Denaturation and Renaturation of Deoxyribonucleic Acid[1,2]

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[2] In discussing the denaturation and renaturation of nucleic acids, particular emphasis has been placed on the biological implications. In many cases, only the more recent publications have been cited, since they include the earlier observations and references, and no attempt has been made to be exhaustive with respect to the literature.

I. Properties of the Native and Denatured States of DNA

Detailed diffraction data from crystalline fibers of DNA (Wilkins *et al.*, 1953) have helped greatly in elucidating its structure as well as in the building of the Watson–Crick (1953a) model. The double-stranded model accounts very well for the observed properties of native DNA. The two antiparallel chains are interwoven in a double helix of planar stacked bases about a common axis. X-ray diffraction analyses of DNA isolated from a wide variety of sources exhibit similar patterns (Hamilton *et al.*, 1959; Wilkins, 1961). Luzzati *et al.* (1961, 1962) have found, using small-angle-scattering X-rays, that the mass per unit length of DNA of bacterial and animal origin in solution agrees well with the Watson–Crick structure.

The molecular weight of DNA *in vivo* remains uncertain. This arises from its ease of shear degradation (Davison, 1959) during extraction and purification as well as from the difficulty in assessing its size by the methods which are currently available. Chromatographic, autoradiographic (Davison *et al.*, 1961; Rubenstein *et al.*, 1961; Cairns, 1961; 1962a,b), and electronmicrographic (Beer, 1961; Bendet *et al.*, 1962) techniques have proven to be extremely useful in estimating the molecular size of DNA to be greater than 10^7 where other techniques such as light scattering have been found wanting (Butler *et al.*, 1959b). The fact that the genetic units of *Escherichia coli* (Jacob and Wollman, 1958) as well as bacteriophage T2 (Streisinger and Bruce, 1960) are situated on single linkage groups is suggestive evidence that the chromosome consists of a continuous physical entity. This is supported by the work of Kleinschmidt *et al.* (1961) and Kleinschmidt (1962) with *Micrococcus lysodeikticus* and *Bacillus megaterium* and of Cairns (1961; 1962a) who has carefully isolated T2 bacteriophage DNA with average lengths of 49 μ as well as *E. coli* DNA with some molecules up to 400 μ in length. If the latter molecules are double-stranded, their lengths correspond to a molecular weight of 10^9. Cairns (1962b) has also shown that λ bacteriophage DNA, when carefully isolated, has a length ranging up to 23 μ. However, since even the most highly purified DNA preparations still possess approximately 0.1–0.2% by weight of either proteins, peptides, or amino acids (Borenfreund *et al.*, 1961; Van Vunakis, personal communication), it still remains to be shown that the large DNA molecules are not held together by non-DNA links (see, for example,

Kirby, 1957; Sadron, 1961; Welsh, 1962). (See also Bendich and Rosenkranz, this volume.)

The native DNA molecule in solution is relatively insensitive to changes in shape when exposed to wide ranges of ionic strength, and thus does not behave as a typical polyelectrolyte. Its hydrodynamic configuration is neither a rod nor a typical randomly coiled polymer but can be described qualitatively as "a highly extended, stiff coil of moderate permeability" (Eigner and Doty, 1963). Essentially the same conclusion, namely, that DNA (in physiological salt solution) is a "worm-like chain" (Doty, 1957), has been arrived at by Hearst and Stockmayer from their analyses (1962). Supporting this view of DNA is its high viscosity compared with other typical polymers with the same degree of polymerization (Flory, 1953) as well as the large extension of the native molecule in space (Doty, 1957).

The relationship between sedimentation coefficient (s_0) and intrinsic viscosity $[\eta]$ for a large number of DNA samples from various sources has been examined by Eigner and Doty (1963). The values for s_0 and $[\eta]$, when plotted for a series of DNA samples varying in source, base composition, compositional heterogeneity, and the presence or absence of hydroxymethylcytosine and glucose, all fall on a curve relating the two parameters as a continuous function. This indicates that DNA's in solution in the *native* state, isolated from various sources, are homogeneous in configuration and supports the concept of a common (two-stranded) structure for all. This relationship between s_0 and $[\eta]$ can be altered by denaturation of the DNA or by what is believed to be the intercalation of dye molecules, which impose a high rigidity and viscosity on the DNA molecule (Lerman, 1961).

Although the native DNA preparations from bacterial and animal sources listed by Cavalieri *et al.* (1961) have not been fully characterized, it appears that the behavior of their higher molecular weight preparations in solution is somewhat different from those studied in other laboratories. This is apparent when one compares the radii of gyration of the DNA by light scattering (which exhibit anomalous, low values) with those listed by Steiner and Beers (1961, p. 203). This might be explained by the aggregation of the DNA molecules in their preparations and might help rationalize the persistence of DNA structures which behave as though they were four-stranded.

The denaturation of DNA is brought about by the collapse of its one-dimensional, uniquely ordered secondary structure. In this review, partially denatured molecules are considered to be collapsed DNA structures having both helical and nonhelical regions ranging between the native and single-stranded configurations. The collapsed molecules can

be derived either from native molecules that have not separated completely or from the partial renaturation of separated, complementary strands. Aggregation refers to the intermolecular interaction of the noncomplementary strands over short, intermittent helical regions. Complete denaturation should yield the two complementary strands of the Watson–Crick double helix (equivalent to irreversible denaturation in the earlier literature). When DNA exposed to elevated temperatures at high ionic strengths is rapidly cooled, denaturation takes place in a cooperative manner (Schildkraut *et al.*, 1961a; Freifelder and Davison, 1962a). When the heating is carried out at lower ionic strengths, disruption of the double helical structure takes place over a broader temperature range, is less cooperative, and is believed to lead to the formation of partially denatured molecules in a metastable state (Dove and Davidson, 1962a; Rownd *et al.*, 1963c). Degradation refers to the breakage of covalent bonds, usually the backbone phosphate ester linkages (as would occur in shear degradation) or glycosyl bonds (during heating, acidification, or exposure to various chemicals). Denaturation and degradation (Doty *et al.*, 1958) can essentially occur independently of one another.

Denatured DNA behaves as a flexible, loosely coiled polyelectrolyte chain, very much dependent in its hydrodynamic properties on the ionic environment (May, 1962). In this respect, it is very similar qualitatively in many ways to RNA, the single-stranded synthetic polynucleotides (Steiner and Beers, 1961), and the noncircular form of single-stranded ϕX174 DNA (Sinsheimer, 1959; Eigner and Doty, 1963).

The secondary structure of denatured DNA is dependent not only on ionic strength but also on the base composition. High guanine plus cytosine (G + C) results in a higher extent of *intra*chain base pairing (Eigner and Doty, 1963) in a manner similar to that proposed for RNA (Fresco and Alberts, 1960; Fresco *et al.*, 1960). Regions of the DNA molecule are folded back on themselves to form double helices and are joined by unorganized polynucleotide chains. Those nucleotides that do not pair could be "looped out" of the helical structure as in the synthetic polynucleotide interactions. The quantitative differences that do exist between denatured DNA and RNA (greater tendency on the part of DNA to aggregate and to be influenced by changes in counter-ion concentration) can be accounted for by the fact that RNA has a more stable and ordered secondary structure, which most likely arises from the helical regions being longer and containing fewer defects (Doty *et al.*, 1959a; Timasheff *et al.*, 1961; Steiner and Beers, 1961; Fresco *et al.*, 1961; Spencer *et al.*, 1962; Eigner and Doty, 1963) than denatured DNA.

The term "renaturation" in the case of DNA is used to signify the reunion of the specific complementary strands leading to the formation

of molecules with native-like properties. Intramolecular renaturation can proceed from collapsed DNA molecules of any source that are held together by residual base pairs or cross-links when the DNA is exposed to appropriate conditions (Cavalieri and Rosenberg, 1957; Geiduschek, 1958; Doty *et al.*, 1959b). It can also take place from separated complementary strands, in which case it has the properties of an intermolecular process (Schildkraut *et al.*, 1961a; Geiduschek, 1962). Renaturation of DNA can be followed in a number of ways discussed in this review.

The term renaturation has also been employed to indicate the interaction between strands of DNA and RNA, arising from the presence of complementary base sequences in the two nucleic acids, to form RNA: DNA hybrids. Renaturation will take place most readily between such polynucleotides as poly A and poly U where the requirements for sequence complementarity are most readily met.

II. Early Denaturation Studies on DNA

The proposal by Watson and Crick (1953a) of the two-strand helical structure for DNA not only provided a structural model that explained many of its observed chemical and physical properties but also suggested a mechanism for self-duplication or replication. The union of the two constituent strands of the DNA double helix is not mediated by covalent linkages, but rather by complementary base pair interactions. Hence, it was plausible to propose a template mechanism for replication whereby each strand, by unwinding, could serve as a template for the assembly and subsequent polymerization of the complementary nucleotides. Thus, by a mechanism involving unwinding or strand separation of its DNA, the genetic information of an organism encoded in the sequence of bases could be passed on to its progeny.

This mechanism is certainly aesthetically pleasing on the biological level, but contains difficulties when visualized in molecular terms. Not only is it necessary to account for the rather large energy requirement to unwind the two strands of large DNA molecules through a large number of turns, but it is also difficult to imagine the separated strands within the cell other than in a state of hopeless entanglement. However, more recent theoretical considerations have indicated that the energy requirement can be satisfied if the synthesis of the new material goes on simultaneously with the separation of the two strands of the old (Levinthal and Crane, 1956; Levinthal, 1960). The entanglement problem seems to suggest that not all of the DNA in the cell exists in the single-stranded state at any one time (Watson and Crick, 1953b; Paigen, 1962). The demonstration of strand separation in solution lends consid-

erable support to the replication scheme proposed by Watson and Crick (1953b) and raises the hope that the structure and function of DNA may be reconciled.

The obvious importance of understanding the changes in the physicochemical properties of DNA when exposed to denaturing conditions has prompted many workers in the field to use a variety of physicochemical as well as biological techniques to study the denaturation reaction. Space does not allow an intensive survey of the early literature on this subject. These results have been amply discussed in a number of other reviews (Shooter, 1957; Peacocke, 1960; Drysdale and Peacocke, 1961; Doty, 1955, 1957, 1959–1960; Sinsheimer, 1962; Zimm and Kallenbach, 1962; Cavalieri and Rosenberg, 1962a,b) as well as in several books (Jordan, 1960; Steiner and Beers, 1961).

The denaturation of DNA has been studied by a wide variety of experimental techniques based, for the most part, on its optical, hydrodynamic, electrometric, and biological properties (see Section IV,A). The concept of denaturation that emerged from the large body of experimental data accumulated over the first decade of intensive study (the period 1947–1957) was the collapse of the DNA molecule with no concomitant change in its molecular weight (Sturtevant *et al.*, 1958; Peacocke, 1960). The contraction of the molecule from the loss of its secondary structure was attributed to the irreversible rupture of the numerous hydrogen bonds between the complementary base pairs of the polynucleotide chains making up the twin helix. However, the failure to observe a halving of the molecular weight upon denaturation argued against the complete separation of the strands although it was generally conceded from the concomitant viscosity changes that the majority of the native hydrogen bonds had been disrupted.

The first observations on the denatured state of highly polymerized DNA were made by Gulland *et al.* (1947) who studied the titration curve of the native structure. When DNA has been treated previously either by acid or by alkali, it no longer gives rise to its characteristic initial titration curve. This "hysteresis" effect was the first indication that groups involved in hydrogen bonding, and hence not accessible to protonation, are released upon denaturation. Moreover, it was also shown that treatment with acid or with alkali results in a decrease in viscosity and a loss of streaming birefringence, which occurs over a relatively narrow pH range. It could thus be inferred that hydrogen bonds are involved in the maintenance of the rigid secondary structure of DNA.

In the following decade, these initial observations were amply confirmed and extended by many workers who used a variety of physico-

chemical and biological techniques to characterize the denaturation of DNA. Thomas (1954) showed that the hypochromicity of the native structure can be reduced under denaturing conditions. This result was interpreted in terms of the breakdown of the uniquely ordered secondary structure of DNA. Reichmann *et al.* (1953) examined the effects of exposure to acid pH on the molecular weight and shape of DNA. They concluded that, although the molecule is extensively collapsed under these conditions, there is no apparent change in its light-scattering molecular weight. Most surprising, in view of later investigations, was the observation that the radius of gyration (a measure of chain extension) and the viscosity return to the native values upon reneutralization of the solution. This suggested that the DNA had been reversibly denatured since it assumed the highly asymmetric native configuration when the denaturing conditions were removed. The failure of many investigators to observe a drop in the molecular weight of DNA (see Steiner and Beers, 1961) at low pH values *before* reneutralization might be explained by the presence of resistant nuclei holding the strands together. It is difficult to interpret any drop in the light-scattering molecular weight under these conditions, since it is necessary to distinguish it from the acidic degradation that occurs during the course of the measurements.

The titration studies by Cavalieri and Rosenberg (1956, 1957) and by Cox and Peacocke (1957, 1958a,b) showed that denaturation by acid or by alkali occurs during the steep portion of the forward titration curve. It was also shown that it is possible to dissociate reversibly the first 10–20% of the hydrogen bonds at the start of the denaturation process. Denaturation results in the reduction of the asymmetry of the DNA molecule, but this occurs at constant molecular weight (Cavalieri and Rosenberg, 1956). These studies did not establish whether denaturation resulted from an irreversible collapse of the molecule to form a *partially* denatured structure or whether a molecular species is denatured only after the *complete* disruption of the double helical structure (strand separation) occurs. For reasons to be discussed in the following section on the mechanism of denaturation, it seems that the latter is the more highly preferred means.

Most of the earlier studies on the denaturation of DNA added little precise knowledge to the conformation of the denatured state in solution. Most often only one physical method was employed to characterize the denatured state, or the denaturing conditions employed often led to either severely degraded or aggregated DNA samples. It could be established only that exposure of solutions of DNA to denaturing conditions effected considerable alteration in its secondary structure. More exact

information was obtained, however, when several physicochemical methods were employed simultaneously to characterize the denatured state, thereby permitting a more rigorous structural assignment. It then became possible to follow in a more systematic fashion the combined effects resulting from the denaturation process.

The first comprehensive study of the effects of denaturation on the macromolecular properties of DNA was undertaken by Doty and his collaborators (Doty and Bunce, 1952; Reichmann *et al.*, 1953; Thomas and Doty, 1956; Doty, 1956; Rice and Doty, 1957; Doty and Rice, 1955; Ehrlich and Doty, 1958; Doty *et al.*, 1959a). They employed a number of optical and hydrodynamic techniques to characterize DNA denatured under mild conditions by exposure to acid, to alkali, and to elevated temperature. Intrinsic viscosity measurements confirmed the observation of Zamenhof *et al.* (1953, 1954) of a precipitous and parallel fall in both the viscosity and transforming activity of *Hemophilus influenzae* DNA in the vicinity of 80°C. The sharpness of the transition suggested a configurational change with the characteristics of the melting of a periodic intramolecular structure (Doty, 1957; Bresler, 1958). The interpretation of this apparent phase transition in molecular terms, however, depended on whether any molecular weight change had occurred during the heating procedure. The molecular weights of the heated samples were determined by light scattering. This method was considered valuable in the macromolecular characterization of the DNA polymer for two reasons. Not only does it provide a technique for the absolute determination of the molecular weight, but also, unlike any other physical method, it provides a measure of the dimensions of a macromolecular particle without any assumptions regarding its general form or shape. Light-scattering measurements of denatured DNA samples by Doty and co-workers, as well as by others, showed that the molecular weight had not increased or decreased, but, rather, remained unchanged. The radius of gyration of the denatured DNA, however, had decreased by a factor of three, corresponding to a decrease in the effective hydrodynamic volume by a factor of 27. The light-scattering results thus confirmed the viscosity measurements that showed an extensive collapse of the DNA secondary structure but at constant molecular weight. A further consequence of these studies was an anomaly in the sedimentation coefficient of denatured DNA. When a polymer has decreased in effective hydrodynamic volume, but not in its molecular weight, the sedimentation coefficient should correspondingly increase because of a reduction of the frictional coefficient. The sedimentation coefficient of denatured DNA did not differ from that of the native state (in approximately 0.2 M Na^+), although there was a broadening of the

sedimenting boundary (Shooter *et al.*, 1956; Rice and Doty, 1957; Ehrlich and Doty, 1958; Sturtevant *et al.*, 1958; Inman and Jordan, 1960).

Since the expected halving of the molecular weight as a consequence of strand separation was not observed, the following conclusions can now be drawn from the early studies on the denaturation of DNA. Denaturing conditions result in the irreversible rupture of a substantial fraction of the hydrogen bonds between complementary base pairs, which leads to a collapse of the rigid secondary structure of the native molecule. However, either not every base pair is dissociated, thus preventing the complete separation of the polynucleotide chains, or the potential base pairing capacity between the two chains is sufficiently great so that the separating strands entangle to form aggregates and give rise to double the mass of the individual strands. Since the expected increase in the sedimentation coefficient upon denaturation is not observed, it appears, moreover, as if the collapsed molecules display atypical polymer behavior. This latter possibility became untenable in the light of later results on the hydrodynamic behavior of RNA and the synthetic polyribonucleotides (Boedtker, 1959, 1960; Hall and Doty, 1958, 1959; Fresco and Doty, 1957; Fresco and Klemperer, 1959; Haselkorn, 1959). Although this group of polymers differs from DNA because of the presence of a hydroxyl group at the 2 position of the sugar moiety, this difference should not give rise to any appreciable variation in their general hydrodynamic behavior. It was found that these close analogs of the DNA single strand exhibited the behavior of randomly coiled polymers.

The very fact that denatured DNA exhibits anomalous hydrodynamic behavior leads one to suspect that uncontrolled variables were still operative in the earlier characterizations of the denatured state of DNA. Since it now appears certain that the polynucleotide chains are separable, it seems possible that these same variables were responsible for the failure to observe a halving of the molecular weight. In retrospect, it seems that in some experiments the strands did separate upon denaturation, but that short, unorganized base pair interactions led to an association of the separated chains, which was promoted by the high ionic strength and DNA concentration as well as by the method of cooling. When DNA that has gone through one cycle of denaturation is taken through a second heating cycle, it is obvious from the broad nature of the absorbance-temperature curve that the reformed regions being melted out consist of short, imperfect helices. The extent of these base pair interactions that are melted out in subsequent thermal exposures is approximately 70–80%, estimated from absorbance measurements (Doty *et al.*, 1959a). Although attempts were made to exclude aggregation as

the basis of the failure to observe a halving of the molecular weight by light scattering (Rice and Doty, 1957; Geiduschek, 1958), this may not have been excluded. The earlier studies on denaturation were carried out with DNA from animal sources; this is very heterogeneous in composition. The separated strands would be less likely to renature but more likely to aggregate since the likelihood of two noncomplementary strands finding short regions that can pair would be much higher than for DNA from viral or bacterial sources. The more recent demonstrations of a halving of the molecular weight of homogeneous DNA after denaturation under conditions that minimize or exclude aggregation would seem to settle the issue. Hence, in the early denaturation studies, it appears that nonspecific aggregation led fortuitously to a molecular weight value of the denatured DNA approximately double that expected for the separated single strands.

III. Denaturation and Evidence for Strand Separation

A. Semiconservative Replication

The demonstration of the separation of the DNA subunits during replication and under denaturing conditions came from the experiment of Meselson and Stahl (1958). In their investigation of the mode of replication of DNA, cells of *E. coli* B were grown on a medium containing N^{15} as the sole nitrogen source, then transferred to one containing N^{14}. Aliquots of the cells were removed at various times after the media shift; these were lysed and centrifuged in a CsCl density gradient in order to analyze the amounts of N^{15}, N^{14}, and hybrid DNA. The first new species of DNA synthesized one generation after the media shift was N^{14}–N^{15} DNA, which was found to band in CsCl exactly midway between the fully labeled (N^{15}) and the unlabeled (N^{14}) DNA species. At the second generation of growth, two DNA species were present; one consisted of half-labeled molecules and the other completely unlabeled. These results demonstrate that the replication of DNA in *E. coli* most likely proceeds by a semiconservative mechanism (Delbruck and Stent, 1957) in which only one half of the parental structure is passed on to the daughter molecules. The later experiments of Rolfe (1962), who degraded the half-labeled or biological hybrid DNA with ultrasonic waves, showed that the subunits were arranged side by side, rather than end to end.

When the *E. coli* biological hybrid DNA was heated at 100°C for 30 minutes at a low concentration of the nucleic acid in approximately 5.7 *M* CsCl, it was found that two DNA species, equal in amount, banded at different positions in the density gradient. Furthermore, the mean band width of the denatured DNA increased, indicating a lowering of

the molecular weight induced by the denaturation (Meselson *et al.*, 1957). In a reconstruction experiment it was found that an equal weight mixture of N^{14}- and N^{15}-labeled DNA, after denaturation, bands in the same position in the density gradient as the separated strands discussed above.

In experiments with biological hybrid *E. coli* DNA (in this case half-labeled with bromouracil), Shooter and Baldwin (1962) observed that the alkaline denaturation of bromouracil-containing DNA occurs at a lower alkaline pH than that for thymine-containing DNA. By carefully controlling the pH in the denaturation of hybrid bromouracil-containing DNA, it was found that the two denatured species banding in CsCl represented the dissociation of hybrid molecules of the Watson–Crick type where one strand is labeled with bromouracil and the other unlabeled, excluding a four-stranded ("biunial") structure in which two strands are labeled and two unlabeled (Cavalieri and Rosenberg, 1962b).

The combined implications of these experiments are extensive. They clearly and simply demonstrate the semiconservative replication of DNA in microorganisms and the experiments also show that under appropriate conditions (replication as well as denaturation) it is possible to demonstrate the separation of the DNA subunits. It should be emphasized that the denaturing conditions differed in an important way from those previously reported. The concentration of the DNA during denaturation was at least 40-fold less than that employed in the earlier investigations. The DNA concentrations used in the earlier light-scattering studies ranged from 40 to 100 μg/ml (Rice and Doty, 1957; Geiduschek, 1958; Geiduschek and Holtzer, 1958); Meselson and Stahl (personal communication) used only 1–2 μg/ml. Hence, intermolecular aggregation of denatured DNA, which is a function of the DNA concentration, ionic strength, and rate of cooling after denaturation (Eigner, 1960; Eigner and Doty, 1963), occurred to a lesser extent, if at all. Moreover, the differential sedimenting forces in the ultracentrifuge, because of the density differences, may have effected the separation of a few residual bonds that would otherwise have held the chains together. This latter explanation appears not to be likely (Rownd, 1963; Rownd and Doty, 1963) and will be discussed in the section on renaturation.

The extension of the heavy isotope labeling technique to the study of DNA replication in *Chlamydomonas* (Sueoka, 1960) as well as the use of 5-bromouracil to follow the replication of mammalian cell DNA in tissue culture (Djordjevic and Szybalski, 1960; Chun and Littlefield, 1961; Simon, 1961) have shown, in all cases where such experiments are possible, that DNA replication takes place in a manner similar to that observed by Meselson and Stahl (1958) for *E. coli*. It has not been

rigorously demonstrated in many of the cases that denaturation of the biological hybrid DNA results in the separation of the component subunits. It has been shown, however, as in the case of *E. coli* and employing the technique of CsCl density gradient centrifugation, that denaturation of biological hybrid DNA isolated from *B. subtilis* also results in the separation of the subunits (Rownd and Green, unpublished).

Another approach to answering the question whether replication of DNA involves strand separation has been that of Cairns (1962b). His conclusion that the strands of λ bacteriophage DNA do separate is based partly on the observation by Meselson and Weigle (1961) that the DNA from this bacteriophage forms hybrids on replication and partly on his own data that λ DNA is two-stranded, using autoradiography to calculate the ratio of the total length to the total mass. The argument used by Cairns is based on the assumption that the DNA isolated from λ represents its native length. It would be of interest to see whether the exposure of biological hybrid λ DNA to denaturing conditions also results in the separation of the strands.

B. Hydrodynamic Evidence

With the demonstration by Meselson and Stahl (1958) that the molecules of *E. coli* DNA can be dissociated into two equal subunits under denaturing conditions, and, moreover, that the separated subunits corresponded to those conserved during replication, experiments were designed to demonstrate the halving of the molecular weight by other techniques. Since the earlier light-scattering molecular weight determinations had not demonstrated the separation of the polynucleotide chains, other physical methods were employed and the denaturing conditions modified to minimize the aggregation of the separated single strands. This was accomplished by inhibiting the reformation of the short helical regions after the reversal of the denaturing conditions.

It should be pointed out that Alexander and Stacey (1955a,b) had reported a halving of the molecular weight as measured by light scattering. They reported that a reduction to one half of the original molecular weight could be brought about by (1) exposure to pH 2.2 followed by reneutralization and (2) exposure to 4 *M* urea at room temperature. Their interpretations were criticized by Doty and Rice (1955; Rice and Doty, 1957), who showed that chemical degradation occurred at this low pH (Thomas and Doty, 1956) and that light scattering measurements in a three-component system could not properly be interpreted in terms of the molecular weight. In addition, 4 *M* urea would not be expected to denature DNA at room temperature (Rice and Doty, 1957; Ts'o *et al.* 1962a). In any event, the results and conclusions of Alexander and

Stacey were equivocal and did not provide evidence for strand separation.

In order to demonstrate the halving of the molecular weight under denaturing conditions by hydrodynamic methods, it was necessary to standardize an approach whereby the complicating features of aggregation and depolymerization could be understood and eliminated as far as possible. When light scattering has been used to determine molecular weights after denaturation, spurious results have been obtained due to aggregation of the denatured DNA. Since the light scattering technique is valid within a certain molecular weight range it can be used to calibrate secondary methods such as sedimentation and viscosity. Eigner (1960) and Eigner and Doty (1963) have used light scattering measurements in combination with viscosity and sedimentation measurements to evaluate β of native DNA. The validity of the original sedimentation and viscosity molecular weight equations is restricted to the validity of the light scattering measurements as far as the absolute values of the molecular weights are concerned. Eigner's experience has shown that changes in molecular weight upon denaturation are more easily assessed by sedimentation-viscosity measurements.

Eigner (1960) showed that significant aggregation occurs upon cooling denatured DNA in solutions of moderate ionic strength ($\sim$0.2 M Na^+). Hence, to avoid the complicating effects of interchain aggregation, a low ionic strength solvent was selected ($\sim$0.01 M Na^+); aggregation is minimized under these conditions since electrostatic repulsion of the charged phosphates of the polynucleotide chains prevents close interchain association. These results indicate a rather highly swollen chain configuration for denatured DNA at low ionic strength, in agreement with earlier observations (Ehrlich and Doty, 1958). For a given chain length, the molecular dimensions correspond to those expected for a typical polyelectrolyte. Since heating DNA at elevated temperatures for extended periods causes hydrolytic degradation of phosphate ester bonds (Doty *et al.*, 1960; Eigner *et al.*, 1961; Fiers and Sinsheimer, 1962), backbone scission must be considered in the evaluation of the molecular weight determined by sedimentation-viscosity measurements. Since thermal denaturation occurs at decreased temperatures as the ionic strength of the solvent is lowered (Thomas, 1954; Rice and Doty, 1957; Doty *et al.*, 1959a; Marmur and Doty, 1959, 1962), chemical degradation, an undesirable side effect, is minimized under the conditions selected by Eigner and Doty (1963).

These authors then followed the effects of heating *E. coli* and *Diplococcus pneumoniae* DNA at a temperature 15° higher than the midpoint of the absorbance-temperature transition profile (the T_m). The effects of depolymerization were eliminated by extrapolation to zero

time of heating. The s_0 and $[\eta]$ were measured after the heated and rapidly cooled solutions were returned to room temperature. Since the ionic strength of the solvent was sufficiently low, aggregation did not occur after the DNA solutions were cooled. The extrapolation to zero time of heating showed that the molecular weights had decreased by factors of at least two as the result of the thermal denaturation. Thus, with aggregation eliminated and thermal depolymerization taken into account, strand separation must have occurred in the early stages of the exposure to the elevated temperature.

The data of Eigner (1960; Eigner and Doty, 1963) also explain the earlier discrepancies between the molecular weight and the sedimentation coefficient that suggested anomalous hydrodynamic behavior for denatured DNA. The constant value of the molecular weight as measured by light scattering is most readily described as arising from the aggregation of the separated chains to form a product of twice the mass of the individual strands. This is most likely because of the high concentrations of DNA employed in the light scattering determinations and the lack of control of the rate of cooling after thermal denaturation and/or the high ionic strength of the solution in which the denatured DNA is dissolved, each or all of which would favor aggregation (Eigner and Doty, 1963). When aggregation is minimized by lowering the DNA concentration and the ionic strength of the solvent, normal coiled polymer hydrodynamic behavior is found for denatured DNA.

Although it has now been well established that the molecular weight of the DNA isolated from bacterial sources is halved when exposed to denaturing conditions (Doty *et al.*, 1960; Eigner, 1960; Eigner and Doty, 1963) and, moreover, that the subunits that are separated are those conserved during replication *in vivo* (Meselson and Stahl, 1958), some recent evidence suggests that the subunits do not represent the DNA single strands (Cavalieri and Rosenberg, 1961; Cavalieri *et al.*, 1962). Their conclusions are that rapidly dividing cells (e.g., *E. coli* in its logarithmic phase of growth) have their DNA in a four-strand state consisting of two Watson–Crick double helices lying side by side, held together by "biunial" bonds (see the discussion by Luzzati in this volume). It is the disruption of these bonds that results in the drop in molecular weight, whereas the complementary strands of either Watson–Crick double helix *cannot* completely separate although their registration can be disrupted, leading to a collapsed structure. Cavalieri and Rosenberg (1961) conclude, however, that DNA extracted from nondividing or "resting" cells, e.g., calf thymus, is double-stranded, according to the Watson–Crick model. Although biunial bonds as described by Cavalieri are still ill-defined, recent studies on the separation of the subunits of the

biological hybrid DNA of *E. coli* have shown that the subunits separate under conditions known to rupture the complementary base pairs of the DNA molecule (Schildkraut *et al.*, 1961a; Freifelder and Davison, 1962; Shooter and Baldwin, 1962).

It is now fairly well documented that the DNA isolated from the *E. coli* bacteriophages T2, T3, T4, and λ are double-helical molecules of the structure proposed by Watson and Crick. The evidence, based on the mass per unit length (reviewed in Section IV,A), suggests that the DNA structure is a continuous molecule consisting of a single Watson–Crick type of structure. The separation of the polynucleotide chains of one of these molecules would be established if it could be demonstrated that the molecular weight is halved under denaturing conditions. The problem of aggregation is especially acute with molecules of such large physical size. Therefore, special precautions must be taken to insure that aggregation of the denatured DNA is completely eliminated. The elimination of aggregation can be accomplished by denaturing in the presence of formaldehyde, which reacts rapidly with the amino groups exposed by the dissociation of the interchain hydrogen bonds (Grossman *et al.*, 1961; Haselkorn and Doty, 1961; Thomas and Berns, 1961). The reaction is generally followed by changes in ultraviolet absorbance. That intrachain hydrogen bonding is also blocked by the formaldehyde treatment is evident from the fact that, upon cooling the denatured sample to room temperature, very little of the native hypochromicity is regained.

Thomas and Berns (1961) have studied the denaturation of unbroken T2 and T4 DNA molecules by CsCl density gradient centrifugation in the presence of formaldehyde. The unbroken molecules were denatured by three methods in the presence of 1% formaldehyde. The molecular weight of denatured whole molecules was found to be one half of that of the native molecule, irrespective of the mode of denaturation. Thus, denaturation of the T2 or T4 DNA molecule, known to possess a double-strand structure on the basis of mass per unit length considerations, resulted in a halving of the molecular weight. This could only be accomplished by the separation of the polynucleotide chains under the denaturing conditions employed. These experiments further showed that there are no preformed breaks in the single polynucleotide chains. If a few single-chain breaks were produced by occasional enzymatic scission by an endonuclease, the molecular weight of the denatured product would be more than halved. The studies of Thomas and Berns (1961) argue against the possibility that the denatured T2 or T4 DNA consists of double helices as suggested by Cavalieri and co-workers.

Freifelder and Davison (1962a,b) have also studied the denaturation

of DNA using formaldehyde to prevent the reformation of base pairs after cooling the solution. Although much of their work is more appropriately discussed below in the section on the mechanism of denaturation, there are several points that are pertinent to the present problem of whether denaturing conditions result in the complete separation of the DNA strands. By exposure of solutions of DNA to increasing temperatures, the dissociation of the interstrand hydrogen bonds is seen to occur over a narrow, but measurable, temperature range. Although it had been thought that the denaturation reaction was an all-or-none phenomenon (Rice and Doty, 1957; Doty, 1959–1960), it now appears that the weaker base-paired regions within individual molecules [i.e., those regions with a lower G + C content—and hence a lower thermal stability—than other regions within the same molecule (Marmur and Doty, 1959)], melt out at temperatures insufficient to denature the entire molecule. Since the other regions of the molecule are still intact, the base sequences remain potentially in register. If the denaturing conditions are then removed, the disrupted hydrogen bonds can subsequently reform. If, however, the partially disrupted molecule at the elevated temperature has reacted with formaldehyde, the complementary base interactions of the dissociated regions are removed. Since the strands have not separated because of the residual helical regions, the molecular weight of the partially disrupted molecule should not have changed. Those regions of the molecule prevented from reforming the rigid nativelike helical structure, however, will add flexibility to the molecule, which will assume a more compact shape in solution. Thus, the frictional coefficient should decrease and, since the molecular weight of the polymer remains unchanged, the sedimentation coefficient should correspondingly increase. It would be expected to rise to a maximum as more and more flexible regions are introduced into the molecule. However, when strand separation occurs, the molecular weight should fall to one half of its original value and this should be reflected in an abrupt drop in the sedimentation coefficient. In their study of the reaction of formaldehyde with T7 DNA, Freifelder and Davison (1963) found that, when all of the interstrand base pairs had been disrupted by thermal treatment and reaction with formaldehyde, the sedimentation coefficient fell precipitously over a very narrow temperature range. In a similar study on *E. coli* biological hybrid DNA, Freifelder and Davison (1962) found that not all the base pairs are equally sensitive to disruption and that the polynucleotide chains separate only after the last few residual bonds holding the two strands together are dissociated.

Kit (1960) has found that the thermal degradation of denatured DNA at 95°C was kinetically similar to that of RNA and hence reflected

the depolymerization of a single polynucleotide chain. Since these measurements were made at an elevated temperature, at which intramolecular hydrogen bonds are dissociated, the complications from intramolecular aggregation were eliminated. Bernardi (personal communication) has shown that the degradation of denatured DNA under controlled conditions at elevated temperatures can be explained by the scission of single-stranded chains. This is at variance with the interpretation of the elevated temperature DNA degradation described by Applequist (1961).

C. Biological Experiments

The denaturation, as well as the renaturation, of DNA can be followed readily by either of two biological methods currently in use. First, when bacterial-transforming DNA is exposed to elevated temperatures for brief periods of time, most of its biological activity is lost over a relatively narrow temperature range. Infectious bacteriophage λ DNA (Kaiser and Hogness, 1960) also loses a substantial fraction of its biological activity at elevated temperatures. Second, the immunological reactivity of DNA is increased upon denaturation (Levine *et al.*, 1960). With each of these methods, the abrupt changes in the biological properties of DNA parallel the collapse of the native secondary structure.

The thermal denaturation of DNA, as measured by the loss of its ability to participate in bacterial transformation, has been investigated in great detail (Zamenhof *et al.*, 1953; Lerman and Tolmach, 1959; Doty *et al.*, 1959b; Marmur and Lane, 1960; Ginoza and Zimm, 1961; Roger and Hotchkiss, 1961; Guild, 1961; Rownd *et al.*, 1961, 1963c). Thermal denaturation has proved to be very useful, as the denaturing conditions can be carefully controlled and then rapidly removed by quenching in ice water; moreover, in the time required to produce thermal denaturation of the DNA molecule, undesirable chemical degradation is minimized. The general features of thermal inactivation are as follows: Solutions of transforming DNA are exposed to elevated temperatures for brief time periods (usually 10–30 minutes), the samples are rapidly cooled in ice water, and the per cent inactivation as a function of temperature is then determined by assay in a transformation experiment. Over a temperature range of only a few degrees, there occurs a precipitous fall in biological activity to a level which is just a few per cent of that of the native DNA (Roger and Hotchkiss, 1961; Marmur and Lane, 1960; Ginoza and Zimm, 1961; Herriott, 1961a,b). When multiply marked DNA is used in this type of experiment, it is found that the unlinked genetic markers are inactivated at distinct temperatures above the critical denaturation temperature (Marmur and Lane, 1960; Doty *et al.*, 1959b; Ginoza and Zimm, 1961; Roger and Hotchkiss, 1961; Guild, 1962; Opara-Kubinska

and Szybalski, 1962); linked markers are inactivated simultaneously (Roger and Hotchkiss, 1961). This was interpreted previously to mean that the thermal stability of a given marker is related to the base composition of the DNA segment regulating that particular marker; thus, those markers having a higher average G + C content would be more resistant to thermal denaturation (Doty *et al.*, 1959b; Marmur and Lane, 1960; Ginoza and Zimm, 1961). More recent studies, in which the average base compositions of the individual, genetically marked molecules were determined from their buoyant densities in CsCl (Rolfe and Ephrussi-Taylor, 1961), indicate that this is probably not the case (Guild, 1962; Opara-Kubinska and Szybalski, 1962), but rather that the critical inactivation temperature is determined by the distribution of the G + C sequences in the genetically marked molecule (see Section III,F).

Prolonged exposure of transforming DNA to elevated temperatures may produce inactivation of its biological activity by mechanisms other than the breakdown of its secondary structure. These changes occur even at subcritical temperatures, i.e., below the temperature causing denaturation, where the molecule has not been altered detectably in its physical properties. The rate constants for inactivation depend on whether the inactivation occurs just slightly above or below the melting temperature (Ginoza and Zimm, 1961; Roger and Hotchkiss, 1961). This type of inactivation appears to arise from chemical alterations in the DNA molecule such as depurination (Ginoza and Zimm, 1961; Roger and Hotchkiss, 1961; Greer and Zamenhof, 1962; Fiers and Sinsheimer, 1962) and/or backbone scission of either the single-stranded or less likely, the double-stranded helical polynucleotide chains (Eigner *et al.*, 1961) rather than from denaturation. Such inactivation would be expected to be related to the size of the genetic marker or that region of the molecule within which damage would prevent genetic exchange of the particular marker in question. This is the conclusion arrived at by Lacks and Hotchkiss (1960) and by Roger and Hotchkiss (1961), who found different orders of inactivation for a series of genetic markers when the DNA was inactivated either at subcritical temperatures or by heating to temperatures sufficient to cause the collapse of the secondary structure of the molecule. Hence, the two types of inactivation must proceed by different mechanisms.

The biological activity of native DNA may be reduced by means of denaturation other than thermal exposure. Acid or base denaturation (Marmur and Lane, unpublished) as well as denaturation by organic solvents, such as formamide (Marmur and Ts'o, 1961), result in a substantial reduction of the biological activity of transforming DNA.

No matter what the denaturing conditions employed, it has always

been found that a small residual biological activity is associated with denatured transforming DNA (Marmur and Lane, 1960; Ginoza and Zimm, 1961; Roger and Hotchkiss, 1961; Herriott, 1961a,b). The origin of this residual activity has been a subject of considerable interest, particularly in the case of thermal denaturation. Marmur and Lane (1960) and Marmur *et al.* (1962) considered that the origin of the residual activity might arise from a small fraction of single strands that specifically paired to form an active renatured molecule. It is also possible that a heat-resistant fraction of molecules is not denatured by the thermal treatment, or that denatured or single-stranded DNA possesses intrinsic biological activity. The presence of a heat-resistant fraction seems unlikely since transforming DNA which has been heated to 120°C in an autoclave still possesses the same residual activity (Marmur and Lane, 1960; Herriott, 1961b); this temperature is sufficiently high to melt even the synthetic poly (dG): (dC). The occurrence of a small amount of renaturation seems untenable in view of the findings of Ginoza and Zimm (1961) that the level of the residual activity is independent of the DNA concentration at denaturation over a hundred-fold range; since renaturation involves the complementary reunion of two separated strands (Marmur and Lane, 1960; Doty *et al.*, 1960; Marmur and Doty, 1962), it should depend on the square of the concentration. Ginoza and Zimm (1961) presented evidence suggesting that the residual activity is an intrinsic property of denatured DNA. The experiments of Roger and Hotchkiss (1961) provide strong evidence that the residual activity of *D. pneumoniae* DNA, heated above the critical temperature, arises from denatured molecules. The concentration-response curve of heated DNA displays a lower saturation level than that of native DNA and is thus highly suggestive that the biological activity is associated with collapsed molecules. Also, the residual activity was independent of the ionic strength at which the DNA was denatured, eliminating the possibility that the biological activity comes from renatured molecules. Their specific reformation is dependent upon ionic strength (Marmur and Lane, 1960).

Independent studies by Guild (1961) with *D. pneumoniae*–transforming DNA and by Rownd *et al.* (1961) with *H. influenzae* DNA show that the residual activity bands in the denatured DNA region in the CsCl density gradient. This led to the conclusion that the observed residual biological activity can indeed be associated with denatured DNA. Hence, the single or collapsed double strands must be absorbed and integrated by competent transforming cells in both transforming systems. Lerman and Tolmach (1959) had previously reported a poor uptake of denatured P^{32}-labeled *D. pneumoniae* DNA by transformable

cells. Since the uptake of denatured DNA by bacterial cells is impaired, it would seem that the denatured DNA, on the basis of P^{32}-labeled DNA taken up, is just as efficient, if not better, than the native structure in carrying out the steps of the transformation between irreversible DNA uptake and the process leading to integration and expression.

Barnhart and Herriott (1962) used a highly purified preparation of the *E. coli* phosphodiesterase (Lehman, 1960) to study the nature of the residual transforming activity of denatured *H. influenzae* DNA. This enzyme acts specifically on denatured DNA, initiating sequential hydrolysis of nucleotides from the 3′-hydroxyl end of the DNA molecule. Although most of the denatured DNA was digested by the enzyme as measured by the release of P^{32}-labeled nucleotides, the residual specific transforming activities of several genetic markers remained essentially the same. Preparative CsCl density gradient centrifugation showed that the undigested, biologically active DNA had a higher buoyant density than native DNA and, therefore, retained its denatured physical properties (Herriott, personal communication). Hence, it seems likely that the remaining biologically-active fragments have a high degree of intrastrand hydrogen bonding or possess some discontinuity that makes the active region resistant to enzymatic digestion (Fiers and Sinsheimer, 1962).

A study of the biological properties of denatured *B. subtilis* DNA has shown that, in this transformation system, the origin of the residual activity of denatured DNA is different than that found in the *D. pneumoniae* and *Hemophilus* transformation systems (Rownd *et al.*, 1963b). Studies with this system have an added advantage, since biological hybrid DNA consisting of heavy and light complementary strands can be more readily prepared and, hence, it can be demonstrated unequivocally that the denatured product is single-stranded. When *B. subtilis* DNA is thermally denatured and then fractionated by preparative CsCl density gradient centrifugation, the residual transforming activity is found to band in a broad density range between native and denatured DNA. The DNA banding in the denatured region is biologically inactive. If biological hybrid *B. subtilis* DNA is thermally denatured and then fractionated with respect to buoyant density, the residual activity appears in that region of the density gradient corresponding only to the buoyant density expected for native and partially denatured *hybrid* molecules. The DNA banding in the regions expected for denatured labeled and unlabeled DNA is biologically inactive. These observations indicate that the residual biological activity of denatured *B. subtilis* DNA resides in those molecules whose strands have collapsed but not completely separated. The activity does not come from a heat-

resistant fraction as reheating the biologically active DNA after fractionation decreases its activity to only a few per cent of its original value. Since this partially denatured, collapsed species would be expected to possess specific biological activities less than that of native DNA, this fraction that did not separate after heating should be greater in concentration than expected from its biological activity. This is in agreement with the physical evidence (discussed below), which indicates that approximately 10% of the strands do not completely separate when DNA is thermally denatured.

Although the *B. subtilis* DNA found in the denatured band is biologically inactive, its activity may be restored to a few per cent of that of the native DNA by annealing at an elevated temperature for a very short time interval (only 30–60 seconds). This reactivation (different from the renaturation of complementary strands) of the biologically inactive *B. subtilis* DNA is concentration-independent and thus appears to result from an intramolecular rearrangement of single strands. The simplest interpretation of these results is that short, imperfect helical regions are formed when the thermally denatured product is rapidly cooled, resulting in biologically inactive molecules. Brief exposure to elevated temperatures, however, melts out the weaker short helical regions, thus allowing the single strands to explore more stable conformations that then satisfy the requirements for activity in bacterial transformation. The specific activity of the denatured sample so treated can be still further increased twentyfold by the usual renaturation procedure. These results raise the question of the importance of intramolecular hydrogen bonding in the determination and stabilization of single-strand conformations in denatured DNA, a question which is as yet poorly understood.

Recent experiments with an endonuclease isolated from brain tissue that acts only on denatured DNA (Healy *et al.*, 1963) show that the residual transforming activity of unfractionated, thermally denatured *B. subtilis* DNA is rapidly destroyed (Marmur and Cahoon, unpublished results). After the action of the enzyme, approximately 1% of the denatured specific activity remains. This is in agreement with the results of Rownd *et al.* (1963b) on the existence of partially denatured, but biologically active, DNA molecules in thermally denatured *B. subtilis* DNA; these species would be expected to be subject to endonucleolytic attack. Recent work by Rownd and Lanyi (unpublished results) shows that it is also possible to isolate *H. influenzae* DNA molecules containing both native and denatured regions; these partially denatured molecules have specific biological activities that depend on the extent of helicity and lie between those of native and denatured DNA.

D. Studies with Synthetic Polynucleotides

The feeling that the long, intertwined chains of the DNA double helix could not readily unwind and separate is not supported by the studies with enzymatically synthesized polyribonucleotides. Fresco and Straus (1962) have reviewed the studies carried out with the homopolymers as well as with homopolymer-copolymer pairs and point out that the interacting strands can explore one another to fulfill their complete hydrogen-bonding capacities and, furthermore, are separable under denaturing conditions. Thus these studies provide models for investigating both strand separation and renaturation.

E. Studies with Bacteriophage Alpha

The DNA from bacteriophage alpha (host: *Bacillus tiberius*) offers a unique advantage for strand separation studies because its complementary strands have different buoyant densities in CsCl and can thus be readily identified (Cordes *et al.*, 1961). By fractionating the denatured DNA on a methylated bovine serum albumin column (Mandell and Hershey, 1961; Cordes *et al.*, 1962), Cordes (unpublished results) has shown that the base composition, although complementary in the unfractionated native or denatured state, displays a bias such that A and T, and to a lesser extent G and C, are unequal in each of the complementary strands. This is similar to the single strand of ϕX174 DNA (Sinsheimer, 1959; Daems *et al.*, 1962). However, a very interesting result is that the per cent of A (or of T) of one of the bacteriophage alpha DNA strands is equal to the complementary base of the other strand. The same is true for the case of G and C. This can only be the case if the separated units contain an odd number of strands (probably one) and this finding provides strong evidence that the strands can indeed be separated.

F. The Mechanism of Denaturation

With the accumulation of evidence that the denaturation of DNA leads to the separation of the polynucleotide chains, it is of considerable interest to inquire into the mechanisms involved in this process. Granted that the end result of the denaturation reaction is the dissociation of essentially all the interchain base pairs, several important questions still remain to be answered. First, are forces other than hydrogen bonding involved in the stabilization of the helix in the native state? Second, what fraction of the base pairs must be broken to cause denaturation of the DNA molecule? This second question may be phrased in another way: Is it possible to break a substantial fraction of the base pairs of

the native structure and still obtain reformation when the denaturing conditions are removed? This, of course, is tantamount to asking whether the denaturation of DNA is an all-or-none phenomenon. Finally, if the majority, but not all, of the hydrogen bonds are broken under certain denaturing conditions, is it possible to "freeze" in metastable, partially denatured states possessing varying proportions of helix and random coil within the same DNA molecule?

1. Forces Involved in Helix Stability

Until recently it was thought that hydrogen bonds supply the main forces responsible for holding together the strands of the DNA double helix. This seemed reasonable because the hydrogen bonds, which dictate the specificity of base pairing in DNA, were considered to be an important factor in the secondary structure of proteins. For example, urea, an agent that causes changes in the conformational properties of polypeptides and proteins, aids in the denaturation of DNA (Rice and Doty, 1957) and it seemed likely that it acted by disrupting the hydrogen-bonded base pairs. It was also found that DNA containing more GC than AT base pairs exhibited higher thermal stability (Marmur and Doty, 1959, 1962). Since it was suggested (Pauling and Corey, 1956) that the GC base pair could form three hydrogen bonds and the AT pair only two, it seemed to account reasonably well for the observed results in terms of hydrogen bonding. The strongest evidence to date of the involvement of hydrogen bonds in maintaining helix stability comes from the experiments on synthetic polynucleotides by Szer and Shugar (1961). They found that, in contrast to poly U, poly methyl U exhibits no secondary structure nor will the latter polymer form any complexes with poly A. Thus, by blocking the formation of one hydrogen bond in one of the aromatic rings, interstrand hydrogen bond interactions are prevented. Other than the possibility that the introduction of the methyl group causes structural distortions due to steric hindrance, it is difficult to explain Szer and Shugar's results in terms except by hydrogen bonding.

During the past few years, it has been found that many substances that are not considered to be hydrogen bond–breaking agents can provoke the denaturation of DNA and bring about strand separation. The evidence for these conclusions has emerged from studies on the stability of the DNA secondary structure in various organic solvents (Helmkamp and Ts'o, 1961; Herskovits *et al.*, 1961; Geiduschek and Herskovits, 1961) and also in concentrated solutions of 1:1 electrolytes (Emanuel, 1960; Hamaguchi and Geiduschek, 1962). It has been shown, too, that some of the usual protein denaturing agents are relatively ineffective in denaturing DNA (Rice and Doty, 1957; Gordon and Jencks, 1963; Levine *et al.*,

1963). Consideration of the relative destabilizing effects of various organic solvents on DNA showed that these effects did not parallel their hydrogen-bonding capacities (Helmkamp and Ts'o, 1961; Herskovits *et al.*, 1961; Geiduschek and Herskovits, 1961; Levine *et al.*, 1963) but rather the converse was found. It was also found that purine and pyrimidine bases can serve as destabilizing agents and hence lower the temperature of thermal denaturation by several degrees (Ts'o *et al.*, 1962a). Other findings indicate that increasing the number of alkyl substituents on amides, ureas, carbamates, and alcohols enhanced their denaturing effectiveness toward DNA (Levine *et al.*, 1963). Anions such as Cl_3CCOO^-, CF_3COO^-, CNS^-, and ClO_4^- lower the T_m of DNA by as much as 60° at neutral pH (Hamaguchi and Geiduschek, 1962).

Hamaguchi and Geiduschek (1962) were also able to use these anions to vary the contributions of the AT and GC pairs toward helix stability. This is seen by the variation in the slope of the line obtained in a plot of the T_m versus G + C content for DNA in various salt solutions. The slope may be varied in both directions from that originally reported by Marmur and Doty (1959, 1962) for the saline-citrate solvent. This suggested to these authors that the differences in the stabilities of AT- and GC-rich DNA helices do not arise exclusively from hydrogen bond contributions. They then were able to make some correlation between the effects of these salts on the structure of DNA and their effects on the structure of water and on the water solubility of nonelectrolytes and concluded that the denaturation of DNA arises from the effect of these electrolytes on the structure of water. They referred to these substances as hydrophobic bond-breaking agents. A thermodynamic treatment of how such agents may affect the structure of water has been presented recently by Némethy and Scheraga (1962). The statistical mechanical treatment of denaturation implies that forces other than hydrogen bonding are involved in maintaining the stability of the DNA helix (Gibbs and DiMarzio, 1959; Zimm, 1960).

Hydrophobic bonding is rather a vague term implying that, in the case of DNA for example, the bases attract each other more than they do the water molecules that normally surround them when the DNA molecule is in the denatured state. DeVoe and Tinoco (1962) calculated what they consider to be the main base-base attractive forces in DNA. These may be called hydrophobic bonds, although they did not use this term. They found that dipole-dipole, dipole-induced-dipole, and London force interactions among the bases in the helix are large and make the free energy of the helix depend on base composition and sequence. The helix stability is calculated to be proportional to the G + C content. Finally, they suggest that the base sequence arrangements in native DNA may be influenced by the free energy.

The calculations of DeVoe and Tinoco indicate that the hydrogen bond energy of the bases probably does not contribute very much toward holding the strands of the double helix together although they may insure specific base pairing. The authors do not calculate the energy involved in the release of water molecules normally bound to the bases when the DNA chain is in the single-stranded state. The recent work of Némethy and Scheraga (1962) may aid in the calculation of these water energy terms. Although the studies of DeVoe and Tinoco may undergo some modification, they do seem to provide a rational explanation for the effects of the denaturing agents on DNA mentioned previously in this section. In addition, they show how to calculate the effect of different base sequences on helix stability. The fact that poly d(A,B), (B = bromouridylate) has a higher T_m than poly d(A-T) under certain conditions can be explained by the polarizability of 5-bromouracil being higher than that of thymine. This work represents an important initial step in the calculation of what may be the major force holding the helices together.

2. Reversible and Irreversible Denaturation

The denaturation of DNA may be followed by a variety of physical-chemical, as well as biological, techniques. However, before the results obtained by these various methods can be compared strictly, it is important to establish whether each of these assay systems reflects the same changes in the secondary structure of the DNA molecule. Moreover, it is necessary to establish that physical or biological measurements made on DNA after the removal of the denaturing conditions reflect the same state of the molecule that existed in the presence of the denaturing conditions. If the changes produced in the physical state of the DNA molecule by thermal denaturation are largely reversible after cooling the solution from the elevated temperature, then the measurements made after cooling will not truly reflect the equilibrium state at the elevated temperature.

Viscosity, light-scattering, or sedimentation measurements are more conveniently made at room temperature than at the ambient temperature. Hence, the effects of exposure of solutions of DNA to elevated temperatures using these assay systems are assessed only after the sample has been cooled and the denaturing conditions have been removed. Biological measurements can, of course, be made only under the rather rigorously defined conditions of transformation, enzymatic, or immunological experiments. Optical measurements, on the other hand, are conveniently carried out at the elevated temperature since spectrophotometers or polarimeters may easily be equipped with thermal spacers for heating the cell compartment (Doty *et al.*, 1959a; Marmur and Doty,

1959; Szybalski and Mennigmann, 1962; Ts'o *et al.*, 1962a). Hence, in this latter case, the physical measurement actually reflects the equilibrium state of the molecule at the elevated temperature.

Geiduschek (1962) has carried out a systematic study on the factors affecting the irreversible denaturation of DNA. The experimental approach has been to use changes in the absorbance of denatured DNA at the ambient temperature and 25°C using heat as the denaturing agent. When DNA is exposed to continuously increasing temperatures, a sharp increase in its absorbance is noted at an elevated temperature reflecting the disruption of the helical structure of the nucleic acid. The ambient temperature denaturation curve, however, is different from the curve obtained if the heated DNA solution is rapidly cooled, re-equilibrated to 25°C, and its absorbance plotted as a function of the temperature to which the DNA was exposed before it was cooled. The midpoint of the ambient temperature denaturation curve ($T_{m,a}$) is several degrees lower than the midpoint of the ultraviolet absorbance-temperature profile measured at 25°C ($T_{m,25}$) (Doty *et al.*, 1959a; Geiduschek, 1962). The denaturation of DNA recorded at the ambient temperature is a measure of the disruption of the helical structure without necessarily involving the separation of the polynucleotide strands. Geiduschek (1962) found that T2 DNA can be heated at a temperature that causes a 37% increase in absorbance at the ambient temperature, but rapid cooling, followed by absorbance measurements at 25°C, indicates that very little, if any, denaturation has taken place. That this reversible denaturation involves the recovery of helical order is indicated by the absence of viscosity changes (Geiduschek and Herskovits, 1961). A similar result, namely, that partial denaturation can be reversed, has been described by Schildkraut *et al.* (1961a) using *E. coli* biological hybrid DNA. They found that, to obtain strand separation by heating and fast cooling, the solution of DNA must be heated several degrees above the $T_{m,a}$. Freifelder and Davison (1962) have related strand separation in *E. coli* biological hybrid DNA to the extent of thermally induced hyperchromic change; they found that full hyperchromic change was a necessary, but not a sufficient, condition for strand separation and concluded that the strands may be prevented from separating by just a few residual base pairs within a DNA-molecule.

Geiduschek (1962) has proposed that the difference (ΔT) between the $T_{m,a}$ and the $T_{m,25}$ can be explained by invoking the presence of thermally resistant G + C rich "nuclei," which can hold the base sequences of collapsed DNA molecules in register until the temperature is reduced, whereupon the molecules, independent of source, can renature. The temperature-absorbance curve of DNA re-equilibrated to 25°C

would reflect the existence of those denatured molecules that suffered strand separation after melting out the last base pair (Freifelder and Davison, 1962) or, to a smaller extent, those DNA molecules that are collapsed and in a partially denatured, metastable state. The presence of the G-C rich nuclei would explain why: (1) the ΔT is greater for higher molecular weight DNA, since larger molecules would have a higher probability of possessing a greater number of more stable nuclei; (2) ΔT is greater the lower the G + C content of the DNA, since the incremental stability of the nuclei would be more apparent with DNA of low *average* G + C content; and (3) there is very little or no correlation between the buoyant density of transforming DNA markers and their inactivation (denaturation) temperatures (Guild, 1962; Opara-Kubinska and Szybalski, 1962). The buoyant density of purified DNA is a measure only of the average base composition, whereas the inactivation temperature reflects in addition the presence of G + C rich nuclei. The existence of such nuclei could also be invoked to explain the partial denaturation of DNA by acid and by heat (Cavalieri and Rosenberg, 1957; Geiduschek and Holtzer, 1958).

The results of Doty *et al.* (1959a), Schildkraut *et al.* (1961a), and Geiduschek (1962) make it necessary to re-evaluate the concept of "all-or-none" denaturation. It would seem obvious that DNA molecules can be *partially* denatured when they are kept in the presence of the denaturing conditions such as elevated temperature (or when heated and cooled in the presence of reagents that prevent the reformation of base pairs) (Beer and Thomas, 1961).

Rownd (1963) has investigated the effects of rapidly cooling T4 DNA in low ionic strength solvents following denaturation, since it might be expected that metastable partially denatured states could be stabilized by such treatment (Dove and Davidson, 1962a,b). A distribution of partially denatured molecules having densities ranging from that of the completely denatured to that of the native DNA can be demonstrated by preparative CsCl density gradient centrifugation. The effects of partial denaturation on the biological properties of *H. influenzae*-transforming DNA have shown an exponential dependence of the remaining biological activity on the per cent helix of the partially denatured molecule (Rownd *et al.*, 1963).

IV. General Aspects of Denaturation and Renaturation

A. Methodology

Essentially similar methods can be employed for studying both the denaturation and renaturation of DNA. Whereas some methods are more

readily adapted to laboratories specializing in certain techniques, it is desirable to employ several different approaches so as not to be misled by such artifacts as aggregation and nonspecific base pair interactions. Both these troublesome aspects are encouraged by denaturation at high ionic strengths and high DNA concentration as well as by a slow reversal of the denaturing treatment (such as cooling after thermal denaturation). This makes it difficult to ascertain the efficacy of certain methods that have been employed in the past where these variables have not been controlled.

Biological, physical, and chemical methods have been used to study the denaturation, and sometimes the renaturation, of DNA. The following three methods belong to the first category.

1. Transformation

The use of bacterial transformation in studying denaturation and renaturation is discussed in Section II.

2. Immunology

Since denatured DNA can react more effectively than native DNA in complement fixation with anti-DNA antibodies, this technique has been used as a means of studying the denaturation and renaturation of DNA (Levine *et al.*, 1960, 1963). The antisera to DNA that have been used are those produced in rabbits in response to injections with disrupted T-even *E. coli* bacteriophages as well as serum from patients with systemic lupus erythematosus (Stollar and Levine, 1961). Whereas the former antibodies react specifically with the glucosylated hydroxymethylcytosine residues of T-even bacteriophage DNA, antisera from different lupus erythematosus patients react with all denatured DNA samples but vary in their complement fixing ability with DNA from different sources (Stollar *et al.*, 1962). The different reactivities most likely arise from the ability of these sera to react with specific exposed DNA nucleotide sequences in denatured DNA (Stollar *et al.*, 1962).

3. Enzymology

The phosphodiesterase from *E. coli* (Lehman, 1960) that acts exonucleolytically on denatured DNA makes it possible to use this enzyme to study the denaturation and renaturation of DNA (Dolbeare and Grossman, 1962). Another enzyme, isolated from brain tissue (Healy *et al.*, 1963) and acting endonucleolytically primarily on denatured DNA, has been useful in studying the thermal transitions of DNA. Thus, while the residual transforming activity of denatured *B. subtilis* DNA is rapidly and almost completely destroyed by this enzyme, renatured

DNA is acted upon only very slightly and native DNA hardly at all. Both enzymes have proven to be very useful in eliminating denatured DNA in the presence of native DNA and also in the digestion of the unpaired regions of renatured DNA (Schildkraut *et al.*, 1961a; Dolbeare and Grossman, unpublished results). By employing P^{32}-labeled DNA and enzymes that specifically digest denatured regions, this method offers the greatest potential in studying limited renaturation.

The physical methods listed below have been used to study the denaturation of DNA and can also be adapted to follow its renaturation.

4. Density Gradient Centrifugation

By far the most useful and versatile method at the present time for studying the transitions of the secondary structure of DNA is the technique of density gradient centrifugation (Meselson *et al.*, 1957).[3] The buoyant densities of native and denatured DNA in CsCl are linearly related to the mole per cent G + C, the denatured state (for DNA possessing 40–60% G + C) being 0.015 gm/ml more dense than native DNA (Sueoka *et al.*, 1959). Small amounts of DNA, even in the unpurified state, can be used for the determination of buoyant density before and after denaturation and to give an accurate picture of its secondary structure. Thus, the exposure of bacteriophage ϕX174 DNA to denaturing or renaturing conditions does not alter its density (Schildkraut *et al.*, 1961a), a finding consistent with the proposal that the DNA isolated from this bacteriophage is single-stranded (Sinsheimer, 1959; Fiers and Sinsheimer, 1962). A modification has recently been introduced that magnifies the buoyant density difference between native and denatured DNA (Vinograd *et al.*, 1962). Native bacteriophage T4 DNA has the same density at or below pH 10.5 in CsCl solution. Denaturation causes T4 DNA to increase in buoyant density at pH 9.5, and at pH 10.5 its density is 0.44 gm/cc denser than native DNA. The extent of the density shift in alkaline solutions is related to the mole per cent G + C.

Preparative CsCl density gradient centrifugation has been found to be very useful in the separation and isolation of native from denatured DNA as well as from molecules displaying the density characteristics of the partially renatured state (Rownd *et al.*, 1961; 1963). The banding of DNA in the CsCl gradient not only gives its buoyant density, but also, from the variance of the gaussian distribution of the DNA molecules at equilibrium, it is possible to calculate the molecular weight (Meselson *et al.*, 1957; Sueoka, 1960; Hearst and Vinograd, 1962). The calculations,

[3] An excellent review on density gradient centrifugation has been written by Vinograd and Hearst (1962).

however, are complicated by the density heterogeneity of the population of DNA molecules. Eigner and Doty (1963) have shown that molecular weights for denatured DNA obtained from CsCl band widths agree with those values obtained by other methods.

5. Light Scattering

Although light scattering would be the most definitive method of studying the molecular weight and shape changes on denaturation and renaturation of DNA, its use is limited by the large amounts and high concentrations of purified DNA required (and thus subject to the hazards of aggregation) and its inapplicability for general and routine use. Earlier light-scattering results on the denaturation of DNA have been reviewed by Geiduschek and Holtzer (1958). In recent years, the method has been used most extensively by Cavalieri and co-workers (Cavalieri and Rosenberg, 1962a,b) in their studies of the thermal denaturation of DNA from various sources and to follow the drop in molecular weight after DNase and X-ray treatments.

According to Cavalieri and co-workers (Cavalieri and Rosenberg, 1962b), the biunial DNA molecules isolated from rapidly growing cells are reduced in molecular weight by a factor of two on denaturation, not by a separation of the two strands of the Watson–Crick type, but rather by the separation of two double helices. The evidence relies mainly on the kinetics of the enzymatic and X-ray degradation of DNA (Cavalieri and Rosenberg, 1961, 1962b). Since neither agent is known to act in a random manner (Doskočil and Šorm, 1961; Vanecko and Laskowski, 1962; Guda *et al.*, 1962), there may be complications involved in the use of kinetics of the light-scattering molecular weight decay to determine the number of strands per DNA molecule (Luzzati *et al.*, 1962). Also, in these experiments, samples to be treated with DNase II were denatured *before* the enzyme was added. Since recooling at high concentrations of DNA causes extensive aggregation, it would tend to complicate the interpretations of the ensuing kinetic experiments.

Luzzati and co-workers (see the article by Luzzati in this volume) developed a method of measuring the mass per unit length of rodlike structures from the absolute intensity of the small-angle scattering of X-rays from dilute solutions. Their results show that the mass per unit length of DNA isolated from animal tissues as well as bacteria (Luzzati *et al.*, 1962) is in good agreement with the Watson–Crick model (see also article by Bendich and Rosenkranz in this volume).

6. Spectrophotometry

The spectrophotometric method of studying the denaturation and renaturation of DNA is the most readily employed and is accessible to

most laboratories. The increase in the ultraviolet absorbance (hyperchromic change) on denaturation results in little or no shift in wave length of the absorption maximum and only small changes in the shape of the absorption curves (discussed by Zimm and Kallenbach, 1962). The sharpness in the helix-to-random-coil transition has been interpreted as indicative of a cooperative phase transition (Peacocke, 1960; Gibbs and DiMarzio, 1959). The hyperchromic change from the native to the denatured state as well as from the completely denatured state to nucleotides probably comes from the disruption of the characteristic stacking of the bases maintained in native DNA as a result of hydrogen bond and/or neighboring base interactions (Bolton and Weiss, 1962; Michelson, 1962; Rhodes, 1961; Kasha *et al.*, 1961; Zimm and Kallenbach, 1962). Assuming the change in ultraviolet absorbance to be linearly related to the helix content, hyperchromicity would appear to be a valid measure of the extent of disruption if the helical regions contain at least seven or eight residues (Tinoco, 1960, 1961).

Little is known about the changes that occur in the DNA structure when it is exposed to temperatures below the onset of hyperchromicity. It is possible that the methods now available (such as ultraviolet absorbance) are not delicate enough to detect any changes that might occur at such a level and that could result in disruption to the extent of several per cent DNA.

The T_m depends somewhat on the wavelength employed, since the presence of the more thermally resistant G + C base pairs, which raise the melting point, have a different absorption spectrum than the A + T pairs (Felsenfeld and von Hippel, 1962).

The renaturation of denatured DNA can be properly followed spectrophotometrically only at temperatures at which short, imperfect inter- and intramolecular helical regions have been melted out (Marmur and Doty, 1961; Cavalieri *et al.*, 1962). By exposing denatured DNA to constant temperatures below the T_m, renaturation can be followed by the hypochromic changes and plotted as a function of time. Renaturation can also be shown to take place when homogeneous denatured samples of DNA (isolated from bacteriophage and bacteria but not from higher plants and animals) are exposed to a slow rate of temperature increase from room temperature. The initial rise in the temperature (resulting in hyperchromicity) melts the weak, short sequences, allowing the complementary strands to renature (Doty *et al.*, 1960). Renaturation cannot be used to describe all the hypochromic changes that occur when denatured DNA is returned to room temperature, since much of the absorbance change could arise from the reformation of short sequences (intra-and interstrand) of base pairs and possibly other factors.

Szybalski and Mennigmann (1962) have described the construction

of an automatic recording thermospectrophotometer for determining the melting temperatures of nucleic acids.

7. Sedimentation and Viscosity

The denaturation of DNA can be followed by the changes in the sedimentation coefficient and viscosity, the measurements being carried out preferably at ionic strengths favoring the greater difference between the helical and random coil configurations (Doty *et al.*, 1959a; Eigner, 1960; Eigner and Doty, 1963). Renaturation results in a partial restoration of the viscosity and a change in the sedimentation coefficient to values similar to those of native DNA. By denaturing DNA in the presence of formaldehyde at slightly elevated temperatures, Freifelder and Davison (1962b) followed the increase in the sedimentation coefficient as a function of time at a constant temperature. After the maximum increase is attained, further increase in temperature results in a sharp drop in the sedimentation coefficient from the melting out of the last base pairs, resulting in a separation of the DNA into two units and most likely representing a single-stranded species. The combination of sedimentation and viscosity data has also proved useful in assigning molecular weights to DNA samples in their native, denatured, and renatured states (Eigner and Doty, 1963).

8. Optical Rotation

The studies of Levedahl (1959), Fresco (1961), and Ts'o *et al.* (1962a,b) show that the helical form of polynucleotides is associated with a large positive contribution to its optical rotation. They also found that the change in the optical rotation of DNA exposed to increasing temperatures qualitatively parallels the observed increase in absorbance. Helmkamp and Ts'o (1962) find that rotation is more sensitive than hypochromicity to the length of the helical regions and is strongly dependent on secondary structure. The combined studies of optical rotation and absorbance strengthen the contention that there is a conformational change during denaturation of DNA from the helical to the random coil form during the sharp change in either parameter (Doty *et al.*, 1959a; Fresco, 1961; Fresco *et al.*, 1961). The optical rotatory properties of DNA are especially useful in studies of the changes in secondary structure by reagents that absorb very strongly in the ultraviolet.

9. Electron Microscopy

Whereas the electron microscope photographs of native and renatured DNA samples are essentially similar, displaying typical rod-shaped particles, denatured DNA appears as flattened patches or "pud-

dles" (Hall and Litt, 1958a; Doty *et al.*, 1960). Electron micrographs of DNA partially denatured in the presence of formaldehyde to prevent the renaturation of the localized regions exhibit both native- and denatured-like regions alternating along the same molecule (Beer and Thomas, 1961). The cross section of native DNA rods in the electron microscope is about 20 Å, in good agreement with the dimensions derived from the X-ray diffraction patterns of the Watson–Crick double helical structure (Hall and Litt, 1958b; Hall and Cavalieri, 1961; Beer and Zobel, 1961; Daems *et al.*, 1962). However, in the preparations of DNA from *D. pneumoniae* examined by Hall and Cavalieri, about 10% of the molecules were branched, each arm of the Y having the same diameter as the trunk. Estimating the weight average molecular weight from light scattering and the average length from the electron microscope photographs on the same preparations, they concluded that the DNA (molecular weight 1.6×10^6) isolated from this organism has 3.6 strands per molecule on the average. This would represent two double helices lying side by side. It remains to be proven conclusively whether these DNA structures exist *in vivo* in rapidly dividing cells or are formed as a result of the DNA isolation and/or preparation for light scattering and electron microscope observations. Using a different source, Beer (1961, personal communication) calculated the total length of whole molecules of T2 bacteriophage DNA appearing in the electron microscope. He found that the average length is approximately 50 μ, which agrees well with that estimated by Cairns (1962a) from radioautography of tritium-labeled T2 DNA and the length expected for a single Watson–Crick double helix. Bendet *et al.* (1962) obtained a mean average length of 14.0 ± 0.6 μ for bacteriophage T3 DNA, which agrees well with that calculated (12.9 μ) from the nucleic acid content, assuming that all the isolated DNA is one double-stranded piece.

10. Column Chromatography

This method offers the greatest potential aid for large-scale preparative separation and identification of the native, denatured, and partially renatured states of DNA. Sueoka and Cheng (1962) have carried out experiments using a chromatographic column consisting of methylated bovine serum albumin and kieselguhr, slightly modified from that described by Mandell and Hershey (1960). Native DNA is eluted at lower ionic strengths (depending on the base composition) than denatured DNA. Sueoka and Cheng have used this column successfully for the fractionation, in the native state or after subjection to denaturing conditions, of the two species of DNA present in the marine crab, *Cancer borealis*. Cordes *et al.* (1962) have used the same column to fractionate

the complementary strands of denatured bacteriophage alpha DNA. Smith *et al.* (1960) made use of a cationic starch column to fractionate nucleic acids.

11. Chemical Methods

Formaldehyde reacts more readily with denatured than native DNA (Haselkorn, 1959; Grossman *et al.*, 1961; Berns and Thomas, 1961). It has been useful in detecting and confirming the single-strand character of ϕX174 bacteriophage DNA (Sinsheimer, 1959) and of the DNA associated with yeast lactic dehydrogenase (Mahler and da Silva Pereira, 1962). The use of acridine orange for detecting denatured DNA might prove to be useful. This is based on the observations of Mayor and Hill (1961) that bacteriophage ϕX174 particles stain bright red whereas T2 bacteriophage stain yellow-green (Anderson *et al.*, 1959). Stone and Bradley (1961) have attempted to characterize spectroscopically the interaction of acridine orange with DNA in the native and denatured states.

Other methods, some quite specialized, which have been shown to distinguish native from denatured DNA and to detect denaturation include oscillographic polarography (Paleček, 1961), calorimetry (Sturtevant *et al.*, 1958), electrophoresis (Matsubara and Takagi, 1962), precipitation with divalent ions (Stevens and Duggan, 1957; Sinsheimer, 1959; Mahler and da Silva Pereira, 1962; Aldridge, 1962), infrared spectrophotometry (Kyogoku *et al.*, 1961), flow dichroism (Cavalieri *et al.*, 1956a), and conductometric titrations (Felsenfeld, 1962).

B. Conditions Affecting Denaturation and Renaturation

Although renaturation is affected considerably by the source of DNA, be it microorganisms or higher plants and animals, denaturation is in general unaffected, depending mainly on the base composition. The optimal renaturation temperature is G + C dependent. Also, renaturation depends to a greater extent upon the molecular weight of the DNA than does denaturation (Marmur and Doty, 1962).

In the past several years it has become apparent that several important factors must be considered in a study of the denaturation and renaturation of DNA. One is aggregation, which is encouraged by high DNA concentrations (greater than 10–20 μg/ml of bacteriophage DNA or 20–40 μg/ml bacterial DNA) and high ionic strength solvents during the annealing process. Aggregates can be recognized by their high s_0 and especially $[\eta]$ values (Eigner, 1960; Eigner and Doty, 1963) and by the formation of complexes of intermediate density between renatured DNA samples that display no sequence homologies (Schildkraut *et al.*, 1961a).

Although the denaturation of DNA is related to its average base composition, the separation of the DNA strands at various stages in the denaturation process depends on the melting out of the most resistant (i.e., G + C rich) regions or "nuclei." The presence of such nuclei can allow partially denatured DNA to renature when it is brought back to room temperature.

Some factors that affect denaturation of DNA are considered below.

1. Ionic Strength

The stability of synthetic polynucleotides and of the DNA structure necessitates that a large proportion of the charged phosphate groups be neutralized or screened (Cavalieri *et al.*, 1956b; Felsenfeld, 1962) and is thus very much dependent upon ionic strength (Marmur and Doty, 1962). The T_m is very nearly a linear function of the logarithm of the ionic concentration (Ts'o *et al.*, 1962a; Dove and Davidson, 1962a; Inman and Baldwin, 1962). Zimmer and Venner (1962) have shown that the T_m is also related, inversely, to the ionic radius of the cation in the suspending medium. Although increasing the ionic strength increases the thermal stability of DNA, the effect tends to level off at high ionic strengths. With large anions at high concentration (e.g., 7.2 *M* $NaClO_4$), the T_m is approximately 40°C lower than in 0.2 *M* NaCl (Hamaguchi and Geiduschek, 1962; Geiduschek, 1962). DNA is unstable at room temperature at ion concentrations less than 10^{-4} *M*. Some divalent ions (Mg^{++}, Ba^{++}, Mn^{++}, Co^{++}, Ni^{++}, Zn^{++}) increase the T_m of calf thymus DNA (Eichhorn, 1962), whereas others decrease its melting temperature (Cu^{++}, Cd^{++}, Pb^{++}). The former act by neutralizing the charged phosphate groups and the latter divalent ions are believed to displace the H-bonds by their coordination to electron donor groups on the nucleotide bases.

2. pH

The stability of DNA is dependent upon the pH, denaturing outside the pH range of 2.7–12 at room temperature (Gulland *et al.*, 1947; Doty, 1957; Bunville and Geiduschek, 1960; Zimmer and Venner, 1962; Dove and Davidson, 1962a). The pH at which DNA is denatured depends upon the temperature, ionic strength, and base composition (Cox and Peacocke, 1957a,b; Bunville and Geiduschek, 1960; Marmur *et al.*, 1961a). Low temperatures and high G + C favor resistance of DNA to acid denaturation, but denaturation by high pH, while G + C dependent, is not much affected by thermal exposures below room temperature (Dove and Davidson, 1962a). At values between 5.5 and 8.5, the T_m of DNA

is not much affected by change of pH (Ts'o *et al.*, 1962a; Zimmer and Venner, 1962; Marmur, Rownd, and Doty, unpublished results).

3. Base Composition

The presence of many G + C base pairs increases the thermal stability of DNA at neutral pH, but not at pH 3 (Zimmer and Venner, 1962; Cox, Marmur and Doty, unpublished results). In 0.15 *M* NaCl plus 0.015 *M* Na citrate at neutral pH the T_m increases 0.41° per mole per cent G + C (Marmur and Doty, 1959, 1962), 0.56° in 7.2 *M* $NaClO_4$, 0.35° in 51% methanol containing 10^{-3} *M* NaCl and 10^{-3} *M* Tris (Geiduschek, 1962), and 0.52° in 0.1 *M* $NaClO_4$ at pH 7 (Dove and Davidson, 1962a). The T_m for poly d(A-T) is lower than that for the extrapolated value from the analysis of the naturally occurring DNA's and could arise from the diminished nearest-neighbor base-base interactions because of the alternating A and T residues (DeVoe and Tinoco, 1962). The differences in the melting temperatures (ΔT), measured both at the ambient ($T_{m,a}$) and room temperature ($T_{m,25}$) depend on the molecular weight of DNA, its base composition, and probably also the distribution of the bases (Geiduschek, 1962). The distribution of the base pairs along the DNA molecule, although very important, is difficult to determine or evaluate quantitatively. The existence of inhomogeneities in base distributions along the DNA molecule is indicated by the chemical analyses of the pyrimidine sequences (Shapiro and Chargaff, 1960; Burton, 1960; Petersen, 1961), and by statistical analysis (Simha and Zimmerman, 1962). In their analyses of the pyrimidine fragments of DNA, Burton and Peterson (personal communication) have found up to four cytosine residues in a row. If such a grouping is adjacent to one or more guanine residues, the resulting sequence (Ralph *et al.*, 1962; Fresco, personal communication) would provide a thermally resistant nucleus that would require very high temperatures for complete strand separation. The T_m of the poly d(A-B) copolymer (B = bromouridylate) at low ionic strengths is higher than that of poly d(A-T), but the difference is minimized at high ionic strengths (Inman and Baldwin, 1962). The substitution of the thymine residues by bromouracil or iodouracil increases the T_m of DNA (Kit and Hsu, 1961; Szybalski, 1962; Szybalski and Mennigmann, 1962) and lowers the pH of the helix to random coil transition in the alkaline region (Shooter and Baldwin, 1962). The melting temperature of high molecular weight RNA varying in G + C between 44 and 61% has also been shown (Spirin, 1961, 1962) to bear a linear relation to the occurrence of these two bases. Pullman and Pullman (1959), from molecular orbital calculations, concluded that the GC pair is both a better electron donor and electron acceptor than

the AT pair. They believe that the former base pair exhibits a greater resonance energy stabilization from hydrogen bonding and could thus, in part, account for the increased helix stability from high proportions of GC.

4. Molecular Weight

Whereas the melting temperature of DNA measured at the ambient temperature ($T_{m,a}$) would appear to be independent of the molecular weight of DNA (Marmur and Doty, 1961), it is size-dependent when measured after the heated DNA solutions are rapidly chilled and returned to room temperature ($T_{m,25}$) (Geiduschek, 1962). This could be attributed to the inhomogeneities in base sequences, so that larger molecules are more likely to possess thermally resistant G + C rich "nuclei" that would more readily allow their reversible denaturation than smaller DNA molecules.

5. Denaturing Agents

Many organic molecules lower the denaturation temperature of DNA. The effects of formaldehyde in lowering T_m and preventing renaturation have already been noted (Stollar and Grossman, 1962; Haselkorn and Doty, 1961; Berns and Thomas, 1961; Thomas and Berns, 1962). Urea, guanidinium chloride, salicylate, formamide, dimethylformamide, sulfoxide, aromatic compounds, some mutagenic agents, nucleosides, and a variety of alcohols lower the denaturation temperature of DNA in varying degrees (Rice and Doty, 1957; Marmur and Ts'o, 1961; Helmkamp and Ts'o, 1961; Ts'o *et al.*, 1962a,b; Belman *et al.*, 1962; Hamaguchi and Geiduschek, 1962). Formamide at high concentrations can denature DNA at room temperature or 37°C without causing backbone scission (Ts'o *et al.*, 1962b) and thus is very useful for denaturing DNA without the introduction of undesirable side effects. Ts'o *et al.* (1962b) have also shown that nucleic acid polymers in formamide solution appear devoid of all secondary structure when measured by optical rotation studies. Some divalent metal ions can also lower the T_m of DNA (Eichhorn, 1962). Concentrated solutions of certain anions (trichloracetate, perchlorate, thiocyanate) are effective in lowering the T_m of DNA; this is considered to arise from an effect on the structure of water (Hamaguchi and Geiduschek, 1962). The most extensive study to date on the denaturation of DNA by organic compounds is that of Levine *et al.* (1963). From their results, they concluded that, contrary to expectations, amides, ureas, carbamates, and alcohols act as denaturing agents not because of their hydrogen-bonding capacity, but rather because they stabilize denatured DNA relative to native DNA. This is accomplished

by decreasing the ion-solvating power and increasing the hydrophobic character of the solvent. They point out that the forces involved in the denaturation of DNA are not necessarily the same as those that are important in maintaining the native structure of the DNA molecule. Thus, they consider that the decrease in the T_m of DNA in the presence of organic denaturing agents cannot be used as evidence for the existence of hydrophobic interactions in maintaining helix stability.

6. Physical and Chemical Agents

Exposure of DNA to ultraviolet light, X-rays, and the photodynamic action of dyes lowers the T_m of DNA (Marmur *et al.*, 1961a; Belman *et al.*, 1962; Simon and Van Vunakis, 1962; Freifelder *et al.*, 1961). The lowering of the denaturation temperature is explicable in terms of a weakening of the DNA double helical structure as a result of some chemical damage inflicted on the molecule, with concomitant localized denaturations along the DNA helix. Hydroxylamine also lowers the T_m, probably by destruction of the cytosine residues (Bendich, personal communication).

7. Diamines and Polyamines

Some diamines and polyamines increase the T_m of DNA, the increase depending on the chain length of the polyamine (Mahler *et al.*, 1961), the ionic strength (Tabor, 1962), and the base composition of the DNA. Mahler and Mehrota (1962) and Mandel (1962a) found that the degree of thermal stabilization is linearly related to the A + T content and extrapolates linearly to very little if any stabilization at zero A + T. Tabor (1961, 1962) found that spermine protected *B. subtilis*–transforming DNA as well as other DNA's from thermal denaturation. These results should caution investigators working with the thermal resistance of DNA to make sure that their DNA preparations are free of polyamines, which exist in high concentrations in some cells (Tabor *et al.*, 1961). Added polyamines can be removed by dialysis (Mandel, 1962a). The action of spermine on DNA involves its interaction with the phosphate groups, neutralizing their charge. The same can be said about the action of divalent ions such as Mg^{++}, Mn^{++}, etc. (Felsenfeld, 1962).

8. Cross-Linking

The introduction of cross-links by means of chemical or physical means will still allow the DNA double helix to be disrupted, but the DNA will return to a renatured configuration when the denaturing agent is removed (Marmur and Grossman, 1961; Marmur *et al.*, 1961a; Geiduschek, 1961, 1962). Cross-linking agents used include ultraviolet light,

nitrous acid, and bifunctional alkylating agents. It is very likely that other physical and chemical agents will also cross-link DNA, and also that some naturally occurring DNA's will possess covalent cross-links.

C. Renaturation Factors

Experiments on conditions affecting the renaturation of DNA are limited. The factors affecting DNA renaturation are outlined briefly, and the reader is referred to the details that are described in the papers by Marmur and Doty (1961), Schildkraut *et al.* (1961b), Geiduschek (1961), Cavalieri *et al.* (1962), and Subirana *et al.* (1963).

1. Concentration

The concentration dependence of the renaturation of *D. pneumoniae*–transforming DNA is one of the most important facts favoring the hypothesis of strand separation and reformation (Doty *et al.*, 1960). Second-order kinetics have been obtained with *H. influenzae*–transforming DNA by measuring the restoration of biological activity (Lanyi, 1963; Herriott, personal communication). The concentration-independent renaturation results of Cavalieri *et al.* (1962), who used several different DNA samples of bacterial origin, are difficult to reconcile with the above observations. Since aggregation is encouraged at high concentrations of DNA, these should be avoided. The aggregated molecules can specifically be removed, e.g., by use of the *E. coli* phosphodiesterase (Lehman, 1960).

2. Source

Since renaturation depends upon the concentration of specific complementary strands, the process would be expected to depend on the homogeneity of the DNA preparation. The optimal homogeneity is attained with DNA isolated from a bacteriophage possessing one molecule per bacteriophage particle. Thus, DNA from bacteriophages renatures extremely well, DNA from bacterial sources reasonably well, and DNA from higher plants and animals hardly at all (Marmur and Doty, 1961) [because of its heterogeneity (Bendich *et al.*, 1956; Sueoka, 1961)] unless first fractionated (Rownd, unpublished results). The ease of renaturation can be used as a measure of DNA homogeneity. With this criterion, DNA from *Mycoplasma* (PPLO) would be considered to be about as homogeneous as bacteriophage DNA (Marmur and Doty, 1961).

3. Temperature

The optimum temperature for the renaturation of bacterial DNA in 0.4 M Na^+ is approximately 25° below its T_m. Studies on the T-even

bacteriophage DNA indicate that 55° is the optimal renaturation temperature (in 0.5 *M* NaCl) (Murakami and Levine, personal communication). The optimum renaturation temperature for the formation of hybrid molecules between DNA and RNA is the result of two competing interactions—the renaturation of DNA strands as well as the formation of the hybrid DNA-RNA molecules. In order to favor the latter process, a temperature causing minimal renaturation of the DNA must be selected. Bautz and Hall (1962) renatured T4 messenger RNA with T4 DNA linked to cellulose in 0.4 *M* Na^+ at 55°C. Yankofsky and Spiegelman (1962), in some of their experiments, used lower temperatures to hybridize ribosomal RNA from *E. coli* with DNA from the same organism.

4. Molecular Weight

The renaturation of DNA appears to be dependent upon molecular weight when measured both by the recovery of transforming activity and by spectrophotometric methods (Marmur and Doty, 1961; Geiduschek, 1962). This is in accord with the nucleation hypothesis, since it would be expected that, for higher molecular weight DNA, fewer nuclei are required to renature a given amount of DNA.

5. Ionic Strength

The ionic strength dependence was determined in the earlier studies of DNA renaturation when denatured DNA was annealed by allowing it to be slowly cooled in a large water bath. With the restoration of transforming activity as a measure of renaturation, it was found that the biological activity of *D. pneumoniae* DNA increased linearly with ionic strength until 0.4 *M* Na^+ was reached and then leveled off (Marmur and Lane, 1960). To obtain the maximum *amount* of renaturation, denatured DNA should be annealed at the optimum ionic strength (0.4 *M* Na^+) and temperature (65 ± 5°C for bacterial samples, 10°C lower for bacteriophage DNA) for 3–4 hours and the DNA concentration should be kept low (4 ± 1 μg/ml for bacteriophage DNA and 6 ± 2 μg/ml for bacterial DNA) to minimize aggregation. These conditions give optimal renaturation of T-even bacteriophage DNA as well as DNA with transforming activity isolated from *D. pneumoniae, H. influenzae,* and *B. subtilis.* Some aggregation most likely takes place during any renaturation; this can be eliminated or reduced to a great extent by enzymes that digest denatured DNA specifically or by exposing renatured DNA to temperatures at or near the T_m in solvents of low ionic strength. The latter method has been found by Rownd (unpublished results) to melt out the more thermally sensitive, aggregated molecules. Rownd (unpub-

lished) has shown that the rate of renaturation at constant temperature is greatly increased at ionic strengths greater than 0.4 M Na^+, using the technique of CsCl density gradient centrifugation to follow conformational changes.

V. Mechanism of Renaturation

The more recent studies on the mechanism of denaturation have given considerable insight into the reformation of specific base pairs between complementary polynucleotide chains when residual interactions maintain the base sequences of the two chains in register. It is of considerable interest to determine the mechanism by which separated single strands may interact with their complementary mates to form renatured molecules with the restoration of many of the properties of native DNA.

A. Helix Formation with Synthetic Polynucleotides

It has now been established that the synthetic polynucleotides can react under appropriate conditions to form double- and triple-stranded helices, the former group being similar in important respects in structure and physical properties to that of naturally occurring DNA (Warner, 1956, 1957; Rich and Davies, 1956; Felsenfeld and Rich, 1957; Fresco and Doty, 1957; Rich, 1958; Fresco and Alberts, 1960; Fresco and Straus, 1962; Steiner and Kitzinger, 1962). The synthetic ribo- and deoxyribopolynucleotides will interact to form "hybrid" helices (Rich, 1960; Schildkraut *et al.*, 1961b) analogous to those observed between DNA and messenger RNA (Hall and Spiegelman, 1961; Gros *et al.*, 1961a,b). Under physiological conditions these polynucleotide homopolymers behave as randomly coiled, single polymer chains and, hence, are close analogs of the DNA single strand; however, they differ in an important way from the latter since, being homopolymers, they do not have the complexity of base sequences along the chain.

Rich and his collaborators (1956) measured the stoichiometry of the interaction between poly A and poly U and found that two- (A + U) or three-stranded (A + 2U) helices could be formed depending on the nature of the solvent. Hence, the interaction of these two polymers may be taken as a prototype for the renaturation reaction of DNA, with the important reservation that any contact between the two interacting polymer chains automatically results in complementarity of base sequence. Ross and Sturtevant (1960) have studied the kinetics of helix formation between poly A and poly U. Since the reaction is too rapid for study by the usual spectrophotometric methods, they employed the stop-flow technique (Chance, 1953). They found that the reaction initially is second-order, but deviates in the later stages toward first-

order kinetics. The initial rate is first-order with respect to each of the polymer components and is strongly dependent on the ionic strength of the solvent, as would be expected for polyelectrolytes carrying charges of the same sign. Thus, at the start of the reaction it appears that the nucleation of the helix is the rate-controlling step. Since this involves two kinetic units, the initial rate would be second-order. However, the later stages of the reaction result from the rearrangement of mismatched regions between the two polymer chains by a process of annealing. Since the annealing procedure would not be expected to follow any simple rate law, it gives rise to the complex kinetics observed during the later stages of the reaction. It should be noted that Rich (1959) had studied the interaction of poly A and poly I to form a double-stranded helical structure and concluded from his kinetic data that the reaction followed first-order kinetics. Ross and Sturtevant (1960) pointed out, however, that with conventional spectrophotometric methods it is not possible to follow the first 40% of the reaction. Some of the reactions examined by Ross and Sturtevant could be interpreted as following first-order kinetics if the first half of the reaction were omitted. Thus, the annealing process can be complex even for such relatively simple molecules as homopolymers.

The enthalpy of reaction of poly A and poly U to form the poly (A + U) helix has been found by Steiner and Kitzinger (1962) to be a well-defined quantity ($-\Delta H$ of about 6000 calories per mole of uracil) that is independent of the molecular weight of the interacting species over a wide range of sizes.

B. Kinetics of Renaturation of DNA

Although the renaturation of DNA is somewhat analogous to the interaction between the synthetic polynucleotides discussed above, there are a number of complicating features that make it fundamentally different. Whereas the synthetic polynucleotides used in the kinetic studies were homopolymers, DNA molecules contain highly complex and specific base sequences along the polynucleotide chain. Moreover, the DNA molecules from bacterial and higher sources show considerable intermolecular heterogeneity. Thus, the complexity of base sequence of the single polynucleotide chains imposes the restriction that not every interaction between the single strands in solution will be fruitful as a nucleation step in helix formation. Even relatively homogeneous DNA such as that isolated from viral sources would be expected to have abortive nucleation steps, since only the pairing of specific regions between complementary chains would result in a nucleus sufficiently stable for subsequent growth of the helix. Unstable nuclei resulting from insufficient sequence com-

plementarity between the interacting chains would only exist as transient states during the renaturation reaction; these would be replaced by other nuclei with greater stability because of the increased number of bases in register.

Once a successful nucleation step occurs, further helix formation can proceed. Although the unwinding of the two strands in denaturation occurs rather rapidly—in a matter of minutes at temperatures just a few degrees above the $T_{m,a}$—it is not yet clear how long the reverse process requires during the *re*naturation reaction. For example, intrachain base pair formation may occur and impede the formation of the double-stranded helix in this region (Stollar and Grossman, 1962; Rownd *et al.*, 1963b), or mismatched regions may occur further along the two chains and require annealing before exact registration can proceed. Since theoretical considerations (Flory, 1961; Saunders and Ross, 1960) as well as experimental observations (Marmur and Doty, 1961; Cavalieri *et al.*, 1962) show that an annealing reaction, such as renaturation, should occur at a maximal rate at temperatures 10–20°C below the helix-coil transition temperature, most of the random *intra*chain base pairs would not be stable at this elevated temperature. However, it would be expected that only the most stable of the intrachain base interactions would seriously impede the formation of helix after the nucleation step.

The over-all kinetics of renaturation would thus depend on whether the nucleation or the "zippering up" stage of the reaction is slower and, hence, rate-determining. If the nucleation step is rate-determining, then the over-all kinetics of renaturation should be second order. If, however, nucleation is rapid and the subsequent growth of helical regions rate-limiting, then complex kinetics would be expected, since the growth of helical regions in the annealing process would not be expected to follow any simple rate law.

The kinetics of renaturation of bacteriophage and bacterial DNA have been studied by the differences in the optical, hydrodynamic, and biological properties of denatured and renatured DNA.

1. Spectrophotometry

Since the denaturation of DNA is accompanied by a hyperchromic shift because of the disruption of its ordered secondary structure, renaturation or the restoration of the native properties can be followed conveniently by measuring the changes in hypochromicity at an elevated temperature. Marmur and Doty (1961) showed qualitatively that the kinetics of renaturation of DNA depend in an important way on the source of the DNA. DNA from viral sources renatures more rapidly and to a much greater extent than bacterial DNA. The DNA isolated

from mammalian sources did not renature at all after several hours of annealing. It was concluded that the rate of renaturation depends on the concentration of *complementary strands*. This is consistent with the hypothesis that the separated single strands renature only with complementary strands, although not necessarily with their original mates.

The kinetics of renaturation have been studied in a more quantitative fashion by Cavalieri *et al.* (1962) with bacterial DNA and by Subirana *et al.* (1963) with both viral and bacterial DNA. The results of these independent studies are in apparent conflict. Cavalieri and co-workers showed that renaturation in 1.0 *M* NaCl is essentially first-order at 60°C, whereas at 70°C and 80°C there is a second-order component of increasing magnitude. They state that renaturation at 85°C proceeds mainly by a bimolecular mechanism; however, it was not demonstrated how the molecularity of the reaction was determined from the data. They concluded from their observations that renaturation does not involve the reaction of kinetically separate strands and suggested that renaturation results in the "zippering up" of collapsed and disordered, but not separated, double-stranded denatured molecules (Cavalieri and Rosenberg, 1961). They attributed the bimolecular kinetics to the interaction between the ends of two double-stranded molecules; this they state would explain the observations of density hybrids (Schildkraut *et al.*, 1961) and genetic hybrids (Herriott, 1961a,b) formed by heating and annealing DNA.

Subirana *et al.* (1963), on the other hand, found that renaturation follows second-order kinetics with bacteriophage T4 (56°C) or bacterial (68°C) DNA at low concentrations (less than 10 μg/ml) in 0.15 *M* NaCl plus 0.015 *M* Na citrate, where the complicating features of aggregation are minimized. At higher DNA concentrations, as well as in 1.0 *M* NaCl, the first part of the reaction follows second-order kinetics whereas in the later stages a different second-order reaction occurs which may arise from further interaction between renatured species to form aggregated-renatured DNA. Thus, these kinetic studies are consistent with the view that the renaturation of DNA involves the specific interaction of separated single strands. At a given concentration viral DNA renatures much more rapidly than bacterial DNA, indicating that the rate of renaturation depends on the homogeneity of the DNA preparation or, in other words, on the concentration of complementary strands, rather than on the total DNA concentration. This helps to explain why such heterogeneous DNA as that isolated from higher plants and animals does not renature. However, once a cross-link is introduced between the complementary strands, renaturation is possible (Geiduschek, 1961). The dependence of the rate of renaturation on the relative homogeneity

of the DNA sample is certainly consistent with the hypothesis of strand separation and specific recombination between complementary mates, but it is difficult to reconcile this behavior with the model of Cavalieri *et al.* (1962) in which the strands are postulated never to have been separated

2. Density Gradient

Since the renaturation of DNA results in a decrease in its buoyant density to a value approaching that of the native sample, the kinetics of renaturation can be followed by density gradient studies as well. When solutions of denatured DNA are annealed for various time intervals, it is found that the relative proportions of the denatured and renatured bands in the CsCl density gradient vary as a function of the time of annealing. At shorter annealing times, the bulk of the DNA remains in the denatured band whereas at longer times renatured DNA is found to be the predominant species. It should be noted that the renatured band does not consist of DNA molecules all renatured to the same extent; rather, partially renatured states of buoyant density intermediate between denatured and renatured DNA are found (Rownd *et al.*, 1961, 1963) and are discussed in further detail below. Because of the existence of these metastable states, only semiquantitative information is obtainable from density gradient experiments. However, the information obtained from studies on the buoyant density properties of renatured DNA confirms the evidence for renaturation based upon spectrophotometric measurements. Thus, viral DNA under optimal renaturation conditions is found to shift in buoyant density toward that of the native DNA very rapidly, whereas bacterial DNA renatures more slowly. The buoyant density of mammalian DNA has not been found to shift to any appreciable extent. If calf thymus DNA is first fractionated by CsCl preparative density gradient centrifugation, which reduces the heterogeneity of the renaturing samples, then by following the change in the buoyant density, the DNA is found to renature (Rownd, unpubl.). Such behavior is consistent with the earlier hypothesis that mammalian DNA does not renature because of its heterogeneity. These results refute the notion (Cavalieri *et al.*, 1962) that there is something special about the base sequences of animal DNA that interferes with its capacity to renature.

When biological hybrid DNA is denatured, the complementary strands of each hybrid molecule band at different positions in the density gradient because of the difference in their isotope content. Thus, single strands may be fractionated by the use of preparative density gradient centrifugation. If the fractionated single strands (both the heavy and light bands will possess equal quantities of the complementary single strands)

are annealed under conditions appropriate for renaturation, it is found that there is a density shift in renaturation as a function of time toward that of native DNA (Rownd, 1963). The kinetics of renaturation of the single strands, followed by the buoyant density measurements, are identical to those found when a homogeneously labeled DNA preparation is used.

Degradation by ultrasonic waves of the *in vitro* hybrid formed by heating and annealing a mixture of heavy isotope labeled and unlabeled DNA has suggested that this hybrid consists mainly of a homogeneous structure and that the heavy labeled and light components of the hybrid are side-to-side [as in the case of *in vivo* hybrids (Rolfe, 1962)] rather than end-to-end structures (Rownd, 1963; Kozinski and Beer, 1962). More conclusive experiments have been carried out with biological hybrid DNA isolated from *E. coli* and *B. subtilis* to show that denaturation and renaturation proceed by a mechanism of strand separation and recombination. When biological hybrid DNA is heated and annealed, and *then* centrifuged in CsCl, both the renatured heavy and renatured light DNA components can be readily recognized by the bands that they form in the gradient. Both bands can form only if the biological hybrid is first dissociated upon heating and the complementary strands with similar labels then recombined to form either the renatured heavy or light DNA. A hybrid density band resulting from the renaturation of strands that have different labels also appears, as well as the unrenatured heavy and light components (Rownd, 1963; Rownd and Doty, 1963). In addition to the five bands discussed above, a sixth band also appears in the density gradient when biological hybrid DNA is heated and *annealed.* This DNA component has the buoyant density of the original native biological hybrid DNA and is present to the extent of about 10% of the total DNA. Since two strands must be properly matched in length in order to give rise to a renatured species of native buoyant density, it seems likely that the sixth band has its origin in biological hybrid DNA molecules whose strands have not completely separated under the conditions of thermal denaturation. However, this band of native biological hybrid density is not observed when the DNA is heated at 100°C for 10 minutes and then quickly cooled. The molecules giving rise to this band must therefore be spread out over a broad density range because of the existence of different proportions of denatured and nativelike regions. This interpretation is in apparent agreement with the observations of Rownd *et al.* (1963b) on the density distribution of the residual transforming activity of denatured *B. subtilis* biological hybrid DNA. The kinetics of renaturation of biological hybrid DNA (Rownd, 1963; Rownd and Doty, 1963) is

essentially the same as that observed by Schildkraut *et al.* (1961a), who used heavy and light DNA as the starting materials.

3. Biological Transformation and Immunology

Lanyi (1963) has made a detailed study of the kinetics of renaturation of several genetic markers in *H. influenzae* DNA. The restoration of biological activity follows second-order kinetics when the order of the reaction is calculated by both the initial velocity method and the reciprocal plot method. Although it is not entirely clear to what extent the restoration of biological activity reflects changes in the molecular conformation of DNA, it is interesting that the second-order rate constants obtained by both spectrophotometric and biological measurements in *H. influenzae* DNA are in close agreement.

The restoration of the native helical structure of T-even bacteriophage DNA may be followed by the decrease of the ability of renatured DNA to react with specific antibodies (Levine *et al.*, 1960). As a function of the time of renaturation, the ability to fix complement levels off at a value about 30% of that of denatured DNA. This is consistent with the extent of renaturation as measured by physical techniques, and with the observation of denatured regions at the ends of renatured molecules as seen under the electron microscope (Doty *et al.*, 1960). Dolbeare and Grossman (1962) have shown by preparative CsCl density gradient centrifugation studies that the residual immunological activity is actually associated with renatured molecules possessing unmatched denatured regions.

C. Partially Renatured States

If the rate of helix formation after the nucleation step in renaturation is sufficiently slow, it should be possible to "freeze in" metastable partially renatured states in which helix formation has not proceeded to completion (Dove and Davidson, 1962a,b). These states should be recognizable by a buoyant density in CsCl intermediate between that of denatured and maximally renatured DNA. Fractionation by CsCl density gradient centrifugation of DNA which has not been completely renatured should show a distribution of DNA densities ranging from the denatured to the maximally renatured density. This is the case with T4 DNA (Rownd, 1963), with *H. influenzae* DNA (Rownd *et al.*, 1961, 1963), and with *B. subtilis* DNA (Rownd, 1963). It appears that the zippering up of renatured molecules must be sufficiently slow so that molecules of low helical content have more than just a transient existence during the renaturation process. When these molecular species of low helical content

are removed from optimal renaturing conditions, they are prevented from renaturing to completion. If, however, partially renatured states obtained by fractionation in CsCl are annealed further under optimal temperature conditions at concentrations sufficiently low to minimize intermolecular contacts, it is found, by the buoyant density criterion, that they renature further. It seems plausible that at high concentrations, where interstrand contacts are more frequent, the zippering up of the renatured molecule could be rate-limiting and, hence, could result in complex renaturation kinetics.

Rownd *et al.* (1961, 1963) isolated partially renatured states in *H. influenzae* DNA and studied the effects of varying amounts of helix and coil on the biological activity of this DNA. The restoration of biological activity of single genetic markers is linearly dependent on the helical content of the renatured molecule. For two linked markers, however, no significant increases in biological activity over that of denatured DNA were found until 50–60% helix had been restored, as estimated from buoyant density measurements. This value of helix content is in excellent agreement with the known distance between the two linked genetic markers and suggests that the remaining steps of the transformation process after uptake of the DNA are carried out most efficiently when both markers are in the helical conformation (Fox, 1962).

VI. Hybrid Formation

A. Requirements

Aside from the environmental conditions suitable for renaturation (outlined in Section IV,B), there are several requirements permitting strands originating from different DNA molecules to form hybrids with a nativelike structure, after denaturation and annealing (here referred to as *"in vitro"* hybrids). The main requirements are that the over-all base compositions of the two interacting "parental" DNA samples be similar and that the DNA originate from microorganisms that are genetically related (Marmur *et al.*, 1961c, 1962). The similarity in base composition would seem to be a minimum requirement in order to have the fairly extensive base sequence homologies needed to form the hybrid, nativelike molecules. It does not follow, of course, that any two heterologous DNA samples with identical average base compositions will be able to form *in vitro* hybrids.

The methods thus far developed to study the formation of hybrids have not yet been developed to such a degree that small changes in base composition or base sequences resulting from mutations can be detected. Thus, the DNA from various *E. coli* strains (B, C, W, K-12, I, TAU$^-$,

and 44B), which vary among themselves by at least several genetic traits, behave in an almost indistinguishable manner when examined for their ability to form *in vitro* hybrids with heavy isotope-labeled *E. coli* B DNA (Schildkraut *et al.*, 1961a).

It is not known at present how far the average DNA base composition of the parental DNA samples can deviate and not affect, or to what extent it would affect, *in vitro* hybrid formation. Such studies may be possible with the DNA isolated from a group of related organisms such as those that belong to the Enterobacteriaceae (Marmur and Doty, 1962; Falkow *et al.*, 1962b). These include some species related taxonomically and physiologically but with slightly different G + C compositions when analyzed by CsCl density gradient centrifugation (Schildkraut *et al.*, 1962a) and/or by their thermal denaturation temperature (Marmur and Doty, 1962; Mandel, 1962b). If these organisms have been derived from a common strain by mutation and selection, they may still possess extensive sequence homologies. The recent finding of Weed (1962) that some copper-induced mutants of *B. subtilis* have altered base compositions has opened up new possibilities for study of comparative base compositions and base sequence homologies.

The requirements for *in vitro* DNA hybrid formation are that the bacteria be genetically related when tested by transformation or transduction or, in the case of the bacteriophages, by recombination (Marmur *et al.*, 1962). However, microorganisms that can exchange genetic material by conjugation or F-duction only do not necessarily contain DNA molecules possessing extensive base sequence homologies permitting *in vitro* hybrid formation. In fact, F-duction can take place between organisms having quite dissimilar DNA base compositions (e.g., *E. coli* and *Serratia marcescens*), suggesting that the transferred genetic material is not integrated with the host DNA but exists and reproduces in unison with it (Marmur *et al.*, 1961b).

The question can be asked whether transduction, transformation, or *in vitro* hybrid formation is the most stringent test for homology. Since the genetic exchange may probe the homologies of small parts of individual molecules and marker regions, it would be expected to require a greater base sequence compatibility than does *in vitro* hybrid formation, which tests the homologies of many DNA molecules. This has been shown to be the case in a study of the DNA from the Bacillaceae (Marmur *et al.*, 1963). Transforming DNA's from species related to *B. subtilis* with the same base composition yield higher proportions of hybrid with the DNA from the recipient organism than can be predicted from the transformation efficiencies.

The observations of Schildkraut *et al.* (1961a) are consistent with

the hypothesis that the strands that combine in renaturation are not the same strands that were united in the native DNA but rather may be complementary strands originating in different cells. The denaturation and renaturation of the DNA is accomplished by heating and annealing a solution of two homologous DNA samples, one of which carries a heavy isotope label. If the pairing of DNA strands depends only on the complementarity between them, one would expect that renaturation would lead to three bands in density gradient centrifugation; one heavy, one light, and one intermediate, corresponding in density to a hybrid composed of one normal and one heavy strand. Moreover, the amount of the hybrid would be expected to be double that of either the heavy or light component, provided that equal amounts of the two DNA samples had been heated and annealed.

Experiments have been carried out by Schildkraut *et al.* (1961a) that support the hypothesis that the strands combining during renaturation are not the identical strands that were united in the native DNA, but rather are complementary strands originating from different cells. The use of N^{15} and deuterium-labeled DNA provides a distinctive density label that is readily resolved in the CsCl gradient from unlabeled DNA. If an equal weight mixture (5 μg/ml) each of native labeled and unlabeled bacterial DNA is thermally denatured and then annealed under optimal conditions, the interaction of the separated strands should result in renatured labeled, hybrid, and renatured unlabeled DNA in the proportions 1:2:1 as expected from the random recombination of the separated strands. If renaturation is not carried out to completion, the denatured forms of the labeled and unlabeled DNA will also appear in the density gradient pattern and give rise to five density species of DNA corresponding to the five-band pattern discussed above. Longer times of annealing lead to complete renaturation, so that the denatured bands are no longer apparent, but these conditions (even at low DNA concentrations) encourage the nonspecific aggregation of the DNA, resulting in a distortion of the proportions of the renatured species predicted from the model of random recombination of separated strands. Incubation of such an apparently aggregated mixture with *E. coli* phosphodiesterase (Lehman, 1960) results in the elimination of these aggregates. This is shown by the restoration of the expected proportions of the three renatured species as well as by their slight decrease in density due to digestion of the unpaired regions. A further consequence of the enzymatic digestion is the elimination of the denatured species, resulting in three instead of five bands. Thus, the results obtained using labeled and unlabeled DNA, as well as biological hybrid DNA (Rownd, 1963), lead to the conclusion that the major

portion of the strands recombining during renaturation is not the original native complementary mates.

B. Nature of the Hybrid

1. Physicochemical Studies

A number of objections might be made that the hybrid does not consist of uniformly labeled strands united by complementary base pairing. First it might be argued that the DNA of intermediate density is an aggregate formed by physical entangling of light and heavy strands. However, a number of examples have been given by Schildkraut *et al.* (1961a) showing that molecules of different base composition, or even of the same composition but different genetic relatedness, would not form hybrids. It seems likely that physical aggregates can be produced and, when the concentration of DNA in the annealing mixture is raised tenfold (to about 50–100 μg/ml), material of intermediate density can be produced from DNA samples with different base composition. When such material is treated with the *E. coli* phosphodiesterase, however, these aggregates disappear. Thus, aggregates formed by physical entanglement can be distinguished from true hybrid molecules. However, the DNA of intermediate density might consist of a uniformly labeled double helix and a completely unlabeled double helix held together by lateral bonds whose nature is as yet unknown (Cavalieri and Rosenberg, 1961). Or, it could be imagined that the hybrid consists of two double helical DNA molecules aggregated end to end. This latter possibility has been examined in the case of the *in vivo* hybrids of *E. coli* DNA by Rolfe (1962). Following several sonically induced lesions, a molecule consisting of an unlabeled double strand connected end to end to a molecule having both strands labeled would produce two new bands in the CsCl gradient at positions corresponding to those normally found for the unlabeled and fully labeled components. This, however, was not found to be the case. Similarly, *in vitro* hybrid *B. subtilis* and *E. coli* DNA have been prepared and fractionated by preparative CsCl density gradient centrifugation from the other DNA species formed simultaneously during the annealing process. The isolated hybrid DNA was then fragmented by sonic degradation. Banding the sonicated *in vitro*–formed hybrids did not indicate the appearance of any significant amount of DNA possessing the densities of uniformly labeled or unlabeled DNA, thus eliminating the possibility of end-to-end aggregation (Rownd, 1963).

The occurrence of a side-by-side aggregate appears to be unlikely from considerations of the strong species specificity of hybrid formation. This high degree of specificity was revealed by the lack of hybridization

in two systems. First, a hybrid could not be formed between the DNA of *E. coli* and *Salmonella typhimurium* or *S. typhosa*. These organisms are known to be genetically related (by conjugation) and they even have similar orders for the genes studied (Zinder, 1960). However, when specific regions of the *E. coli* chromosomes are transferred to *S. typhosa* by bacterial conjugation (Baron *et al.*, 1960; Falkow *et al.*, 1962a), the recombinant strains form *in vitro* hybrids with *E. coli* DNA, the extent of hybrid formation being in good agreement with the extent of genetic transfer as estimated from the mating kinetics (Rownd *et al.*, 1962, 1963a; Schildkraut and Marmur, unpublished results). Second, a hybrid cannot be formed between DNA from temperate bacteriophage λ and unfractionated DNA from *E. coli*, even though Josse *et al.* (1961) showed that their nearest neighbor base frequencies are very similar.

Further specificity has been demonstrated among the DNA of the bacteriophages of the T-series (Schildkraut *et al.*, 1962b). Hybrids can be formed between pairs of T-even bacteriophage DNA molecules or between T3 and T7 DNA. No hybrids, however, could be formed between DNA from T-even and T-odd phages nor between these and *E. coli* DNA.

Closer examination of the T-even DNA hybrids has indicated that, even in this case, differences can be detected, therefore demonstrating a lack of complete homology (Rownd and Wierzchowski, unpublished results). T4 bacteriophage DNA labeled with N^{15} and deuterium was used to form hybrids with unlabeled DNA from T2, T4, and T6 bacteriophages, by the method described by Schildkraut *et al.* (1962b). The hybrid DNA molecules were isolated by preparative CsCl density gradient centrifugation and the T_m determined. Since T2, T4, and T6 DNA have quite similar melting temperatures, this physical property can be used to determine the relative stability of hybrid DNA molecules among the bacteriophages of the T-even series, and thus estimate the extent of homology in the hybrid. It was found that the hybrid formed between the DNA of T4 and T4 had a higher degree of thermal stability than that formed between T4 and T2 or T6.

The studies summarized above on the highly specific nature of hybrid formation indicate that if some unknown bonds are responsible for the molecules associating in a side-by-side fashion during the annealing process, they are sequence specific. If these are some type of protein or hydrogen bonds they in turn must be regulated by the base sequence of the DNA molecule. It also appears that whenever the hydrogen bonds connecting the strands of the Watson–Crick double helix are broken, the hypothetical bonds also break. Instead of postulating a new type of linkage, it seems more reasonable to identify the subunits that separate

when the bonds between the single strands of the double helix are broken with the single strands themselves.

In our discussion of hybrid formation we should also mention the *in vitro* production of hybrid molecules by the DNA polymerase isolated from *E. coli*. It would be predicted that after the primer DNA had been doubled by synthesis, most of the molecules present would contain one new strand and one old. These hybrids should be easily observed in the CsCl density gradient if either the newly synthesized DNA or the primer DNA has been heavy labeled. The production of hybrids by both of these methods was achieved by Baldwin and co-workers using 5-bromouracil (B) as a label. Wake and Baldwin (1962) used poly d(A-T) as a primer for the synthesis of poly d(A-B) and vice versa. They showed that during synthesis hybrid molecules are initially formed but subsequently a product containing only newly synthesized DNA appears. If the reaction is stopped at the hybrid stage and the hybrid DNA is heated and banded in Cs_2SO_4, bands appear at the positions of d(A-T) and d(A-B) polymers, indicating strand separation. The time of incubation at elevated temperatures required to produce strand separation was longer for this enzymatically formed hybrid than for similar hybrids formed by heating and annealing of the hybrid DNA of *E. coli* produced *in vivo* and studied by Schildkraut *et al.* (1961a). Inman and Baldwin (1962) have shown that hybrid molecules can be formed from these same alternating copolymers [d(A-T) and d(A-B)] by slowly cooling a solution in high salt, where both polymers have nearly the same melting temperature. Very high concentrations are needed to form the hybrids, suggesting that at low concentrations the single strands fold back on themselves.

2. Biological Studies

The nature of hybrid DNA molecules can be approached in yet another way by studying the transforming activity of heterozygous molecules in which each of the complementary strands carry one of a pair of linked markers. Consider the case in which DNA is isolated from a cell carrying markers A and B that show linkage when used to transform cells carrying the corresponding alleles a and b. Suppose now that the DNA isolated from a strain carrying only one of the two markers is denatured and annealed with the DNA from a strain carrying the other marker. Designating the "parental" DNA's as

$$\frac{a\ B}{a\ B} \quad \text{and} \quad \frac{A\ b}{A\ b}$$

strand separation and renaturation would result in renatured molecules of the parental type as well as hybrid molecules ("heterozygous") which can be designated as

$$\frac{A \quad b}{a \quad B}$$

Such hybrid molecules might behave in transformation in one (or more) of at least three different ways:

(*a*) The hybrid molecule, after entering the transformable cell, might dissociate at cell division and segregate so that only one parental strand of DNA would appear in each cell. This would give rise to mixed clones containing transformed cells carrying only one marker (*A* or *B*). This would happen if both of the complementary strands are phenotypically competent.

(*b*) The hybrid (heterozygous) molecules may behave like native DNA molecules carrying the dominant markers *A* and *B* on both strands and give rise to linked transformants. It is possible that some discontinuity in the secondary structure between *A* and *b* or *a* and *B* (or on both strands) is a necessary prerequisite to display linkage.

(*c*) If only one of the two strands is potentially capable of giving rise to cells with a transformed trait, then the hybrid DNA molecule will give rise to clones containing only one of the two markers (*A or B*).

The results thus far obtained on the transforming activity of heterozygous DNA molecules are meager. Herriott (1961a,b) has shown that the hybrids give rise to double transformants (case *b* above) in *H. influenzae,* albeit about ten times lower than expected on the basis of their product resulting from a bimolecular reaction in the formation of the heterozygous DNA molecules. He finds that the yield of double transformants by the heterologous DNA depends on the genetic map distance between the linkage groups: the shorter the distance, the smaller the yield of double transformants.

Different results (Marmur, Lane, and Doty, unpublished results) have been obtained with the linked markers of *D. pneumoniae.* By heating and annealing DNA carrying the erythromycin resistance marker ery-r2 with DNA carrying the marker ery-r3 [linked when present in the same cell (Ravin and Iyer, 1962)], the hybrid molecules give rise to transformed clones carrying either the ery-r2 or ery-r3 markers, but not both (case *c* above). The heterozygous DNA molecules did not produce double transformants as in the case with *Hemophilus.* The results obtained with *D. pneumoniae* are consistent with the idea that only one strand of the DNA double helix is active in the final expression of the marker in the transformed cell. Supporting this is the finding that when

genetically marked *D. pneumoniae* DNA carrying the marker for streptomycin resistance is denatured and annealed with a large excess of unmarked, wild-type DNA, the number of transformants per unit weight of marked DNA annealed is no greater than when an equivalent concentration of marked DNA goes through the same thermal process alone. A similar result has recently been reported by Suzuki *et al.* (1962). Were each strand capable of phenotypic expression with 100% efficiency, one might expect that each marked DNA molecule would give rise to two hybrid molecules and thus increase the yield of transformants by a factor of two. These results (Marmur, Lane, and Doty, unpublished) are contrary to those published earlier (Marmur and Lane, 1960) in which the effects of increasing the concentration by the unmarked DNA were erroneously neglected.

The differences between the results with *Hemophilus* and *D. pneumoniae* remain unexplained. DNA isolated from cells carrying both markers (ery-r2 and ery-r3) in the *D. pneumoniae* studies shows approximately the same degree of linkage as the two closest markers (streptomycin, *S*, and kanomycin, *K*) used in the *Hemophilus* work. As has already been pointed out, the yield of double transformants depends on the genetic (and thus probably the physical) distance between the markers in the heterozygous DNA molecules. The yield of transformants to *S* and *K* resistance in *Hemophilus* by annealed hybrid molecules is very low. If all the pairs of linked markers can form heterozygous molecules to the same extent, it would be expected that the closer the linkage of the markers, the *greater* might be the probability of transformation by heterozygous DNA to yield linked transformants. However, the relative frequency of transformants by annealed heterozygous DNA is the inverse of that obtained with the unheated native DNA extracted from cells carrying both markers (D. Luzzati *et al.*, 1961). It is possible that the active heterozygous molecules that eventually result in double transformants arise from one single unbroken strand pairing with two segments (each carrying one of the dominant alleles) of the complementary strand that has suffered a scission between the two markers in question: e.g.,

$$\frac{A \;\big|\; \big\lfloor B}{A \quad b} \qquad \text{or} \qquad \frac{A \;\big|\; \big\lfloor B}{a \quad B}$$

These structures, with a discontinuity in the primary structure of one strand, might be formed before (by renaturation) or after entry into the cell has occurred. Such a mechanism would predict that the shorter the distance between *A* and *B*, the less likely would be the possibility of obtaining heterozygous DNA molecules active in double transformation.

This is the case in *Hemophilus*. In the case of *D. pneumoniae* the yield of active heterozygous molecules might have been too low to be detected by the number of transformants assayed.

C. DNA-RNA Hybrids

It has been demonstrated that hybrid formation by strand separation and recombination provides a means to test sequence homologies in bacterial and bacteriophage DNA samples. This technique also provides an experimental approach for examining one of the characteristic features expected of "messenger RNA," the RNA that carries the genetic information for coding amino acid sequences in protein molecules. The DNA-DNA hybrids are formed only if the interacting samples are isolated from closely related species (Schildkraut *et al.*, 1961a). The conditions (e.g., low DNA concentrations, optimum temperature, etc.) for the formation of these hybrids have been carefully selected to reduce nonspecific aggregation. In the case of the formation of the mixed DNA-RNA hybrids, this has not always been taken into account. In several instances, relatively high concentrations of DNA have been employed. Aggregation of the DNA during renaturation might readily occlude RNA and could be taken for the existence of base sequence homologies. Where homologies might exist, the extent of the related sequences is unknown. The application of enzymes that would specifically digest the unpaired regions would be extremely useful in selecting the regions which have paired specifically.

The properties of messenger RNA have been discussed by Brenner *et al.* (1961) and Spiegelman (1962). If information is transferred from DNA to messenger RNA by complementary base pairing, then it would be expected that an RNA-DNA complex could be formed by heating and annealing a mixture of DNA and messenger RNA from the same organism. RNA has a buoyant density of about 2 gm/ml, while that of DNA is about 1.7 gm/ml. A double-stranded DNA-RNA hybrid molecule (1:1) probably has the average density of 1.85 gm/ml, but it might band at any intermediate density, depending on the ratio of DNA/RNA in the hybrid.

To examine the properties of such a hybrid, Schildkraut *et al.* (1961b) prepared a hybrid consisting of poly dG and poly rC. They did this by heating and quickly cooling a mixture of poly (rC) and (dG):(dC). The hybrid banded in CsCl at a density intermediate between that of poly (rC) and (dG):(dC). Now that the density of poly dG in alkaline Cs_2SO_4 and that expected in neutral CsCl have been calculated (Inman and Baldwin, personal communication), it is apparent that the density of the hybrid was approximately the average of that of poly (rC) and poly (dG). The poly (dC), which did not appear in the photographs of

Schildkraut *et al.* (1961b), has been shown by Inman and Baldwin (personal communication) to have a comparatively low density in Cs_2SO_4, and would not be expected to band in the high-density CsCl solution used in the studies of the former authors. The (dG): (rC) complex had a T_m that was 18°C higher than that of (dG): (dC). In addition, the complex was resistant to both pancreatic ribonuclease and deoxyribonuclease for a period of up to 24 hours. A similar hybrid, between dT oligonucleotides and poly rA, was obtained by Rich (1960), who studied its formation by ultraviolet absorbance measurements.

Schildkraut *et al.* (1961b) have also heated and annealed a mixture of DNA from *E. coli* and ribosomal RNA from various sources. *E. coli* RNA produced a skewing of the DNA band in the direction of higher density. RNA from *Pseudomonas aeruginosa, D. pneumoniae,* and *Mycobacterium phlei* did not produce this effect. More recent studies from other laboratories have shown that modification of the band profile was probably produced by messenger RNA present in the ribosomal RNA fraction or by the microsomal RNA itself (Yankofsky and Spiegelman, 1962).

Hall and Spiegelman (1961) and Spiegelman (1962) have shown that the specific RNA produced after T2 infection forms a complex with denatured T2 DNA when the two are annealed. The complex bands in CsCl at the same position as denatured T2 DNA. When the T2-specific RNA is annealed with DNA from *Ps. aeruginosa* or T5 bacteriophage, no complex is formed. The authors estimated that the ratio of DNA to RNA in the complex was 5 to 1.

Hayashi and Spiegelman (1961) and Spiegelman (1962) have formed what appears to be an RNA-DNA hybrid, using messenger RNA from uninfected cells. They transferred cells growing in a rich medium to a synthetic medium and showed that this led to a temporary cessation of ribosomal RNA synthesis. RNA produced after such a "step down" transfer is enriched with messenger RNA, identified by base ratio determinations. They then annealed total RNA isolated by the phenol method with homologous denatured DNA and found the labeled RNA in the high-density region of the DNA band. Nonhomologous DNA did not interact with the RNA. These observations are similar to those made by Schildkraut *et al.* (1961b). Spiegelman (1961) discusses evidence supporting the claim that the skewing of the DNA is caused by messenger RNA.

The first example of an RNA-DNA complex that bands at a density considerably higher than that of the DNA was presented by Geiduschek *et al.* (1961). Weiss and Nakamoto (1961) studied some of the properties of RNA synthesized by the RNA polymerase that uses DNA as primer and were able to show that the average base composition and the

average nearest-neighbor frequency of the enzymatically synthesized RNA and the DNA primer are complementary. Geiduschek *et al.*, (1961) showed that, on heating and annealing, a complex is formed between the synthesized RNA and the primer DNA, the density of this RNA-DNA complex depending on the ratio of complementary RNA to DNA in the annealing mixture. When the ratio of moles of RNA-P to DNA-P was 1:5, the complex was only slightly heavier than DNA. When approximately equal amounts of complementary RNA and DNA were annealed, the density of the complex was greatly increased. Since all of the DNA employed reacted with the newly synthesized RNA to form a DNA-RNA hybrid, the conclusion was drawn that both the DNA strands could act as templates for RNA synthesis *in vitro*.

Many of the RNA-DNA complexes studied thus far are resistant to low levels of ribonuclease (see, for example, Gros *et al.*, 1961a). The (dG):(dC) complex of Schildkraut *et al.* (1961b) is resistant to both ribonuclease and deoxyribonuclease. Another distinguishing feature of this complex formed with homopolymers is that its T_m was much higher than that of (dG):(dC). Schulman and Bonner (1962), however, reported a naturally occurring RNA-DNA complex isolated from *Neurospora* whose properties differ from those just discussed. The undegraded complex is susceptible to ribonuclease and its T_m is lower than that of the DNA component. The authors suggest this complex may consist of a double strand of DNA with an RNA strand wrapped around it. Yankofsky and Spiegelman (1962) used the resistance of the DNA-RNA hybrids to ribonuclease to enrich and isolate the complex formed on annealing of DNA and ribosomal RNA. Nonspecific interactions between the two nucleic acids were digested readily by RNase and DNase. Similar studies have been carried out by Giacomoni and Spiegelman (1962) on the annealing of DNA with soluble RNA.

DNA-RNA hybrids have been detected *in vivo* in higher plants and animals (Venkataraman and Coe, 1961; Bonner *et al.*, 1961; Rho and Bonner, 1961); however, their structure has not been fully investigated. The use of the specific interaction between the two nucleic acids (Bautz and Hall, 1962) for the isolation of specific messenger RNA is outlined below.

VII. Applications of Renaturation

A. Genetic and Taxonomic Relationships

It has recently been pointed out that there is a correlation between the genetic as well as taxonomic relatedness of microorganisms and the average base compositions of their DNA (Lee *et al.*, 1956; Belozersky and Spirin, 1960; Lanni, 1960; Sueoka, 1961; Marmur *et al.*, 1961c,

1962). A more demanding criterion of the relatedness of microorganisms would be that their DNA bases have sequence homologies; a minimum requirement for an extensive homology between two DNA samples is that their base compositions be similar. Since DNA hybrid formation by denaturation and annealing requires extensive base sequence homologies (Schildkraut *et al.*, 1961a), it can be used as a tool in studying, *in vitro*, the relationship between the organisms from which the DNA is isolated. Such a study was undertaken (Marmur *et al.*, 1961c) and it was shown that any two microorganisms that can exchange genetic markers by transformation or transduction possess DNA with similar base compositions and can form hybrids on denaturation and annealing. The same is true for the T-even *E. coli* bacteriophages which have been shown to be genetically related. However, when genetic exchanges can take place between organisms with different average base compositions (*Serratia marcescens* and *E. coli*) by F-duction (Falkow *et al.*, 1961) or by conjugation (Zinder, 1960; Baron *et al.*, 1960) only (*S. typhimurium* $\times$ *E. coli*), then the DNA isolated from the parental strains do not form extensive *in vitro* hybrids.

Since there is a close relationship between *in vitro* molecular hybrid formation, taxonomy, and genetic compatibility of microorganisms, we would expect organisms yielding DNA that can interact to form hybrid molecules to belong to the same taxonomic group and very likely to be genetically related. The most demanding test in the classification of higher organisms into species is that they be genetically related. Thus, when there is a difficulty in finding the proper conditions for routes of genetic transfer, the ability of the DNA from the parental organisms to form hybrids *in vitro* could be substituted as the genetic test.

It should be pointed out that since unfractionated samples of DNA of higher plants and animals do not renature under conditions where microbial DNA does, the principles discussed above are applicable only to organisms having relatively homogeneous populations of DNA. It is possible, however, that if the DNA of higher species were first fractionated, then the more homogeneous DNA could be used in *in vitro* molecular hybridization experiments with corresponding fractions of the DNA of a closely related species. The possibility also exists in studying the homology of the DNA of such tumor-inducing viruses as polyoma and Shope papilloma with fractionated DNA from the host cells.

B. Fractionation, Isolation, and Identification of Specific Nucleic Acids

The homologous base sequence relationship in nucleic acids has been extended to that existing between DNA and "messenger" RNA isolated

in vivo, naturally occurring DNA-RNA hybrids, and the RNA synthesized by the RNA polymerase using DNA as the primer. By heating and annealing the nucleic acids (and if nonspecific aggregation is eliminated), the RNA with a complementary base sequence to that of DNA can be recognized by examining the mixed hybrids formed. Thus, Hall and Spiegelman (1961) recognized the presence of *in vivo* synthesized messenger RNA as well as naturally occurring DNA-RNA hybrids. Geiduschek *et al.* (1961) showed that the RNA synthesized *in vitro* by the RNA polymerase isolated from *M. lysodeikticus* contained molecular species that were complementary to either of the DNA strands used as the primer. It would seem possible that, by heating and annealing a mixture of DNA and RNA and adjusting conditions to favor the interaction of the two different nucleic acids rather than each type by itself, one could concentrate and isolate only those molecules of RNA and DNA exhibiting base sequence complementarity.

Such an approach was utilized by Bautz and Hall (1962). Denatured T4 bacteriophage was first coupled (through the glucose residues) in order to immobilize it to form a column. T4 messenger RNA isolated from *E. coli* after bacteriophage infection was then passed through the column and the complementary messenger RNA that specifically combined with, and was retained by, the denatured DNA could then be eluted by adjusting the ionic strength and/or raising the temperature. By using a column containing denatured DNA obtained from deletion mutants, it was possible to isolate from the total messenger RNA primed by wild-type bacteriophage that complementary RNA corresponding to the deletion. A modification of this technique which can employ any high molecular weight, denatured DNA in the column, has been introduced by Bolton and McCarthy (1962). It was found that when the DNA was embedded in agar, a column could be prepared that would specifically adsorb messenger RNA and that the latter could be specifically eluted under proper conditions of temperature and ionic strength. Yankofsky and Spiegelman (1962) have made use of the homology requirements for the annealing of the two nucleic acids to demonstrate that *E. coli* ribosomal RNA can form specific hybrids with 0.1–0.2% of the host genome. They made use of the observation that specific hybrids, but not aggregates, are relatively insensitive to RNase and DNase.

C. Reactions of Denatured DNA

Since denaturation followed by annealing allows the DNA to be treated first in the denatured state, conditions are provided for reactions that proceed more readily with denatured DNA. It has been found recently (Horn and Herriott, 1962) that denatured *H. influenzae* DNA

treated with nitrous acid and then renatured will result in a high yield of mutants, whereas native DNA so treated yields few or none. Such experiments, employing partially denatured and denatured DNA have also been performed with the *B. subtilis*–transforming system (Freese and Strack, 1962) using nitrous acid and hydroxylamine.

Denaturation and renaturation have also been used to show that certain enzymatic reactions proceed more readily with either the native or denatured state of DNA. Thus, Zimmerman *et al.* (1962) found that the enzymatic glucosidation of T-even bacteriophage DNA was extremely slow and limited in extent when denatured DNA was the substrate. Renatured DNA behaved in a manner similar to that of native DNA.

VIII. Summary

The data that has been accumulated to date on the structure of native DNA is consistent with the double stranded model proposed by Watson and Crick. This does not imply that subtle differences do not exist in the secondary and tertiary structure of DNA preparations isolated from widely varying sources. A very pressing problem is the molecular state of the DNA molecule *in vivo*. An insight may come about as a result of two important recent developments in studying the properties of DNA. These have been (1) the isolation of high molecular weight material from bacteriophages and bacteria by methods that avoid shear degradation and (2) the introduction of new techniques such as CsCl density gradient centrifugation and refinements of radioautography as well as electron microscopy. The latter two methods have proven to be extremely promising for ascertaining the molecular weight of large DNA molecules whose dimensions could not be accurately ascertained by previous physicochemical means. The immediate consequence of the isolation and characterization of large DNA molecules is to make it possible to study the relationship between DNA structure and genetic linkage maps. By assaying the distribution of genetic units (employing transformation by bacterial or bacteriophage DNA) along the entire or sheared fragments of the DNA molecule, it is now possible to decide whether there is a colinearity between genetic sequences and DNA structure (Kaiser, 1962). It has also made it possible to calculate the length per unit mass of the entire DNA complement of several bacteriophages. Not only has this data been consistent with the two-stranded model but it has been possible to conclude (Cairns, 1962b) that the strands of coliphage λ DNA separate during replication. This is consistent with the previous physicochemical evidence that the semiconserved DNA subunits (which can dissociate under denaturing conditions)

are the single strands. The DNA strands can separate upon denaturation. The double-stranded synthetic polynucleotides readily do so, as do the DNA molecules isolated from bacteriophage alpha (Cordes *et al.*, 1962) as well as other DNA samples. Following through with this reasoning, one can accept the phenomenon of renaturation, which occurs under appropriate conditions, from the collapsed or single-stranded states of DNA.

The main interest in the denaturation and renaturation of isolated, purified nucleic acids is in their applications. Renaturation of denatured DNA, isolated from closely related organisms, can be used to investigate their taxonomic and evolutionary relationships. Renaturation can also be employed to prepare hybrid molecules between closely related strains or species that differ in a few or many genetic markers and then used in conjunction with transformation experiments to ascertain the genetic and phenotypic potential of each of the strands of the renatured hybrid.

Thus, renaturation cannot only be used to detect base sequence homologies, but also makes it possible, by adding a nucleic acid "reactant," to isolate from a population of molecules those showing complementarity to it. This has made it feasible to prepare columns, as did Bautz and Hall (1962), consisting of an immobilized nucleic acid species that can fractionate structures with complementary sequences. Using the technique of preparative density gradient centrifugation, it should also be possible, for instance, to recover the DNA of a lysogenic bacteriophage from its bacterial host after heating and annealing with isotopically labeled DNA from independently isolated homologous bacteriophages.

The renaturation of DNA and RNA makes it possible to examine how the two nucleic acids are related structurally as well as metabolically. For instance, it is not only potentially possible to answer the question as to which DNA strand codes for the various RNA species synthesized on a DNA template (Bautz and Hall, 1962), but also, once an RNA molecule forms hybrids by annealing with DNA, to isolate (by preparative CsCl density gradient centrifugation, for example) those DNA sequences that are homologous to the RNA. The chemical and enzymatic methods that are thus far available for studying the sequences of the bases in DNA and RNA are in the very early stages of development, but it would be of great interest to see whether the short sequences that can be assayed show a complementary relationship between the two nucleic acids of the DNA-RNA hybrid region.

The denaturation and renaturation of nucleic acids might provide some clues as to the behavior of these macromolecules *in vivo*. The replication of DNA, the mechanism of recombination, the interactions

of DNA and RNA, as well as the interplay of the various RNA species during protein synthesis, all might conceivably involve base pair interactions that mimic in many respects those observed *in vitro* (see, for example, the statistical thermodynamic treatment of denaturation and replication by Vol'kenshtein and Yel'yashevich, 1961). A clearer understanding of denaturation and renaturation and the factors influencing these processes would contribute significantly to a rational approach in designing experiments for gaining an insight into the interactions of nucleic acids during the growth of cells and viruses.

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References

Aldridge, W. G. (1962). *Nature* **195**, 284.
Alexander, P., and Stacey, K. A. (1955a). *Biochem. J.* **60**, 194.
Alexander, P., and Stacey, K. A. (1955b). *Nature* **176**, 162.
Anderson, E. S., Armstrong, J. A., and Niven, J. S. F. (1959). *Symposium Soc. Gen. Microbiol.* **9**, 224.
Applequist, J. (1961). *Arch. Biochem. Biophys.* **95**, 42.
Barnhart, J., and Herriott, R. M. (1962). *Federation Proc.* **21**, 374.
Baron, L. S., Spilman, W. M., and Carey, W. F. (1960). *J. Exptl. Med.* **112**, 361.
Bautz, E. K. F., and Hall, B. D. (1962). *Proc. Natl. Acad. Sci. U.S.* **48**, 400.
Beer, M. (1961). *J. Mol. Biol.* **3**, 263.
Beer, M., and Thomas, C. A. (1961). *J. Mol. Biol.* **3**, 699.
Beer, M., and Zobel, R. (1961). *J. Mol. Biol.* **3**, 717.
Belman, S., Levine, E., and Troll, W. (1962). *Federation Proc.* **21**, 374.
Belozersky, A. N., and Spirin, A. S. (1960). *In* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. III, pp. 147–185. Academic Press, New York.
Bendet, I., Schachter, E., and Lauffer, M. A. (1962). *J. Mol. Biol.* **5**, 76.
Bendich, A., Pahl, H. B., and Beiser, S. M. (1956). *Cold Spring Harbor Symposia Quant. Biol.* **21**, 31.
Berns, K. I., and Thomas, C. A. (1961). *J. Mol. Biol.* **3**, 289.
Boedtker, H. (1959). *Biochim. et Biophys. Acta* **32**, 519.
Boedtker, H. (1960). *J. Mol. Biol.* **2**, 171.
Bolton, A. C., and Weiss, J. J. (1962). *Nature* **195**, 666.
Bolton, E. T., and McCarthy, B. J. (1962). *Proc. Natl. Acad. Sci. U.S.* **48**, 1390.
Bonner, J., Huang, R. C., and Maheshwari, N. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 1548.
Borenfreund, E., Fitt, E., and Bendich, A. (1961). *Nature* **191**, 1375.
Brenner, S., Jacob, F., and Meselson, M. (1961). *Nature* **190**, 576.

Bresler, S. E. (1958). *Discussions Faraday Soc.* No. **25**, 209.
Bunville, L. G., and Geiduschek, E. P. (1960). *Biochem. Biophys. Research Communs.* **2**, 287.
Burgi, E., and Hershey, A. D. (1961). *J. Mol. Biol.* **3**, 458.
Burton, K. (1960). *Biochem. J.* **77**, 547.
Butler, J. A. V., Laurence, D. J. R., Robins, A. B., and Shooter, K. V. (1959a). *Trans. Faraday Soc.* **55**, 489.
Butler, J. A. V., Laurence, D. J. R., Robins, A. B., and Shooter, K. V. (1959b). *Proc. Roy. Soc.* **A250**, 1.
Cairns, J. (1961). *J. Mol. Biol.* **3**, 756.
Cairns, J. (1962a). *J. Mol. Biol.* **5**, 407.
Cairns, J. (1962b). *Nature* **194**, 1274.
Cavalieri, L. F., and Rosenberg, B. H. (1956). *Biochim. et Biophys. Acta* **21**, 202.
Cavalieri, L. F., and Rosenberg, B. H. (1957). *J. Am. Chem. Soc.* **79**, 5352.
Cavalieri, L. F., and Rosenberg, B. H. (1961). *Biophys. J.* **1**, 317, 323, 337.
Cavalieri, L. F., and Rosenberg, B. H. (1962a). *Ann. Rev. Biochem.* **31**, 247.
Cavalieri, L. F., and Rosenberg, B. H. (1962b). *In* "The Molecular Basis of Neoplasia," pp. 44–58. Univ. of Texas Press, Austin, Texas.
Cavalieri, L. F., Rosenberg, B. H., and Rosoff, M. (1956a). *J. Am. Chem. Soc.* **78**, 5235.
Cavalieri, L. F., Rosoff, M., and Rosenberg, B. H. (1956b). *J. Am. Chem. Soc.* **78**, 5239.
Cavalieri, L. F., Deutsch, J. F., and Rosenberg, B. H. (1961). *Biophys. J.* **1**, 301.
Cavalieri, L. F., Small, T., and Sarkar, N. (1962). *Biophys. J.* **2**, 339.
Chance, B. (1953). *In* "Investigations of Rates and Mechanisms of Reactions" (S. L. Friess and A. Weissburger, eds.), pp. 690–710. Interscience, New York.
Chun, E. H. L., and Littlefield, J. W. (1961). *J. Mol. Biol.* **3**, 668.
Conway, B. E. (1956). *J. Polymer Sci.* **20**, 299.
Cordes, S., Epstein, H. T., and Marmur, J. (1961). *Nature* **191**, 1097.
Cordes, S., Epstein, H. T., and Marmur, J. (1962). *Federation Proc.* **21**, 375.
Cox, R. A., and Peacocke, A. R. (1957a). *J. Polymer Sci.* **23**, 765.
Cox, R. A., and Peacocke, A. R. (1957b). *J. Chem. Soc.*, p. 4724.
Cox, R. A., and Peacocke, A. R. (1958a). *J. Chem. Soc.*, p. 4117.
Cox, R. A., and Peacocke, A. R. (1958b). *Discussions Faraday Soc.* No. **25**, 211, 213.
Daems, W. T., Eigner, J., Sluys, I., and Cohen, J. A. (1962). *Biochim. et Biophys. Acta* **55**, 801.
Davison, P. F. (1959). *Proc. Natl. Acad. Sci. U.S.* **45**, 1560.
Davison, P. F., Freifelder, D., Hede, R., and Levinthal, C. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 1123.
Delbruck, M., and Stent, G. (1957). *In* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), pp. 699–736. Johns Hopkins Press, Baltimore, Maryland.
DeVoe, H., and Tinoco, I., Jr. (1962). *J. Mol. Biol.* **4**, 500.
Djordjevic, B., and Szybalski, W. (1960). *J. Exptl. Med.* **112**, 509.
Dolbeare, F., and Grossman, L. (1962). *Federation Proc.* **21**, 373.
Doskočil, J., and Šorm, F. (1961). *Biochim. et Biophys. Acta* **48**, 211.
Doty, P. (1955). *Biochim. et Biophys. Acta* **16**, 446.
Doty, P. (1956). *Proc. 3rd Intern. Congr. Biochem., Brussels*, p. 135.
Doty, P. (1957). *J. Cellular Comp. Physiol.* **49**, *Suppl.* 1, 27.
Doty, P. (1959–1960). *Harvey Lectures* **55**, 103.

Doty, P., and Bunce, B. H. (1952). *J. Am. Chem. Soc.* **74**, 5029.
Doty, P., and Rice, S. A. (1955). *Biochim. et Biophys. Acta* **16**, 446.
Doty, P., McGill, B. B., and Rice, S. A. (1958). *Proc. Natl. Acad. Sci. US.* **44**, 432.
Doty, P., Boedtker, H., Fresco, J. R., Haselkorn, R., and Litt, M. (1959a). *Proc. Natl. Acad. Sci. US.* **45**, 482.
Doty, P., Marmur, J., and Sueoka, N. (1959b). *Brookhaven Symposia in Biol.* **No. 12**, 1.
Doty, P., Marmur, J., Eigner, J., and Schildkraut, C. (1960). *Proc. Natl. Acad. Sci. US.* **46**, 461.
Dove, W. F., and Davidson, N. (1962a). *J. Mol. Biol.* **5**, 467.
Dove, W. F., and Davidson, N. (1962b). *J. Mol. Biol.* **5**, 479.
Drysdale, R. B., and Peacocke, A. R. (1961). *Biol. Revs.* **36**, 537.
Duggan, E. L. (1961). *Biochem. Biophys. Research Communs.* **6**, 93.
Ehrlich, P., and Doty, P. (1958). *J. Am. Chem. Soc.* **80**, 4251.
Eichhorn, G. L. (1962). *Nature* **194**, 474.
Eigner, J. (1960). Ph.D. Dissertation, Harvard University, Cambridge, Massachusetts.
Eigner, J., and Doty, P. (1963). In preparation.
Eigner, J., Boedtker, H., and Michaels, G. (1961). *Biochim. et Biophys. Acta* **51**, 165.
Eigner, J., Schildkraut, C., and Doty, P. (1962). *Biochim. et Biophys. Acta* **55**, 13.
Emanuel, C. F. (1960). *Biochim. et Biophys. Acta* **42**, 91.
Falkow, S., Marmur, J., Carey, W. F., Spilman, A. S., and Baron, L. S. (1961). *Genetics* **46**, 703.
Falkow, S., Rownd, R., and Baron, L. S. (1962a). *J. Bacteriol.* **84**, 1303.
Falkow, S., Ryman, I. R., and Washington, O. (1962b). *J. Bacteriol.* **83**, 1318.
Felsenfeld, G. (1962). *In* "The Molecular Basis of Neoplasia," pp. 104–119. Univ. of Texas Press, Austin.
Felsenfeld, G., and von Hippel, P. (1962). *Abstr. Biophys. Soc.* **FA7**; *J. Mol. Biol.* **5**, 587.
Felsenfeld, G., and Rich, A. (1957). *Biochim. et Biophys. Acta* **26**, 457.
Fiers, W., and Sinsheimer, R. L. (1962). *J. Mol. Biol.* **5**, 408, 420, 424.
Flory, P. J. (1953). "Principles of Polymer Chemistry." Cornell Univ. Press, Ithaca, New York.
Flory, P. J. (1961). *J. Polymer Sci.* **49**, 105.
Fox, M. S. (1962). *Proc. Natl. Acad. Sci.* **48**, 1048.
Freese, E., and Strack, H. B. (1962). *Proc. Natl. Acad. Sci. US.* **48**, 1796.
Freifelder, D., and Davison, P. F. (1962). *Biophys. J.* **2**, 249.
Freifelder, D., and Davison, P. F. (1963). *Biophys. J.* **3**, 49.
Freifelder, D., Davison, P. F., and Geiduschek, E. P. (1961). *Biophys. J.* **1**, 389.
Fresco, J. R. (1961). *Tetrahedron* **13**, 185.
Fresco, J. R., and Alberts, B. M. (1960). *Proc. Natl. Acad. Sci. US.* **46**, 311.
Fresco, J. R., and Doty, P. (1957). *J. Am. Chem. Soc.* **79**, 3928.
Fresco, J. R., and Klemperer, E. (1959). *Ann. N.Y. Acad. Sci.* **81**, 730.
Fresco, J. R., and Straus, D. B. (1962). *Am. Scientist* **50**, 158.
Fresco, J. R., Alberts, B. M., and Doty, P. (1960). *Nature* **188**, 98.
Fresco, J. R., Lesk, A. M., Gorn, R., and Doty, P. (1961). *J. Am. Chem. Soc.* **83**, 3155.
Geiduschek, E. P. (1958). *J. Polymer Sci.* **31**, 67.
Geiduschek, E. P. (1961). *Proc. Natl. Acad. Sci. US.* **47**, 950.
Geiduschek, E. P. (1962). *J. Mol. Biol.* **4**, 467.
Geiduschek, E. P., and Herskovits, T. T. (1961). *Arch. Biochem. Biophys.* **65**, 99.

Geiduschek, E. P., and Holtzer, A. (1958). *Advances in Biol. and Med. Phys.* **6**, 432.
Geiduschek, E. P., Nakamoto, T., and Weiss, S. B. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 1405.
Geiduschek, E. P., Moohr, J. W., and Weiss, S. B. (1962). *Proc. Natl. Acad. Sci.* **48**, 1078.
Giacomoni, D., and Spiegelman, S. (1962). *Science* **138**, 1328.
Gibbs, J. A., and DiMarzio, E. A. (1959). *J. Chem. Phys.* **30**, 271.
Ginoza, W., and Zimm, B. H. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 639.
Gordon, J. A., and Jencks, W. P. (1963). *Biochemistry* **2**, 47.
Greer, S., and Zamenhof, S. (1962). *J. Mol. Biol.* **4**, 123.
Gros, F., Gilbert, W., Hiatt, H., Attardi, G., Spahr, P. F., and Watson, J. D. (1961a). *Cold Spring Harbor Symposia Quant. Biol.* **26**, 111.
Gros, F., Gilbert, W., Hiatt, H., Kurland, C., Risebrough, R. W., and Watson, J. D. (1961b). *Nature* **190**, 581.
Grossman, L., Levine, S. S., and Allison, W. S. (1961). *J. Mol. Biol.* **3**, 47.
Guda, H. E., Frajola, J., and Lessler, M. A. (1962). *Science* **137**, 607.
Guild, W. R. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 1560.
Guild, W. R. (1962). *Abstr. Biophys. Soc.* WA12.
Gulland, J. M., Jordan, D. O., and Taylor, H. F. W. (1947). *J. Chem. Soc.*, p. 1131.
Hall, B. D., and Doty, P (1958). *In* "Microsomal Particles and Protein Synthesis" (R. Roberts, ed.), pp. 27–35. Pergamon, New York.
Hall, B. D., and Doty, P. (1959). *J. Mol. Biol.* **1**, 111.
Hall, B. D., and Spiegelman, S. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 137.
Hall, C. E., and Cavalieri, L. F. (1961). *J. Biophys. Biochem. Cytol.* **10**, 347.
Hall, C. E., and Litt, M. (1958a). *J. Am. Chem. Soc.* **42**, 801.
Hall, C. E., and Litt, M. (1958b). *J. Biophys. Biochem. Cytol.* **4**, 1.
Hamaguchi, K., and Geiduschek, E. P. (1962). *J. Am. Chem. Soc.* **84**, 1329.
Hamilton, L. D., Barclay, R. K., Wilkins, M. H. F., Brown, G. L., Wilson, H. R., Marvin, D. A., Ephrussi-Taylor, H., and Simmons, N. S. (1959). *J. Biophys. Biochem. Cytol.* **5**, 397.
Haselkorn, R. (1959). Ph.D. Dissertation. Harvard University, Cambridge, Massachusetts.
Haselkorn, R., and Doty, P. (1961). *J. Biol. Chem.* **236**, 2738.
Hayashi, M., and Spiegelman, S. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 1564.
Healy, W. J., Stollar, D., and Levine, L. (1963). In preparation.
Hearst, J. E., and Stockmayer, W. H. (1962). *J. Chem. Phys.* **37**, 1425.
Hearst, J. E., and Vinograd, J. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 1005.
Helmkamp, G. K., and Ts'o, P. O. P. (1961). *J. Am. Chem. Soc.* **83**, 138.
Helmkamp, G. K., and Ts'o, P. O. P. (1962). *Biochim. et Biophys. Acta* **55**, 601.
Herriott, R. M. (1961a). *Proc. Natl. Acad. Sci. U.S.* **47**, 146.
Herriott, R. M. (1961b). *J. chim. phys.* **58**, 1101.
Herskovits, T. T., Singer, S. J., and Geiduschek, E. P. (1961). *Arch. Biochem. Biophys.* **94**, 99.
Horn, E. E., and Herriott, R. M. (1962). *Proc. Natl. Acad. Sci. U.S.* **48**, 1409.
Inman, R. B., and Baldwin, R. (1962). *J. Mol. Biol.* **5**, 172, 185.
Inman, R. B., and Jordan, D. O. (1960). *Biochim. et Biophys. Acta* **37**, 162.
Jacob, F., and Wollman, E. (1958). *Symposia Soc. Exptl. Biol.* **12**, 75.
Jordan, D. O. (1960). "The Chemistry of Nucleic Acids." Butterworths, London.
Josse, J., Kaiser, A. D., and Kornberg, A. (1961). *J. Biol. Chem.* **236**, 864.

Kaiser, A. D. (1962). *J. Mol. Biol.* **4**, 275.
Kaiser, A. D., and Hogness, D. S. (1960). *J. Mol. Biol.* **2**, 392.
Kasha, M., El-Bayoumi, M. A., and Rhodes, W. (1961). *J. chim. phys.* **58**, 916.
Kirby, K. S. (1957). *Biochem. J.* **66**, 495.
Kit, S. (1960). *Biochem. Biophys. Research Communs.* **3**, 377.
Kit, S., and Hsu, T. C. (1961). *Biochem. Biophys. Research Communs.* **5**, 120.
Kleinschmidt, A. K. (1962). *Intern. Congr. Microbiol., 8th Congr.* p. 38.
Kleinschmidt, A., Lang, D., Plescher, C., Hellman, W., Haas, J., Zahn, R. K., and Hagedorn, A. (1961). *Z. Naturforschung* **16**, 730.
Kozinski, A. W., and Beer, M. (1962) *Biophys. J.* **2**, 129.
Kyogoku, Y., Tsuboi, M., Shimanouchi, T., and Watanabe, I. (1961). *Nature* **189**, 120.
Lacks, S., and Hotchkiss, R. D. (1960). *Biochim. et Biophys. Acta* **39**, 508.
Lanni, F. (1960). *Perspectives in Biol. Med.* **3**, 418.
Lanyi, J. (1963). Ph.D. Dissertation, Harvard University, Cambridge, Massachusetts.
Lee, K. Y., Wahl, R., and Barbu, R. (1956). *Ann. inst. Pasteur* **91**, 212.
Lehman, I. R. (1960). *J. Biol. Chem.* **235**, 1479.
Lerman, L. S. (1961). *J. Mol. Biol.* **3**, 18.
Lerman, L. S., and Tolmach, L. J. (1959). *Biochim. et Biophys. Acta* **33**, 371.
Levedahl, B. H. (1959). *Proc. Natl. Biophys. Conf., 1st Conf., Columbus, Ohio, 1957*, pp. 210–216.
Levine, L., Murakami, W. T., Van Vunakis, H., and Grossman, L. (1960). *Proc. Natl. Acad. Sci. US.* **46**, 1038.
Levine, L., Gordon, J. A., and Jencks, W. P. (1963). *Biochemistry* **2**, 168.
Levinthal, C. (1960). Genetics Trans. Conf. Josiah Macy, Jr. Foundation 1st Conf., p. 229.
Levinthal, C., and Crane, H. R. (1956). *Proc. Natl. Acad. Sci. US.* **42**, 436.
Luzzati, D., Schweitz, H., Bach, M.-L., and Chevallier, M.-R. (1961). *J. chim. phys.* **58**, 1021.
Luzzati, V., Luzzati, D., and Masson, F. (1962). *J. Mol. Biol.* **5**, 375.
Luzzati, V., Nicolaieff, A., and Masson, F. (1961). *J. Mol. Biol.* **3**, 185.
Mahler, H. R., and da Silva Pereira, A. (1962). *J. Mol. Biol.* **5**, 325.
Mahler, H. R., and Mehrotra, B. D. (1962). *Biochim. et Biophys. Acta* **55**, 252.
Mahler, H. R., Mehrotra, B. D., and Sharp, C. W. (1961). *Biochem. Biophys. Research Comuns.* **4**, 79.
Mandel, M. (1962a). *J. Mol. Biol.* **5**, 435.
Mandel, M. (1962b). *Bacteriol. Proc. (Soc. Am. Bacteriologists)* **62**, 51.
Mandell, J. D., and Hershey, A. D. (1960). *Anal. Biochem.* **1**, 66.
Marmur, J., Anderson, W. F., Matthews, L., Berns, K., Gajewska, E., and Doty, P. (1961a). *J. Cellular Comp. Physiol.* **58**, *Suppl.* 1, 33.
Marmur, J., and Doty, P. (1959). *Nature* **183**, 1427.
Marmur, J., and Doty, P. (1961). *J. Mol. Biol.* **3**, 585.
Marmur, J., and Doty, P. (1962). *J. Mol. Biol.* **5**, 109.
Marmur, J., and Grossman, L. (1961). *Proc. Natl. Acad. Sci. US.* **47**, 778.
Marmur, J., and Lane, D. (1960). *Proc. Natl. Acad. Sci. US.* **46**, 453.
Marmur, J., and Ts'o, P. O. P. (1961). *Biochim. et Biophys. Acta* **51**, 32.
Marmur, J., Rownd, R., Falkow, S., Baron, L. S., Schildkraut, C. L., and Doty, P. (1961b). *Proc. Natl. Acad. Sci. US.* **47**, 972.
Marmur, J., Schildkraut, C. L., and Doty, P. (1961c). *J. chim. phys.* **58**, 945.

Marmur, J., Schildkraut, C. L., and Doty, P. (1962). *In* "The Molecular Basis of Neoplasia," pp. 9–43. Univ. of Texas Press, Austin, Texas.
Marmur, J., Seaman, E., and Levine, J. (1963). *J. Bacteriol.* (in press).
Matsubara, K., and Takagi, Y. (1962). *Biochim. et Biophys. Acta* **55**, 389.
May, P. (1962). *Compt. rend. acad. sci.* **254**, 4086.
Mayor, H. D., and Hill, N. O. (1961). *Virology* **14**, 264.
Meselson, M., and Stahl, F. (1958). *Proc. Natl. Acad. Sci. U.S.* **44**, 671.
Meselson, M., and Weigle, J. J. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 857.
Meselson, M., Stahl, F. W., and Vinograd, J. (1957). *Proc. Natl. Acad. Sci. U.S.* **43**, 581.
Michelson, A. M. (1962). *Biochim. et Biophys. Acta* **55**, 841.
Némethy, G., and Scheraga, H. A. (1962). *J. Chem. Phys.* **36**, 3382, 3401; *J. Phys. Chem.* **66**, 1773.
Opara-Kubinska, Z., and Szybalski, W. (1962). *Abstr. Biophys. Soc.* W48.
Paigen, K. (1962). *J. Theoret. Biol.* **3**, 268.
Paleček, E. (1961). *Biochim. et Biophys. Acta* **51**, 1.
Pauling, L., and Corey, R. B. (1956). *Arch. Biochem. Biophys.* **65**, 164.
Peacocke, A. R. (1960). *Progr. in Biophys. and Biophys. Chem.* **10**, 55.
Petersen, G. B. (1961). *Biochim. et Biophys. Acta* **51**, 212.
Pullman, B., and Pullman, A. (1959). *Biochim. et Biophys. Acta* **36**, 343.
Ralph, R. K., Connors, W. J., and Khorana, H. G. (1962). *J. Am. Chem. Soc.* **84**, 2265.
Ravin, A. W., and Iyer, V. N. (1962). *Genetics* **47**, 1355, 1369.
Reichmann, M. E., Bunce, B. H., and Doty, P. (1953). *J. Polymer Sci.* **10**, 109.
Rho, J. H., and Bonner, J. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 1611.
Rhodes, W. (1961). *J. Am. Chem. Soc.* **83**, 3609.
Rice, S. A., and Doty, P. (1957). *J. Am. Chem. Soc.* **79**, 3937.
Rich, A. (1958). *Biochim. et Biophys. Acta* **29**, 502.
Rich, A. (1959). *In* "Nucleoproteins: Proceedings of the 11th Solvay Conference on Chemistry, Brussels," pp. 273–298. Wiley (Interscience), New York.
Rich, A. (1960). *Proc. Natl. Acad. Sci. U.S.* **46**, 1044.
Rich, A., and Davies, D. (1956). *J. Am. Chem. Soc.* **78**, 3548.
Roger, M., and Hotchkiss, R. D. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 653.
Rolfe, R. (1962). *J. Mol. Biol.* **4**, 22.
Rolfe, R., and Ephrussi-Taylor, H. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 1450.
Rolfe, R., and Meselson, M. (1959). *Proc. Natl. Acad. Sci. U.S.* **45**, 1039.
Ross, P. D., and Sturtevant, J. M. (1960). *Proc. Natl. Acad. Sci. U.S.* **46**, 1360.
Rownd, R. (1963). Ph.D. Dissertation, Harvard University, Cambridge, Massachusetts.
Rownd, R., and Doty, P. (1963). (In preparation.)
Rownd, R., Lanyi, J., and Doty, P. (1961). *Biochim. et Biophys. Acta* **53**, 225.
Rownd, R., Falkow, S., and Baron, L. S. (1962). *Abstr. Biophys. Soc.* FB10.
Rownd, R., Falkow, S., and Baron, L. S. (1963a). (In preparation.)
Rownd, R., Green, D. M., and Doty, P. (1963b). *Abstr. Biophys. Soc. TB7.*
Rownd, R., Lanyi, J., and Doty, P. (1963c). (In preparation.)
Rubenstein, I., Thomas, C. A., and Hershey, A. D. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 1113.
Sadron, C. (1961). *J. chim. phys.* **58**, 877.
Saunders, M., and Ross, P. (1960). *Biochem. Biophys. Research Communs.* **3**, 314.
Scheraga, H. A., and Mandelkern, L. (1953). *J. Am. Chem. Soc.* **75**, 179.

Schildkraut, C. L., Marmur, J., and Doty, P. (1961a). *J. Mol. Biol.* **3**, 595.
Schildkraut, C. L., Marmur, J., Fresco, J., and Doty, P. (1961b). *J. Biol. Chem.* **236**, PC4.
Schildkraut, C. L., Marmur, J., and Doty, P. (1962a). *J. Mol. Biol.* **4**, 430.
Schildkraut, C. L., Wierzchowski, L., Marmur, J., Green, D. M., and Doty, P. (1962b). *Virology* **18**, 43.
Schulman, H. M., and Bonner, D. M. (1962). *Proc. Natl. Acad. Sci. US.* **48**, 53.
Shapiro, H. S., and Chargaff, E. (1960). *Biochim. et Biophys. Acta* **39**, 68.
Shooter, E. M., and Baldwin, R. L. (1962). *Federation Proc.* **21**, 374.
Shooter, K. V. (1957). *Progr. in Biophys. and Biophys. Chem.* **7**, 309.
Shooter, K. V., Pain, R. H., and Butler, J. A. V. (1956). *Biochim. et Biophys. Acta* **20**, 497.
Simha, R., and Zimmerman, J. M. (1962). *J. Theoret. Biol.* **2**, 87.
Simon, E. H. (1961). *J. Mol. Biol.* **3**, 101.
Simon, M. I., and Van Vunakis, H. (1962). *J. Mol. Biol.* **4**, 488.
Sinsheimer, R. L. (1959). *J. Mol. Biol.* **1**, 43.
Sinsheimer, R. L. (1962). *In* "The Molecular Control of Cellular Activity" (J. M. Allen, ed.), pp. 221–243. McGraw-Hill, New York.
Smith, K. C., Rebhun, S., and Kaplan, H. S. (1960). *Anal. Biochem.* **1**, 202.
Spencer, M., Fuller, W., Wilkins, M. H. F., and Brown, G. L. (1962). *Nature* **194**, 1014.
Spiegelman, S. (1961). *Cold Spring Harbor Symposia Quant. Biol.* **26**, 75.
Spiegelman, S. (1962). *In* "Acides ribonucléiques et polyphosphates: Structure, synthèse et functions," pp. 407–434. Centre National de la Recherche Scientifique, Paris.
Spirin, A. S. (1961). *Biokhimiya* **26**, 511.
Spirin, A. S. (1962). *In* "Acides ribonucléiques et polyphosphates: Structure synthèse et functions," pp. 73–87. Centre National de la Recherche Scientifique, Paris.
Steiner, R. F., and Beers, R. F. (1961). *In* "Polynucleotides." Elsevier, Amsterdam.
Steiner, R. F., and Kitzinger, C. (1962). *Nature* **194**, 1172.
Stevens, V. L., and Duggan, E. L. (1957). *J. Am. Chem. Soc.* **79**, 160.
Stollar, D., and Grossman, L. (1962). *J. Mol. Biol.* **4**, 31.
Stollar, D., and Levine, L. (1961). *J. Immunol.* **87**, 477.
Stollar, D., Levine, L., Lehrer, H. I., and Van Vunakis, H. (1962). *Proc. Natl. Acad. Sci. US.* **48**, 874.
Stollar, D., Levine, L., and Marmur, J. (1962). *Biochim. et Biophys. Acta* **61**, 7.
Stone, A. L., and Bradley, D. F. (1961). *J. Am. Chem. Soc.* **83**, 3627.
Streisinger, G., and Bruce, V. (1960). *Genetics* **45**, 1290.
Sturtevant, J., Rice, S., and Geiduschek, E. (1958). *Discussions Faraday Soc.* **No. 25**, 138.
Subirana, J., Rownd, R., and Doty, P. (1963). (In preparation.)
Sueoka, N. (1959). *Proc. Natl. Acad. Sci. US.* **45**, 1480.
Sueoka, N. (1960). *Proc. Natl. Acad. Sci. US.* **46**, 83.
Sueoka, N. (1961). *J. Mol. Biol.* **3**, 31.
Sueoka, N., and Cheng, T.-Y. (1962). *J. Mol. Biol.* **4**, 161.
Sueoka, N., Marmur, J., and Doty, P. (1959). *Nature* **183**, 1429.
Suzuki, K., Yamagami, H., and Rebeyrotte, N. (1962). *J. Mol. Biol.* **5**, 577.
Szer, W., and Shugar, D. (1961). *Acta Biochim. Polon.* **8**, 235.

Szybalski, W. (1962). *In* "The Molecular Basis of Neoplasia," pp. 147–171. Univ. of Texas Press, Austin, Texas.
Szybalski, W., and Mennigmann, H.-D. (1962). *Anal. Biochem.* **3**, 267.
Tabor, H. (1961). *Biochem. Biophys. Research Communs.* **4**, 228.
Tabor, H. (1962). *Biochemistry* **1**, 496.
Tabor, H., Tabor, C. W., and Rosenthal, S. M. (1961). *Ann. Rev. Biochem.* **30**, 579.
Tanford, C. (1961). *In* "The Physical Chemistry of Macromolecules," p. 522. Wiley, New York.
Thomas, C. A., and Berns, K. I. (1961). *J. Mol. Biol.* **3**, 277.
Thomas, C. A., and Berns, K. I. (1962). *J. Mol. Biol.* **4**, 309.
Thomas, C. A., and Doty, P. (1956). *J. Am. Chem. Soc.* **78**, 1854.
Thomas, R. (1954). *Biochim. et Biophys. Acta* **14**, 231.
Timasheff, S. N., Witz, J., and Luzzati, V. (1961). *Biophys. J.* **1**, 525.
Tinoco, I., Jr. (1960). *J. Am. Chem. Soc.* **82**, 4785.
Tinoco, I., Jr. (1961). *J. Chem. Phys.* **34**, 1067.
Ts'o, P. O. P., Helmkamp, G. K., and Sander, C. (1962a). *Proc. Natl. Acad. Sci. U.S.* **48**, 686.
Ts'o, P. O. P., Helmkamp, G. K., and Sander, C. (1962b). *Biochim. et Biophys. Acta* **55**, 584.
Vanecko, S., and Laskowski, M., Sr. (1962). *Biochim. et Biophys. Acta* **61**, 547.
Venkataraman, P. R., and Coe, E. L. (1961). *Federation Proc.* **20**, 359.
Vinograd, J., and Hearst, J. E. (1962). *Fortschr. Chem. Org. Naturstoffe* **20**, 372.
Vinograd, J., Morris, J., Dove, W. F., and Davidson, N. (1962). *Abstr. Biophys. Soc.* FA3.
Vol'kenshtein, M. V., and Yel'yashevich, A. M. (1961). *Biophysics (U.S.S.R.—English Translation)* **6**, 569.
Wake, J., and Baldwin, R. (1962). *J. Mol. Biol.* **5**, 201.
Warner, R. C. (1956). *Federation Proc.* **15**, 379.
Warner, R. C. (1957). *J. Biol. Chem.* **229**, 711.
Watson, J. D., and Crick, F. H. C. (1953a). *Nature* **171**, 737.
Watson, J. D., and Crick, F. H. C. (1953b). *Nature* **171**, 964.
Weed, L. L. (1962). *Federation Proc.* **21**, 371.
Weiss, S. B., and Nakamoto, T. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 1400.
Welsh, R. S. (1962). *Proc. Natl. Acad. Sci. U.S.* **48**, 887.
Wilkins, M. H. F. (1961). *J. chim. phys.* **58**, 891.
Wilkins, M. H. F., Stokes, A., and Wilson, H. (1953). *Nature* **171**, 738.
Yankofsky, S. A., and Spiegelman, S. (1962). *Proc. Natl. Acad. Sci. U.S.* **48**, 1069, 1466.
Zamenhof, S., Alexander, H. E., and Leidy, G. (1953). *J. Exptl. Med.* **98**, 373.
Zamenhof, S., Griboff, G., and Marullo, N. (1954). *Biochim. et Biophys. Acta* **13**, 459.
Zimm, B. H. (1960). *J. Chem. Phys.* **33**, 1349.
Zimm, B. H., and Kallenbach, N. R. (1962). *Ann. Rev. Phys. Chem.* **13**, 171.
Zimmer, C., and Venner, H. (1962). *Naturwissenschaften* **49**, 86.
Zimmerman, S. B., Kornberg, S. R., and Kornberg, A. (1962). *J. Biol. Chem.* **237**, 512.
Zinder, N. (1960). *Science* **131**, 813.