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Studies on Acid Deoxyribonuclease. I. Kinetics of the Initial Degradation of Deoxyribonucleic Acid by Acid Deoxyribonuclease*

The mechanism of degradation of deoxyribonucleic acid from different sources (chicken erythrocytes, calf thymus, and *Escherichia coli*) by acid deoxyribonuclease preparations from chicken erythrocytes, calf thymus, calf spleen, and hog spleen has been investigated by following the molecular-weight decrease by light scattering. Native deoxyribonucleic acid is degraded according to both "single-hit" and "double-hit" kinetics. The "single-hit" degradation appears to take place through the simultaneous splitting of both strands at the same level. The "double-hit" degradation, which resembles the degradation by pancreatic deoxyribonuclease, becomes evident only after a time lag. Heat-denatured deoxyribonucleic acid is attacked essentially according to the "double-hit" mechanism.

Work carried out in this laboratory in 1960 (Bernardi *et al.*, 1960, 1961; Sadron, 1961) showed that DNA from calf thymus ($M_w = 6.5 \times 10^6$; $s_{20,w} = 20.0$ S) or chicken erythrocytes ($M_w = 8.0 \times 10^6$; $s_{20,w} = 27.5$ S) can be degraded with crude enzyme preparations from chicken erythrocytes.[1] The enzymic activity showed a *p*H optimum close to 5.5 and was strongly inhibited by $Mg.^{2+}$ No appreciable amounts of dialyzable nucleotides were formed during the digestion. The degraded DNA from both sources so formed appeared to consist of particles having a molecular weight of $M_w = (5.5 \pm 0.5) \times 10^5$. The light-scattering data were compatible with a solution of stiff rods having a weight per unit length $M/L = 200 \pm 20$ daltons/A. The sedimentation coefficient $s_{20,w}$ was equal to 5.8 S, and the distribution function of the sedimentation coefficients was very narrow.

In further work (Bernardi and Sadron, 1961), the crude enzyme preparation from chicken erythrocytes was purified 200 times according to a procedure reported in the present paper, and used at concentrations about

* This work has been aided by a grant (UR-E9-[10,60]-80) from the U. S. Department of Agriculture.

[1] Abbreviations used in this work: M_w, weight-average molecular weight; M_n, number-average molecular weight.

100 times higher than those of the crude preparation used before.

Degradation of chicken erythrocyte DNA by the more active enzyme showed (a) a first phase exhibiting all the features previously found in the degradation of DNA by the crude enzymic preparation; and (b) a second phase in which the molecular weight of DNA continued to drop and there occurred a liberation of acid-soluble nucleotides and an increase in the ultraviolet absorption at 260 mμ. This phase had not been detected when using the crude enzymic preparation, probably because the enzyme was inactivated before the later stage was reached. The finding of this second phase strongly suggested that the chicken erythrocyte enzyme was a DNAase. This interpretation was supported by the fact that an acid DNAase preparation from calf thymus (Bernardi, 1961) degraded DNA in much the same way as the purified chicken erythrocytes preparation.

A kinetic study of the enzymic degradation suggested that this was proceeding according to two different yet superimposed mechanisms: (a) a "single-hit"[2] degradation which is operative from the very start of the digestion and which implies, if DNA molecules are continuous double-stranded structures, that double scissions on opposite strands occur simultaneously; (b) a "double-hit" degradation, of the type already known for pancreatic DNAase (Schumaker *et al.*, 1956; Thomas, 1956), which becomes effective in splitting DNA molecules only after a time lag; during this time single scissions occur on one or the other strand but they do not cause any decrease in molecular weight.

A presentation of a first part of this kinetic study is given here. A brief summary of this work has been recently presented elsewhere (Bernardi and Sadron, 1964).

Materials

Chicken Erythrocytes DNA.—The following preparation procedure, already used in previous work (Bernardi *et al.*, 1960, 1961; Bernardi and Sadron, 1961) and partially derived from that of Zubay and Doty (1959), was used. All operations were done at 1–4° and all centrifugations were carried out for 10 minutes at 8000 $\times g$ in a Lourdes centrifuge. Chicken blood (800 ml) was collected in 600 ml of ice-cold standard solvent of Zubay and Doty (1959), 0.075 M NaCl + 0.024 M Versene, pH = 8.0, and centrifuged. The sediment was dispersed in 600 ml of standard solvent, added to 600 ml of 0.2% saponin (Merck) in standard solvent, and centrifuged. This was repeated five more times without saponin in the solvent. At the end of this treatment the supernatant was clear and very pale yellow. The sediment was then dispersed in 800 ml of standard solvent, homogenized in a Waring Blendor in 200-ml portions (to each of which 1 ml of isooctanol was added) for 1 minute at high speed and 5 minutes at low speed, and centrifuged. This was repeated five to seven times with the difference that 0.5 ml isooctanol was added to each portion and homogenization was carried out for 30 seconds at high speed and 2.5 minutes at low speed. The sediment was then dispersed by hand-shaking into 800 ml of distilled water and centrifuged. The sediment was homogenized in 1 liter of distilled water and then mechanically stirred for 3 hours; during this time the volume was increased to 3 liters with distilled water. The opalescent, colorless nucleoprotein gel was then saturated with NaCl by addition of crystals, under very vigorous stirring which was continued for 12 hours. After at least 24 hours (sometimes after many days or weeks) the suspension was clarified by centrifugation at 30,000 rpm in a Spinco Model L preparative ultracentrifuge. The clear, viscous supernatant was then mechanically shaken overnight with 0.5 volume of chloroform–isoamyl alcohol, v/v 5:1 (Sevag, 1934), and centrifuged for 1 hour at 20,000 rpm. The aqueous phase was treated four to five more times as before and then stored. This preparation procedure will be indicated as *method A*. More recently, following Cavalieri *et al.* (1961), several further chloroform–isoamyl alcohol treatments were done using acetate buffer containing 0.01 M Versene, μ = 0.15, pH 5.0 (standard acetate buffer) as the DNA solvent. By this procedure, DNA samples were obtained which showed lower molecular weights ($3.5-4.5 \times 10^6$) than those prepared according to method A.

Calf Thymus DNA.—Essentially the same procedure as described was used starting with the blending step. Other DNA preparations from the aforementioned sources were obtained (1) by treating the nucleoprotein gel or the homogenate in standard solvent according to Kay *et al.* (1952), which will be called *method B;* (2) by dissolving the nucleoprotein into 1 M NaCl instead of water, then using the further steps of method A (*method C*); (3) by treating DNA solutions in 0.15 M NaCl prepared by method B with 1 volume of freshly distilled phenol (90% solution in water) (*method D*).

ϕ X 174 DNA was a gift of Dr. R. Wahl, Institut Pasteur, Paris.

The preparations of *E. coli DNA* used in this work were obtained from Dr. D. Luzzati in this laboratory, according to a procedure described elsewhere (Luzzati *et al.*, 1962).

Chicken Erythrocytes Acid DNAase—The supernatants obtained during the DNA preparation described a (0.15 M NaCl was used instead of the standard solvent of Zubay and Doty, 1959) were checked for enzymic activity by following the decrease in DNA molecular weight by light scattering. The supernatant obtained by centrifuging the first homogenate was the richest source of enzyme. The supernatants obtained from the following homogenates contained decreasing amounts of enzyme, the fourth one showing no more activity. When using calf thymus a longer series of washings (up to eight) was necessary to remove DNAase activity from the nucleoprotein.

The supernatant from the first homogenate was clarified by centrifugation (2 hours at 30,000 rpm), dialyzed against distilled water, clarified again, and freeze-dried. The fluff was dissolved in acetate buffer, μ = 0.15, pH 5.4, and treated with $(NH_4)_2SO_4$. The precipitate collected between 0.5 and 0.8 saturation was dissolved in water, dialyzed against distilled water, and freeze-dried. On the basis of nitrogen content and the rate of DNA depolymerization, this product was about 200 times more active than the crude enzyme previously used (Bernardi *et al.*, 1960, 1961), yet it was less active than the crude hog spleen DNAase (*vide infra*).

Calf thymus acid DNAase was prepared according to the procedure of Bernardi (1961). Unless otherwise indicated, the preparation was used before any chromatographic purification. Treatment of this product with diisopropylfluorophosphate was carried out according to Naughton *et al.* (1960). *Calf spleen acid DNAase* was a commercial product (Worthington Biochemical Corp., Freehold, N.J.; lot 6010). *Hog spleen acid DNAase* was prepared as described by

[2] By "single-hit" kinetics we mean the degradation kinetics shown by a linear, single-stranded polymer when it is split at random; the "double-hit" kinetics is that shown by a linear double-stranded polymer when bonds on one or another of its two chains are split at random.

Bernardi *et al.*, (1963), and Bernardi and Griffé (1964). Both crude and highly purified enzyme preparations were used.

Papain was twice-crystallized Worthington papain; 0.2 ml of the suspension was dissolved in 3 ml of distilled water; 0.02 or 0.05 ml of this solution was added to 30 ml DNA solutions (50–100 μg/ml) in acetate buffer, *p*H 5.4, $\mu = 0.15$. The final enzyme concentrations were about 1 or 2.5 μg/ml, respectively.

Chymotrypsin was three-times-crystallized Worthington α-chymotrypsin. A 0.1% aqueous solution (0.03 ml) was added to 30 ml DNA solutions (50–100 μg/ml) in phosphate buffer, $\mu = 0.1$, *p*H 6.8. The final enzyme concentration was therefore about 1 μg/ml.

Pancreatic DNAase was once-crystallized Worthington DNAase (lot 932). The enzyme was dissolved in acetate buffer, $\mu = 0.2$, *p*H 5.5, containing 0.02 M $MgCl_2$, at a concentration of about 0.5 mg/ml. Aliquots of 0.05–0.01 ml of freshly prepared solution were added to 30 ml of DNA (90–140 μg/ml) in the same solvent. The final enzyme concentration was of the order of 0.1 μg/ml.

Methods

Nitrogen was determined by the micro-Kjeldahl procedure, and phosphorus by the method of Martin and Doty (1949). Ultraviolet absorption, sedimentation and light-scattering measurements were performed as already described (Bernardi *et al.*, 1961).

In the more recent light-scattering work, a new clarification technique (Bernardi, 1964), also successfully used in a low-angle light-scattering investigation of DNA solutions (Froelich *et al.*, 1963), was employed. Couette viscometry was performed, as in previous work (Bernardi *et al.*, 1961), using an instrument built by Dr. G. Scheibling of this laboratory.

Enzymatic digestion was performed, at least in duplicate, directly in the light-scattering cell at room temperature (20–22°). Unless otherwise stated, 0.01 or 0.02 ml of crude or commercial DNAase solution (1 mg/ml) was added to 30 ml of DNA (50–150 μg/ml), the final enzyme concentration being about 0.3 μg/ml. Much lower concentrations were used for the pure enzyme preparations; bovine serum albumin was added to these preparations in order to stabilize them. Routinely, readings were taken at only six angles between 30° and 90°. When plotting Kc/R versus $\sin^2(\theta/2)$, the points belonging to the four lower angles (often all the points) were on a straight line and extrapolation to zero angle was performed linearly to obtain $1/M_w$.

Acid-soluble oligonucleotides were determined as described in the following paper (Bernardi and Griffé, 1964). For heat denaturation of intact and partially digested DNA, in a typical experiment, a large DNA sample (for instance 500 ml of a 0.5 mg/ml solution) was digested in standard acetate buffer at 37° and aliquots were removed at different times. Digestion was stopped by cooling these in an ice-bath and shaking them with chloroform-isoamyl alcohol. An intact sample of the starting DNA solution was treated in the same way. All samples were dialyzed against Na acetate, $\mu = 0.2$, *p*H 8.0, and diluted to give a DNA concentration of 10 μg/ml. For heat denaturation the samples were put in a boiling-water bath for different times, then rapidly cooled in an ice bath, shaken with chloroform-isoamyl alcohol, dialyzed back to standard acetate buffer, shaken once more with chloroform-isoamyl alcohol, and used for the light-scattering experiments. This rather complicated procedure was the only one capable of giving solutions which were perfectly stable for at least 24 hours at room temperature, as judged from the light-scattering envelopes. The same solutions in neutral or slightly alkaline buffers invariably showed a rapid aggregation. The same solutions were used for the determinations of optical density, phosphorus, and acid-soluble-oligonucleotide content.

Treatment of Kinetic Data

The statistical theory of random degradation of linear polymers of any initial molecular-weight distribution predicts that $1/M_n$ is a linear function of time t when the number of chain scissions is proportional to (Mark and Tobolsky, 1950). It has been shown by Charlesby (1954) that any initial molecular-weight distribution yields the most probable one after at small extent of degradation (three to eight chain fractures per molecule). For the most probable molecular-weight distribution, $M_w = 2M_n$, and $1/M_w$ will also be a linear function of t.

Schumaker *et al.* (1956) 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:

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

Here, $R = M_t/M_0$ (M_t and M_0 are 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 is obtained by replacing, as proposed by Cavalieri and Rosenberg (1961), $(1 - R)$ by $(1 - R)/R$ in equation (1). Then, assuming that $p = kt$ (k being a proportionality constant), equation (1) becomes:

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

The advantage of this representation is 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:

$$\frac{1}{M_t} - \frac{1}{M_0} = kt \qquad (3)$$

that is, the reciprocal of molecular weight is a linear function of t, as indicated.

Two more treatments of the kinetic data obtained during the degradation of DNA have been proposed by Thomas (1956) and Applequist (1961), respectively. Both methods require data obtained during a rather extensive degradation, down to values of R of the order of 0.1 or lower; in the former method titration data are also required. In the present work the treatment of Schumaker *et al.* (1956) was preferred because it utilizes data obtained during the very early stage of the enzymic degradation, a period where the assumption $p = kt$ is justified, and interference by other enzymes or inhibition phenomena are less likely to occur. These advantages were, of course, especially important when titration data and pure enzyme preparations were not yet available.

Results

Degradation of Native DNA with Pancreatic DNAase —These experiments were performed mainly in order to check our technique and to compare our results with those of Thomas (1956) and Schumaker *et al.* (1956). The data obtained (Figs. 1 and 2; Table I) are in agreement with those already published by the above

TABLE I
DEGRADATION OF NATIVE DNA BY PANCREATIC DNAASE[a]

DNA Sample	Source	Preparation Method	DNA Concn (μg/ml)	M_w 10^{-6}	R_z (A)	n
(1) B 11/2	E	A	96.5	7.5	1540	2.0
(2) B 13/5	E	B	140	6.6	2470	1.7

[a] See footnotes to Table II.

TABLE II
DEGRADATION OF NATIVE DNA BY ACID DNAASE PREPARATIONS[a]

DNA Sample	Source[b]	Preparation Method[c]	DNA Concn (μg/ml)	M_w 10^{-6}	R_z (A)	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	ET3/3	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	113	4.6	2400	ET2	1.0

[a] All degradations were performed at room temperature (20–22°) in the light-scattering cell, using standard acetate buffer as the solvent, except for samples 7 (acetate buffer, pH 5.4), 11 (0.005 M 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 Methods. [d] ET3, calf thymus DNAase; ERW, calf spleen DNAase (Worthington); ERP/3, hog spleen pure enzyme; ET3/3, purified calf thymus DNAase of specific activity 50 (see Bernardi and Griffé, 1964); (t)HL1, calf thymus DNAase; 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 (Schumaker *et al.*, 1956).

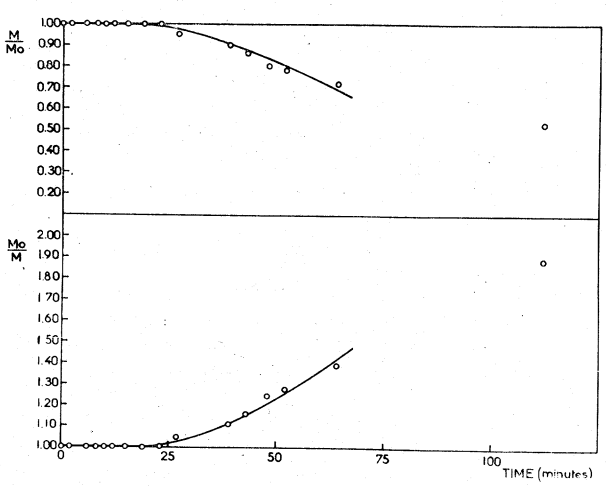

FIG. 1.—Digestion of DNA sample B 11/2 with pancreatic DNAase (see Table I).

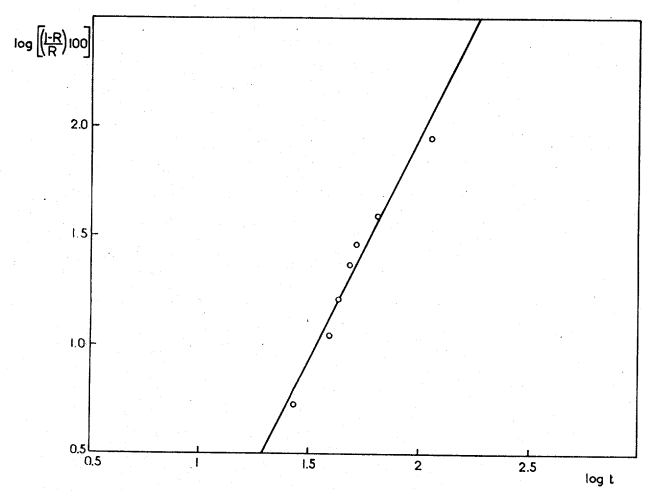

FIG. 2.—Digestion of DNA sample B 11/2 with pancreatic DNAase. Data of Fig. 1 are plotted according to Schumaker *et al.* (1956). See also Table I.

mentioned authors. The molecular weight decrease showed the typical time lag (Fig. 1), and the apparent number of strands was found to be equal to 1.7–2.0 (Fig. 2), as expected. Aggregation phenomena invariably occurred at an early stage, a result in agreement with a similar finding by Thomas (1956).

Degradation of Native DNA with Acid DNAase Preparations.—Many different DNA preparations from calf thymus, chicken erythrocytes, and *E. coli*, obtained using the different preparative procedures described in the experimental section, were digested with acid-DNAase preparations from calf thymus, calf spleen, hog spleen, and chicken erythrocytes.

Some typical results are shown in Table II. They indicate that in all cases the apparent number of strands was found to be equal to 1 ∓ 0.1, in spite of differences in the source, preparation, and concentration of both DNA and enzyme samples used. No differences were found that could be related to the molecular weight and radius of gyration of DNA. Digestions performed with neutral low-molarity buffers as the solvents (Shack, 1957) instead of standard acetate buffer, with enzyme preparations treated with diisopropylfluorophosphate, or with preparations having very different degrees of purity also gave the same result. Figures 3 and 4 show the kinetic data obtained in a typical case.

In all cases, enzymic degradations could be followed

TABLE III
HEAT DENATURATION OF INTACT AND PARTIALLY DIGESTED DNA[a]

DNA Sample	$M_w\ 10^{-6}$		$\frac{M_{den}}{M_{nat}}$	$\epsilon(P)^b$		$\frac{\epsilon(P)_{den}}{\epsilon(P)_{nat}}$
	Native	Denatured		Native	Denatured	
B6z/0	3.80	2.20	0.58	6440	6950	1.08
B6z/1	2.35	1.16	0.49	6500	7080	1.09
B6z/2	1.75	0.75	0.43	6450	7280	1.13
B6z/3	1.45	0.54	0.37	6500	7450	1.15

[a] The DNA sample used in this experiment was from chicken erythrocytes. The DNAase preparation used in this digestion was from calf thymus. Heat denaturation was performed as described in the experimental part. Acid-soluble oligonucleotides were not detected in any of the samples, both native and denatured. [b] See Chargaff and Zamenhof (1948).

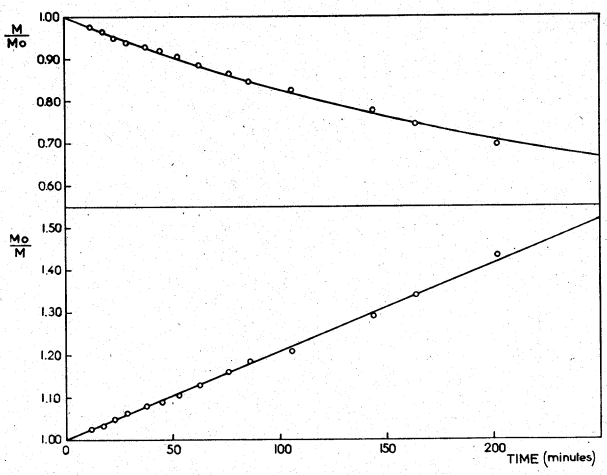

FIG. 3.—Digestion of DNA sample B6zN/1 with acid DNAase (see Table II).

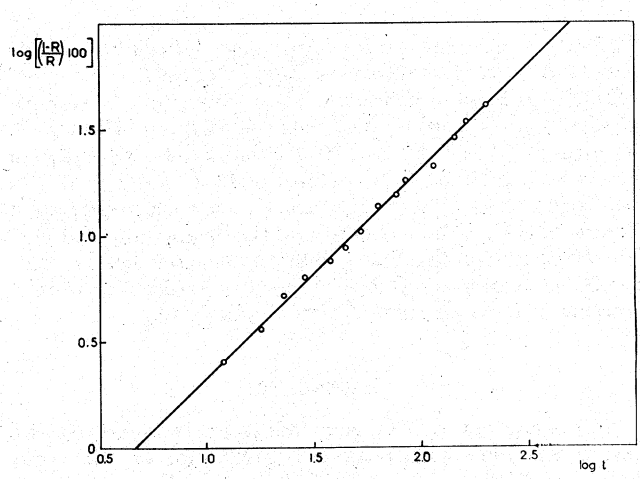

FIG. 4.—Digestion of DNA sample B6zN/1 with acid DNAase. Data of Fig. 3 are plotted according to Schumaker *et al.* (1956). See also Table II.

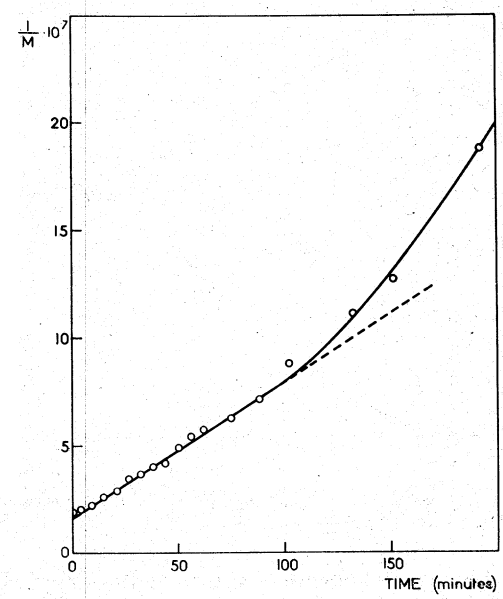

FIG. 5.—Digestion of DNA sample B 13/46 with acid DNAase (see Table II).

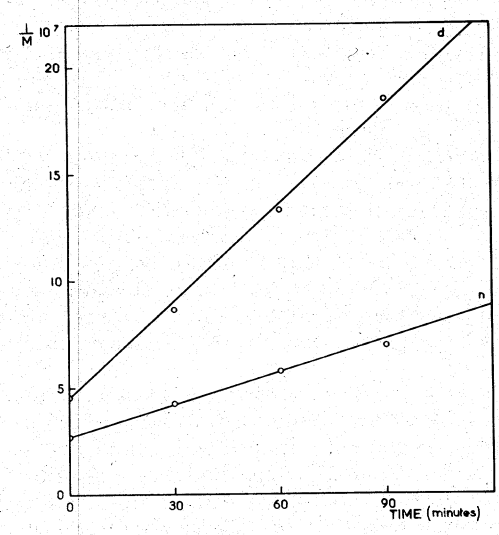

FIG. 6.—Heat denaturation of intact and partially digested DNA (see Table III). A large DNA sample was digested with acid DNAase, aliquots were removed at different times, and digestion was stopped. The reciprocal molecular weights of these samples are given by the lower set of points. Samples were then heat denatured; their reciprocal molecular weights after thermal treatment are given by the upper set of points. See text for further details.

down to extremely low molecular weights; we did not find aggregation phenomena such as those obtained when using pancreatic DNAase nor enzyme-inhibition effects, of the type found by Cavalieri and Rosenberg (1961) in their digestions of DNA, with very crude acid-DNAase preparations from mouse sarcoma 180.

When the molecular-weight decrease was studied for a long enough time, it was observed that the initial linear increase of $1/M$ with digestion time was followed by a higher-order relationship (Fig. 5); the change took place when a molecular weight of about 1×10^6 was reached. This phenomenon was found without exception in all degradations investigated so far.

Degradation of Single-stranded DNA Samples with Acid-DNAase Preparations.—Samples of ϕ X 174 DNA were also degraded by calf thymus–acid DNAase, as indicated by acid-soluble oligonucleotide liberation. The similar phage fd DNA was also digested by pure hog spleen–acid DNAase.[3] No kinetic data are avail-

[3] H. E. Schaller, personal communication.

TABLE IV
DEGRADATION OF DENATURED DNA BY ACID-DNAASE PREPARATIONS[a]

DNA Sample	Source	Preparation Method	DNA Concn (μg/ml)	M_w 10^{-6}	R_z	Enzyme	n
(1) B6z-td/1	E	A	121	2.4	590	ET3	2.0
(2) B6z-td/2	E	A	121	2.4	590	ET3	2.0
(3) B3/64	T	A	41.5	2.3	740	ET3	1.6
(4) DL2/27	C		77	2.5	525	ET3	1.4
(5) DL2/28	C		77	2.5	525	ET3	1.8

[a] All degradations were performed in standard acetate buffer. For explanations see footnotes to Table II.

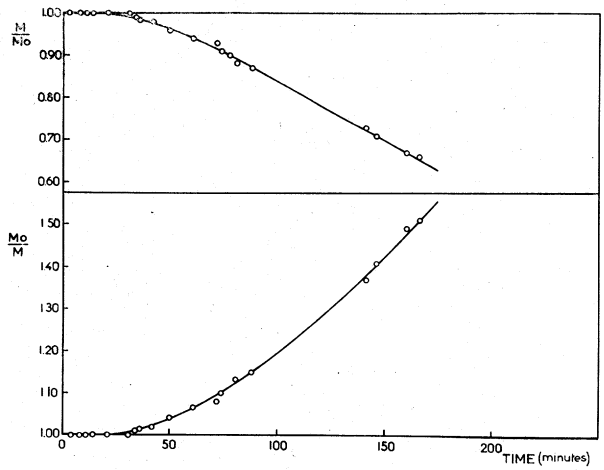

FIG. 7.—Digestion of heat-denatured DNA sample B6-td/1 with acid DNAase (see Table IV).

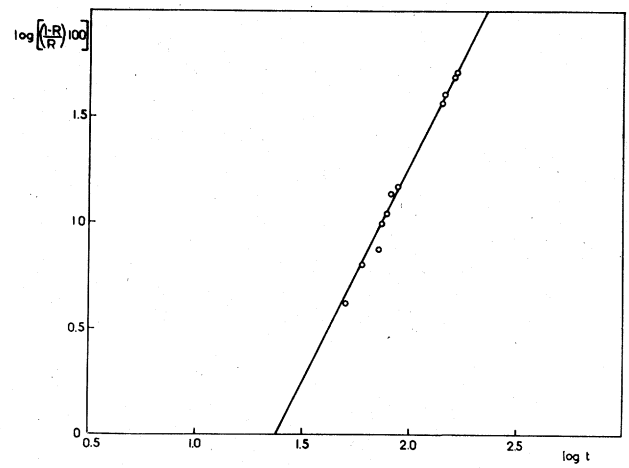

FIG. 8.—Digestion of heat-denatured DNA sample with acid DNAase. Data of Fig. 7 are plotted according to Schumaker *et al.* (1956).

able in these cases because of the scarcity of these DNA preparations.

Heat-Denaturation of Partially Digested DNA.—When DNA samples, partially digested by acid DNAase and ranging in molecular weight from 4 to 1 $\times$ 10^6, were thermally denatured, the percentage molecular-weight decrease caused by heating was found to be larger for the samples of smaller initial molecular weight (Fig. 6; Table III).

Degradation of Heat-denatured DNA by Acid DNAase.—DNA samples, heated for 10 minutes at 100° and fast-cooled showed, upon digestion with acid DNAase, a double-hit kinetic (Figs. 7 and 8; Table IV). The values of n, however, were found to be generally in the range 1.4–1.7. The degradation, as measured by acid-soluble-oligonucleotide liberation, is slower for heat-denatured than for native DNA (Bernardi and Griffé, 1964).

Degradation with Proteolytic Enzymes.—These experiments, as well as those with diisopropylfluorophosphate-treated enzyme, mentioned above, were performed, before acid DNAase had been obtained in a pure form, to rule out the possibility that a proteolytic enzyme was responsible for the single-hit degradation by breaking hypothetical protein "links." They are reported here because they are at variance with results published by Cavalieri *et al.* (1961). Four different samples derived from preparation B6 (from chicken erythrocytes) were used in these experiments: (a) DNA B6 A was obtained by submitting DNA in the saturated NaCl stage (see Materials) to only one chloroform–isoamyl alcohol treatment; (b) DNA B6 B received four more chloroform–isoamyl alcohol treatments in saturated NaCl solution; (c) DNA B6 C was obtained from B6 A by shaking it five more times with chloroform–isoamyl alcohol in Na acetate, μ = 0.15, *p*H 8.6; (d) DNA B6 D was a B6 C sample reaggregated by allowing it to stand 20 days in acetate buffer, *p*H 5.4, μ = 0.15, at room temperature (the solution was saturated with chloroform–isoamyl alcohol).

The results of digestion of the above samples with papain and chymotrypsin are shown in Figure 9. Samples B6 A, B6 B, and B6 D displayed a decrease in molecular weight to final values in the range 4–6 $\times$ 10^6, the lower values being obtained with chymotrypsin. Sample B6 C, which showed at the beginning of digestion the same molecular weight as sample B6 D after papain treatment, did not show any molecular-weight decrease upon incubation with the enzyme.

DISCUSSION

No special comments seem necessary concerning the results obtained with pancreatic DNAase or with proteolytic enzymes. As indicated earlier, all native DNA samples investigated in our laboratory were initially degraded by acid DNAase according to a single-hit kinetics. In spite of all changes introduced into the variable factors involved, it was not possible to find any exception to this rule. The same conclusion was reached when using viscometry instead of light scattering to follow the degradation.[4] These results firmly establish the existence of a single-hit kinetics, a point first suggested on the basis of qualitative evidence by Oth *et al.* (1958).

Using their acid DNAase preparation from mouse sarcoma in order to degrade native calf thymus DNA, Cavalieri and Rosenberg (1961) obtained values of n between 1.5 and 1.7. This result is not necessarily in disagreement with ours, since digestion was performed on sheared DNA samples, where we also find values of n equal to 1.3–1.5.[5]

[4] E. G. Richards, personal communication.
[5] G. Bernardi and C. Sadron, to be published.

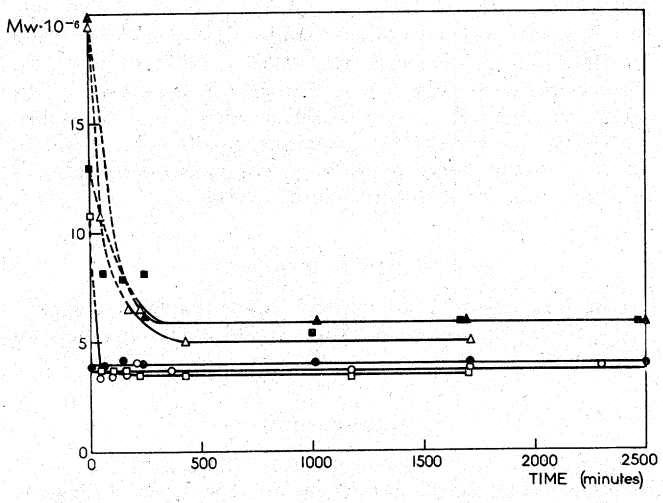

FIG. 9.—Digestion of DNA samples B6 A (triangles), B6 B (squares), and B6 C (circles) with chymotrypsin (open symbols) and papain (solid symbols). DNA concentration was 50 μg/ml for DNA B6 A, 67 μg/ml for DNA B6 B, and 125 μg/ml for DNA B6 C. Molecular weights higher than 6.10^6 are highly doubtful (Froelich *et al.*, 1963). See text for further details.

That the enzyme not only degrades DNA by a single-hit but also by a double-hit mechanism is indicated by the following findings: (a) Titration data obtained by Dr. E. G. Richards in our laboratory,[4] and to be reported elsewhere in detail, show that phosphodiester bond splitting is linear with time down to extremely low molecular weights, a result which justifies the replacement of p with t in equation (2). For a given decrease 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. In fact, about ten to twenty breaks (a value likely to be overestimated) were found to be necessary to halve the molecular weight of each parent molecule of $M_w = 6 \times 10^6$, whereas 200 breaks are needed in a double-hit degradation (Thomas, 1956) and about 3 in pure single-hit kinetics in order to obtain the same result.

(b) It has been reported here that $1/M$ increases faster with time after a value of about 1×10^6 has been reached. Since bond splitting is linear with time, this phenomenon is incompatible with the existence of pure single-hit kinetics, and is most easily explained by the superposition of two different mechanisms of degradation. One of them, the single-hit mechanism, is immediately effective in causing a decrease in molecular weight, whereas the second, the double-hit mechanism, also starting immediately after the addition of enzyme, becomes effective only after a time lag.

This conclusion was confirmed by MacHattie *et al.* (1963) using a completely different experimental approach DNA from T5 bacteriophage was digested with acid DNAase and the fragment-size distribution at three time intervals was studied by electron microscopy. If the number of scissions found by MacHattie *et al.* (1963) is plotted against digestion time, a curve is obtained whose shape is very similar to that shown in Figure 5.

(c) The results obtained upon heat-denaturation of partially digested DNA samples (Fig. 6) may be explained by the fact that strand separation, occurring at a high temperature (Doty *et al.*, 1960), liberates more single-stranded fragments from partially digested DNA than from intact DNA because of the presence in partially digested DNA of a certain number of "nicks"

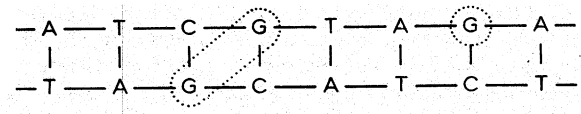

FIG. 10.—Scheme of degradation of DNA by difunctional alkylating agents. From Brookes and Lawley (1961).

introduced by the double-hit action of the enzyme. An interesting finding is the higher degree of residual hyperchromicity (following the rapid cooling of the samples) for the longer-digested samples (Table III). This seems to indicate that the difficulty of re-formation of double-stranded structures upon fast cooling varies inversely with the size of the single-stranded fragments present in the solution at high temperature. These results are similar to those reported by Geiduschek (1962) for his type I reversibility. A dependence of renaturation (type II reversibility of Geiduschek) upon DNA molecular weight was also reported by Marmur and Doty (1961).

The data presented in Figure 6 permit one to estimate the ratio of total bonds broken to bonds broken by the single-hit kinetics as equal to about 3, which means two breaks by the double-hit kinetics per one break by the single-hit kinetics. However, since this calculation requires several unwarranted assumptions such as complete strand separation upon heating, no interchain association upon cooling, and no DNA hydrolysis, the foregoing result may be considered only as being in rough agreement with titration data.

(d) The degradation of DNA from phages ϕ X 174 and fd indicates that the enzyme is also able to hydrolyze single-stranded molecules. (e) Heat-denatured DNA samples are digested according to double-hit kinetics. This apparently puzzling result is not so surprising if one considers that heat-denatured DNA regains a very largely double-stranded structure upon cooling. A certain number of single-hit attacks seem to be present as suggested by generally low values found for n. This point will be considered in more detail elsewhere.[5]

While the double-hit mechanism is essentially identical with that exhibited by pancreatic DNAase and does not need any special comment, several hypotheses may be put forward to explain the single-hit mechanism of degradation: (a) DNA has some extent of strand separation at the acid pH used in the enzymatic digestion. This explanation (Oth *et al.*, 1958) certainly is incorrect, because no difference in the intrinsic viscosity or in the radius of gyration can be found for the same DNA solution at pH 7 or 5 ($\mu = 0.15$).[5] Furthermore the same kinetics is found at pH 7.0, $\mu = 0.005$, as at pH 5.0, $\mu = 0.15$.

(b) DNA has some interruptions on one or the other strand, which may be primary or secondary to enzymatic attack during the extraction procedure. The single-hit degradation would then correspond to the breakage of bonds opposite the interruptions (these bonds also should not be susceptible to pancreatic DNAase; otherwise the degradation by this enzyme would give a single-hit kinetics). This hypothesis also appears to be incorrect, as judged from the following evidence. First, it is unlikely that all the DNA samples used in this work had undergone, roughly at the same extent, an enzymatic degradation during the preparation. Second, when random breaks were purposely introduced by mild enzymic digestion, their existence became evident upon heat denaturation (Table III), whereas no intact DNA sample used in this work showed more than a 40% decrease in molecular weight upon thermal treatment.

(c) DNA is made up of two continuous filaments, and both are broken at the same level (Oth *et al.*, 1958). Two cases may be considered, depending whether the two breaks are simultaneous or successive. The case of two independent successive breaks is excluded because, unless the two breaks follow each other extremely closely in time, this would show up as a double-hit kinetics. The second hypothesis of a simultaneous breakage of both DNA strands at the same level appears to be the correct one.

In relationship with a possible visualization of the process involved in the single-hit action, it may be interesting to consider a chemical degradation of DNA, which is formally identical to that provoked by acid DNAase. This is the degradation of DNA by difunctional alkylating agents, as studied by Brookes and Lawley (1961). Under their 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 are found on opposite strands, as in Figure 10. 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 DNAase, whereas alkylation by difunctional agents causes a degradation which involves both single-hit and double-hit kinetics, therefore simulating the action of acid DNAase.[6]

These data led Bernardi and Sadron (1964) to put forward the hypothesis that acid DNAase might be a protein with two active sites. The enzyme would then be able to split both DNA strands simultaneously at sites where susceptible nucleotide sequences exist at the same level on opposite strands, or to break only one strand when a susceptible site exists on one chain only. The almost complete disappearance of the single-hit kinetics in heat-denatured DNA might indicate that the re-formation of sites susceptible to double breaks (by rematching of the sequences on the opposite strands) had not occurred to a significant extent upon fast cooling. Another possibility is that the double-stranded structure of fast-cooled heat-denatured DNA is sufficiently different from that of native DNA to make the single-hit attack impossible. This latter interpretation deserves some consideration in view of the finding that in the recently discovered competitive inhibition of acid DNAase by polyribonucleotides[7] double stranded poly-A or poly-C do not exert any action whereas soluble RNA or poly-A–poly-U are very powerful inhibitors, a phenomenon possibly related to the structural differences existing among the polyribonucleotides mentioned (see Tomita and Rich, 1964, for example).

Alternatively, one might think that the single-hit degradation is due to the splitting of special bonds like the phosphoserine-nucleoside linkages postulated by Bendich and Rosenkranz (1963), for example. In connection with this second hypothesis it may be interesting to recall that acid DNAase also has a phosphodiesterase activity on Ca[bis-(*p*-nitrophenyl)phosphate]$_2$ and on the *p*-nitrophenyl esters of nucleoside-3′-phosphates (Bernardi and Griffé, 1964).

[6] It should be pointed out that this description of the DNA degradation by alkylating agents gives a rather idealized picture of the process involved; in fact, for example, the double-hit kinetics expected with monofunctional agents was not found experimentally (J. A. V. Butler, personal communication).

[7] G. Bernardi, to be published

Acknowledgments

The authors wish to express their gratitude to Drs. E. G. Richards and C. A. Thomas, Jr., for many helpful discussions.

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