MECHANISM OF ACTION AND STRUCTURE OF ACID DEOXYRIBONUCLEASE

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I. Introduction

The present article will review mainly the investigations on acid deoxyribonuclease (DNase) carried out in the author's laboratory during the past eight years. This choice seems to be justified by the following considerations: (*1*) the work performed on acid DNase between the discovery of this enzymic activity in 1947 and 1959 has already been reviewed a number of times (1–12); (*2*) during the past eight years, only a very small number of investigations on acid DNase have been reported from other laboratories, and we have been in the position of working almost alone in the field.

The situation at the time we started our work has been summarized by Laskowski (11). Briefly, the facts known at that time were the following. The existence of an acid DNase activity, first reported independently by Catcheside and Holmes (13) and by Maver and

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Greco (14–16), had been confirmed by other authors (17–44). Several methods for the partial purification of the enzyme had been proposed (45–53) and its ionic requirements had been investigated to some extent (54–60). The terminal oligonucleotides and the specificity of the enzyme had been studied (54) and it had been shown that all fragments were terminated in 3′-phosphates (54). The relevant findings of previous workers will be mentioned, in the proper context, in the following sections.

As far as the nomenclature is concerned, the early authors referred to the enzymic activity as to an acid DNase activity. Later the name DNase II, intended to mean the second type of animal DNase, was suggested for the acid DNase activity (23) to contrast it with pancreatic DNase, which was called DNase I. At the time of introduction of numbers for DNases, little was known about their mechanism of action and nothing about their specificity (11). In retrospect, it appears that this terminology, chosen at the wrong moment and lacking any clear definition, has only added confusion to the literature. More recently, DNases have been classified 5′-monoester formers and 3′-monoester formers (11), a division which is not identical with that of DNases I and II, even if frequently it is assumed to be so. In the present article, we will stick to the original nomenclature.

Our main interests in acid DNase may be briefly summarized as follows:

1. Acid DNase is an enzyme of particular interest in the study of the anatomy of DNA molecules at the polynucleotide and oligonucleotide levels since it is endowed with a very special mechanism of action—namely, the ability to break simultaneously both DNA strands at the same level—as well as with a very narrow specificity toward the base sequences in DNA. Along this line of research, we also have developed new fractionation techniques [such as chromatography of nucleic acids on hydroxyapatite (61–68) and of nucleotides, nucleosides, and bases on polyacrylamide gels (69)] and obtained new preparations of enzymes useful in sequence studies, such as spleen acid phosphomonoesterases (70–72) and spleen exonuclease (73–74).

2. Acid DNase, a small protein molecule well characterized in its physical and chemical properties, is a very appealing model, because of its dimeric structure, symmetry, and allosteric properties, in the study of a basic biological problem, the recognition by proteins of nucleic acid structures, both primary and secondary.

3. Enzymes with properties very similar to those of spleen acid DNase seem to be present in all cells of eukariotic organisms (75–77); furthermore, enzymes possessing the characteristic ability of acid DNase of splitting both DNA strands also exist in bacteria (78,79,79a). It would obviously be very interesting to understand the biological role of these enzymes.

Our investigations have been developed along the three lines of research just mentioned. This review will deal, however, only with the mechanism of action and the structure of acid DNase. We will not deal here with the biological role of the enzyme, its distribution, or its intracellular localization.

II. Mechanism of Action of Acid DNase

A. INTRODUCTION

Three different phases may be distinguished in the degradation of native DNA by acid DNase:

1. The initial phase, which is defined as the phase in which the macromolecular and biological properties of DNA are dramatically modified, whereas no change occurs in the spectral properties and no acid-soluble fragments are formed. In terms of molecular weight this phase extends from the initial molecular weight (which may range from 10^8 to 10^6 daltons) to about 10^5 daltons. This phase will be dealt with in Section II-C.

2. The middle phase, which is characterized by a hyperchromic shift and the formation of acid-soluble oligonucleotides; monoesterified phosphate may be detected in this phase. The molecular weight of DNA in the middle phase is between 10^5 and about 5×10^3 daltons.

3. The terminal phase, which shows an extremely slow, further increase in the hyperchromic shift and acid-soluble oligonucleotide release. No definite end point can be obtained for this phase. The middle and the terminal phase will be discussed in Section II-F.

The degradation of native DNA by acid DNase may be investigated, like any other DNA degradation, using the following methods:

Physical methods: (*1*) Methods based on the macromolecular properties of DNA: light-scattering, viscosity, sedimentation, electron microscopy. (*2*) Methods based on structural properties of DNA: ultraviolet spectroscopy, optical rotatory dispersion, chromatography on hydroxyapatite.

Biological methods: Inactivation of infectious or transforming activity.

Chemical methods: Titration of secondary acidity of phosphate groups; determination of acid-soluble oligonucleotides.

Two main difficulties are encountered in investigations on DNA degradations: (*1*) No single method can be used to follow the entire course of the degradation; and (*2*) as far as the methods based on the macromolecular properties of DNA are concerned, it is imperative to know the limitations of each method, for instance the molecular weight range in which light-scattering measurements are valid, or the concentration and velocity gradient dependence of viscosity; unawareness of these limitations has often led to the publication of results of doubtful validity.

B. EARLY WORK

Our work on acid DNase started from the observation made in our laboratory that DNA preparations having molecular weights close to 1×10^6 could be obtained by water extraction of the nucleoprotein from chicken erythrocytes (M. Champagne, unpublished results, 1959). This finding was reminiscent of other reports on low molecular weight DNA's existing in the literature, yet the reproducibility of the molecular weights obtained suggested that these small DNA's might be typical of nonreproducing cells such as chicken erythrocytes. This possibility was ruled out by two experiments (G. Bernardi, unpublished results, 1959) which showed that the low molecular weight DNA preparations were the result of an enzymic degradation: (*1*) By using standard preparation procedures, high molecular weight DNA preparations could be obtained from chicken erythrocytes; (*2*) incubation of the high molecular weight DNA with the crude nucleoprotein extract resulted in DNA degradation.

Work carried out in our laboratory in 1960 (80,81) showed that DNA from calf thymus ($S_{20,w} = 20.0S$; $M_w = 6.5 \times 10^6$) and chicken erythrocytes ($S_{20,w} = 27.5S$; $M_w = 8.0 \times 10^6$) could be degraded by crude enzyme preparations from chicken erythrocytes. The enzymic activity showed a pH optimum close to 5.5 and was strongly inhibited by Mg^{++}. No appreciable amounts of dialyzable nucleotides were formed during the digestion. The degraded DNA from both sources appeared to consist of particles having a molecular weight of $5.5\ (\pm 0.5) \times 10^5$. The light-scattering data were compatible with a solution of rods or wormlike chains with a mass per unit length, M/L, of 200 ± 20 daltons/Å, a value corresponding to that of the B form of DNA (82). The sedimentation coefficient was 5.8S and the distribution function of the sedimentation coefficients was very narrow.

In further work (83–85) the crude enzyme preparation from chicken erythrocytes was purified 200 times. Degradation of chicken erythrocyte DNA by the more active enzyme exhibited all the features previously found in the degradation of DNA by the crude enzymic preparations except that now the molecular weight of DNA continued to drop with simultaneous liberation of acid-soluble nucleotides and an increase in the ultraviolet absorption at 260 mμ. This phenomenon, not detected when using the crude enzymic preparation, probably because of enzyme inactivation before the later stage could be reached, strongly suggested that the chicken erythrocyte enzyme was an acid DNase, in spite of the negative report of Allfrey and Mirsky (19). This interpretation was supported by the finding that an acid DNase preparation from calf thymus (86) degraded DNA in much the same way as the purified chicken erythrocytes preparation. Later work done in our laboratory (75–77) confirmed that chicken erythrocytes do contain acid DNase at an extremely low level.

The most important feature of the degradation of DNA by acid DNase shown by these early results was that the standard deviation of the distribution of the sedimentation coefficients was narrower at the 5×10^5 molecular weight level than at the starting molecular weight, indicating that degradation was occurring according to a mechanism different from that of pancreatic DNase (see below). This finding encouraged us to carry out a detailed kinetic study which will be summarized in the next section.

C. KINETICS OF THE INITIAL DEGRADATION OF NATIVE DNA

The initial degradation of native DNA can be followed by the physical methods based on the macromolecular properties of DNA mentioned on p. 3, for instance by light scattering and viscosity (87,88).

Kinetic data can be treated according to Schumaker, Richards, and Schachman (88). These authors have given a general theory

for the degradation of multistranded polymers of any initial distribution of molecular weights. They have shown that for the initial stage of degradation the following equation holds:

$$\log (1 - R) = n \log p + \text{constant} \qquad (1)$$

where $R = M_t/M_0$, M_t and M_0 being the molecular weights at time t and at time 0, respectively; n is the apparent number of strands, and p is the probability that any given bond be broken at time t. Equation 1 shows that a plot of log $(1 - R)$ versus log p will have a slope equal to n. A more convenient plot (89) is obtained by replacing log $(1 - R)$ by log $[(1 - R)/R]$ in equation 1, the latter being a linear function of log p over a wider range of R values. Then, if $p = kt$, k being a proportionality constant, equation 1 becomes

$$\log [(1 - R)/R] = n \log t + \text{constant} \qquad (2)$$

For the particular case where $n = 1$, equation 2 becomes

$$1/M_t - 1/M_0 = kt \qquad (3)$$

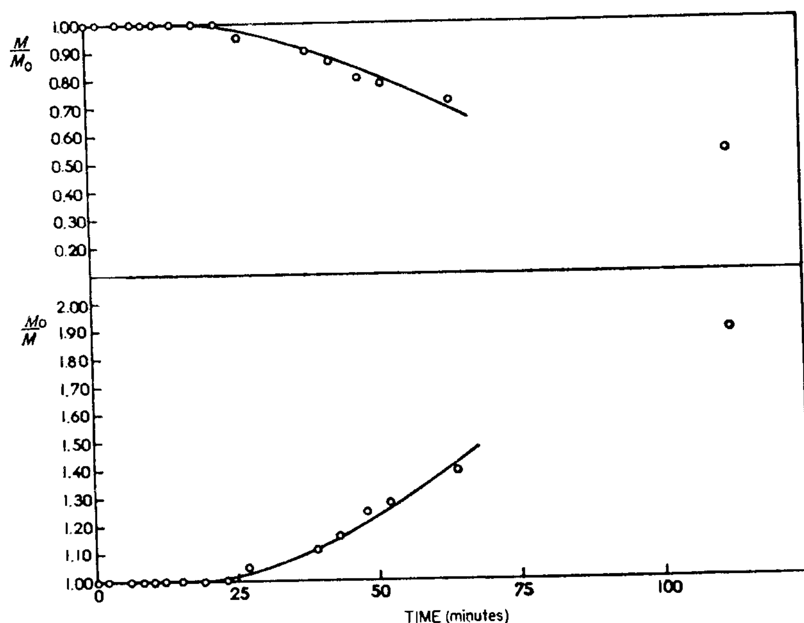

Fig. 1. Digestion of DNA sample B11/2 with pancreatic DNase. From Bernardi and Sadron (85).

that is, the reciprocal of molecular weight is a linear function of t, as predicted by the statistical theory of random degradation of linear polymers of any initial molecular weight distribution. When native DNA is digested by pancreatic DNase, the initial phase of degradation is characterized by a lag period during which bond splitting occurs, as indicated by titration, but molecular weight and radius of gyration do not change, as shown, for instance, by light scattering and viscosity (87,88; see Fig. 1). By plotting experimental data according to equation 2, it can be shown that, in the case of the degradation of native DNA by pancreatic DNase, the apparent number of strands is 1.7–2.0 (Fig. 2). This means that the enzyme splits native DNA according to a double-hit kinetics, introducing breaks at random on one strand or the other. The lag period is explained by the fact that no molecular scission occurs until two breaks take place opposite (or almost opposite) to one another. According to the usual nomenclature, this type of degradation is a "double hit" degradation. In order to avoid the ambiguity involved in the use of

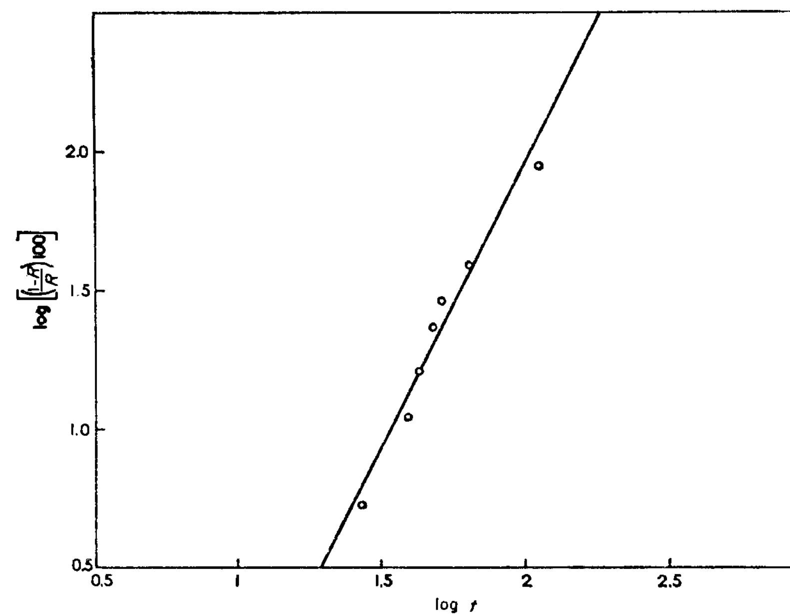

Fig. 2. Digestion of DNA sample B11/2 with pancreatic DNase. Data of Figure 3 are plotted according to Schumaker et al. (88). From Bernardi and Sadron (85).

this term, which actually refers to breakage in a single strand, it is suggested that this mechanism be called "haplotomic" [from the Greek 'απλοσ (haplos), single, and τομη (tome), break]; a mechanism involving the double breakage of the two strands at the same level (a "single hit" degradation according to the usual nomenclature) would then be called a "diplotomic" mechanism (from the Greek διπλοσ (diplos), double).

In sharp contrast with pancreatic DNase, acid DNase initially degrades native DNA according to a diplotomic mechanism as unequivocally shown by our work (83–85; see Table I and Figs. 3 and 4). Indeed, the degradation takes place without any time lag (Fig. 3) and, more significantly, when data are plotted according to equation 2, the apparent number of strands, n, is found to be equal to 1 ± 0.1 (Fig. 4 and Table I). The diplotomic degradation by acid DNase was found, without exception, in all cases studied so far in our laboratory, in spite of differences in the sources, preparation procedures, and concentrations of both DNA and enzyme samples used (76,85,90) as well as in the techiques used: these were essentially light scattering (used in the proper operating range, ref. 91) and viscosity (used under conditions such that both concentration and shear dependence could be neglected or corrected). Interestingly enough, the same initial kinetics was found when using the enzyme at low ionic strength and neutral pH (see Table I and p. 31).

The diplotomic mechanism of degradation of acid DNase first suggested, on the basis of qualitative evidence, by Oth, Fredericq, and Hacha (92), has been subsequently confirmed by the work of Young and Sinsheimer (93), who investigated the degradation of lambda phage DNA by zone sedimentation, and by Kates and McAuslan (94).

Contrary to the suggestions of some authors (93,95) that acid DNase only splits DNA according to a diplotomic mechanism, the enzyme does degrade DNA according to both diplotomic and haplotomic mechanisms, as indicated by the following findings.

1. If the molecular weight decrease caused by acid DNase is studied for a sufficiently long time, it can be seen that the initial linear increase of $1/M$ with digestion time is followed by a higher order relationship (Fig. 5) when a molecular weight of the order of 10^6 is reached. Since bond splitting is linear with time (Richards and Bernardi, unpublished data), this finding is incompatible with the existence of a purely diplotomic mechanism of degradation and is best

explained by the superposition of two different mechanisms of degradation; one of them, the diplotomic mechanism, is effective immediately in causing a decrease in molecular weight, whereas the second, the haplotomic mechanism, which also starts immediately upon the addition of the enzyme, becomes effective only after a lag time. A curve similar to that of Figure 5 was obtained by plotting the number of breaks, as determined from the size distribution of degraded T5 DNA, against digestion time (see p. 16).

TABLE I

Degradation of Native DNA by Acid DNase Preparations[a] (85)

DNA sample	Source[b]	Preparation method[c]	DNA concn. (μg/ml)	$M_w \times 10^{-6}$	R_z, Å	Enzyme[d]	n[e]
(1) B6z-N/1	E	A	115	3.8	2080	ET3	1.0
(2) B13/24	E	B	90	7.2	2900	ET3	0.9
(3) B13/30	E	B	86	6.9	2900	ET3	1.0
(4) B13/46	E	B	133	5.5	2570	ERW	1.1
(5) B13/52	E	B	133	5.5	2570	ERP/3	0.9
(6) B6z-N/11	E	A	65	3.8	2080	ETS/3	1.0
(7) B6/15	E	A	75	4.9	2160	(t)HL1	0.9
(8) B3/57	T	A	140	4.6	2120	ERW	1.1
(9) DL1/176	C				2100	(t)HL1	0.9
(10) B6z-N/12	E	A	62.5	3.8	2080	ET3/DFP	1.0
(11) B6/185	E	A		3.4	2330	(t)HL1	0.9
(12) B13/74	E	B			1930	ERW	0.9
(13) B11a/19	E	D	133	4.6	2400	ET2	1.0

[a] All degradations were performed at room temperature (20–22°) in the light-scattering cell, using $0.15M$ acetate–$0.01M$ EDTA as the solvent, except for samples 7 ($0.15M$ acetate buffer, pH 5.4), 11 ($0.005M$ EDTA, pH 7.2), and 12 (phosphate buffer, pH 6.8, $\mu = 0.004$, containing $10^{-4}M$ EDTA).

[b] E, chicken erythrocytes; T, calf thymus; C, *E. coli*.

[c] See reference 85.

[d] ET3, calf thymus DNase, ERW, calf spleen DNase (Worthington); ERP/3, hog spleen pure enzyme; ET/3, purified calf thymus DNase of specific activity 50; (t)HL 1, calf thymus DNase; ET3/DFP, ET3 treated with diisopropylfluorophosphate.

[e] n is the apparent number of strands as calculated from the slope of log $[(1 - R)/R]$ versus log t (88,89).

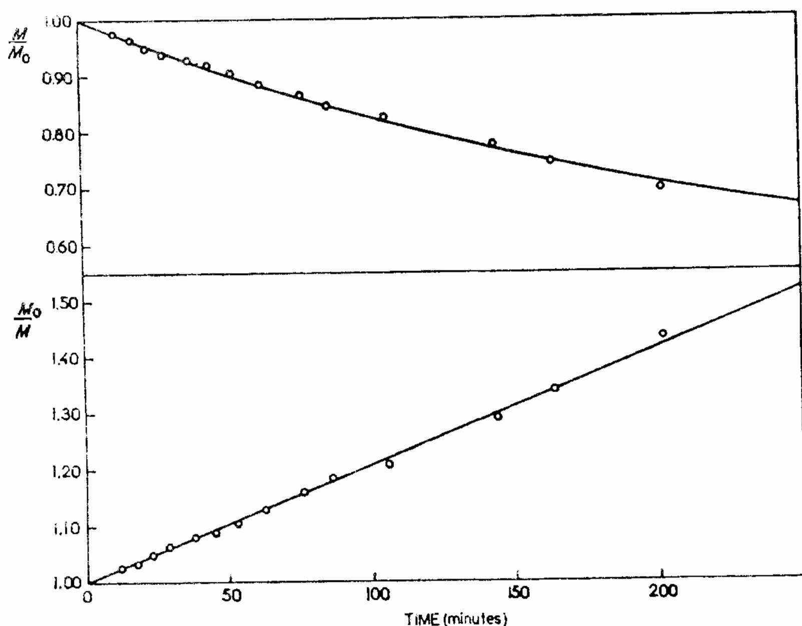

Fig. 3. Digestion of DNA sample B6zN/1 with acid DNase (see Table I). From Bernardi and Sadron (85).

2. Titration data (Richards and Bernardi, unpublished data) show that, for a given decrease in molecular weight (down to 5×10^5), the number of bonds broken is larger than that expected for a purely diplotomic degradation, but much smaller than for haplotomic degradation; in fact, 10–20 breaks (a value likely to be overestimated for technical reasons) were found to be necessary to halve the weight-average molecular weight of each parent molecule of $M_w = 6 \times 10^6$, whereas 200 breaks are needed in a haplotomic degradation (87) and 6 breaks (3 scissions) in a diplotomic degradation in order to obtain the same result. From titration data obtained during acid DNase degradation at pH 5.0, one can estimate the ratio of total bonds broken to bonds broken by diplotomic mechanism to lie between 1.7 and 3.3.

3. If DNA samples, partially digested by acid DNase and ranging in molecular weight from 4×10^6 to 1×10^6, are thermally denatured, the relative molecular weight decrease caused by heating is found to be larger for the samples with a lower initial molecular

weight (Fig. 6; Table II). These results may be explained by the fact that strand separation, occurring at high temperature, forms more single-stranded fragments from partially digested DNA than from intact DNA, because of the presence in the former of a certain number of "nicks," introduced by the haplotomic action of the enzyme. The data of Table II allow an estimate of the ratio of total bonds broken to bonds broken by the diplotomic mechanism. This ratio is 1.45–1.9, and seems to increase with decreasing molecular weight. Interestingly enough, very similar values, 1.2–1.6, can be calculated from the data of Young and Sinsheimer (93; also shown in Table II). The agreement between the two sets of data suggests that, in our case, the error due to incomplete strand separation was compensated for by thermal hydrolysis. Both sets of data are in reasonable agreement with the titration data, and indicate a ratio of total bonds broken to bonds broken by the diplotomic mechanism of the order of 1.5–3.0.

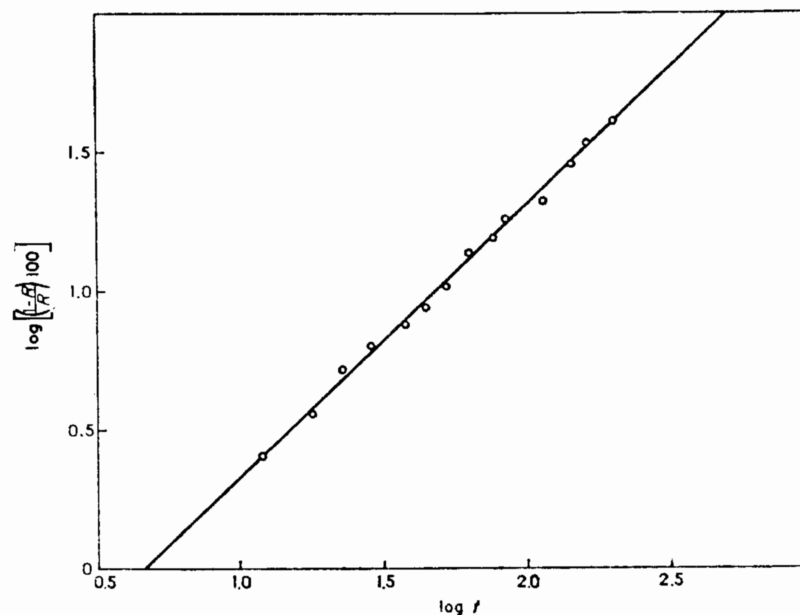

Fig. 4. Digestion of DNA sample B6zN/1 with acid DNase. Data of Figure 1 are plotted according to Shumaker et al. (88). See also Table I. From Bernardi and Sadron (85).

TABLE II
Degradation of DNA by Acid DNase (90)

Digestion time (min)	Native DNA		Denatured DNA		Hits den. DNA[b] / Hits native DNA
	$M_w \times 10^{-6}$	Hits[a]	$M_w \times 10^{-6}$	Hits[a]	
Chicken erythrocytes DNA (ref. 85; Table III)					
0	3.80	—	2.20	—	—
30	2.35	1.85	1.16	2.70	1.45
60	1.75	3.51	0.75	5.82	1.66
90	1.45	4.86	0.54	9.20	1.90
Lambda phage DNA (ref. 93; Table I)					
0	27.3	—	14.5	—	—
5	24.9	0.29	12.5	0.47	1.62
10	24.2	0.36	9.3	1.52	4.22
15	20.3	1.00	9.7	1.35	1.35
20	16.3	1.80	8.0	2.14	1.19
45	10.9	3.65	4.5	5.60	1.53

[a] Hits (scissions) were calculated according to Charlesby's (96) equations:

$$(M_w)_t/(M_w)_0 = 1/(1 + \tfrac{1}{3}p) \quad (4a)$$

$$(M_w)_t/(M_w)_0 = 2(e^{-p} + p - 1)/p^2 \quad (4b)$$

Equation 4a is valid when the initial distribution of molecular weights is the most probable one and was used in the case of chicken erythrocytes DNA. Equation 4b is valid when the initial distribution of molecular weights is uniform and was used for calculating the data of lambda DNA. In both cases p is the average number of scissions.

[b] This column gives the ratio of total bonds broken to bonds broken by diplotomic degradation, per parent native molecule.

While the haplotomic mechanism is essentially identical with that exhibited by pancreatic DNase and does not need any special comments, several hypotheses may be put forward to explain the diplotomic mechanism of degradation:

1. The enzyme splits at random one or the other DNA strand and the single-hit kinetics is due to the presence of some strand separation at the acid pH used in the enzymic digestion (92) or of interruptions in the DNA strands; these possibilities may be ruled out (85,93).

2. The enzyme is able to split simultaneously and at the same level both DNA strands (if the two breaks are successive, this would show up as double-hit kinetics). Two possibilities may be considered: (*a*) The enzyme has one active site and splits the two strands at the same level in extremely close succession; or (*b*) the enzyme has two active sites that split the two chains simultaneously.

We thought that the latter explanation was the correct one and put forward the hypothesis that acid DNase might be a dimeric protein molecule with two active sites, one on each subunit (84,85). The enzyme would then be able to split one DNA strand only at sites where the susceptible sequence exists on one strand, whereas its complementary sequence is resistant, and to split both DNA strands where susceptible sequences exist at the same level on both

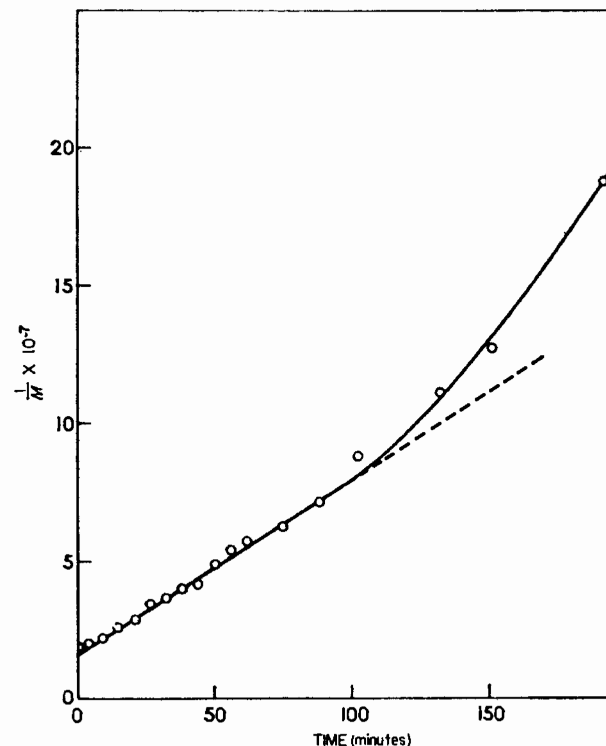

Fig. 5. Digestion of DNA sample B13/45 with acid DNAse (see Table I). From Bernardi and Sadron (85).

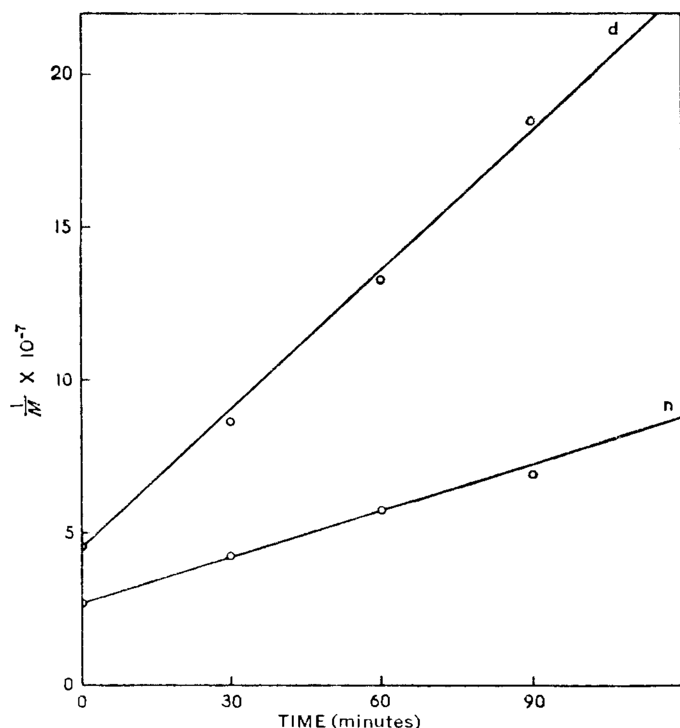

Fig. 6. Heat denaturation of intact and partially digested DNA (see Table II). A large DNA sample was digested with acid DNase, aliquots were removed at different times, and digestion was stopped. The reciprocal molecular weights of these samples are given by the lower set of points. Samples were then heat denatured; their reciprocal molecular weights after thermal treatment are given by the upper set of points. From Bernardi and Sadron (85).

strands; among these latter sequences one would expect to find A-T and/or G-C sequences, since these have identical complements.

In suggesting our model for acid DNase, we were inspired by the results of Lawley and Brookes (97,98) on the degradation of DNA by bifunctional alkylating agents. These authors showed that, under their experimental conditions, alkylation occurs at N_7 of guanine, monofunctional agents yielding 7-alkylguanines, and bifunctional agents yielding *in addition*, di(guaninyl) derivatives. This latter event only occurs when two guanines are located as shown in Figure 7. Alkylated DNA decomposes with a loss of alkylated guanines and subsequently the corresponding phosphodiester bonds are hydrolyzed.

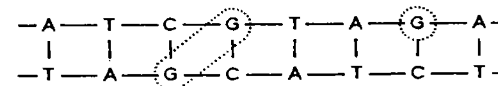

Fig. 7. Scheme of degradation of DNA by difunctional alkylating agents. From Brookes and Lawley (97).

The net result is that alkylation by monofunctional agents gives rise to a DNA degradation of the type obtained with pancreatic DNase, whereas alkylation by bifunctional agents causes a degradation which involves both single and double breaks, thus simulating the action of acid DNase.

A model for acid DNase including two active sites per enzyme molecule, each of which binds a native DNA molecule and catalyzes its hydrolysis by both single-hit and double-hit degradation, has been recently proposed by Kates and McAuslan (94); however, this complicated model is difficult to reconcile with the size of the enzyme (pp. 36 and 44).

D. STATISTICS OF DEGRADATION OF NATIVE DNA

As a complement to the kinetic work just reported, a statistical study of the molecular weight distribution during the degradation of native DNA by acid DNase was carried out. This investigation (99) was done using chromatographically purified "whole" DNA molecules from phage T5. The distribution of the molecular lengths was studied by electron microscopy at three different digestion times and shown to fit what would be expected from calculations according to the theory of Montroll and Simha (100). This is a statistical treatment for the size distributions arising from the degradation of a sharp fraction of polymer molecules of finite size n. At a degree of hydrolysis such that r links have been broken, the number of s-mers, N_s, arising from the hydrolysis of an n-mer (where s-mer and n-mer refer to chains of s and n links, respectively) is:

$$N_s = p^{s-1}(1 - p) 2 + (n - s)(1 - p) \qquad (5)$$

where $1 - p = r/n$. Theoretical distributions calculated for several different numbers of breaks for each original T5 molecule were tried against each observed distribution, assessing the goodness of fit by the χ^2 test. In each case χ^2 passed through a sharp minimum, indicating best-fitting values of 97, 231, and 832 breaks for each parent 40-μ molecule at 30, 90, and 160 min of digestion (Fig. 8). The

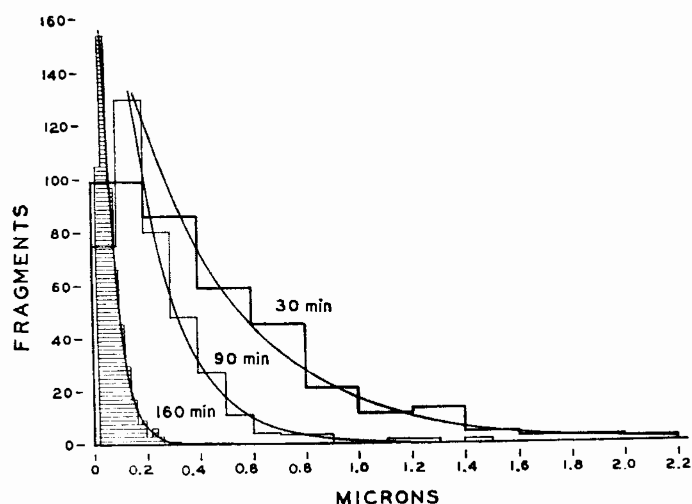

Fig. 8. Fragment size distributions in the 30-, 90-, and 160- min digestion products of "whole" T5 DNA molecules. The smooth curves are the best-fit theoretical distributions calculated on the basis of random scission corresponding to 97, 231, and 832 scissions per original 40-μ molecule. For the 160-min histogram the number scale is three times that shown. From MacHattie, Bernardi, and Thomas (99).

probabilities of fit were high: 50, 60, and 83%, respectively. Thus the number of breaks required to fit the data increases proportionally with the time of digestion up to 90 min. The last point shows more splitting than expected from a linear relationship of breaks versus time. Since fragments shorter than 0.05–0.1 μ could not reliably be seen, this size was neglected in fitting the distributions.

In conclusion, the statistics of degradation of native DNA by acid DNase show that the action of the enzyme gives a random distribution of sizes, as expected on the basis of the kinetic data already described. That no preferred sub-unit size was found confirms that, in the digestion of DNA with crude enzyme preparation (p. 4), degradation did not continue below a molecular weight of 5×10^5 probably because of enzyme inactivation.

Interesting additional results obtained from the electron-microscopic work were: (*1*) the confirmation that the fragments have a double-stranded structure; (*2*) the finding that a plot of the number of breaks, as determined from the size distribution, against digestion time showed an upward curvature similar to that of Fig. 5, indicating the coexistence of a haplotomic and a diplotomic mechanism (p. 8).

E. RELATIONSHIP BETWEEN R_z, S, $[\eta]$, AND M_w OF THE DNA FRAGMENTS OBTAINED BY ACID DNase DIGESTION

Except for a small number of random "nicks," the DNA fragments obtained by acid DNase digestion have the same structure as native DNA, at least when their molecular weight is higher than 10^5. They show the hyperchromicity, the melting curve, the diameter (99), the mass/unit length ratio (80,81) and the chromatographic behavior on hydroxyapatite (65) typical of native DNA. These fragments are therefore a very useful material for a number of physicochemical measurements. Figures 9 and 10 show the relationships which were established in the molecular weight range 0.4 to 4×10^6 by Richards and Bernardi (84,101). These fit the following equations:

$$S_{20,w} = 0.057 \times M^{0.382} \quad (S \text{ in Svedberg units}) \qquad (6)$$

$$[\eta] = 0.835 \times 10^{-4} M^{1.175} \quad ([\eta] \text{ in cgs units}) \qquad (7)$$

$$R_z = 1.1 \times M^{0.50} \quad (R \text{ in Angstrom units}) \qquad (8)$$

Interestingly enough, these relationships are not very different from those established by Doty, McGill, and Rice (102) using sonicated DNA samples; it is known that sonication, like acid DNase degradation, causes double breaks in the DNA molecules. More recently the problem of the relationships among M_w, R_z, S, and $[\eta]$ has been reconsidered (104–107) and slightly different equations have been proposed.

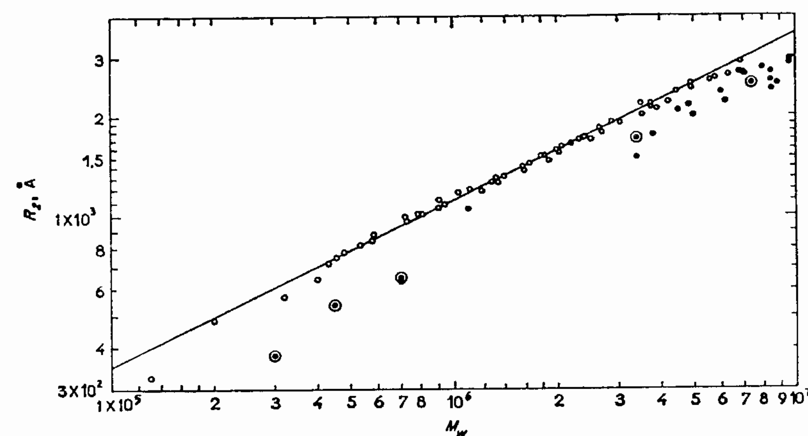

Fig. 9. Variation of radius of gyration (R_z) with weight-average molecular weight (M_w) for DNA samples digested with acid DNase (circles). Circled points are data by Doty et al. (102); points data of Lett and Stacey (103). From Bernardi and Sadron (84).

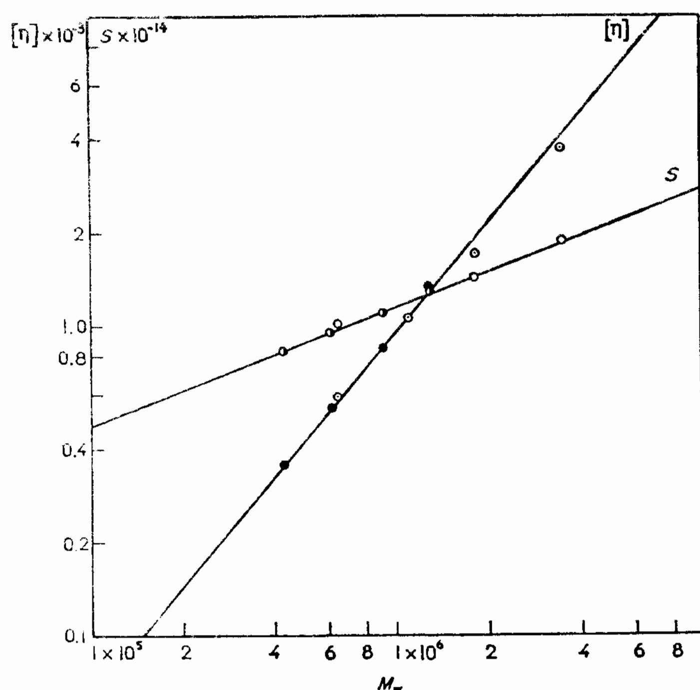

Fig. 10. Variation of intrinsic viscosity (η) and sedimentation constants (S) with weight-average molecular weight (M_w) for DNA samples digested with acid DNase. From Bernardi and Sadron (84).

The interest of the relationships established by Richards and Bernardi (84,101) lies in the fact that they were determined on DNA samples obtained by a degradation whose kinetics and statistics are known.

F. THE OLIGONUCLEOTIDES OBTAINED FROM DNA BY ACID DNase DIGESTION. SPECIFICITY OF ACID DNase

Having studied in the previous sections the initial phase of the acid DNase digestion, we will now consider the middle and terminal phases and the related problem of the enzyme specificity.

A number of investigations have been devoted to this subject between 1954 and 1962 (108–112), but in spite of the remarkable

efforts accomplished no clear picture of the average size and the size distribution of the fragments present in the final digest emerged; furthermore, the results concerning the terminal nucleotides were quite variable. A thorough investigation of this very difficult problem has been carried out recently by Carrara and Bernardi (113, 114) and Torti and Bernardi (115). In this work, the homogeneous preparations of acid DNase, obtained according to the method of Bernardi and Griffé (116,117), as modified by Bernardi, Bernardi, and Chersi (118,119), were used instead of the partially purified preparations used by previous authors. Furthermore, new techniques were developed (69) and new enzyme preparations were obtained (70–74) in order to study the digest and the specificity of acid DNase; in the source of this work, some artifacts incurred by previous authors were recognized. For these reasons, we will report here the results obtained in our laboratory; a comparison with the older data will then be presented.

When acid DNase digestions are followed by measuring the absorption increase at 260 mμ (hyperchromic shift) and the release of acid-soluble oligonucleotides, it is evident (Fig. 11) that both phenomena are characterized by an initial steep slope (middle phase) followed by a much shallower one (terminal or slow phase), the ratio of the two slopes being close to 100:1. The slowing down of the absorption increase starts at a hyperchromicity of about 30%, and independent experiments have shown that this is not due to inhibition of the enzyme by the reaction products nor to enzyme inactivation.

When small enzyme concentrations are used, it is possible to show (Fig. 11) an initial lag time in both the hyperchromic shift and the formation of acid-soluble oligonucleotides [first reported by Fredericq (95)]. As already pointed out (p. 3), it is during this time that the drastic macromolecular changes of the initial digestion phase take place in DNA.

As far as the terminal or slow phase is concerned, we agree with Koerner and Sinsheimer (54), who first observed it, that this is due to acid DNase itself, since the presence of a trace contamination of exonuclease in the enzyme (which might, alternatively, explain the slow phase) seems to be ruled out by the fact that no increase in the very small mononucleotide fraction (see below) is evident when digestion times are longer and/or enzyme concentrations are higher. It seems, therefore, that acid DNase can split very slowly, but still

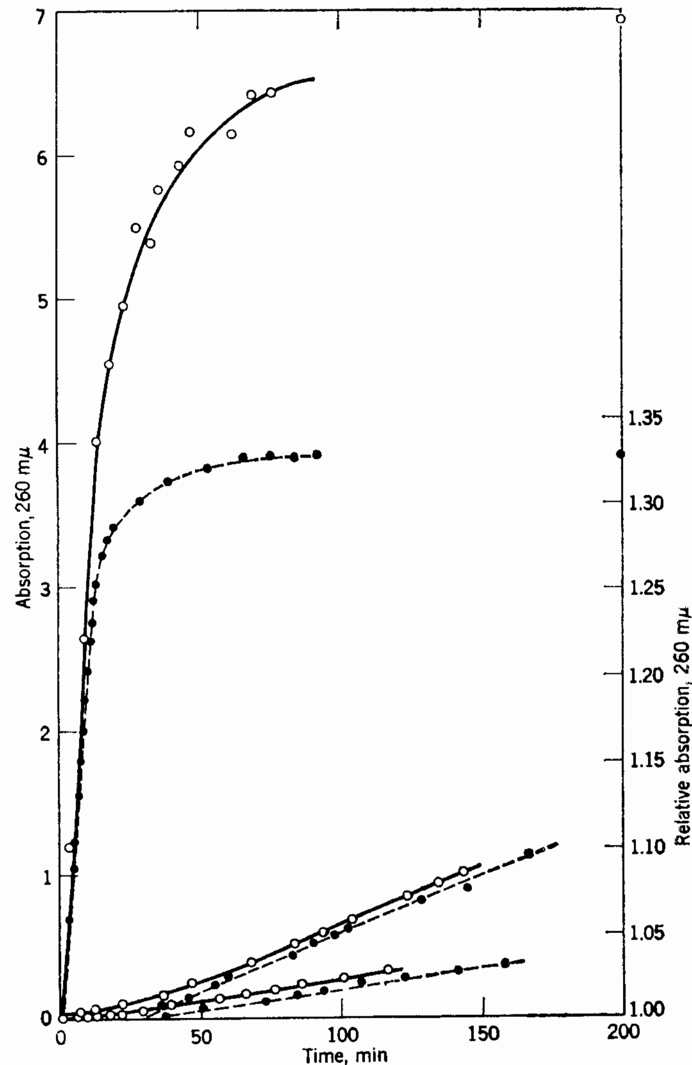

Fig. 11. Acid-soluble oligonucleotide liberation (○, left-hand ordinate; values not corrected for the 2:3 dilution with perchloric acid), and hyperchromicity (●, right-hand ordinate), obtained upon digestion of calf thymus DNA with acid DNase. Digestion was carried out at 30°. Silica cells with a 0.1-cm optical path were used in the experiment on hyperchromicity, in order to work with the same DNA solution (A_{260} = 7.52) used in the acid-soluble oligonucleotide determination. The three sets of curves refer to three different enzyme concentrations. From Bernardi and Sadron (84).

in an endonucleolytic way, some linkages of the fragments present in the digest.

The average size of the oligonucleotides present in the early terminal phase (32–36% hyperchromic shift) was estimated by determining the total phosphorus/terminal phosphorus ratio, using spleen acid phosphomonoesterases I and II (70–72), or the total nucleotides/terminal nucleotides ratio after spleen exonuclease (73,74) digestion of the dephosphorylated acid DNase digests. The average size was found to range from 10 to 12 (114). The size distribution of the oligonucleotides was investigated by chromatography of the digests on DEAE-cellulose columns, which showed the following features (114; Fig. 12):

1. An initial region of small and ill-defined peaks, representing about 3% of the total ultraviolet absorption recovered; when loaded on DEAE-cellulose columns equilibrated with 0.01*M* NH-carbonate pH 8.6, only about half of this material was retained. Generally, three peaks could be seen in this region of the chromatogram: a first sharp peak (not shown in Fig. 12) was formed by opalescent fractions corresponding to the breakthrough of the urea solutions; two subsequent peaks, indicated by *a* and *b* in Figure 12 were identified as (probably pyrimidine and purine) mononucleotides;

2. A series of well-defined peaks, formed by tri- to heptanucleotides, as shown by total phosphorus/monoesterified phosphorus and total nucleotides/terminal nucleotides ratios; these peaks are labeled I–V in Figure 12; the total amount of fractions I–V is about 25–35% of the digest;

3. A very large, unresolved fraction, forming 65–75% of the digest; the average size of the oligonucleotides present in this fraction is certainly higher than 10, since it follows the poorly resolved peaks of octa- and nonanucleotides, and can be estimated as close to 14–15, if the average size of the total digest is 10–12.

Our results on the average size and the size distribution of the "terminal" digest differ very significantly from those previously reported. The average oligonucleotide size of 10–12 found by Carrara and Bernardi (114) is not far from that, 10, obtained by Koerner and Sinsheimer (54) at the end of the fast phase, but is much higher than that reported by other authors: Doskocil and Šorm (112) found a size of about 4, and Vanecko and Laskowski found sizes

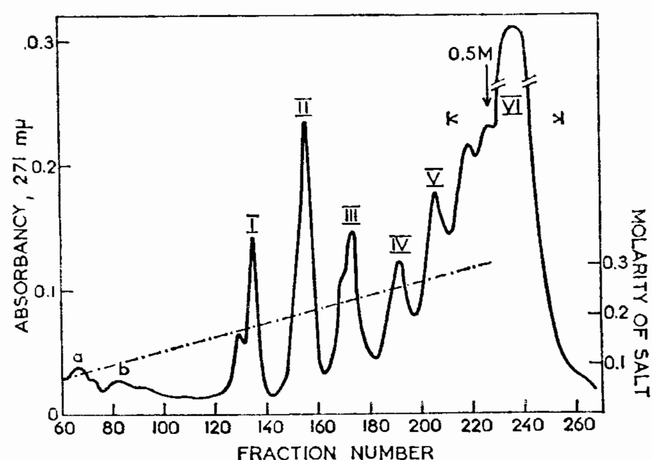

Fig. 12. Chromatography of an acid DNase digest (A: see Table I and II of ref. 114) of calf thymus DNA (170 A_{271} units) on a DEAE-cellulose (chloride) column (1.8 × 25.5 cm). 650 ml of DNA solution (A_{260} = 8.88) in 0.15*M* acetate buffer–0.01*M* EDTA pH 5.0 were digested with 20 μl of DNase HS 11 (undiluted; A_{280} = 1.14) for 1 hr at room temperature; the sample was further digested with 10 μl of enzyme for 15 hr at room temperature. The digest was diluted with four volumes of water, adjusted to pH 8.0, and loaded. Elution was carried out with a linear molarity gradient of NaCl 0–0.3*M* (2000 ml) in 7*M* urea, pH 7.5. Fraction size, 8.5 ml; flow rate 50 ml/hr. The continuous line indicates the absorption at 271 mμ; the broken line the molarity gradient. Loading was started at fraction 0. From Carrara and Bernardi (114).

of 4.5 (108) and 6.2 (109), after elimination of mono- and dinucleotides from the digest. The difference between these results and ours is particularly striking if the large fragments are considered: for instance, Doskocil and Šorm (112) report that their highest fraction (average size 7) formed 4% of the digest, whereas in our case the large fragments (average size higher than 7) formed 65–75% of the digest.

The low average sizes of the digests investigated by previous authors and the presence of large amounts of mono- and dinucleotides in those digests are the two features which differentiate them from the digests studied in our laboratory. Both phenomena seem to have originated from one or both of the following artifacts: (*1*) enzyme contamination: the presence of exonuclease in acid DNase and in

phosphomonoesterase, and of phosphomonoesterase in exonuclease seem to be responsible to a large extent for the previous results; (*2*) dephosphorylation of 3′P-deoxyribonucleotides and breakdown of 3′P-oligonucleotides during the concentration procedures from the volatile solvents used: the occurrence of these phenomena, which apparently have escaped the attention of previous workers, has been clearly established by Carrara and Bernardi (in preparation).

In view of the above, differences between the results on the enzyme specificity obtained previously and those obtained by us must be expected. The results obtained in our laboratory are compared with those of other authors in Table III. Very relevant differences are evident: for instance, we have found that pyrimidine nucleotides form at most 20% of the 3′ termini, whereas a value almost twice as high was reported by previous investigators. This result is not surprising in view of the various artifacts incurred by them, since it corresponds to the expected randomization of the termini.

TABLE III

3′-Phosphate Terminal Nucleotides in Acid DNase Digests of Calf Thymus DNA

Authors	Koerner & Sinsheimer (54)	Vanecko & Laskowski (108)	Doskocil & Šorm (112)	Vanecko & Laskowski (109)		Carrara & Bernardi (114)
Average size	10	4.5	4	6.7		10–12
Method[a]	A	B	C	A	C	C
3′P termini						
G	33.3	23.3	36.3	34	31	36–43
A	30.3	38.4	27.3	35	32	34–43
T	28.8	26.9	24.7	21	25	10–15
C	7.6	11.4	11.7	10	12	5–7

[a] Methods:
A. After venom exonuclease digestion.
B. After pancreatic DNase digestion.
C. After phosphomonoesterase and spleen exonuclease digestion. Data of Doskocil and Šorm were calculated from the results given by those authors.

III. Structure of Acid DNase

A. PURIFICATION

A homogeneous acid DNase preparation was first obtained from hog spleen by Bernardi and Griffé (116,117). This preparation had a specific activity which was estimated (117) to be about three times as high as that of the best partially purified preparations previously obtained, namely those of Koerner and Sinsheimer (49), Shimomura and Laskowski (51), and Fredericq and Oth (52).

The method of Bernardi and Griffé (117) was later modified by Bernardi, Bernardi, and Chersi (118,119) in order to reduce the labor involved and to increase the enzyme yield. In both methods, the isolation of the enzymes involves the preparation of a crude enzyme and its chromatographic purification. A brief description of the more recent method (118) follows.

The preparation of the crude enzyme [called crude spleen nuclease II to distinguish it from the crude spleen nuclease I obtained by the original method of Bernardi and Griffé (117)] involved the following steps: trimming, grinding, and homogenization of hog spleens with $0.05M$ H_2SO_4; acidification of the homogenate to pH 2.5 with $0.1M$ H_2SO_4; fractionation between 40 and 80% saturation of $(NH_4)_2SO_4$ of the supernatant from the previous step; dissolution of the precipitate so obtained in distilled water; dialysis against distilled water, clarification, concentration by freeze-drying, and dialysis against $0.05M$ phosphate buffer at pH 6.8.

Acid DNase activity was determined at the various steps leading to the crude enzyme preparation, and the results are shown in Table IV. Two modifications of the procedure in which $0.1M$ HCl and $0.15M$ NaCl, respectively, replaced $0.05M$ H_2SO_4 in the extraction mixture were also studied and the enzymic activities are also reported in Table IV. $0.1M$ HCl or $0.05M$ H_2SO_4 are evidently more effective than $0.15M$ NaCl in extracting acid deoxyribonuclease; this is very probably due to the fact that contact with the acid solution is effective in breaking down the subcellular particles to which the enzyme is bound in the cell. It is important to stress that the difference is, at least in part, due to the extraction of exonuclease by the acidic solutions. The acidification step to pH 2.5 is very effective in releasing more acid deoxyribonuclease; this increase is smaller for the extracts obtained with $0.1M$ HCl or $0.05M$ H_2SO_4, but it

should not be forgotten that spleen exonuclease is almost completely inactivated by this step; the real increase in the amount of extracted enzyme is, therefore, larger than that apparent from the table.

Crude nuclease II is obtained in a yield of 0.2–0.3 g (dry weight) per kg of ground spleen. Its total DNase activity is about 3000 units/kg of ground tissue, the specific activity being about 10. The A_{280}/A_{260} ratio of the crude enzyme is 1.3–1.5.

The chromatographic purification [procedure C developed by Bernardi, Bernardi, and Chersi (118) to replace procedures A and B of Bernardi and Griffé (117)] is summarized in Table V. Figures 13–16 show the three chromatographic steps, DEAE-Sephadex, Hydroxyapatite, and CM-Sephadex and the rechromatography of the two activity peaks, A and B, obtained from CM-Sephadex. The central parts of the rechromatographed peaks were loaded on Sephadex G-25 columns equilibrated with $0.001M$ acetate buffer, pH 5.0.

TABLE IV

Preparation of Spleen Acid Deoxyribonuclease (118)

Values show total activities per kg of trimmed spleen as determined on aliquots taken from a preparation at the consecutive steps indicated in the first column. The supernatants obtained by centrifuging products 1, 2, and 3 at 8000 × *g* for 1 hr, and the aqueous solution of precipitate 4, were dialyzed against $0.15M$ NaCl and assayed.

Preparation step	Extraction procedure			Dry weight[a] g
	$0.15M$ NaCl	$0.1M$ HCl	$0.05M$ H_2SO_4	
1. Extraction	1270	3890	3880	4.1
2. Acidification to pH 2.5[b]	3170	4540	4100	2.9
3. $0.4(NH_4)_2SO_4$ saturation[c]	2670	2940	3080	0.7
4. $0.8(NH_4)_2SO_4$ saturation	3040	3600	3570	—

[a] This column gives the dry weight of undialyzable material per kg of trimmed spleen, as determined on the dialyzed products mentioned above ($0.05M$ H_2SO_4 extraction).

[b] This was done with $0.2M$ HCl for the extracts obtained with $0.15M$ NaCl and $0.1M$ HCl.

[c] The low values found at this step are probably due to the presence of residual SO_4^{2-}; this is an inhibitor of acid deoxyribonuclease (54,117).

The enzyme fractions were then concentrated by freeze-drying down to solutions having $A_{280} = 3$, frozen, and stored at $-60°$.

The physical, chemical, and enzymological properties of acid DNase (main or B component) will be given in the following section. They were found to be indistinguishable from those of enzyme preparations obtained with the older procedure of Bernardi and Griffé (117). The purity is also identical to that of the preparations by Bernardi and Griffé (117); these were free of phosphomonoesterase, exonuclease, and nucleoside polyphosphatase activity; the only contaminating impurity detected in the preparation is RNase activity, which is present, however, at a trace level and may be removed by an additional chromatographic step on Sephadex G-100. When testing the activity of the enzyme on bis(*p*-nitrophenyl)phosphate we discovered its phosphodiesterase activity (117; see below).

The properties of the two acid DNase components A and B were compared. No differences were found in the sedimentation velocities, elution volume from Sephadex G-100 columns, ultraviolet spectra, orcinol reactions, or enzymological properties (DNase and phosphodiesterase activities). The only difference between the two

TABLE V

Chromatographic Purification of Spleen Acid Deoxyribonuclease (Procedure C) (118)

The reported data refer to preparation HS 11. All values quoted refer to the fractions which were processed further or to the final product; the sides of the activity peaks were processed separately. Preparation HS 11 was obtained from 60 kg of spleen.

Fraction	Weight, g	Volume, ml	Total units	Total $A_{280\,m\mu}$	Specific activity $A_{280\,m\mu}$	Specific activity Weight[a]
Crude spleen nuclease II	10	400	160,000	125,000	12.8	16
I. DEAE-Sephadex	0.935	725	105,000	1,625	64.5	112
II. Hydroxyapatite	0.193	425	61,500	225	273	318
III. CM-Sephadex A[b]	0.0185	20	7,850	22.4	350	425
CM-Sephadex B[b]	0.073	30	31,000	88.5	350	425

[a] Specific activity data given in this column were obtained by dividing the activity (total units) by the dry weights (in mg) of the enzyme preparations.

[b] Values reported refer to fractions A and B, respectively.

components found so far (besides their different behavior on CM-Sephadex columns, of course), was that one particular single tryptic peptide spot of component A was resolved into two spots in the map of component B.

The comparative results obtained for fractions A and B of spleen acid deoxyribonuclease indicate that the two products do not differ in their macromolecular and enzymological properties. Component A is likely to be slightly more acidic than component B since it is eluted from a carboxylic ion exchanger by a lower phosphate molarity. The peptide maps of the two fractions suggest that component B has one more peptide bond susceptible to splitting by trypsin than component A.

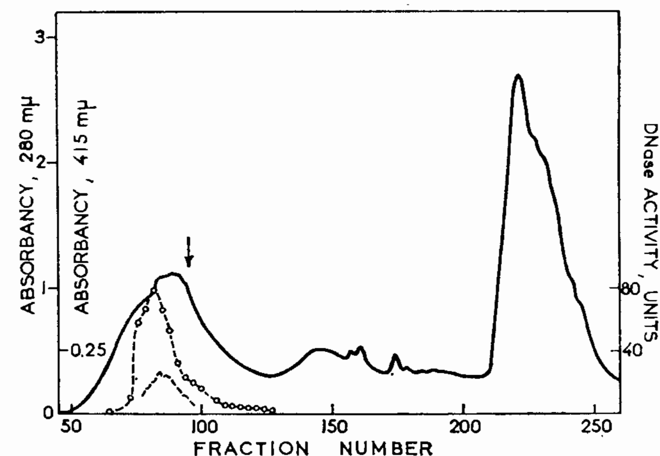

Fig. 13. Chromatography of crude spleen nuclease II on DEAE-Sephadex A-50 (procedure C, step 1). 330 ml of preparation HS9 ($A_{280\,m\mu} = 10.3$; $A_{260\,m\mu} = 6.9$) were loaded on a 8×80 cm column of DEAE-Sephadex A-50 equilibrated with $0.05M$ phosphate buffer (pH 6.8). This buffer was also used to elute the first protein peak. $0.5M$ phosphate buffer (pH 6.8) was loaded at the fraction indicated by the arrow. 24-ml fractions were collected. The continuous line indicates the adsorption at 280 mμ. Circles indicate the acid deoxyribonuclease activity (right-hand scale). The broken line indicates the absorption at 415 mμ of cytochrome *c* (left-hand inner scale). Fractions 50–65 were processed further. Acid and basic ribonuclease, acid phosphomonoesterase and phosphodiesterase, and phosphodiesterase were also assayed; the results are shown elsewhere (70,120). From Bernardi, Bernardi, and Chersi (118).

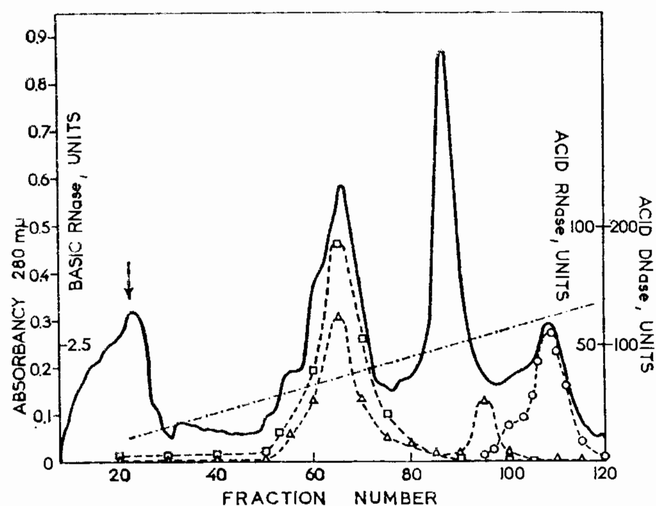

Fig. 14. Chromatography of fractions 50–65 from step I on hydroxyapatite (procedure C: step II). 370 ml ($A_{280\ m\mu}$ = 1.48) were loaded on a 2 × 40 cm column of hydroxyapatite equilibrated with 0.05*M* phosphate buffer (pH 6.8). A molarity gradient (0.05–0.5*M*) was started at the fraction indicated by the arrow; at fraction 120 the molarity of the effluent was 0.35. 24-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles (○) indicate the acid deoxyribonuclease activity (right-hand scale). Cytochrome *c* was eluted as a sharp peak centered on fraction 86 ($A_{415\ m\mu}$ = 0.82; not shown in the figure). Acid ribonuclease (□; right-hand inner scale) and basic ribonuclease (△; left-hand inner scale) are also shown. Fractions 100–115 were concentrated by freeze-drying to about 70 ml, filtered through a Sephadex G-25 column equilibrated with 0.075*M* phosphate buffer (pH 6.8), and processed further. From Bernardi, Bernardi, and Chersi (118).

These findings may be explained tentatively by assuming that the difference between A and B is due to the deamination 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; the resulting peptide bond would be resistant to trypsin. It is known that acid DNase is very rich in amide groups (121) and that deamidation can occur at low pH in a number of proteins.

To test this hypothesis, three acid deoxyribonuclease preparations were made using modifications of the procedure described above. In preparation 1, HCl replaced H_2SO_4; in preparation 2, the acidification step to pH 2.5 was omitted and 0.1*M* H_2SO_4 was replaced by the same amount of 0.15*M* NaCl. Preparation 3 was like preparation 2, except that 0.15*M* NaCl replaced 0.05*M* H_2SO_4 in the tissue-homogenization step. All these preparations were carried through the chromatographic purification (procedure C) and the amount of A and B components were estimated from the elution curve obtained in the CM-Sephadex chromatography. Component A, which represents about 20% of total deoxyribonuclease when the enzyme is prepared according to the usual procedure, decreased to 15–19% in preparation 1, to 7% in preparation 2, and was not present at all in preparation 3.

In the light of the above results, it seems possible that the two chromatographic components of spleen acid DNase separated by Koszalka, Falkenheim, and Altman (50) on Amberlite IRC-50 columns had an origin similar to that of our components A and B, since these authors adjusted the tissue extract to pH 4.0 with 5*N* H_2SO_4.

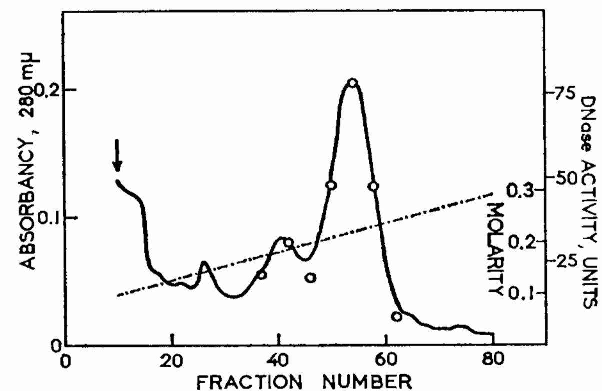

Fig. 15. Chromatography of fractions 100–115 from step II on CM-Sephadex C-50 (procedure C; step III). 115 ml ($A_{280\ m\mu}$ = 0.490) was loaded on a column equilibrated with 0.05*M* phosphate buffer (pH 6.8). A molarity gradient (0.1–0.4*M*) of phosphate buffer (pH 6.8) was started at the fraction indicated with an arrow (right-hand inner scale). 11-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid deoxyribonuclease activity (right-hand outer scale). Fractions 37–48 and 49–61 were processed further. From Bernardi, Bernardi, and Chersi (118).

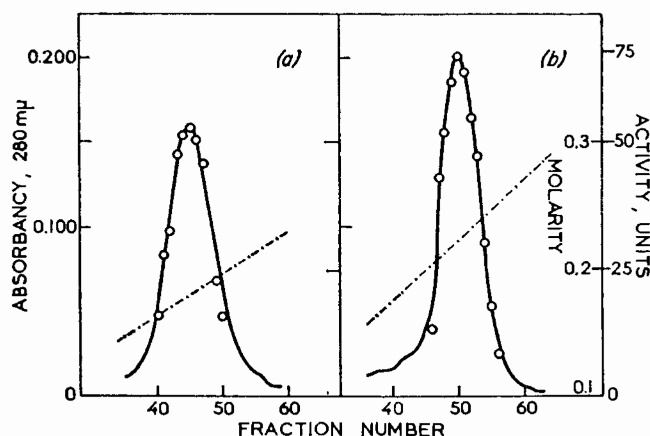

Fig. 16. Rechromatography of acid deoxyribonuclease fractions A and B on CM-Sephadex C-50. 8 $A_{280\ m\mu}$ units of each acid deoxyribonuclease (preparation HS 10) fractions A and B were loaded on two 1 × 100 cm CM-Sephadex C-50 columns. A molarity gradient (0.1–0.4*M*) of phosphate buffer (pH 6.8) was started at fraction I (right-hand inner scale). 3-ml fractions were collected. The continuous line shows the adsorption at 280 mμ. Circles indicate the deoxyribonuclease activity (right-hand outer scale). From Bernardi, Bernardi, and Chersi (118).

B. CATALYTIC PROPERTIES (117)

As already mentioned, acid DNase is active on both DNA and a series of *p*-nitrophenyl phosphodiesters.

1. *DNase Activity*

The pH–activity curves and the ionic requirements of acid DNase have been studied in several laboratories with rather strikingly different results (see, for instance, refs. 54–57). The differences in the pH–activity curves seem to be due to their strong dependence upon the ionic strength and the nature of the cations present in the incubation mixture (11), whereas the differences found in the effects of Mg^{++}, SO_4^{2-}, and EDTA appear to be associated with the widely different amounts of foreign proteins in the enzyme preparations used (76,77).

Figure 17 shows the results obtained by Bernardi and Griffé (117) with hog spleen acid DNase at $\mu = 0.15$. No protecting proteins were added when the enzyme solutions were diluted for this experiment.

The pH optimum was found to be close to 4.8. At a 0.01*M* level, Mg^{++} is slightly inhibitory above pH 4.5, whereas EDTA is an activator. HPO_4^{2-} is slightly inhibitory above pH 5.0, 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, $\mu = 0.15$. We have confirmed the finding of Shack (37) that at low ionic strength acid DNase is active at neutral pH.

The occurrence of a dialyzable, heat-stable inhibitor in human urine has been reported (122); this has since been identified as sulfate ion (123,76). An activation of acid DNase by cysteine was reported by Maver and Greco (15) but was not found by Brown, Jacobs, and Laskowski (20). We have confirmed that cysteine activates acid DNase preparations of specific activity higher than 50 (117). This effect, however, was no longer apparent when protecting proteins (serum albumin and, especially, cytochrome *c*) were added to the

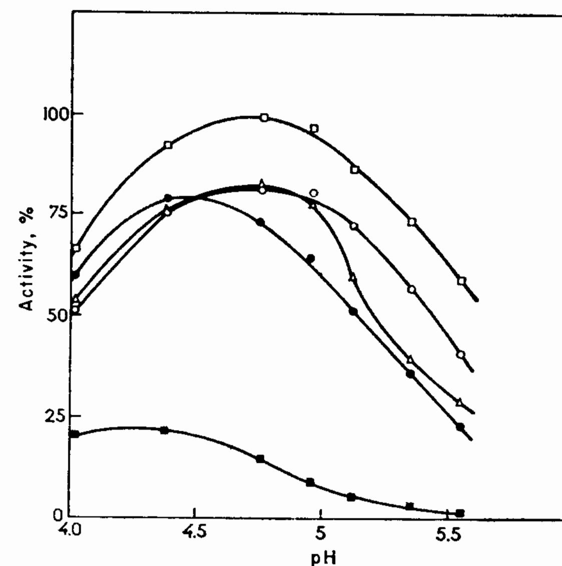

Fig. 17. DNase activity of acid DNase, as assayed by the acid-soluble oligonucleotide formation at $\mu = 0.15$. (○) Acetate buffer; (●) acetate buffer + 0.01*M* $MgCl_2$; (□) acetate buffer + 0.01*M* EDTA; (■) acetate buffer + 0.01*M* Na_2SO_4; (△) acetate buffer + 0.01*M* KH_2PO_4. From Bernardi and Griffé (117).

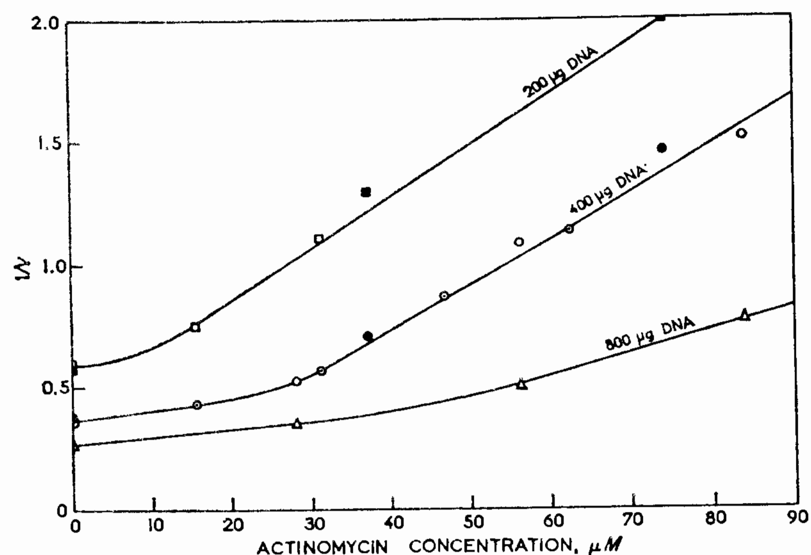

Fig. 18. Digestion of native calf thymus DNA by hog spleen acid DNase in the presence of different actinomycin concentrations. V = Absorbancy of liberated oligonucleotides at 260 mμ. The three curves correspond to three different DNA concentrations, 200 μg/2.5 ml (290 μM as DNA-P), 400 μg/2.5 ml (480 μM as DNA-P), and 800 μg/2.5 ml (960 μM as DNA-P). Different symbols on the curves refer to different experiments. (G. Bernardi, unpublished observations, 1964).

enzyme solutions. Since acid DNase has no free sulfhydryl groups (see Section III-C) it is probable that cysteine protects the enzyme from traces of heavy metals. As protecting protein, cytochrome c is particularly effective and we have used it regularly for this purpose since we observed that chromatographic fractions in which acid DNase was contaminated by cytochrome c were particularly stable.

Acid DNase is strongly inhibited by actinomycin D (G. Bernardi, unpublished observations 1964); in contrast with a recent report (124) claiming that actinomycin causes the same extent of inhibition of both pancreatic and acid DNase, we found that acid DNase is much more inhibited by the chromopeptide than either pancreatic or *E. coli* DNase. The type of inhibition of actinomycin upon acid DNase was studied and Figure 18 shows a plot of reciprocal velocity versus inhibitor concentration. Both this type of plot and the double reciprocal plot of Lineweaver-Burk are those theoretically expected for the case of inhibition by coupling of the inhibitor with the substrate but not with the enzyme. This is not unexpected in view of the strong binding of actinomycin by guanylic acid residues in DNA. The strong effect on acid DNase is quite understandable since guanylic acid residues are found in almost 50% of the 3′P terminal positions of the oligonucleotides (see Table III).

2. *Phosphodiesterase Activity*

The enzyme shows a weak hydrolytic activity on calcium (bis-p-nitrophenyl) phosphate and the p-nitrophenyl esters of thymidine-, deoxyguanosine-, and deoxycytidine-3′-phosphates (the deoxyadenosine derivative was not assayed) which are split with liberation of p-nitrophenol; the derivative of thymidine-5′-phosphate is resistant. The enzyme activity on the p-nitrophenyl esters of deoxyribonucleoside phosphates mimics, therefore, its specificity toward the natural substrate. To our knowledge, this is the first case in which synthetic substrates for an endonuclease have been described.

Using as a substrate Ca(bis-(p-nitrophenyl)phosphate)$_2$, the pH-optimum was found to be between 5.6 and 5.9. In the 5.1–5.6 pH range, the activity in acetate is about twice as large as in succinate buffer. In the pH range 4.0–5.6, no significant changes occur upon addition of Mg^{2+} or Versene (Fig. 19); in the pH range 4.0–7.0, SO_4^{2-} and HPO_4^{2-} give a very strong inhibition at a level of 0.01. It seems possible that the phosphodiesterase activity found by Koerner and Sinsheimer (49) to elute from Celite columns together with acid DNase was due to this intrinsic phosphodiesterase activity of the enzyme.

The following results show that the DNase and the phosphodiesterase activities are carried by the same protein molecule.

(a) Chromatography on Amberlite IRC-50, CM-Sephadex, and Sephadex G-50 and G-100. When running acid DNase prepared according to Bernardi and Griffé (117) on these columns, only one symmetrical peak was obtained and the ratio of the two activities was constant through the peak. On CM-Sephadex the enzyme was eluted by a molarity gradient (0.1–0.3M) of phosphate buffer, pH 6.8. The runs on Sephadex G-50 and G-100 were performed using 0.01M acetate buffer, pH 5.0, and 0.001M potassium phosphate buffer, pH 6.8, respectively, as solvents. We have mentioned already

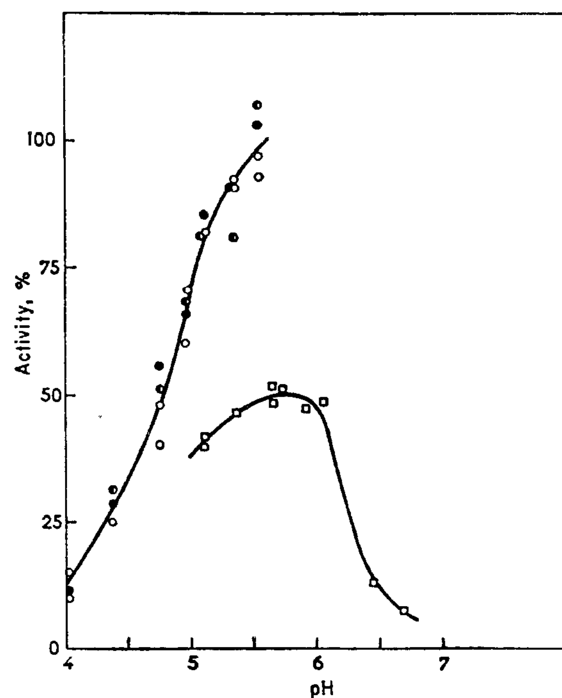

Fig. 19. Phosphodiesterase activity of acid DNase as assayed on Ca(bis(*p*-nitrophenyl)phosphate)$_2$ at μ = 0.15. (○) Acetate buffer; (◐) acetate buffer + 0.01M $MgCl_2$; (●) acetate buffer + 0.01 M EDTA; (□) succinate buffer. From Bernardi and Griffé (117).

that the A and B components (see p. 26) obtained by the method of Bernardi, Bernardi, and Chersi (118) do not differ in their activities on bis(*p*-nitrophenyl)phosphate and DNA.

(b) Thermal Inactivation. Enzyme samples (0.4 ml; A_{280} = 0.148) were kept for 20 min at several temperatures ranging from 25° to 75° in 0.15M acetate buffer–0.01M EDTA, pH 5.0, chilled in an ice bath, and used in digestion experiments at 37°. The inactivation curves of DNase and phosphodiesterase activities were identical. The thermal stability of the enzyme was found to be lower at higher pH values, in agreement with results of other authors (26,49).

(c) Sucrose-Gradient Centrifugation. A constant ratio of the two activities was found through the enzyme peak. When spleen

exonuclease was added to acid DNase, it showed up as a heavier component. This behavior suggests that spleen exonuclease has a higher molecular weight than acid DNase; this is also indicated by the fact that both on Sephadex G-50 and on Sephadex G-100 spleen exonuclease is eluted before acid DNase. More recent experiments (74) confirmed this conclusion (see also Table VI).

TABLE VI

Properties of Enzymes Active on Bis(*p*-nitrophenyl)phosphate (125)

	Acid DNase	Spleen exonuclease	Nucleoside polyphosphatase
1. Sedimentation coefficient[a]	3.4	4.6	3.2
2. pH optimum[b]	5.6–5.9	5.8	6.8
3. Substrates			
Bis(*p*-nitrophenyl)phosphate	+	+	+
p-Nitrophenyl esters of			
thymidine-5′ phosphate	−	−	−
thymidine-3′ phosphate	+	+	+
Native DNA	+	−	−
ATP, ADP, etc.	−	−	+
3′-Phosphate oligonucleotides	−	+	−
4. Inhibitors[b]			
HPO_4^{2-}	+	−	+
SO_4^{2-}	+	−	−
Polyribonucleotides	+	+[c]	−
5. Thermal inactivation (50%)	60°	57°	
6. Chromatographic properties			
DEAE-Sephadex (pH 6.8)	0.05M KP[d]	0.05–0.1M	0.05M KP
Hydroxyapatite (pH 6.8)	0.3M KP	0.12M KP	0.12M KP
CM-Sephadex	pH 6.8; 0.2M KP	pH 6.3; 0.2M KCl	pH 5.7; 0.11M KP

[a] As determined by sedimentation in sucrose gradient, using cytochrome *c* as a reference protein; enzymic assays were done on both bis(*p*-nitrophenyl)-phosphate and the natural substrates: the results were the same.

[b] Using bis(*p*-nitrophenyl)phosphate in 0.25M succinate buffer as the substrate.

[c] The enzyme degrades polyribonucleotides to 3′P mononucleosides; these are inhibitory.

[d] Eluting molarity. KP is potassium phosphate buffer.

(d) **Inhibition by Polyribonucleotides.** Some natural and biosynthetic polyribonucleotides behaved as competitive inhibitors with respect to both enzymic activities (see also p. 38).

The activity on bis(p-nitrophenyl)phosphate seems to be a nonspecific activity of acid DNase; this activity is displayed by at least two other spleen enzymes, which also have different natural substrates: exonuclease and nucleoside polyphosphatase. Table VI summarizes the properties of the phosphodiesterase activities of these three enzymes (125; see also p. 41).

C. PHYSICAL AND CHEMICAL PROPERTIES (116,121)

The physical and chemical properties of hog spleen acid DNase were investigated using enzyme preparations obtained according to the methods of Bernardi and Griffé (117) and Bernardi, Bernardi, and Chersi (118). The physical results are summarized in Table VII. They indicate that acid DNase is a small globular protein of molecular weight 38,000; its sedimentation coefficient is 3.4 as determined both by measurements in the analytical ultracentrifuge after extrapolation to zero concentration and by sucrose gradient centrifugation, using cytochrome c as a reference protein. In the latter case it was checked that recovery of activity was higher than 90%. This indicates that the protein does not dissociate at high dilution. The sedimentation coefficient found by us is significantly higher than that, 2.7S, reported by Kates and McAuslan (94) for both spleen and HeLa cell acid DNase; we have no explanation for this lower value. The isoelectric point is close to 10.2, as estimated by zone electrophoresis on cellulose acetate.

TABLE VII

Physical Properties of Hog Spleen Acid Deoxyribonuclease (121)

$S_{20,w}$ (Svedbergs)	3.4
$D_{20,w}$, 10^{-7} cm^2/sec[a]	7.8
$\bar{v}$, ml/g[b]	0.72
Mol. weight	3.8×10^4
f/f_0	1.34
$E^{1\%\ 1\,cm}_{280\ m\mu}$	12.1

[a] This value was obtained at concentrations of 0.5% and about 0.1%.
[b] Value calculated from amino acid composition.

The amino acid analysis is reported in Table VIII. It shows a high level of aromatic amino acids, as expected from the high extinction coefficient at 280 mμ. The high ammonia content of the acid hydrolysate suggests that a large percentage of the dicarboxylic

TABLE VIII

Amino Acid Analysis of Hog Spleen Deoxyribonuclease (121)

	Grams of amino acid residues per 100 g of protein[a] in hydrolysis time of:				Moles of amino acid per mole of protein M.W. 38,000	Nearest integral number of residues per mole of protein[c]
	22 hr	48 hr	72 hr	Corrected values[b]		
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[d]	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[e]	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] Total N is 17.2%; total S is 1.0%.
[b] In calculating the corrected values, the criteria given by Tristram and Smith (127) have been followed.
[c] The selection of the integral numbers of residues has been done taking into account also results from other analyses.
[d] After performic acid oxidation, cysteic acid 8.2 residues.
[e] From N-bromosuccinimide titration.

acids might be present in the protein as the corresponding amides. Some preliminary work on pronase digests of the protein confirmed its high amide level. These findings explain the alkaline isoelectric point of the enzyme. An unexpected result is the presence of glucosamine in the protein; in the tryptic digests, the glucosamine residues (and also some as yet unidentified neutral sugars) are found in a single peptide spot. Interestingly enough for the hypothesis of the dimeric structure of the enzyme, all amino acids present at low levels were found to be present in an even number of residues per protein molecule. Since no free sulfhydryl groups can be detected in acid DNase, both native and denatured, the eight half-cystine residues must form four disulfide bridges (126).

D. INHIBITION BY POLYRIBONUCLEOTIDES

Inhibition by natural and biosynthetic polyribonucleotides, of the type previously found for some bacterial DNases (128–131) has been demonstrated to occur in the case of spleen acid DNase (132,133). The inhibition is, as in the case of *E. coli* DNase (131), of the competitive type (Fig. 20).

In our experiments, which were performed at 37° in 0.15M acetate buffer-0.01M EDTA, pH 5.0, the following products were assayed for their inhibitory activity: yeast-transfer RNA, ribosomal RNA from Ehrlich ascites tumor cells, polyadenylic, polyuridylic, polycytidylic, and polyinosinic acid and the poly A–poly U and poly I–poly C 1:1 complexes. All these substances were generally used at a 10–100 μg level, the DNA present in the incubation mixture varying from 200 to 800 μg. With the remarkable exceptions of poly A and poly C, which did not show any effect on the DNase activity, all polyribonucleotides tested exhibited an inhibitory activity. This was very weak with the single-stranded (134) polymer poly U. Inhibition 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 excess of cytochrome c, a strongly basic protein, in the incubation mixture does not interfere with inhibition.

If one considers the structures of the polyribonucleotides studied, it appears that those presenting co-parallel, double-stranded structures, like poly A and poly C at pH 5.0 (135–137), do not inhibit, whereas those which have anti-parallel, double-stranded structures

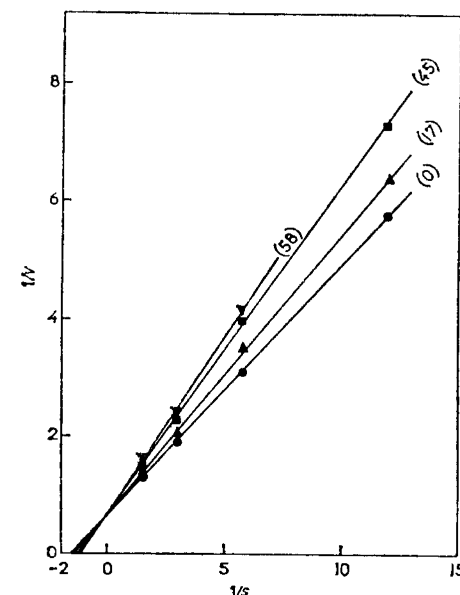

Fig. 20. Competitive inhibition of hog spleen acid DNase by yeast tRNA. The number in parentheses indicate the mμmoles of tRNAP/ml. v = μmoles DNA-P liberated in 15 min, s = μmoles DNA-P/ml. From Bernardi (132).

like poly A–poly U and poly I–poly C, or regions with this type of structure, like tRNA and ribosomal RNA do inhibit acid DNase (138,139). It seems, therefore, that structures simulating that of native DNA are endowed with the highest affinity for the enzyme. This conclusion has some bearing on the symmetry of the enzyme molecule (see p. 43).

The findings 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 (117) underline the weaker binding of the enzyme by single-stranded structures.

In the case of tRNA, stoichiometric formation of enzyme–inhibitor complexes may be shown; the interaction between seryl-tRNA and acid DNase has been studied in detail and a 1:1 strong complex has been demonstrated (work to be published).

E. DIMERIC STRUCTURE AND ALLOSTERIC PROPERTIES (138,139)

Evidence supporting a dimeric structure of hog spleen acid DNase was obtained in our laboratory (138) from both physical and chemical investigations. Physical evidence for the dimeric structure of the enzyme was provided by sedimentation studies using urea- and guanidine-containing solvents. Table IX shows the sedimentation coefficients obtained in several dissociating solvents. At a 1% concentration, the sedimentation coefficient, which is 2.8S in acetate or phosphate buffers, drops to 1.75S and 2.1S in 6M guanidine at pH 5.6 and 8.6, respectively; these values support the idea that in these solvents the enzyme molecule is dissociated into two subunits; in the presence of reducing agents, like β-mercaptoethanol, the sedimentation coefficient is still lower, as expected: 1.5S in 6M guanidine and only 0.8S in 8M urea; in 4M urea both the dissociated and the undissociated protein boundaries can be seen (Fig. 21). The fact that dissociation takes place also in the absence of reducing agents indicates that none of the four disulfide bridges found in the enzyme crosslink the two subunits, which, therefore, contain two disulfide bridges each.

TABLE IX
Sedimentation Coefficients of Acid DNase

Solvents	$S_{20°,w}$ (c = 1%)
1. 0.15M Acetate buffer–0.01M EDTA, pH 5.0	2.8
2. 0.25M Phosphate buffer, pH 6.8	2.8
3. 0.15M Acetate buffer–0.01M EDTA, pH 5.0	
0.05M β-Mercaptoethanol	2.6
4M Urea	0.8
4. 0.15M Acetate buffer–0.01M EDTA, pH 5.0	0.8
0.1M β-Mercaptoethanol	
8M Urea	
5. 0.1M Tris-acetate buffer, pH 8.6	2.1
0.001M EDTA	
6M Guanidine	
6. 0.1M Tris-acetate buffer, pH 8.6	1.5
0.001M EDTA	
0.1M β-Mercaptoethanol	
6M Guanidine	
7. 0.1M Acetate buffer, pH 5.6	1.75
0.001M EDTA	
6M [illegible]	

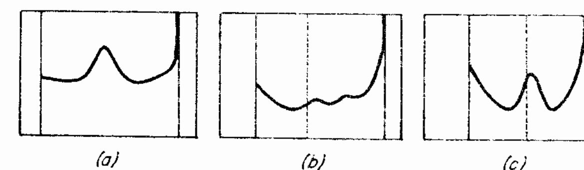

Fig. 21. Sedimentation patterns of hog spleen acid DNase centrifuged at 59,780 rpm in a Spinco Model E apparatus (*a*) In 0.15M acetate buffer pH 5.0 + 0.01M EDTA (after 122 min). (*b*) In acetate-EDTA + 4M urea–0.05M 2-mercaptoethanol (after 76 min). (*c*) In acetate-EDTA + 8M urea–0.1M 2-mercaptoethanol (after 93 min). Experiment (*a*) was carried out in a conventional cell; experiments (*b*) and (*c*) in synthetic boundary cells, the position of the starting boundary is indicated by the broken line. The sedimentation coefficients of the enzyme in acetate–EDTA and of the fast component in 4M urea were close to 2.8S (c = 1%); those of the enzyme in 8M urea and of the slow component in 4M urea were close to 0.8S. From Bernardi (138).

Chemical evidence for the dimeric structure of acid DNase came from an investigation of the tryptic hydrolysate of the enzyme. This was reduced, carboxymethylated, and digested with crystalline trypsin treated with L-1-tosylamido-2-phenethyl-chloromethyl-ketone to inactivate contaminating chymotrypsin (an indispensable precaution in view of the abundance of aromatic amino acid residues in the enzyme). In the map of the digest, 17–19 peptides were found (Fig. 22) as opposed to 32–34 arginine + lysine residues found by Bernardi, Appella, and Zito (121) in the enzyme of molecular weight 38,000. Arginine, tryptophan, and histidine peptides were found to be present in half, or less than half, the number of the respective amino acids in the dimeric protein.

A study of the hydrolysis of bis(p-nitrophenyl) phosphate provided additional, although indirect, evidence for the dimeric structure of acid DNase. In fact, plots of the initial velocity of hydrolysis versus substrate concentration have a sigmoidal shape, a phenomenon which is not found when using DNA as substrate (Fig. 23). In contrast, spleen exonuclease and nucleoside polyphosphatase (p. 35) split bis(p-nitrophenyl)phosphate according to a Michaelis kinetics. Figure 23 indicates that the system bis(p-nitrophenyl)phosphate–acid DNase exhibits a cooperative type of substrate–enzyme interaction or that, to use the terminology of Monod, Wyman, and Changeux (140), bis(p-nitrophenyl) phosphate behaves as a homotropic allosteric effector with respect to acid DNase. It is well known that allosteric phenomena are characteristically found in oligomeric enzymes.

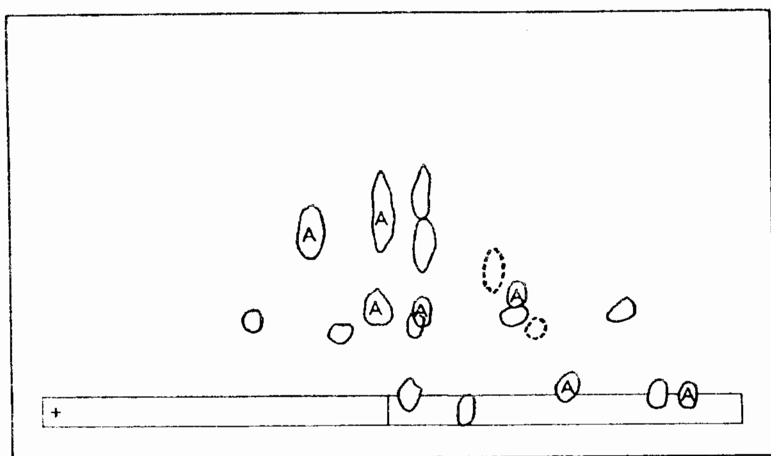

Fig. 22. Electrophoresis chromatography of trypsin peptides obtained from reduced and carboxymethylated hog spleen acid DNase. Arginine-containing peptides are marked A. From Bernardi, Appella, and Zito (126).

It appears, therefore, that, in agreement with our original hypothesis based on purely kinetic results (84,85), acid DNase is, indeed, a dimeric protein molecule with two (very probably) identical subunits, each of them carrying an active site.

Three types of acid DNase-DNA complexes may be thought to take place: (*1*) An enzyme-inhibitor complex can be formed every time the enzyme molecule meets sequences, on both strands, which cannot be cleaved because of the specificity of the enzyme. Perhaps the phenomenon of inhibition of acid DNase by excess substrate (138) is due to the formation of such complexes; these are essentially identical to those formed between some polyribonucleotides and acid DNase. (*2*) An enzyme-substrate complex I, formed by acid DNase and a site of native DNA in which a sequence on one strand may be split, but not its complement on the opposite strand. (*3*) An enzyme-substrate complex II, formed by acid DNase and a site of native DNA in which two complementary sequences may be split; among these sequences one would expect to find primarily A-T and G-C sequences. Enzyme-substrate complexes I and II would be, obviously, associated with the haplotomic and diplotomic mechanism of degradation, respectively.

As far as the possible significance of the allosteric properties of acid DNase in connection with its mechanism of action on DNA is concerned, it is conceivable that an allosteric transition in the quaternary structure of the enzyme may play a role in facilitating the formation of the enzyme-DNA complex; alternatively it may be thought that it favors the "second splitting" in the double-breakage mechanism. An important implication of the mechanism of action of acid DNase and of the antiparallel arrangement of the DNA strands is that the enzyme molecule itself must have a dyad axis of symmetry (139). The observation that acid DNase is competitively inhibited by antiparallel, but not by parallel, double-stranded polyribonucleotides (see p. 38) lends additional support to this prediction,

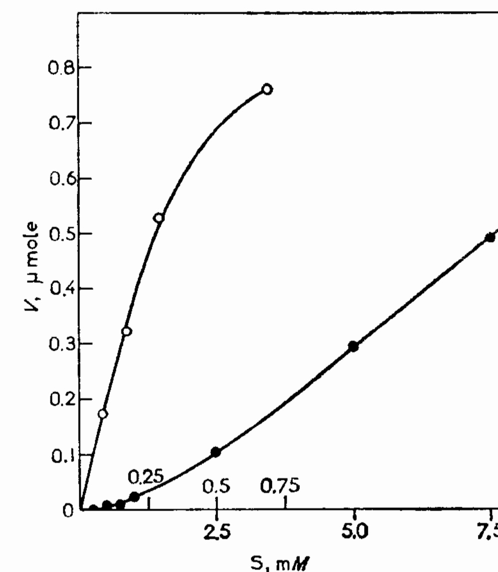

Fig. 23. Velocity, V, of hydrolysis at 37°C. of DNA (○) and bis(p-nitrophenyl) phosphate (●) by hog spleen acid DNase at different substrate (S) concentrations. V is given in moles of acid-soluble nucleotide phosphorus liberated in 15 min (DNA) or of p-nitrophenol liberated in 120 min (synthetic substrate). The enzyme concentration used in the DNase assay was about 100 times lower than that in the phosphodiesterase assay. Substrate concentrations higher than those shown in the figure were not used because of substrate inhibition in the case of DNA, or low solubility in the case of bis(p-nitrophenyl) phosphate. From Bernardi (138).

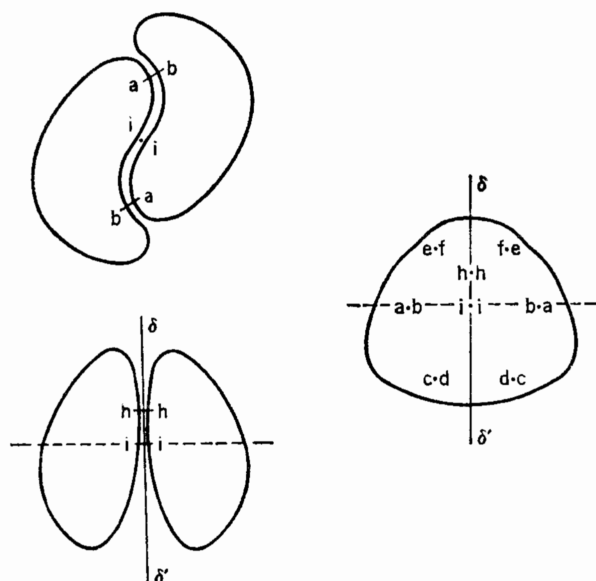

Fig. 24. Model of acid DNase. From Monod et al. (140).

which is in agreement with the model postulated by Monod, Wyman, and Changeux (140). As already mentioned, single-stranded DNA's are much poorer substrates and single-stranded polyribonucleotides are much poorer inhibitors than their double-stranded, antiparallel, counterparts.

In conclusion, a schematic model of the acid DNase molecule may be that of Figure 24, which is taken from the paper of Monod, Wyman, and Changeux (140). In connection with this model, it may be relevant to recall that the dimer of β-lactoglobulin, which has a molecular weight, 36,000, very close to that of acid DNase, has a distance between the two centers of the monomer units equal to 18 Å, as determined by x-ray crystallography (141,142). These data suggest that the dimensions of acid DNase certainly are such as to permit the formation of an enzyme–substrate complex with native DNA (diameter 20 Å) such as postulated by our model.

Note Added in Proof. After this manuscript had been submitted for publication, an article by Prof. M. Laskowski on "DNases and their use in the studies of primary structure of nucleic acids" has appeared (12a). The reader is referred to it for an extensive survey of recent literature in the area of deoxyribonucleases.

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