The Rate of DNA Strand Separation

ELISABETH BAUTZ FREESE AND ERNST FREESE

From the Laboratory of Molecular Biology, NINDB, National Institutes of Health, Bethesda 14, Maryland

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The melting temperatures T_m of *B. subtilis* DNA have been measured at several values of high and low *p*H in the *pK* range of the DNA bases. At temperatures sufficiently high above T_m transforming DNA is rapidly inactivated by uncoiling. The initial rates of this inactivation have been determined at *p*H 3.65, 10, and 11; they increase rapidly with the temperature. In 0.5 M NaOH, however, in which this inactivation occurs within less than 3 seconds, the initial rate is practically independent of the temperature. This rate is much faster than that predicted by previous hypotheses; it is, however, of the same order of magnitude as the rate predicted by a new hypothesis, which assumes that the necessary rotation is performed by a double-stranded portion remaining unseparated at one end. The proposed mode of spontaneous uncoiling is so similar to the mechanism of DNA duplication that essentially the same minimal times are required for either process. Since these times increase with the square of the molecular weight the possibility has been excluded that DNA in large chromosomes exists as one long uninterrupted double strand.

When chromosomes duplicate, DNA double helices have to unwind. This requires 2×10^4 turns for phage T2, whose DNA is contained in one uninterrupted double strand (Rubenstein *et al.*, 1961; Berns and Thomas, 1961). In larger chromosomes much longer continuous double strands might occur, up to the length of the total DNA complement of the chromosome. Since rotation is impeded by a viscous drag, the rate of strand separation must decrease with the length of the strands. The molecular weight dependence of this rate defines an upper limit for the length of uninterrupted double strands in a chromosome, because the time required for strand separation cannot be longer than the duplication time of the chromosome in which the double strand occurs.

Three different theories have attempted to estimate the rates of strand separation. (Levinthal and Crane, 1956; Kuhn, 1957; Longuet-Higgins and Zimm, 1960). Experimental data, however, are still missing. One could attempt to measure the duplication time of chromosomes *in vivo* and try to decide by physical chemical experiments whether or not one of the strands is occasionally interrupted. However, it is difficult to determine the duplication time of very small chromosomes (e.g., phages), for which physical measurements are possible, and it is hard to unravel the total length of uninterrupted double strands in large chromosomes, for which duplication may be followed individually.

We have therefore used another approach. Theoretical considerations show that the maximal rate of strand unwinding during DNA duplication should have about the same value and the same molecular weight dependence as the rate of spontaneous uncoiling, provided the latter is determined under conditions under which the DNA bases cannot even transiently attach to one another. Using such conditions, we have measured the rate of spontaneous strand separation in transforming DNA. When we equate the observed rate with the maximal rate of DNA duplication, for duplexes of the same length, we can estimate the minimal DNA duplication times for larger uninterrupted double strands and thus obtain an upper limit for their length.

Experimentally, this paper reports DNA melting points at different *p*H values and shows that conditions of negligible base interaction can be obtained only at very high *p*H. At other *p*H values the rate of spontaneous strand separation, measured by the inactivation of transforming activity, depends strongly on the temperature, even quite high above the melting temperature.

MATERIALS AND METHODS

Bacteria.—DNA donor, *Bacillus subtilis* SB 19 (W. R. Romig), prototroph. Recipient strain T_3 (C. Anagnostopoulos), blocked in tryptophan synthetase, requires tryptophan.

Isolation of DNA.—Twenty liters bacteria grown to 3×10^8 cells/ml were spun and resuspended in 400 ml 0.1 M Tris + 0.01 M EDTA, *p*H 8.0 (= Tris-Versene). Mercaptoethanol (to give 10^{-3} M) and lysozyme (to give 100 μg/ml) were added and the cells incubated for 15 minutes at 37°. The partially lysed cells were slowly stirred at room temperature and the following compounds were slowly added in this sequence: 32 ml sodium dodecyl sulfate (= dupanol, saturated in 45% ethanol), which lysed the cells completely; 80 ml sodium-*p*-aminosalicylate (30% solution) (Kirby, 1957); and 250 ml sodium perchlorate (7.1 M solution). The mixture was shaken for 1 hour and, if desirable, left overnight in the cold. Seven hundred ml phenol (Merck liquified) was added and the mixture slowly stirred for 1 hour. After centrifugation most of the DNA was found in the interphase; only when the stirring was too vigorous did some DNA appear in the lower aqueous phase. The interphase was washed with 80% ethanol, removing some protein, and resuspended in 30 ml TV plus 2 ml sodium dodecyl sulfate. The solution was then deproteinized with chloroform–isoamyl alcohol (20:1), treated with RNAase, and the DNA isolated with isopropanol as described by Marmur (1961). However, the DNA was at no stage exposed to more than 80% ethanol, because it then dissolved more readily, even in undiluted buffers, probably because it kept more of its aqueous layer. The DNA was finally dissolved in 2 M NaCl + 5×10^{-3} M Tris + 5×10^{-4} M EDTA, *p*H 8.

The concentration of the DNA used for the experiments in this paper was 360 μg/ml. The sedimentation constant of this DNA was kindly determined by Dr. D. J. Cummings using the conditions given by Thomas and Pinkerton (1962), adding the solution to a liquid boundary cell, and centrifuging at 35,600 rpm at 25°. The midpoint of the sedimentation profile gave a sedimentation constant of 30.65 which corresponds to a molecular weight of about 35×10^6 if one uses the formula of Thomas and Pinkerton (1962).

Melting temperatures were determined by the increase of the UV absorption at 260 mμ, read in a Zeiss spectrophotometer against the same buffer.

Inactivation of Transforming DNA.—The initial rapid inactivation was usually measured by adding DNA to sodium acetate–acetic acid (0.2 M Na) or sodium carbonate–bicarbonate (0.2 M) at the final temperature. Aliquots were diluted into ice-cold Tris (1 M, *p*H 7) after different times. It took less than 10 seconds to transfer the sample from the experimental tube into the neutral buffer; the recorded time was that at which the DNA was blown into the ice-cold Tris. As control, DNA was diluted directly into the Tris.

In order to measure the much more rapid inactivation in 0.5 M NaOH, aliquots of 10 μl DNA were carefully added to the bottom of several tubes with a lambda pipette having a constant internal diameter. The tubes, pipettes, and solutions were equilibrated at the temperature of the experiment. Then 0.5 ml of 0.5 M NaOH was injected using a rubber bulb on a 1-ml blow-out pipette whose end was close to the DNA; 4.5 ml ice-cold Tris (1 M, *p*H 6.8) was squirted in shortly afterwards by means of a pro-pipette. We measured the time between the instant at which NaOH came into contact with the DNA and that at which Tris was mixed into the DNA + NaOH. Working with two persons the shortest time recorded was about 0.5 second.

For the long-term inactivation, DNA was mixed with the ice-cold reaction mixture (below the melting point), a sample was withdrawn as the zero time control, and the rest was placed in the water bath at the temperature of the experiment.

The same batch of transforming bacteria was used to determine all points of an inactivation curve.

Transformation procedure.—To 1 ml bacteria, grown for 4 hours, spun, and resuspended according to the method of Anagnostopoulos and Spizizen (1961) 0.1 ml of the DNA solution in 1 M Tris, was added. The final concentration of DNA was 0.05 μg/ml or less, which is below the saturation level. After 2 hours slow shaking, the bacteria were diluted in minimal medium and plated on minimal agar containing 250 μg/ml of vitamin-free casein hydrolysate, acid (Nutritional Biochemicals Co.) The number of transformed bacteria was counted after about 16 hours incubation at 37°.

Results

A. *Melting Temperatures at Different pH's and in Different Buffers.*

The partial or complete separation of DNA strands is accompanied by an increase of the extinction at 260 mμ. The melting temperature T_m is defined as the midpoint of this melting curve (Doty *et al.*, 1960). T_m remains nearly constant in the *p*H range from 5 to 8, at a sodium concentration of 0.2 M, and decreases outside this *p*H range, as can be seen for low *p*H in Figure 1 and for high *p*H in Figure 2. It should be noted that all *p*H values have been measured at room temperature (25°) and must be corrected for higher temperatures. For the buffers used here the correction is negligible at low *p*H but drastic at high *p*H (see Fig. 2). Cations with two amino groups protect DNA against melting at low *p*H but not at high *p*H.

1. Low pH.—The change of T_m with *p*H is shown in Figure 1. In 0.2 M sodium acetate the T_m drops sharply when the *p*H goes below 4. When the monoamines glycine and ethanolamine (0.2 M) are used in place of sodium acetate the T_m values are slightly higher, while tributylamine (0.2 M), which has a smaller surface charge, shows no protection. The diamines lysine and putrescine are very effective protecting agents, putrescine slightly more than lysine, both at 0.02 M (see Table I), probably because the carboxyl group in lysine partially neutralizes the effect of the adjacent amino group.

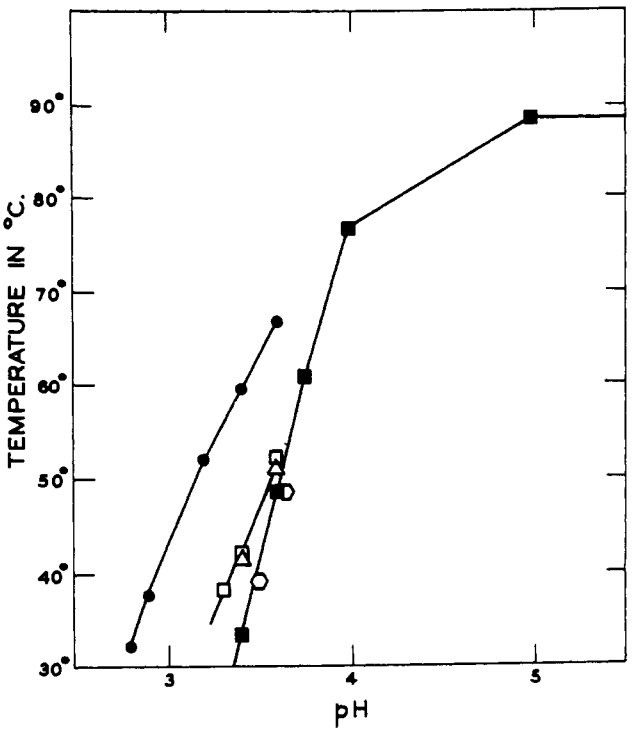

FIG. 1.—Melting temperatures (T_m) of *B. subtilis* DNA at acid *p*H: ■ sodium acetate, ⬡ tributylamine, □ glycine, △ ethanolamine, ● lysine; 0.2 M each, *p*H measured at 25°.

In order to estimate the change of *p*H with temperature, we have measured the actual *p*H at various temperatures for both lysine and sodium acetate, using

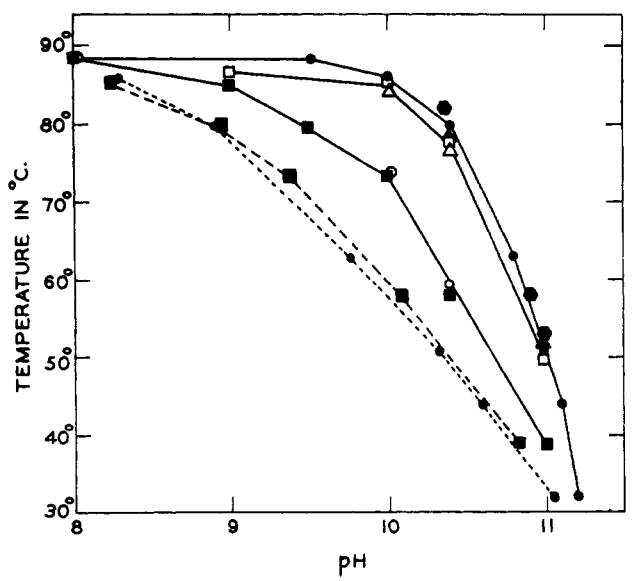

FIG. 2.—Melting temperatures (T_m) of *B. subtilis* DNA at alkaline *p*H: ● lysine, ⬤ putrescine, □ glycine, △ ethanolamine, ▲ γ-aminobutyrate, ■ sodium carbonate, ○ sodium phosphate; 0.2 M each. The measurements were made in solutions of which the *p*H was measured at 25° (solid lines). The dashed lines connect the points that were obtained by using the actual *p*H values of the buffer at the observed T_m.

TABLE I

MELTING TEMPERATURES (T_m IN °C) OF *B. subtilis* DNA IN SOME BUFFERS OF *p*H 3.6 (AT 25°)

Concentration of Compound	0.02 M	0.2 M
Sodium acetate	47	48.5
Tributylamine	47	48.5
Ethanolamine	47	51.2
Glycine	47	52.0
Lysine	51	67.0
Putrescine	58	—

as standard potassium hydrogen phthalate (0.05 M) for which the *p*H change with temperature is known. Even at 70° the *p*H change was not more than 0.1 *p*H unit; this effect is negligible for our purpose (it actually increases the protection slightly).

2. *High* *p*H.—Figure 2 (solid lines) gives the T_m for buffers, whose *p*H was determined at room temperature (25°). At first sight mono- and diamines seem to protect efficiently against melting; but the *p*H change of the buffers with temperature is very large under these conditions. We have measured the actual *p*H of the sodium carbonate and the lysine buffers at different temperatures by using as standard a glycine-NaCl-NaOH buffer for which the *p*H at different temperatures is given in the International Critical Tables (1926). The dashed lines in Figure 2 show the melting temperatures for the corrected *p*H values. There remains no significant difference between the melting points in lysine and sodium carbonate. Glycine has a *p*H dependence similar to lysine and should approximately coincide with the corrected curves. For putrescine *p*H changes with temperature have not been measured, but it should behave similarly to lysine. The absence of a protective effect is understandable because the *pK* values of the amino compounds decrease so much with temperature that the amino groups are essentially uncharged within the melting range. Glycine, for example, has a *pK* of 9.78 at 25° and one can calculate (see Edsall and Wyman, 1958) or directly see from the International Critical Tables (1926) that its *pK* is about 8.0 at 90°. A similar decrease of about 1.8 *p*H units would be expected (according to our measurements) for the *pK* values of lysine which at 25° are 8.95 for the α- and 10.53 for the ϵ-amino group.

B. *Inactivation of Transforming Principle*

When transforming DNA is exposed to temperatures several degrees above the T_m its transforming activity rapidly decreases. In order to measure the rate of this decrease at different temperatures we used buffers at *p*H 3.65, 10.0, and 11.0 at which the T_m are sufficiently low. Since the inactivation rates at these *p*H's still increased rapidly with the temperature, we employed, in addition, a very high *p*H.

1. *p*H *10 and 11.*—The inactivation of transforming activity was studied by exposing DNA to 0.2 M sodium carbonate–bicarbonate buffers of *p*H 10.0 (T_m = 73°) and 11.0 (T_m = 39°). The *p*H was measured at room temperature (25°). At temperatures below 40° no inactivation was observed; this shows that the chemical effect of *p*H 11 itself is negligible at low temperatures. At this *p*H, the transforming activity remained constant even up to about 4° above the T_m. However, when DNA was exposed to temperatures above 45° the transforming activity rapidly decreased initially and then remained constant at about 1–2% of the original activity (see Fig. 3); only at higher temperature did it slowly continue to decrease. The same

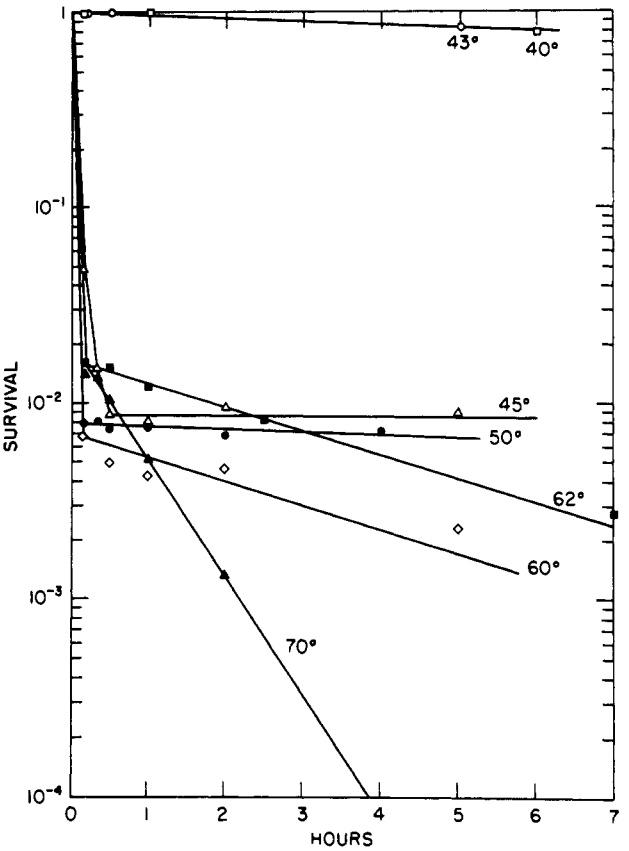

FIG. 3.—Long-term survival of transforming DNA in 0.2 M sodium carbonate–bicarbonate, *p*H 11.0, at different temperatures: □ 40°, ○ 43°, △ 45°, ● 50°, ◇ 60°, ■ 62°, ▲70°

survival of 1–2% was found when one-fifth the concentration of DNA was used in the reaction tube.

The initial inactivation was followed more closely in order to measure its rate at different temperatures. The results in Figure 4 show that the rate of uncoiling is temperature dependent. The initial rate

$$k = \frac{1}{t_2 - t_1} \ln \frac{N_1}{N_2}$$

(t = time in hours, N = number of transformants/ml)

decreases linearly with increasing $1/T$ (T = absolute temperature) (see Fig. 8).

Since the *p*H of the sodium carbonate buffer decreases with increasing temperature (see Fig. 2) the actual *p*H values at high temperatures were lower than those measured at 25°. If the actual *p*H had been kept constant for the various temperatures, the slopes of the high *p*H curves in Figure 8 would very likely be steeper, because the binding of DNA bases decreases with increasing *p*H.

2. *pH 3.65.*—The T_m of *B. subtilis* DNA in 0.2 M sodium acetate at *p*H 3.65 is about 50°. At this *p*H the transforming activity decreases even when the temperature is below T_m as shown in Figure 5. The "activation energy" of this process is about 20 kcal/mole (see Fig. 8).

Above the T_m, however, the transforming activity decreases much more rapidly than below (see Fig. 6). Again, the initial inactivation rate increases with the temperature (see Fig. 8) with an "activation energy" of about 49 kcal/mole. After the initial rapid drop of transforming activity a slower inactivation follows (see Fig. 6) with a rate similar to that observed below the melting temperature. The residual transforming

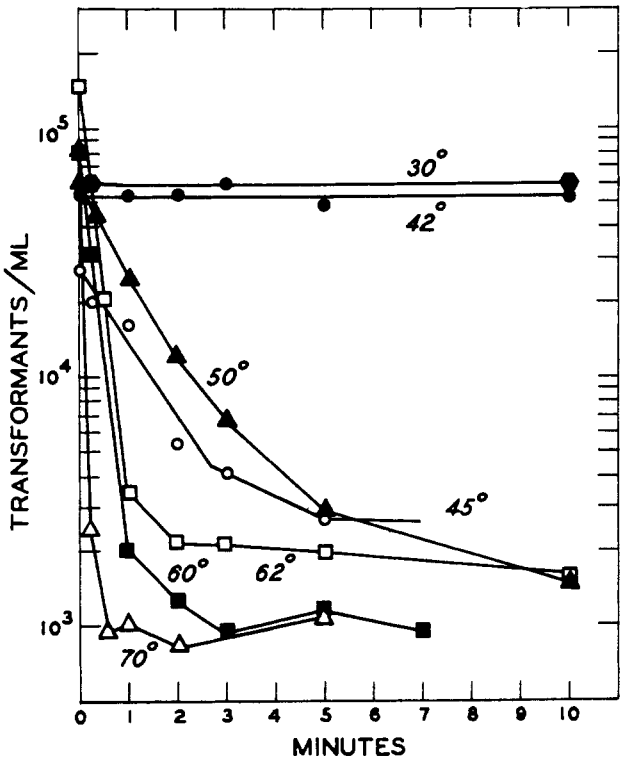

FIG. 4.—Short-term inactivation of transforming DNA in 0.2 M sodium carbonate–bicarbonate, *p*H 11.0, at different temperatures: ⬬ 30°, ● 42°, ○ 45°, ▲ 50°, ■ 60°, □ 62°, △ 70°.

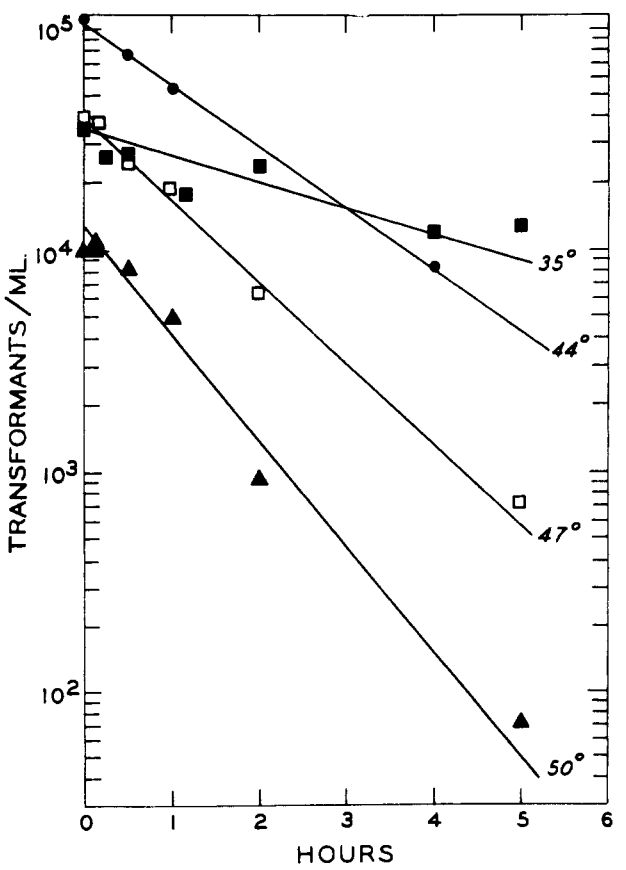

FIG. 5.—Inactivation of transforming DNA in 0.2 M sodium acetate *p*H 3.65 below the melting temperature: ■ 35°, ● 44°, □ 47°, ▲ 50°.

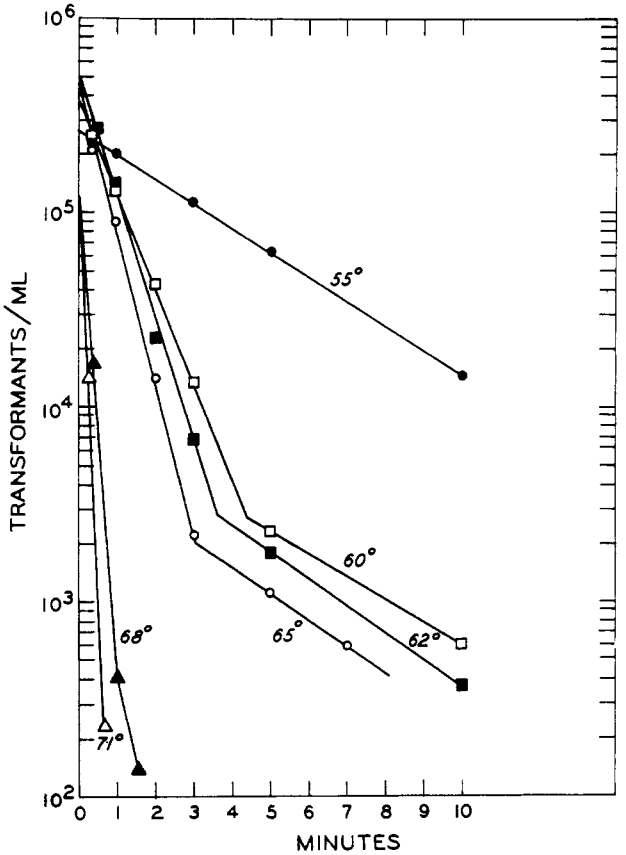

FIG. 6.—Short-term inactivation of transforming DNA in 0.2 M sodium acetate, *p*H 3.65, above the melting temperature: ● 55°, □ 60°, ■ 62°, ○ 65°, ▲ 68°, △ 71°.

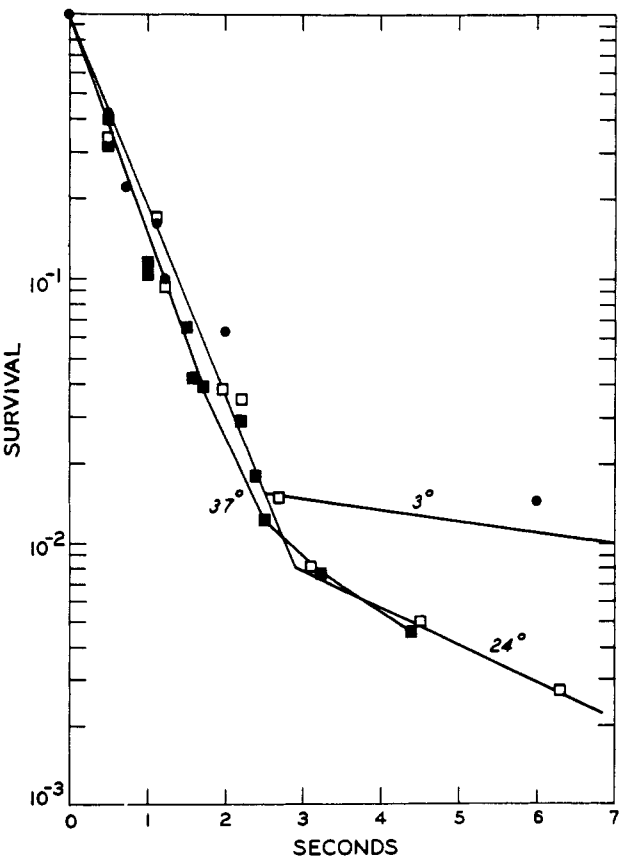

FIG. 7.—Survival of transforming DNA in 0.5 M NaOH: ● 3°, □ 24°, ■ 37°.

activity is about 1–2% of the original value, when the ultimate slope is extrapolated to zero time.

3. 0.5 M NaOH.—In 0.5 M NaOH (= *p*H 13.5 at 25°) DNA melts even at 0°. Figure 7 shows that the transforming activity rapidly decreases, within 3 seconds, and then continues to decrease much more slowly. We have followed the decrease at 3° for 60 seconds and still found a survival of about 2×10^{-3}. The rate of inactivation depends very little on the temperature as can be seen from Figure 7 as well as from the plot of rates against $1/T$ in Figure 8.

Discussion

A. *The Melting Temperature of DNA*

In the *p*H range from 5 to 8 the melting temperature is nearly constant; outside this range it decreases because the DNA bases become charged and lose their ability to make hydrogen bonds. The *p*H dependence of DNA denaturation at room temperature has been often described (e.g., Jordan, 1960). At present it is not possible to correlate quantitatively the melting points at different *p*H's to the fraction of charged bases, because the *pK* values of the bases in DNA differ from those of the free nucleotides and they depend on both salt concentration and temperature (e.g., Jordan *et al.*, 1956). For example cytidine (in 0.1 M NaCl) has a *pK* of 4.1 at 26° and 3.8 at 80° (Miles, 1962); no such information seems to be known for the DNA nucleotides.

*At acidic p*H DNA keeps stable down to rather low *p*H values. For example, in 0.2 M sodium acetate at *p*H 3.5, *B. subtilis* DNA melts at about 39°. The melting temperature at a given *p*H is higher when cations with one or two amino groups are present. This effect has already been reported for neutral *p*H by Mahler *et al.* (1961) and by Tabor (1962); it is more pronounced at low *p*H. The larger the number of positively charged amino groups on the molecule the more efficient is the protection. This is probably not only due to the larger charge density of the positive ions but also is caused by the spatial separation of the charges. The more amino groups a molecule has, the larger is the probability that at least one of them remains attached to DNA when the others separate; this makes reattachment of the separated amino groups very probable and, on the average, keeps the molecule attached for longer times.

*At basic p*H the *pK* values seem to decrease with increasing temperature, because the melting temperature of DNA begins to decrease above *p*H 8. Mono- or diamines offer little or no protection against melting at high *p*H. These compounds are essentially uncharged in the *p*H range in which the T_n drops, because their *p*K values decrease rapidly with increasing temperature.

B. *Inactivation of Transforming DNA*

When the temperature is below T_m the transforming activity remains almost constant or decreases slowly, depending on *p*H and temperature. At low *p*H the decrease is significant and its rate rapidly increases with the temperature; the inactivation is probably caused by depurination (e.g., Tamm *et al.*, 1952). However, barely any inactivation can be observed at high *p*H, even when the temperature is raised slightly above T_m. This resistance of DNA against strand separation above the T_m is well known. Geiduschek (1961) and Freifelder and Davison (1962) showed that strand separation starts only in the upper portion of the melting curve and that some DNA strands separate only when the temperature is well above the T_m. In addition, Roger and Hotchkiss (1961) as well as Ginoza and Zimm (1961) found that the critical denaturation temperature of transforming DNA differs for different markers. The strands of the DNA pieces, containing the tryptophan marker for which we have assayed, undoubtedly uncoil partially when the temperature is above T_m. Loops within a single DNA molecule have actually been seen in the electron microscope (Beer and Thomas, 1961). But when the DNA is returned to neutral *p*H and low temperature the partially separated strands can apparently rewind sufficiently to retain full transforming activity.

1. The Residual Transforming Activity of Denatured DNA.—At temperatures sufficiently high above the T_m the transforming activity decreases at first rapidly and then much more slowly. The initial decrease must be caused by the uncoiling of the two DNA strands, whereas the later slow decrease must be due to some chemical reaction (e.g., depurination, chain scission, etc.). The density labeling experiments, e.g., of Schildkraut *et al.* (1961), have shown that *most* DNA strands actually separate completely when DNA is exposed to temperatures sufficiently high above the melting point. The only remaining question is why the rapid inactivation of transforming activity does not go to completion; the slow decrease can be extrapolated to zero time yielding survival values of about 1–2%. Similar observations have been made at neutral *p*H by Ginoza and Zimm (1961) and by Marmur *et al.* (1962). Either all strands separate completely and the single strands (one or both of a double strand) are able to transform with about 1–2% the efficiency of double strands, or a few per cent of the double strands remain somehow attached and transform together. (The residual transformation cannot be explained by renaturation of completely separated strands, for the same survival is observed at different DNA concentrations.) We have not investigated this alternative any further since the decision is unimportant to our problem of the rate of uncoiling. But our results indicate that any bonds keeping the two strands together would have to be covalent bonds since the residual transforming activity is retained for rather long times, even at *p*H 13.5 at which all ionic and most hydrogen bonds must be eliminated.

2. Temperature Dependence of the Rates of DNA Uncoiling.—Above the melting point, the rate of the rapid initial inactivation of transforming DNA, i.e., the rate of DNA uncoiling, increases with the temperature. The temperature dependence can be formally described by the Arrhenius equation $k \sim e^{-E/RT}$ (see Fig. 8); the Boltzmann factor shows that the two DNA strands continue to interact with each other, even high above the melting point, until they are separated. This separation is rather slow and should be faster with shorter G-C regions.

This picture shows immediately that measurements at neutral *p*H, even at high temperatures, do not permit a decision as to how fast two DNA strands would unwind if no bonds kept the DNA molecules together. For it is not known to what extent the residual binding between the DNA bases influences the rate of strand separation.

One can, however, approximate conditions under which the residual binding of the bases is eliminated, by exposing DNA to very high *p*H (0.5 N NaOH), at which hydrogen bonding is virtually impossible. The DNA strands then uncoil rapidly (within 3 seconds) (see Fig. 7), with a rate that depends very little, if at all, on the temperature (Fig. 8), indicating that binding forces between the strands are negligible.

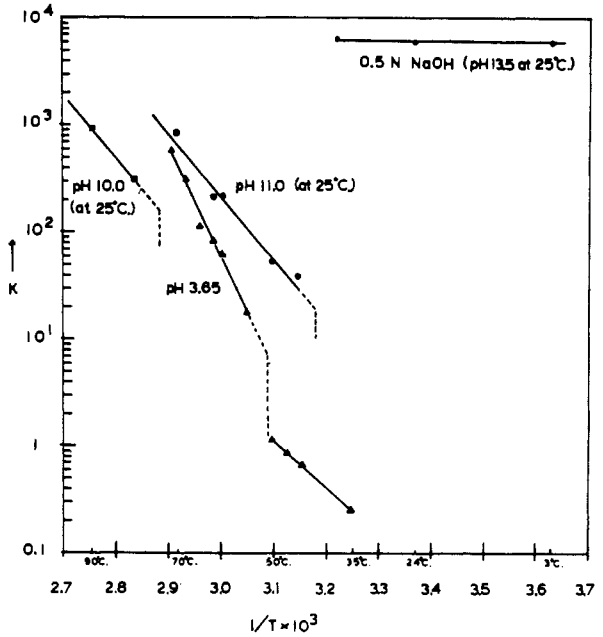

FIG. 8.—Initial rates K of inactivation of transforming DNA plotted against $10^3/T$ (T = absolute temperature): △ *p*H 3.65, □ *p*H 10.0, ○ *p*H 11.0, and ● = 0.5 N NaOH (*p*H 13.5). All *p*H values measured at 25°.

The rapid initial inactivation of transforming activity caused by strand uncoiling, occurs exponentially. At *p*H values at which the uncoiling rates strongly depend on the temperature, the exponential curve is mainly the result of the thermodynamic quasi equilibrium of binding and separating DNA bases. The present discussion shall be limited, however, to very high *p*H values at which any residual binding between the bases appears to be very small. Under these conditions, the exponential inactivation may be mainly the result of two factors. First, the DNA sample, prepared by a rather rough method, contains a broad distribution of molecular weights. Second, DNA pieces of a given molecular weight separate at different times, because their uncoiling is subject to Brownian motion; the mean value of this separation time increases with the molecular weight. A superposition of these two distribution functions may approximately produce an exponential inactivation curve.

Our DNA sample had an average molecular weight of 35×10^6; the molecular weight distribution about the mean is not known. We estimate about 0.5 second for the time at which the average molecules of 35×10^6 mw separate their strands at very high *p*H, because at this time about 60% of the transforming molecules have been inactivated by strand uncoiling.

C. *Comparison with Hypotheses on Spontaneous Strand Separation*

The separation of the two intertwined DNA strands requires their rotation about each other for as many times as there are turns in an uninterrupted double helix. Regarding conditions under which no hydrogen bonds can be made, the maximal rate of strand separation can be calculated by equating the free energy, available to "drive" the rotation, with the energy that is dissipated by the viscous friction of the strands rotating at a rate to be determined.

1. Available Free Energy.—A torque (= free energy per rotation) driving the spontaneous strand separation can be created by both energy and entropy changes which result from uncoiling. The entropy increases because the ordered double strand is converted into two random coils; this contributes about 10 kT (k = Boltzmann constant; T = absolute temperature) to the free energy per base pair uncoiled (Longuet-Higgins and Zimm, 1960). The energy decreases because the distance of two highly negatively charged strands increases. The coulomb energy of two unit charges in the distance of 20 A is 1.15×10^{-12} erg or about 29 kT (for T = 300). The actual energy gain per base pair uncoiled is smaller because a large portion of the negative charge is neutralized by surrounding free and attached cations of the solvent. Owing to charge repulsion the rate of uncoiling at high *p*H, at which even the DNA bases are negatively charged, may be slightly larger than the rate which would be obtained at neutral *p*H if all base interaction could be eliminated.

2. Viscous Drag and the Time of Spontaneous Strand Separation.—The spontaneous separation of DNA strands could come about by several modes of uncoiling differing in the amount of viscous drag. The fastest process should determine the predominant mode and the separation time actually required.

Kuhn (1957, 1961) assumed that the two noninteracting strands initially separate by a rotational translation in opposite direction (as if pulled from opposite ends). Later, they were supposed to increase their relative distance, which requires more time and shall here be neglected. Treating the strands as rigid ellipsoids rotating by Brownian motion, Kuhn calculated for the time of complete unwinding:

$$\tau = \frac{43}{T} \times M^3 \qquad (1)$$

(M = molecular weight in units 10^6, τ in seconds)

Longuet-Higgins and Zimm (1960, 1961) proposed that DNA uncoils from *both* ends. The double stranded portion would then be at rest while the two randomly coiled portions at each end would rotate about each other. Using the entropy change to drive the rotation the authors calculated:

$$\tau = \frac{1.4}{T} \times M^{5/2} \qquad (2)$$

The initial uncoiling may proceed by this mode since the flailing motion of two short strands may dissipate less energy than the rotation of a long double helix. But the random coils soon become so bulky that their viscous drag is larger than that of unseparated double strands rotating about their axis.

A third mode of uncoiling is illustrated in Figures 9a and 9b. Uncoiling would start, by chance, most likely at one end (Fig. 9a) but perhaps occasionally within the double helix (Fig. 9b). As the single stranded portions increase in size their rotation about each other would slow down while any double stranded portion, having an unseparated end, would rotate at an increasing rate about its own axis and eventually take over most of the necessary rotation. If both ends should have started to uncoil, by flailing of the single strands, one of them would become bulkier by chance; this end would continue to unravel while the other end would soon be forced to stop because the double stranded portion started rotating in a sense opposite to that required for uncoiling of that end. Since the torque of this rotation would tend to rewind the strands, some loops or coils formed previously might thus become partially or completely eliminated again. In contrast to the second mode, at least one of the ends of the double helix would remain coiled (or nearly so) and take up the rotation, while the random coils would

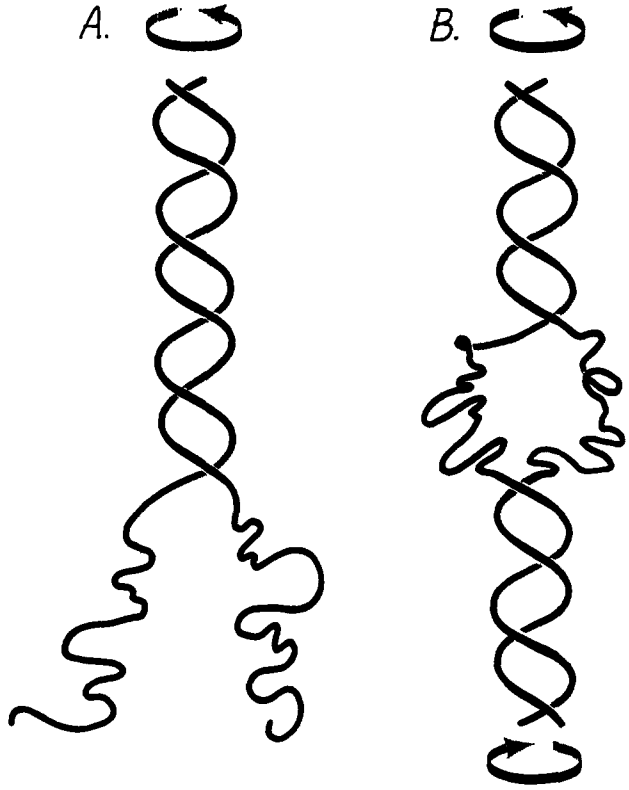

FIG. 9.—Proposed model of DNA strand separation: (a) Spontaneous uncoiling from one end. The other end takes up the rotation while the random coils rotate little or not at all about each other. (b) Spontaneous uncoiling starting within the duplex. Both ends rotate, in opposite direction.

remain nearly at rest as soon as they become large. In most cases only one large uncoiling center should occur in an uninterrupted double helix. The time dt required for dn rotations of the double helix is obtained by equating the torque of a cylinder rotating in a viscous medium (e.g., Page, 1955) with the torque created by the process of uncoiling (= free energy ΔF per radian):

$$dt = \frac{16\pi^3\eta p r^2}{10 \times \Delta F} n dn \qquad (3)$$

with $\Delta F = C + 10\,kT$

(η = viscosity coefficient, p = pitch of the helix = 3.4×10^{-7} cm, r = radius = 10^{-7} cm, n = number of turns in the rotating double stranded portion, C = coulomb term). Integrating over all turns in the double helix and inserting $n = 10^3\,M/6.18$ and η = 1 centipoise, we get:

$$\tau = \frac{8\pi^3\eta p r^2}{6.18^2 \times \Delta F} 10\,M^2 = \frac{1.6 \times 10^{-2}}{T\left(1 + \frac{C}{10kT}\right)} M^2 \qquad (4)$$

This would be the separation time for the mode of uncoiling shown in Figure 9a. In the case of Figure 9b both ends could rotate and the separation would occur slightly faster (by a factor two if uncoiling started in the middle).

A comparison of (1), (2), and (4) shows that the third mode of uncoiling is the fastest and therefore should be the predominant one, at least for large DNA pieces.[1] The three modes differ also in their molecular weight dependence; this could be checked for different homogeneous DNA samples of known molecular weight.

[1] See note added in proof.

TABLE II

HYPOTHETICAL TIMES (IN SECONDS) OF SPONTANEOUS STRAND SEPARATION FOR UNINTERRUPTED DNA DOUBLE HELICES OF DIFFERENT MOLECULAR WEIGHT M

Calculated for three modes of uncoiling discussed in the text. M in units 10^6, T = 300, C small compared to 10 kT.

Formula	M = 35	M = 130	M = 10^3
(1) $\tau = 1.4 \times 10^{-1} \times M^3$	6.0×10^3	3.1×10^5	1.4×10^8
(2) $\tau = 4.6 \times 10^{-3} \times M^{5/2}$	33	870	1.5×10^5
(4) $\tau = 5.3 \times 10^{-5} \times M^2$	0.07	0.9	53

Table II gives the strand separation times predicted by the three hypotheses for different molecular weights. A comparison with our experimental value of 0.5 second for DNA of 35×10^6 mw shows that the first two hypotheses give far too long times while the third hypothesis predicts a shorter time than observed. Since the calculated times were minimal estimates the first two hypotheses seem to be experimentally ruled out.

D. *Comparison of DNA Duplication with Spontaneous Strand Separation*

1. The Mechanism of DNA Duplication.—Watson and Crick (1953) originally proposed that DNA duplication proceeds zipperlike from one end by separation of the strands and the formation of new complements along *both* parental strands, immediately after they have separated. (For a detailed discussion of this and other mechanisms see Delbrück and Stent, 1957). The finding of Kornberg (1957) that DNA polymerase attaches nucleotides only to the 3′ end of a growing DNA strand suggested that the two new strands may have to grow in opposite directions. This possibility can be further subdivided into at least two modes, one in which strand unwinding concurs with the onset of DNA duplication and another in which it takes place later.

In the first mode the two parental strands would unwind wherever their hydrogen bonds are broken in the course of duplication. Since only one strand is copied, in the area in which the strands separate, both a daughter double strand and a free single strand would be formed intermediately. The single strand would be copied later. However, the unseparated end of the parental double helix could not start duplicating before the strand separation were complete since it would rapidly rotate, as explained below.

In the second mode a new strand would also be formed by breaking the existing hydrogen bonds and forming new ones between the new strand and one parent. The other parent, however, would not unwind but remain coiled about its partners until later. Since the strands would not yet unwind no DNA rotation would be required at this stage. DNA duplication could therefore start from both ends (copying always the strand with the 3′-hydroxyl end). The unwinding of the parental strands would start later in that DNA region (end or middle) in which one parental strand had already been copied and the copying of the second strand would begin.

Whatever the actual mechanism of DNA duplication may be, it is clear that the parental strands have to rotate about each other, whenever they begin to separate completely. It is very unlikely that this rotation would be performed by the already unwound portions of the parental strands, since this would lead to their entanglement: chromosomes (even ring chro-

TABLE III

MINIMAL TIMES NEEDED FOR STRAND SEPARATION IN DNA DUPLICATION

M = molecular weight of an uninterrupted double strand; length = total length of such a strand. The times have been calculated under the assumption that a DNA double helix of 35×10^6 mw needs at least 0.5 second for strand separation by DNA duplication and that τ increases proportional to M^2.

M in 10^6	Length in cm	Minimal Separation Time, τ	Representative Organism in Which Chromosomes of Such DNA *Content* Occur	DNA Content per Cell in 10^{-13} g	No. of Chromosomes per Cell Assumed	Maximal Time Available for the Duplication of Chromosomes	Literature
1.3×10^2	7.1×10^{-3}	7 sec	phage T2	—	1	3 min	Rubenstein *et al.*, 1961 Berns and Thoma s,1961
2.4×10^3	0.13	39 min	*Salmonella typhi-murium*	0.004 (nuclear body)	1 (nuclear body)	30 min	Schaechter *et al.*, 1958
6×10^4	3.3	400 hr	Human	5	46	10 hr	Vendrely, 1955
4×10^5	22	2×10^4 hr	Frog	15	26	1 hr	Vendrely, 1955 Pollister & Moore, 1937
1.2×10^6	59	2×10^5 hr	*Vicia faba*	24	12	6 hr	Ishida *et al.*, 1961 Taylor, 1962
6×10^6	330	4×10^6 hr	*Amphiuma*	168	30	?	Vendrely, 1955 Taylor, 1962

mosomes) usually duplicate without entangling. The unwinding of the strands would rather be achieved by the rotation of the helical portions about their own axes, whether they are double or triple stranded. Irrespective of the detailed mechanism a second round of duplication could not be started until the unwinding of the parental strands were complete, because rotating strands cannot separate.

2. *Calculation of the Minimal Time Required for DNA Duplication.*—DNA cannot duplicate faster than its strands can unwind. Since the rotation of DNA, necessary for the unwinding process, requires energy its rate is limited by the energy available. This allows one to calculate the minimum time required for the duplication of an uninterrupted double strand of a given molecular weight. The actual duplication time may be larger, because the DNA polymerase may not act as fast as the strands can rotate, or the DNA precursors may not be available at the necessary rate. These features depend on the particular organism and shall not concern us here.

The minimal time of strand separation during DNA duplication can be calculated from the torque of a rotating cylinder, as shown by Levinthal and Crane (1956). These authors have argued that the free energy needed for the rotation of duplicating DNA must come somehow (directly or indirectly) from the energy liberated during the polymerization process.

The hydrolysis of a high-energy phosphate bond yields about 8 kcal/mole. We shall assume that the same energy is liberated when a nucleotide is incorporated into a polynucleotide strand. For the modes of DNA duplication, for which only one strand is copied at the place at which the strands unwind, the energy available to drive the unwinding would therefore be about $6.6 \times 10^{23} = 1.3 \times 10^{-23}$ kcal $= 5.6 \times 10^{-13}$ erg, or about 13 kT (for $T = 300$) per nucleotide pair unwound. If both strands would be copied in one unwinding area the available energy would be twice this amount. But the exact value is not as important, for the following comparison, as the fact that the free energy, maximally available to drive the rotation of DNA during strand separation, is about the same for DNA duplication as for spontaneous uncoiling.

We shall use for the free energy available to drive strand unwinding $\Delta F = 5.6 \times 10^{-13}$ erg and for the other constants the same values as in (4) (this assumes that an intermediate triple strand would still have the radius of about 10A). Inserting these values in the formula of Levinthal and Crane we obtain:

$$\tau = 8.0 \times 10^{-5} \times M^2 \qquad (5)$$

(The τ values, obtained under the assumptions that either all helices or only the unseparated parental strand rotate, or that duplication starts either at one or at both ends, differ from [5] only by a factor 2.)

A comparison of (4) and (5) shows that the hypothetical minimal strand separation times have about the same value for spontaneous uncoiling as for DNA duplication, and they both increase with the square of the molecular weight. We may therefore assume that the experimental times observed for the spontaneous separation of DNA pieces with a given molecular weight are approximately also the minimal times at which uninterrupted DNA helices of the same molecular weight could duplicate. If we adjust the constant in (5) (multiply by 7) to give our experimental value of 0.5 second for a molecular weight of 35×10^6, we can estimate how fast double helices with larger molecular weight can maximally separate their strands in DNA duplication. Such values are summarized in Table III.

3. *Consequences for the Structure of DNA in Chromosomes.*—The smallest double strand mentioned in Table III is that of phage T2 whose DNA is contained in an uninterrupted double strand of a molecular weight of about 130×10^6 (Rubenstein *et al.*, 1961; Berns and Thomas, 1961). It is unimportant for this discussion whether the strands contain any non-DNA links or not. According to Table III the T2 chromosome would need at least 7 seconds for its duplication. The actual duplication time is not known, but it cannot be larger than 3 minutes.

A bacterial chromosome could also consist of one uninterrupted double strand and still duplicate within about 30 minutes. Larger chromosomes, however, cannot contain all their DNA in a few large uninterrupted strands, at least not throughout DNA duplication, because the minimal duplication times would become excessively large. According to Table III the upper limit for the molecular weight of uninterrupted double strands would be about 10^{10}, several powers of 10 smaller than the total amount of DNA in some chromosomes.

The major factor in (1) to (5) determining an upper limit of the molecular weight is the exponent of M. It is noteworthy that the minimal strand separation time must increase with at least the square of the molecular weight, irrespective of the particular theory employed. The friction, which opposes each rotation of the strands about each other, is proportional at least to the first power of the length of the strands, i.e., to M. Since the number of rotations required for complete strand separation is also proportional to M, the time increases at least with M^2.

Both experimental and theoretical reasons induce us therefore to state that in larger chromosomes swivel points must exist, at least during DNA duplication, at which one of the DNA strands is interrupted and can rotate about the other. This leaves it open whether the interruptions are made at random, during or before DNA duplication, and can be repaired at some later time, or whether they occur at specific places in the genome. It also leaves it undecided whether or not any non-DNA links exist (covalent or others).

Added in Proof

Essentially the same conclusion, derived independently, has been published recently by M. Fixman (1963), *J. Mol. Biol. 6*, 39.

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