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DEGRADATION OF DEOXYRIBONUCLEIC ACID BY ACID DEOXYRIBONUCLEASE

GIORGIO BERNARDI and CHARLES SADRON
Centre de Recherches sur les Macromolécules, Strasbourg (France)

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Introduction

Enzymology of polynucleotides has been a very fast moving field of investigation during the past few years. Undoubtedly the most significantt advances have been obtained in the area of polynucleotide-synthesizing enzymes. The progress of our knowledge in the field of polynucleotide-hydrolyzing enzymes has been much less dramatic, yet very exciting findings have been reported recently. These enzymes generally are valuable tools in elucidating polynucleotide structures; when carefully studied, some of them have shown unexpected specificities in relationship not only with the primary but also with the secondary structure of nucleic acids. Acid deoxyribonuclease is perhaps a good example of an enzyme which has shown, upon a detailed kinetic study, a surprising mechanism of action.

Extensive investigations have been carried out in Strasbourg during the past three years on the degradation of deoxyribonucleic acid (DNA) by acid deoxyribonuclease (DNase). The early part of this work has already been described elsewhere [1 -] and therefore will not be presented here. In subsequent work, two main lines of investigation have been followed: 1) isolation of pure acid DNase and its physical and chemical characterization (see ref. 4 for a preliminary report); 2) a study of the kinetics of degradation of DNA by acid DNase. In this communication a concise description of the kinetic results will be given. A preliminary account of the early part of this work has already been published [5] and a detailed presentation of the kinetic data is now in preparation and will appear elsewhere in due time [6].

Treatment of kinetic data

Schumaker, Richards and Schachman [7] 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:

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

Here $R = M_t/M_o$; (M_t and M_o are the molecular weights at time t and at time zero, respectively); n is the number of strands and p is the probability that any given bond is broken. Equation (1) shows that a plot of $\log (1 - R)$ *vs.* $\log p$ will have a slope equal to n. A more convenient plot is obtained by replacing, as proposed by Cavalieri and Rosenberg [8], $(1 - R)$ by $(1 - R)/R$ in equation (1). Then, assuming that $p = k\,t$, (k being a proportionality constant) equation (1) becomes:

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

The use of equation(2) has the advantage that $\log(1-R)/R$ is a linear function of $\log t$ over a wider range of R values. For the particular case where $n = 1$, equation (2) becomes

$$\frac{1}{M_t} = k\,t + \text{const} \qquad (3)$$

which is exactly what the statistical theory of random degradation of linear polymers predicts [9].

Methods and Materials

The enzymatic degradation of DNA has been followed by digesting DNA solutions directly in the cell of a Wippler and Scheibling light-scattering apparatus [10] and measuring at different time intervals the intensities of the light scattered at six angles comprised between 30° and 90°. The amounts of enzymes added were such as to obtain a change of R from 1.0 to 0.7 in about 2 hours. The validity of the light-scattering technique in assessing

molecular weights of DNA up to 6.10^6 has been recently demonstrated by Froelich, Strazielle, Bernardi and Benoit [11] using a low-angle light-scattering instrument.

Alternatively, low-gradient viscometry has been used in order to follow the enzymatic degradation. A Couette apparatus built by Dr. Scheibling in our laboratory has been used. The results

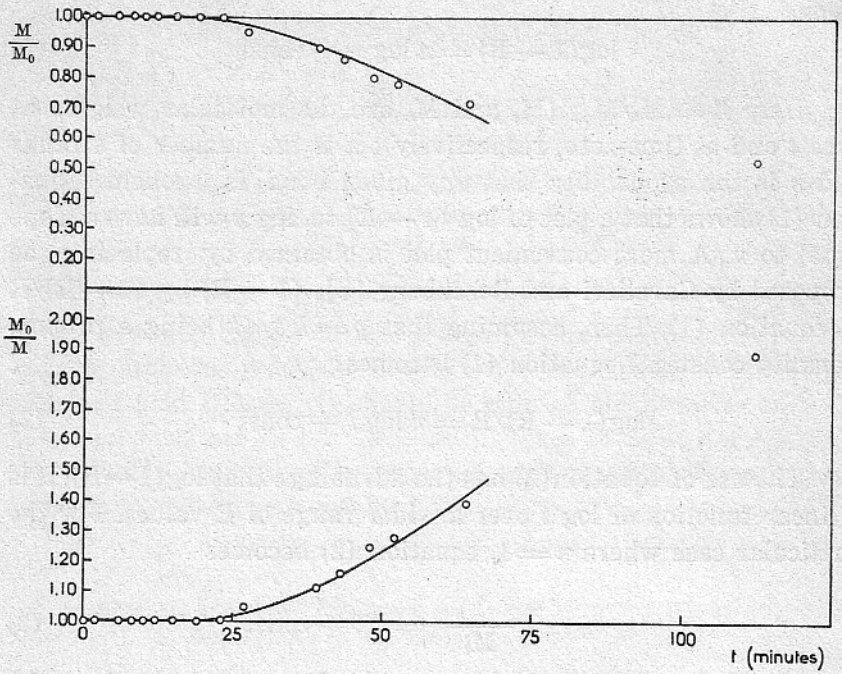

Fig. 1. — Degradation of native DNA by pancreatic DNase.

obtained with this technique have always been in full agreement with those derived from light-scattering.

The enzymatic digestion has also been followed by sedimentation, by titration and, in the case of T5 DNA, by electron microscopy [12].

DNA prepared according to different techniques from different sources (calf thymus, chicken erythrocytes, *E. coli*) has been degraded with enzymatic preparations also derived from different sources (chicken erythrocytes, calf thymus, calf spleen, hog spleen). No differences in the kinetic results could be found according to the DNA or the enzyme source.

Results

As a term of comparison, let us consider first the results we obtained when digesting DNA with pancreatic (neutral) DNase.

The kinetics of degradation of DNA by pancreatic DNase has been shown by Thomas [13] and by Schumaker *et al.* [7] to be

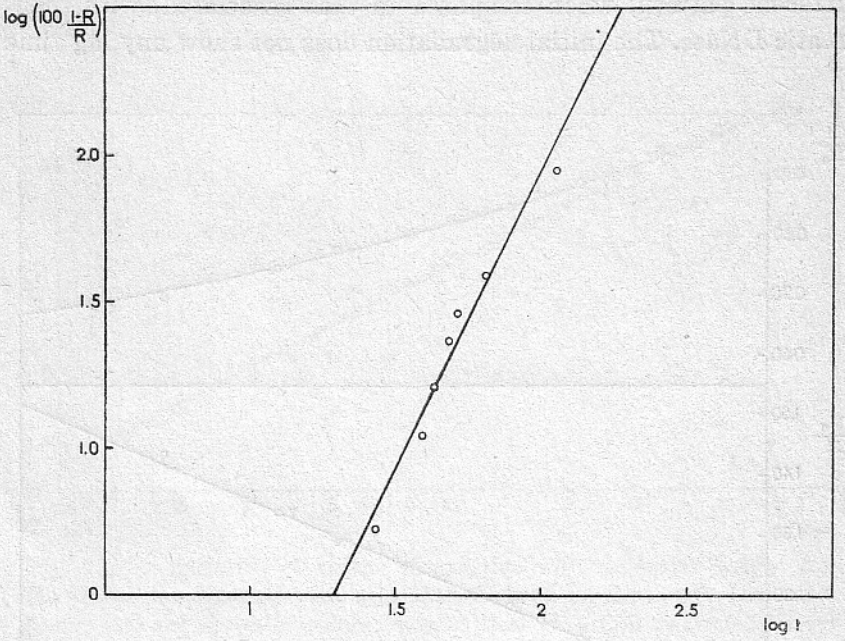

Fig. 2. — Degradation of native DNA by pancreatic DNase.

a « double hit » process. The enzyme attacks at random phosphodiester bonds on both DNA strands; a decrease in molecular weight takes place only when opposite breaks occur; this happens after a lag time during which random breaks accumulate on each parent molecule.

Accordingly, in the degradation by pancreatic DNase we find a definite lag time and when the molecular weight starts to decrease, (1/M) increases quadratically with time. In the double logarithmic plot we find $n = 2$ (figures 1 and 2).

Let us now consider the degradation of DNA by acid DNase. We will briefly describe the results of several different series of experiments carried out, respectively, on 1) native DNA 2) denatured DNA 3) sheared DNA.

1) Native DNA.

The degradation of native DNA by acid DNase shows a quite different pattern, when compared to that associated with pancreatic DNase. The initial degradation does not show any lag time

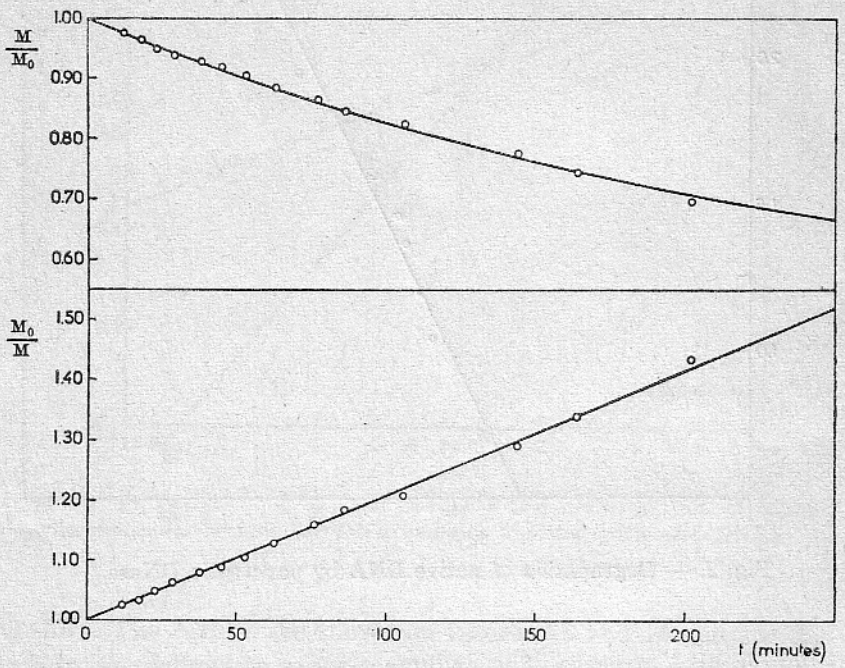

Fig. 3. — Degradation of native DNA by acid DNase.

and a « single hit » kinetics is found instead of the « double hit » kinetics obtained when digesting the same DNA samples with pancreatic DNase; $1/M_t$ is linear with time, at least down to molecular weights of $1 - 0.5 \times 10^6$ (figure 3) and in the Schumaker *et al.* [7] plot, $n = 1$ (figure 4).

However, the « single hit » degradation is not the only active process during the digestion, as is shown by the following lines of evidence:

a) Titration data obtained by Dr. Richards in our laboratory show that phosphodiester bond splitting is linear with time down to extremely low molecular weights (this finding justifies the replacement of p with t in equation (2)). For a given decrease

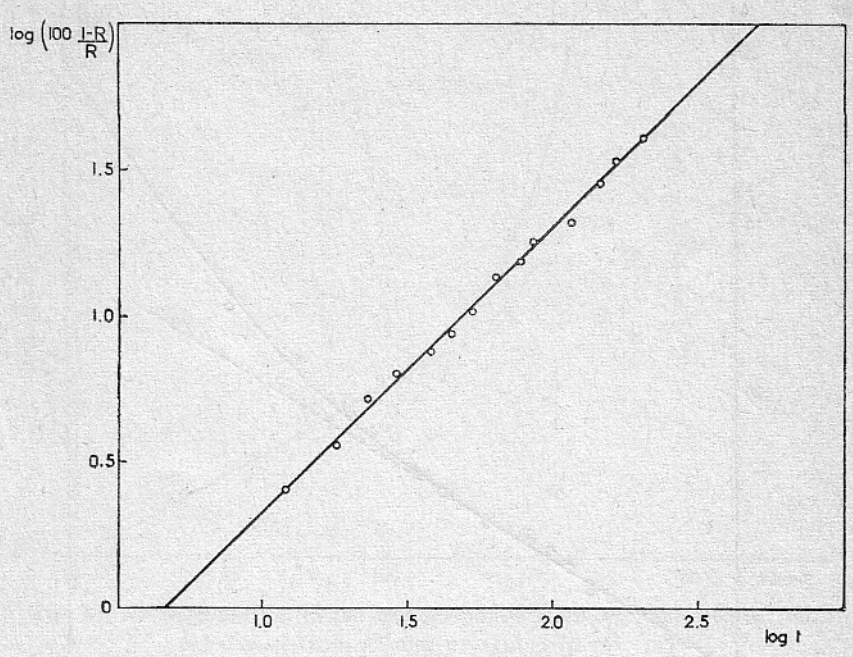

Fig. 4. — Degradation of native DNA by acid DNase.

in molecular weight (down to 0.5×10^6) the number of bonds broken is larger than expected for a pure « single hit » kinetics, but much smaller than for a « double hit » kinetics. Our data show that we need at most 10-20 breaks per parent molecule of 6.10^6 molecular weight to reduce it by a factor of two, whereas 200 breaks are needed in a « double hit » degradation [13] and about 3 in a pure « single hit » degradation in order to obtain the same result.

b) When the degradation is followed for a long enough time it becomes evident that the rate of decrease of molecular weight is accelerated after a molecular weight of $1 - 0{,}5.\ 10^6$ has been reached. Since bond splitting is linear with time, this pheno-

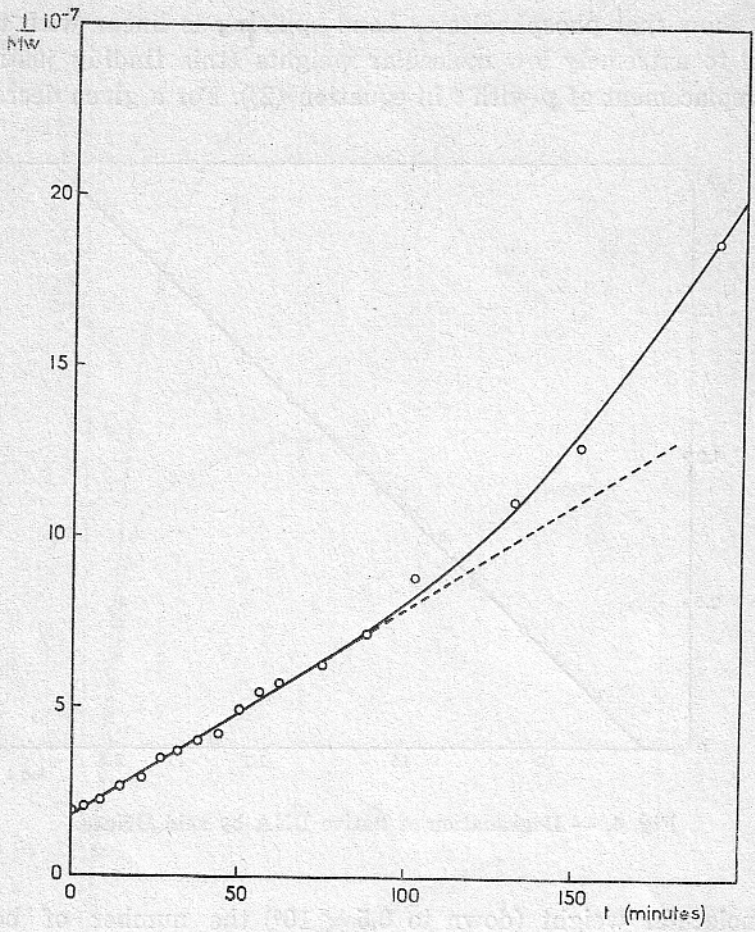

Fig. 5. — Degradation of native DNA by acid DNase.

menon is incompatible with the existence of a pure « single hit » kinetics (figure 5).

c) When DNA samples, partially digested by acid DNase, and ranging in molecular weights from 4 to $1.\ 10^6$ are thermally denatured, the percentage molecular weight decrease caused by heating is larger for samples of smaller initial molecular weight (figure 6).

d) A fourth line of evidence is provided by the degradation of heat denatured DNA.

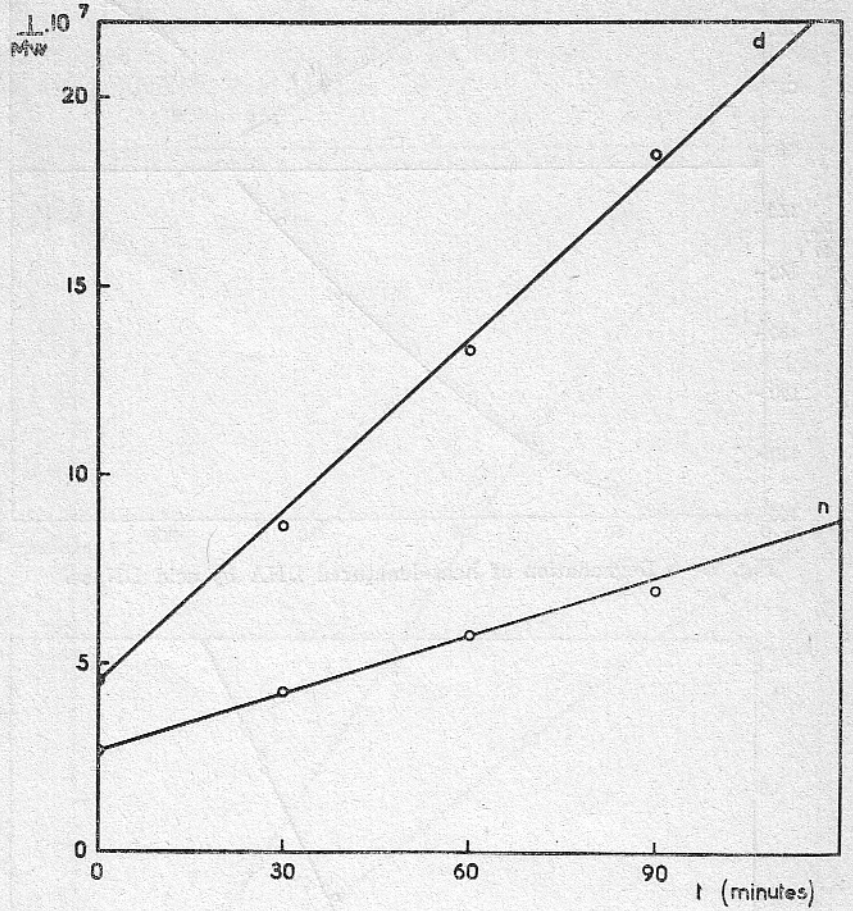

Fig. 6 — Effect of heat denaturation upon the molecular weight of DNA samples obtained at different times of digestion with acid DNase. Upper line: denatured samples; lower line: native samples.

2) Denatured DNA.

DNA samples, heated for 10 minutes at 100°C, and fast cooled, showed, upon digestion with acid DNase, a « double hit » kinetics (figures 7 and 8). This result is in agreement with similar results obtained by Cavalieri *et al.* [8]. Apparently, the sites susceptible to the « single hit » action do not exist any more in heat

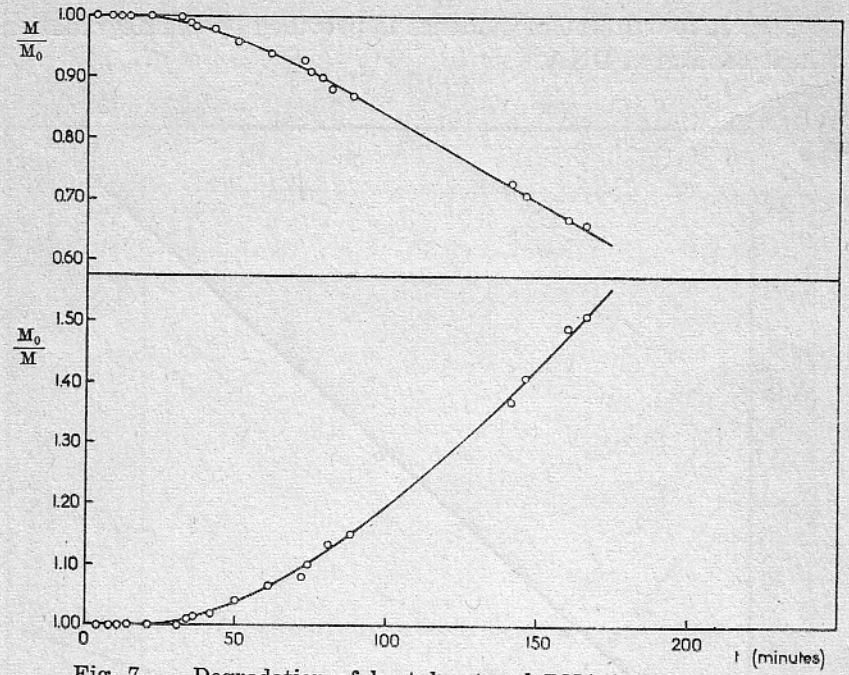

Fig. 7. — Degradation of heat-denatured DNA by acid DNase.

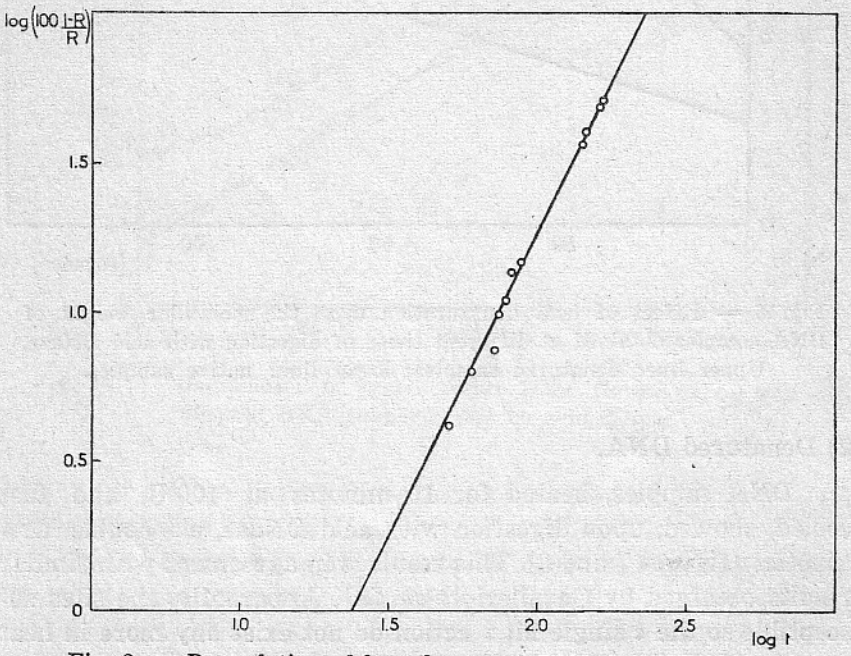

Fig. 8. — Degradation of heat-denatured DNA by acid DNase.

denatured DNA. The degradation, as measured by acid-soluble nucleotide formation, is slower, by a factor of two, for heat denatured DNA than for native DNA (figure 9).

On the other hand, DNA denatured by electrolyte dilution at 20°, once brought back to 0.15 ionic strength, behaves like native DNA towards acid DNase.

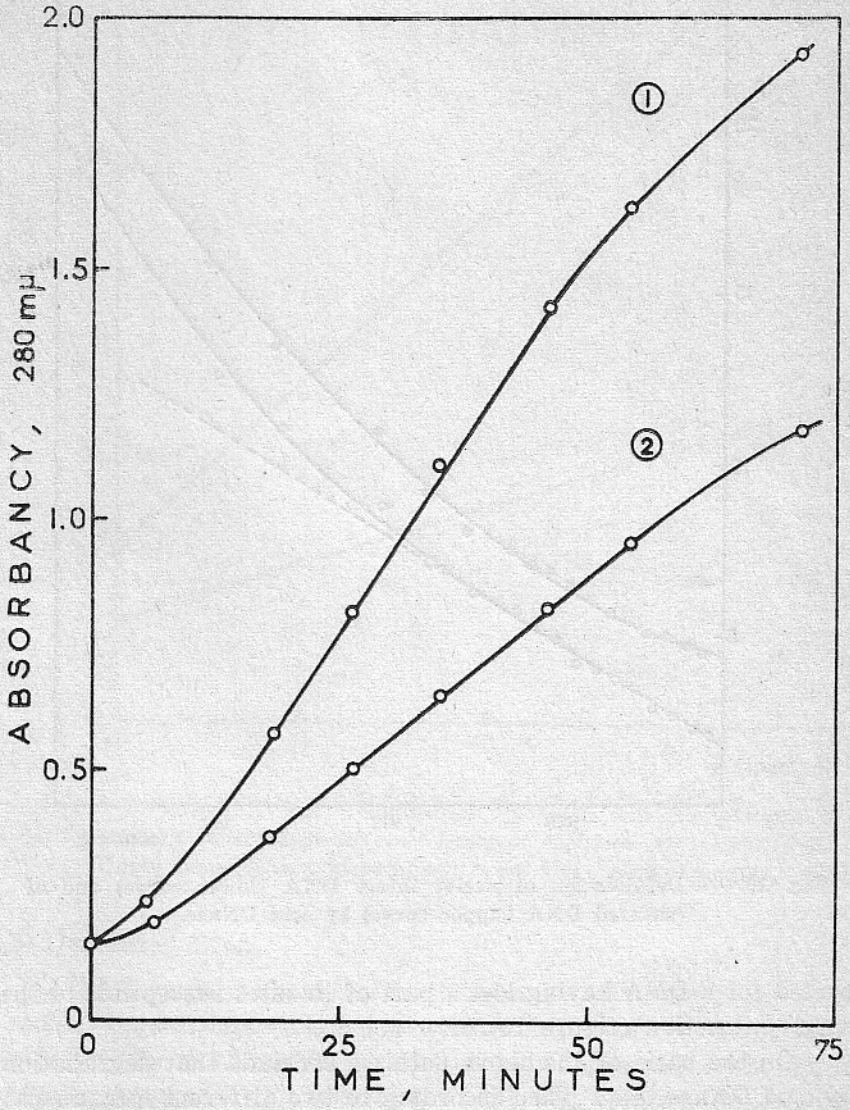

Fig. 9. — Acid-soluble nucleotide formation plotted *vs* digestion time by acid DNase. Upper curve: native DNA; lower line: heat-denatured DNA.

3) Sheared DNA.

Sheared DNA shows a behaviour intermediate between those of native and heat denatured DNA. Values of n ranging from 1.3 to 1.5 are generally found. The behaviour is therefore the one ex-

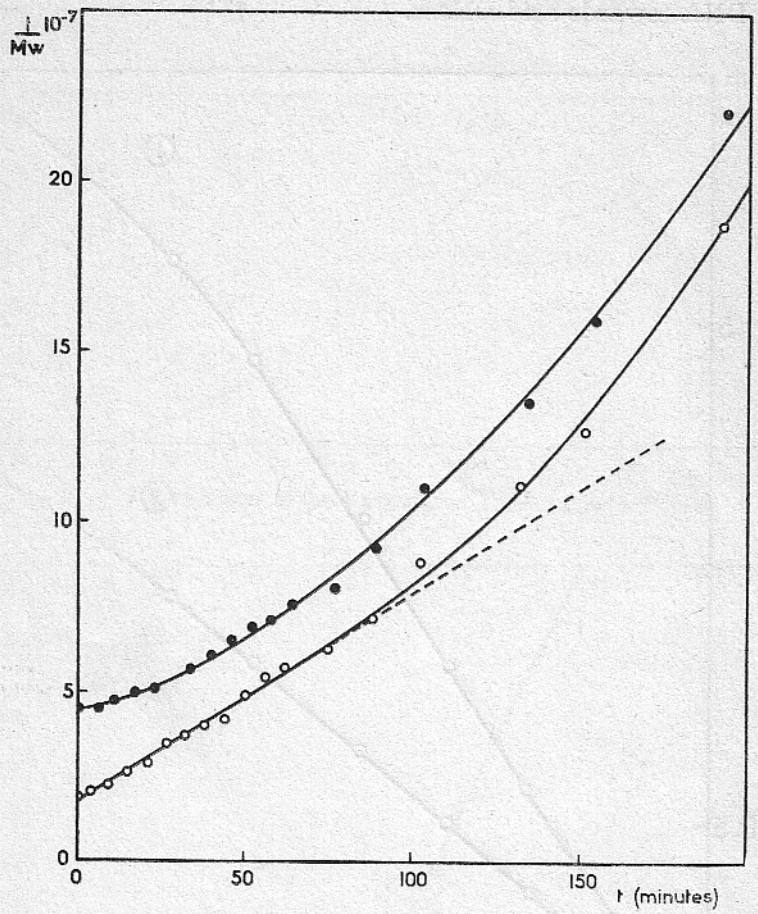

Fig. 10. — Degradation of native intact DNA (lower curve) and of sheared DNA (upper curve) by acid DNase.

pected for a DNA having lost a part of its sites susceptible to the single hit action (figure 10).

On the basis of the above data we conclude that degradation by acid DNase takes place according to two different mecanisms:

1) a « single hit » kinetics in which (if DNA molecules are made up of two uninterrupted filaments), both strands are simultaneously split at the same site;

2) a « double hit » kinetics in which random breaks occur on one strand at a time as in the case of pancreatic DNase. This second type of kinetics is not evident in the early stage of degra-

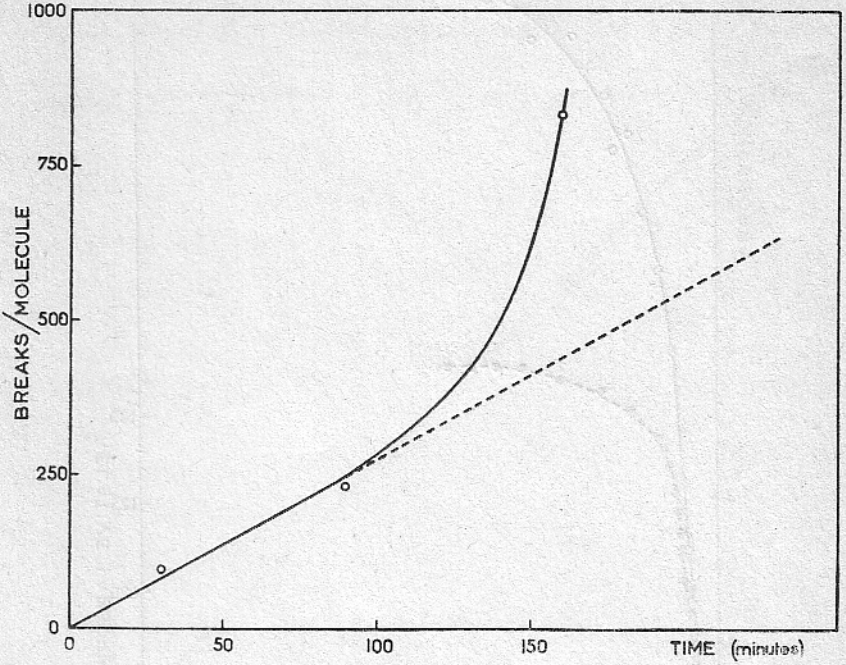

Fig. 11. — Degradation of T5 bacteriophage DNA by acid DNase (data calculated from results presented in ref. 12).

dation because of its own lag time, and only becomes apparent in a later stage.

Interestingly enough, these conclusions were confirmed last year in Baltimore by Mac Hattie, Bernardi and Thomas [12] using a completely different experimental approach. DNA from T5 bacteriophage was digested with acid DNase and the fragment size distribution at three time intervals was studied by electron microscopy. When plotted against the digestion time, the number of scissions showed, after an initial linear part, an upward curvature; therefore the plot (figure 11) was very similar to that shown

in figure 5. Electron microscopy also showed that the fragment size distribution was random, and that the fragments had the same width as native DNA.

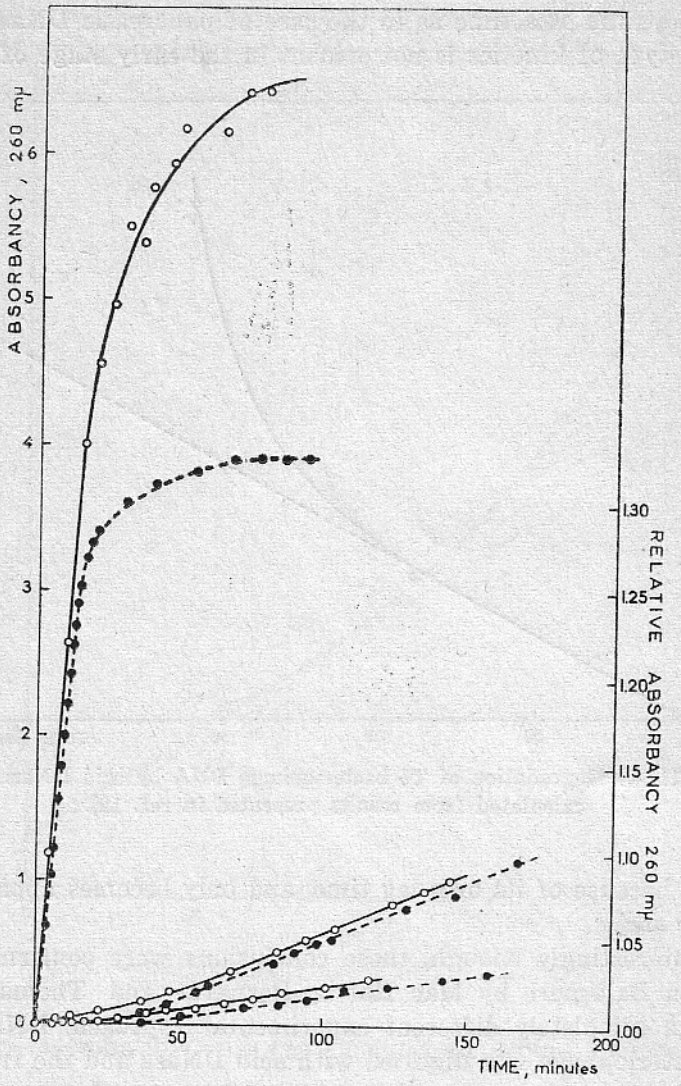

Fig. 12. — Acid-soluble nucleotide formation (circles; left-hand ordinate) and hyperchromic effect (points; right-hand ordinate) plotted *vs* digestion time of DNA by acid DNase. The three sets of curves refer to three different enzyme concentrations.

We would like to discuss briefly here only some of the points raised by the results reported above.

Except for a few « nicks » caused by the « double hit » action, the DNA fragments obtained by acid DNase digestion (at least down to a molecular weight of 0.5×10^6), have the structure of native DNA. They show no hyperchromicity, no acid-soluble oligonucleotides formation (both of these phenomena showing a lag time) (figure 12), a melting curve which does not differ from that

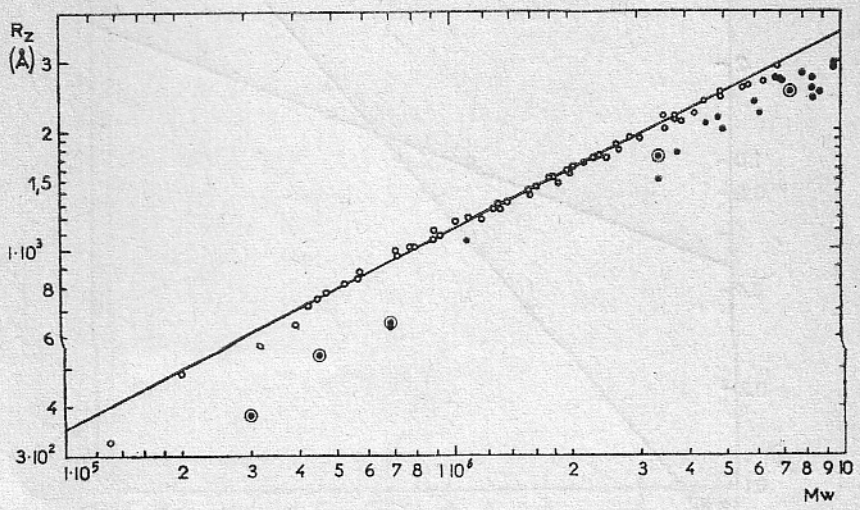

Fig. 13. — Variation of radius of gyration with molecular weight for DNA samples digested with acid DNase (circles). Data of Lett and Stacey (14) are shown as points; data of Doty *et al.* (15) as circled points.

of undigested DNA, a particle width close to 20 Å, and a molecular weight per unit length of 200 daltons/A. Therefore DNA fragmented by acid DNase should be a useful material for measurement of physical properties which are difficult to assess when using the very viscous solutions of high molecular weight DNA and, in addition, a host of physico-chemical investigations could also be profitably performed on this material.

On the other hand, the fact that by acid DNase digestion double stranded fragments of DNA of any molecular weight between 6×10^6 and 0.5×10^6 may be obtained, allowed us to esta-

blish the relationships between molecular weight (by light scattering) and viscosity, sedimentation and radius of gyration. Many of these measurements were performed by Dr. Richards in our la-

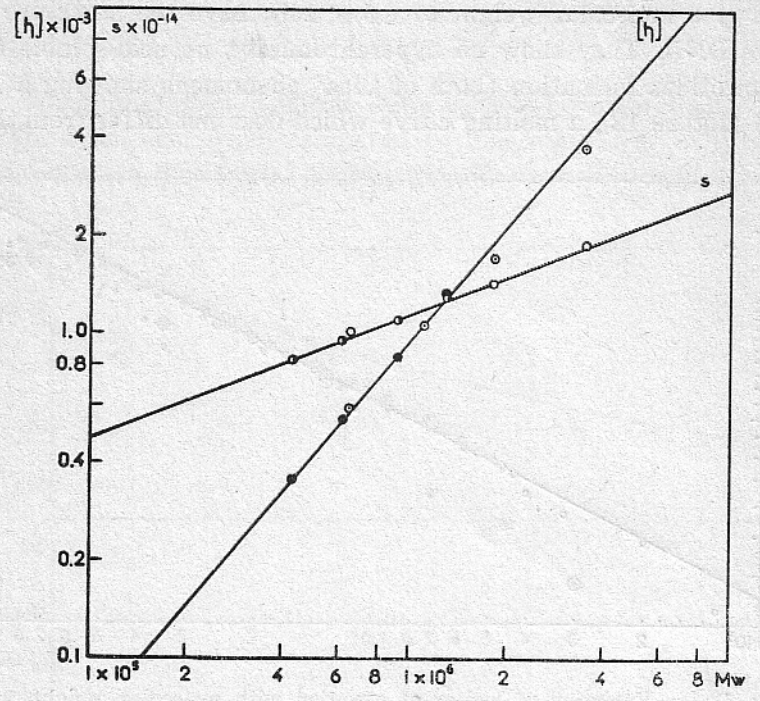

Fig. 14. — Variation of intrinsic viscosity and sedimentation constant with molecular weight for DNA samples digested with acid DNase.

boratory. The results are shown on figures 13 and 14 and summarized in the following equations:

$$S_{20,w} = 0.057 \times M^{0.382} \quad \text{(S in Svedberg Units)} \tag{4}$$

$$[\eta] = 0.835 \times 10^{-4} M^{1.175} \quad ([\eta] \text{ in CGS units}) \tag{5}$$

$$R = 0.11 \times M^{0.50} \quad \text{(R in Angstrom units)} \tag{6}$$

It may be interesting to compare these relationships with those obtained by Doty *et al.* [15] on sonicated DNA (sonication is

thought to cause double breaks on the DNA molecules):

$$S_{20,w} = 0.063 \times M^{0.37} \quad (7)$$

$$[\eta] = 1.45 \times 10^{-4} M^{1.12} \quad (8)$$

$$R = 0.27 \times M^{0.58} \quad (9)$$

As far as the mechanism of the enzymatic degradation is concerned, much work remains to be done before we can reach a complete understanding of the processes involved.

At the present moment we think that two working hypotheses deserve some consideration:

1) DNA molecules are formed by sub-units connected by « linkers », for instance, phosphoserine residues as suggested by Welsh [16] and, more recently, by Bendich and Rosenkranz [17]. Acid DNase would be able to split phosphodiester bonds involving serine, thus providing a « single hit » action; the « double hit » action would take place, instead, on the normal internucleotidic links. Since it has been shown very recently in our laboratory [18] that pure acid DNase also has a phosphodiesterase activity and is able to split even synthetic substrates like Ca [bis(*p*-nitro-phenyl-phosphate]$_2$, an activity on an hypothetical nucleotidyl-serine linkage cannot be ruled out a priori. The main trouble with this hypothesis is therefore the lack of a definite evidence for the existence of serine « linkers ».

2) Acid DNase is a dimeric protein molecule and its mechanism of action is formally analogous to that of difunctional alkylating agents. Alternatively the enzyme might have two active sites.

This second hypothesis does not involve any arbitrary assumption of non-nucleotidic material interpolated along the DNA duplex. It is based upon a chemical model, namely the degradation of DNA by alkylating agents, as studied by Lawley and Brookes [19]. We will briefly recall the main points of this work. Under a given set of experimental conditions, alkylation has been shown to occur at N_7 of guanine, monofunctional agents yielding 7-alkylguanines, and difunctional agents yielding, in addition, di(guaninyl) derivatives. This latter event has been shown to occur only when two guanines find themselves on opposite strands

as in figure 15. Alkylated DNA decomposes with loss of the alkylated guanines and subsequently the corresponding phosphodiester bonds are hydrolyzed. Therefore the net result is that alkylation by monofunctional agents gives rise to a random degradation of DNA of the type obtainable with pancreatic DNase. Alkylation

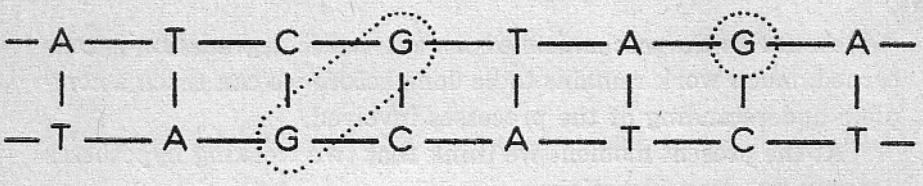

Fig. 15. — Diagrammatic representation of the possible scissions of a DNA chain operated by a difunctional alkylating agent (modified from ref. 19).

by difunctional agents causes a degradation which involves both « single hit » and « double hit » kinetics, simulating therefore the action of acid DNase. (It should be pointed out however that the above schemes are rather idealized pictures because, for example, the « double hit » kinetics expected with monofunctional agents was not found experimentally [20].

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Discussion following Dr. BERNARDI's paper.

Dr. Dulbecco: I want to recall one finding we observed recently while working with the polyoma virus. We studied the kinetics of the change of the ring into the rod by DNAase. One cut produces a rod out of the ring. To our great surprise we found that it was a first order kinetic. This is DNAase I at pH 8.1 in sodium chloride 0.1 M. This shows that there are special factors involvd in determining the kinetics. The striking fact in this particular system is that, although the kinetics of the opening of the ring is single hit, the kinetics of further breaks is 2 hit. It is really the configuration of the molecule which, in this case, determines the kinetics of DNAase digestion as single hit rather than 2 hit. We have, furthermore, evidence that, while this first break is produced, there are no single chain braks. This was obtained by sedimenting the material at pH 12.5. This result is rather surprising and I do not really know how to explain it.

Dr. Bernardi: I do not know if I really caught your point. You have a ring DNA, you treat this ring DNA with pancreatic DNAase and you get a single hit action. Which way was the single hit kinetics determined?

Dr. Dulbecco: By determining the proportion of the ring surviving as a function of time, in the regular kinetic way.

Dr. Bernardi: I do not know if it is enough to follow the two peaks in the ultracentrifuge as a function of digestion. In order to study this kinetics you would really need to work between R values of 1 and 0.5. R = 0.5 corresponds to one hit per molecule, roughly speaking. So I really do not know if you can do it by sedimentation.

Dr. Dulbecco: You have every reason to believe my data.

Dr. Bernardi: I believe your data. It is the interpretation which I doubt.

Dr. Dulbecco: I think the data are all right and you can see them published, but the fact is that if you plot the log of the surviving proportion of what remains as a ring vrs. the time of action of DNAase, the curve is not limited to 0.5, because one can follow the conversion of the rings to rods until the band of the rings completely disappears. As it receives one hit this material passes into the other band and therefore the measurements are very accurate. But the material which has been transformed into S, or the native rods, show the classical kinetics. So, it is the same DNA, with the same molecular weight, the only difference is in the configuration; in the case of the ring, one hit breaks both chains; in the case of the rod we have the usual kinetics.

Dr. Bernardi: I think these data are very puzzling. The ring shape is by itself not a factor, because Sinsheimer has shown that the ØX DNA, which is ring shaped and single stranded, behaves as expected using pancreatic DNAase.

Dr. Thomas: I would like to continue this argument. If I understand your suggestion correctly, Dr. Dulbecco, the picture in your mind is that the pancreatic DNAase is breaking the phosphodiester linkages. Therefore, if you were to say that it is single hit, this supposes that there is a single chain region in the polyoma circle, and breaks are directed preferentially towards this region where it is effective immediately. I believe you brought up this question because you are suggesting to Dr. Bernardi that there are single chain regions in the DNA molecule which may, in fact, be nicked, the nick resulting in complete cleavage. Am I a mind reader?

Dr. Dulbecco: May I just answer the facts. Actually, there is very good evidence that there are no single strand fragments in this ring; it is entirely double strand.

Dr. Thomas: But what is the confidence for that statement; are there only 2 or 3 open splices?

Dr. Dulbecco: The rate at which these rings are broken is of the same order of which single strands are broken after the first break is made. If there were a single strand area it would be a very large one and we can exclude this in a most catagoric way. The only thing we can not exclude would be something which is less that 1% of the total lengths, but then the kinetics, the rate, would be much smaller.

Dr. Thomas: May I ask one more question? In one of your slides, Dr. Bernardi, you had the change of molecular weight of the duplex and of the single chain form, and the slopes of these two lines were not parallel, but widely different as a function of time. This presumably would allow you to calculate the number of "nicks per chop" and I wanted to know how that agreed with the titration data.

Dr. Bernardi: From the data shown in Fig. 6, one would estimate less "nicks per chop" than from titration.