THE REPLICATION OF DNA

II. The Number of Polynucleotide Strands in the Conserved Unit of DNA

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ABSTRACT The kinetics of degradation of DNA by deoxyribonuclease II have been studied, using the techniques of light scattering, viscosity, and titration. Theoretical equations have been derived for both random and non-random attacks, and all assumptions have been evaluated. It has been shown that these equations permit a valid calculation of the number of polynucleotide strands per molecule. The results have been verified by two independent experimental methods. DNA from proliferating sources was found to be four-stranded; DNA from non-proliferating sources was found to be two-stranded. The implications of these findings are discussed.

We have just presented inferential evidence that the unit of DNA conserved during replication is a double helix (1). No other observations bearing directly on the nature of the conserved unit have been reported in the literature, save that of the possible existence in nature of a single-stranded DNA capable of replication (2). However, conclusive evidence regarding the number of strands in the conserved unit can be obtained only by methods which permit one actually to count the strands in the unit itself. This can be done, for example, by determining the ratio of the contour length to the mass, using a technique such as electron microscopy (3), or by studying the kinetics of attack upon the DNA by some degradative agent. We have chosen the second approach in this paper, with deoxyribonuclease II (DNase II) (4) as the agent which attacks polynucleotide strands and light scattering or viscometry as the method of observing the effect on the molecule as a whole. If a molecule possesses only one strand, its molecular weight will start to decay as soon as it comes in contact with the agent; if it possesses two or more strands, there will be an initial lag until all the strands have been cleaved at or near the same place. One can thus calculate the number of strands from the molecular-weight decay.

EXPERIMENTAL CONSIDERATIONS

The DNA samples, all of which were disaggregated, have been described previously (1, 5). A DNase II extract was prepared from mouse sarcoma-180 as follows:

nuclei were isolated by a sucrose method (6), and the supernatant, containing the remainder of the cell components, was centrifuged to remove particulate debris. The soluble fraction was then exhaustively dialyzed against 0.15 M NaCl, and finally lyophilized. It was shown that there were no enzymes present which attack mixtures of nucleosides or nucleotides. This was done by incubating these mixtures under the conditions of the kinetic experiments and showing that the titer did not change with time. DNase II was chosen because it requires no divalent ions, which cause aggregation of denatured DNA.

Light-scattering procedure has already been described (5). In the light-scattering experiments reported here, the DNA concentration was approximately 0.1 mg/ml and the solvent was 0.2 M sodium acetate at pH 5.1. The enzyme was freed of dust by centrifuging a concentrated solution (14 mg of lyophilized material per ml), which was then drawn into a microburet previously cleaned with millipore-filtered solvent. After an initial measurement of the complete scattering envelope and adjustment of the temperature to 33° or 38°, a volume of about 0.003 ml of enzyme solution was added to the 11 ml DNA solution in the scattering cell. Readings of the scattering at from 3 to 6 angles between 35° and 90° were then made rapidly and periodically. Since the angular dependence of the Zimm plots was linear in this region, the curves could easily be extrapolated to zero angle. Accuracy was increased by the fact that many of the samples studied had been denatured, and the curves therefore had both low and constant slopes. It has previously been shown that the extrapolation to zero concentration is horizontal (5). The error in R, the ratio of the weight-average molecular weights at times t and 0, is about 1 per cent, and is determined by the precision of the galvanometer (<0.5 per cent). It should be noted that the actual observation is the change in galvanometer deflections with time. For this reason, the precision of this procedure is many times greater than in the usual molecular weight determination.

Before many of the light-scattering experiments, the DNA was denatured by heating it to 100° at pH 8 for 10 minutes, followed by rapidly cooling to room temperature and shaking with chloroform-octanol (5). In addition to the advantage mentioned above, denaturation of the DNA decreases the inhibition of the enzyme by the products of the reaction. The initial light-scattering measurement, performed at room temperature shortly before addition of the enzyme, provided proof that aggregation was not occurring under the experimental conditions and that the molecular weight had been unchanged by denaturation. Furthermore, some of the DNA samples were allowed to stand at 38° for 1 hour before the initial scattering measurement with no effect on their molecular weights.

Viscosities were measured continuously during the reaction in a Couette viscometer at a shear rate of 10 sec.$^{-1}$ and a concentration of 0.06 mg/ml DNA, after it had been ascertained that there is no shear- or concentration-dependence under these conditions. The temperature, solvent, and enzyme concentration were the same as in the light-scattering experiments.

Titrations were performed with a Beckman pH meter equipped with external electrodes. Aliquots of a 0.15 to 0.20 mg/ml DNA solution in 0.2 M NaCl at pH 5.1 and 38°, containing 0.01 to 0.02 mg/ml of the enzyme preparation, were withdrawn periodically, cooled rapidly, and titrated at 4° to pH 8 with 0.01 M NaOH in a microburet. When the titer at time 0 was subtracted from the titer at a later time, the result (T_t) could be interpreted as the number of sugar–phosphate ester bonds cleaved in the given interval. The change in pH during the reaction was less than 0.1 pH unit. The rate of reaction was found to be proportional to both enzyme and DNA concentrations within the range studied, so that it was possible to calculate the number of bonds cleaved at any time under the conditions of the light-scattering and viscosity experiments. The estimated error in the determination of this quantity is 5 per cent.

THEORETICAL CONSIDERATIONS

The equation used in calculating the number of polynucleotide strands per molecule from the molecular-weight decay is derived rigorously below. All restrictions or assumptions will be numbered throughout the derivation and discussed in a later section.

At time t after addition of the enzyme, the number of times a given DNA molecule has been cleaved is $M_0/\overline{M}_t - 1$, in which M_0 is the initial molecular weight and $\overline{M}_t$ is the number–average molecular weight of the pieces derived from the original molecule during time t. If the molecule consists of one long polynucleotide strand, the expected number of cleavages can also be expressed as mp_t, in which m is the initial number of enzyme-sensitive internucleotide bonds per strand and p_t is the probability that any given bond will be cleaved during time t. For a molecule consisting of n strands, all equally sensitive to enzymatic attack, the number of molecular cleavages is then

$$m(p_t)_1\,(p_t)_2\,(p_t)_3 \cdots (p_t)_n = mp_t^n,$$

and

$$M_0/\overline{M}_t - 1 = mp_t^n. \qquad [1]$$

This equation holds true for $n > 1$ only when the enzymatic attacks on internucleotide bonds occur randomly (restriction 1). It will later be shown that such was the case in our experiments.

If scission of the molecule can result even when the attacks on the n strands do not occur directly opposite one another, the number of molecular cleavages at time t will be greater than mp_t^n. For example, in a two-stranded molecule, in which the hydrogen-bonded base pairs may be thought of schematically as the rungs of a ladder, after an initial enzymatic attack the molecule can be cleaved by a second attack on the opposite strand even though this attack is 1, 2, 3 . . . base pairs removed, in either direction, from the initial attack. In our example let us imagine that a sec-

ond attack, producing molecular cleavage, may occur no more than three "rungs" away from the initial attack, in either direction. Then there are seven "inter-rung" positions (or internucleotide bonds) whose scission will produce molecular cleavage. This number of positions or bonds is denoted by B_2. The number of molecular cleavages is then given in this case by $m(p_t)_1 \cdot (B_2p_t)_2$. This treatment can be generalized to n strands. Thus, the total number of molecular cleavages will be

$$m(p_t)_1 (B_2p_t)_2 (B_3p_t)_3 \cdots (B_np_t)_n = m(\prod B)p_t^n,$$

in which $\prod B = B_2B_3 \ldots B_n$, and equation [1] becomes

$$M_0/\overline{M}_t - 1 = m(\prod B)p_t^n. \qquad [2]$$

This equation is restricted to the range in which $p_t \leqslant 1/B_L$, in which B_L is the largest member of the set of B's (restriction 2). If this limitation were not imposed, the probabilities $(B_2p_t)_2$, $(B_3p_t)_3$, etc., would eventually become greater than 1, which is impossible. However, for our purposes, this restriction is of no consequence since it merely limits the number of useful molecular cleavages to m/B; this is certain to be far beyond the range of our experiments.

Since each B is a function of the number and position of attacks, it is also to some extent a function of time. $\prod B$ must therefore be considered a statistical average for time t under the conditions of the experiment. In order to employ $\prod B$ as a constant, we assume that its variation is negligible during the time of the experiments (assumption 1).

Let us now consider a polydisperse collection of n-stranded molecules, each of which decays in molecular weight according to an equation homologous with equation [2]. Number averages of the variables are derived as follows:—

$$\frac{\sum_{i=1}^{X_0}\left(\frac{M_0}{\overline{M}_t}\right)_i}{X_0} = \frac{\sum_{i=1}^{X_0}[1 + m(\prod B)p_t^n]_i}{X_0} = 1 + (m\prod B)_N p_t^n, \qquad [3]$$

in which X_0 is the total number of molecules at time 0, and the subscript N signifies a number-average quantity. Since $(M_0/\overline{M}_t)_i$ is the number of molecules derived during time t from the i^{th} original molecule,

$$\sum_{i=1}^{X_0}\left(\frac{M_0}{\overline{M}_t}\right)_i = X_t = \text{the total number of molecules present at time } t.$$

Then X_t/X_0 is the average number of molecules derived during time t from each original molecule. This quantity can also be expressed as $(M_0)_N/(M_t)_N$. Thus,

$$\frac{\sum_{i=1}^{X_0}\left(\frac{M_0}{\overline{M}_t}\right)_i}{X_0} = \frac{X_t}{X_0} = \frac{(M_0)_N}{(M_t)_N} = 1 + (m\prod B)_N p_t^n. \qquad [4]$$

We must now make the approximation (assumption 2) that

$$(M_0)_N/(M_t)_N \approx (M_0)_w/(M_t)_w,$$

since only the latter quantity is obtainable by light scattering. The subscript w de-

notes a weight-average quantity. To enable the calculation of n, equation [4] is converted to the form

$$\log \frac{1 - R}{R} = \log (m \prod B)_N + n \log p_t, \qquad [5]$$

in which R stands for $(M_t)_w/(M_0)_w$. When the left-hand side of equation [5] is plotted against log p_t, the slope of the curve gives the number of strands per molecule.

There are two approaches to the evaluation of p_t. First, it can easily be demonstrated by means of the probability theory that $p_t = kt$ when p_t is small (*i.e.*, when less than about 20 per cent of the bonds have been cleaved). This implies constant enzyme activity—there can be no increase in inhibition during the reaction. If the extent of the reaction employed for calculating n is sufficiently small to meet these requirements (assumption 3), one can then modify equation [5] to

$$\log \frac{1 - R}{R} = \log (m \prod B)_N k^* + n \log t. \qquad [6]$$

The other approach, which involves no assumptions, utilizes titration data for direct calculation of p_t. Since one proton is released for every internucleotide bond that is hydrolyzed, the net titer T_t is the number (in mols) of bonds cleaved in a given sample during time t. If m_0 is the total number (in mols) of internucleotide bonds initially present in the sample,

$$\frac{T_t}{m_0} = p_t \qquad [7]$$

and

$$\log \frac{1 - R}{R} = \log (m \prod B)_N + n \log \frac{T_t}{m_0}. \qquad [8]$$

A similar equation has been derived by Schumaker, Richards, and Schachman (7).

Approximate values of R can be obtained from intrinsic viscosities, using the equation

$$R \approx \frac{[\eta]_t}{[\eta]_0} = \frac{K_t (M_t)_w^{\alpha}}{K_0 (M_0)_w^{\alpha}}. \qquad [9]$$

The value of α has been found to remain close to 1 during the random degradation of DNA (8). K does not change significantly during the early part of the reaction.

The foregoing theoretical considerations apply to the random degradation of n-stranded DNA molecules. There are no practical restrictions on the use of equation [8] in such cases, save the two assumptions mentioned in the derivation. These assumptions and their justification will be discussed in a later section of this paper.

It is possible to derive an even more general equation which will cover both random and non-random degradation. The procedure differs from that outlined above in that, once the first attack in some area has occurred, the probability of an

attack on *(e.g.)* the second strand in that area is $B_2\ (p_t + \phi)$. The probability of cleavage of any given bond, p_t, is now an average over all non-random cleavages with the exception of the case in which an enzyme molecule moves directly from a first attack to an attack on one of the $B_2 + B_3 + \ldots$ neighboring bonds in the adjacent strands. The average probability of these attacks is ϕ, which has a fixed value expressing the instantaneous probability of another scission leading to molecular cleavage. The approximation is hereby made that these preferential attacks in the cross-wise direction are accomplished in a negligible length of time. Although ϕ may differ for each strand and may depend on the order of attack, for our purposes it can be shown that there is no loss in generality and much gain in simplicity in considering an average ϕ. The number of molecular cleavages is then

$$m(p_t)_1\ (B_2[p_t + \phi])_2\ (B_3[p_t + \phi])_3 \cdots (B_n[p_t + \phi])_n,$$

and

$$\frac{M_0}{M_t} - 1 = m(\prod B)p_t\ (p_t + \phi)^{n-1}. \qquad [10]$$

This equation, analogous to equation [2], is restricted to the range in which

$$(p_t + \phi) \lesssim 1/B_L.$$

It is next converted to the form of equation [5]:

$$\log \frac{1 - R}{R} = \log\ (m\prod B)_N + n \log^{1/n} p_t \quad (p_t + \phi)^{(n-1)/n}. \qquad [11]$$

Equation [11] reduces to equation [5], derived for random degradation, as $\phi \to 0$.

Titration of a molecule during non-random degradation measures both the nmp_t bonds cleaved with probability p_t and those cleaved with probability ϕ. The latter are among the

$$B_2 + B_3 + \cdots + B_n = \sum_{i=2}^{n} B_i$$

bonds adjacent to each first cleavage, of which there are mp_t. Thus the number of bonds cleaved per molecule during time t is

$$nmp_t + mp_t \left(\sum_{i=2}^{n} B_i\right)\phi, \quad \text{when} \quad (p_t + \phi) \lesssim \frac{1}{B_L},$$

and the titer T_t for a sample of X_0 (original) molecules is

$$\sum_{i=1}^{X_0} \left(nmp_t + mp_t \sum_{j=2}^{n} B_j\phi\right)_i.$$

Dividing by $m_0 = X_0 n m_N$, the total number of internucleotide bonds in the sample, gives

$$\frac{T_t}{m_0} = \frac{p_t \sum_{i=1}^{X_0} m_i}{m_N X_0} + \frac{p_t\phi \sum_{i=1}^{X_0} \left(m \sum_{j=2}^{n} B_j\right)_i}{n m_N X_0},$$

and, taking number averages,

$$\frac{T_t}{m_0} = p_t\left[\frac{m_N}{m_N} + \phi\frac{\left(m\sum_{i=2}^{n} B_i\right)_N}{nm_N}\right] = p_t\,(1 + \phi K). \qquad [12]$$

K, a constant, stands for

$$\frac{\left(m\sum_{i=2}^{n} B_i\right)_N}{nm_N}.$$

In the case of random degradation ($\phi = 0$), this equation reduces to equation [7]. However, when $\phi > 0$, T_t/m_0 is no longer equivalent to the probability term in equation [11]; experimental data of some other kind are needed in order to evaluate n.

If one determines $(1 - R)/R$ and T_t/m_0 at various intervals for a sample undergoing non-random degradation, and, wrongly assuming random attacks, plots the logarithms of these quantities (as in equation [8]), one obtains a curve of slope σ; this slope, the apparent number of strands per molecule, can be calculated using the right-hand sides of equations [11] and [12]:

$$\sigma = \frac{[\log p_t\,(p_t + \phi)^{n-1}]_2 - [\log p_t\,(p_t + \phi)^{n-1}]_1}{[\log p_t\,(1 + \phi K)]_2 - [\log p_t\,(1 + \phi K)]_1}$$

$$= \frac{[\log (p_t)_2 - \log (p_t)_1] + (n - 1)\,[\log (p_t + \phi)_2 - \log (p_t + \phi)_1]}{\log (p_t)_2 - \log (p_t)_1}. \qquad [13]$$

On the other hand, if one could determine $p_t^{1/n}\,(p_t + \phi)^{(n-1)/n}$ at various intervals for the same sample and plot the logarithm against $\log (1 - R)/R$ as ordinate, the result would be a straight line of slope n, as equation [11] indicates. Dividing equation [13] by n,

$$\frac{\sigma}{n} = \frac{\frac{1}{n}[\log (p_t)_2 - \log (p_t)_1] + \frac{n-1}{n}[\log (p_t + \phi)_2 - \log (p_t + \phi)_1]}{\log (p_t)_2 - \log (p_t)_1}. \qquad [14]$$

since

$$[\log (p_t + \phi)_2 - \log (p_t + \phi)_1] < [\log (p_t)_2 - \log (p_t)_1],$$

and

$$\frac{1}{n} + \frac{n-1}{n} = 1,$$

the denominator of equation [14] is always larger than the numerator, for any positive value of ϕ. Therefore n, the true number of strands, is always greater than σ, the slope obtained when data from non-random degradation are wrongly assumed to fit the equation for random degradation; σ provides a *minimum* estimate of n.

Using the equations already derived and the experimentally available data, it is

possible to estimate the degree to which the enzymatic attacks depart from random. The parameter of interest is ϕ, the value of which ranges from 0 for completely random attacks to 1 for the case in which all strands are inevitably cleaved in sequence at the same point. Dividing equation [11], in the antilog form, by equation [12], and substituting the value of p_t from equation [12], gives

$$\left(\frac{1-R}{R}\right)\left(\frac{1}{T_t/m_0}\right) = \frac{(m\prod B)_N}{1+\phi K}\left(\frac{T_t/m_0}{1+\phi K}+\phi\right)^{n-1}. \qquad [15]$$

When the experimentally derived quantity on the left side of equation [15] is plotted against T_t/m_0, the intercept (at $T_t/m_0 = 0$) is

$$\frac{(m\prod B)_N^n \phi^1}{1+\phi K}.$$

If $\phi = 1$, it can be shown that the intercept is greater than $m/2$; if $\phi = 0$, the intercept is 0, as also predicted by equation [8]. In other words, the intercept increases greatly for non-random reactions and thus provides a sensitive test; an intercept of 0 is excellent evidence for random enzymatic attacks.

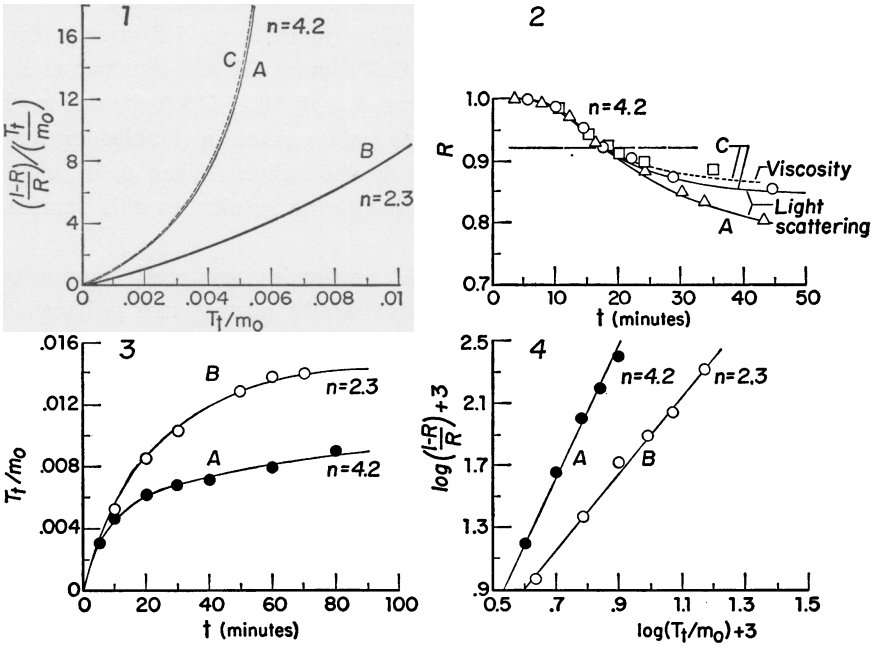
RESULTS

The validity of the equations ([7] and [8]) derived for the degration of DNA by random enzyme attacks was established by plotting the light-scattering and titration data as shown in Fig. 1. According to equation [15], the intercept of such a curve will be 0 only when the bonds are cleaved at random. This was found to be the case for both two- and four-stranded DNA, denatured and undenatured. It is pertinent to note here that since the intercept is zero, the number of preformed breaks in the polynucleotide chains must also be zero (see reference 7, equation 19A).

Fig. 2 shows the kinetics of degradation of undenatured *Escherichia coli* 15_{T-} DNA as observed by both light scattering and viscometry; the values of R [$= (M_t)_w/(M_0)_w$] obtained by the two methods are in close agreement. The degradation of denatured pneumococcus DNA, observed by light scattering, is also included in the figure. In Fig. 3, the fraction of internucleotide bonds cleaved, obtained by titration, is plotted against time. This fraction is equivalent to p_t, the cumulative probability of cleavage of any given bond. In order to calculate the number of strands per molecule one must plot log p_t *versus* log $(1 - R)/R$ (see equation [5]). Accordingly, *time* in Fig. 2 can be converted to T_t/m_0, or p_t, from the data in Fig. 3. Then a plot of log $(1 - R)/R$ *versus* log T_t/m_0, as in Fig. 4, gives the number of strands per molecule directly from the slope. When p_t is obtained in this way it is theoretically possible to utilize data from most of the degradation span for calculating the number (n) of strands per molecule; practically, however, inhibition causes the reaction to stop almost completely when the molecular weight has decreased approximately 25 per cent, for denatured DNA, and

somewhat less, for undenatured DNA (see Fig. 2). It is for this reason that we have performed many of these experiments with denatured DNA.

The value of n can also be calculated independently of titration data, using the relation $p_t = kt$. The degradation data are then plotted as in Fig. 4, except that log t is substituted for log (T_t/m_0) on the abscissa (see equation [6]). Calculation of n by this method is limited to data from the early part of the reaction, where the enzyme activity is constant. We have therefore confined such calculations to the range $1 \geqslant R \geqslant 0.92$ (above the dashed horizontal line in Fig. 2). Variation of the



Curves A and B, pneumococcus DNA (sample LC-I); A ($n = 4.2$) has been heated without cesium chloride, B ($n = 2.3$) has been heated in cesium chloride. Curve C, *E. coli* 15_T- DNA (sample Wθ), undenatured. T_t/m_0 is the fraction of bonds hydrolyzed by the enzyme in time t, and is equivalent to the probability of hydrolysis, p_t; R is the ratio of the molecular weight at time t to that at time 0; $(1 - R)/R$ is the number of molecular cleavages at time t; n is the number of strands per molecule. FIGURE 1 The intercept of 0 indicates that the enzymatic attacks are random (see equation [15]). FIGURE 2 The molecular-weight decay during enzymatic degradation, as determined by light scattering (unbroken curves) and by viscometry (broken curve). Because of increasing enzyme inhibition at later times, only the portion of the curves above the dashed horizontal line was employed when calculating n using the relation $p_t = kt$ (see equation [6]); however, the entire curves were utilized when p_t was evaluated from titration data (equations [7] and [8]). FIGURE 3 The fraction of bonds hydrolyzed *versus* time. FIGURE 4 Logarithmic plots of the number of molecular cleavages *versus* the fraction of individual bonds cleaved. The slopes of the curves give the number of strands per molecule (see equation [8]).

enzyme concentration over a 2.5-fold range did not affect the value of n calculated in this way.

The most reliable n values in Table I were calculated from light-scattering and titration data; the maximum experimental error in n is $\pm$ 0.3. Within these limits, the results are the same for denatured and undenatured DNA, indicating that no interactions occur, after denaturation, which might disturb the degradation kinetics. The experimental error is somewhat greater when titration data are not used, since n must then be calculated only from the early points. However, the use in equation [6] of R from viscometry and p_t from kt provides a totally independent estimate of n for comparison with that derived from light scattering and titration. The results for undenatured calf thymus DNA are in close agreement: 1.5 strands for the former and 1.7 strands by the latter method. Calf thymus DNA is thus two-stranded, within the experimental error. For undenatured *E. coli* 15_{T-} DNA the two methods gave 3.3 and 4.2 strands, respectively. These values agree in establishing the fact that *E. coli* DNA molecules are *not* simple double helices. When p_t is calculated from titration data, the viscosity and light-scattering results for this sample are almost identical (4.1 and 4.2 strands).

The preceding paper predicted that DNA molecules apparently composed of only one replicating unit (unitary molecules) would possess two polynucleotide strands, and that DNA molecules composed of two units (biunial molecules) would possess four strands. These predictions have been confirmed in every case studied: *E. coli* B and 15_{T-}, pneumococcus, and mouse sarcoma-180 all yield biunial DNA of approximately four strands per molecule, while calf thymus yields two-stranded, unitary DNA. When biunial molecules are heated in cesium chloride they separate into units possessing two strands each.

Using the data already presented, it is possible to make a rough estimate of the strength of the bonding between the various strands of the molecule. This is given by B, in equation [8]. Specifically, $B/2$ is a distance, in terms of nucleotides, which is indicative of the number of hydrogen or other bonds required to hold the strands of the molecule together. Substituting the calculated n and experimental values of $(1 - R)/R$ and T_t/m_0 into equation [8], one can calculate $(m\prod B)_N$. If this quantity is set equal to $m_w B_{Av}{}^{n-1}$, then $B_{Av} \approx (\prod B)_N{}^{1/(n-1)}$, in which m_w is the number of nucleotides per weight-average strand and $(\prod B)_N$ is the number-average of the product of the B values for each of the $n - 1$ pairs of strands (see equation [2]). One obtains a B_{Av} of the order of 2 for two-stranded molecules and 50 for four-stranded molecules. This difference is indicative of the lesser strength of the biunial bonding, compared to the hydrogen bonding within double helices. The value obtained for (unitary) calf thymus DNA agrees with the estimate (using DNase I) of Schumaker *et al.* (7), whose $\beta = (B - 1)/2 = 0.5$ to 5. It should be emphasized that these values of B are not very accurate, since they are calculated as the $(n - 1)$th root; a small error in n thus results in a large error in B. The true values of B for denatured and undenatured molecules may therefore differ several-fold (9).

TABLE I

THE NUMBER OF STRANDS (n) IN VARIOUS DNA MOLECULES

Source	Sample code	Class*	Treatment	Molecular weight‡	*n* from light scattering and titration§	*n* from light scattering alone‖	*n* from viscosity
E. coli B	I	Biunial	Heat-denatured	2.4×10^6	3.9	4.3	
E. cloi B	I		Heated in CsCl¶	1.3×10^6	2.2	2.3	
E. coli 15_{T-}	Wθ	Biunial	Undenatured	1.2×10^6	4.2	3.1	4.1§ 3.3‖
E. coli 15_{T-}	J	Biunial	Heat-denatured	1.1×10^6	3.7		
Pneumococcus R6	LC–I	Biunial	Heat-denatured	1.5×10^6	4.2	4.5	
Pneumococcus R6	LC–I		Heated in CsCl	0.84×10^6	2.3	2.5	
Mouse sarcoma-180	LC–SI	Biunial	Heat-denatured	1.3×10^6	3.8	4.8	
Mouse sarcoma-180	LC–SI		Heated in CsCl	0.61×10^6	2.2	2.2	
Calf thymus	ALS	Unitary	Acid-denatured**	1.7×10^6	2.3	2.2	
Calf thymus	JD–CN	Unitary	Undenatured		1.7	1.7	1.5‖

* Based on the criteria discussed in the preceding paper (1).

‡ Measured by light scattering at the start of the kinetic experiment.

§ Calculated using $p_t = T_t/m_0$. This is the more reliable method.

‖ Calculated using $p_t = kt$.

¶ Treatment described in the preceding paper (1).

** Denatured by lowering the pH to 2 and reneutralizing immediately.

DISCUSSION

The equation used to calculate the number of strands in the DNA molecule is restricted in two ways, both of which have been justified experimentally. First, equation [15] and Fig. 1 establish that the enzymatic attacks on DNA are random in our experiments. If this had not been the case, equation [14] shows that the computed number of strands would have constituted a *minimum* estimate. Second, the equation for n is valid only when the maximum number of molecular cleavages per molecule during the experiment is less than m/B (see the discussion following equation [2]). The experimental maximum is 0.28, whereas m/B, also evaluated experimentally, is of the order of 20 to 4000 (depending on the molecular weight and the number of strands). Thus, provided that the following two assumptions employed in the derivation of equation [8] can be justified, it is unequivocally established that the experimental approach employed in this investigation yields the correct number of strands per molecule.

The first assumption is that the change in $\prod B$ during the experiments is negligible. In other words, the number of hydrogen bonds or other links required to hold the strands together in considered to remain constant. If it did not, $\prod B$ would increase, causing the plot of $\log (1 - R)/R$ *vs.* $\log T_t/m_0$ (see equation [8]) to curve upward; only the initial slope would be valid for calculating the number of strands. The assumption is justified by the actual linearity of the curves, as well as by the fact that termination of the experiments when only about 0.28 cleavage per molecule had occurred eliminated the possibility of a significant increase in end effects.

The second assumption is that the number-average ratio $(M_t)_N/(M_0)_N$ can be replaced by the weight-average ratio $(M_t)_w/(M_0)_w$, in the relevant equations. If the nature of the DNA molecular-weight distribution does not change between time 0 and time t, the two quantities will be identical. However, if there *is* a change in the nature of the distribution, they diverge. Now the maximum error thus incurred can be determined by considering the most extreme case; namely, that in which the starting material is monodisperse and is degraded randomly. For this case, Charlesby (10) has developed equations which connect the weight- and number-average molecular weights as a function of the average number of molecular cleavages per initial molecule. We have thus been able to compare, for the extreme case, the slopes of $\log (1 - R)/R$ with respect to $\log p_t$, when

$$R = (M_t)_w/(M_0)_w \quad \text{and} \quad R = (M_t)_N/(M_0)_N.$$

In the region corresponding to our experiments, in which

$$1 \geqslant (M_t)_w/(M_0)_w \geqslant 0.78,$$

and far beyond that region, these slopes are within 3 per cent of one another, although the quantities themselves differ. Thus n, which is determined (see equation [8]) by the rate of change of $\log (1 - R)/R$ rather than by its magnitude, will appear the same regardless of whether number- or weight-average molecular weights

are used in its calculation. If this is true when the starting material is monodisperse, it is true *a fortiori* in our experiments, in which the initial molecular-weight distribution was more nearly random than uniform.

The third assumption employed relates only to the less reliable (see equation [6]) of the two methods for evaluating p_t (the probability of cleavage of any given bond during time t). More accurately, it is a restriction of the experiment to the early part of the degradation reaction, when $p_t = kt$ and the enzyme activity is constant. Since the values of p_t (obtained by titration) in our experiments are all less than 0.02, whereas p_t is proportional to time at values as high as 0.2, the first part of the restriction is no problem. The insensitivity of the calculated n to variation of the enzyme concentration indicates that inhibition of the enzyme by products formed during the part of the reaction when $R \gtrsim 0.92$ is not great. Furthermore, the log $(1 - R)/R$ *vs.* log t curves are linear in this range, as predicted. As the reaction proceeds, however, the slopes of these curves eventually decrease to zero because of enzyme inhibition. Thus, any error caused by the inapplicability of this assumption will decrease the parent number of strands.

CONCLUSIONS

The numbers of polynucleotide strands in DNA molecules from various sources have been calculated from the kinetics of degradation of the DNA by DNase II. The results confirm the hypothesis, developed in the preceding paper, that there are two classes of DNA molecules: those (biunial molecules) possessing two conserved units and those (unitary molecules) possessing one unit. We have demonstrated that there are four strands in biunial molecules, two strands in unitary molecules, and two strands in the units into which biunial molecules separate when heated in cesium chloride. The conserved unit is therefore two-stranded, and the two units within biunial molecules must be laterally associated. These conclusions are shown diagramatically in Fig. 3 of the following paper. The kinetic results also show that the two strands comprising the conserved unit are more strongly bound together than are the units themselves; in other words, the biunial bonding is comparatively weak.

The equations employed to calculate the number of strands have been shown, experimentally and theoretically, to be entirely valid for this purpose. Two experimentally independent procedures have produced concordant results. The distinction in number of strands between the two classes of DNA is therefore unequivocal.

The observations presented in this and the two preceding papers lead to the following conclusions. The DNA units conserved during replication (at least in *E. coli*) are two-stranded, as are DNA molecules from non-proliferating sources (11). It is probable that these are equivalent; *i.e.*, that non-proliferating DNA molecules consist of single conserved units that have not yet replicated themselves.

In DNA from proliferating cells, two conserved units, one parental and one newly synthesized, are laterally attached by some weak type of connection (the biunial bond), thereby forming a four-stranded molecule. The molecules in turn, whether biunial or unitary, are laterally associated, *in vivo;* remnants of this association are present *in vitro* before complete deproteinization of the sample.

This hierarchy of strands might be organized as two sheets, each composed of a row of double helices held together laterally by protein, with each double helix in the parental sheet attached *via* the biunial bond to its newly synthesized counterpart in the opposing sheet (see Fig. 3 of the following paper). Such a model would permit (*a*) disaggregation, producing biunial molecules, by removal of protein; and (*b*) separation of units, either before or after disaggregation, producing unitary molecules (or aggregates thereof) by breaking the biunial bond.

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