MECHANISM OF ACTION AND STRUCTURE
OF ACID DEOXYRIBONUCLEASE

By GIORGIO BERNARDI, Strasbourg, France

CONTENTS

I. Introduction ......................................................... 1
II. Mechanism of Action of Acid DNase ................................ 3
   A. Introduction ...................................................... 3
   B. Early Work ...................................................... 4
   C. Kinetics of the Initial Degradation by Native DNA .......... 5
   D. Statistics of Degradation of Native DNA .................. 15
   E. Relationship between $R_0$, $[e]$, and $M_F$ of the DNA Fragments Obtained by Acid DNase Digestion ........... 17
   F. The Oligonucleotides Obtained from DNA by Acid DNase Digestion. Specificity of Acid DNase .................. 18
III. Structures of Acid DNase ........................................ 24
    A. Purification ................................................... 24
    B. Catalytic Properties ......................................... 30
    C. Physical and Chemical Properties ........................ 36
    D. Inhibition by Polynucleotides .............................. 38
    E. Dimeric Structure and Allosteric Properties ............ 40

References ............................................................ 45

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 Lasowski (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 Navey and
Acid DNase has been extensively studied and characterized. Several methods have been developed for the partial purification of the enzyme, and its interaction with DNA has been investigated in detail. The enzyme can be isolated from various sources, including bacteria, fungi, and mammalian cells. Acid DNase is a non-specific endonuclease that cleaves DNA at multiple sites, leading to the formation of single- and double-stranded fragments. The enzyme is activated by low pH, typically ranging from 5.0 to 6.5.

III. Mechanism of Action of Acid DNase

A. Introduction

Three different phases may be distinguished: the degradation of native DNA, the degradation of denatured DNA, and the degradation of denatured DNA in the presence of a chaotropic agent.

1. The initial phase, which is characterized by the rapid degradation of native DNA, occurs at pH values below 5.5. The enzyme cleaves the DNA at multiple sites, leading to the formation of large fragments.

2. The middle phase, which is characterized by the degradation of denatured DNA, occurs at pH values between 5.5 and 7.5. The enzyme cleaves the DNA at multiple sites, leading to the formation of smaller fragments.

3. The terminal phase, which is characterized by the degradation of denatured DNA in the presence of a chaotropic agent, occurs at pH values above 7.5. The enzyme cleaves the DNA at multiple sites, leading to the formation of even smaller fragments.

The degradation of native DNA by acid DNase is an important process in a variety of biological systems, including DNA transcription, DNA replication, and DNA repair.
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 x 10^6 could be obtained by water extraction of the nucleoprotein from chicken erythrocytes (M. Charnavyje, unpublished results, 1950). 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, 1950) 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 (90.61) showed that DNA from calf thymus (S_{20, w} = 20.08; M_n = 6.5 x 10^9) and chicken erythrocytes (S_{20, w} = 27.38; M_n = 5.0 x 10^9) 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 (40.5) x 10^9. 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/A, a value corresponding to that of the B form of DNA (82). The sedimentation coefficient was 5.58 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 nm. 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 (96) degraded DNA in much the same way as the purified chicken erythrocyte 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 x 10^9 molecular weight level than at the starting molecular weight, indicating that degradation was occurring according to a mechanism different from that of pancreatic DNA (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 multi-stranded polymers of any initial distribution of molecular weights. They have shown that for the initial stage of degradation the following equation holds:

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

(1)

where $R = M_t / M_i$, $M_t$ and $M_i$ 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 (90) 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 $p = ct$, $k$ being a proportionality constant, equation 1 becomes

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

(2)

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

$$1/M_t - 1/M_i = kt$$

(3)

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 dissection 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. 1. Digestion of DNA sample B1/2 with pancreatic DNase. From Bernardi and Salmen (85).

Fig. 2. Digestion of DNA sample B1/2 with pancreatic DNase. Data of Figures 2 are plotted according to Schmacker et al. (88) From Bernardi and Salmen (85).
this term, which actually refers to breakage in a single strand, it is suggested that this mechanism be called "haploptome" (from the Greek ἁπλός [haplos], single, and ὁμαδή [homadē], 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 "diploptome" mechanism (from the Greek ἰδίωμα [idiouma], double).

In sharp contrast with pancreatic DNase, acid DNase initially degrades native DNA according to a diploptome mechanism as unequivocally shown by our work (80-81); 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, , is found to be equal to 1 ± 0.1 (Fig. 4 and Table I). The diploptome degradation by acid DNase was found, with the exception of 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 (76,80-81) as in the technique 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 diploptome mechanism of degradation of acid DNase first suggested, on the basis of qualitative evidence, by Otth, Fredericq, and Hsia (92), has been subsequently confirmed by the work of Young and Stinson (93), who investigated the degradation of lambda phage DNA by non-sedimenting, and by Katz and McEwan (94).

Contrary to the suggestions of some authors (80,85) that acid DNase only splits DNA according to a diploptome mechanism, the enzyme does degrade DNA according to both diploptome and haploptome 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 106 is reached. Since bond splitting is linear with time (Richards and Bernardi, unpublished data), this finding is incompatible with the existence of a purely diploptome mechanism of degradation and is best explained by the superposition of two different mechanisms of degradation: one of them, the diploptome mechanism, is effective immediately in causing a decrease in molecular weight, whereas the second, the haploptome 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
Degrading of Native DNA by Acid DNase Preparations* (85)

<table>
<thead>
<tr>
<th>DNA range</th>
<th>Preparation method</th>
<th>DNA concn. (µg/ml)</th>
<th>M0 x 10³</th>
<th>R0 Å</th>
<th>Enzyme units</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) B6a-N/1</td>
<td>E</td>
<td>A</td>
<td>115</td>
<td>3.8</td>
<td>2090</td>
</tr>
<tr>
<td>(2) B6b/24</td>
<td>E</td>
<td>B</td>
<td>50</td>
<td>7.2</td>
<td>2000</td>
</tr>
<tr>
<td>(3) B6/30</td>
<td>E</td>
<td>B</td>
<td>86</td>
<td>9.6</td>
<td>2000</td>
</tr>
<tr>
<td>(4) B6/46</td>
<td>E</td>
<td>B</td>
<td>133</td>
<td>5.5</td>
<td>2530</td>
</tr>
<tr>
<td>(5) B6/52</td>
<td>E</td>
<td>B</td>
<td>133</td>
<td>5.5</td>
<td>2530</td>
</tr>
<tr>
<td>(6) B6a-N/1</td>
<td>E</td>
<td>A</td>
<td>65</td>
<td>8.4</td>
<td>2000</td>
</tr>
<tr>
<td>(7) B6/15</td>
<td>E</td>
<td>A</td>
<td>74</td>
<td>4.9</td>
<td>2100</td>
</tr>
<tr>
<td>(8) B6/37</td>
<td>T</td>
<td>A</td>
<td>140</td>
<td>4.6</td>
<td>2100</td>
</tr>
<tr>
<td>(9) D4/176</td>
<td>C</td>
<td>A</td>
<td>65.5</td>
<td>3.8</td>
<td>2000</td>
</tr>
<tr>
<td>(10) B6b-N/12</td>
<td>E</td>
<td>A</td>
<td>62.5</td>
<td>3.8</td>
<td>2000</td>
</tr>
<tr>
<td>(11) B6/185</td>
<td>E</td>
<td>A</td>
<td>24</td>
<td>2.3</td>
<td>2530</td>
</tr>
<tr>
<td>(12) B6/74</td>
<td>E</td>
<td>B</td>
<td>1900</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>(13) B6a/59</td>
<td>E</td>
<td>D</td>
<td>133</td>
<td>4.6</td>
<td>2000</td>
</tr>
</tbody>
</table>

* All degradations were performed at room temperature (20-22°C) in the light-scattering cell, using 0.15M NaCl, 0.01M EDTA in the absence, except for samples 7, 10, 15, 18, 22, and EDTA in the presence of EDTA, pH 7.4, and 72°C (phosphate buffer, pH 8.0, α = 0.005, containing 10-5M EDTA).

* E, chicken erythrocytes; T, calf thymus; C, calf colo.

* See reference 85.

* EDTA, calf thymus DNase, ERW, calf thymus DNase ( Worthington); EDT/3, hog spleen pure enzyme; EC/2, purified calf thymus DNase of specific activity 50; (1/HL) 1, calf thymus DNase; EC/3, ET treated with disopropylfluorophosphate.

* x is the apparent number of strands as calculated from the slope of log (1 - R/R0) versus log t (80,96).
2. Titrating data (Richards and Bernadi, unpublished data) show that, for a given decrease in molecular weight (down to $5 \times 10^6$), the number of bonds broken is larger than that expected for a purely dipotassium degradation, but much smaller than for high-molecular degradation; in fact, 10-20 breaks (a value likely to be underestimated for technical reasons) were found to be necessary to halve the weight-average molecular weight of each parent molecule at $M_a = 6 \times 10^6$, whereas 280 breaks are needed in a high-molecular degradation (87) and 6 breaks (3 sessions) in a dipotassium 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 dipotassium mechanism to lie between 1.7 and 3.3.

3. If DNA samples, partially digested by acid DNase and ranging in molecular weight from $1 \times 10^6$ to $4 \times 10^6$, 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 high-molecular action of the enzyme. The data of Table II allow an estimate of the ratio of total bonds broken to bonds broken by the dipotassium 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 Simonsen (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 dipotassium mechanism of the order of 1.5-3.0.

Fig. 3. Digestion of DNA sample B6N/1 with acid DNase (see Table 1). From Bernardi and Salomon (85).

Fig. 4. Digestion of DNA sample B6N/1 with acid DNase. Data of Figure 6 are plotted according to Shmoozor et al. (88). See also Table I. From Bernardi and Salomon (85).
TABLE II
Degradation of DNA by And DNase (96)

<table>
<thead>
<tr>
<th>Digestion time (min)</th>
<th>Native DNA</th>
<th>Deradened DNA</th>
<th>Hit den. DNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$M_w \times 10^{-6}$</td>
<td>$M_w \times 10^{-6}$</td>
<td>$M_w \times 10^{-6}$</td>
</tr>
<tr>
<td>0</td>
<td>3.88</td>
<td>2.20</td>
<td>1.45</td>
</tr>
<tr>
<td>20</td>
<td>3.32</td>
<td>1.56</td>
<td>0.55</td>
</tr>
<tr>
<td>60</td>
<td>1.25</td>
<td>0.75</td>
<td>0.20</td>
</tr>
<tr>
<td>90</td>
<td>1.45</td>
<td>0.54</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Chicken erythrocytes DNA (ref. 85; Table III)

<table>
<thead>
<tr>
<th>Lambda phage DNA (ref. 92; Table I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>15</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>45</td>
</tr>
</tbody>
</table>

* Hit (solutions) were calculated according to Charlesby’s (96) equations:

$$\frac{(M_{w,1}/M_{lw}) - 1}{(M_{w,2}/M_{lw}) - 1} = \frac{1}{1 + \gamma p}$$

$$\frac{(M_{w,1}/M_{lw}) - 1}{(M_{w,2}/M_{lw}) - 1} = 2(\alpha + p - 1)/\alpha$$

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 subunits.

This column gives the ratio of total bonds broken to bonds broken by tRNA digestion, per parent native molecule.

While the lysobisotonic mechanism is essentially identical with that exhibited by pancreatic DNA and does not need any special comments, several hypotheses may be put forward to explain the tRNA digestion mechanism:

1. The enzyme splits at random one or the other DNA strand and the single-lot 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-lot 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 (84,95). 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. B: Digestion of DNA sample D3/45 with acid DNase (96).
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 Bates and McAulay (94); however, this complicated model is difficult to reconcile with the size of the enzyme (pp. 36 and 44).

D. STATISTICAL 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 $n$ links have been broken, the number of $n$-mers, $N_n$ arising from the hydrolysis of an $n$-mer (where $n$-mer and $n$-mer refer to chains of $n$ and $n$ links, respectively) is:

$$N_n = \frac{p^{n-1}}{(1 - p)} 2 + (n - a)(1 - p)$$

(5)

where $1 - p = r/n$. Theoretical distributions were calculated for several different numbers of breaks for each original T5 molecule and were fitted against each observed distribution, assessing the goodness of fit by the $x^2$ test. In each case $x^2$ passed through a sharp minimum, indicating best-fitting values of 97, 231, and 832 breaks for each parent 40-mer molecule at 30, 90, and 160 min of digestion (Fig. 8).
Fig. 8. Fragment size distributions in the 30-90, and 100- min digestion products of "whole" T5 DNA molecules. The smooth curves are the best-fit theoretical distributions calculated on the basis of random coalescence corresponding to 57, 231, and 572 coalescences per original 40,000 molecules. For the 100-min histogram the number scale is three times that shown. From Machliss, Bernardi, and Thomas (90).

probabilities of fit were high: 30, 60, and 83%, respectively. Thus the number of brazees 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 0.03-0.1 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 D Nase 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 an preferred sub-unit size was found confirms that, in the destruction of DNA with crude enzyme preparation (p. 4), degradation did not continue below a molecular weight of 3 X 10^6 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. 4, indicating the coexistence of a haplotoxic and a diplotomic mechanism (p. 8).

E. RELATIONSHIP BETWEEN Rg, S, N, AND Mw 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 10^6. They show the hyperchromicity, the melting curve, the diameter (90), the mass/length ratio (80,81), and the chromatographic behavior on hydroxypatite (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 X 10^6 by Richards and Bernardi (84,101).

These fit the following equations:

\[ S_{11, \nu} = 0.057 \times M_w^{0.242} \] (S in Svedberg units)  
\[ \eta = 0.353 \times 10^{-4} M_w^{1.175} \] (η in cgs units)  
\[ R_g = 1.1 \times M_w^{0.55} \] (R in Angstrom units)

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

Fig. 9. Variation of radius of gyration (Rg) with weight-average molecular weight (Mw) for DNA samples digested with acid DNase (circle). Closed points are data by Doty et al. (102); points data of Last and Stacey (103). From Bernardi and Sadron (84).
Fig. 10. Variation of intrinsic viscosity (η) and sedimentation constant (S) with weight-average molecular weight (Mw) for DNA samples digested with acid DNase. From Bernardi and Saito (84).

The interest of the relationships established by Richards and Bernardi (84, 114) lies in the fact that they were determined on DNA samples obtained by a degradation whose kinetics and statistics are known.

IV. 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 1953 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 Torri and Bernardi (115). In this work, the homogeneous preparations of acid DNase, obtained according to the method of Bernardi and Griffe (116, 117), as modified by Bernardi, Bernardi, and Cheesi (118, 119), were used instead of the partially purified preparations used by previous authors. Furthermore, new techniques were developed (90) and new enzyme preparations were obtained (70-74) in order to study the digest and the specificity of acid DNase: in the course 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 Frederieq (85)). 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
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 (22-36%) 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 ecornuclease (73,74) digestion of the depolymerized acid DNase digest. 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 0.1 M NH4-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 Czerara and Bernardi (114) is not far from that, 10, obtained by Koerner and Sinzheimen (74) at the end of the fast phase, but is much higher than that reported by other authors: Daskoelj and Förm (112) found a size of about 4, and Vacecko and Laskowski found sizes
Fig. 12. Chromatography of an acid D.Nase digest (A); see Table 1 and II of ref. 111 of calf thymus DNA (105 A₂₆₀ units) on a 105-A₂₆₀ column (chloride column 1.8 × 23 cm). 650 ml of 10% solution (A2₆₀ = 0.38) in 0.1M acetic acid buffer pH 3.5 (2.5 M KCl) was digested with 20 A₂₆₀ units of D.Nase I (111 ammonium). A₂₆₀ = 1.14 for 1 br at room temperature; the sample was further digested with 11 A₂₆₀ units for 11 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 salinity gradient of NaClO·0.3M (2000 ml in 7M urea, pH 7.5). Fraction size: 5.5 ml; flow rate 30 ml/hr. The continuous line indicates the absorbance at 251 nm, the broken line the salt gradient. Loading was started at fraction 0. From Carreira and Bernardi (111).

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, Dossaill and Sorm (112) report their highest fraction average size 7; formed 4% of the digest, whereas in our case the large fragments average size higher than 7 formed 65.7% 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 these 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 exo- nuclease in acid D.Nase and n

TABLE III

<table>
<thead>
<tr>
<th>Author</th>
<th>Kornberg &amp; Bloch (54)</th>
<th>Yanisch &amp; Laskowski (108)</th>
<th>Dossaill &amp; Sorm (112)</th>
<th>Yanisch &amp; Laskowski (108)</th>
<th>Carreira &amp; Bernardi (111)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average size</td>
<td>10</td>
<td>4.5</td>
<td>4</td>
<td>6.7</td>
<td>10-12</td>
</tr>
<tr>
<td>Method</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A</td>
<td>C</td>
</tr>
<tr>
<td>SP terminal</td>
<td>G</td>
<td>33.1</td>
<td>23.3</td>
<td>36.3</td>
<td>34.3</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>39.3</td>
<td>38.4</td>
<td>27.3</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>28.8</td>
<td>26.9</td>
<td>39.7</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>7.6</td>
<td>11.4</td>
<td>11.7</td>
<td>10</td>
</tr>
</tbody>
</table>

* Methods:
A. After venom endonuclease digestion.
B. After pancreatic D.Nase digestion.
C. After phosphononuclease and spleen exonuclease digestion. Data of Dossaill and Sorm were calculated from the results given by these authors.
III. Structure of Acid DNase

A. PURIFICATION

A homogeneous acid DNase preparation was first obtained from hog spleen by Bernardi and Griffi (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 Sinheimer (49), Shimomura and Lasowski (53), and Fredriech and Oth (52).

The method of Bernardi and Griffi (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 (jailed) crude spleen nuclease II to distinguish it from the crude spleen nuclease I obtained by the original method of Bernardi and Griffi (117) involved the following steps: trimming, grinding, and homogenization of hog spleens with 0.05 M H2SO4; acidification of the homogenate to pH 2.5 with 0.1 M H3PO4; fractionation between 40 and 80%; saturation of (NH4)2SO4 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 0.05 M 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 0.1 M HCl and 0.15 M NaCl, respectively, replaced 0.05 M H2SO4 in the extraction mixture were also studied and the enzyme activities are also reported in Table IV. 0.1 M HCl or 0.05 M H2SO4 are evidently more effective than 0.15 M NaCl in extracting acid deoxyribonuclease; this is very probably due to the fact that contact with the acid solution is effective in bringing 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 acetic 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 0.1 M HCl or 0.05 M H2SO4, 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 0.2-0.5 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 As/l/As 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 Griffi (117)) is summarized in Table V. Figures 13-16 show the three chromatographic steps, DEAE-Sephadas, Hydroxypatite, and CM-Sephadex and the rechromatography of the two activity peaks, A and B, obtained from CM-Sephtades. The central part of the rechromatographed peaks were loaded on Sepha-
dex G-25 columns equilibrated with 0.001 M acetate buffer, pH 5.0.

TABLE IV

Preparation of Spleen Acid Deoxyribonuclease (118)

<table>
<thead>
<tr>
<th>Preparation step</th>
<th>0.15M NaCl</th>
<th>0.1 M HCl</th>
<th>0.05 M H2SO4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extraction</td>
<td>1270</td>
<td>3200</td>
<td>3800</td>
</tr>
<tr>
<td>2. Acidification to pH 2.5</td>
<td>3170</td>
<td>4500</td>
<td>4100</td>
</tr>
<tr>
<td>3. 0.4(NH4)2SO4 saturation</td>
<td>2670</td>
<td>2940</td>
<td>3090</td>
</tr>
<tr>
<td>4. 0.15(NH4)2SO4 saturation</td>
<td>3040</td>
<td>3600</td>
<td>3570</td>
</tr>
</tbody>
</table>

- Extraction procedure
- Dry weight g

- This column gives the dry weight of undialyzable material per kg of trimed spleen, as determined on the dialyzable products mentioned above 0.05 M H2SO4 extraction.

- This was done with 0.2M HCl for the extracts obtained with 0.15 M NaCl and 0.1 M HCl.

- The low values found at this step are probably due to the presence of residual SO4<sup>2-</sup>; this is an inhibitor of acid deoxyribonuclease (54,117).
The enzyme fractions were then concentrated by freeze-drying down to solutions having A440 = 3, frozen, and stored at -80°.

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 Griffe (117). The purity is also identical to that of the preparations by Bernardi and Griffe (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-nitrophenylphosphate 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 volumes from Sephadex G-100 columns, ultraviolet spectra, optical rotations, or enzymological properties (DNase and phosphodiesterase activities). The only difference between the two 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.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Weight (mg)</th>
<th>Volume (ml)</th>
<th>Activity (units)</th>
<th>Specific Activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>400</td>
<td>160,000</td>
<td>125,000</td>
</tr>
<tr>
<td>I. DEAE-Sephadex</td>
<td>0.36</td>
<td>725</td>
<td>105,000</td>
<td>145,000</td>
</tr>
<tr>
<td>II. Hydroxyapatite</td>
<td>0.18</td>
<td>425</td>
<td>81,500</td>
<td>95,500</td>
</tr>
<tr>
<td>III. CM-Sephadex A</td>
<td>0.18</td>
<td>20</td>
<td>7,850</td>
<td>22,4</td>
</tr>
<tr>
<td>IV. CM-Sephadex B</td>
<td>0.08</td>
<td>20</td>
<td>10,000</td>
<td>58,5</td>
</tr>
</tbody>
</table>

Specific activity data given in the columns were obtained by dividing the activity (total units) by the dry weight (in mg) of the enzyme preparations.

Values reported refer to fractions A and B, respectively.

![Fig. 13. Chromatography of crude spleen nuclease II on DEAE-Sephadex A-50 (procedure C, step 1). 400 ml of preparation B40 (A440 = 104; A280 = 9) were loaded on a 8 x 20 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-h fractions were collected. The continuous line indicates the absorbance at 280 nm. Circles indicate the acid deoxyribonuclease activity (right-hand scale). The broken line indicates the absorbance at 415 nm of cytochrome c (left-hand insert scale). Fractions 40-60 were pooled further. Acid and basic ribonuclease, acid phosphomonoesterase and phosphodiesterase, and phosphomonoesterase were also assayed; the results are shown elsewhere (70,20). From Bernardi, Bernardi, and Chemi (118).]
Fig. 14. Chromatography of fractions 50-65 from step I on hydroxypatite (procedure C, step II). 370 ml (A_w w = 0.48) were loaded on a 2 × 80 cm column of hydroxypatite equilibrated with 0.05M phosphate buffer (pH 6.8). A modality gradient 0.05-0.5M was started at the fraction indicated by the arrow at fraction 120 the modality of the effluent was 0.35. 24 ml fractions were collected. The continuous line indicates the absorption at 290 nm. Circles (○) indicate the acid deoxyribonuclease activity (right-hand scale). Cytocence c was eluted as a sharp peak centered on fraction 86 (A_w w = 0.52; not shown in the figure). Acid ribonuclease (□, right-hand inner scale) and basic ribonuclease (A, right-hand outer scale) are also shown. Fractions 100-115 were concentrated by freeze-drying to about 30 ml, filtered through a Sephadex G-25 column equilibrated with 0.025M phosphate buffer (pH 6.8), and processed further. From Bernardi, Bernardi, and Cesur (138).

These findings may be explained tentatively by assuming that the disappearance of A and B is due to the denaturation 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 tryptic digestion. It is known that acid DNase is very rich in asparagine (127) and that denaturation 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 HSO₄, in preparation 2, the acidification step to pH 2.5 was omitted and 0.1M H₂SO₄ was replaced by the same amount of 0.15M NaCl. Preparation 3 was like preparation 2, except that 0.15M NaCl replaced 0.05M H₂SO₄ in the tissue-homogenization step. All three 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 17% 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 (150) on Amberite 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 5N H₂SO₄.

Fig. 15. Chromatography of fractions 100-115 from step II on CM-Sephadex C-50 (procedure C, step III). 115 ml (A_w w = 0.496) were loaded on a column equilibrated with 0.05M phosphate buffer (pH 6.8). A modality gradient 0.1-0.4M of phosphate buffer (pH 6.8) was started at the fraction indicated by the arrow (right-hand inner scale). 44 ml fractions were collected. The continuous line indicates the absorption at 290 nm. Circles indicate the acid deoxyribonuclease activity (right-hand outer scale). Fractions 37-48 and 49-61 were processed further. From Bernardi, Bernardi, and Cesur (138).
B. CATALYTIC PROPERTIES (117)

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

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\(^{2+}\), 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 Griffith (117) with hog spleen acid DNase at \( \mu = 0.15 \). No proteolytic proteins were added when the enzyme solutions were diluted for this experiment.
enzyme solutions. Since acid DNase has no free sulfhydryl groups (see Section II-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' terminal positions of the oligonucleotides (see Table H1).

2. Phosphodiesterase Activity

The enzyme shows a weak hydrolytic activity on calcium (bis-p-nitrophenoxy) phosphate and the p-nitrophenoxy 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-nitrophenoxy 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 Calcium (bis-p-nitrophenoxy) phosphate, the pH optimum was found to be between 5.0 and 5.5. In the 5.1-5.6 pH range, the activity in acetate is about twice as large as in sucrose buffer. In the pH range 4.0-5.6, no significant changes occur upon addition of Mg²⁺ or Versene (Fig. 19); in the pH range 4.0-7.6, SO₄³⁻ and HPO₄²⁻ give a very strong inhibition at a level of 0.01. It seems possible that the phosphodiesterase activity found by Kornmer and Sinzheimer (49) to elute from Ultrex 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 Griffo (117) on these columns, only one symmetrical peak was obtained and the ratio of the two activities was constant throughout the peak. On CM-Sephadex the enzyme was eluted by a mortality 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
that the A and B components (see p. 26) obtained by the method of Bernardi, Bernardi, and Chierri (118) do not differ in their activities on bis(p-nitrophenyl)phosphate and DNA.

(b) Thermal Inactivation. Enzyme samples (0.4 ml; $A_{420} = 0.14$) were kept for 20 min at several temperatures ranging from 25° to 75° in 0.10 M acetate buffer - 0.01 M EDTA, pH 5.0, chilled in an ice bath, and used in digestion experiments at 37°. The activation 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)

<table>
<thead>
<tr>
<th>Acid DNase</th>
<th>Spleen exonuclease</th>
<th>Nucleoside polyphosphates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sedimentation co.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH optimum $^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>4.6</td>
</tr>
<tr>
<td>5.6</td>
<td>5.9</td>
<td>5.8</td>
</tr>
<tr>
<td>3.</td>
<td>Bis(p-nitrophenyl)phosphate</td>
<td></td>
</tr>
<tr>
<td>$p$-Nitrophenyl</td>
<td>$^a+$</td>
<td>$^a+$</td>
</tr>
<tr>
<td>Phosphate</td>
<td>$^a$-</td>
<td>$^a$-</td>
</tr>
<tr>
<td>Thymidine-3' phosphate</td>
<td>$^a+$</td>
<td>$^a$-</td>
</tr>
<tr>
<td>Adenosine-3' phosphate</td>
<td>$^a+$</td>
<td>$^a$-</td>
</tr>
<tr>
<td>Native $5^a$</td>
<td>$^a+$</td>
<td>$^a$-</td>
</tr>
<tr>
<td>ATP, ADP, etc.</td>
<td>$^a$-</td>
<td>$^a$-</td>
</tr>
<tr>
<td>3'Phosphate sugar monoesters</td>
<td>$^a$-</td>
<td>$^a$-</td>
</tr>
<tr>
<td>4.</td>
<td>Inhibitors $^a$</td>
<td></td>
</tr>
<tr>
<td>HSA $^a$</td>
<td>$^a+$</td>
<td>$^a$-</td>
</tr>
<tr>
<td>NPS $^a$</td>
<td>$^a$-</td>
<td>$^a$-</td>
</tr>
<tr>
<td>Polysine $^a$</td>
<td>$^a+$</td>
<td>$^a$-</td>
</tr>
<tr>
<td>5.</td>
<td>Thermal inactivation $^a$</td>
<td></td>
</tr>
<tr>
<td>60°</td>
<td>25°</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Chromatographic properties</td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephadex (pH 6.8)</td>
<td>0.05 M KP</td>
<td>0.05 M 0.3 M</td>
</tr>
<tr>
<td>DEAE-Sephadex (pH 6.8)</td>
<td>0.3 M KP</td>
<td>0.12 M KP</td>
</tr>
<tr>
<td>CM-Sephadex</td>
<td>pH 6.8:</td>
<td>pH 6.2;</td>
</tr>
<tr>
<td>0.2 M KP</td>
<td>0.23 M KCl</td>
<td>0.14 M KCl</td>
</tr>
</tbody>
</table>

$^a$ As determined by sedimentation in sucrose gradient, using cytorhene $c$ as a reference protein; enzyme 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 0.25 M sucrose buffer as the substrate.

$^c$ The enzyme digests polyribonucleotides to 3'P mononucleotides; there are inhibitors.

$^d$ Elutingolarity. KP is potassium phosphate buffer.
(d) inhibition by Polyribonucleotides. Some natural and
bio-synthetic polyribonucleotides behaved as competitive inhibitors
with respect to both enzyme activities (see also p. 38).
The activity on benzoylphenylphosphate seems to be a non-
specific activity of acid DNase; this activity is displayed by at least
two other spleen enzymes, which also have different natural sub-
strates, covalence and nucleoside polyphosphates. Table VI
summarizes the properties of the phosphatase 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 Grossi (117) and Bernardi, Bernardi,
and Chiari (118). The physical results are summarized in Table
VII. They indicate that acid DNase is a small globular protein of
molecular weight 30,000; its sedimentation coefficient is 6.4 as
determined both by refractometry in the analytical ultracentrifuge
after extrapolation to zero concentration and by curve 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, 2.78, reported by Kats and McEwan (94) for
both spleen and HeLa cell DNases; we have no explanation for this
lower value. The isoelectric point is close to pH 6.2, as estimated by
zone electrophoresis on cellulose acetate.

| TABLE VII |
| Physical Properties of Hog Spleen Acid Deoxyribonuclease (121) |
| Protein content | 3.4 |
| Gelatin clumping | 7.8 |
| pH opt | 6.72 |
| Wheat gluten | 0.6 x 10^-4 |
| FDC/L | 0.76 |

* This value was obtained at concentrations of 0.5% and about 0.1%.  
* 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 mg. The high ornithine content of the
acid hydrolysate suggests that a large percentage of the deoxyribosyl

| TABLE VIII |
| Amino Acid Analysis of Hog Spleen Deoxyribonuclease (121) |
| Molar extinction coefficient of protein of residuum | M.W. (98,000) |
| 22 hr | 48 hr | 72 hr | Corrected value* |
| Lys | 6.94 | 6.80 | 6.95 | 6.89 | 29.44 | 20 |
| His | 2.34 | 2.17 | 2.31 | 2.29 | 6.08 | 6 |
| Asn (NH2) | 21.05 (21.65) (24.06) (25.20) (49.4) (49) |
| Arg | 5.04 | 5.55 | 5.37 | 5.46 | 13.30 | 13 |
| Asp | 10.19 | 10.54 | 9.36 | 9.80 | 22.68 | 23 |
| Thr | 5.44 | 5.20 | 4.80 | 3.63 | 21.16 | 21 |
| Ser | 6.30 | 6.10 | 7.18 | 8.46 | 36.93 | 37 |
| Glu | 11.03 | 11.09 | 10.66 | 10.91 | 32.19 | 32 |
| Pro | 7.10 | 7.14 | 6.51 | 6.91 | 25.07 | 25 |
| Gly | 3.88 | 3.97 | 3.87 | 3.98 | 23.99 | 23 |
| Ala | 4.84 | 4.72 | 4.33 | 4.63 | 21.77 | 25 |
| Leu | 1.77 | 1.70 | 1.79 | 1.79 | 6.65 | 8 |
| Val | 2.62 | 2.53 | 3.35 | 3.35 | 19.94 | 13 |
| Met | 1.65 | 1.65 | 1.50 | 1.52 | 4.45 | 4 |
| Ileu | 2.28 | 2.40 | 2.52 | 2.52 | 8.47 | 8 |
| Leu | 10.47 | 11.13 | 10.49 | 10.67 | 35.87 | 36 |
| Tyr | 3.23 | 3.17 | 4.83 | 5.28 | 12.33 | 12 |
| Phe | 6.30 | 6.56 | 6.97 | 6.31 | 16.39 | 16 |
| Glycine | 3.07 | 3.32 | 3.66 | 3.15 | 7.45 | 8 |
| Total | 88.46 | 100.32 | 94.75 | 99.30 | 334 |
| N recovery, % | 98.9 | 98.7 | 97.4 |

* Total N is 17.2%; total S is 1.8%.
* In calculating the corrected values, the criteria given by Travis and Smith
(127) have been followed.
* The selection of the integral numbers of residues has been done taking into
account also results from other analyses.
* After performic acid oxidation, cysteic acid 8.2 residues.
* From Schramm's chromium 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 dinergic structure of the enzyme, all amine acid present at low levels were found to be present in an even number of residues per protein moleule. 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).

10. 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 0.001M 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 polyadenosinic acid and the poly A-poly U and poly I-poly C 1:1 complex. 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 U, 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 polynucleotide, a polyribonucleotide, or a complex 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 a parallel, double-stranded structure, like poly A and poly C at pH 5.0 (135-137), do not inhibit, whereas those which have anti-parallel, double-stranded structures 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. 41).

The findings that polyribonucleotides having single-stranded structures like poly U have very weak inhibitory properties, and that a 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 polyribo- and acid DNA has been studied in detail and a 1:1 strong complex has been demonstrated (work to be published).
Evidence supporting a dimeric structure of hog sperm acid DNase was obtained in our laboratory (18) from both physical and chemical investigations. Physical evidence for the dimeric structure of the enzyme was provided by sedimentation studies using aqueous-guanidine-containing solvents. Table IX shows the sedimentation coefficients obtained in several dissociating solvents. At a 1.0 M concentration, the sedimentation coefficient, which is 2.88 in acetate, phosphate buffer, drops to 1.708 and 2.18 in 6 M guanidine at pH 6.0 and 5.0, 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 N-mercaptoethanol, the sedimentation coefficient is still lower, as expected: 1.28 in guanidine and only 0.85 in 6 M urea; 1.6 M urea both the dissociated and the associated forms of the enzyme can be seen (Fig. 21). The fact that dissociation takes place also in the absence of reducing agents indicates that most of the four disulfide bridges found in the enzyme crosslink the two subunits, which, therefore, can form two disulfide bridges each.

Table IX: Sedimentation Coefficients of Acid DNase

<table>
<thead>
<tr>
<th>Solvent Conditions</th>
<th>Sedimentation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 M acetate buffer (0.01 M EDTA, pH 5.0)</td>
<td>2.8</td>
</tr>
<tr>
<td>0.05 M phosphate buffer, pH 5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>0.1 M acetate buffer (0.01 M EDTA, pH 5.0)</td>
<td>2.0</td>
</tr>
<tr>
<td>0.1 M N-mercaptoethanol</td>
<td>2.0</td>
</tr>
<tr>
<td>4 M urea</td>
<td>0.8</td>
</tr>
<tr>
<td>6 M guanidine</td>
<td>0.8</td>
</tr>
<tr>
<td>6 M guanidine (0.1 M EDTA, pH 5.0)</td>
<td>2.4</td>
</tr>
<tr>
<td>6 M guanidine (0.1 M EDTA, pH 5.0)</td>
<td>2.4</td>
</tr>
<tr>
<td>6 M guanidine (0.1 M EDTA, pH 5.0)</td>
<td>2.4</td>
</tr>
<tr>
<td>6 M guanidine (0.1 M EDTA, pH 5.0)</td>
<td>2.4</td>
</tr>
<tr>
<td>6 M guanidine (0.1 M EDTA, pH 5.0)</td>
<td>2.4</td>
</tr>
<tr>
<td>6 M guanidine (0.1 M EDTA, pH 5.0)</td>
<td>2.4</td>
</tr>
<tr>
<td>6 M guanidine (0.1 M EDTA, pH 5.0)</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Chemical evidence for the dimeric structure of acid DNase comes from an investigation of the tryptic hydrolysis of the enzyme. This was reduced, carboxymethylated, and digested with crystalline trypsin treated with 1-tosylamide-2-phenyltetrahydromethane 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, 19, 099 peptides were found (Fig. 22) as opposed to 32, 000 arginine and lysine residues found by Bernardi, Appels, and Zito (121) in the enzyme of molecular weight 88,000. 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 or substrate (Fig. 23). In contrast, spleen exonuclease and nucleotide-polynucleotidease (p. 55) 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 Molot, Wyman, and Chang (140), bis(p-nitrophenyl) phosphate behaves as a homotropic allosteric effecter with respect to acid DNase. It is well known that allosteric phenomena are characterizedly found in oligomeric enzymes.
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 (128). 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.
which 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 polynucleotides 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 adenosine-5'-diphosphate, 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 8 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. Laszukowski on DNAse and their use in the studies of primary structure of polynucleotides has appeared (12a). The reader is referred to it for an extensive survey of recent literature in the area of deoxyribonuclease.

Acknowledgments

I wish to thank Prof. Charles Sanger for his continuous support and encouragement, and my colleagues Madeleine Champagne, Glen Richards, Maurice Cottle, Claude Cordeau, Helia Schaller, Lorne MacHattie, Charles Thomas, Ettore Appella, Romano Zito, Alberto Bernardi, Alberto Orelli, and Mariano Curtasi for their collaboration in various parts of this work. The technical help of Hunt Shackle, Iets Nadel, and Alfred Schirrer is gratefully acknowledged.

These investigations were aided in part by a grant (TEC-28-46-4863) from the U.S. Department of Agriculture.

References