide release since the number of breaks per unit length of DNA is lower. If a direct method of estimating enzymic activity is used, such as the determination of phosphatase-sensitive phosphate, it can be shown that the inhibition by high substrate seen by the acid solubility method is only apparent (34).

The effect of pH and ions on acid DNase activity has been investigated in several laboratories, and rather different results have been reported. It appears now that many discrepancies result from a rather poor understanding of the complexity of pH and ion effects. In fact, it has been shown (34) that electrolytes and pH modify the acid DNase activity not only by affecting the enzyme itself but also by stabilizing or destabilizing the secondary structure of native DNA. Since the enzyme has a quite different affinity for the native vs. the denatured structure

34. R. Rosenbluth and S.-C. Sung, *Can. J. Biochem.* **47**, 1081 (1969).

of DNA (9), any change in the secondary structure of the substrate will indirectly affect the enzymic activity. Another complicating factor is the presence or absence of contaminating proteins. The effects of Mg^{2+} and SO_4^{2-} are quite different at different levels of enzyme purity (35).

Using very highly purified hog spleen acid DNase at $\mu = 0.15$ (9) the pH optimum is close to 4.8. At a 0.01 M level, Mg^{2+} is slightly inhibitory above pH 4.5, whereas EDTA is an activator. Above pH 5.0, HPO_4^{2-} is slightly inhibitory and SO_4^{2-} is very strongly inhibitory, particularly above pH 4.5. In succinate buffer, $\mu = 0.15$, pH 6.7, the activity is less than 3% of that in acetate buffer, pH 5.0, $\mu = 0.15$. At low ionic strength, acid DNase is active at neutral pH (24, 33, 36).

Activation of acid DNase by cysteine was reported by Maver and Greco (37), but was not found by Brown *et al.* (38). Bernardi and Griffé (9) found an activating effect only on rather highly purified enzyme preparations. This effect was no longer apparent when protecting proteins were added to the enzyme solutions. Since acid DNase has no free sulfhydryl groups (see Section II,B), it is possible that cysteine protects the enzyme against traces of heavy metals. It should be mentioned that cytochrome c is particularly effective as a protecting protein.

2. Inhibitors

The occurrence of a dialyzable, heat-stable inhibitor in human urine has been reported (39, 40). Inhibition results from urinary sulfate (41) as well as from other salts (42).

Iodoacetic acid, *N*-bromosuccinimide, and H_2O_2 were found to be strongly inhibitory, whereas iodoacetamide was only slightly inhibitory and diisopropylfluorophosphate was not inhibitory. These results suggest that tryptophan, methionine, and/or histidine, but not serine, are involved in the enzymic activity (43).

Acid DNase is strongly inhibited by actinomycin D. In contrast with the claim (44) that actinomycin causes the same extent of inhibition of

35. C. Cordonnier and G. Bernardi, *Can. J. Biochem.* **46**, 989 (1968).

36. J. Shack, *JBC* **226**, 573 (1957).

37. M. E. Maver and A. Greco, *JBC* **181**, 853 (1949).

38. K. D. Brown, G. Jacobs, and M. Laskowski, *JBC* **194**, 445 (1952).

39. T. R. Koszalka, K. Schreier, and K. I. Altman, *BBA* **15**, 194 (1954).

40. O. D. Kowlessar, S. Okada, J. L. Potter, and K. I. Altman, *ABB* **68**, 231 (1957).

41. E. C. Rauenbusch and K. I. Altman, *Proc. Soc. Exptl. Biol. Med.* **104**, 385 (1960).

42. P. Ove, O. E. Brown, and J. Laszlo, *ABB* **131**, 652 (1969).

43. M. S. Melzer, *Can. J. Biochem.* **47**, 987 (1969).

44. N. R. Sarkar, *BBA* **145**, 174 (1967).

both pancreatic and acid DNase, the inhibition on acid DNase is much stronger than that on either pancreatic or *E. coli* DNase (6). The type of inhibition of actinomycin upon acid DNase is that expected for the case of inhibition by coupling of the inhibitor with the substrate but not with the enzyme (6). This is not surprising in view of the strong binding of actinomycin by guanylic acid residues in DNA and of the high guanylic acid level in the sequence split by acid DNase (see below).

A protein inhibitor has been extracted and partially purified from mouse liver by Lesca and Paoletti (45). This protein inhibits acid DNases from different tissues and species but not pancreatic or *E. coli* DNases. Very interestingly, V vs. substrate concentration plots become sigmoid in the presence of the inhibitor provided that pH is lower than 5.6. The existence of a DNase-inhibitor complex is suggested by sucrose-gradient results. An unusual feature of the inhibitor is its ability to reactivate acid DNase preparations treated with 8 *M* urea.

A weak competitive inhibitory effect of bases, nucleosides, and nucleoside mono- and polyphosphates has been reported (46). 3'P-oligonucleotides are very weakly inhibitory.

Inhibition by natural and biosynthetic polyribonucleotides of the type previously found for some bacterial DNases (47-50) has been demonstrated to occur in the case of spleen acid DNase (51, 52). The inhibition is, as in the case of *E. coli* DNase (50), of the competitive type. With the remarkable exceptions of poly A and poly C, which did not show any effect on the DNase activity, all synthetic polyribonucleotides tested, transfer RNA and ribosomal RNA exhibited an inhibitory activity. This was very weak with the single-stranded polymer poly U. The finding that polyribonucleotides having single-stranded structures, like poly U, have very weak inhibitory properties and that single-stranded DNA is a poorer substrate than double-stranded DNA underlines the weaker binding of the enzyme by single-stranded structures. It should be stressed that the competitive inhibition by polyribonucleotides is specific and does not simply represent the binding of a polyanion by a basic protein; in fact, some polyribonucleotides are ineffective as inhibitors, and an

45. P. Lesca and C. Paoletti, *Proc. Natl. Acad. Sci. U. S.* **64**, 913 (1969).

46. H. Slor. Ph.D. Thesis, Indiana University, 1966.

47. A. W. Bernheimer and N. K. Ruffier, *J. Exptl. Med.* **93**, 399 (1951).

48. A. W. Bernheimer, *BJ* **53**, 53 (1953).

49. L. Kozloff. *Cold Spring Harbor Symp. Quant. Biol.* **18**, 209 (1953).

50. I. R. Lehman, G. G. Roussos, and E. A. Pratt, *JBC* **237**, 819 (1962).

51. G. Bernardi, *BBRC* **17**, 573 (1964).

52. A. Jacquemin-Sablon, J. Laval, J.-Y. Le Talaer, J.-B. Le Pecq. and C. Paoletti, *Compt. Rend.* **259**, 2551 (1964).

excess of cytochrome c, a strongly basic protein, in the incubation mixture does not interfere with inhibition.

3. "Phosphodiesterase" Activity

Acid DNase from hog spleen catalyzes the slow hydrolysis of *p*-nitrophenol from bis(*p*-nitrophenyl) phosphate and the *p*-nitrophenyl esters of deoxyribonucleoside 3'-phosphates, but not from those of deoxyribonucleoside 5'-phosphates (9). Using bis(*p*-nitrophenyl) phosphate as the substrate, the pH optimum was found to be between 5.6 and 5.9. The activity in acetate is about twice as large as in succinate buffer. In acetate buffer, no significant changes occur upon addition of Mg^{2+} or EDTA. In the 4-7 pH range, 0.01 *M* SO_4^{2-} and 0.01 *M* HPO_4^{2-} give a very strong inhibition. Interestingly, plots of the initial velocity of hydrolysis of bis(*p*-nitrophenyl) phosphate vs. substrate concentration have an initial upward curvature, whereas they are hyperbolic when native DNA is used as the substrate.

The conclusion of Bernardi and Griffé (9) that the "phosphodiesterase" activity of acid DNase is an intrinsic property of the enzyme molecule has been recently challenged by Slor (46, 53), Swenson and Hodes (54), and Slor and Hodes (55), who claimed to have obtained a separation of the two activities. In fact, none of the reported results proves an actual separation of the two activities and constitutes an acceptable evidence against the two activities being carried by the same protein molecule. Some data suggest, however, that the "phosphodiesterase" activity may be inactivated preferentially by some treatments. In connection with the phosphodiesterase activity of acid DNase, see also Tables I and II in reference (56) and the related discussion (56a).

E. SPECIFICITY

Determining the specificity of a DNase is a problem of great complexity since not only the enzyme itself must be extremely pure but also the other enzymes (exonucleases and phosphatases) used in chain length and terminal nucleotide determinations must be extremely pure; in addition, extremely accurate analytical methods are needed. In retrospect, it appears that these requirements were only partially met in some

53. H. Slor, *BBRC* **38**, 1084 (1970).

54. M. R. Swenson and M. E. Hodes, *JBC* **244**, 1803 (1969).

55. H. Slor and M. E. Hodes, *ABB* **139**, 172 (1970).

56. A. Bernardi and G. Bernardi, this volume, p. 329.

56a. P. J. Sicard, A. Obrenovitch, and G. Aubel-Sadron, *FEBS Letters* **12**, 41 (1970) could not confirm the claim of Hodes *et al.* (54, 55).

investigations (57-60) devoted to the specificity of acid DNase (61). A series of investigations in some crucial areas such as the purification of ancillary enzymes (62-65), base analysis (66, 67), and kinetics of the middle and terminal phases of acid DNase digestion (19, 65) were therefore made in the author's laboratory. As a result of this work, a new picture of the acid DNase specificity is emerging. The events characterizing the middle and terminal phase may be briefly described as follows.

During the middle phase the composition of the 3'-P terminal and of the 5'-OH terminal and penultimate nucleotides of oligonucleotides obtained from calf thymus DNA by acid DNase digestion, in the incubation conditions of Fig. 1, is practically constant. Some results obtained in this phase are given in Table III. Purine nucleotides form about 75% of the 3'-terminals and of the 5'-OH penultimates with a predominance of G in

TABLE III
SPECIFICITY OF SPLEEN ACID DNase ON CALF THYMUS DNA

	3'-Phosphate terminal nucleotide ^a	5'-OH terminal nucleotide ^b	5'-OH penultimate nucleotide ^b
A	28	22	52
G	44	34	23
T	20	13	16
C	8	31	9
Purines	72	56	75
Pyrimidines	28	44	25
Average size	14-20	9.6	9.6

^a From Soave *et al.* (19).

^b From Ehrlich *et al.* (65).

57. S. Vanecko and M. Laskowski, *JBC* **236**, 1135 (1961).
 58. S. Vanecko and M. Laskowski, *BBA* **61**, 547 (1962).
 59. J. Doskocil and F. Sorm, *Collection Czech. Chem. Commun.* **26**, 2739 (1961).
 60. J. Doskocil and F. Sorm, *Collection Czech. Chem. Commun.* **27**, 1476 (1962).
 61. M. Carrara and G. Bernardi, *Biochemistry* **7**, 1121 (1968).
 62. A. Chersi, A. Bernardi, and G. Bernardi, *BBA* **129**, 11 (1966).
 63. A. Bernardi and G. Bernardi, *BBA* **155**, 360 (1968).
 64. A. Chersi, A. Bernardi, and G. Bernardi, *BBA*, submitted for publication.
 65. S. Ehrlich, G. Torti, and G. Bernardi, *Biochemistry* (in press).
 66. M. Carrara and G. Bernardi, *BBA* **155**, 1 (1968).
 67. G. Piperno and G. Bernardi, *BBA* (in press).

the first case and of A in the second one. This indicates not only that enzyme splittings are very far from random, but also that the enzyme is able to recognize a sequence of at least three nucleotides in DNA. It is possible, therefore, that acid DNase may be used to assess the relative concentrations of recognized sequences in different DNA's.

During the terminal phase a drift in the composition of terminal nucleotides takes place, leading to a more random distribution of terminals.

IV. Distribution, Intracellular Localization, and Biological Role

An acid DNase activity has been found in the cells of a number of animal tissues and species (see references 1-6 for reviews of the literature). An interesting problem is whether this activity is carried by similar protein molecules. An unequivocal answer could be obtained by comparing the chemical, physical, and enzymic properties of highly purified preparations obtained from different sources. This being a very long and difficult task, Cordonnier and Bernardi (35) compared the chromatographic and enzymic properties, the sedimentation coefficients, and the mechanism of action on native DNA exhibited by partially purified acid DNase preparations obtained from 15 different animal sources: (a) epithelial tissues—hog liver, hog kidney, and hog pancreas; (b) lymphatic tissues—hog spleen, calf spleen, and calf thymus; (c) tumor tissues—a fibroblastic sarcoma from AKR mice and a mammary epithelioma from C₃H mice; (d) nonmultiplying cells—chicken erythrocytes, hog erythrocytes, and mackerel sperms; (e) biological fluids—hog serum, bull seminal plasma, and human urine; and (f) an invertebrate, the clam *Mercenaria mercenaria*. The results obtained strongly indicated that the enzymic activity is associated with protein molecules endowed with very similar properties.

The enzyme levels in the different tissues examined by Cordonnier and Bernardi (35) were found to vary by as much as three orders of magnitude. The highest acid DNase levels were found in lymphatic and tumoral tissues; the lowest were found in cells (sperms and erythrocytes) that do not reproduce themselves anymore. This relationship between levels of acid DNase activity and capacity for proliferation or regeneration of a given tissue had already been observed by Allfrey and Mirsky (68).

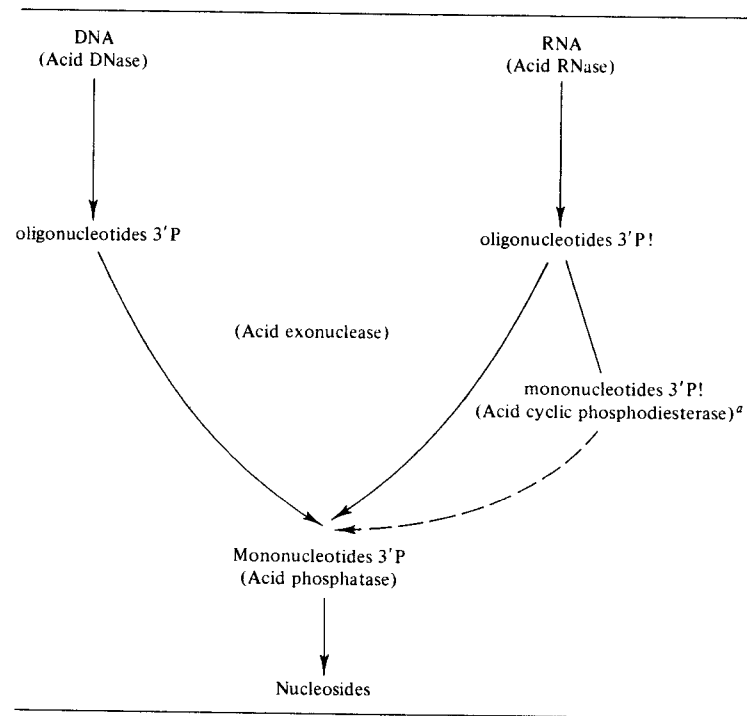
As far as the intracellular localization of acid DNase is concerned,

68. V. G. Allfrey and A. E. Mirsky, *J. Gen. Physiol.* **36**, 227 (1952).

such an activity was found to be associated with lysosomes by several authors (69). On the other hand, it has been recognized very early that acid DNase activity is latent and that tissue autolysis or acidic treatment is necessary to release it. J. M. Van Dyck and G. Bernardi (unpublished experiments) found that the enzyme from rat liver tritosomes has the same sedimentation coefficient as spleen acid DNase. It may be interesting to recall that acid DNase has been the first lysosomal enzyme obtained as a homogeneous protein.

Concerning the biological role of acid DNase, it has been suggested by several authors that this enzyme might be involved in some essential biological mechanism, like DNA replication, where it might play some accessory role, and DNA recombination. If, however, one considers that lysosomes appear to contain all enzymes needed to degrade nucleic acids to nucleosides (Table IV), it seems more likely that, at least under

TABLE IV
DEGRADATION OF NUCLEIC ACIDS BY LYSOSOMAL HYDROLASES



^a Hypothetical.

69. J. T. Dingle and H. B. Fell, eds., "Lysosome in Biology and Pathology." North-Holland Publ., Amsterdam, 1969.

normal conditions, the biological role of acid DNase is a degradative one. Its diplotomic mechanism of action might be an extremely effective way of degrading foreign DNA. From the information gathered so far on acid DNase and other lysosomal hydrolases, it appears that these enzymes share some properties, like the basic character and the absence of SH groups, of digestive enzymes which are excreted from pancreatic cells into the intestinal tract. In this connection, it may be pertinent to mention here that a set of hydrolases of nucleic acids, recalling those of lysosomes, are found in bacterial cells such as *E. coli* and that they are localized between cell wall and cell membrane (70).

70. C. Cordonnier and G. Bernardi, *BBRC* 20, 555 (1965).