type DNA are active in carrying out transformations with respect to the mutant property.

The authors would like to thank Dr. Paul Doty for valuable suggestions and discussions as well as Mr. C. Schildkraut and Mr. J. Eigner for their help and stimulation during the course of many of the experiments. We would like to thank Drs. M. Roger and R. D. Hotchkiss for numerous helpful discussions. This work was supported by a grant from the United States Public Health Service (C-2170).

[1] Doty, P., J. Marmur, J. Eigner, and C. Schildkraut, these PROCEEDINGS, **46**, 461 (1960).
[2] Rice, S. A., and P. Doty, *J. Am. Chem. Soc.*, **79**, 3937 (1957).
[3] Doty, P., H. Boedtker, J. R. Fresco, R. Haselkorn, and M. Litt, these PROCEEDINGS, **45**, 482 (1959).
[4] Marmur, J., and P. Doty, *Nature*, **183**, 1427 (1959).
[5] Doty, P., J. Marmur, and N. Sueoka, in *Brookhaven Symposia in Biology*, **12**, 1 (1959).
[6] Meselson, M., and F. Stahl, these PROCEEDINGS, **44**, 671 (1958).
[7] Zamenhof, S., H. E. Alexander, and G. Leidy, *J. Exptl. Med.*, **98**, 373 (1953); Zamehhof, S., in *The Chemical Basis of Heredity* (Baltimore: Johns Hopkins Press, 1957), p. 373.
[8] Roger, M., and R. D. Hotchkiss (personal communication).
[9] Fox, M. S., and R. D. Hotchkiss, *Nature*, **179**, 1322 (1957).
[10] Litt, M., J. Marmur, H. Ephrussi-Taylor, and P. Doty, these PROCEEDINGS, **44**, 144 (1958).
[11] Hotchkiss, R. D., and A. Evans, *Cold Spring Harbor Symp. Quant. Biol.*, **23**, 85 (1958).
[12] Marmur, J. (in preparation.)
[13] Lerman, L. S., and L. J. Tolmach, *Biochim. Biophys. Acta*, **33**, 371 (1959).
[14] Pratt, D., and G. S. Stent, these PROCEEDINGS, **45**, 1507 (1959).
[15] Tessman, I., *Virology*, **9**, 375 (1959).
[16] Sinsheimer, R. L., *J. Mol. Biol.*, **1**, 45 (1959).
[17] Bracco, R. M., M. R. Krauss, A. S. Roe, and C. M. MacLeod, *J. Exptl. Med.*, **106**, 247 (1957).
[18] Fox, M. S., *Biochem. Biophys. Acta*, **26**, 83 (1957); Lerman, L .S., and L. J. Tolmach, *Biochim. Biophys. Acta*, **26**, 68 (1957).
[19] Goodgal, S. H., and R. M. Herriott, in *The Chemical Basis of Heredity* (Baltimore: Johns Hopkins Press, 1957), p. 336.
[20] Schaeffer, P., *Symp. Soc. Exptl. Biol.*, **12**, 60 (1958).

STRAND SEPARATION AND SPECIFIC RECOMBINATION IN DEOXYRIBONUCLEIC ACIDS: PHYSICAL CHEMICAL STUDIES

BY P. DOTY, J. MARMUR, J. EIGNER, AND C. SCHILDKRAUT*

CONANT LABORATORY, DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY

Communicated February 25, 1960

The separation and reunification of the complementary molecular strands of DNA, so clearly indicated by the restoration of biological activity,[1] can be demonstrated by physical chemical techniques as well. Such studies permit a more quantitative description of the phenomenon, make possible the inclusion of DNA samples that do not participate in bacterial transformation and lead to a better insight into the controlling features of the reaction. This, in turn, should provide a better basis for understanding the possibilities and limitations that DNA has

in vivo for the macromolecular reactions that are the counterpart of its genetic function.

In summarizing here our current work in this direction we begin by showing how strand separation and recombination is reflected in three physical chemical methods in the order in which we took them up. To proceed from these to more quantitative studies it became necessary to find reliable, routine means for determining the molecular weight of DNA in the various forms with which we were confronted, so that the complicating problems of aggregation and depolymerization could be assessed and minimized. Success along these lines made it possible to follow the molecular weight changes accompanying the processes being studied and to examine the effect of molecular weight thereon. In the final section we present the results of a study of recombination between strands which differ either in isotopic label or in species of origin. In both cases "hybrid" or "heterozygous" reformed molecules can be demonstrated.

OBSERVATION OF STRAND RECOMBINATION

Absorbance-Temperature Curves.—It has been established that when DNA solutions are slowly heated a dramatic macromolecular change occurs in a very restricted temperature range. The change is a cooperative melting out of the one-dimensional helical structure yielding disorganized, coiled polynucleotide chains. At 0.2 molar sodium ions the midpoint of this transition, T_m, lies in the interval of 80 to 100° depending on the guanine-cytosine content of the DNA.[2] The change, which involves a 40 per cent increase in absorbance, can be easily and accurately followed by measuring the absorbance at 260 mμ as a function of temperature.[3] When the solution is cooled to 25° the absorbance decreases until it is about 12 per cent above that of the original solution at room temperature. This appears to be due to the formation of short, imperfect, intrachain helical regions in which a major portion of the bases are paired. Upon reheating such solutions, in the case of calf thymus and other mammalian DNA, the absorbance increases gradually without a region of rapid rise since short, imperfect helices have a broad range of transition temperatures.[3]

When DNA of *Diplococcus pneumoniae* was thermally denatured and then reheated it was found, in contrast to the general behavior just described, that a distinct maximum occurred in the middle of the temperature range; beyond this there was a sharp rise coincident with the latter half of the original curve for native DNA. This behavior suggested that a typical helix-coil transition was taking place involving the denaturation of long, perfect helices containing about half of the bases in the DNA sample.

The meaning of this became more clear when the absorbance-temperature curves of quickly and slowly cooled samples were measured. Such results are shown in Figure 1. The rate of cooling of these samples is the same as that described in reference 1. The absorbance of the slowly cooled sample is seen to rise to a plateau and then continue to the abrupt region coincident with the thermal denaturation of the native DNA. This sample had retained a large degree of its original transforming activity as described in the foregoing paper.[1] From this it is concluded that the slowly cooled sample contained substantial amounts of the Watson–Crick helix. The curve for the quickly cooled sample, which had very low transforming

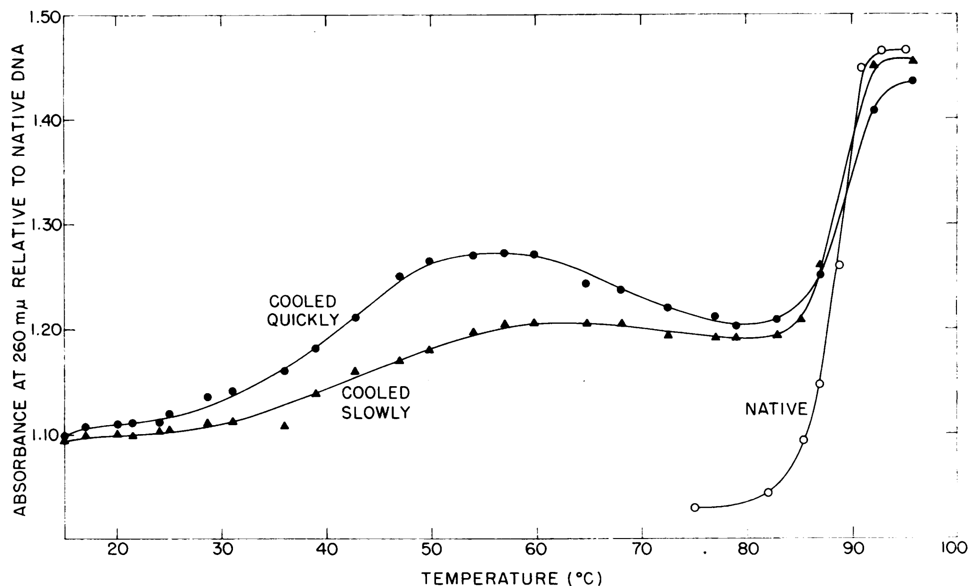

Fig. 1.—Thermal transitions of native, slowly cooled and quickly cooled *D. pneumoniae* DNA. *D. pneumoniae* DNA was heated at 100° for 10 min in standard saline-citrate. Hot concentrated saline-citrate was then added to a final concentration of 0.27 *M* NaCl plus 0.02 *M* Na citrate and subdivided into two portions, one cooled quickly in ice-water, the other cooled slowly. The native DNA was an aliquot of the same sample. The absorbance at 260 mμ, corrected for thermal expansion, was recorded after each solution was exposed for 15 min to the indicated temperature. The ordinate gives this reading relative to the absorbance of the native sample at 25°.

activity, can now be understood. At room temperature it is devoid of long, complementary helical regions, but during the gradual heating period short, imperfect helical regions melt out, releasing chains that then have time to develop long, complementary helices: these then melt in the vicinity of the characteristic temperature, T_m. Thus the display of the sharply melting region by the quickly cooled sample is seen to be an artifact in that this is not evidence that long, complementary helices exist in the same DNA sample at room temperature. This is consistent with its near absence of biological activity. We propose to call this form of DNA *denatured* and refer to the slowly cooled DNA with substantial amounts of reformed complementary helical regions as *renatured.*

Density Gradient Ultracentrifugation.—When a very dilute solution of DNA (∼ 2 γ/ml) in about 7.8 molal cesium chloride is centrifuged at high speed in an analytical ultracentrifuge for 30–40 hours it is found that a density gradient has been set up and the DNA has migrated to a band at a position corresponding to its effective density. This is the basis of avaluable new technique[4] which permits the very accurate assessment of the density of DNA and, through the band profile, reflects in a combined fashion the molecular weight and the density heterogeneity of the DNA sample.[5] It has recently been shown that the density of DNA varies linearly with the guanine-cytosine content[6, 7] and that the distribution of guanine-cytosine among the molecules making up a DNA sample is relatively narrow, particularly for bacterial DNA.[6–8] It has been also shown that the density of DNA increases about 0.016 units upon denaturation.

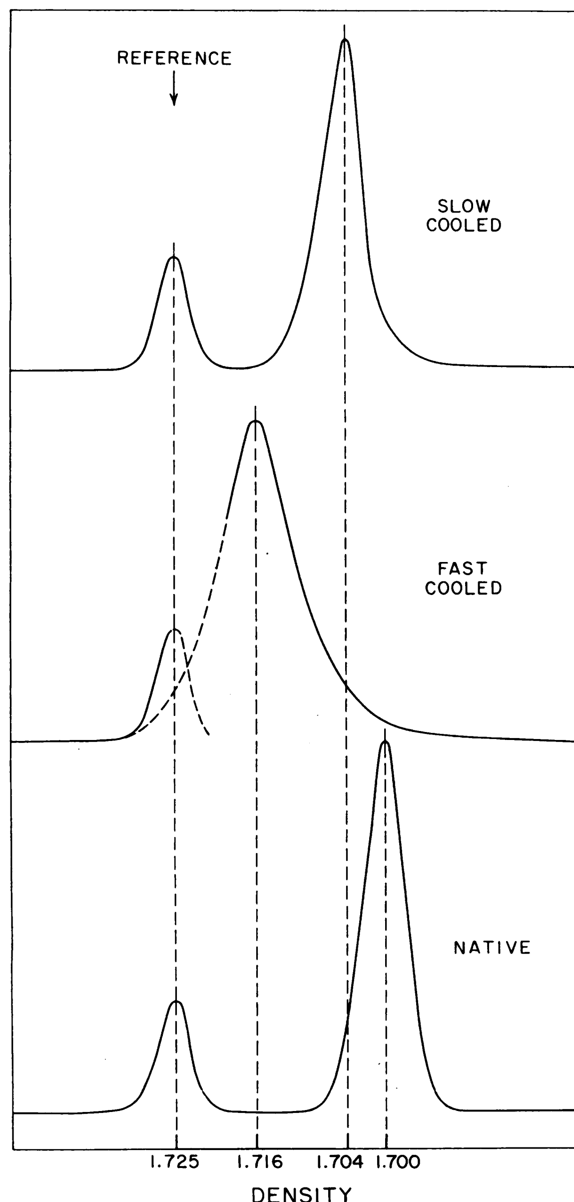

FIG. 2.—Molecular reconstitution in *D. pneumoniae* DNA.

Equilibrium concentration distributions of DNA samples banded in CsCl gradient. See text for a description of samples. Centrifugation at 44,770 rpm. The ordinate represents the DNA concentration as a function of the distance from the axis of rotation. Density increases towards the left. Conditions of heating and cooling are as described for Figure 1 of the preceding paper.[1]

In this situation it was obvious that the density of denatured and renatured pneumococcal DNA should be determined because our view of renaturation, brought about by slowly cooling thermally denatured DNA, predicts that such material should have a density close to the original native DNA. The photometric traces of the ultraviolet photographs of the appropriate density gradient ultracentrifugations are shown in Figure 2. At the bottom of the figure one sees the band for native pneumococcal DNA with a density of 1.700 together with a small band of DNA from *Pseudomonas aeruginosa* which is present in each case to provide a reference density. The trace for the quickly cooled, denatured pneumococcal DNA is shown in the middle exhibiting the expected higher density of 1.716. At the top is seen the tracing for the renatured DNA: it has a density of 1.704. Thus the renatured DNA is found to have a density close to that of native DNA supporting the view that it consists predominately of reformed, complementary helices of the same character, and density, as the native DNA.

Although the molecular weights of these three forms will be discussed at a later point it may be of interest to mention here that the pronounced widening of the band for the denatured DNA reflects an approximately five-fold reduction in molecular weight; hence only a part of this can be due to strand separation alone. The

near equivalence of the profile for the renatured DNA to the native DNA involves, therefore, not only a recombination of two strands but, under the conditions employed, some aggregation which by chance increases the molecular weight to about that of the original native DNA.

Electron Microscopy.—The evidence presented in the two previous sections clearly predicts that electron microscopy should reveal in the renatured DNA the long, cylindrical threads of 20 A. diameter that are characteristic of native DNA: these should be absent in the denatured (quickly cooled) DNA. Accordingly, pneumococcal DNA was heated in saline-citrate at 100° for 10 minutes, diluted with more concentrated saline-citrate to a final concentration of 18 γ/ml in 0.30 *M* NaCl + 0.03 *M* Na citrate, and cooled in two parts, one quickly and one slowly. Both solutions were then dialysed into 0.05 *M* ammonium carbonate plus 0.1 *M* ammonium acetate. Thereafter Professor C. E. Hall sprayed the solutions on freshly cleaved mica and, proceeding in a manner previously described[9, 10] obtained electron micrographs shown in Figure 3 (slowly cooled) and Figure 4 (quickly cooled). The former shows the structure characteristic of native DNA. The only difference between this and micrographs of native DNA is the more frequent occurrence of irregular patches at the ends of cylindrical threads. These are probably regions of denatured DNA arising from incomplete recombination or the inequality in length of the two strands that are paired. The micrograph for the quickly cooled sample (Fig. 4) shows only irregularly coiled molecules with clustered regions. These results are very similar to those found for ribonucleic acid.[11] Thus the identification of renatured DNA as being for the most part similar to native DNA and denatured DNA as being an irregular chain with considerable base pairing in short regions seems to be complete.

Dependence of Molecular Recombination on Concentration.—In the foregoing report[1] it is shown that the transforming activity of renatured pneumococcal DNA increases with the concentration at which it is slowly cooled (see Fig. 4 of ref. 1). This suggests that the extent of molecular recombination is quite low at concentrations considerably below those employed in the experiments described in the foregoing sections, that is, ~ 20 γ/ml. In order to find out if the biological activity reflected the state of the DNA sample itself we heated and slowly cooled a pneumococcal DNA sample in the usual manner at a concentration of 1 γ/ml. The density of this sample was found to be 1.715. This value matches that of quickly cooled pneumococcal DNA (1.716) prepared either at the usual concentration, 20 γ/ml, or at this very low concentration, 1 γ/ml. Thus cooling at what we have described as a slow rate, that is from 90 to 60° in 80 minutes, does not lead to substantial recombination of strands when the concentration is quite low.

With the physical exhibition of the molecular recombination, as well as the biological, shown to be quite concentration dependent it seemed permissible to attempt to account for the concentration dependence observed (Fig. 4 of ref. 1) by assuming a bimolecular reaction, $A + A \leftrightarrows A_2$, which reaches effective equilibrium at a particular temperature that lies within the region where slow cooling takes place. If α represents the mole fraction of strands that are combined in the helical form, the equilibrium constant $K = \alpha/[(1 - \alpha)^2c]$ where c represents the DNA concentration. This can be applied to the data in Figure 4 of reference 1 by

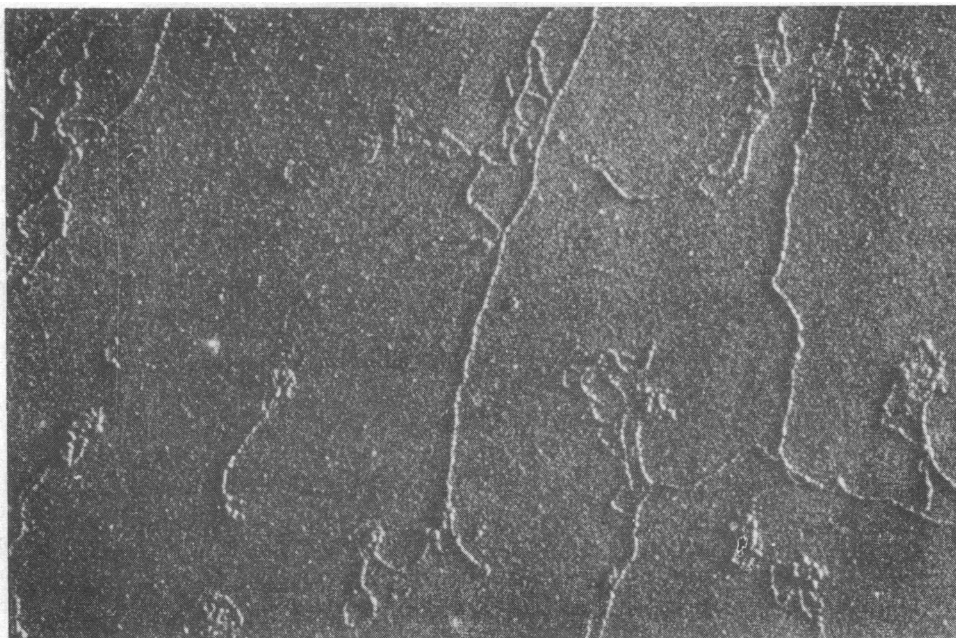

FIG. 3.—Electron micrograph of renatured *D. pneumoniae* DNA. Magnification 95,000 X. Shadow casting is from the right at a shadow to height ratio of 10:1. See text for heating and cooling conditions.

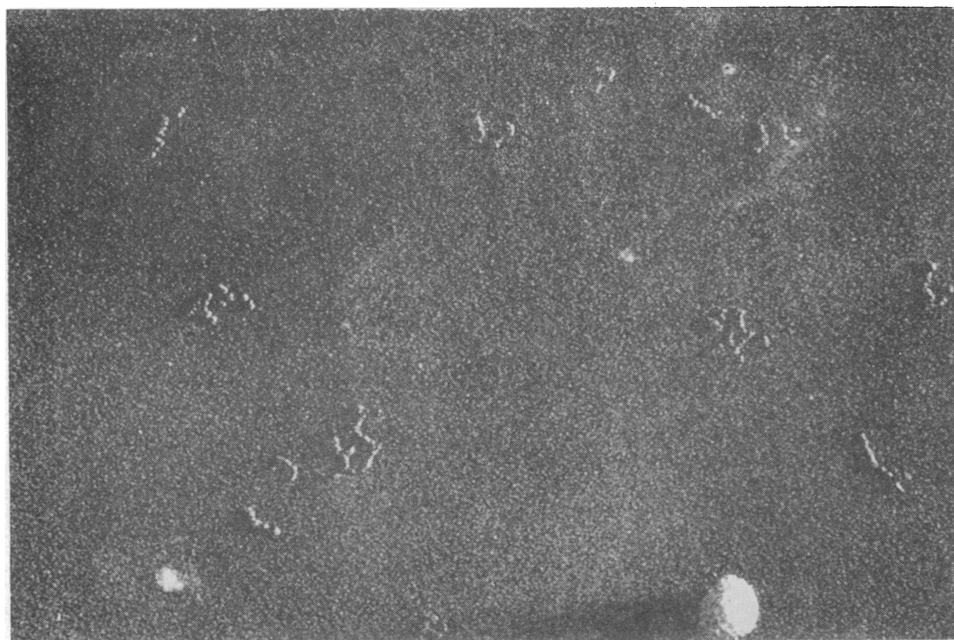

FIG. 4.—Electron micrograph of denatured *D. pneumoniae* DNA. Magnification 95,000 ×. The polystyrene reference sphere at the lower right of the micrograph has a diameter of 880 Å. Shadow casting is from the right at a shadow to height ratio of 10:1. See text for heating and cooling conditions.

assuming that α is equal to the fractional activity of the renatured DNA. By choosing one point to permit an evaluation of the product Kc, α can be calculated as a function of c. The dashed line drawn in Figure 4 of reference 1 is the result calculated in this way. Since it fits the experimental data there is reason to conclude that the molecular recombination is indeed bimolecular as the character of the Watson–Crick structure for DNA would demand. Moreover, the success of this simple approach suggests that properly designed experiments which yield K as a function of temperature should lead to the determination of the heat and entropy of the recombination as well as the free energy change involved in this process at the temperature of cellular division.

Dependence of Molecular Recombination on the Source of DNA.—With the view that molecular recombination in DNA consists simply of two complementary strands coming together with the fraction of strands complexed under control of an equilibrium constant it follows that the manifestation of molecular recombination depends critically on the concentration of complementary strands. Now the concentration of complementary strands is not identical with the concentration of DNA, when DNA from different sources is considered. Take the extreme cases, of typical bacterial DNA and the DNA of mammalian cells. Here the DNA content per cell differs by the order of a thousand fold. If we take the average molecular weight to be the same in both cases and if it is assumed that each DNA molecule in a given cell is different, it follows that at the same weight concentration, the concentration of complementary pairs will be a thousand fold greater for the bacterial than for the animal DNA. From this it follows that molecular recombination will not be expected for animal DNA under the conditions that are just sufficient to permit its occurrence in bacterial DNA.

The expectation that animal DNA will not show complementary reformation when bacterial DNA will do so has been borne out in two kinds of experiments.[12] In one set the absorbance temperature profiles for a number of denatured DNA samples have been determined. The majority of bacterial DNA samples showed some of the character seen in Figure 1, that is a sharply rising region at high temperature approximately coincident with the curve for native DNA. By contrast calf thymus and salmon sperm DNA showed essentially a continuous and gradual rise with temperature. This shows that on the time scale of the heating curve there was some molecular reconstitution in most of the bacterial samples but not in the cellular DNA.

In another experiment samples of thermally denatured DNA were cooled quickly and then brought to 80° and the absorbance followed as a function of time in 0.30 M NaCl plus 0.03 M sodium citrate. The pneumococcal DNA fell from 1.40 times the absorbance of the native DNA at 25° to 1.08 whereas the calf thymus showed no drop at all. This again demonstrates the inability of calf thymus DNA to reform the helical configuration under the conditions which do permit pneumococcal DNA to do so. Consequently, the great difference in concentration of complementary strands in the denatured DNA appears to offer the explanation.

THE MACROMOLECULAR PROPERTIES OF NATIVE, DENATURED, AND RENATURED DNA

A means of making reasonably accurate molecular weight assignments to DNA in each of the forms under study is necessary in order to examine quantitatively

the phenomenon of molecular recombination in DNA and to enable the complicating features of aggregation and depolymerization to be understood and eliminated in so far as possible. Although light scattering measurements were the first method to satisfactorily establish the molecular weight and shape of DNA in solution,[13, 14] its accuracy and applicability in routine use, particularly for molecular weights greater than 5 million, has been found wanting elsewhere[15] as well as in this Laboratory. Consequently, a systematic investigation of sedimentation constants, evaluated by extrapolation to zero concentration, and intrinsic viscosities evaluated by extrapolation to zero gradients, has been undertaken[16] and molecular weights have been derived by a careful application of the Mandelkern–Flory equation:[17]

$$M = \left[\frac{s^{\circ}[\eta]^{1/3}\eta_0 N}{\beta(1 - \bar{v}\rho)}\right]^{3/2}$$

In this equation, s° = the sedimentation constant at zero concentration, $[\eta]$ = the intrinsic viscosity at zero gradient, η_0 = the solvent viscosity, $N = 6.03 \times 10^{23}$, $(1 - \bar{v}\rho)$ = the buoyancy factor and β = a constant which has a value which ranges from 2×10^6 to about 4×10^6 as the permeability of a coiled molecule or the axial ratio of a rigid ellipsoid increase. For most flexible polymers the value of β has been found to be about 2.6×10^6.

Native DNA.—An investigation[18] of DNA samples in which the molecular weight had been varied by ultrasonic radiation and the molecular weights measured by light scattering showed β to increase monotonically from 2.56 at 300,000 to 3.29 at 8,000,000. Other light scattering experiments, and an independent study[19, 20] in which molecular weights were determined by sedimentation-diffusion, have shown good agreement with this result. Such an empirical evaluation of β, based as it is on absolute methods, permits us to use the Mandelkern–Flory equation to derive weight average molecular weights from sedimentation-viscosity data.

TABLE 1

VALUES OF s° AND $[\eta]$ FOR SELECTED MOLECULAR WEIGHTS OF NATIVE DNA

Molecular Weight	$s^{0}_{20,w}$ in HMP	$[\eta]$ in HMP	$s^{0}_{20,w}$ in SSC	$[\eta]$ in SSC
1,000,000	8.8 S	9.1 dl/gm	10.0 S	8.3 dl/gm
2,000,000	11.5	21.0	−13.1	18.7
4,000,000	15.4	45.5	17.5	38.0
10,000,000	22.9	120	26.4	82.5
16,000,000	28.2	190	32.8	119

For a number of samples s° and $[\eta]$ have been measured in a solvent of lower ionic strength, 0.011 M in Na^+ ions, as well as in the more common solvent that is 0.195 M in Na^+ ions. The former solvent contains 0.0025 M Na_2HPO_4, 0.0050 M NaH_2PO_4 and 0.001 M sodium ethylenediaminetetraacetate and has a pH of 6.8. For convenience this is referred to as HMP (hundredth molar phosphate). The more common solvent consists of 0.15 M NaCl and 0.015 M $Na_3(C_6H_5O_7)$ with a pH of about 7. This will be referred to as SSC (standard saline-citrate). On a double logarithmic plot of s° against M the data in these two solvents fall on two parallel lines exhibiting slight upward curvature. Similarly, log $[\eta]$ against log M yields lines with downward curvature. Table 1 summarizes the smoothed $s^{0}_{20,w}$ and $[\eta]$ values in these two solvents for selected molecular weights.

Denatured DNA and the Problem of Aggregation.—When DNA is thermally denatured and cooled, a large fraction of the bases become paired.[3] While most of this pair formation can occur within each chain, some interchain bonding can be expected and this would increase with DNA concentration and molecular weight. Consequently, the determination of the molecular weight of denatured DNA requires proof that aggregation has been eliminated. Early light scattering studies showed no significant change in molecular weight on thermal denaturation,[21] and thereby suggested that the two strands had not separated. Other reports claimed a decrease to one-half the molecular weight but they were not convincing.[22] Moreover, the $s°$ and $[\eta]$ values for such denatured DNA did not yield a molecular weight in agreement with the light scattering values by a wide margin. Upon further investigation of this dilemma we have been able to show that significant aggregation occurs upon cooling denatured DNA in SSC at concentrations in excess of 40–100 γ/ml. Since light scattering and viscosity measurements require concentrations in this range or higher we were able to conclude that aggregation had occurred in most samples previously studied.

In order to eliminate this aggregation in the useful range of concentration we have gone to a solvent of lower ionic strength, HMP. Although there is some reformation of base pairs (about 25 per cent) in this solvent at room temperature no aggregation is evident by any of the methods employed and a satisfactory dependence of $s°$ and $[\eta]$ on molecular weight has been established by using the Mandelkern-Flory equation with a value of 2.6×10^6 for β. The results of such studies can be fitted with the usual empirical relations: $s° = K_s M^{a_s}$ and $[\eta] = KM^a$.

Now it has been shown that the guanine-cytosine bond is stronger than the adenine-thymine bond[2, 8] and as a result it is to be expected that the amount of base pairs in denatured DNA will increase with the guanine content. Indeed this has been shown to be the case with RNA samples of different composition.[3] Consequently it is possible that the dependence of $s°$ and $[\eta]$ on molecular weight for denatured DNA will depend on the composition of the DNA. This has been found to be the case and it is illustrated in Table 2 where the constants for the empirical equations are listed for denatured DNA (quickly cooled) from *D. pneumoniae* and *Escherichia coli* (K-12).

TABLE 2

CONSTANTS FOR THE EMPIRICAL SEDIMENTATION AND VISCOSITY RELATIONS FOR DENATURED DNA IN PHOSPHATE-VERSENE SOLUTION 0.011 M IN Na^+ (HMP)

Source of DNA	K_s	a_s	K	a
D. pneumoniae	0.054_5	0.35_4	$3.4_6 \times 10^{-5}$	0.93_3
E. coli K-12	0.055_8	0.36_1	$3.1_1 \times 10^{-5}$	0.91_2

These results indicate a rather highly swollen chain configuration for DNA, despite some base pair interaction. For a given chain length the molecular dimensions that can be deduced from this are in the range expected for typical polyelectrolytes. These dimensions are much larger than those for RNA of the same chain length and in the same solvent: this is probably due to the additional contraction of the RNA coil due to additional hydrogen bonding made possible by the 2-OH of the ribose ring.

With these results at hand it is possible to assess the molecular weight as a function of time of heating at a given temperature and to demonstrate whether or not strand separation has occurred at the early stages of heating.

Strand Separation and Depolymerization.—In the low ionic strength solvent (HMP) the midpoint of the thermal denaturation profile, T_m, for *D. pneumoniae* is shifted downward from 85° in SSC to 64° and the transition is complete at 70°. For *E. coli* DNA the corresponding values are 5° higher. A temperature 15° above the T_m is well beyond the melting out region for DNA in this solvent, indeed by about the same amount as 100° is for the higher ionic strength solvent (SSC). Consequently 79° and 84° were chosen as the temperatures at which to follow the thermal degradation of these two DNA samples. Aliquots were removed periodically from a large amount of the solution that had been brought to

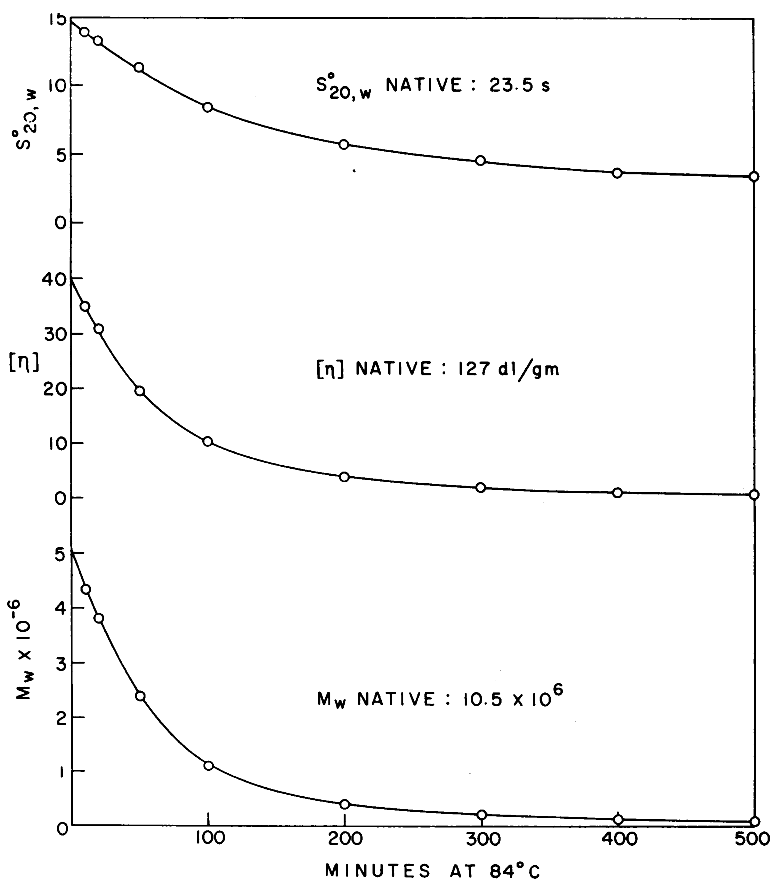

FIG. 5.—Thermal degradation of *E. coli* DNA in HMP at 84°. Molecular weights derived from Mandelkern-Flory equation[17] using $\beta = 2.6 \times 10^6$.

this temperature, and cooled quickly. The $s°$ and $[\eta]$ were measured at 25° and the molecular weight calculated as indicated above. The results for *E. coli* DNA are shown in Figure 5. It is seen that the molecular weight falls continuously but rather slowly through the period of exposure to elevated temperature. The ex-

trapolation back to zero time is obvious and the molecular weight value there is 5.0 million. The molecular weight of the native DNA was 10.5 million ($s^{\circ}_{20,w}$ = 23.5 S and $[\eta]$ = 126). In this case we have, therefore, a clear example of the molecular weight decreasing by a factor of two upon thermal denaturation, and with aggregation eliminated and depolymerization taken into account it can be said that strand separation did occur in the very early stages of the exposure to the elevated temperature.

The behavior of pneumococcal DNA in a similar experiment was roughly the same except that in this case the molecular weight fell from 8.2 million for the native DNA to 2.9 million for the denatured DNA at zero time. This change by a factor of somewhat more than 2, in this case 2.83, probably reflects an occasional enzymatically induced single chain scission in the original DNA preparation.

From the data obtained during the first 100 minutes of the above experiments the rate of thermal depolymerization of the polynucleotide chains of DNA can be evaluated. In practical units this can be expressed as 0.165 scission per 10 million molecular weight per 10 minutes at 79°. Similar experiments at 100° yields 1.41 in the same units. These and other results lead to a value of about 20 kcal for the activation energy for hydrolytic scission of individual strands of DNA, a value similar to that found in a quite different study of ribonucleic acid.[23] With this evaluation of the rate of bond scission it is possible to assess the extent of fragmentation induced by the exposure to 100° for 10 minutes followed by fast or slow cooling. For fast cooling the value stated above is the answer desired since the units have been chosen for convenience in this situation. Since pneumococcal DNA is often in the range of 10 million molecular weight each native molecule will receive on the average between one and two scissions as a result of the heating to 100°. Slow cooling will involve a longer exposure to temperatures where degradation is significant: in the case of our particular cooling rate the depolymerization would be about doubled.

Renatured DNA.—Renatured DNA was prepared by heating pneumococcal DNA of molecular weight 8.2 million at 100° for 10 minutes in standard saline-citrate at a concentration of 20 γ/ml and cooling slowly in double the saline-citrate concentration. In standard saline-citrate this material had a $S^{\circ}_{20,w}$ of 23.5 S and an $[\eta]$ of 21.6 from which a molecular weight of 6.0 million was deduced. An aliquot of the same heated solution, quickly cooled, had a molecular weight of 2.0 million. When dialyzed into the lower ionic strength solvent (HMP), the renatured DNA had a $s^{\circ}_{20,w}$ of 15.4 S and an $[\eta]$ of 43. A molecular weight of 4.0 million was obtained using these results. The denatured DNA showed no change in molecular weight when similarly transferred to the lower ionic strength solvent.

From these results it appears that a small amount of aggregation occurs when renaturation is carried out at 20 γ/ml in 0.3 M NaCl plus 0.03 M Na citrate and that the molecular weight falls by about the amount expected from the depolymerization rates reported above. In the lower ionic strength solvent, where the aggregation appears to have been eliminated, it is interesting to note that the s° and $[\eta]$ values are essentially equal to those given in Table 1 for 4 million molecular weight native DNA. This supplies further evidence of the close structural similarity of the renatured to the native DNA.

The results have an important bearing on the transforming activity that can be

expected for renatured pneumococcal DNA. The loss in activity to be expected from the molecular weight reduction can be obtained directly from an earlier study[24] of the molecular weight dependence of transforming activity. For a reduction of 10 to 4 million the activity should fall to 70 per cent of its original value. However, the activity would be expected to be lowered still further due to the fact that the complementary strands that recombine will generally be of different length as a result of the mild thermal depolymerization. If there was no selection according to chain length in recombination there would be a loss of about 50 per cent of the nucleotides from helical regions due to this effect and a drop to 35 per cent would be expected for the maximum activity. However, there is probably some preference for complementary chains of more nearly equal molecular weight to recombine and the evidence from the absorbance-temperature profile and DNA density in CsCl indicates that about 75 per cent of the nucleotides do reform in the helical configuration. Consequently the maximum biological activity of renatured transforming DNA under the conditions employed may be placed at about 50 per cent. The highest observed[1] has been approximately 50 per cent.

RECOMBINATION OF DNA STRANDS OF DIFFERENT DENSITY AND FROM DIFFERENT SPECIES

In this final section we return to the technique of density gradient ultracentrifugation and ask if by its use recombination between strands of different density can be observed. Two cases are of interest. In one we mix two DNA samples from the same species, one of which is denser by virtue of having N^{15} instead of N^{14}. In the other we mix N^{15} *E. coli* DNA with N^{14} DNA from other bacteria to see if renatured molecules of intermediate density can be observed. Such "heterozygous" molecules may be expected from closely related bacteria but not from distantly related ones.

From what has been stated in the previous section concerning aggregation and strands of unequal length in the renatured molecules, it is not surprising to find that the optimal resolution of hybrid molecules has proved somewhat elusive. Thus, while maximum helix development in the renatured sample is favored by high DNA concentrations, so is aggregation. Obviously aggregation of renatured molecules of different density will smear out the hybrid band one seeks to resolve. Likewise the inequality of chain lengths in the hybrid molecules will spread out their densities with a corresponding broadening of the hybrid band.

Against this background we present the results obtained with a mixture of N^{14} and N^{15} *E. coli* DNA each at a concentration of 5 γ/ml. This concentration is low enough to prevent aggregation but at the price of only limited recombination to the helical form. The results are collected in Figure 6. At the top (*A*) is shown the tracing for the quickly cooled, denatured DNA. The bands are seen to be completely separated without any hybrid formation. Thus, the reforming of base pairs has been entirely intrachain.

If an aliquot of the same heated mixture is slowly cooled the results are as shown in the second tracing (*B*). Here the peaks have remained at the same densities as in the denatured DNA but about one-third of the total DNA has shifted to a lighter density distributed about the value of 1.724. While this is indicative of hybrid formation it is far from proof since the densities of the peaks are essentially

those of denatured N^{14} and N^{15} DNA and do not approach that of renatured material which should be about 0.008–0.012 density units lighter than the denatured materials.

In this situation it is necessary to provide a control consisting of the mixture of the two separately cooled DNA samples, that is, samples that have had the same thermal history as the slowly cooled mixture (*B*) but have been prevented from forming hybrids. The tracing of this control is shown in the Figure as (*C*). This provides a key to our interpretation since peaks corresponding to the renatured molecules are clearly evident at the right of each of the denatured peaks.

If the control *C* is now subtracted from the trace for the slowly cooled mixture (*B*) the migration of material to the density corresponding to renatured hybrids should be evident. This is shown at the bottom as (*D*). A shift of material from extremities to the density of the hybrid is clearly evident. About 20 per cent of the total DNA is involved: this is about that expected from (*C*) since there it can be seen that about 40 per cent of the material is in the bands corresponding to the renatured form.

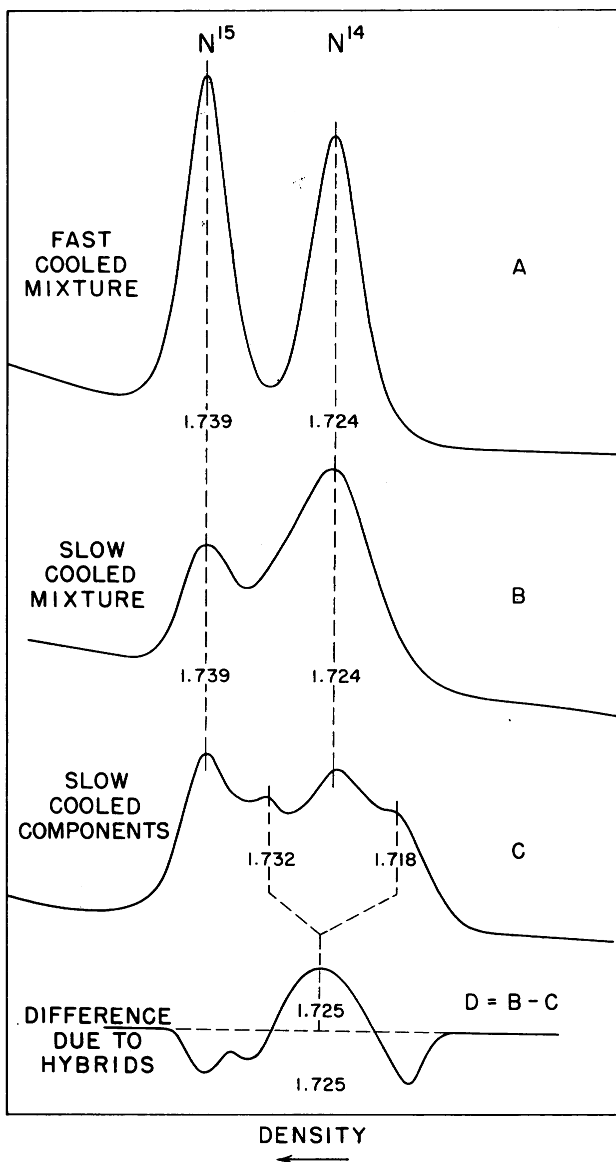

FIG. 6.—Hybrid formation in *E. coli* B DNA.
Equilibrium concentration distributions of DNA samples banded in CsCl gradient. See text for a description of samples. Centrifugation at 44,770 rpm. The ordinate represents the concentration of DNA in the centrifuge cell. The area of each band is proportional to the amount of DNA it contains. N^{15} and N^{14} labeled *E. coli* B DNA were prepared by growing wild-type cells in a synthetic medium containing $N^{15}H_4Cl$ or $N^{14}H_4Cl$, respectively, as the sole nitrogen source. The DNA was isolated by a procedure to be published (J. Marmur, in preparation).

By using 4 times the concentrations employed in the above experiment the de-

natured species can be essentially eliminated but the additional spreading introduced by aggregation results in only a single band of somewhat irregular shape, and a proper correction cannot be made.

Despite this difficulty we have made some preliminary observations on mixtures of DNA from two different species. In these cases the peak densities appear to be sufficient for interpretation.

The results of Baron *et al.*[25] showing that *E. coli*, *Shigella* and *Salmonella* are closely related genetically, as well as the report of the similarity of their base compositions[26] prompted an experiment to see whether N^{15} labeled DNA strands from *E. coli* would recombine with N^{14} DNA strands from the other enteric organisms.

N^{15} *E. coli* B DNA and N^{14} *Shigella dysenteriae* (obtained from Dr. S. E. Luria) DNA were mixed, heated as usual and slowly cooled. The densities of the native samples were 1.725 and 1.710 respectively. If we assume 50 per cent of the resultant mixture is composed of hybrid molecules in which helical regions have formed to the extent of 75 per cent of the native helical content we would expect that, as was the case for *E. coli* and *D. pneumoniae*, the density of the resultant hybrid band should not be the mean of the native values but 0.004 density units heavier. A band centered at a density of 1.722 was indeed found and thus hybrid, or heterozygous molecules appear to have formed.

A similar prediction can be made for N^{15} *E. coli* B DNA and N^{14} *Salmonella typhimurium*(LT-2) (obtained from Dr. M. Demerec) since the base compositions are the same. However, a density of 1.729 was observed. Thus it appears that heterozygous renatured molecules have not formed. Since the observed density is midway between that predicted for denatured molecules and renatured, homogenous molecules, it is possible that occasional heterozygous regions have combined preventing a full development of homogeneous renatured molecules. Perhaps under optimal conditions interspecies molecular hybridization of the DNA would be observed in this case as well.

In another experiment, N^{14} *D. pneumoniae* DNA was heated and slowly cooled with N^{14} *Serratia marcescens* DNA. The density of the native samples are 1.700 and 1.717 respectively. The resulting trace showed two clearly separated bands of reformed *D. pneumoniae* of density 1.704 and reformed *Serratia* of density 1.721. There was no evidence of a component of intermediate density indicating that neither aggregation nor interspecies hybridization had occurred.

This serves as an ideal control to show that molecules having approximately the same density difference (0.017 density unit) but not the genetic similarity discussed above, show a strong preference for renaturing only with members of their homologous species. Only in the case where similarity in base composition has been correlated with genetic interaction has it been possible to demonstrate the appearance of heterozygous molecules.

Discussion.—Our aim in this paper has been to present a series of preliminary reports of closely related work on strand separation and molecular recombination in DNA. Consequently, the discussion will be postponed until the fuller accounts are published. Nevertheless, perhaps two points may be emphasized briefly.

The demonstration that strand separation actually occurs in a matter of a few minutes or less eliminates some of the objections[27] pertaining to the unwinding of DNA that have been put forward as a criticism of the Watson–Crick mechanism[28]

for DNA duplication. What has been observed here favors the more recent calculations of the time required for this process.[29, 30] Also, although it is not as yet apparent whether the reformation of denatured DNA during slow cooling has a biological counterpart, the demonstration that it does occur suggests a mechanism for genetic exchange in closely related DNA molecules.

The second point is the recognition that the routine reduction of DNA into single strands and their specific reformation into essentially native molecules provides a means of creating entirely new DNA molecules. The study of the progeny of such heterozygous molecules will be of obvious interest. A particular instance is that of a heterozygous molecule each strand of which contains a different marker. The progeny from cells having a single nucleus may be pure clones, mixed clones or transformants with markers normally linked. Any of these results would, of course, illuminate the molecular aspects of the replication act. If linkage can be found, not only will crossing-over be demonstrated but the possibility of making *in vitro* new forms of viable DNA not previously existent will be assured. It seems likely that heterozygous DNA molecules will find other uses as well. For example, active hybrid molecules containing a greater variety of alterations in one strand. New possibilities of testing whether or not homologous ribonucleic acid has sequences in common with DNA can now be explored. The formation of molecular hybrids between closely related organisms should be a useful tool for plotting homologies in base sequences where no genetic exchanges have yet been demonstrated.

Summary.—When solutions of bacterial DNA are denatured by heating and then cooled, two different molecular states can be obtained in essentially pure form depending on the choice of conditions, that is, rate of cooling, DNA concentration, and ionic strength. One state corresponding to fast cooling consists of single stranded DNA having about half the molecular weight of the original DNA. The other state corresponding to slow cooling consists of recombined strands united by complementary base pairing over most of their length. This form has as much as 50 per cent of its original transforming activity and is called renatured. The quickly cooled, single stranded form is essentially inactive and is called denatured. These two forms are clearly identified by differences in (1) absorbance-temperature curves, (2) density, (3) appearance in electron micrographs and (4) hydrodynamic properties. The recombination depends on concentration in the manner expected for an equilibrium between two independent strands and a bimolecular complex.

Molecular weight determinations of the native and renatured forms have been based on an extension of an earlier calibration of sedimentation and intrinsic viscosity in terms of light scattering measurements. For the denatured form molecular weights were determined by the use of the Mandelkern-Flory relation. By these means thermal degradation was accurately assessed. To avoid aggregation it was necessary to substantially lower the cation concentration over that previously used. In this way denatured DNA has been characterized as a single-chain, unaggregated polymer and strand separation demonstrated.

Density gradient experiments on N^{14} and N^{15} *E. coli* DNA have shown the the existence of hybrids in the DNA renatured from the mixture. Similarly, hybrids have been shown to form between the strands of bacteria that are closely related genetically. Thus the possibility of forming by renaturation heterozygous DNA

molecules with different genetic markers or chemical modifications in the two strands seems assured.

The authors would like to thank Miss D. Lane for valuable assistance and Professor A. Peterlin, Professor J. D. Watson, and Dr. N. Sueoka for helpful advice and discussions. We are very grateful to Professor C. E. Hall of the Massachusetts Institute of Technology for the electron micrographs and to Drs. S. E. Luria and M. Demerec for supplying us with strains of *Shigella dysenteriae* and *Salmonella typhimurium* (LT-2). This work was supported by a grant from the United States Public Health Service (C-2170).

* Predoctoral Fellow of the National Science Foundation.

[1] Marmur, J., and D. Lane, these PROCEEDINGS, **46**, 453 (1960).

[2] Marmur, J., and P. Doty, *Nature*, **183**, 1427 (1959).

[3] Doty, P., H. Boedtker, J. R. Fresco, R. Haselkorn, and M. Litt, these PROCEEDINGS, **45**, 482 (1959).

[4] Meselson, M., F. W. Stahl, and J. Vinograd, these PROCEEDINGS, **43**, 581 (1957).

[5] Sueoka, N., these PROCEEDINGS, **45**, 1480 (1959).

[6] Sueoka, N., J. Marmur, and P. Doty, *Nature*, **183**, 1429 (1959).

[7] Rolfe, R., and M. Meselson, these PROCEEDINGS, **45**, 1039 (1959).

[8] Doty, P., J. Marmur, and N. Sueoka, *Brookhaven Symposia in Biology*, **12**, 1 (1959).

[9] Hall, C. E., and M. Litt, *J. Biophys. and Biochem. Cytology*, **4**, 1 (1958).

[10] Hall, C. E., and P. Doty, *J. Am. Chem. Soc.*, **80**, 1269 (1958).

[11] Hall, C. E., and H. Boedtker (unpublished results).

[12] Wright, G., senior thesis, Harvard University, 1960.

[13] Doty, P., and B. H. Bunce, *J. Am. Chem. Soc.*, **74**, 5029 (1952).

[14] Reichmann, M. E., S. A. Rice, C. A. Thomas, and P. Doty, *J. Am. Chem. Soc.*, **76**, 3407 (1954).

[15] Butler, J. A. V., D. J. R. Laurence, A. B. Robins, and K. V. Shooter, *Proc. Roy. Soc.*, **A250**, 1 (1959).

[16] Eigner, J., doctoral thesis, Harvard University, 1960.

[17] Mandelkern, L., H. A. Sheraga, W. R. Krigbaum, and P. J. Flory, *J. Chem. Phys.*, **20**, 1392 (1952).

[18] Doty, P., B. B. McGill, and S. A. Rice, these PROCEEDINGS, **44**, 432 (1958).

[19] Kawade, Y., and I. Watanabe, *Biochim. Biophys. Acta*, **19**, 513 (1956).

[20] Iso, K., and Watanabe, I., *J. Chem. Soc. Japan, Pure Chem. Section*, **78**, 1268 (1957), in Japanese.

[21] Rice, S. A., and P. Doty, *J. Am. Chem. Soc.*, **79**, 3937 (1957).

[22] Alexander, P., and K. A. Stacey, *Biochem. J.*, **60**, 194 (1955).

[23] Bacher, J. E., and W. Kauzmann, *J. Amer. Chem. Soc.*, **74**, 3779 (1952).

[24] Litt, M., J. Marmur, H. Ephrussi-Taylor, and P. Doty, these PROCEEDINGS, **44**, 144 (1958).

[25] Baron, L. S., W. F. Carey, and W. M. Spilman, these PROCEEDINGS, **45**, 1752 (1959).

[26] Lee, K. Y., R. Wahl, and E. Barbu, *Ann. Inst. Pasteur*, **91**, 212 (1956).

[27] Delbrück, M., and G. S. Stent, in *The Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins Press, 1957), p. 699.

[28] Watson, J. D., and F. H. C. Crick, *Nature*, **171**, 964 (1953).

[29] Levinthal, C., and H. R. Crane, these PROCEEDINGS, **42**, 436 (1956).

[30] Longuet-Higgins, H. C., and B. H. Zimm, *J. Mol. Biol.* (in press).