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The Formation of Hybrid DNA Molecules and their use in Studies of DNA Homologies

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Heavy-isotope-labeled DNA has been used to study strand separation and recombination. The rate at which ^{14}N–^{15}N biologically half-labeled DNA can be made to separate into subunits corresponds very closely to what has been predicted for the rate of unwinding of the strands of a double helix.

The use of a phosphodiesterase from *E. coli* (Lehman, 1960) which selectively attacks single-stranded DNA has made it possible to remove unmatched single chain ends from renatured DNA and reduce the remaining differences between renatured and native DNA.

A mixture of heavy-isotope-labeled and normal bacterial DNA was taken through a heating and annealing cycle, treated with the phosphodiesterase and examined by cesium chloride density-gradient centrifugation. Three bands were observed, corresponding to heavy renatured, hybrid, and light renatured DNA. As would be expected for random pairing of complementary strands, the amount of the hybrid was double that of either the heavy or the light component. It has thus been demonstrated that the strands which unite in renaturation are not the same strands that were united in the native DNA but instead are complementary strands originating in different bacterial cells.

The formation of hybrids has been possible only where the heavy and normal DNA samples have a similar overall base composition. It has also been shown for DNA samples isolated from bacteria of different genera in a case where genetic exchange by conjugation has been demonstrated. The evidence for the parallelism between genetic compatibility and the formation of DNA hybrids *in vitro* has led to the proposal that organisms yielding DNA which forms hybrid molecules are genetically and taxonomically related.

1. Introduction

It has been shown recently that it is possible to separate the two strands of DNA molecules and then to reunite these strands so as to restore to a large degree the original helical structure and biological activity (Marmur & Lane, 1960; Doty, Marmur, Eigner & Schildkraut, 1960). The optimum conditions for bringing about maximum restoration, that is, renaturation, have been presented in the preceding paper (Marmur & Doty, 1961).

The work presented in this paper is aimed at the detailed examination of the process of strand separation and recombination, the formation of hybrid DNA molecules with

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each of the strands coming from a different source, and the use of such hybrids in detecting overlap in nucleotide sequences in the two samples. If complementarity is the condition for maximum renaturation then it is proper to enquire to what extent deviations from complementarity are required to interfere with the renaturation. The most modest deviation is probably that which occurs between a point mutant and wild-type DNA. In a following paper (Marmur, Lane & Doty, 1961) this kind of recombination is studied with bacterial transformation as the criterion and it is concluded that such small differences do not interfere with renaturation.

The method of study in the present report was density-gradient centrifugation (Meselson, Stahl & Vinograd, 1957) coupled with the use of heavy-isotope-labeled DNA. This proves to be the best technique for following the interaction between homologous and heterologous DNA. Native DNA samples of different GC (guanine-cytosine) contents can be observed separately when banded in the density gradient (Sueoka, Marmur & Doty, 1959; Rolfe & Meselson, 1959). Two native samples having the same GC content can also be observed and their interaction studied if one of them is labeled with heavy isotopes such as ^{15}N, ^{13}C and deuterium (Meselson & Stahl, 1958; Davern & Meselson, 1960; Marmur & Schildkraut, 1961*a*).

A double helix resulting from the union of a heavy-labeled and non-labeled strand will have an intermediate density. It can be clearly distinguished from the original molecules if they are separated by a sufficient distance in the density gradient. The amount of hybrid molecules formed between different pairs of DNA samples expected to be homologous should vary with the degree of homology.

In order to examine the possibilities of renaturation occurring between DNA from different species that may be genetically related, more quantitative work was necessary to obtain a proper base line for comparison. The removal of non-renatured regions by means of a new phosphodiesterase, specific to single-stranded DNA (Lehman, 1960) provided a means of eliminating artifacts and improving resolution so that hybrid DNA molecules could be clearly and quantitatively resolved. As a result it was possible to examine in some detail the renaturation that was possible between pairs of DNA samples from different sources. This appears to offer a new means of estimating the extent of overlap in DNA sequence between DNA samples from genetically related species.

2. Materials and Methods

(a) *Bacterial strains.* The organisms used in this study together with the base composition and buoyant density of their DNA are listed in Table 1.

(b) *Preparation of DNA.* The isolation procedure of Marmur (1961) was used to extract DNA from cells grown in the exponential phase in Difco brain heart infusion medium. When heavy-isotope-labeled DNA was required the cells were grown in a synthetic medium containing $^{15}NH_4Cl$ as the only nitrogen source and D_2O of greater than 99% purity (Marmur & Schildkraut, 1961*a*).

(c) *Solvents.* In order to eliminate spurious effects due to divalent metal ion contamination, and to inhibit possible attack by nucleases, a chelating agent was always present in the DNA solutions. Citrate ions have proved most convenient in this respect and the standard saline solution (0·15 M-NaCl was used most often) contained 0·015 M Na-citrate. This solvent, standard saline-citrate, will be designated by the abbreviation SSC. Reference will also be made to various multiples of SSC. For example, 2 × SSC is 0·30 M-NaCl and 0·030 M-Na citrate.

(d) *Heating and Annealing.* Solutions containing equal amounts (by weight) of heavy-isotope-labeled and normal DNA in 1·9 × SSC were prepared. In the usual preparation of

renatured DNA, 1 ml. was placed in a 2 ml. glass stoppered volumetric flask, immersed in a bath of boiling water for 10 min and immediately transferred to a bath thermostated at 68°C. After 2 hr the temperature was lowered in approximately 5°C steps at intervals of 15 min until 25°C was reached. Although all results reported here were obtained by this cooling procedure, it has been found that the salt concentration and the rate of cooling from 68°C to room temperature can be varied somewhat without noticeable change in the final results.

TABLE 1

Bacteria from which DNA was isolated for use in studies of hybrid formation

Species	Strain	Source	%GC†	Density (g/cm³)
Bacillus brevis	9999	ATCC‡		1·704
Bacillus macerans	7069	ATCC		1·713
Bacillus megaterium		Univ. of Penn.	38	1·697
Bacillus natto	MB-275	A Demain		1·703
Bacillus subtilis	168	Yale University	42	1·703
Clostridium perfringens	87b	M. Mandel	31	1·691
Diplococcus pneumoniae	R-36A	R. Hotchkiss	39	1·701
Erwinia carotovora	8061	ATCC	54	1·709
Escherichia coli	B	S. Luria	50	1·710
Escherichia coli	44 B	M. Mandel		1·710
Escherichia coli	C-600	R. Appleyard		
Escherichia coli	K12 (W678)	J. Lederberg	50	1·710
Escherichia coli	TAU⁻	S. Cohen	50	1·710
Escherichia coli	W-3110	M. Yarmolinsky		1·710
Escherichia coli	I	A. N. Belozersky	52	
Escherichia coli	II-IV-4	A. N. Belozersky	67	
Escherichia freundii	17	H. Blechman		1·710
Escherichia freundii	5610-52	M. Mandel		
Salmonella arizona	PC145	Walter Reed Hosp.		1·712
Salmonella ballerup	ETS107	Walter Reed Hosp.		
Salmonella typhimurium	LT-2	M. Demerec	50, 54	1·712
Salmonella typhimurium	ETS9	Walter Reed Hosp.		
Salmonella typhosa	643	Walter Reed Hosp.	53	1·711
Shigella dysenteriae	15	S. Luria	53	1·710

† The GC contents of the DNA samples were all obtained from Belozersky & Spirin (1960).
‡ American Type Culture Collection.

(e) *CsCl.* Optical grade CsCl was obtained from the Maywood Chemical Co., Maywood, N.J. A concentrated stock solution was prepared by dissolving 130 g CsCl in 70 ml. of 0·02 M-tris buffer (2-amino-2-hydroxymethylpropane-1:3-diol) pH 8·5. The final solution was passed through a medium grade sintered glass filter to remove large amounts of solid material that seemed to contaminate the solid CsCl. When solutions in which the DNA had been annealed at low concentrations had to be brought up to the correct density for centrifugation, the solid CsCl was added directly. These solutions were not subsequently filtered.

(f) *Density-gradient centrifugation.* The technique described by Meselson *et al.* (1957) was followed. CsCl was used to bring the density of the DNA solution being examined to values between 1·71 g/cm³ and 1·75 g/cm³, depending on the specific sample involved. This could be done most easily by mixing the DNA solution with the concentrated CsCl stock solution. If the DNA solution was not concentrated enough, as was generally the case, this procedure would diminish the final concentration of the DNA below that required for accurate observation. To avoid this problem it was possible to use solid CsCl to give the proper density. To 1·03 g

CsCl was added 0·80 ml. of the slow cooled DNA solution (still in the same saline citrate solution) and 0·01 ml. of a stock solution of 50 μg/ml. of a DNA sample whose density is well established and can be used as a standard. DNA from *Cl. perfringens* is generally used since its density is the lowest observed thus far. This places it in a position where its band cannot coincide with any heavy-isotope-labeled DNA samples. The final adjustment of density was made by the addition of a small amount of water or solid CsCl. The measurement of the density at various stages of the adjustment is facilitated by the use of the linear relation between refractive index and density (Meselson, 1958, personal communication; Ifft, Voet & Vinograd, 1961)

$$\rho^{25\cdot0°C} = 10\cdot8601\ n_D^{25\cdot0°C} - 13\cdot4974.$$

Approximately 0·75 ml. of the final CsCl solution was placed in a cell containing a plastic (Kel-F) centerpiece and centrifuged in a Spinco model E analytical ultracentrifuge at 44,770 rev/min at 25°C. After 20 hr of centrifugation, ultraviolet absorption photographs were taken on Kodak commercial film. It was clear that equilibrium had been closely approached in 20 hr since there was no difference in calculated densities when photographs taken after 48 hr were used. Moreover, the variances did not decrease by more than 5% between 20 and 48 hr. Tracings were made with a Joyce-Loebl double-beam recording microdensitometer with an effective slit width of 50 microns in the film dimension.

Densities were calculated by using the position of the standard DNA as a reference. The CsCl density gradient was obtained from the data of Ifft *et al.* (1961).

(g) E. Coli *phosphodiesterase.* This enzyme, which preferentially hydrolyses single-stranded DNA, was kindly supplied by Dr. L. Grossman and had been prepared in highly purified form by the method of Lehman (1961, personal communication). The enzymatic hydrolysis was carried out by the following modification (L. Grossman, 1961, personal communication) of the method of Lehman (1960). The annealed sample, usually at a total DNA concentration of 10 μg/ml., was dialysed against two changes of 0·067 M-glycine buffer, pH 9·2. To 0·7 ml. of the dialysed DNA solution was added 1·6 μmoles of $MgCl_2$, 2·4 μmoles of 2-mercaptoethanol, and approximately 25 to 75 units of crystallized *E. coli* phosphodiesterase. The final mixture (about 0·8 ml.) was incubated at 37°C for 3 hr. Solid CsCl and the standard DNA were then added and the solution was made up to the proper density.

3. Strand Separation

In order to form hybrid molecules by renaturation it is first necessary to examine in some detail the conditions necessary for strand separation. A very useful material for these studies has been the ^{14}N-^{15}N-half-labeled or biological "hybrid" DNA molecules first described by Meselson & Stahl (1958). DNA having one strand ^{15}N-labeled and the other strand normal was isolated from *E. coli* B according to the method of Marmur (1961). It was found that the biologically formed "hybrid" DNA banded at a density of 1·717 g/cm^3 in the CsCl gradient, exactly between that of fully ^{15}N-labeled and normal DNA from *E. coli*. The band profile is shown in the top tracing of Fig. 1.

(a) *Methods for inducing strand separation*

In terms of the Watson-Crick model (1953) for DNA, the dissociation of the two strands obviously requires first the rupture of the hydrogen bonds uniting the base pairs and second the uncoiling and diffusing apart of the two strands. The rupture of the hydrogen bonds has been shown to occur in a relatively narrow temperature range at elevated temperatures but the diffusion apart of the strands did not automatically follow (Rice & Doty, 1957). Later it was found that lower ionic strengths and more dilute solutions did permit the diffusing apart to occur (Doty *et al.*, 1960;

Eigner & Doty, 1961) and prevented the non-specific re-association from occurring at lower temperatures where numerous hydrogen bonds re-form.

Meanwhile it had been found by Meselson & Stahl (1958) that the DNAs containing ^{14}N and ^{15}N subunits, each of which replicate in a conservative manner, produced two bands on heating in CsCl for 30 min. Thus they demonstrated that the two molecular subunits dissociated on heating. Meselson & Stahl left the question of the molecular structure of the subunits open to further investigation. This prompted us to isolate DNA from cells grown according to the procedure described by Meselson & Stahl (1958), and to see how the hybrid DNA reacted to the various methods that produce strand separation. The evidence that these methods do produce strand separation can be found in the papers to which we will refer. We are concerned here only with their effect on the biological hybrids and the detailed investigation of this effect in order to learn more about strand separation.

First we sought to reproduce the Meselson-Stahl results. They had observed the two-band pattern after heating a cell lysate in CsCl for 30 min. We found a similar pattern was produced by heating a sample of our purified biological hybrid at 100°C for only 10 min at a concentration of 20 μg/ml. in SSC. The tracings of the bands before and after heat treatment can be seen at the top and bottom frames of Fig. 1. The apparent density of the native, hybrid DNA is 1·717 g/cm^3; that of the two denatured bands is greater by 0·007 and 0·023 g/cm^3 respectively. The average of these is, of course, greater than that of the native hybrid DNA by the 0·015 g/cm^3 characteristic of denatured DNA. As a check, it was demonstrated that the same two-band pattern results when ^{14}N and ^{15}N DNA samples are separately denatured and then mixed.

Three other methods which have recently been shown to bring about strand separation have also caused the disappearance of the native hybrid band and the formation of two heavier bands. This additional information, combined with the demonstration of Doty *et al.* (1960) that the above heating procedure produces strand separation in bacterial DNA samples, now leads us to conclude that Meselson & Stahl were indeed observing the separation of single strands. We shall return to this point in the discussion, and now proceed to summarize the experimental observations on the biological hybrids.

By the addition of 8 M-urea the temperature for thermal denaturation can be reduced by nearly 20°C (Rice & Doty, 1957). Consequently, by using 0·01 M-salt and 8 M-urea it was found possible to bring about strand separation by heating DNA only to 65°C (Eigner & Doty, 1961). When this procedure was applied to the biological hybrid DNA, two bands formed in the CsCl density gradient, exactly as was the case after heating at 100°C for 30 min in CsCl or for 10 min in SSC.

Formamide, being a more potent hydrogen bonding agent, and being miscible with water in all proportions, offers a substantial improvement over urea. Marmur & Ts'o (1961) have shown that in 95% formamide strand separation readily occurs at room temperature. Again, when this is applied to the biological hybrids the typical two-band pattern is produced.

Acid and base titration, by virtue of their ability to disrupt hydrogen bonding, offer another route to strand separation. It has now been shown (Cox, Marmur & Doty, 1961) that strand separation occurs when the pH is lowered to 2·5 or raised to 12·0. These two pH values lie just outside the region in which the hypochromic shift (at 260 mμ) indicates that the helix-coil transition is complete. At pH values on

the other side of the hypochromic shift, strand separation has not occurred. When the biological hybrids are exposed to conditions in which the pH is below 2·5 or above 12·0, two bands again result in the CsCl gradient.

Thus it is seen that several different procedures lead to the formation of the same two-band pattern in the CsCl density gradient, and that all of them are a consequence of the prior breaking of hydrogen bonds uniting the two DNA strands. While the present work is not aimed at establishing how many subunits make up DNA molecules, it does appear that the extension to the biological hybrid DNA of the consistent finding that strand separation occurs whenever hydrogen bonds are cooperatively broken, shows that only one type of bonding is uniting the subunits. Since this can only be identified with that assumed in the Watson-Crick structure, there seems to be no alternative to equating the subunits, observed as two bands, with the two strands in the native DNA molecule.

(b) *Temperature dependence of strand separation*

With the demonstration that the two-band pattern of the biological hybrid DNA is indicative of strand separation we now attempt to use this material to obtain further details about the process of strand separation. The studies of Marmur & Doty (1959) demonstrated that it should be possible to melt out the molecules having a high AT (adenine-thymine) content leaving GC (guanine-cytosine)-rich molecules undenatured. This can be observed to occur with the biological hybrids by exposing them to temperatures only slightly above the temperature, T_m, of the midpoint of the absorbance rise. T_m is the same for all *E. coli* DNA samples, including the biological hybrids (Marmur & Doty, 1961). The band profile obtained after heating the latter at T_m for 20 min is shown as the second tracing in Fig. 1. Since there is no difference from the profile of the native sample, it is concluded that all the molecules remain in the helical form. To melt selectively the AT-rich molecules, it is necessary to expose them to temperatures a few degrees above T_m. The results of doing this are shown as the third tracing of Fig. 1, where it is seen that approximately 25% of the molecules undergo strand separation. The molecules showing the greater resistance to heat denaturation are seen to have a higher density, in agreement with the hypothesis that they should be GC-rich.

(c) *Kinetics of strand separation*

Since a small but detectable thermal depolymerization always accompanies strand separation it is desirable to know the minimum time at the elevated temperature required to obtain complete separation, and thus avoid this undesirable side effect. In addition, since a few theoretical calculations of the rate of uncoiling of the DNA molecule have been reported (Kuhn, 1957; Longuet-Higgins & Zimm, 1960), some comparison with experimental findings seems to be in order. The rate of separation into subunits can be observed experimentally by following the disappearance of the hybrid band or the appearance of the two heavier bands. The results presented in Plate I and Fig. 2 show that the complete disappearance of material of density 1·717 g/cm^3 takes slightly over 60 sec at 100°C. This is very close indeed to the time calculated by Kuhn (1957) for the unwinding of a double helix of comparable length in which no bonds are holding the strands together. As is evident from the absorbance-temperature profile, the rise in absorbance is complete at 95°C. When the temperature of a DNA solution

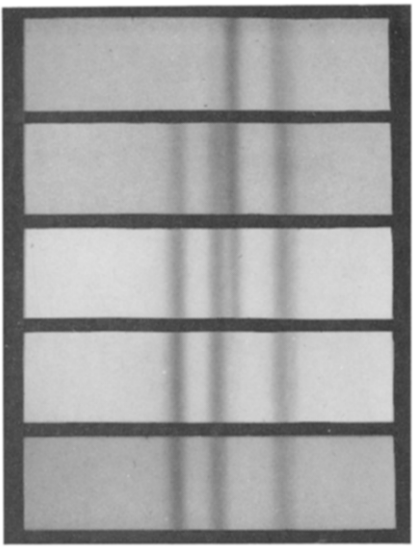

PLATE I. Ultraviolet absorption photographs of ^{14}N-^{15}N-labeled DNA showing different stages of the thermally induced separation into subunits. The band at the far right has been used as a standard and is DNA isolated from *D. pneumoniae*. The other band in the top photograph is the biologically formed hybrid DNA. The second photograph shows the stability of the hybrid to a 20 min exposure at 93·8°C. At 100°C the number of molecules separating increases rapidly with time of exposure as shown in the next 3 photographs. The samples were heated in SSC at 20 μg/ml. for 30 sec, 1 min and 10 min, respectively.

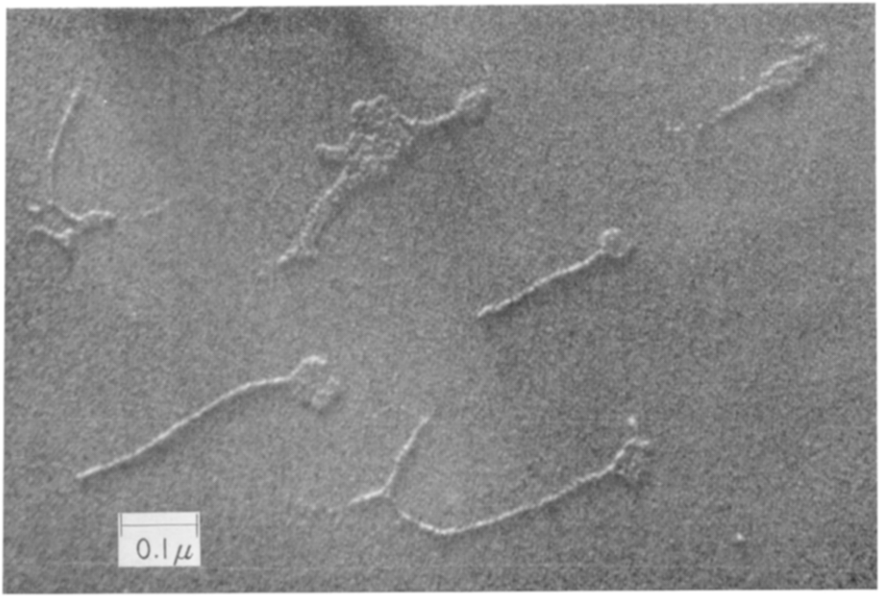

PLATE II. Electron micrograph of renatured *D. pneumoniae* DNA showing an unusually large concentration of renatured molecules with circular regions at one or both ends. Magnification $\times$ 100,000.

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is raised to 100°C few hydrogen bonds should exist between strands and Kuhn's model seems applicable to such a molecule.

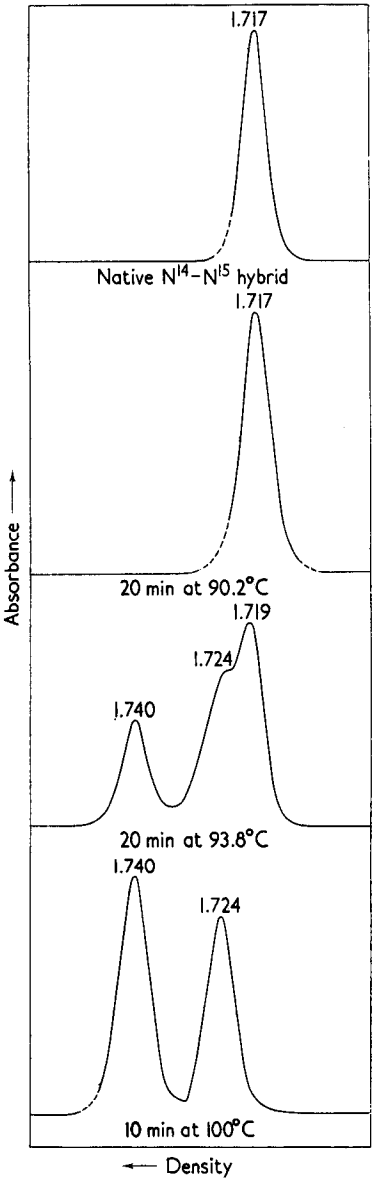

FIG. 1. The temperature dependence of strand separation. Microdensitometer tracings of ultraviolet absorption photographs of samples equilibrated in a CsCl density gradient. Each sample was heated in SSC at a concentration of 20 μg/ml.

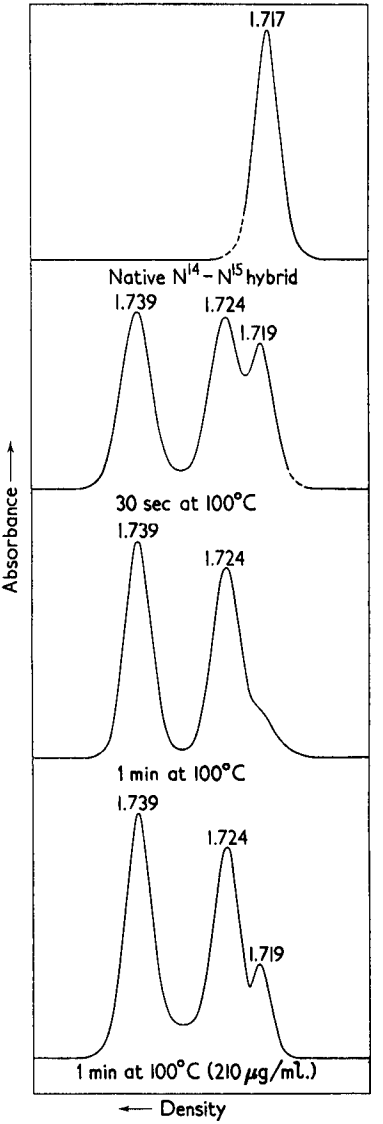

FIG. 2. Kinetics of strand separation. The results shown in the second and third tracings were obtained by heating at a concentration of 20 μg/ml. in SSC.

(d) *Dependence of the time of strand separation on viscosity*

In the theoretical considerations just mentioned in connection with the kinetics of strand separation, the variation of the rate of unwinding with the viscosity of the

medium is also considered (Longuet-Higgins & Zimm, 1960). An equation is derived which predicts that strand separation will take longer as the viscosity of the medium is increased. In the tracing shown at the bottom of Fig. 2 the time necessary for separation into subunits is shown to have increased with increased DNA concentration. Similar results have also been obtained by using concentrated sucrose solutions to produce a medium of higher viscosity (Schildkraut, Wierzchowski & Doty, 1961).

(e) *DNA from bacteriophage ΦX174*

The results of the studies with the biological hybrids indicate that the most convenient way to obtain single-stranded DNA is to heat for 10 min in SSC at a concentration of from 10 to 20 μg/ml. and quickly cool. Since one kind of DNA, that from bacteriophage ΦX174†, is known to be single-stranded (Sinsheimer, 1959), it is expected that our thermal treatment would produce no density change in this material. This has, indeed, been found to be the case.

4. Renaturation

The general features of the specific recombination of single DNA strands to form the native helical structure have been described in the first publications (Marmur & Lane, 1960; Doty *et al.*, 1960), and methods of optimizing conditions to ensure the maximum renaturation were described in the preceding paper (Marmur & Doty, 1961). Here we have only one point to add to this: it has to do with the reduction or elimination of the remaining differences between renatured and native DNA. In the earlier work it was evident that renaturation was not complete. For example, the density had returned only about 75% of the way from the denatured to the native density. It was thought that this was due to the inequality in length of the recombined strands. DNA molecules are apt to be broken during isolation and the single strands suffer some hydrolysis during the thermal treatment. Using the measurements on rate of bond scission by Eigner, Boedtker & Michaels (1961), we would estimate that strands originally in molecules of 10,000,000 molecular weight would undergo about three scissions each as a result of a typical heat treatment: 10 min at 100°C and 100 min at 68°C. This would mean that two strands meeting in a complementary region may differ in length by as much as one quarter to one half, on the average. If they renatured completely, there would still remain 20 to 33% of the weight unrenatured and the incomplete recovery of the characteristics of the native DNA would be explained.

In the course of electron microscope studies with Professor C. E. Hall we frequently saw renatured molecules with circular regions at one or both ends that would be consistent with the protrusion of a single chain end beyond the re-formed helical region just described. One electron micrograph showing an unusually large concentration of these in a single photograph is shown in Plate II. While this interpretation lacks proof, the observation is nevertheless strikingly similar to what was expected from the point of view outlined.

From this evidence it appeared that in order to improve the extent of renaturation the unmatched single chain ends would have to be removed. The possibility of doing this suddenly became available with the discovery by Lehman (1960) of a phosphodiesterase from *E. coli* that selectively attacked single-stranded DNA. This was tested

† This material was kindly provided by Professor Robert L. Sinsheimer.

on renatured DNA from *B. subtilis* as shown in Fig. 3. The denatured DNA showed the characteristic increase in density. Upon renaturation at a concentration low enough to ensure that some denatured DNA remained, the result shown in the third frame was obtained. The density of the renatured DNA is, as expected, 0·004 g/cm^3 higher than that of the native DNA. After treatment of this sample with the phosphodiesterase it is seen, in the bottom frame, that the denatured shoulder has been

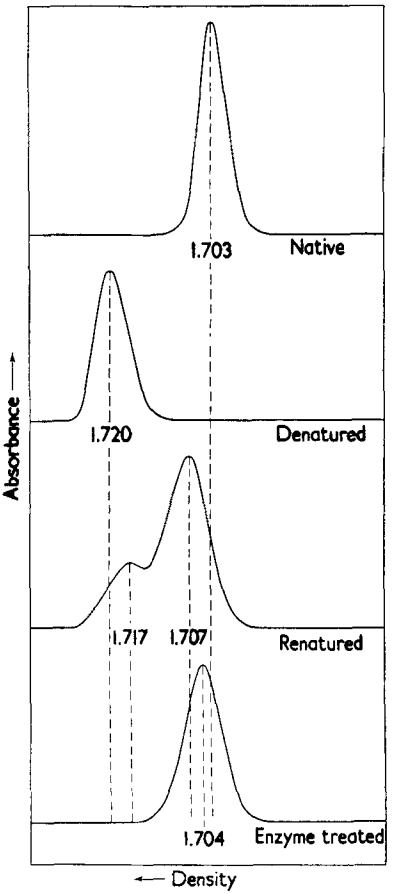

FIG. 3. Renaturation of *B. subtilis* DNA. When a solution of native DNA (band profile shown in top tracing) is heated for 10 min at 10 μg/ml. at 100°C in 1·9 × SSC and quickly cooled, the density increases 0·017 g/cm^3 (second tracing). A portion of this solution was annealed and, as is evident from the third tracing, about 80% of the DNA renatures. This concentration (10 μg/ml.) was chosen so that some denatured material would still be present. Treatment with the *E. coli* phosphodiesterase causes the complete disappearance of the denatured band and a decrease in the buoyant density of the renatured band (bottom tracing).

completely removed and the density of the renatured band has been reduced to within 0·001 g/cm^3 of the native DNA density. Thus the possibility of removing the denatured unmatched ends from renatured molecules has been demonstrated. As will be seen, this procedure greatly improves the resolution of bands encountered in the study of artificially produced hybrid molecules.

It may be of interest to mention at this point our failure thus far to produce renaturation of DNA from bacterial sources without thermal treatment of the type described here and in the preceding papers. We have employed urea and moderate

temperatures, formamide, and low pH to produce strand separation; and then, by gradual withdrawal of the hydrogen-bond breaking agent attempted to renature the strands. Such attempts have thus far failed. While our experiments have not been exhaustive, it appears quite possible that renaturation cannot be achieved in this way. If so, a likely explanation would be that at room temperature the decreased Brownian motion of the chain segments is no longer sufficient to provide the mobility required for the exploration necessary to create nuclei, that is, for complementary regions to find each other rather than become frozen in mismatched pairings.

In connection with renaturation, we might return just briefly to the case of ΦX DNA and mention that the density does not decrease but remains exactly 1·723 g/cm^3 after heating and annealing. This is as should be expected for single-stranded material whose complementary strands are not present during the annealing process.

5. Hybrid DNA Molecules produced by Renaturation

We now turn to the problem of demonstrating that the strands which unite in renaturation are not the same strands that were united in the native DNA but instead are complementary strands originating in different cells. This requires that renaturation be studied in a solution of two homologous DNA samples, one of which carries a distinctive label. With density gradient ultracentrifugation offering such good resolution it was natural to turn to the introduction of heavy atoms or to heavy isotope substitution. If the pairing of DNA strands is restrained only by the condition of complementarity one would expect that renaturation would lead to three bands, 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 intermediate 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 mixed originally.

If this situation is found, then it will be of interest to attempt to form hybrid molecules from other pairs of DNA samples in which some differences exist. In order to have sufficient resolution to exploit this means of searching for such effects it is necessary to have a considerable density difference between the two samples. Practical considerations indicate that it should be at least 0·040 g/cm^3. Thus the use of ^{15}N label in one sample, such as first used by Meselson & Stahl (1958), is not sufficient since this gives a separation of only about 0·015 g/cm^3.

(a) *Preliminary experiments*

Our first attempts to produce DNA with substantially higher density were with 5-bromouracil substitution for thymine inasmuch as this had been accomplished in both bacteria and bacteriophage (Dunn & Smith, 1954; Zamenhof & Griboff, 1954). A sample of such DNA from *B. subtilis* (Ephrati-Elizur & Zamenhof, 1959) was kindly supplied to us by Professor W. Szybalski. Unfortunately, when this sample was carried through the heating and annealing cycle it displayed a quite broad band with little indication of renaturation. The molecular weight had evidently been substantially reduced either by enzymatic attack during isolation or as a result of the greater heat sensitivity of this substituted DNA.

Nevertheless this material was mixed with high molecular weight, normal *B. subtilis* DNA, and put through the heating and annealing cycle. The banding of the mixture before and after heating is shown in Fig. 4. It is seen that the thermal treatment has

produced a broad band without any resolution of the expected hybrid. Indeed the result is such as to indicate that the smaller heavy-labeled chains have been bound in a random manner to the larger, normal density chains producing a complete spectrum of densities which hides what little renaturation may have occurred.

Initial attempts to study 5-bromodeoxyuridine-labeled DNA from T4r^+ bacteriophage led to similar disappointing results.

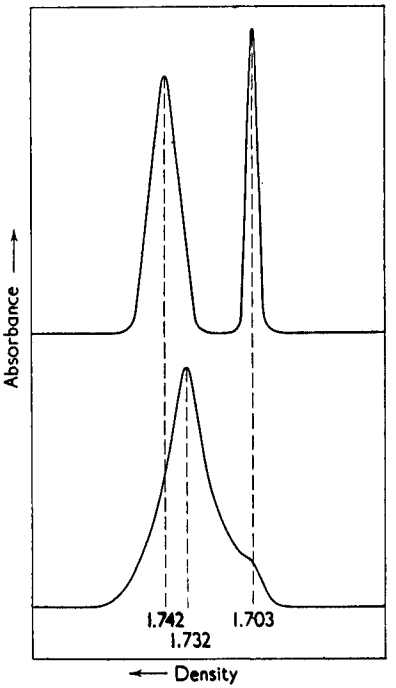

FIG. 4. Hybrid formation using 5-bromodeoxyuridine-labeled DNA. The upper tracing shows native normal and 5-bromodeoxyuridine-labeled DNA isolated from *B. subtilis*. The properties of the latter DNA have been described in detail elsewhere (Szybalski, Opara-Kubinska, Lorkiewicz, Ephrati-Elizur & Zamenhof, 1960). Portions of these two samples were heated together in 1·9 × SSC at a concentration of 20 μg/ml. each for 5 min at 100°C. The solution was then allowed to cool to room temperature over a period of about 5 hr and CsCl was added to obtain the proper density. The lower tracing suggests that the expected renatured species are present in solution, but the broadening of bands due to degradation and aggregation obscures the results.

(b) ^{15}N-*deuterated DNA*

Under these circumstances we turned to a combination of ^{15}N and deuterium to provide the density increase. After considerable manipulation this combined labeling has been shown to be successful in producing DNA with a density increase of about 0·040 g/cm^3 and the details have now been published elsewhere (Marmur & Schildkraut, 1961*a*).

We were, therefore, in a position to proceed with the experiment that would demonstrate whether or not hybrid DNA molecules form from random mating of complementary strands. The steps are shown for *B. subtilis* DNA in Fig. 5. In the first two frames the density profiles of the normal and labeled native DNA samples are shown. These were then mixed so that the concentration of each was 5 μg/ml. in 1·9 × SSC, and the heating and annealing cycle was carried out. At this concentration only partial renaturation is expected. So low a concentration was chosen in order to

minimize the association of renatured DNA molecules since this would lead to an unnecessary smearing of the pattern. Consequently, at this concentration, one would expect to find 5 species of different density: heavy denatured DNA, heavy renatured DNA, hybrid DNA, light denatured DNA and light renatured DNA. The result, seen in the third frame of Fig. 5, is of precisely this character.

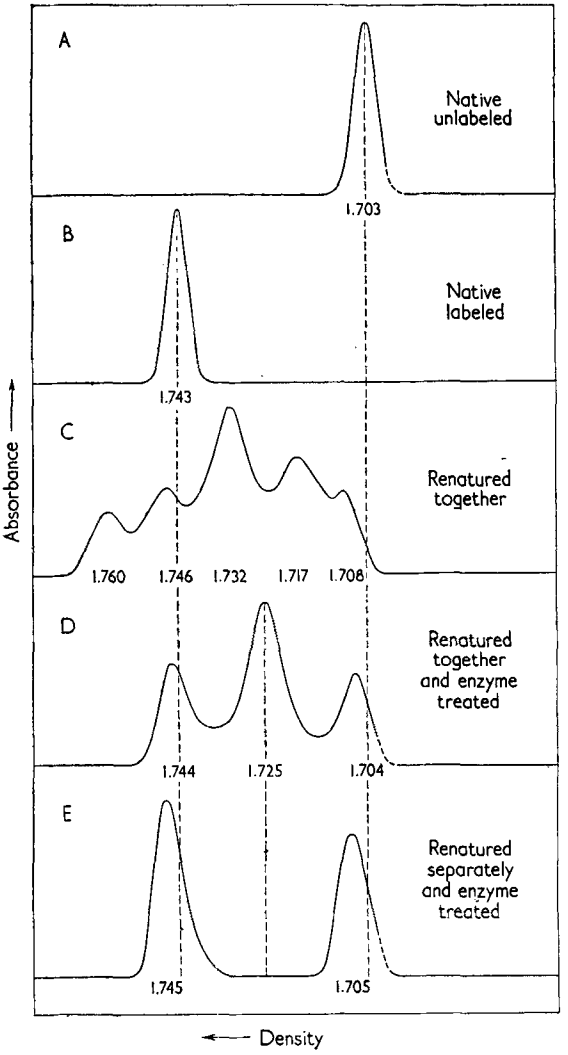

FIG. 5. The effect of *E. coli* phosphodiesterase on a heated and annealed mixture of heavy-labeled and normal *B. subtilis* DNA.

(A) and (B) Native samples.

(C) Mixed so that the final concentration of each was 5 μg/ml. and then heated and annealed.

(D) The sample shown in (C) was dialysed against 0·067 M-glycine buffer and incubated with the *E. coli* phosphodiesterase.

(E) Heated and annealed separately, treated with the phosphodiesterase and then mixed.

The application of the phosphodiesterase previously described would be expected to remove the first and fourth bands as well as shift the density of the remaining species back toward the native values. The band profile after this treatment is shown in the fourth frame and it is seen that the expected changes had been brought about.

As a control, the two native DNA samples were carried through the identical operations described above and then mixed and banded. The result is shown in the last frame where it is seen that the densities match within 0·001 g/cm^3 that of the outer bands of the frame above and that no hybrid is evident.

Similar experiments were carried out on *E. coli* DNA: the band profiles corresponding to frames C and D of Fig. 5 are shown in Fig. 6. The result is seen to be the same.

It is to be noted that the area under the hybrid band in these two experiments is about equal in area to that of the two outer bands.† Thus the conclusion can be drawn that the earlier and much more primitive experiment along these lines (Doty *et al.*, 1960) has been substantiated and the formation of hybrid DNA molecules by random pairing of complementary strands can be taken as proved.

It is useful to note in passing that the phosphodiesterase treatment had one other effect not evident in the band profiles. Without enzyme treatment part of the hybrid band would begin to form much earlier than the other, indicating high molecular weight material suggestive of some aggregation despite our efforts to avoid it. However, after enzyme treatment all three bands appear at the same rate. Thus, the enzymatic treatment not only improves the resolution by removing denatured material but appears to break up aggregated renatured DNA molecules as well.

(c) *Further investigation of 5-bromodeoxyuridine-substituted DNA*

With the successful conclusion of the above work, we have returned again to the 5-bromodeoxyuridine-labeled bacteriophage DNA. With the guide that the above work provides it has been possible to overcome some of the problems that appear to be associated with the greater inherent instability of this material. As a consequence results nearly as satisfactory as those shown in Fig. 5 and 6 are being obtained at present. This system has been used to study DNA homologies among the T-even bacteriophages (Schildkraut, Marmur, Wierzchowski, Green & Doty, 1961).

(d) *Kinetics of hybrid formation*

As a further check on the above interpretation and in order to show further details of the process of hybrid formation, samples were withdrawn from the annealing mixtures, held at 68°C, during renaturation. These were quickly cooled, in order to "freeze" the distribution present and band profiles were obtained in the ultracentrifuge. A number of profiles are shown in Fig. 7. From these, the progress of the renaturation can be followed and it is seen that it reaches about 50% completion in the first hour.

6. Hybrid DNA Formation among Various Strains of *E. coli*

It is to be expected from their close taxonomic, physiological and genetic relationships that all strains identified as *E. coli* should yield DNA which will form the 5-band pattern upon the renaturation discussed in the previous section. Hybrid formation in a number of pairs has been examined. The results are summarized in Plate III where the ultraviolet absorption photographs are reproduced. It is seen that the 5-band pattern is produced in all but the one case where sequence homology did not exist.

† The linearity of response of our optical system is not yet such as to justify a precise measurement of the area under these curves.

This DNA isolated from *E. coli* 11-IV-4, an alkali-producing form, had a GC content of 67%, which is far from that characteristic of *E. coli* DNA. Belozersky (1957) has reported that these cells were produced by an alteration of the properties of *E. coli* CM caused by growing it together with Breslau bacteria No. 70 killed by heat.

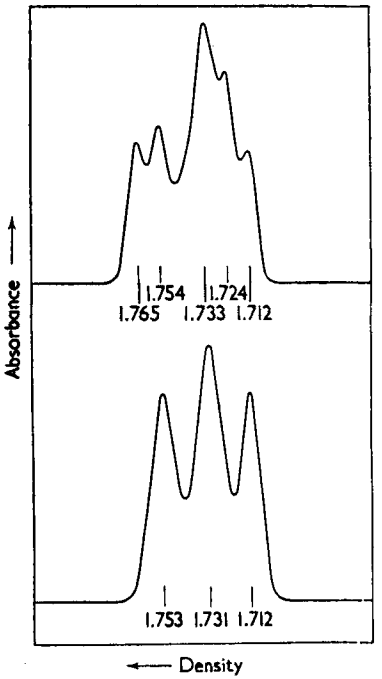

FIG. 6. The effect of *E. coli* phosphodiesterase on a heated and annealed mixture of heavy-labeled DNA from *E. coli* B and normal DNA from *E. coli* K12. The concentration of each sample was 5 μg/ml. each during the heating and annealing cycle.

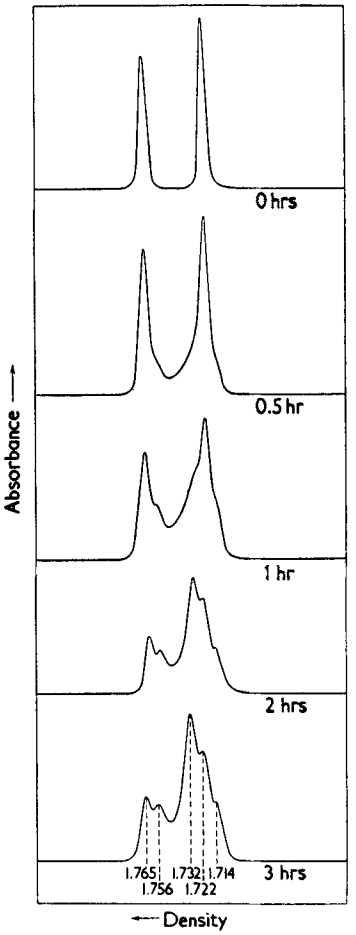

FIG. 7. Kinetics of hybrid formation. Heavy-labeled and unlabeled DNA from *E. coli* B were heated together at 100°C for 10 min under the usual conditions of 5 μg/ml. each and in 1·9 × SSC. The mixture was placed at 68°C and quickly cooled portions were centrifuged after the addition of CsCl. The tracings are shown above. These samples have, of course, not been treated with the phosphodiesterase.

The failure to obtain a hybrid band confirms the gross difference from *E. coli* DNA, but of course does not help to eliminate the argument that these special cells may have been a contaminant rather than a variant. As seen in the photograph the location of the peaks and their intensities is variable. For this reason three of the samples were treated with phosphodiesterase. When this was done the patterns

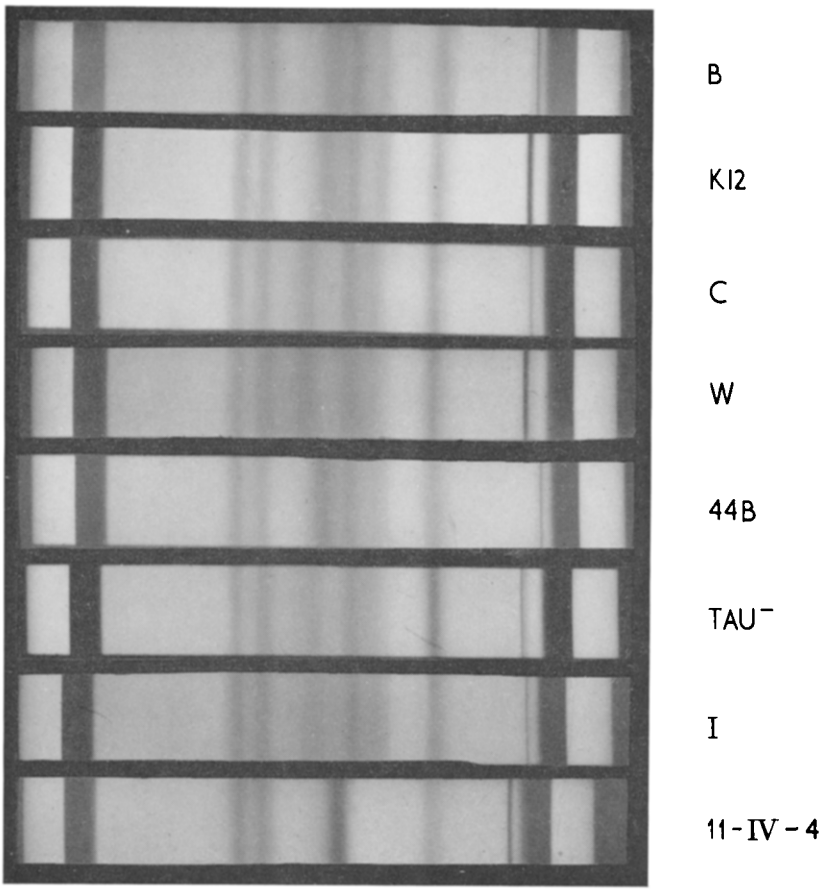

PLATE III. Hybrid formation between *E. coli* B and other *E. coli* strains. *E. coli* B DNA, labeled with ^{15}N and deuterium, was mixed with DNA from each of the strains listed above and heated and annealed in separate experiments. The concentrations were 5 μg/ml. each, and the other conditions were as described in the section on methods. Each of 8 different ultracentrifuge runs is represented above by a typical ultraviolet absorption photograph. Six DNA bands appear in all but the last example. The photographs have been lined up according to the position of the standard band at the far right, which is DNA from *Cl. perfringens*.

[*To face page* 608

gave three bands and showed greatly reduced differentiation. We are, therefore, now in a position to decide if there are any small reproducible differences in the renaturation of various *E. coli* pairs that may indicate small differences in sequence, but the effect is at most quite small. We can conclude that the homology is essentially complete.

7. The Aggregation of Renatured DNA

In exploring further the extent to which hybrids can form between somewhat different DNA strands we encountered more examples of the way in which renatured DNA molecules could aggregate so as to indicate hybrid formation even though it was indeed an artifact. While these difficulties were always removed by phosphodiesterase treatment it is of interest to record a few examples in order to indicate the nature of this process and to emphasize the need for phosphodiesterase treatment.

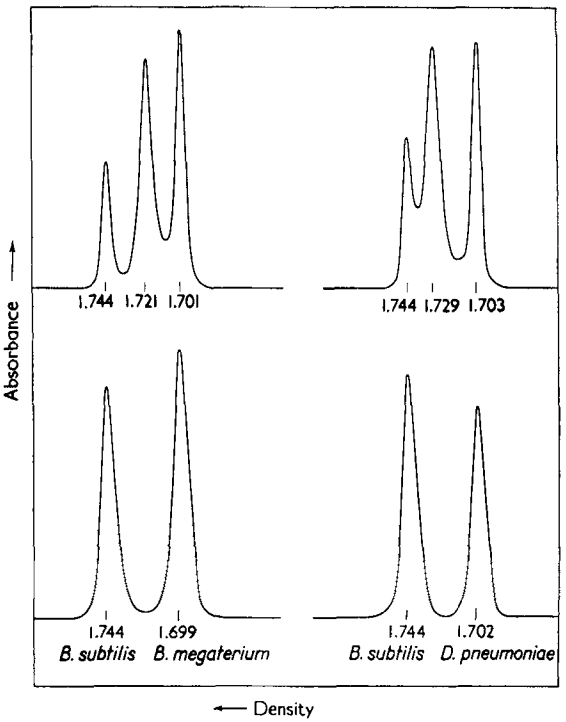

FIG. 8. Non-specific aggregation. ^{15}N-deuterated DNA from *B. subtilis* was heated with either DNA from *B. megaterium* or *D. pneumoniae* at a concentration of 50 μg/ml. each in 1·9 × SSC. The mixtures were annealed under the usual conditions. Portions were centrifuged in CsCl before (upper tracings) and after (lower tracings) treatment with the *E. coli* phosphodiesterase.

A number of experiments were carried out using the heavy-labeled *B. subtilis* DNA and various normal DNA samples at concentrations of about 50 μg/ml. where no denatured strands remain after renaturation. (This is in contrast to the standard procedure which involves tenfold lower concentrations.) Results are shown in Fig. 8 for renatured mixtures of *B. subtilis* DNA with either *B. megaterium* or *D. pneumoniae* DNA. The band profiles before enzyme treatment are shown at the top. Here we see three well-resolved band patterns highly suggestive of hybrid formation. However, the enzyme treatment completely eliminates the central band and increases the amount

of material in the outer bands. Thus the center band must have been the result of homogeneous renatured DNA molecules being held together by the association of unpaired, protruding chains. The rather remarkable feature is that the aggregation must consist mostly of two such renatured molecules, one of each type, since otherwise a whole range of densities would be displayed.

It was also possible to form aggregates between *B. subtilis* and *B. brevis* DNA by heating and annealing at a concentration of 40 μg/ml. each, and between *B. subtilis* and *B. macerans* DNA by heating and annealing at 50 μg/ml. each. The GC content of *B. subtilis* DNA is similar to that of *B. brevis* while that of *B. macerans* is somewhat higher. In this case the density of the aggregates was considerably higher than the average of the two renatured "parent" samples. This was true for practically every case of aggregation except two, one shown in Fig. 8 and the other in Fig. 13. When *B. subtilis* and calf thymus DNA were heated together and annealed at a total concentration of 100 μg/ml., the heavy labeled *B. subtilis* DNA reformed completely while the calf thymus DNA maintained the denatured density. No intermediate band was visible.

As a final example we report the rather puzzling case of aggregation occurring upon mixing renatured solutions at room temperature. Heavy and light *B. subtilis* DNA at a concentration of 10 μg/ml. were heated and annealed separately; the band profiles are shown in the first 4 frames of Fig. 9. The two renatured solutions (C and D) were then mixed at room temperature and examined in the density gradient. The result is shown in Fig. 9 (E); it is seen that a central band has formed, apparently at the expense of the renatured molecules. Thus it would appear that unpaired chain ends which cannot be satisfied in the separated solutions can pair in the mixture. However, this artifact too was removed by phosphodiesterase treatment, as shown in the final frame of Fig. 9. It is important to note that this aggregation of renatured molecules at room temperature occurred between homologous molecules, not molecules from two different sources.

Thus the formation of bands of intermediate density that have the appearance of true hybrid DNA molecules has been clarified, and the importance of eliminating such false bands with phosphodiesterase treatment has been emphasized.

8. Interspecies Hybridization of DNA

We are now in a position to examine the possibility of forming DNA hybrids between strands of DNA coming from different bacterial species. By heating and annealing each of several DNA samples with the heavy-labeled *B. subtilis* DNA, it became evident, even without recourse to the enzyme treatment, that hybrid DNA molecules did not form. Such results are shown in Fig. 10. These and other results could be summarized by stating that hybrid DNA formation was not observed, even at double the usual DNA concentration, if the two DNA samples had significantly different base-compositions.

With the search for hybrid formation narrowed to DNA samples having essentially the same composition, it was natural to look first at DNA from two organisms of the same species which were known to be genetically related by virtue of their ability to undergo transformation with each other's DNA. This is true for *B. subtilis* and *B. natto* (Marmur, Seaman & Levine, 1961). The results for heating, annealing and enzyme treating this pair of DNA samples are shown in Fig. 11. It is seen that substantial

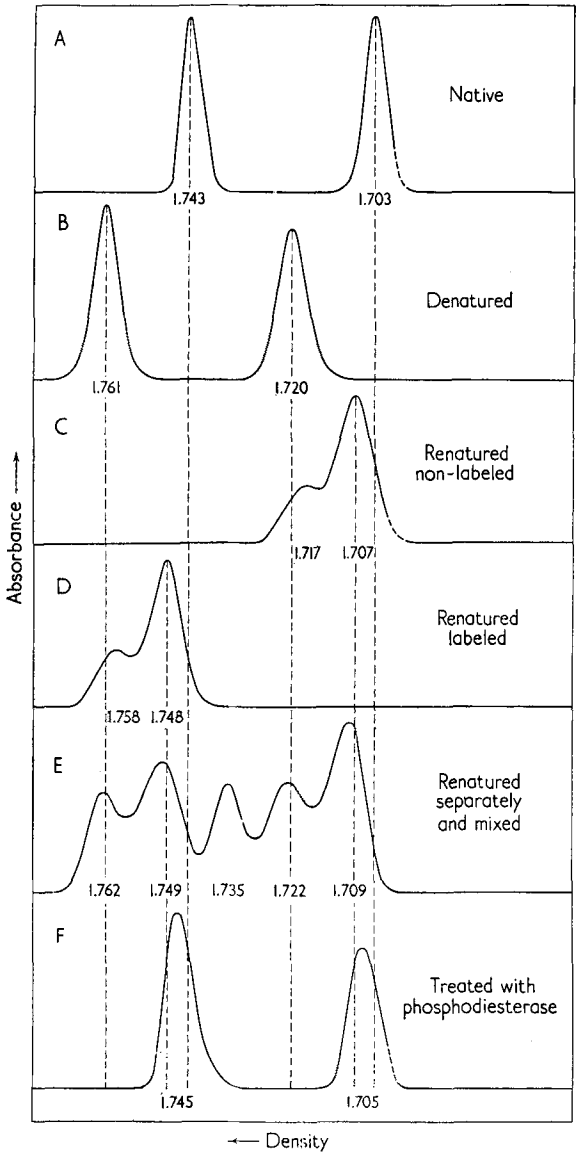

FIG. 9. Control experiment for hybrid formation with *B. subtilis* DNA. A stock solution in 1·9 × SSC of normal *B. subtilis* DNA was prepared so that the concentration was 10 μg/ml. The same was done for an ^{15}N-deuterated sample. After portions of these solutions received the following treatments, they were made up to the proper density with CsCl and centrifuged. The tracings are shown above.

(A) Mixed while still native.

(B) Heated separately for 10 min at 100°C, quickly cooled and mixed.

(C) and (D) Heated separately, annealed under identical conditions and banded separately.

(E) Mixed immediately after annealing while still in the 1·9 × SSC solvent. If the mixture was dialysed against a lower salt concentration (0·067 M-glycine buffer) before addition of CsCl or if each annealed sample was dialysed against 0·067 M-glycine buffer before mixing, 5 bands resulted.

(F) Mixed, dialysed against 0·067 M-glycine buffer and treated with the *E. coli* phosphodiesterase.

The same picture also resulted when portions of the annealed samples shown in B and C were separately treated with the enzyme and then mixed.

hybrid formation did occur although it may not have been as much as expected from random pairing of complementary strands since the three bands appear to have about equal areas.

For the next experiment we chose three members of the family Enterobacteriaceae which have similar base compositions (Lee, Wahl & Barbu, 1956) and which are genetically related. *E. coli* K12 shows a high degree of genetic exchange by conjugation and transduction with *E. coli* B and *Sh. dysenteriae* (Lennox, 1955; Luria & Burrous, 1957; Luria, Adams & Ting, 1960) whereas *Salm. typhimurium* mates well with K12 but is transduced only to a very limited extent, if at all (Zinder, 1960).

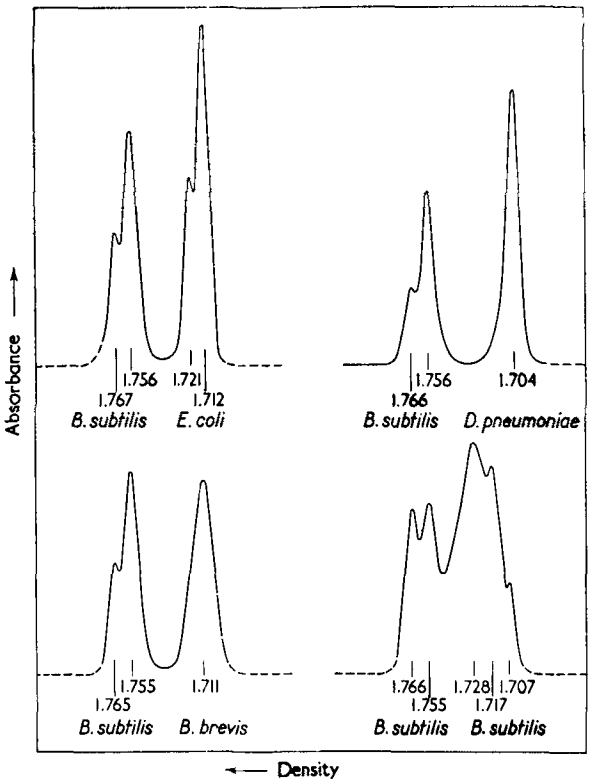

FIG. 10. The specific requirement for hybrid formation using heavy-labeled *B. subtilis* DNA. The three non-homologous samples were heated and annealed at a concentration of 10 μg/ml. each, under the same conditions already described. In the case where both samples were *B. subtilis* DNA, the concentrations were 5 μg/ml. each. Neither the labeled nor the unlabeled *B. subtilis* preparations were the same ones used to obtain the results shown in Fig. 5.

In this context, attempts were begun to prepare hybrid DNA molecules between ^{15}N-deuterated *E. coli* DNA and normal *Shigella* or *Salmonella* DNA. Unlabeled DNA isolated from *Sh. dysenteriae* was substituted for the unlabeled *E. coli* DNA in the procedure discussed in Section 5, and the heated and annealed mixture was treated with *E. coli* phosphodiesterase. Three bands were observed as shown in Fig. 12. The hybrid band does not contain twice as much DNA as either of the uniformly labeled renatured bands, as was observed in most previous examples. This indicates that not every *Sh. dysenteriae* DNA molecule is homologous to a corresponding *E. coli* DNA molecule. Genetic evidence also supports this partial degree of homology (Luria & Burrous, 1957).

Similar studies were carried out with DNA isolated from different strains and species of *Salmonella*. Heating and annealing was carried out with DNA isolated from each *Salmonella* organism listed in Table 1. The results for all samples were similar to those shown in Fig. 13 for the *Salm. typhimurium* and the heavy-labeled *E. coli* B DNA. No hybrid appears at 10 μg/ml. (top tracing) but an intermediate band does appear at 20 μg/ml. (middle tracing). It is, however, removed by enzyme treatment. The experiments were also repeated using heavy-labeled DNA from *E. coli* K12 since this strain has been used most successfully in the studies of genetic recombination. So far,

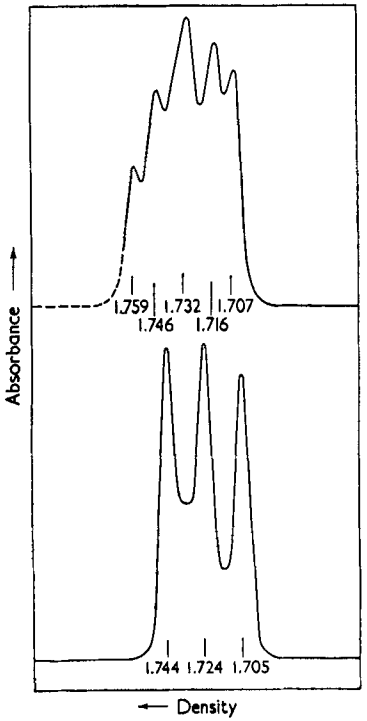

FIG. 11. Interspecies hybrid formation with *B. natto* DNA. Heavy-labeled *B. subtilis* DNA and unlabeled DNA from *B. natto* were heated and annealed at 5 μg/ml. each in 1·9 × SSC. The lower tracing shows the three bands produced when the mixture is treated with the *E. coli* phosphodiesterase.

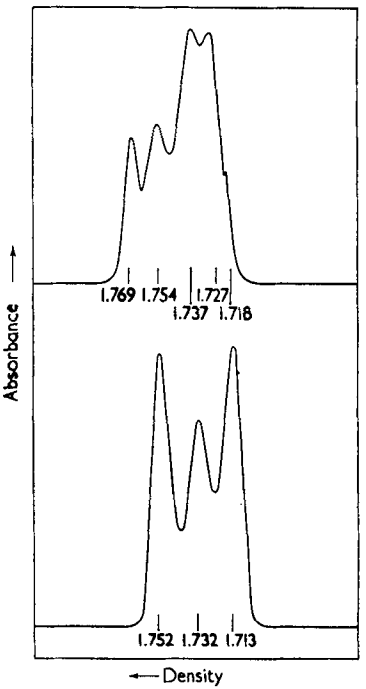

FIG. 12. Interspecies hybrid formation with *Sh. dysenteriae* DNA. Heavy-labeled *E. coli* DNA and unlabeled DNA from *Sh. dysenteriae* were heated and annealed at 5 μg/ml. each in 1·9 × SSC. When the mixture is treated with the phosphodiesterase and centrifuged in CsCl, only three bands appear.

the DNA of only one of the strains of *Salmonella* (*Salm. typhimurium*) has been tried but no hybrid was formed in this case either. We may then conclude that, in general, there seems to be no indication of sequence complementarity between the DNA of *Salmonella* and *E. coli* as measured by hybrid formation.

Thus in this case, where some homology was to be expected, none was found. It is likely that there is homology in some regions of the DNA molecules, but its failure to be displayed suggests that the homologous regions are dispersed or exist in only a few of the several hundred different molecules. Low concentrations of hybrid molecules would not be observed in the analytical ultracentrifuge, but could be isolated by using larger amounts of interacting DNA and working with the preparative swinging-bucket

rotor. In this way we plan to search very carefully for small amounts of hybrid that may be formed between ^{15}N-deuterated DNA from *E. coli* K12 and DNA from any one of the *Salmonella* strains.

Hybrid formation among other members of the family Enterobacteriaceae is now being investigated. Preliminary results with heavy *E. coli* B DNA and normal DNA from a strain of *E. freundii* (5610–52) or of *Erwinia carotovora* (ATCC 8061) show no

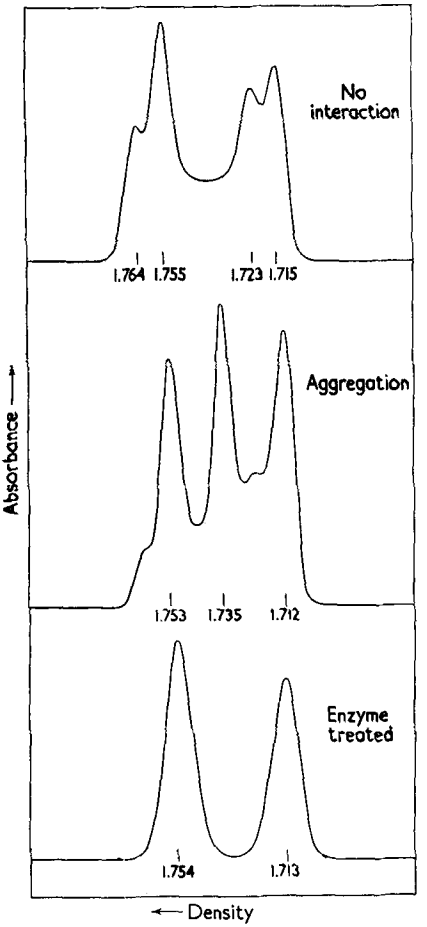

FIG. 13. Attempts at hybrid formation between *Salm. typhimurium* and *E. coli* DNA. The top tracing shows the results of heating and annealing DNA from *Salm. typhimurium* with ^{15}N-deuterated DNA from *E. coli*. Even at 10 μg/ml., which is double the usual concentration, no hybrids have been formed. In the central tracing an intermediate band appears as a result of heating at 20 μg/ml. each. This must be due to aggregates rather than double-stranded molecules since the band disappears after treatment with the phosphodiesterase (bottom tracing).

tendency toward hybrid formation. Unlabeled DNA isolated from another strain (17) of *E. freundii* did form hybrids with labeled *E. coli* B DNA. Since the classification of *E. freundii* is difficult, further studies with DNA of strains of this organism are necessary. Heavily-labeled DNA from *E. coli* K12, *Erwinia carotovora*, and *Salm. typhimurium* have also been prepared, allowing the study of other obvious possible relationships.

From this survey it can be concluded that equality of base composition and some genetic relation are necessary but not sufficient requirements for the formation of

hybrid DNA molecules composed of strands from different organisms. The present work is only indicative in nature but it does suggest that, with the further development of this technique, the degree of sequential homology between two different DNA molecules can be quantitatively explored.

9. Discussion

Until now we have been talking strictly in terms of the concept of strand separation and recombination, in accordance with the evidence presented by Doty *et al.* (1960). It has been seen that some important new facts can now be added to this evidence. By using heavy-isotope-labeled and normal DNA for heating and annealing experiments, it has been demonstrated that the units that unite in renaturation could not be the same as those that were united in the native DNA. It has also been demonstrated that the hybrids formed are not simply physically entangled aggregates. The strong species specificity of hybrid formation indicates that the links that hold the subunits of the hybrid molecules together are determined by the base sequence in each subunit. These links break whenever the hydrogen bonded structure of the molecule is broken. In fact, the rate of separation into subunits corresponds very closely to what has been predicted for the rate of unwinding of the strands of a double helix. It is possible that certain unknown links are broken whenever the Watson-Crick structure is disrupted and that the base sequence determines the specific type of linkage. The most natural explanation, however, seems to be that the strands of the Watson-Crick double helix do separate completely and come back together again through the formation of hydrogen bonds between base-pairs of complementary strands.

The hypothesis that genetic information resides in the sequence of bases in DNA has created a demand for techniques that will allow the study of the linear order of nucleotides along the DNA strand. An approach to this problem has been made in the nearest neighbor studies of Josse, Kaiser & Kornberg (1961) which offers a statistical evaluation of sequences displayed in DNA of various sources. By inference it can be assumed that genetic compatibility is also a measure of similarity in sequences between the DNA of the two parental strains. The present report has outlined a method by which it is possible to measure the extent of renaturation between homologous and heterologous DNA strands and has attempted to add a new dimension to the study of sequences of DNA of microbial origin.

The methodology of molecular hybrid formation *in vitro* has been outlined and its use in the study of similarities of DNA of various groups of micro-organisms has been illustrated. The close correlation between genetic compatibility, taxonomy, and hybrid formation has been mentioned. These applications are discussed in much greater detail elsewhere (Marmur & Schildkraut, 1961*b*; Marmur, Schildkraut & Doty, 1961). Organisms whose taxonomic classification is in doubt might readily be classified by first determining their base composition and studying their interaction by heating and annealing. Such experiments are now being extended to other members of the family Enterobacteriaceae (*Aerobacter*, *Klebsiella*, and *Serratia*) as well as the Pseudomonadaceae (*Pseudomonas Xanthomonas* and *Acetobacter*). Moreover, it might also be possible, by collecting homogeneous fractions of DNA of animal or plant origin, to investigate relationships in a similar manner. Aside from its taxonomic importance the technique offers a rational approach to the study of genetic compatibility where genetic exchanges have not yet been demonstrated.

It is a pleasure to express our gratitude to Dr. L. Grossman for his generous gift of the *E. coli* phosphodiesterase and for his valuable advice. We are especially indebted to Mr. William Torrey for his expert technical assistance with many aspects of this work, and to Mrs. F. Seelig for aid in preparation of the manuscript. We would also like to thank Drs. L. Wierzchowski and Donald M. Green, and Mr. Robert Rownd for their aid. The authors are very grateful to Professor C. E. Hall of the Massachusetts Institute of Technology for the electron micrograph. This investigation was supported by a grant (C-2170) from the National Cancer Institute, United States Public Health Service.

Note added in proof

It can be argued that the DNA species of intermediate buoyant density observed in the CsCl density gradient could be formed by non-specific, end-to-end aggregation of heavy isotope labeled and unlabeled DNA. It is evident, however, that biological hybrids could not be used to form DNA species possessing buoyant densities characteristic of either renatured fully labeled or fully unlabeled DNA unless strand separation and subsequent recombination of similarly labeled strands occurs during the heating and annealing procedure. Preliminary experiments by R. Rownd and D. Green using biological hybrids labeled with both ^{15}N and deuterium in only one strand and isolated from either *Escherichia coli* or *Bacillus subtilis* have shown that the heating and annealing procedure does result in renatured labeled and fully unlabeled, as well as hybrid, DNA molecules. The proportions are the same as observed when a mixture of labeled and unlabeled DNA is used as the starting material.

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