THE RENATURATION OF DENATURED DNA

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ABSTRACT The kinetics of renaturation of heat-denatured DNA from *E. coli* and *pneumococcus* have been examined by ultraviolet absorption measurements. The molecularity of the reaction was assessed by three independent treatments of the data, and all lead to the conclusion that renaturation is essentially first order at 60°; at 70° and 80° there is an increasing second order component, resulting in simultaneous unimolecular and bimolecular kinetics. The unimolecular kinetics rule out reaction between two, kinetically separate strands, indicating rather the zippering-up of a single, denatured entity. The bimolecular kinetics can be attributed to the complexing of two such entities; thus, the genetic or density-labeled complexes that have been observed by other investigators can be accounted for without invoking strand separation. Since renaturation at best is never complete, the free ends of *two* renatured molecules permit sufficient bimolecular reaction to produce density hybrids. The observed kinetics can be accounted for if the hydrogen bonds of DNA are broken during heat denaturation but the strands do not separate. Light scattering supports this by showing that the molecular weight is unchanged by denaturation. Since there is no existing evidence that is inconsistent with this hypothesis, it is reasonable to conclude that heat denaturation does not completely separate the entangled strands of the DNA molecule.

When solutions of DNA are heated above a critical temperature, the helical structure collapses as shown by a variety of physical techniques. We wish to know whether this collapse is accompanied by a separation of the polynucleotide chains of the helix. A review of the evidence bearing on this question has been presented elsewhere (1). In this laboratory we have employed several experimental approaches to the problem. One involved the determination of the molecular weight; we carried out this study with disaggregated samples of DNA and found, that while the molecules became more compact on denaturation, there was no change in molecular weight (2; see also Rice and Doty, 3). In addition, we followed the kinetics of DNase degradation of DNA before and after denaturation, and calculated the number of strands per molecule; the number did not change (4). The present approach involves the reverse reaction, *i.e.*, renaturation. If the reformation of helical structures arises from the interaction of two separate complementary chains, the kinetics of the reaction should be second order; on the other hand, they should be first order if the strands never completely separate following structural collapse. In the latter case, the polynucleotide chains of the denatured molecules may be imagined as only partially separated, being held together by entanglements and, at least at lower temperatures, some randomly formed hydrogen bonds. Renaturation would then represent a zippering-up of a single kinetic entity. In this paper we present data which show that renaturation, as determined by the hypochromic shift, can indeed occur by a unimolecular reaction and, therefore, that heat denaturation is probably not accompanied by strand separation as a major mechanism.

It has been shown by Ross and Sturtevant (5) that the single strand polynucleotides poly A and poly U obey second order kinetics to form a complementary helix. The fact that their optical density measurements indicated that two entities known to be single strands react bimolecularly provides a firm and independent experimental basis for the present investigation.

EXPERIMENTAL ARRANGEMENT

Preparation of DNA. DNA was isolated from *E. coli* 15_{T-} using a modified Kay, Simmons, and Dounce procedure (6). For sample Do, the bacteria were lysed with lysozyme at pH 9 in 0.02 M versene–0.02 M tris. The lysate was made 1 M in NaCl, stirred with duponol for one hour, centrifuged (35,000*g*, one hour) and precipitated with alcohol. The fibers were dissolved in the versene-tris solvent, the duponol treatment was repeated and the supernatant, after the high-speed centrifugation, was treated with RNase. After dialysis against 2 M followed by 0.2 M NaCl, the DNA was adjusted to a concentration of 0.35 mg/ml and was finally precipiated with redistilled ethoxyethanol. For sample Re, lysis was accomplished with 1 per cent duponol in 0.02 M versene at pH 6.1. The remainder of the procedure was the same as for sample Do. For sample 108 the lysate was centrifuged at 10,000 RPM for ½ hour and the crude DNA pellet was treated as for sample Do. The DNA from *pneumococcus* was prepared according to the modified method of Meyers and Spizizen (7) given previously (2). Sample LC-I was that used for earlier studies (2), while sample E_2 was prepared later. All DNA samples were repeatedly shaken, before use, with $CHCl_3$-octanol in 0.2 M NaCl until no material appeared at the interface.

Denaturation of Samples. Samples were denatured by heating for 10 minutes at 101.5° in 0.2 M sodium chloride (pH ~ 6) or 0.2 M sodium acetate (pH 7.8) and cooled rapidly by placing in ice water. Solid sodium chloride or sodium acetate was added to make the solution 1 M before renaturation. No significant differences were observed when a denatured sample of relatively high concentration was diluted at this point or when a series of concentrations were separately denatured.

Apparatus. Optical density was measured with a Model DU Beckman spectrophotometer. The DNA solutions to be renatured were placed in a Spinco Model E centrifuge cell the centerpiece of which was replaced by either teflon or painted metal cylinders of height varying from 0.14 to 6.7 cm, depending on the DNA concentration. The cross-section was circular rather than sector-shaped to avoid reflection of the incident beam by the walls. The cell was placed in a wirewound aluminum housing in which three thermistors were embedded for monitoring the temperature, which was maintained to within 0.1°

during renaturation. The aluminum block fit into the standard Beckman housing designed for 10 cm cylindrical cells.

The block was preheated to 4 or 5° above the desired temperature before the cell, containing a denatured DNA solution, was inserted. Thermal equilibrium was reached within 1 to 2 minutes. In general, there is an initial increase in absorbance just before the decrease begins. It is essential, therefore, to reach the desired temperature as rapidly as possible in order for the initial rate to be exact. The air thermostat available commercially is probably not adequate in this respect. In the device described here, the cell is in snug contact with the aluminum housing, giving the maximum rate of heat transfer. The fact that equilibrium is reached sufficiently rapidly is proved by the fact that the initial rates give rate constants which are almost identical to the constants calculated from the integrated equation. In the latter the data are taken over a long time period where equilibration is certainly attained. In addition to this, the time of equilibration to 60° in the 1.1 cm teflon cell was measured with a thermometer to be within 2 minutes.

METHODOLOGY

Optical density measurements were made at frequent intervals until the renaturation was virtually complete. The exact value of the final optical density, however, was obtained by extrapolating a plot of absorbance^{-1} *versus* time^{-1}. The total decrement in optical density, denoted by a_0 in the equations, was always close to 60 per cent of the absorbance increment due to denaturation, irrespective of the DNA concentration or the temperature of renaturation. The extent of renaturation is almost constant, therefore, regardless of the rate or mechanism of the reaction.

Two methods have been used to verify that renaturation, as evidenced by the hypochromic shift, is in fact a reversion of the original helix-to-coil transition. First, the melting curve of the renatured sample always corresponds almost exactly to the original curve. Secondly, when renaturation is carried out in the light-scattering cell, the radius of gyration, which is a measure of stiffness and therefore helicity, rises gradually with time until roughly three-fourths of the original value is attained.

The degrees to which the original radius of gyration and the original hypochromicity are restored are not directly comparable, however, since there is evidence in some cases of aggregation during the annealing period. Aggregation should affect only the radius of gyration, not hypochromicity. We would like to emphasize that any molecular weight increase observed during renaturation constituted an increase *above* that of the original, undenatured DNA.

It has, of course, been known for some time, as the result of denaturation studies, that absorbance changes reflect *structural* changes rather than the formation or breakage of hydrogen bonds *per se,* a large proportion of which can be broken under certain conditions without affecting the absorbance (8). Tinoco (9) has shown theoretically that the absorbance decrement is proportional to the degree of ordering of the bases (*i.e.* helix formation) when the ordered regions are 7 or more nucleotides long. One would expect the formation of hydrogen bonds during renaturation to be a cooperative process, with the formation of the first few in a given

TABLE I

RATE CONSTANTS FOR RENATURATION* OF *E. COLI* and *PNEUMOCOCCUS* DNA

Sample	DNA conc.	Final optical density decrement (a_0)	Temp.	First order rate constant (hr^{-1})		Second order rate constant||	(Initial rate)/$(a_0)^n$	Apparent order (n)
				Initial rate‡	Rate at 75 per cent§			
	mg/ml		°*C*					
E. coli (Do)	0.0006	0.04¶	60	0.22			0.22	1.0
	0.0069	0.025	60	(0.17)**	0.180		0.17	1.0
	0.0176	0.062	60	0.200	0.185		0.200	1.0
	0.027	0.100	60	0.180	0.185		0.180	1.0
	0.022	0.088	70	0.92				
	0.025	0.100	70	0.92	0.93			
	0.030	0.125	70	0.95	0.90			
	0.028	0.120	80	1.01	1.03		3.60	1.6
	0.035	0.160	80	1.25	1.30		3.77	1.6
E. coli (108)	0.0022	0.010	80	0.4**		40.0	0.4	1.0
	0.006	0.027	80	0.37		13.8	0.37	1.0
	0.036	0.160	80	0.37		2.4	0.37	1.0
	0.062	0.250	80	0.51		2.1	2.1	~2
	0.110	0.450	80	1.16		2.5	2.5	~2
E. coli (Re)	0.012	0.045	70	1.20		26.5	2.25	1.2
	0.027	0.102	70	1.40		14.4	2.23	1.2
	0.048	0.180	70	1.67		9.28	2.33	1.2
	0.012	0.049	85	(0.28)**		5.8	3.14	1.8
	0.033	0.125	85	0.64		5.2	3.33	1.8
	0.041	0.165	85	0.73		4.4	3.04	1.8
Pneumococcus (LC-I)	0.030	0.130	60	0.92				
Pneumococcus (E_2)	0.026	0.109	60	1.47				
	0.034	0.140	60	1.36				
	0.053	0.223	60	1.47				
	0.077	0.324	60	1.32				
	0.022	0.090	55	0.100‡‡				
	0.021	0.089	60	0.175‡‡				
	0.022	0.090	80	0.038‡‡				

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* All renaturation experiments, except the last three in Table I, were carried out in 1 M sodium chloride, pH 6.

‡ Calculated from the initial rate as $(dx/dt)_i/a_0$, where x is the optical density decrement at time t, and $(dx/dt)_i$ is the rate of change of the optical density decrement during the first 20 to 25 per cent of the reaction.

§ Taken from the slope of log $((a_0 - x)/a_0)$ *versus* time at 75 per cent reaction, Fig. 1.

** Initial decrement too low for accurate calculation.

‡‡ Renaturation carried out in 0.16 M sodium chloride –0.04 M sodium acetate, pH 7.4.

|| Calculated from initial rates.

¶ Carried out in a 10 cm cylindrical cell.

region proceeding slowly and many subsequent ones following rapidly. Thus, the vast majority of hydrogen bonds formed would contribute directly to helix formation, and would be closely paralleled by hypochromicity. The fact that the first order rate constant calculated from the initial rates is the same as that calculated after 75 per cent reaction (Table I) shows that this must be so, for, if the relationship between hypochromic shift and number of hydrogen bonds formed were to change, the rate constant of the reaction would also appear to change. The final optical density decrement, a_0, is therefore used in the rate equations as the initial concentration of the reactant (denatured DNA), and the optical density decrement, x, at any given time is used as the concentration of product (renatured DNA) at that time.

Apparently, depurination does not greatly affect helix formation. For example, *pneumococcus* DNA, heated for 3 hours in 0.2 M NaCl (pH ~ 6) at 100°, renatured almost as well as a sample heated for only 10 minutes. However, transforming activity is greatly affected by depurination (10), emphasizing the fact that the integrity of the re-formed helical structures is not complete. The effect of prolonged heating was also examined by denaturing the renatured DNA, and then renaturing again. Repeating this cycle several times had no apparent effect on the rate or extent of renaturation.

RESULTS

The experiments discussed here are representative of many others that have been performed in this laboratory. Fig. 1 shows a semilog plot of the fraction unreacted *versus* time for *E. coli* DNA. The initially straight lines suggest that the bulk of the reaction is unimolecular at 60° and 70°. At 85° the curve is non-linear. The data are plotted according to the method of Ross and Sturtevant (5) in Fig. 2. The dashed theoretical curves provide an independent reference to check the conclusions drawn from Fig. 1. Both figures show a deviation from first order kinetics at 70° and 80°. From Fig. 2 it is clear that the reaction becomes progressively bimolecular as the temperature increases.

The most rigorous test of the order of the reaction is the variation of the initial rate with DNA concentration. In the case of *E. coli*, first order rate constants have been calculated from initial rates at a series of initial DNA concentrations for the reaction at 60° (sample Do), 70° (samples Do and Re), and 80° (sample 108). These are essentially constant, at 60° and 70°. At 85°, where the reaction appears to be bimolecular in the concentration range examined, second order rate constants were calculated from initial rates.

More precisely, a small deviation from first order kinetics is observed at temperatures as low as 70°; for example, for *E. coli* at 70° the apparent first order rate constant increases slightly with concentration. On the other hand, the second order rate constant at 85° decreases somewhat, indicating that the reaction is not quite second order. However, in this concentration range constancy may be realized if the

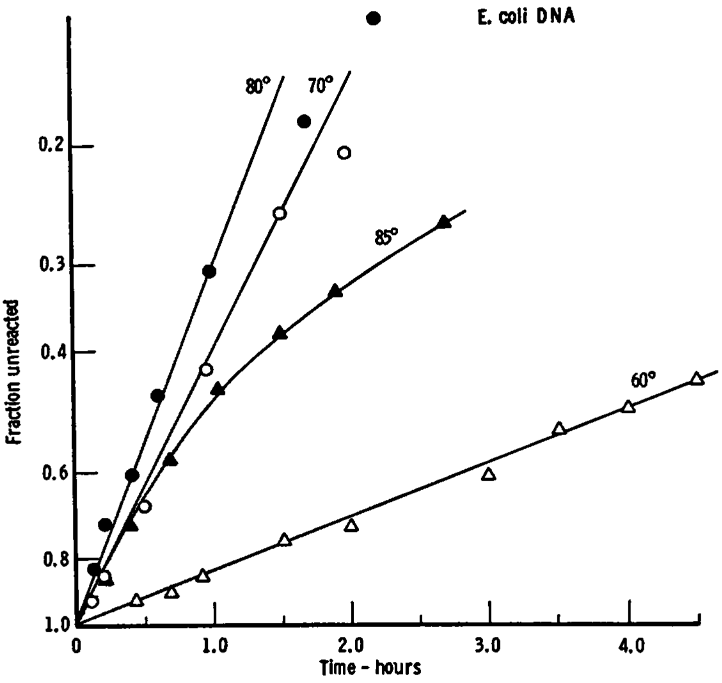

FIGURE 1 —Log fraction unreacted {= $(a_0 - x)/a_0$} *versus* time for *E. coli* DNA. Sample Do at 60°, 70°, 80°; sample Re at 85°. The DNA concentrations at 70°, 80°, and 85° are 0.025, 0.035, and 0.041 mg/ml, and the samples are Do, Do, and Re, respectively.

order is taken as 1.2 instead of 1 at 70°, 1.6 at 80° (sample Do), and 1.8 instead of 2 at 85°. At 60° the first order rate constant does not change, over the entire 45-fold range examined. Thus, the order increases from 1 to 1.8 as the temperature is increased from 60° to 85°.

At 60° there is no shift in order with concentration. At most of the other temperatures studied the concentration range was too limited to ascertain this, but at 80° (*coli* 108) a wide concentration range was also examined. At this temperature the apparent first order rate constant (Table I, column 5) increases at higher concentrations, while the apparent second order rate constant (column 7) first decreases then becomes essentially constant. Thus, the reaction is approximately second order at high DNA concentrations and decreases to first order on decreasing the concentration. This behavior is indicative of simultaneous first and second order kinetics. Doubtless a decrease to first order would also have been observed at 70° and 85° had the concentration range (about fourfold at 70° and 85 *versus* 50-fold at 80°) been larger.

For *pneumococcus* DNA (sample E_2), a first order rate obtains initially over a

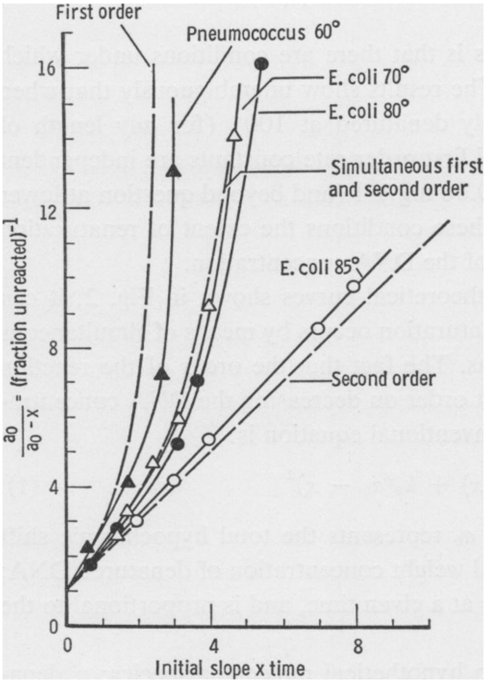

FIGURE 2 The reciprocal of the fraction unreacted was first plotted against time (hours), and the initial slope of this curve × the time was used as the abscissa of Fig. 2. The dashed lines are the theoretical curves for the reaction mechanisms indicated. For the simultaneous first and second order reaction, the theoretical curve was constructed by assuming equal initial rates for the two mechanisms. The *E. coli* DNA concentrations at 70°, 80°, and 85° are 0.025, 0.035, and 0.041 mg/ml, and the samples are Do, Do, and Re, respectively. The *pneumococcus* sample is LC-I, 0.03 mg/ml.

threefold concentration range at 60°. However, after the reaction has progressed somewhat, it is clear that some bimolecular reaction is present (Fig. 2). This behavior is consistent with the conclusion that the free remaining denatured ends of one renatured molecule react with those of a second molecule.

DNA from T2 bacteriophage renatured too fast to allow the kinetics to be followed conveniently. We were able to achieve a small degree of renaturation with salmon sperm DNA, but no significant amount with calf thymus or mouse sarcoma 180 DNA.

The ionic strength was chosen to give the optimal renaturation rate. Table I shows that the rate is faster in 1 M salt than in 0.2 M for *pneumococcus* DNA. The renaturation rate also depends somewhat on the sample itself. This can be seen by comparing the rates at 70° and 80° for the different *E. coli* DNA's. These DNA samples were isolated in a similar but not identical manner (see Experimental Arrangement section). Some of the differences may be due to the state of aggregation at the time of renaturation. If quantitative comparisons are desired, it is therefore essential that aliquots of the same denatured sample be used. As might be anticipated, the source of the DNA also affects the renaturation rate: at 60° *pneumococcus* DNA renatures about 8 times faster than *E. coli* DNA.

As predicted theoretically (11, 12), the initial rates at a given concentration go through a maximum as the annealing temperature increases.

DISCUSSION

The most important feature of our results is that there are conditions under which renaturation follows first order kinetics. The results show unambiguously that when *coli* or *pneumococcus* DNA is completely denatured at 100° (for *any* length of time) and then annealed at 60°, the initial first order rate constants are independent of concentration, at least from 0.0006 to 0.08 mg/ml (and beyond question at lower concentrations). This means that under these conditions the extent of renaturation for a given annealing time is independent of the DNA concentration.

However, from the experimental and theoretical curves shown in Fig. 2, it can be inferred that at higher temperatures renaturation occurs by means of simultaneous unimolecular and bimolecular mechanisms. The fact that the order of the reaction decreases from nearly second order to first order on decreasing the DNA concentration indicates that this is the case. The conventional equation is:

$$\text{rate} = k_1(a_0 - x) + k_2(a_0 - x)^2 \qquad (1).$$

As applied to the present circumstances, a_0 represents the total hypochromic shift observable and is proportional to the initial weight concentration of denatured DNA; x is the observed decrement in absorbance at a given time, and is proportional to the concentration of helical structures.

In this discussion we will focus on two hypothetical molecular species: a denatured DNA molecule whose strands are not completely separated, and a single stranded molecular chain which has separated completely from its partner as a result of denaturation. For convenience, the former will be designated as *NS* (not separated), the latter as *S* (separated). The results can be explained in a straightforward manner by assuming that the solution contains only the *NS* species which, at the lower temperatures, renatures unimolecularly by zippering up. As the temperature is raised, a bimolecular reaction sets in, involving the formation of helical structures between strands attached to two different *NS* molecules. Such strands are probably able to interact because of partial uncoiling of *NS* molecules, *e.g.* at the ends. At higher temperatures the lengths of the free ends would tend to be greater; in effect, there is an increased concentration of long free ends even though they come in pairs in undissociated *NS* molecules. Under these conditions there is an appreciable probability of collision and reaction between two chains attached to separate *NS* molecules.

The change from uni- to bimolecular kinetics as the temperature increases must arise from a difference between the rate of formation of hydrogen bonds within an *NS* molecule and the rate between *two NS* molecules. This is explained quantitatively by the theory of Flory (11) and Saunders and Ross (12). The details are discussed in the Appendix. Briefly, the results require that the two terms on the right side of equation (1) vary with temperature. At low temperatures the second term on the right side must vanish. Clearly, at intermediate temperatures both the first and second order terms will contribute and the apparent order of the reaction will be intermediate. The variation with temperature is brought about by changes in the free energy of activation, which is implicit in the two velocity constants k_1 and k_2 and which is different for the two mechanisms.

A second possible interpretation for the unimolecular kinetic results involves a consecutive reaction of the type

$$2S \underset{k_1'}{\overset{k_1}{\rightleftharpoons}} B \xrightarrow{k_2} C \qquad (2).$$

Here we could imagine that S represents a single strand; B represents a union between complementary strands; C represents a stable helical structure. Since the reaction is first order at low temperatures, it is required that k_2 be much smaller than k_1, making the second step rate-determining. Two cases must then be considered: $k_1 > k_1'$ and $k_1 < k_1'$.

The first of these possibilities means that at low annealing temperatures the concentration of B will be high, approaching the input DNA concentration. This in turn requires the assumption that the formation of B is not accompanied by a significant hypochromic shift, for otherwise the reaction would appear to be instantaneous, or to shift from second to first order with time, which is not observed. In other words, the strands must initially join only in short regions. There are no doubt many base sequences that are complementary to one another over a limited range, but any pairing that might occur in these regions would usually leave the remainder of the two strands out of register. Whenever the two strands involved in such a complex (B) were actually complementary throughout their lengths, a unimolecular rearrangement would be possible in which, for example, the ends would match and the strands would zipper together completely. At higher annealing temperatures longer sequences would be required for the initial bimolecular pairing. This would result in a hypochromic shift accompanying the first step of the reaction; in the limit of high temperature, the second step would be completely eliminated and the reaction would take place entirely in one step, following second order kinetics. Up to this point, this model is consistent with the kinetic results reported here. It can, however, be eliminated on the basis of the concentration dependence of renaturation. We have found that the initial order of the reaction at 80° shifts from 2 to 1 as the concentration decreases (Table I). *Such a shift upon dilution requires alternative rather than consecutive reactions, i.e. the reaction requires simultaneous first and second order reactions.*

In the second case considered above, where $k_1 < k_1'$, the concentration of B could be sufficiently small that, although B were hypochromic, the total hypochromicity would be negligible. This would require considerable hydrogen bond formation, accompanied by hypochromism, in the intermediate B, while at the same time the equilibrium constant k_1/k_1' must still greatly favor dissociation. The second step of the reaction would then consist of irreversible completion of the base pairing.

This implies two groups of specific hydrogen bonds, the *first* more rapidly formed but less stable than the second. There is no basis for such heterogeneity of bonds, however, and there would be no justification for the much *slower* formation, in the second step, of the remaining hydrogen bonds under conditions involving a much smaller decrease in entropy. We therefore eliminate the consecutive mechanism represented in equation (2).

This same argument rules out the possibility that the bimolecular reaction observed at higher temperatures occurs between S molecules. For if the strands were separated at 85° they would surely have been separated at 100° as well, but would have had to rejoin after denaturation to form a two-stranded intermediate when cooled to 60°, in order to exhibit the unimolecular renaturation kinetics observed at that temperature. But such an intermediate has just been ruled out. Thus, aqueous solutions of denatured DNA cannot contain a significant number of S molecules, at *any* temperature.

The work of Ross and Sturtevant (5) shows that when S molecules exist (in their case, poly A and poly U), the kinetics of helix formation are initially second order at all temperatures, as expected. Although a first order component was observed at later times under certain conditions (conducive to initially incomplete helix formation), under no circumstances were the *initial* rates first order.

Some recent results on strand separation in T2 and T4 phage DNA are also of interest. Berns and Thomas (13) have shown that T4 DNA can be denatured in 2 minutes. If the molecular weight is of the order of 120 million (13, 14) then it would take at least 12.4 minutes for each molecule to unwind, according to the equation of Longuet-Higgins and Zimm (15); [also see Kuhn (16, 17)]. It has been emphasized that this is a *minimum* time (15); therefore, it seems unlikely on theoretical grounds that the strands separate upon denaturation. The high degree of entanglement between the two strands of a plectonemic helix should therefore be sufficient to hold them together under ordinary circumstances, even in the absence of cross-linking hydrogen bonds.

The product of the second order renaturation reaction, which we have concluded to be a complex of two or more DNA molecules, each possessing incompletely separated strands, can be identified with the density complexes of Schildkraut (18), Marmur and Doty (19), and the genetic complexes of Herriott (20). In the former study, complexes were formed between various bacterial, isotopically labeled DNA's when they were annealed together; in the latter, certain drug resistance markers of *H. influenzae* became linked during the annealing of mixtures of transforming DNA's. Kozinski and Beer (21) have recently published electron micrographs which show many-branched structures as the product of slow cooling. Since the formation of such structures was specific for homologous DNA, it is clear that multistranded entities can participate in the renaturation reaction. Even when the reaction, as measured by the hypochromic shift, is essentially unimolecular, a small percentage of the hydrogen bonds might be formed bimolecularly (*e.g.* between short unwound segments at the ends of mostly renatured molecules). This would yield complex products whose density and genetic properties would be composites of the various renatured double helices composing them. Thus, the appearance of genetic or density "hybrids" upon renaturation may be looked upon as an aggregation phenomenon, and does not necessarily reflect the major mechanism of the renaturation reaction.

The results with *pneumococcus* DNA follow the same general pattern as *E. coli* DNA (Table I), although the former was not studied in as great detail. With regard to DNA from other sources, the results of Geiduschek (22) on "reversible" DNA are of interest. Treatment of calf thymus or salmon sperm DNA with HNO_2 or nitrogen mustard yielded DNA which could not be permanently heat-denatured; *i.e.*, the DNA reverted spontaneously to "native" DNA on cooling. This would be expected if the two strands were held in register by cross-linking. Perhaps this constitutes a clue as to why DNA's from some sources do not renature: the base sequences might be sufficiently more complex that they are more easily entangled or knocked out of register. It is significant that the d-AT copolymer, which has a regularly alternating sequence of adenylate and thymidylate in each chain, is also reversibly denatured. Bacterial DNA's, in which the base sequences are more complex and more difficult to match, do not renature completely even under optimal conditions. The DNA of higher organisms may be even more complex, thus obviating the argument that its inability to renature arises from the smaller fraction of complementary strands available for participation in a bimolecular renaturation reaction.

In conclusion, it appears that the kinetic results, taken together with the light-scattering evidence (1) that the molecular weight of *disaggregated* DNA does not halve on heating above the denaturation temperature, constitute compelling evidence that heat denaturation does not result in complete strand separation.

APPENDIX

Applying the theory of Flory (11) and Saunders and Ross (12), equation (1) takes the form

$$\text{rate} = k_1(a_0 - x)\,\frac{\Delta H_1}{RT^2}\,(-\Delta T_1) + k_2(a_0 - x)^2\,\frac{\Delta H_2}{RT^2}\,(-\Delta T_2) \qquad (3)$$

where k_1 and k_2 are the average rate constants for the formation of helices from *NS* molecules, and can be replaced by expressions of the form $ce^{-\Delta F^*/RT}$; the k_1 term refers to the zippering up of single *NS* molecules and the k_2 term refers to the bimolecular reaction between two *NS* molecules; ΔH_1 and ΔH_2 are the enthalpies of formation of a single renaturing unit in the unimolecular and bimolecular reaction, respectively; ΔT_1 and ΔT_2 represent the differences between the temperature of the experiment and the melting temperatures of the respective helical structures.

This equation can be used to construct a theoretical curve of rate *versus* temperature with a maximum at the observed temperature, if the free energy of activation ΔF_1^* is of the order of 20 kcal and ΔF_2^* is about 2 kcal larger than ΔF_1^*. It is possible that there might be a small difference between ΔT_1 and ΔT_2, if the melting temperature of the helices formed in the unimolecular and bimolecular reactions were not exactly the same, but such a difference would not have much effect on the magnitude of the ΔF^*'s. Considered separately, then, the first term will go through a maximum at a lower temperature than the second term, and in the sum the relative contributions of each term will therefore change with temperature. This behavior is independent of the exact values of ΔF_1^* and ΔF_2^*. The significant point is that the apparent change from unimolecular to bimolecular kinetics is easily interpretable on the basis of a relatively small difference in free energy of activation between the unimolecular and bimolecular mechanisms, and possibly a small difference in melting temperatures of the products.

There is an interesting speculation which can be made concerning the high value of the free energy of activation. ΔF^* refers to a single renaturing unit. Its high value suggests that many nucleotide pairs are involved in each act. Conceptually this may mean that, say, ten base pairs at a time are renatured; *i.e.* the renaturation unit may be a cluster of base pairs rather than just one. In that case, the energy of activation would be associated with the attainment of an appropriate configuration by sizeable segments of the two chains involved.

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