[CONTRIBUTION FROM GIBBS LABORATORY, DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY, AND THE LILLY RESEARCH LABORATORIES]

The Enzymatic Degradation of Desoxyribose Nucleic Acid

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The enzymatic degradation of DNA has been followed by a continuous titration method and simultaneously by light scattering or viscosity. The resulting data, which are in terms of the number of bonds broken per parent molecule and the molecular weight decay, are compared with the expected molecular weight decay, calculated on the basis of several simple models. The results indicate that the molecule is composed of two polynucleotide strands and that the enzyme cleaves phosphorus–ester linkages at random. The maximum number of contiguous hydrogen bonds which fail to keep two fragments united is estimated at 2 pairs at 25°. Non-coincident ester-bond breaks have little effect on the over-all molecular configuration. The intact double helix appears to be "free-draining" in character, as evidenced by the simple proportionality between intrinsic viscosity and molecular weight; this is in contrast to the impermeability of free polynucleotide chains. Finally, it appears that a double helix in which there are numerous interruptions in the polynucleotide chains is incompatible with the evidence.

In an earlier communication[2] the response of calf thymus, desoxyribose nucleic acid (DNA), to mild acidity has been described. A major feature of DNA in aqueous solutions at *p*H 2.60 is that almost all of the hydrogen bonds which were intact at neutral *p*H have been destroyed, and the hydrolysis of phosphorus diester linkages proceeds very slowly. It is now of interest to explore the response of native DNA to the action of a highly specific phosphodiesterase which might be expected to break only the ester bonds in DNA, leaving the hydrogen-bond structure substantially intact.

The Watson–Crick[3] double-helical model for DNA provides a clear picture of the manner in which ester bonds and hydrogen bonds coöperate in maintaining the structure of the DNA molecule in solution. It is of interest to learn whether such a model is compatible with the observed response of DNA to acid and enzyme. The degradation of a double-stranded molecule, united at each monomer unit by a pair of hydrogen bonds, represents a unique kind of problem in polymer degradation, the solution to which can be compared with the experimental results. Finally, it would be interesting to test the validity of the postulate that there exist random yet non-coincident interruptions in the nucleotide chains forming the double helix.[4]

The action of pancreatic desoxyribonuclease on DNA recently has been reviewed.[5] The salient features of the reaction appear to be: (1) the liberation of hydrogen ion from newly-formed secondary phosphoryl groups,[6,7] (2) a decrease in viscosity and molecular weight[8,9] and (3) the appearance of small nucleotide fragments.[10,11] In the case where the DNA displays transforming ability, this decreases rapidly as the enzymatic degradation proceeds.[12,13]

Our attention was drawn to this problem by some preliminary light scattering experiments done in this Laboratory by Dr. M. E. Reichmann.[14] He found that during the initial stages of the degradation the molecular weight remained sensibly constant, but began to decay more rapidly as time went on. It was clear from this that if the rate of cleavage of the phosphorus–ester bonds were uniform, more than one phosphorus–ester linkage must be broken before a complete scission of the molecule could result. This approach seemed capable of yielding new information about the structure of the DNA molecule in solution when combined with titration measurements which could provide an independent measure of the number of bonds broken by the enzyme.

In this work we shall focus on two features of the action of the enzyme on DNA: namely, the cleavage of phosphorus–diester bonds as evidenced by the production of hydrogen ion, and the fall in weight average molecular weight as determined by simultaneous light scattering or viscosity measurements. These two kinds of measurements were performed continuously on the same solution of DNA, thus we are able to relate molecular weight decay directly to the number of ester bonds broken. The mechanism of the degradation thus becomes our primary interest, while the rate of the reaction, which depends on the variable activity of the enzyme (among other things), becomes of secondary interest.

Experimental Methods

DNA Preparation.—The DNA used in this investigation was a lyophilized portion of a preparation made by Dr. R. Varin which displays the same molecular weight and intrinsic viscosity as the unlyophilized material previously described.[15] Stock solutions were prepared by dissolving approximately 25 mg. of raw fibers in distilled water and stirring overnight in the cold. The resulting solution was made 0.20 *M* in NaCl. The concentration of the stock solution was determined after centrifugation by measuring the optical density of an appropriately diluted sample at

(1) Department of Physics, University of Michigan, Ann Arbor, Mich.

(2) Charles A. Thomas, Jr., and Paul Doty, THIS JOURNAL, **78**, 1854 (1956).

(3) J. D. Watson and F. H. C. Crick, *Nature*, **171**, 737 (1953).

(4) C. A. Dekker and H. K. Schachman, *Proc. Natl. Acad. Sci.*, **40**, 894 (1954).

(5) G. Schmidt, "The Nucleic Acids," Vol. I, E. Chargaff and J. N. Davidson, Ed., Academic Press, Inc., New York, N. Y., 1955, pp. 555–655.

(6) J. Gregoire, *Bull. soc. chim. biol.*, **34**, 284 (1952).

(7) L. F. Cavalieri and B. Hatch, THIS JOURNAL, **75**, 1110 (1953).

(8) J. M. Kunitz, *J. Gen. Physiol.*, **33**, 363 (1950).

(9) J. W. Rowen, *Biochim. Biophys. Acta*, **10**, 391 (1953).

(10) R. L. Sinsheimer, *J. Biol. Chem.*, **208**, 445 (1953).

(11) S. Zamenhof and E. Chargaff, *ibid.*, **187**, 1 (1950).

(12) S. Zamenhof, H. E. Alexander and G. Leidy, *J. Exp. Med.*, **98**, 373 (1953).

(13) R. D. Hotchkiss, "Harvey Lectures, Series XII," Academic Press, Inc., New York, N. Y., 1955.

(14) M. E. Reichmann, *J. Phys. Chem.*, in press.

(15) M. E. Reichmann, S. Rice, C. A. Thomas and Paul Doty, *ibid.*, **76**, 3047 (1954).

259 mμ and employing an extinction coefficient ($E^{1\%}_{1\ cm.}$) of 213.[14]

Desoxyribonuclease.—The crystalline pancreatic DNase (Worthington Biochemical Sales Co.) was dissolved in 0.10 or 0.20 *M* NaCl in the cold to form stock solutions containing 200 or 300 γ/ml. It was found that solutions of this concentration were stable in the cold over a period of days.

Viscometer and "*p*H-stat."—The apparatus which allowed continuous recording of ester bond breakage and viscosity on the same solution is shown in Fig. 1. The viscometer consisted of a helical piece of capillary approximately 0.061 cm. in radius, 65 cm. in length and a bulb 2.25 cm.^{3} in volume, located 11 cm. above the surface of the liquid in the titration vessel. These constants, together with the flow time for water, 25 sec., give an average gradient $\bar{\beta}$ of $340/\eta_{rel}$. No kinetic energy corrections were made, neither was any attempt made to correct for the gradient dependence of the DNA solution. However since $[\eta_{sp.}/c]\bar{\beta}$ for this DNA solution does not depend strongly on c in the range of $0 < \bar{\beta} < 1500$ sec.$^{-1}$,[15,16] we can assume that this quantity is equal to the intrinsic viscosity at the average gradient at which it is measured. The thermostat enclosing the major part of the viscometer could be held to 25.00 ± 0.03°; the temperature of the room was 25.0 ± 0.5°.

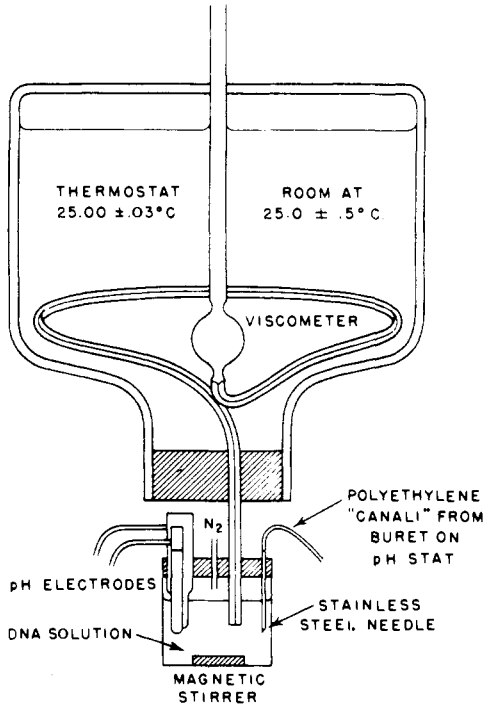

Fig. 1.—Viscometer and titration vessel.

The titration vessel into which the viscometer emptied contained 20 ml. of DNA solution (*ca.* 200 γ/ml. in 0.20 *M* NaCl) and was covered with a tight-fitting rubber stopper which mounted a pair of small Beckmann electrodes (diameter of each about 9 mm.), a stainless steel needle conducting a stream of washed N_2 and another similar needle to introduce 10^{-2} *N* NaOH.[17]

As the degradation proceeds, the addition of NaOH is necessary to hold the *p*H constant. This operation was performed automatically by a "*p*H-stat" constructed by Mr. T. V. Parke.[18] This instrument detects any difference in e.m.f. between the *p*H electrodes and a preset e.m.f. (which for our experiments was an e.m.f. corresponding to a *p*H of 7.0). This difference in e.m.f. is amplified and actuates a servo-motor which delivers the titrant from a 0.10-ml. microburet. The volume delivered could be read to 10^{-4} ml. and estimated to 2×10^{-5} ml. The amount of titrant added was automatically graphed on a Brown recorder, the chart of which was being unrolled at a constant speed. The resulting graph was a convenient plot of OH^- uptake as a function of time. It now remained for the operator to measure the viscosity as the degradation proceeded and record the flow times on the chart opposite the pen position.

The uptake of OH^- ion was converted into a value denoted as $2pu_z$, the average number of bonds broken per parent *z*-average molecule.[2] The *z*-average molecular weight has been assumed to be 3/2 times 6.6×10^6. We have taken 6.6×10^6 as the weight average molecular weight of Varin's DNA, instead of the published value of 6.85×10^6, because this is the average of more recent unpublished determinations by the author. It should be noted that the conclusions we shall attempt to draw from these experiments are not dependent strongly on any assumed value of the molecular weight. We have decided to described the OH^- uptake during the degradation in terms of the average number of bonds broken per parent *z*-average molecule, rather than the fraction of all ester bonds broken, to lend conceptual clarity to the process, as well as for convenient comparison with calculations discussed later. The sensitivity of the apparatus under the conditions used was such that we could easily detect the cleavage of 8 bonds per parent molecule.

$$2pu_z = \frac{\text{moles of NaOH required}}{\text{moles of DNA present}} \times \frac{1}{0.91}$$

The factor 1/0.91 appears as a result of the assumption that the pK_a' of the secondary phosphoryl group is 6.0.[1] Thus we can only titrate 91% of these groups by holding the *p*H of the solution constant at 7.0.

The elimination of CO_2 deserves special attention. It was found that if unbuffered solutions at *p*H 7.0 were exposed to the atmosphere, CO_2 was absorbed rapidly, masking any liberation of hydrogen ion by the degrading DNA. These effects were eliminated by continuously flushing the titration vessel with washed N_2. It was desirable to remove as much dissolved CO_2 from the DNA solution as possible, because its buffering action decreases the shift in *p*H in response to the liberation of hydrogen ion by the degradation. Thus the presence of such a buffer tends to decrease the precision of the measurement, although the magnitude of the OH^- uptake should be the same in the presence of, as in the absence of small quantities of buffer. This is because the buffer itself takes up no titrant if the *p*H remains exactly constant. This is a substantial experimental advantage over those methods which measure the amount of acid liberated by a change in *p*H, because the magnitude of this *p*H change depends strongly on the presence of dissolved CO_2 or any buffer.

In a typical experiment 20 ml. of DNA in 0.20 *M* NaCl was introduced into the titration cell which was then flushed with N_2 until the *p*H of the solution increased to 6.5 or higher. The solution was made 0.020 *M* in Mg^{++} and the flushing continued for an additional 30 minutes. The solution was then gradually brought to *p*H 7 by the addition of 10^{-2} *M* NaOH from the microburet. The *p*H-stat was engaged and the preset potential gradually adjusted to a potential which would just barefuly initiate the addition of titrant. This was a small adjustment and never more than 0.10 *p*H unit from 7.0. The apparatus was allowed to remain thus for 15 to 20 minutes to see if: (1) there was any absorption of CO_2 or (2) if the DNA was spontaneously degrading in the absence of enzyme. Neither 1 nor 2 was ever observed. [Similar control experiments were continued for 2 to 3 hours; there was no demand for OH^- and no change in the viscosity.

A small quantity (10^{-2} to 10^{-3} ml.) of enzyme solution which had been adjusted to *p*H 7 was then added. In all cases the titrant began to be introduced within not more than 30 seconds after the addition of enzyme. This short delay is probably caused by the fact that the preset potential is at a slightly more acid *p*H than the actual *p*H of the solution.

(16) M. E. Reichmann, B. Bunce and Paul Doty, *J. Polymer Sci.*, **10**, 109 (1952).

(17) This dilute NaOH solution was prepared by dilution of a concentrated solution of known normality under paraffin oil to exclude CO_2. The 10^{-2} *N* solution was standardized from time to time and found undepleted by CO_2 absorption. It is interesting to note that KOH was unsatisfactory as a titrant, presumably because of some inhibitory action of K^+ ion on the enzyme reaction.

(18) This device is similar in principle to that reported by Jacobsen and Leonis, *Compt. rend. Lab. Carlsberg*, **27**, 333 (1951).

(19) J. M. Gulland, D. O. Jordan and H. F. W. Taylor, *J. Chem. Soc.*, 1131 (1947).

We have no evidence for any induction period or delay in the initiation of ester-bond breakage, although we could not detect an induction period whose duration was less than 10–30 seconds.

Simultaneous Light Scattering and Titration Experiments.—Continuous light scattering and titration measurements are more difficult than viscosity–titration measurements because it is necessary to rigidly exclude dust particles. It was found almost impossible to clean acceptably a sodium hydroxide solution. It seems likely that basic solutions are able to remove and suspend small particles of silica from glass vessels. Therefore, it was necessary to turn to gaseous ammonia as the titrant. Nitrogen and ammonia were mixed in a low pressure steel tank and the ratio of the two gases adjusted until the ammonia was about 10^{-4} molar in concentration. This gaseous titrant was measured into the light scattering cell by a thermostated silicone oil manometer. Both the titrant gas and the stream of N_2, which continuously flushed the light scattering cell, were passed through Millipore type AA filters.[20]

In order to measure the *p*H of the light scattering solution continuously, a cylindrical cell was fitted with the standard size Beckman electrodes which were mounted in a tightly fitting Nylon cover for the cell. Measurements were performed in a Brice–Phoenix light scattering photometer which had been modified by arranging a magnetic stirrer on the table of the photometer to allow continuous stirring during measurements. The apparatus is depicted in Fig. 2.

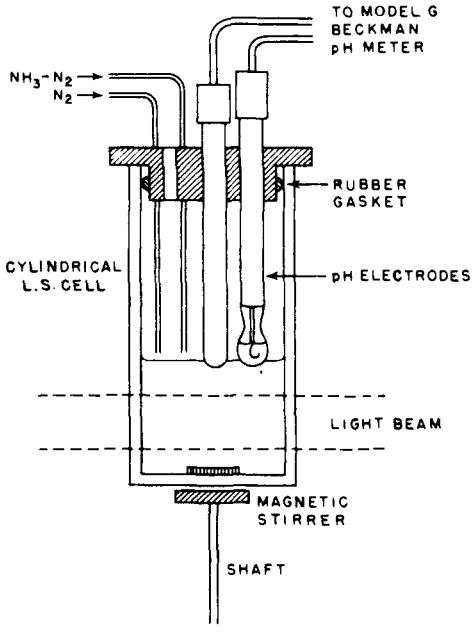

Fig. 2.—Light scattering cell in which NH_3 titrations can be continuously performed.

The titrant gas was standardized against a dilute HCl solution of known molarity and at known atmospheric pressure. It was found that the ammonia was quantitatively absorbed by the liquid *without* bubbling it into the solution. This was fortunate because the presence of air bubbles in the solution makes simultaneous scattering measurements impossible. A centrifuged DNA solution (*ca.* 190 γ/ml.) containing 0.020 *M* Mg^{++} was placed in the cell which was then thoroughly flushed with nitrogen until the *p*H of the solution increased to 6.5 or higher. The solution was then adjusted to a *p*H of 7.0 by the introduction of a small quantity of ammonia. A drop of centrifuged enzyme solution was added through a port in the cell cover making the final solution approximately 10^{-3} γ/ml.; a gradual downward drift in *p*H began. The *p*H was kept as constant as possible by the addition of NH_3 from the manometer. During the course of the experiment, three quantities were measured: (1) time, (2) NH_3 uptake and (3) angular envelopes of scattered light between 30 and 60 degrees. These data were handled in a manner similar to that already described in the viscosity experiments. The limit of sensitivity for the above experiments was about 5 bonds per parent molecule.

(20) Millipore Filter Corp., Watertown, Mass.

Experimental Results

Preliminary Experiments.—Three different kinds of preliminary experiments were performed for the purpose of duplicating some of the known features of the degradation of DNA by DNase.

The first of these was to verify that our DNA preparation displayed the same "delay" in the decrease of viscosity after the addition of enzyme. Duplicating Zamenhof's experiments,[21] it was found that the viscosity of the DNA solution remained constant for 13 to 34 minutes depending on the enzyme concentration. After this initial "induction" period, the viscosity decays in a regular fashion with time. In this case the enzyme was activated with $MnCl_2$, which was present in a final concentration of 0.0053 molar. The viscosity measurements were made with an Ostwald–Fenske viscometer that had a flow time for water of 53 seconds. The results shown in Fig. 3 indicate that the induction period observed by Zamenhof can be duplicated with Varin's DNA.

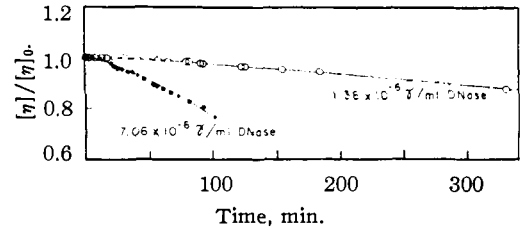

Fig. 3.—Viscosity decay with time in the presence of DNase. Zamenhof's observations can be duplicated with Varin's DNA.

Utilizing the "*p*H-stat" it was found that approximately 25% of all the internucleotide linkages were broken when an excess of Mg^{++}-activated DNase was added. This is in agreement with the findings of others.[7,10]

Lastly, it was found that the initial rate of uptake of NaOH depends roughly on the first power of the enzyme concentration; this is in agreement with the experiments of Gregoire[6] and Cavalieri.[7]

Simultaneous Viscosity–Titration Experiments.—The results of a series of experiments are summarized in Fig. 4. The experimental conditions are listed in Table I.

TABLE I

No.	DNA concn., γ/ml.	Mg^{++} concn., *M*	Enzyme concn., γ/ml.	Initial rate, bonds/min.
4	200	0.02	2×10^{-3}	37.8
5	200	.02	2×10^{-3}	10.2
7	198	.02	2×10^{-3}	87.5
16	182	5.6×10^{-3}	0.15	69.0

In Fig. 4 we see that about 200 ester bonds per molecule must be broken in order for the viscosity to be diminished by a factor of two. If the intrinsic viscosity can be taken as a crude measure of

(21) S. Zamenhof, G. Griboff and N. Marullo, *Biochim. Biophys. Acta*, **13**, 459 (1954).

molecular weight, we shall see later that this observation is not compatible with either random or end-selective bond breakage of a single polynucleotide chain.

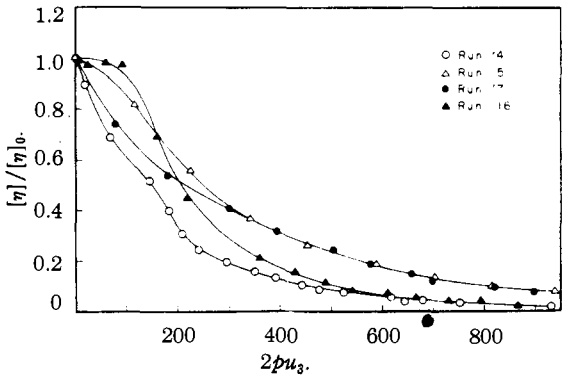

Fig. 4.—Fractional decrease in intrinsic viscosity as a function of the number of bonds broken per parent molecule of z-average molecular weight.

The reasons for the differences between the four runs in Fig. 4 are not known at present. While measurements of this kind would be very sensitive to the initial intactness of phosphorous ester and hydrogen bonds, an equal source of variation lies in the sensitivity of intrinsic viscosity to molecular configuration. This sensitivity is accentuated because these measurements were performed at finite gradients. Thus slight changes in the shape of the DNA molecules would cause a decrease in viscosity even if the molecular weight did not change; however, under these conditions a decrease in molecular weight would invariably result in a decrease in viscosity. Since it is not intrinsic viscosity but molecular weight which is related to the mechanism of the degradation, in the next section light scattering measurements are reported which show the average molecular weight decay. Molecular weights determined by this method are independent of whatever shapes the molecules happen to be.

Simultaneous Light Scattering and Titration Experiments.—In Fig. 5 is shown the fractional decrease of weight average molecular weight as a function of the average number of bonds broken per parent (z-average) molecule, as determined by the ammonia required to keep the pH of the solution constant. Because we are dealing with the fractional decrease in molecular weight, the small error resulting from the fact that these measurements were not extrapolated to zero concentration is neglected here.[22] It is at once clear that the molecular weight decay parallels the viscosity decay, and thus the possibility that the viscosity decay might have come about by an aggregation of the DNA during degradation is excluded. Here is seen the initial "induction period" observed by Reichmann[14] where bonds are being broken and the molecular weight changes only slightly. This is in agreement with Zamenhof,[21] the results shown in Fig. 3 and two runs in Fig. 4. The "induction period" is followed by a more rapid decline in molecular weight reaching one-half its original value when 200 bonds per molecule have been broken. After 1200 bonds per molecule have been broken a rapid aggregation invariably begins. Therefore we shall only consider the character of the molecular weight decay between 0 and 500 bonds broken, which should be safely free from any effects of aggregation. Duplicate runs using Mg^{++} and Mn^{++} as activating cations yielded points which fell slightly above those shown in Fig. 5. These experiments displayed somewhat earlier aggregation, a fact which tends to obscure the fundamental process. The experiment which is reported displays the most rapid molecular weight decay and has been selected as being most free from the affects of aggregation.

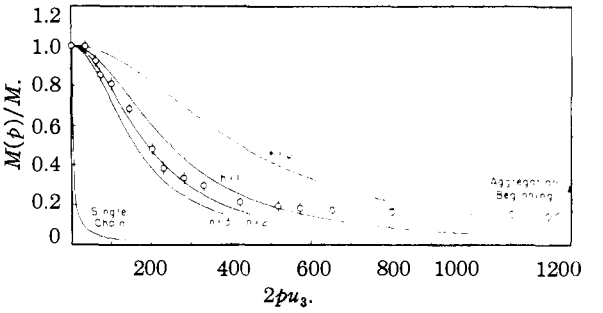

Fig. 5.—Molecular weight decay as a function of the number of bonds broken per parent z-average molecule. The points were determined by the light scattering-titration method, the lines were calculated from the double-chain degradation theory.

The shape of the DNA molecule in solution can best be approximated at present by a very highly extended gaussian coil.[15] From the initial slope of Kc/R_θ *vs.* $\sin^2 \theta/2$ one can calculate the quantity $\overline{R^2}$ which is the mean square distance between the ends of a randomly coiled polymer chain. We note that the quantity $\overline{R^2}/M(p)$, which is a measure of the relative size of the molecule in comparison to its molecular weight, displays (Fig. 6) a gradual downward drift as the degradation proceeds. Thus it appears that the relative extension of the molecule is not changing greatly during the degradation. This stands in contrast to the response of DNA to pH 2.60, where a great contraction takes place.[16] After about 500 bonds have been broken, the experimental points in Fig. 6 show a sudden increase. Since $\overline{R^2}$ is a measure of the dimensions of

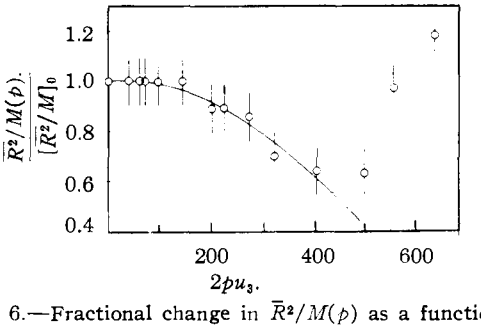

Fig. 6.—Fractional change in $\overline{R^2}/M(p)$ as a function of the number of bonds broken per parent z-average molecule. The gentle decrease indicates very little increase in flexibility resulting from broken ester bonds.

(22) C. A. Thomas, Jr., Thesis, Harvard University, p. 47 (1954).

those molecules having a z-average molecular weight, this quantity is sensitive to the presence of small amounts of aggregates, which presumably begin to form during this period.

Controls.—The unbuffered DNA solutions are stable at pH of 7 and the intensity of scattered light does not change. The presence of metal activator causes some small immediate increase in the scattering, but this remains constant over a period of days. Thus, it appears that DNA in the absence of enzyme is stable and that the presence of Mg^{++} does not cause aggregation in the absence of the enzyme.

Viscosity Molecular Weight Relationship.—In an effort to see if there is any simple relationship between the intrinsic viscosity and molecular weight, the log (fractional decrease in intrinsic viscosity) is plotted against the log (fractional decrease in molecular weight) as shown in Fig. 7. Although there is a great deal of scatter in the points, it is clear that they fall in a band about a line with a slope of unity. It appears that the molecular weight obeys the simple Staudinger relationship $[\eta] = KM$ over a fivefold range in molecular weight. From Table I for Varin's DNA[4] we can calculate a value for K of 7×10^{-6}.

It is important to notice that the viscosity measurements were not extrapolated to zero gradient and further that the average gradient changed during the degradation from an initial value of about 170 sec.$^{-1}$ to about 340 sec.$^{-1}$. The general drift upward of the experimental points in Fig. 7 is probably a result of aggregation.

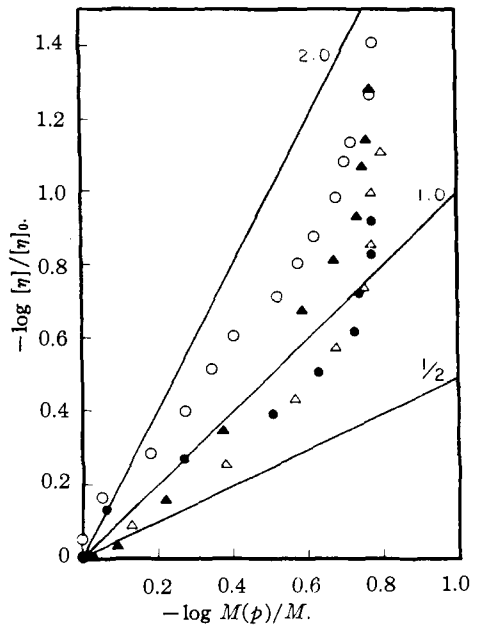

Fig. 7.—Viscosity-molecular weight relationship during the degradation. Slope of 1.0 indicates free-draining character of DNA fragments as opposed to the solvent-entrapping character of single polynucleotide chains (see ref. 2).

The Degradation of a Double Chain

The analysis of the above experimental results requires some conception of how the weight average molecular weight might be expected to decay as a function of the number of broken bonds per parent molecule. The character of the molecular weight decay depends both on the mechanism of degradation and on the kind of polymer that is being degraded. Finally, the character of the molecular weight decay will depend on the degree of polydispersity of the starting material. In an earlier communication[2] the subject of polydisperse starting material has been explored. If a linear molecule is subjected to random scission, the following equations describe the molecular weight decay

$$\frac{M(p)}{M} = \frac{2}{\gamma^2}[e^{-\gamma} + \gamma - 1] \quad \begin{matrix}\text{monodisperse} \\ \text{starting material}\end{matrix} \qquad (1)$$

$$\frac{M(p)}{M} = \frac{1}{1 + \frac{1}{3}\gamma_3} \quad \begin{matrix}\text{polydisperse} \\ \text{starting material} \\ \text{with } {}^1/_3 M_z = \\ {}^1/_2 M_w = M_n\end{matrix} \qquad (2)$$

The variable γ_3 is the average number of times a parent molecule of z-average molecular weight has suffered a complete scission resulting in two fragments, and M_z, M_w and M_n are the z, weight and number averages of the initial molecular weight distribution. The symbol γ is used to denote the number of molecular scissions suffered by any one of the identical starting molecules.

If we are dealing with a *single-chain linear polymer*, a bond breakage results in a molecular scission. In this case the molecular weight falls quite rapidly with the number of bonds broken. In Fig. 5 the curve marked "single chain" was calculated by substituting $\gamma_3 = 2pu_3$ in equation 2. This leads directly to the conclusion that the enzymatic degradation of DNA is not the random breaking of bonds in a single-chain polymer.

We make our next hypothesis by supposing that the Watson–Crick model for DNA describes the structure of the molecule in solution. Such a model is in essence a "ladder-like" molecule wherein the rungs of the ladder represent the pairs of hydrogen bonds between opposite nucleotides, and the side members of the ladder between the rungs represent the phosphorus–ester linkages between nucleotides. This model was considered in attempting to explain the character of the acidic degradation of DNA.[2] At pH 2.60 most of the hydrogen bonds have been broken and so the majority of the rungs in the equivalent ladder model are missing. In the case of DNA at neutral pH, however, it is reasonable to assume that all of the hydrogen bonds are intact. If they are very strong, then the enzyme must break an ester bond immediately opposite an already existing broken bond in order to cause a molecular scission. The probability of breaking both chains in such a way as to produce two fragments is

$$s = p^2$$

where s is the probability of a double-chain scission and p is the probability of an ester-bond breakage. If the hydrogen bonds are weaker, 1, 2, . . . h contiguous hydrogen bonds may fail to hold the two fragments together. In this case the probability of a double-chain scission is greater

$$s = p[1 - (1 - p)^{2h+1}] \cong p^2(2h + 1) \qquad (3)$$

Defining u as the degree of polymerization *of one of the two polynucleotide chains* and u_1, u_2 and u_3 as the number, weight and z-average degree of poly-

merization, respectively, in the polydisperse case, we can write the average number of double-chain scissions per parent molecule thus

$$\gamma_3 = su_3 = (2h + 1)p^2u_3 = \frac{(2pu_3)^2(2h + 1)}{4u_3} \quad (4)$$

On substituting in equations 1 and 2 and plotting in terms of $2pu_3$ (the average number of bonds broken per z-average parent molecule) we arrive at the results shown in Fig. 8. If one actually plots against the quantity $2pu_3(2h + 1)^{1/2}$ the graph can be used to calculate the molecular weight decay for any assumed hydrogen bond strength. Since some degree of polydispersity in DNA undoubtedly exists, we make the assumption that it is like a random distribution in which $^1/_3 M_z = {}^1/_2 M_w = M_n$. Although this is an arbitrary assumption, it is likely to be nearer the truth than the assumption of monodisperse starting material. Therefore, in what follows, we shall be using equation 2.

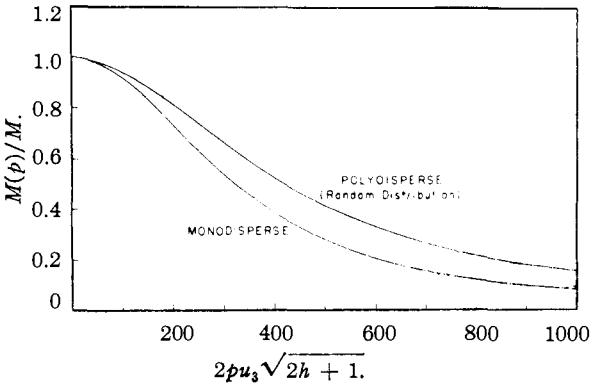

Fig. 8.—Molecular weight decay for the random degradation of a double-chained molecule in terms of the number of bonds broken per parent molecule and the maximum number of contiguous pairs of hydrogen bonds which fail to unite double-chain fragments.

In Fig. 5 we have plotted a family of curves showing the molecular weight decay with increasing values of h. On the same graph are shown the experimental points from light scattering–ammonia uptake experiments. An inspection of this figure will show that the experimental data can be fitted over a substantial range of molecular weight decay by the curve for $h = 2$. The viscosity measurements can also be fitted with the curve for $h = 2$. Thus it appears that all of the molecular weight decay data could be explained by assuming that the DNase breaks ester linkages at random, and if these breaks appear on opposite chains not more than two hydrogen bonds apart, a scission of the molecule will result and the average molecular weight will decrease. If the cuts appear 3, 4, 5 or more hydrogen bonds apart the molecule will hold together by the coöperative effort of the intervening hydrogen bonds.

A Double Chain with Random Yet Non-coincident Interruptions.—We next turn our attention to molecular weight decay expected if there are interruptions in the poynucleotide chain. In a model of this kind, which has been proposed by Dekker and Schachman,[4] there is one missing ester bond in 50. Although this model has been criticized on other grounds[23,2] it is informative to consider this question in the light of these experiments. If the initial missing bonds are not close enough together on opposite chains to cause a spontaneous double-chain scission, then the probability of a double-chain scission under random enzymatic attack is approximately

$$s' \cong (2h + 1)[p^2 + pp_i] \quad (5)$$

where p_i is the probability that an ester bond is missing initially. If p_i is $^1/_{50}$,[4] we can substitute this value of s' into equation 4 and finally back into equation 2. This has been done and the results have been plotted in Fig. 9. It is easily seen that even for an assumed value of $h = 0$ (meaning a single pair of hydrogen bonds between nucleotides is strong enough to unite partially degraded fragments) the experimental data do not fall on the expected curve. Neither does the calculated line describe a salient feature of the enzymatic degradation of DNA, namely, the initial horizontal slope of the viscosity or molecular weight decay seen in Figs. 3 and 5. This horizontal slope has also been observed in independent light scattering experiments by Reichmann.[14] Thus it is clear that the process going on here is probably *not* the random degradation of a double helix possessing frequent non-coincident interruption.

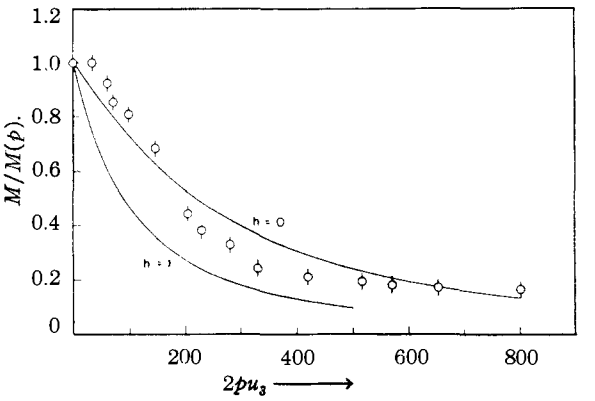

Fig. 9.—Molecular weight decay expected from the random degradation of an interrupted double helix. A z-average molecule is assumed to have 600 interruptions.

Discussion

The experimental results are in good agreement with the concept of a long double-chained molecule united by hydrogen bonds at each monomer to another monomer on the opposite chain. The cleavage of ester linkages which do not lead to a double-chain scission does not appear to increase greatly the flexibility of the DNA molecule, thus the short-range, as well as the long-range, structure probably remains intact in spite of non-coincident ester-bond breaks. The DNA fragments which result from the partial enzymatic digestion of DNA appear to obey the simple Staudinger relationship $[\eta] = KM$, a fact which indicates that they retain their open and "free-draining" hydrodynamic character as opposed to the more compact configuration assumed by polynucleotide chains.[2]

(23) P. Doty and S. A. Rice, *Biochim. Biophys. Acta*, **16**, 446 (1955).

The conclusions presented above have been drawn from a comparison of the molecular weight and viscosity decay with the expected molecular weight decay assuming random cleavage of phosphorous ester linkages. These assumptions are not stringent. Furthermore, the interpretation of the observations along these lines does not require that *all* of the molecular scissions and ester bond cleavages occur at random, but rather that only the first 9 or 10 molecular scissions and the first 2% of the ester-link cleavages occur at random.

The extreme opposite case of end selective double-chain scission is not possible, for in this case about 3000 bonds per parent z-average molecule would have to be broken before the molecular weight would fall to 90% of its original value. This is not observed in Fig. 5. In calculating the probability of a double-chain scission, it is assumed that the enzyme can split interpurine as easily as interpyrimidine linkages. If the converse were true and if purines were always paired with pyrimidines on opposite chains, this would mean that the probability of having opposite, or nearly opposite, cuts would be very much smaller. This would cause a much more gentle decrease in molecular weight as a function of the number of bonds broken. This again is not observed in Fig. 5. In the case of Mg^{++}-activated DNase, the assumption of equal sensitivity of interpurine and interpyrimidine linkages cannot be disproven from the data available. If coincident cuts were more probable than random, then the molecular weight decay would be sharper, and the initial horizontal slope would be eliminated. Thus this possibility is unlikely. Any discussion concerning selectivity of enzyme attack for particular internucleotide linkages can only be interpreted, at present, by assuming that sensitive linkages are distributed over the molecule in an effectively random fashion.

These considerations tend to support the hypothesis that all linkages are equally susceptible to enzymatic cleavage. When this assumption is made, we see in Fig. 5 that the experimental data fall on the calculated curve for $h = 2$. This means that cuts on opposite chains with zero, one or two nucleotides between them will result in a complete scission of the double-chained molecule. Figure 10 shows a schematic drawing of this process. This estimate of the maximum number of unstable pairs of hydrogen bonds is somewhat dependent on the initial degree of polydispersity of the DNA. If the degree of polydispersity has been significantly overestimated by assuming a "most probable" distribution, then the estimate of the unstable pairs must be reduced to one pair. This uncertainty in h is of the same order as the experimental error in these measurements. It must be further noted that the process taking place during the enzymatic degradation of DNA could be a very complicated one in which the kinetics of hydrogen bond rupture could play a large role. For these reasons the value of h obtained from these experiments is at best an *estimate* denoting order of magnitude rather than any discrete value.

In the later stages of each enzymatic degradation experiment, a rapid aggregation begins. This is

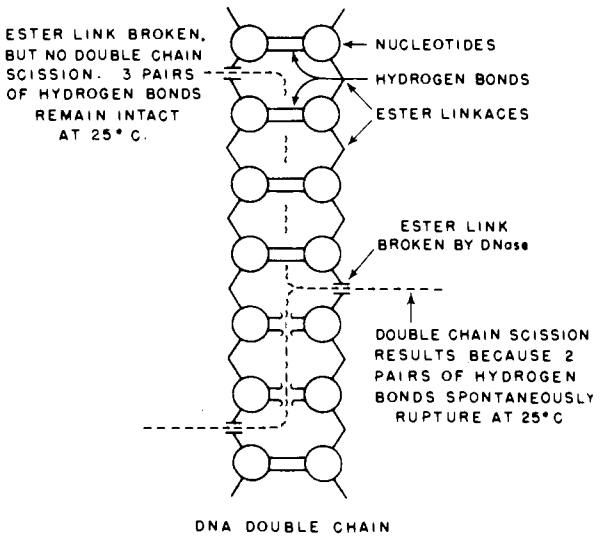

Fig. 10.—A schematic diagram of the enzymatic degradation of DNA by DNase showing random cleavage of ester bonds and a double-chain scission resulting from the spontaneous rupture of two hydrogen bonds.

first reflected by an increase in the $\overline{R^2}/M$ value as seen in Fig. 6 and finally by an increase in molecular weight after about 1200 bonds per molecule have been broken. However, it seems that this phenomenon does not affect the interpretation between 0 and 500 broken bonds per molecule. It is quite possible that this aggregation comes about by the reformation of groups of exposed hydrogen bonds between different fragments, in which case this aggregation would be similar to that observed on reneutralization of a solution of DNA which had undergone partial acidic hydrolysis at pH 2.60.[2]

In most of the experiments reported here, the DNase has been activated by magnesium ion. Although there may be some difference in detail, it has been possible to duplicate the general features of both the molecular weight and viscosity decay using DNase which has been activated with manganese ion.

This picture of the enzymatic degradation process is also well suited for the explanation of some of the other known features of the reaction. It is known that the breakage of the hydrogen bonds in DNA results in a certain increase[24] in the extinction coefficient of DNA.[25] It is also known that during the later stages of the enzymatic digestion of DNA there is a substantial increase in the extinction coefficient.[8] If, as depicted in Fig. 10, isolated pairs of nucleotides, unsupported by ester linkages, soon rupture, then it would appear that a major part of the optical density exaltation during the enzymatic digestion can be related directly to the rupture of hydrogen bonds. Thus the increase in extinction coefficient during the final stages of the enzymatic degradation is easily reconciled with the interpretation of the molecular weight decay given in Fig. 5.

Reversing this reasoning, if the exaltation of the

(24) It is clear that a certain average reduction of extinction coefficient results from the formation of an internucleotide link. See ref. 10.

(25) R. Thomas, *Biochim. Biophys. Acta*, **14**, 231 (1954).

extinction coefficient is evidence for the fact that isolated nucleotides are not stable, then this assumption allows one to compare the experimental data in Fig. 9 with the calculated curve for $h = 1$ or 2 rather than $h = 0$. The wide disagreement between the observed and expected results makes it possible to estimate, under the assumptions made, that there are fewer than one or two initial interruptions in 500. However, it is probable that the number is very much smaller than this in view of the constancy of molecular weight when subjected to acid,[2,16] alkali[26] and heat.[23]

Finally, we note that the estimation of $h = 2$ is made at 25°. It is quite certain that as one increases the temperature, the minimum number of contiguous pairs of hydrogen bonds necessary to unite two fragments increases. Thus if a DNA molecule has suffered a number of ester bond breakages, but relatively few double-chain scissions, the number of double-chain scissions will increase as the temperature increases because larger and larger groups of hydrogen bonds will be able to melt out at higher temperatures. This decreased heat stability of the viscosity of DNA which has been sensitized by DNase has been observed by Zamenhof.[21]

(26) P. Ehrlich and P. Doty, in press.

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