heterologous DNA even if it has the same over-all base composition as T2-DNA. It is concluded that T2-DNA and T2-specific RNA form hybrids because they possess complementary nucleotide sequences. The generality of the existence of complementary RNA and its possible role as a carrier of information from the genetic material to the site of protein synthesis is briefly discussed.

We are indebted to Drs. Noboru Sueoka and John Drake for providing the T5 DNA and *Pseudomonas* DNA used in this work and to Miss Cherith Watson for her assistance in performing the experiments.

* This investigation was aided by grants in aid from the U.S. Public Health Service, National Science Foundation, and the Office of Naval Research.

2 Volkin, E., these *Proceedings*, 46, 1336 (1960).
4 Marmur, J., and D. Lane, these *Proceedings*, 46, 453 (1960).
8 Marmur, J., personal communication.

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**FORMATION OF HETEROZYGOTES BY ANNEALING A MIXTURE OF TRANSFORMING DNAs**

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An important discovery was made when Marmur and Lane observed that the genetic properties of pneumococcal transforming DNA which had been lost through heating to 100°C were restored by slow cooling. The loss of function was considered to be due to denaturation, a rupture of the inter-strand hydrogen bonds followed by a separation of the strands, and Marmur and Lane suggested that restoration occurred by a highly specific pairing of complementary strands—a process favored by slow cooling. Since recovery of genetic function of a small quantity of transforming DNA was enhanced by the addition of unmarked but
homologous DNA from recipient pneumococci and not with heterologous DNA, the authors suggested that a marked strand might pair with the complementary strand of an unmarked but otherwise equal molecule and form a mixed molecule or "heterozygote." Physical chemical evidence in support of this suggestion was presented by Doty, Marmur, Eigner, and Schildkraut.  

To test their suggested mechanism, we decided to attempt the formation of doubly marked heterozygotes by heating and annealing a mixture of differently marked transforming DNAs. Markers known to link were chosen, for they would represent allelic or similar molecules and their strands would be expected to complement each other to a high degree. Although segregation of markers might well have obscured detection of the heterozygotes, the present results show that a physical unit carrying both markers was formed by annealing a heated mixture of these two DNAs.

Materials and Methods.—In general the methods used were described previously. A detailed account of all the methods will appear elsewhere.

Genetically marked DNAs: Four differently marked DNAs were employed in this work. They were isolated from cell stocks of Hemophilus influenzae resistant to 250 \( \mu \)g/ml of streptomycin (S), 2.5 \( \mu \)g/ml cathomycin (C) (novobiocin), 6–10 \( \mu \)g/ml of erythromycin (E), and finally a stock resistant to both streptomycin and cathomycin (S-C). The S-C stock organism was obtained by transforming competent streptomycin-resistant cells with C DNA. It could also have been prepared by the reciprocal transformation. Each DNA preparation, after first lysing the organisms at pH 11, was purified by the chloroform-octanol method of protein removal followed by digestion with RNAase and repeated precipitation from 1.5 M NaCl-35 per cent ethanol solution. The E260/E230 ratio of the preparations ranged from 2.3 to 2.4.

Heating and annealing: In general, a 1-ml sample of purified DNA dissolved in 0.5 M NaCl-0.01 M Na citrate was placed in a 15 ml screw-capped tube and heated in four liters of water at the indicated temperature, after which the tube was transferred rapidly to four liters of water at 90°C, which was then allowed to cool without stirring. For those experiments at temperatures above 100°C, the 1-ml samples were sealed into glass tubes and then heated in a glycol bath. When the bath temperature was 100°C, the temperature in the sample rose to 95°C or higher in one minute. In annealing, the temperature dropped from 90° to 60°C in 100 minutes and to 37°C in another 100 minutes. Cooling from 100°C instead of 90°C gave similar but somewhat lower recoveries of activity.

Transformation procedure: Unless otherwise noted, the transformation reaction was carried out by mixing 0.1-ml competent receptor cells (1.5 \( \times 10^8 \)/ml) with 2.8 ml saline and 0.1 ml of 0.2 \( \mu \)g/ml DNA, a 1/100 dilution of the annealed DNA solution. After thirty minutes' incubation, an aliquot was diluted appropriately and plated with nutrient-agar after which it was incubated...
two hours for development of antibiotic resistance. The plates were then covered with an equal volume of nutrient-agar containing the antibiotic at double strength. The plates were incubated twenty-four to forty-eight hours and the resulting colonies counted. Duplicate plates agreed within ±20 per cent but duplicate experiments were more variable.

Experimental Results.—Doubly marked transformants from a mixture of singly marked units: The results of an experiment in which a mixture of S and C DNAs was heated to 100°C and then annealed are shown in (c) of Table 1. As controls, four additional tubes were included. The first (a) was an unheated sample; (b)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Temperature of heating</th>
<th>Cooling procedure</th>
<th>Marker</th>
<th>Dilution</th>
<th>Count</th>
<th>Titer in reaction mixture</th>
<th>S-C cells expected (product of frequencies of singles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>25°C</td>
<td>Slow</td>
<td>S</td>
<td>1 × 10^3</td>
<td>261</td>
<td>2.7 × 10^4</td>
<td>24</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>2 × 10^4</td>
<td>232</td>
<td>4.7 × 10^4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S-C</td>
<td>9</td>
<td>9</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>90°C</td>
<td>Slow</td>
<td>S</td>
<td>1 × 10^2</td>
<td>229</td>
<td>2.2 × 10^4</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>4.3 × 10^4</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>S-C</td>
<td>9</td>
<td>13</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>100°C</td>
<td>Slow</td>
<td>S</td>
<td>5 × 10^1</td>
<td>259</td>
<td>1.2 × 10^4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>1 × 10^3</td>
<td>232</td>
<td>2.8 × 10^4</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>S-C</td>
<td>252</td>
<td>272</td>
<td>2.7 × 10^4</td>
<td>7</td>
</tr>
<tr>
<td>d</td>
<td>100°C</td>
<td>Rapid</td>
<td>S</td>
<td>2 × 10^1</td>
<td>38</td>
<td>6.8 × 10^3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>5 × 10^1</td>
<td>102</td>
<td>5 × 10^3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S-C</td>
<td>0</td>
<td>105</td>
<td>&lt;1</td>
<td></td>
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<tr>
<td>e</td>
<td>100°C</td>
<td>Slow</td>
<td>S</td>
<td>2 × 10^1</td>
<td>526</td>
<td>1.2 × 10^4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>5 × 10^1</td>
<td>525</td>
<td>2.9 × 10^4</td>
<td>7</td>
</tr>
</tbody>
</table>

was the mixture heated to 90°C and then cooled slowly; in (d), the mixture was
heated and chilled rapidly instead of annealing; and finally in (e), the two DNAs,
S and C, were separately heated to 100°C and cooled before they were mixed. The
number of double transformants expected from the random uptake of the individual
markers was calculated in Table 1 from the product of the individual frequencies.
In the above system, where the cell concentration was 5 × 10^9/ml, the
expected number of doubles equals the product of the S and C transformations
divided by the number of cells.
An examination of the results in Table 1 shows the following:

1. Heating the mixture of S and C DNAs to 100°C followed by a slow cooling (c) produced many more S-C transformations, i.e., to both markers, than expected from a random distribution of S and C units.

2. The number of S-C transformations from the annealed mixture was greater than observed in the unheated mixture (compare c and a); i.e., there was a net increase.

3. Formation of the S-C unit required a temperature above 90°C, for at this temperature no unexpected double transformations were observed (b).

4. Slow cooling is important to formation of this S-C unit for there were few if any unexpected doubles when the heated sample was chilled suddenly (d).

5. S-C units do not form if the DNAs are mixed after heating and annealing. They must anneal together (e).

The Nature of the New Agent Producing Double Transformations.—The effect of concentration of annealed DNA on the number of double transformations:—The results in Table 1 show that the increase in doubles after heating and annealing could not have been produced by the random uptake of residually active individual units, but conceivably some type of reactivation or nonrandom uptake of these markers following the heat treatment might produce the observed effect, although the former seems unlikely in the light of the net increase in doubles over those present in the unheated sample. Except for an extreme case of nonrandom uptake in which the uptake of one marker produced a strong preference for the other, it would be expected that doubles produced by two physical units could be distinguished from those produced by a single unit carrying both markers by the manner in which the double transformations varied with DNA concentration. Doubles produced by two units would vary with the square of the DNA concentration, whereas those produced by a single unit would vary directly. The results of such a test are shown in Figure 1, where the double transformations obtained before and after annealing are plotted against the dilution of DNA. The solid lines are the curves expected if before annealing, the doubles arise from two independent units and after annealing, from a single physical unit. For comparison and to bring out the difference in the two systems, the fraction of the double transformations is plotted on a logarithmic scale against the simple dilution of the DNA.

The results in Figure 1 show clearly that annealing changed the agents responsible for double transformations. After annealing, dilution of the DNA produced the effect expected if the doubles were produced by a single unit. Titers of the doubles obtained after heating were adjusted when the contribution from random doubles was greater than 10 per cent. The results are strong evidence that a single physical unit carrying both genetic markers (a heterozygote) has been produced by heating and annealing a mixture of these two DNAs.

Effect of ultraviolet radiation on the heterozygote: Ultraviolet radiation was used to detect a difference between the hetero- and homozygous doubly marked units. The results are shown in Figure 2. The difference is clear but difficult to interpret other than to state the obvious fact that the heterozygous unit was less sensitive to ultraviolet radiation than was the homozygous S-C.

The mechanism of heterozygote formation: Most of the conditions favoring heterozygote formation in the highest concentration were those which Marmur and
Lane\(^1\) reported for maximal restoration of transforming activity. Thus, about 20 \(\mu g\) DNA/ml in 0.5 \(M\) NaCl heated to 100°C for a few minutes and then cooled slowly from 90°C appeared to be optimal, but some heterozygotes were formed in 0.05 \(M\) NaCl. In studying the mechanism of formation, increasing quantities of one marker were mixed with decreasing quantities of the other marker, so the total DNA was constant, and after heating and annealing, the mixtures were scored for the number of single and double transformations. Figure 3 shows a plot of the fraction of the total of each marker present as a double transformant in the various mixtures of DNA. If the annealing process is highly efficient and the mechanism of reformation is the specific strand pairing suggested by Marmur and Lane, then, when a small quantity of one marker (S) was heated with a relatively large quantity of the second (C), it would be expected that a high proportion of the S would be found in the heterozygotes, for there would be more C strands to unite with S strands than there were complementary S strands. As seen in Figure 3, the proportion of the total S as S-C (SC/S) did increase as the fraction of S in the initial mixture of DNA decreased, but even in the extreme case of 0.5/19.5 or 0.1/19.9, the proportion of S appearing as heterozygote did not exceed 0.05. In a comparable way, the proportion of C appearing as heterozygote did not exceed 0.02. The experiment has been repeated with many variations without exceeding these figures, which suggests that perhaps they are upper limits. Neither the linear effect in Figure 3 nor the low recovery of heterozygotes is in keeping with the simple bimolecular reaction such as specific strand pairing. In view of the relatively high (often 50 per cent or more) recovery of the individual markers it appears that an unrecognized feature of this system must be controlling heterozygote formation. Several possible explanations will now be considered, some of which permit experimental testing.

A. The number of heterozygotes in the annealed solution may be greater than observed with the biological assay responsible for the reduced number. Thus, segregation (separation of the genetic units into different daughter cells) or recombinational events acting on such an unusual unit as the heterozygote may be
responsible for the low recovery. A clonal analysis should throw light on the possibility of segregation.

B. Perhaps due to their complete complementarity, pure singles reform during cooling at a slightly higher temperature than do the heterozygotes, so that when the temperature falls to that permitting heterozygotes to form, the number of separated strands is lower than expected. On this basis, the heterozygotes should melt at a lower temperature than the individual markers on reheating. Experiments thus far on this point are not decisive. This explanation is weak, however, in view of the results in Figure 3.

C. Under the conditions of heating, the strands of most of the molecules may not completely separate despite the nearly complete loss of function and a rise in chromicity on chilling. However, extending the time of heating or, as shown in

Figure 4, raising the temperature above 100°C to separate the strands of more molecules failed to increase heterozygote formation, so that this explanation lacks direct support.

D. If the strand complementarity requirements for reformation are less specific than originally proposed and strands from nonallelic molecules have sufficient complementarity (points of hydrogen bonding) to pair with the marked strands and thereby prevent reunion of the more complementary strands and also to prevent collapse of the marked strands on cooling, this would explain the low recovery of heterozygotes in the face of a high recovery of individual markers. *Hemophilus influenzae* contains about 50 molecules of DNA per cell, only one of which appears to carry a particular marker, so that if all strands are capable of some degree of complementation, a lower limit of a few per cent in recovery of heterozygotes would be expected. If a relatively random pairing of strands is the case, then a recovery of twice the original number of individual markers would be expected after anneal-
ing, which is not observed. However, this assumes that the probability of incorporation of a marker is the same for molecules having one or both strands carrying the marker.

If the pairing is not highly specific, other doubly marked heterozygotes would be expected. A few experiments with mixtures of streptomycin- and erythromycin-resistance markers failed to reveal any doubly marked units over those expected from random uptake of singles, but at least one explanation for this failure may be suggested. The S and C markers under normal circumstances link or recombine together soon after entering the cell, whereas S and E do not, and in the absence of linkage, segregation may obscure the presence of the heterozygote. A clonal analysis of an annealed mixture of S + E DNAs would help clarify the possibility that nonallelic strands can pair up.

E. If, as Doty et al. suggest, a fraction of the strands develop breaks at the high temperatures, then, on annealing, a single unbroken strand could rescue, by pairing up, segments from both S and C strands. In such a model, the low recovery of heterozygotes in the face of high recoveries of individual markers is accounted for, and more important, it provides a possible mechanism for linking the two markers.

Fig. 4.—Effect of high temperature on the recovery of heterozygotes. DNA mixtures at the usual concentration sealed in glass tubing were heated in a glycol bath for 5 minutes at the indicated temperature, after which they were transferred to a bath at 90°C and annealed.
F. Finally, the possibility that the heterozygote consists of two singly marked molecules fused together through partial pairing making a unit composed of four original strands cannot as yet be excluded.

Annealed samples were shaken with 80 per cent phenol, treated with chymotrypsin and shaken with chloroform-octanol to remove any protein which might have bound them together, but none of these treatments altered the ratio of doubles to singles.

With so many possible explanations for the low recovery of heterozygotes, some decisive experiments must precede further consideration of the mechanism. One additional result may be noted, however, in closing. Heated mixtures of markers which had been chilled rapidly (such as d in Table 1), and which consequently showed a low level of both markers, returned to 25–50 per cent of the initial transforming activity on reheating and annealing just as Marmur and Lane had found. More important for this discussion, heterozygotes which were absent from the chilled mixture formed during the annealing of the reheated DNAs. This shows that the rapid chilling which causes collapse of the separated strands,9 does not, however, inflict irreparable damage on either marker or their capacity to form heterozygotes.

Summary.—Heat denaturation of a mixture of genetically different transforming DNAs from different stocks of *Hemophilus influenzae* followed by the annealing treatment recommended by Marmur and Lane led to the formation of heterozygotes, physical units carrying both genetic markers. A number of possible mechanisms for their formation have been considered.

The author is grateful to Drs. Sol H. Goodgal, Claud S. Rupert, and C. A. Thomas, Jr., for their criticisms and suggestions. He also wishes to acknowledge the excellent technical assistance of Miss Stella Mayorga-Nestler.

* This work was supported in part by Public Health Service Research Grant E-1218 and Atomic Energy Commission contract AT(30-1)-1371.

1 Marmur, J., and D. Lane, these PROCEEDINGS, 46, 453–461 (1960).
2 Meselson, M., and F. Stahl, these PROCEEDINGS, 44, 671 (1958).

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**THE KINETICS OF CARRIER-MEDIATED ACTIVE TRANSPORT OF AMINO ACIDS**

**BY JOHN A. JACQUEZ**

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*Communicated by Joseph O. Hirschfelder, December 5, 1960*

Amino acids are concentrated in many types of cells by an active transport process. However, it is only with the cells of Ehrlich ascites carcinoma that sufficient data have accumulated for us to consider setting up models of this process.

We proceed by first setting up some general models of active transport systems