EFFECT OF CONCENTRATION ON THE FORMATION OF MOLECULAR HYBRIDS FROM T_4 DNA

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ABSTRACT When the thymine of T_4 DNA is replaced by 5-BU the melting temperature of T_4 DNA is increased from about 83° to about 93°C. Heating and slow cooling of T_4 DNA at concentrations of about 30 μg/ml leads to aggregates which consist of several polynucleotide chains which appear in the electron microscope as a branched structure. The aggregates have regions which are true hybrids. When the concentration of T_4 DNA is lowered to less than 1 μg/ml the products of hybridization are not aggregates but have the morphology of native DNA molecules and the density labels are distributed as expected from the fusing of two chains of approximately equal length.

INTRODUCTION

Marmur and Lane (1) have shown that DNA molecules denatured by heating can be made to reform into biologically active DNA molecules if the cooling is carried out slowly. It has been further shown (1, 2) that it is possible to form biologically active hybrid molecules in which strands or parts of strands are derived from genotypically different DNA molecules.

Electron micrographs of DNA after heating and slow cooling have already been published (3). In these the DNA did not appear to be single molecules with 20 A diameter but rather intertwined aggregates. In this paper we report on factors relevant to the formation of aggregates during the hybridization process. It is shown that at high concentrations of DNA aggregates with very high molecular weight are formed. In the electron microscope these appear as branched structures. However, at low concentrations pure hybrids seem to be produced and these have a morphology similar to native DNA.

Our analysis of the hybridization process involves the use of DNA in which a high proportion of the thymine is replaced by 5-bromouracil (5-BU). If all the thymine is replaced by 5-BU the DNA has a density of 1.80 as measured in a CsCl density gradient (Kozinski, unpublished). This is considerably higher than the density of 1.69 characteristic of normal T_4 DNA. After hybridization the posi-

tion of the DNA in the density gradient reflects its content of heavy DNA (labeled with 5-BU) and light DNA (without 5-BU label). This procedure is essentially that of Meselson and Stahl (5) who, however, used N^{15} labels.

METHODS

Both the 5-BU T_4 DNA and the light DNA were prepared by the same procedures as were used in a previous study (4).

The sedimentation constant for both the normal and the labeled DNA was about 35S indicating a molecular weight of about 5×10^7 (*Cf.* 5). In all the melting and hybridization experiments DNA was dissolved in 0.15 M sodium chloride and 0.15 M tris buffer at pH 7.4. The DNA solutions in pyrex test tubes were immersed in water at 100°C in a 5 liter beaker. Slow cooling was accomplished by leaving the beaker covered at room temperature overnight.

The CsCl centrifugations were carried out as described previously (4). The swinging bucket head SW 39L of a Spinco preparative ultracentrifuge was spun at 35,000 RPM for 72 hours. Afterwards the contents were drained through a hole punctured in the bottom of the tube and about 30 fractions were collected for ultraviolet light absorption measurements and P^{32} decay counts.

The melting of the DNA was followed spectrophotometrically in a Cary Model 11 recording spectrometer equipped with a cell holder that could be maintained at any temperature below 100°C. Sonication was carried out in a Raytheon sonicator at 10,000 cycles per second for 5 minutes. The resulting DNA had a sedimentation constant of about 8S.

Two types of studies were carried out with the electron microscope. The first was to determine the lengths and diameters of hybrid molecules. This was done by a method previously described for native DNA (7). DNA was diluted to a concentration of 1 to 0.1 μg/ml with volatile ammonium acetate—acetic acid buffer at pH 6.3. Grids covered with styrene-vinylpyridine copolymer or evaporated carbon film were streaked, membrane side down, along the DNA solution. Excess liquid was removed taking care that it should recede in a direction parallel to the streaking. Grids were rinsed in water or ethanol and then shadowed with platinum in a direction perpendicular to the streaking direction.

The second type of study was carried out to check that the streaking procedures used above do not select for molecules of a given morphology. This was done by spreading a drop of 1 μg/ml DNA in volatile buffer solution on freshly cleaved mica, shadowing with platinum and taking a replica as described by Hall (8).

RESULTS

1. *The Melting Temperature of DNA Heavily Labeled with 5-BU*

The temperatures in hybridization experiments must be sufficiently high to melt all the DNA. Since the melting temperature for DNA labeled with 5-BU was not known when this work was begun, we determined it.

The optical density as a function of temperature is plotted in Fig. 1 for both normal T_4 DNA and 5-BU T_4 DNA. It is seen that the two curves are similar in shape but that the melting temperature is $7 - 8°$ higher for 5-BU T_4 DNA.

The DNA used in these experiments had about 80 per cent of its thymine replaced by 5-BU as determined by banding in CsCl density gradient. The optical densities of the fractions obtained from the centrifuge tubes are shown in Fig. 2*a*.

2. *Hybridization in Concentrated Solutions of DNA*

(*a*) *30 μg/ml 5-BU DNA, 0.3 μg/ml P^{32}-Labeled T_4 DNA.* A reaction mixture was made up containing 30 μg/ml of heavy, non-radioactive, DNA and 0.3 μg/ml of light P^{32}-labeled DNA. Prior to heating this mixture, an analysis by

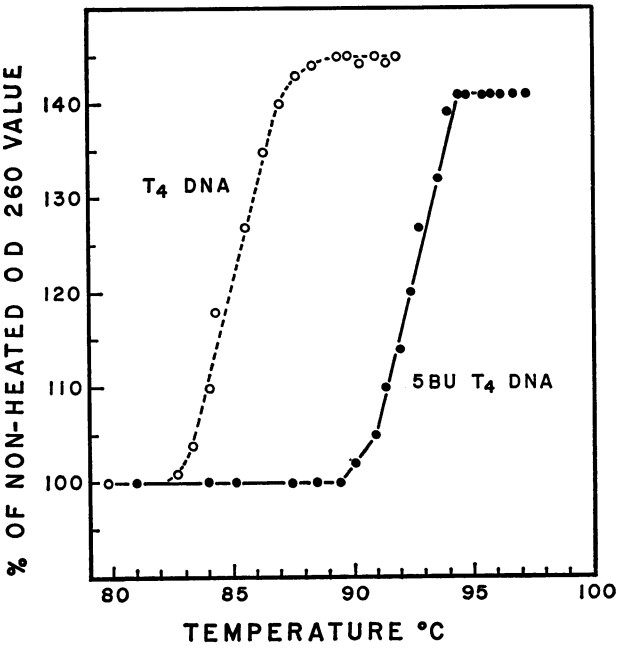

FIGURE 1 Melting of T_4 DNA and 5-BU T_4 DNA.

centrifugation in the CsCl density gradient gave the results shown in Fig. 2*a*. The light DNA marked by the P^{32} activity was centered about 1.70 gm/cc. The heavy DNA, which was responsible for essentially all the UV absorption, formed a band between 1.77 and 1.80 gm/cc. The spread of the band indicated that there was some heterogeneity in the extent of 5-BU labeling.

Fig 2*b* shows the results after heating followed by slow cooling. It is seen that a single narrow band contained both the P^{32} activity and the UV absorption, indicating the presence of only one species with density about 1.78 gm/cc.

If the solution was diluted 100 times after cooling and then banded in a CsCl density gradient, the P^{32} activity was again found in the same narrow band as in the concentrated solution. This indicates that the band position is independent of

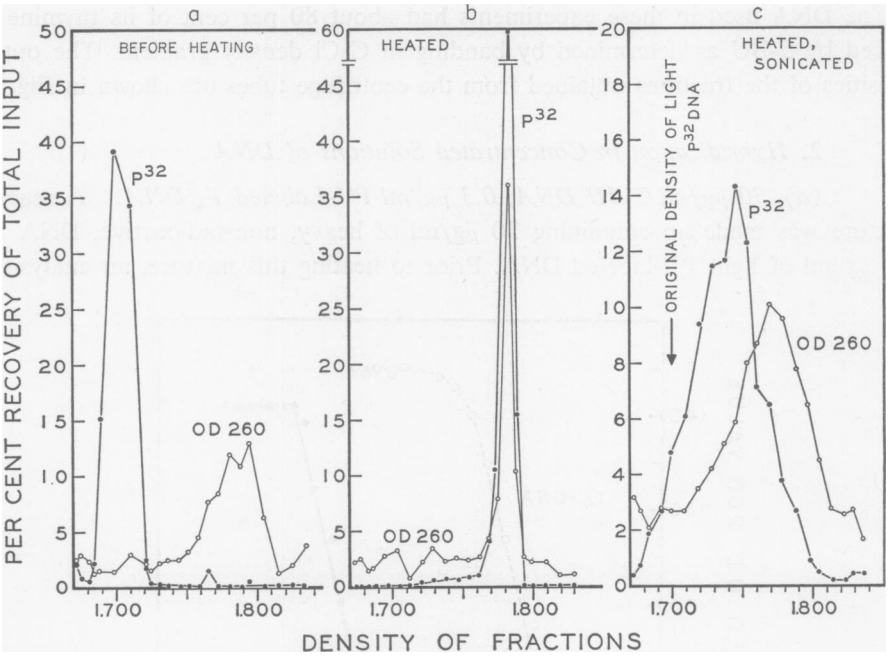

FIGURE 2 Hybrid formation in solution containing 30 μg/ml of non-radioactive 5-BU DNA and 0.3 μg/ml of light P^{32} DNA. (*a*) Distribution of UV-absorbing material and P^{32} activity in CsCl gradient of the mixture before heating. (*b*) The same mixture as in (*a*), but after heating and slow cooling. (*c*) The same as (*b*), but after sonication.

the concentration of the DNA during the centrifugation. The optical density was not determined in this very dilute solution.

If the DNA solution characterized in Fig. 2*b* was disrupted by sonication (under conditions known to produce fragments with molecular weight a quarter to a half million) and the product was subsequently banded in CsCl density gradient, the pattern shown in Figure 2*c* was obtained. Clearly the band containing the P^{32} activity moved to a new position about midway between that of the light DNA and the heavy DNA. In addition the two bands containing the P^{32} activity and the UV absorption became appreciably wider than prior to sonication.

(*b*) *0.2 μg/ml 5-BU DNA with P^{32} Label, 20 μg/ml Light Non-Radioactive DNA.* When this reaction mixture was analyzed after heating and slow cooling the pattern shown in Fig. 3 was obtained. Clearly the minority and the majority DNA again formed a single band near the density of the majority.

In another experiment the same mixture was disrupted by sonication prior to heating. An analysis in CsCl density gradient led to the results shown in Fig. 4. The radioactivity was found near 1.78 while the optical density was near 1.69 showing that sonic disruption did not change the mean densities.

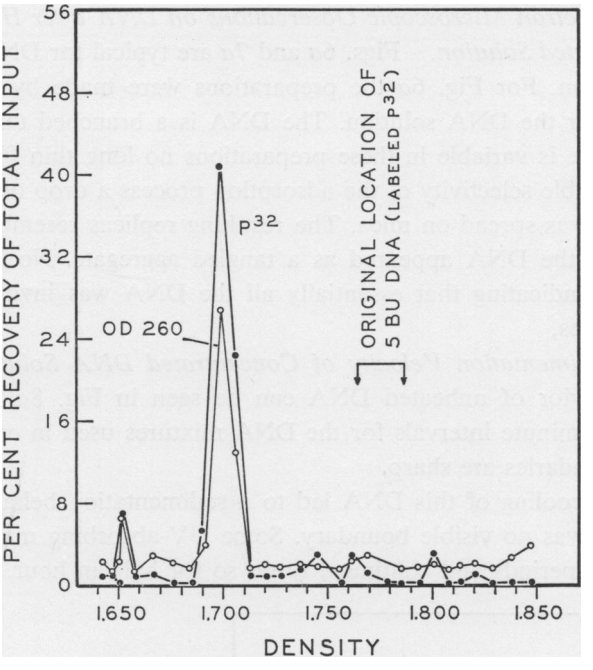

FIGURE 3 Hybrid formation in solution containing 20 μg/ml of non-radioactive light DNA mixed with 0.2 μg/ml of P^{32}-labeled 5-BU DNA. Figure shows UV absorption and P^{32} activity after heating and slow cooling.

Even if the DNA was disrupted by sonication the product obtained by heating and slow cooling had a density essentially that of the majority component. This is shown in Fig. 4. Both the radioactivity and the UV absorption banded near the UV-absorbing material in the unheated preparation (Fig. 4). Thus, the disrupted material behaves in this respect as the high molecular weight DNA.

(*c*) *15 μg/ml 5-BU DNA, 15 μg/ml Light P^{32} Labeled DNA.* When this reaction mixture was analyzed after heating and slow cooling the pattern shown in Fig. 5*a* was obtained. Here the density and P^{32} activity again moved to a single peak. Its position, however, was midway between the densities of light and heavy DNA.

After sonic disruption the analysis obtained was the one shown in Figure 5*b*. It is seen that the P^{32} activity is spread from the mid position between light and heavy DNA to the original position of light DNA.

(*d*) *Hybridization of Mixtures of DNA from Different Sources.* When a mixture of 15 μg/ml of light DNA from H. Influenzae and 15 μg/ml of P^{32} labeled heavy T_4 DNA were heated together, cooled slowly and analyzed in a CsCl density gradient it was found that the P^{32} activity all remained in the density region it occupied before heating.

(*e*) *Electron Microscopic Observations on DNA after Heating and Cooling in Concentrated Solution.* Figs. 6*a* and 7*a* are typical for DNA cooled in concentrated solution. For Fig. 6*a* the preparations were made by streaking an adsorbing film over the DNA solution. The DNA is a branched tangle. Though the size of the tangle is variable in these preparations no long thin fibrils were found. To test the possible selectivity of the adsorption process a drop of DNA in volatile buffer solution was spread on mica. The resulting replicas resembled those in Fig. 7*a*. Here again the DNA appeared as a tangled aggregate. No long thin strands were observed indicating that essentially all the DNA was involved in these extensive aggregates.

(*f*) *Sedimentation Velocity of Concentrated DNA Solutions.* The sedimentation behavior of unheated DNA can be seen in Fig. 8*a* which shows exposures at four minute intervals for the DNA mixtures used in experiments 1, 2*a*, and 3. The boundaries are sharp.

Heating and cooling of this DNA led to a sedimentation behavior as shown in Fig. 8*b*. There was no visible boundary. Some UV-absorbing material sedimented after very short periods and continued to do so for half an hour.

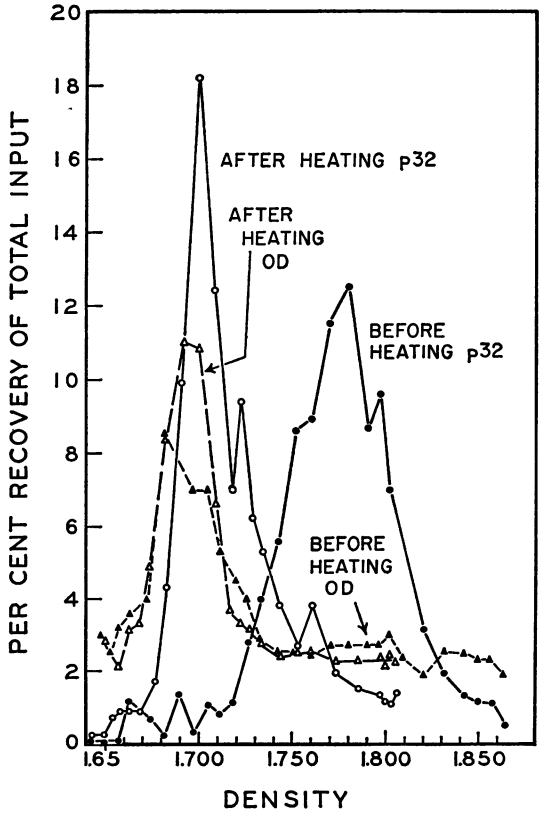

FIGURE 4 Hybrid formation in sonicated solution containing 20 μg/ml of non-radioactive light DNA, and 0.2 μg/ml of P^{32}-labeled 5-BU DNA. After heating both UV absorption and P^{32} activity are found in density range characteristic of majority light DNA.

3. *Hybridization in Dilute Solutions*

(*a*) *0.6 μg/ml 5-BU DNA 0.006 μg/ml P^{32} Labeled T_4 DNA.* The DNA mixture used in these experiments was the same as in experiment 2*a* (Fig. 2) but diluted 50 times. Because of the very low concentrations the optical density in this experiment could not be measured. It is clear however from Fig. 9 that the results were quite different from those obtained in concentrated solutions. Here the P^{32} activity formed a band in a new position centered about 1.74 gm/cc which was approximately midway between light and heavy DNA as shown by the curve marked "after heating." This is to be contrasted with experiment 2*a*, where the P^{32} activity moved to the density value of the majority DNA.

When examined in the electron microscope (Figs. 6*b* and 7*b*) such DNA preparations were found to consist of long fibrils with thickness about 20 ± 10 A as determined from the shadow dimension. The molecules were unbranched and were indistinguishable from native DNA. The mica preparations were made in an attempt to check that the streaking procedure did not select for the unbranched

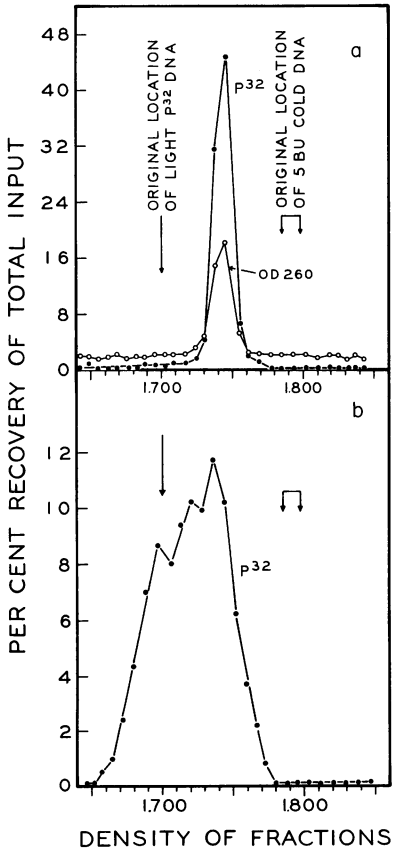

FIGURE 5 Hybrid formation in solution containing 15 μg/ml of light P^{32}-labeled and 15 μg/ml non-radioactive 5-BU DNA. (*a*) After heating and slow cooling P^{32} activity and UV absorption move to intermediate density. (*b*) Same as (*a*), but after sonication P^{32} activity spread from light to intermediate density.

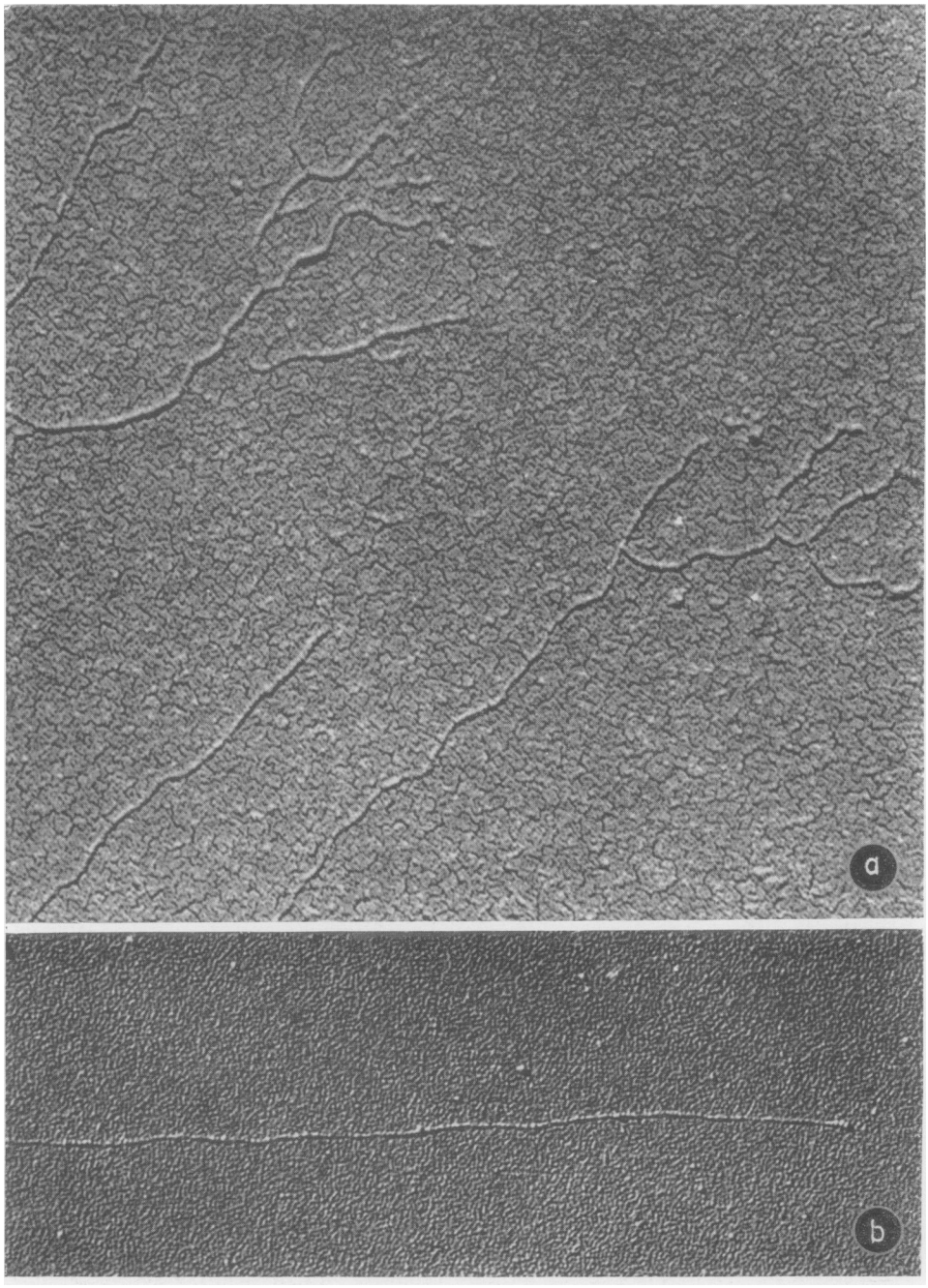

FIGURE 6 DNA streaked on carbon film after heating and slow cooling. The concentration of DNA during heating and slow cooling was (*a*) 30 μg/ml (*b*) 0.6 μg/ml. × 70,000.

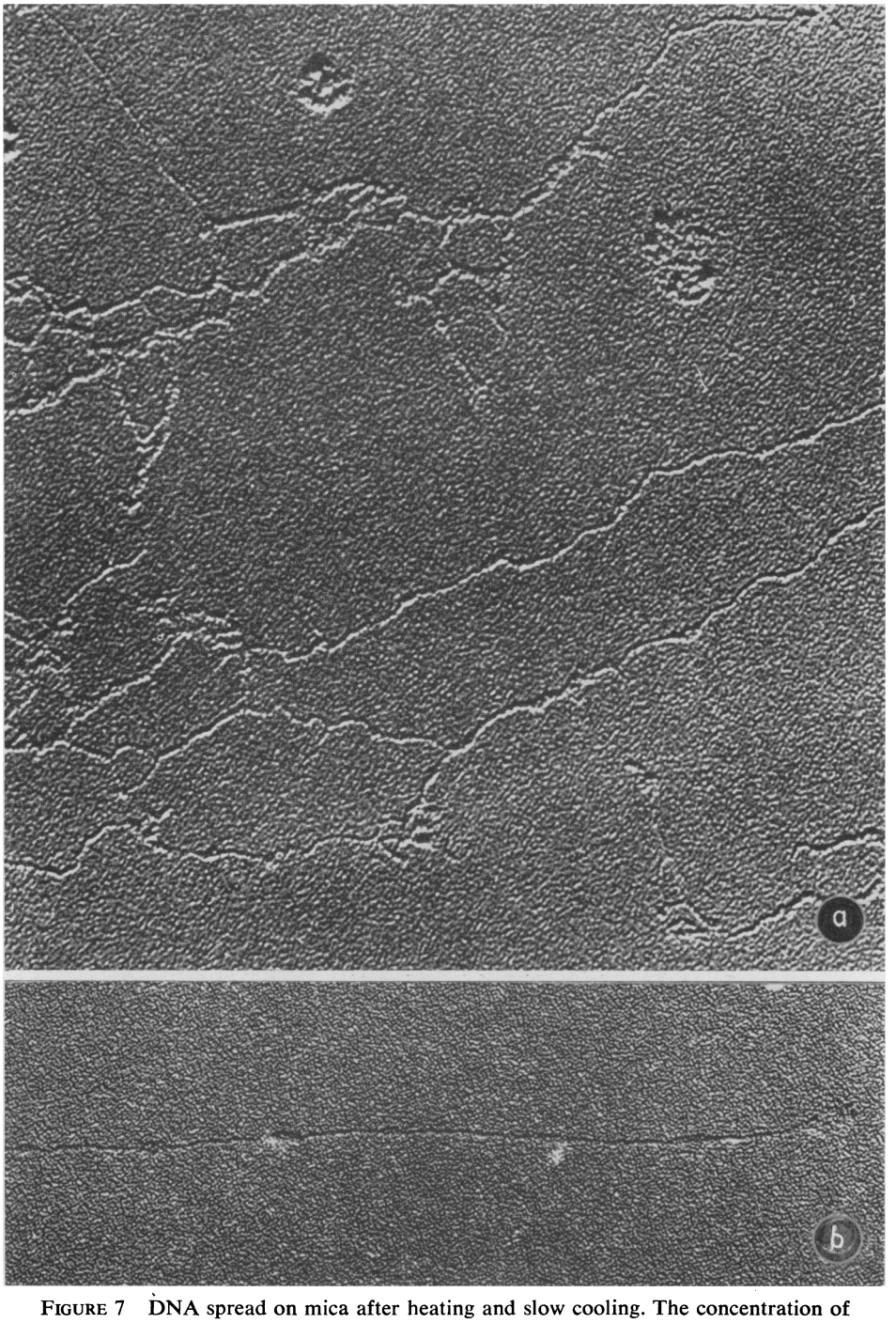

FIGURE 7 DNA spread on mica after heating and slow cooling. The concentration of DNA during heating and slow cooling was (*a*) 30 μg/ml (*b*) 0.6 μg/ml. × 70,000.

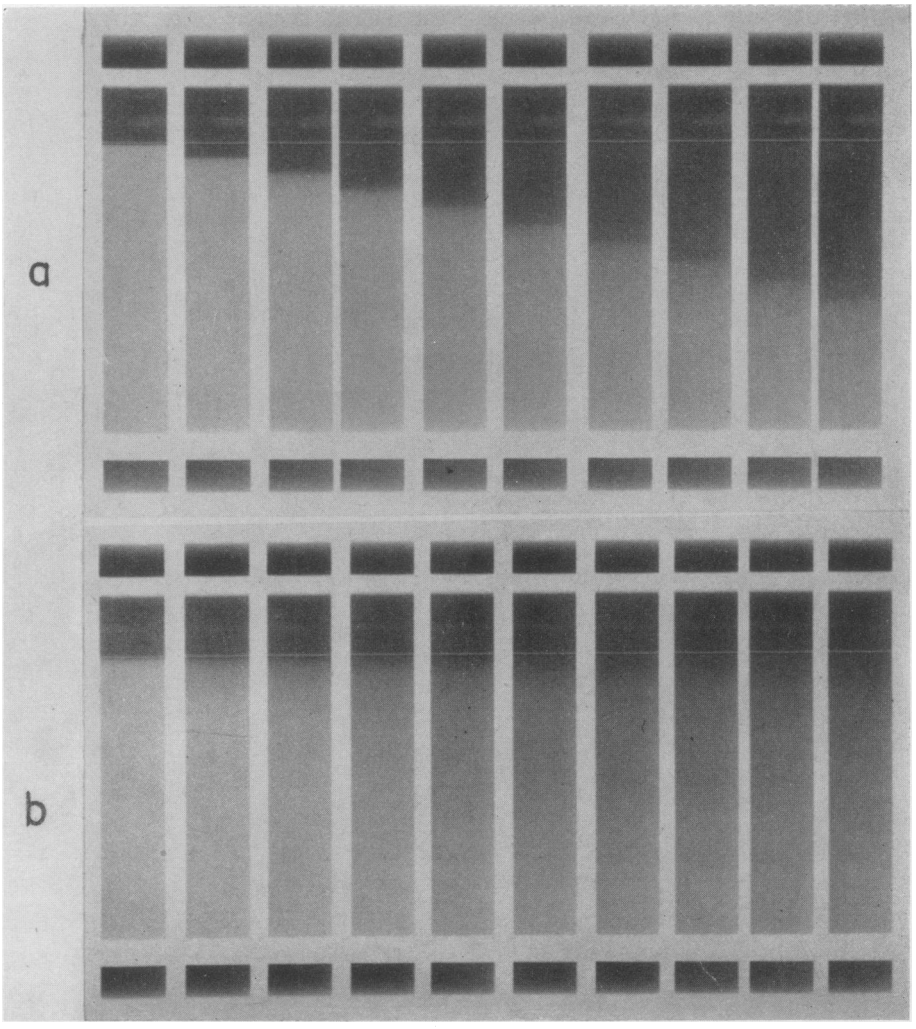

FIGURE 8 Sedimentation pattern of T_4 DNA. (*a*) before heating and cooling. (*b*) after heating and cooling.

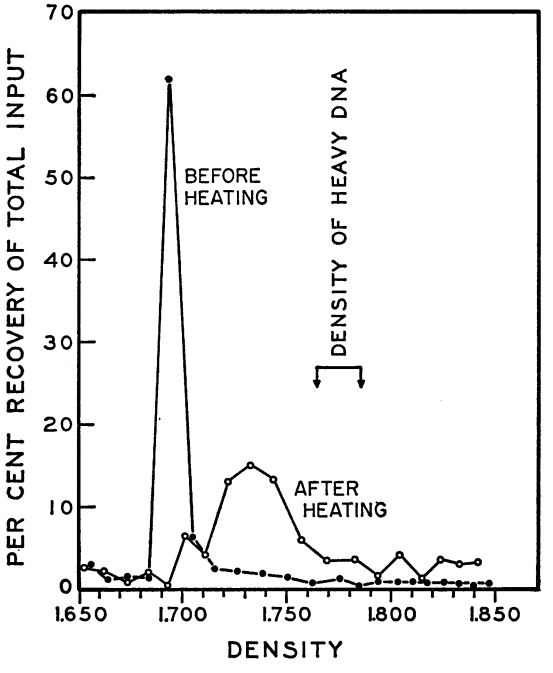

FIGURE 9 Hybrid formation in solution containing 0.6 μg/ml of non-radioactive 5-BU DNA and 0.006 μg/ml of light P^{32} active DNA. The P^{32} activity before heating has the density of light DNA. After heating the P^{32} activity has the density characteristic of equal amounts of heavy and light DNA.

molecules. The DNA on the mica preparations consisted again of fibrils characteristic of DNA. No evidence of branched molecules was found.

The lengths of twelve DNA fibrils were measured after recording on serial exposures in the electron microscope. The average lengths of these was about 10 μ with the longest near 30 μ.

DISCUSSION

1. *Melting Temperature of 5-BU T_4 DNA*

The melting temperature of our 5-BU T_4 DNA was about 8°C higher than for T_4 DNA. The extent of replacement of thymine by 5-BU was about 80 per cent. A reasonable approximation is that the increase in melting temperature is proportional to the extent of substitutions. This is to be expected in analogy with the finding of Marmur and Doty (9) that the melting temperature is a linear function of the abundance of G-C base pairs.

Our observations are consistent with the recent results of Kit and Hsu (10) who also found a rise in the melting point when 5-BU was substituted for the thymine in DNA.

2. *Hybridization of DNA at Various Concentrations*

The three types of experiments in which the DNA concentration exceeded 20 μg/ml all gave rise to a narrow band, the position of which corresponded to the average density of all the DNA in the solution. The simple hybridization of half molecules

must here be ruled out since the minority of radioactive DNA would then have banded at an intermediate density. Our experiments show that the units formed on slow cooling are aggregates, and have the average density of the system.

Much of the aggregate formed at high concentrations of DNA sediments very rapidly showing that its molecular weight is much greater than that of the original molecule. In the electron microscope these products of hybridization appear as a large tangled or branched structure.

The aggregates contain portions of hybrids. This is seen from the results of the sonic disruption, which leads to fragments in which the radioactive minority DNA is always found at the intermediate densities as expected for hybrids formed from equal quantities of heavy and light DNA. Fusion of two minority type strands would occur with very low probability, and therefore light DNA would not be expected after heating and cooling.

When, however, the light radioactive DNA had the same concentration as the 5-BU unlabeled DNA the probability of light-light hybrid formation was appreciable. This was indeed found to be the case as shown in Fig. 5*b*. Here the radioactivity is spread from the density characteristic of light DNA to that of pure hybrid.

The specific nature of the hydrogen bonding is further shown by the fact that when the DNA comes from entirely different sources—T_4 phage and *H. influenzae* —after heating and slow cooling the two types of DNA band separately indicating that there is no entangling between them.

These results can all be interpreted on the assumption that in the products of hybridization at high concentration a polynucleotide chain forms hybrids with different molecules over different regions. This would be expected if the concentration is so high that different regions of one molecule collide almost simultaneously with several other molecules.

In the experiments where the DNA concentration was below 1 μg/ml, the radioactive minority DNA moved to a density range midway between the light and the heavy DNA. This indicates that the units of light radioactive DNA formed during heating became associated with about equal quantities of heavy DNA. The band of the hybrid centering about 1.73 to 1.74 gm/cc is quite broad. This breadth reflects the breadth of the heavy DNA band and is associated with its heterogeneity of density due to variation in label carried by different molecules.

Density heterogeneity can also arise if the two polynucleotide chains involved in forming a hybrid are of unequal length. Then in the resulting hybrid the excess length of the longer chain will remain a single polynucleotide chain. Since single chains have a slightly greater density than the double strands the presence of such a component will increase the density of the hybrid. If the proportion of a single chain is variable the effect on the density would be also variable.

The density change arising from single strand portions of the DNA cannot however account for the density shift observed in our experiments with dilute DNA.

(Fig. 9) This is clear from the fact that when dilute solutions (2 to 0.5 μg/ml) of light, P^{32}-labeled DNA are heated to the melting temperature and cooled slowly the change in density as compared with light non-heated carrier DNA is negligible (12). Thus, the single strand portions remaining during hybrid formation cannot account for the density shift observed. This must therefore be attributed to the formation of hybrids between equal amounts of light and heavy DNA.

The clean fibrils observed in the electron microscope also indicate that at these low concentrations true hybrid molecules, not branched aggregates, are formed.

The sonication of aggregates yields pure hybrids of low molecular weight. This rules out the possibility that hybrid density is due to an end-to-end attachment of unmelted molecules with different densities. If the intermediate density were due to such attachment, sonication would give mostly pure high density or pure low density with perhaps a very small amount of intermediate density of the portion which includes the junction of the heavy and light molecules.

The lengths of the hybrid molecules range up to near 30 microns with the average about 10 microns. The molecular weight of these fragments can be estimated on the assumption that they have the mass to length ratio consistent with the Watson-Crick model of DNA. Such an assumption appears a fair one in the light of measurements on known fragments of T_4 DNA (11). The total phage content of about 12×10^7 molecular weight (6) then corresponds to about 60 microns of DNA. Accordingly the hybrid molecules observed here have lengths up to a half of the total content but mostly somewhat under a quarter. Since the molecular weight of the starting material was about half the phage content it appears that during hybridization the molecular weight dropped by a factor of about 2 to 3.

The competent assistance of Mrs. P. B. Kozinski and Miss Doris Naimark is gratefully acknowledged. The DNA from *H. influenzae* was the generous gift of Dr. R. M. Herriott.

This research was supported by National Science Foundation Grants G-15076 and G-7595.

Received for publication, September 13, 1961.

REFERENCES

1. MARMUR, J., LANE, D., *Proc. Nat. Acad. Sc.*, 1960, **46**, 453.
2. HERRIOTT, R. M., *Proc. Nat. Acad. Sc.*, 1961, **47**, 146.
3. DOTY, P., MARMUR, J., EIGNER, J., and SCHILDKRAUT, C., *Proc. Nat. Acad. Sc.*, 1960, **46**, 461.
4. KOZINSKI, A. W., *Virology*, 1961, **13**, 124.
5. MESELSON, M., and STAHL, F. W., *Proc. Nat. Acad. Sc.*, 1958, **44**, 671.
6. RUBENSTEIN, I., THOMAS, C. A., JR., and HERSHEY, A. D., *Proc. Nat. Acad. Sc.*, 1961, **47**, 1113.
7. BEER, M., *J. Mol. Biol.*, 1961, **3**, 263.
8. HALL, C. E., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 625.
9. MARMUR, J., and DOTY, P., *Nature*, 1959, **183**, 1427.
10. KIT, S., and HSU, T. C., *Biochem. and Biophysic. Research Communications*, 1961, **5**, 120.
11. BEER, M., NAIMARK, D., and HERSHEY, A. D., data to be published.
12. KOZINSKI, A. W., to be published.