Reprinted from *The Journal of General Physiology*, July, 1966
Volume 49, Number 6, Part 2, pp. 197–209
Printed in United States of America

On the Nature of Recombinants Formed during Transformation in *Hemophilus influenzae*

NIHAL NOTANI and SOL H. GOODGAL

From the Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia

ABSTRACT During the process of transformation in *Hemophilus influenzae* integration of donor DNA, i.e. the formation of recombinant DNA, involves the incorporation of single-stranded DNA. Evidence was obtained from cesium chloride density gradient centrifugation of DNA from donor-recipient complexes that integration was accompanied by the formation of hybrid DNA with a density intermediate with respect to heavy, ^{2}H, ^{15}N, donor and light, ^{1}H, ^{14}N recipient DNA. On denaturation the position of the heavy donor DNA moved closer to, but not all the way toward, the density position of the original donor DNA. In addition to supporting the idea of single-stranded incorporation, this evidence suggested that the integrated donor DNA was covalently linked to light recipient DNA. The DNA was taken up in the double-stranded form and no detectable amounts of denatured DNA could be found during the transformation process. However, during the process of integration an amount of donor atoms, equivalent to the amount of hybrid DNA formed, appeared in recipient DNA, and indicated that while one strand of DNA was integrated the other was broken down and resynthesized. The density of the hybrid DNA, as well as rebanding of denatured hybrid, indicated that the size of the integrated piece of DNA was large, approximately 6×10^{6} daltons.

INTRODUCTION

In bacterial transforming systems considerable evidence has been accumulated which demonstrates that the process of transformation involves the incorporation of donor genetic information by a recipient population (1, 2) and consequentially provides an ideal system for studying the process of recombination of genetic factors at a molecular level. Although it has been known for some time that the number of transformations produced is directly proportional to the number of molecules bound irreversibly, it has not been known how much of this material is physically transferred to bacterial chromosomes. In *Hemophilus influenzae* under conditions of growth there is little or no release of the donor DNA atoms into the solution during the transformation process

(3), and the number of cell complements of DNA required to produce a transformation varies from 2 to 5 depending upon the marker tested. The efficiency is high but it is not one. The expectation from these observations is that there should be an association of donor DNA with the bacterial chromosome, but that not all of the donor DNA should become associated as intact macromolecules. In previous studies (4–6) in order to follow the *association* of donor DNA into the bacterial chromosome, sucrose density gradient experiments were utilized. They showed that donor atoms were progressively bound to the recipient DNA along with a concomitant increase in recombinant

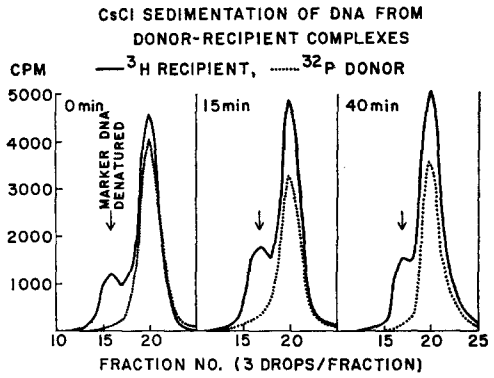

FIGURE 1. Density of donor DNA as a function of time after uptake. Uptake period was 3 min. Donor DNA was labeled with ^{32}P and recipient with ^{3}H. After the times indicated the cells were lysed with 1% SDS and extracted by the procedure of Marmur (16). DNA labeled with ^{3}H was denatured and added as a position marker to the CsCl solution along with the extracted DNA. The material was banded for 60 hr at 32,000 RPM in an SW 39 rotor (Spinco model L). Fractions of 3 drops each were collected and counted for ^{32}P and ^{3}H.

transforming activity. Although donor DNA and transforming activity were transferred to the recipient chromosome, it could not be concluded that this transfer involved a conservative integration of the donor DNA. It should be noted that the association of donor DNA with the recipient was blocked by treatment of the donor DNA or the recipient cell with ultraviolet light (6). Under these conditions the irreversible uptake of donor DNA was normal. Similar results were obtained by subjecting cells to the low postuptake temperature of 18°C (5). Upon returning these cells to 38°C the association ot donor DNA with the recipient chromosome was resumed. All the above evidence suggested that the uptake of DNA could be dissociated from the integration step, and that recombinants were not formed unless the association of donor DNA with the recipient chromosome occurred (5).

EXPERIMENTAL RESULTS AND DISCUSSION

Evidence for the Uptake of Double-Stranded DNA

Following the irreversible uptake of DNA by competent cells in *Pneumococcus* (13, 14) no DNA resembling native donor DNA is found. On the other hand, in *H. influenzae* the unintegrated DNA at no time appeared as denatured material. In Fig. 1 are shown cesium chloride density gradients of ^{32}P-labeled donor DNA extracted from transforming cells at 0, 15, and 40 min after a 3 min uptake period. Tritiated (^{3}H) denatured DNA was added as a reference. There was no evidence of denatured donor material in the transforming cells. Furthermore, the density of the unintegrated donor DNA was the same as the density of the original donor DNA. It may be concluded that if any denatured

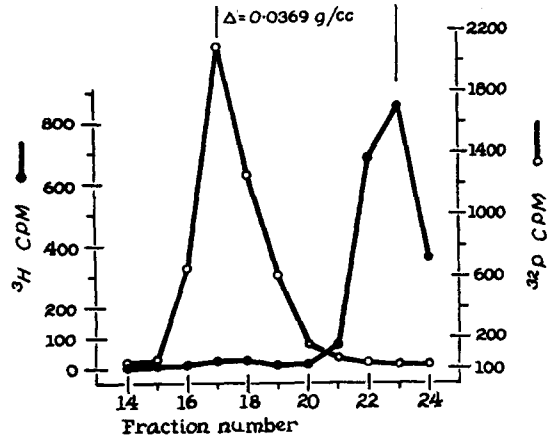

FIGURE 2. Distribution of radioactivity of heavy (open circles) and light (solid circles) DNA following CsCl density gradient centrifugation. The heavy DNA was labeled with ^{32}P and the light DNA with ^{3}H. Centrifugation, 60 hr at 32,000 RPM in an SW39 Spinco rotor.

DNA was generated during the transformation process in *H. influenzae* it was either degraded or rapidly utilized in the recombination reaction.

Following DNA uptake there is a progressive loss of donor transforming activity to a level corresponding to the frequency of transformation for a particular marker (7, 8). A direct comparison of the specific activities of ^{32}P-labeled DNA carrying the cathomycin marker was made before and after DNA uptake. These experiments demonstrated that unintegrated donor DNA after uptake lost approximately 60% of its activity in approximately 10 min. The extent of loss in donor DNA, however, was limited; that is, the specific activity after 40 min was the same as that after 10 min for the unintegrated DNA. The failure to obtain denatured donor DNA from transforming cells suggested that some other form of inactivation must be responsible for this loss. Zone sedimentation analysis of unintegrated donor DNA showed that this DNA was somewhat reduced in size and showed lower frequencies of transformation (9).

Evidence for the Integration of Single-Stranded DNA

Although it could be concluded from the sucrose density gradient experiments that donor atoms were transferred to the recipient, it was still not certain whether or not this association involved discrete pieces of donor DNA. The data in fact were consistent with either a copy-choice or a breakage reunion model of recombination. If recombinants resulted from a copy-choice mechanism they should be relatively free of parental DNA whereas a breakage

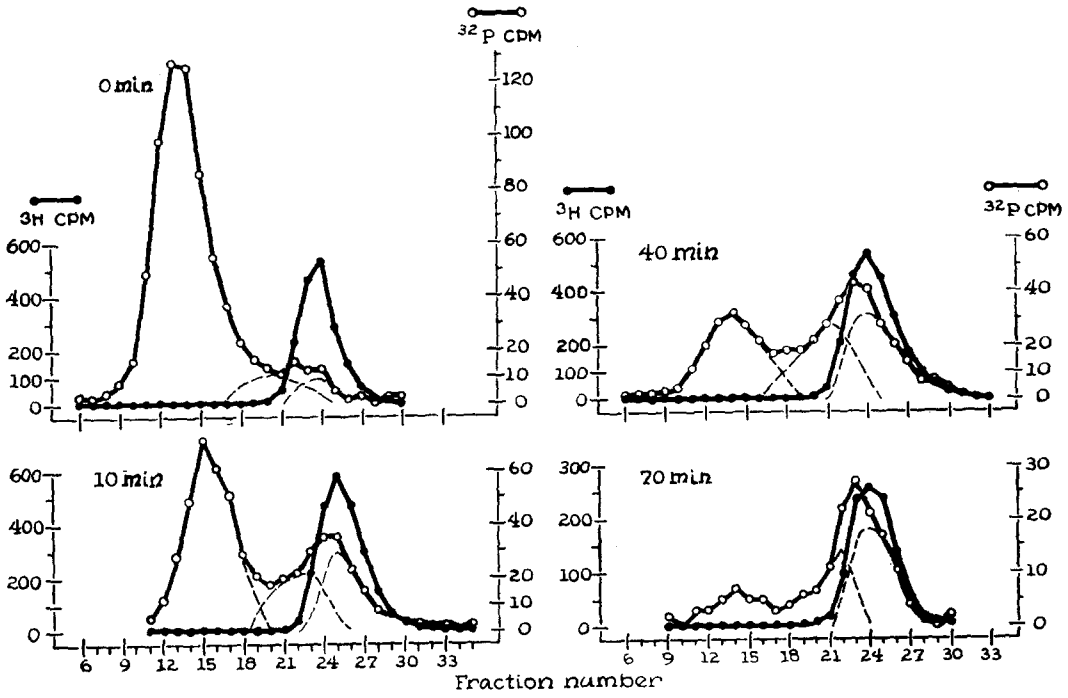

FIGURE 3. CsCl density gradient banding of DNA from donor recipient complexes as a function of time after DNA uptake. The donor DNA was labeled with ^{2}H, ^{15}N, ^{32}P as indicated in Fig. 2. The recipient was light and labeled with ^{3}H prior to DNA uptake. Samples were taken at the times indicated and purified by the method of Marmur (16). After banding 2 drop fractions were collected into 1 ml of citrate saline (0.014 M citrate, 0.15 M NaCl) and aliquots counted in a Packard Tri-carb scintillation counter.

reunion mechanism requires an association between the original parental DNA and recombinants. Evidence has now been obtained to show that in *H. influenzae*, recombinant molecules with genetic information contributed by both donor and recipient DNA's have a structure that suggests direct insertion of discrete single-stranded segments of donor DNA into the recipient chromosome during transformation.

The series of experiments to be reported below utilized donor DNA that had a buoyant density of 0.0310 to 0.0360 g/cc greater than native light DNA.

It was labeled with ^{32}P and carried a genetic marker for resistance to cathomycin (resistant to a concentration of over 20 μg/ml cathomycin). The light recipient DNA was labeled with tritium (^{3}H) and carried the genetic marker for resistance to streptomycin (over 2,000 μg/ml streptomycin). The density distribution in cesium chloride of the two DNA's is shown in Fig. 2. This

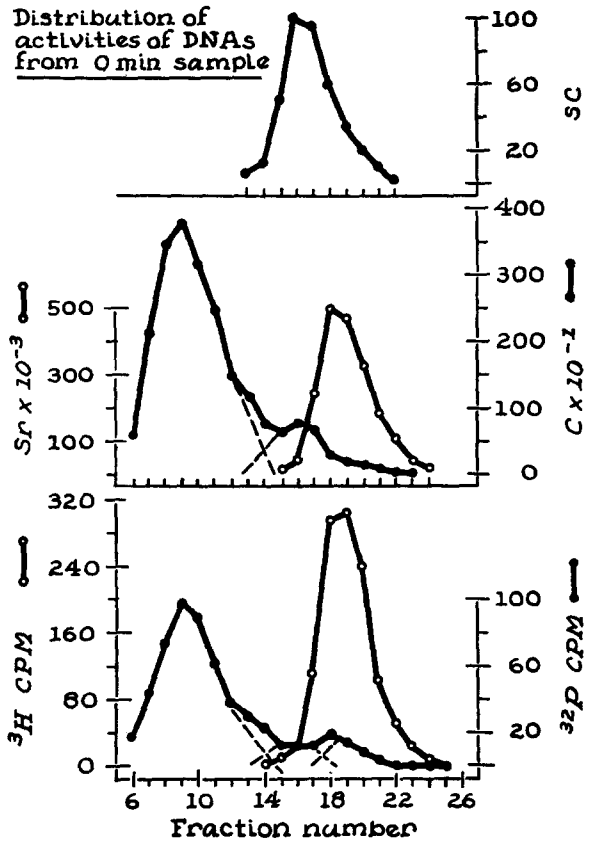

FIGURE 4. CsCl density gradient centrifugation analysis of DNA's extracted from ^{14}N-, ^{1}H-, ^{3}H-labeled streptomycin-resistant cells (N 5 × 10^{8}/ml) transformed by ^{15}N-, ^{2}H-, ^{32}P-labeled, cathomycin resistance-marked DNA (0.1 μg/ml). DNA uptake was for 5 min followed by a 2 min treatment with 5 μg/ml of DNAase at 37°C. Samples were taken without further incubation.

density difference produced an 18 drop (0.3 cc) fractionation between the peaks of native heavy and native light DNA's. Heat denaturation of either DNA moved its position about 9 drops toward the heavier side. It should be emphasized that no DNA of intermediate density was formed when light and heavy DNA's were mixed and banded in cesium chloride. The ^{32}P-labeled, heavy donor DNA was incubated for 5 min with competent cells previously grown in ^{3}H-thymidine. DNA uptake was terminated by a 2 min DNAase

treatment, and the cells were then incubated for various periods of time, lysed with sodium dodecyl sulfate, and subsequently treated with chloroform-octanol. These extracts were banded in cesium chloride for approximately 60 hr.

A series of experiments that demonstrated the kinetics of integration of

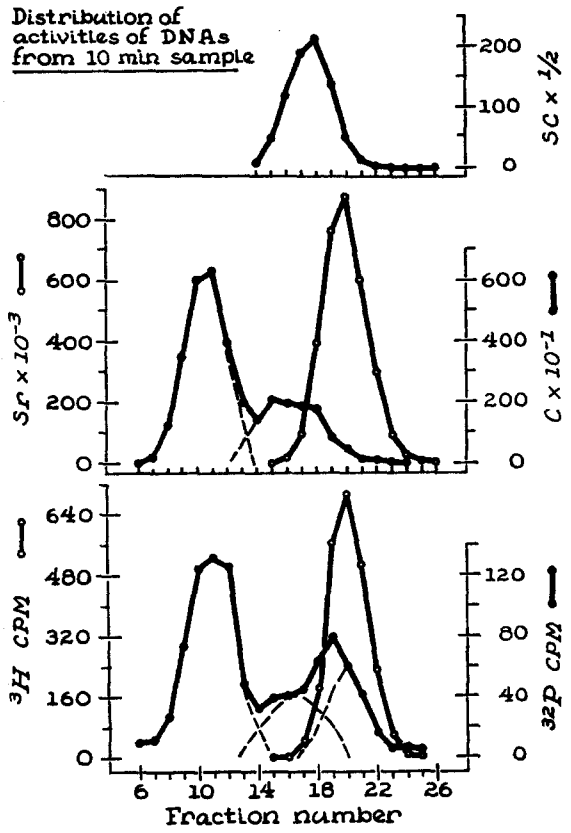

FIGURE 5. CsCl density gradient centrifugation analysis of DNA's extracted from ^{14}N-, ^{1}H-, ^{3}H-labeled streptomycin resistant cells (N 5 × 10^8/ml) transformed by ^{15}N-, ^{2}H-, ^{32}P-labeled, cathomycin-resistance marked DNA (0.1 μg/ml). DNA uptake was for 5 min followed by a 2 min treatment with 5 μg/ml of DNAase at 37°C. Samples were taken after further incubation for 10 min.

donor atoms into recipient DNA are given in Fig. 3. These kinetics were similar to data obtained from sucrose density gradients which showed approximately 75% of donor DNA associated with the recipient 70 min after uptake. After short periods of incubation a large fraction of the donor DNA was at its original density position indicative of unintegrated DNA. Only 10 to 15% of the donor atoms were found at a lighter position. As a function of time there was a progressive transfer of heavy donor DNA to a lighter position until at

70 min only 20% of the donor DNA was at its original position. The donor DNA which had shifted its position was clearly denser than recipient DNA. Although from the density profile it appeared that there were only two peaks of ^{32}P material, from a consideration of the biological activities of this material three regions could be delineated (Figs. 4 to 6): (*a*) a peak of unintegrated donor DNA which decreased as a function of time and banded at the position

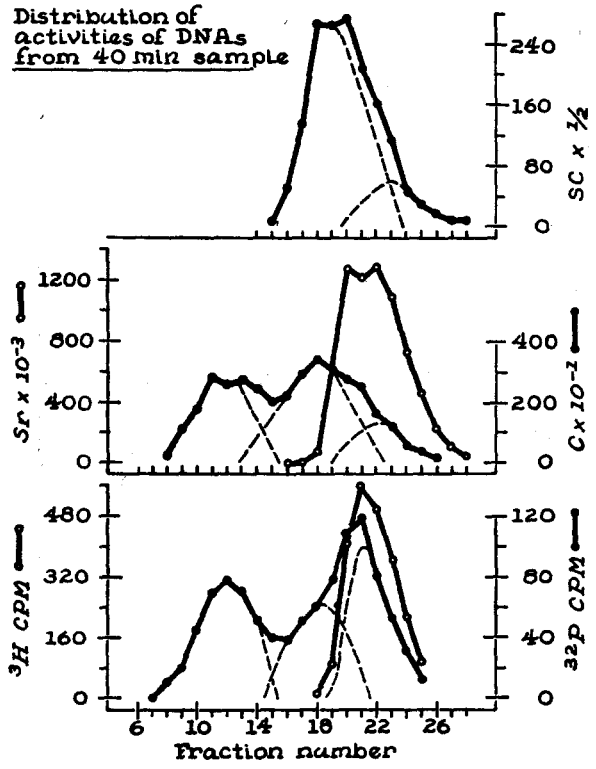

FIGURE 6. CsCl density gradient centrifugation analysis of DNA's extracted from ^{14}N-, ^{1}H-, ^{3}H-labeled streptomycin-resistant cells (N 5 × 10^8/ml) transformed by ^{15}N-, ^{2}H-, ^{32}P-labeled, cathomycin resistance–marked DNA (0.1 μg/ml). DNA uptake was for 5 min followed by a 2 min treatment with 5 μg/ml of DNAase at 37°C. Samples were taken after further incubation for 40 min.

of the native heavy DNA, (*b*) a peak of intermediate density from which recombinant DNA could be obtained, and (*c*) a region corresponding to the recipient light DNA in which there were initially relatively few donor recombinants. In all these samples the donor transforming activity occurred at two positions in the gradient, its original position and a density position approximately 33% denser than recipient DNA. Recombinant transforming activity (SC), which must result from the association of genetic information from both the donor and the recipient, occurred at a position 25% denser than

recipient DNA. The fact that donor DNA was present in a hybrid position and that the specific activity of this DNA was similar to unintegrated DNA supported the notion that discrete pieces of DNA had been integrated into the recipient chrmosome.

Inasmuch as recombinant material did not incorporate more than 50% of the heavy donor DNA it was concluded that the structure of the early recombinants could not result from the incorporation of double-stranded material only, and might be the consequence of the incorporation of single-stranded DNA. In examining this problem two approaches were used. First, if the DNA were incorporated as double strands it must on the next replication

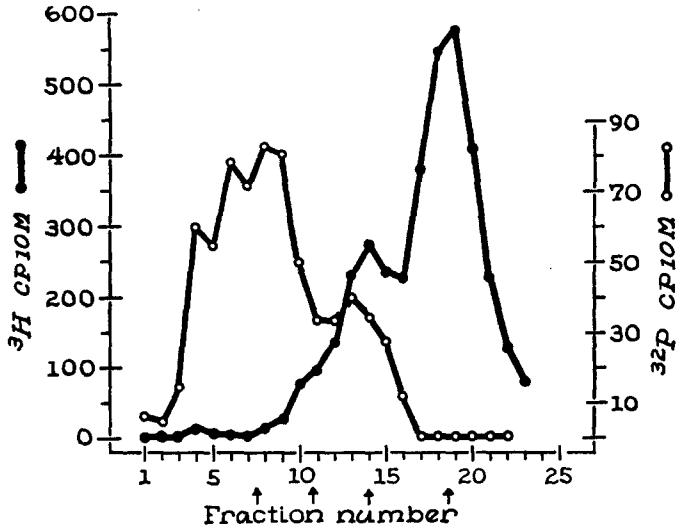

FIGURE 7. Fraction 17 from the 10 min sample shown in Fig. 5 was denatured and rebanded in CsCl to which was added ^3H light native DNA indicated by the arrow to the right, the 2nd arrow indicates the position of light denatured DNA, the 3rd the position expected for the denatured original sample, and the 4th arrow shows the average position of the denatured heavy DNA.

become relatively lighter, that is hybrid material at 10 min compared to material one generation later at 40 min, must reflect the incorporation of light DNA. On the other hand, if single-stranded incorporation had occurred, subsequent replication would yield hybrid material of approximately the same density. In fact, the position of the recombinant molecules did not change. For SC recombinants the initial hybrid position was two and a half fractions from the light peak and for the C recombinants approximately 3 fractions from the light peak. At 40 min some donor transforming activity and recombinant activity have appeared in the light position, but the major portion has remained at the hybrid density. At 70 min the recombinants were positioned half in the light and half in the hybrid fractions. Since a density

shift did not occur in the bulk of the recombinant material at 40 min, one was therefore inclined to accept the notion that incorporation involved single strands. Support for this notion was obtained from experiments in which the hybrid material was denatured and rebanded. If the structure of the hybrid represents the incorporation of segments of double-stranded donor DNA, then upon denaturation the density of the single strands should not increase more than the amount expected due to denaturation alone. Alternatively, if only a single strand of the donor DNA were integrated, on denaturation of the hybrid DNA, the donor radioactivity should band at a position closer to that

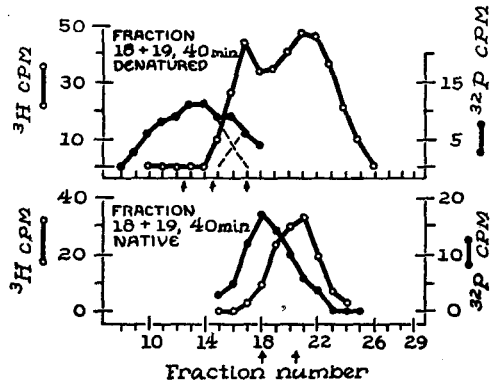

FIGURE 8. Rebanding of fractions 18 and 19 from the CsCl gradient given in Fig. 6. Lower curve, fractions were rebanded as native material, the arrow to the right indicates light native recipient DNA, the 2nd arrow the position of native hybrid DNA. Upper curve, fractions were denatured for 10 min in a boiling water bath, quickly chilled, ^{3}H native DNA added as position indicator, and rebanded for 60 hr at 32,000 RPM. The arrow to the right indicates denatured light recipient DNA; the 2nd arrow, the expected position of denatured hybrid DNA if there were no change in ratio of dense and light DNA in the hybrid. The arrow to the left gives the average density of the denatured hybrid DNA.

of denatured dense DNA. From the data presented in Fig. 7 it is clear that the ^{32}P hybrid DNA shifted to a heavier position than that expected merely from denaturation. In another experiment presented in Fig. 8, the banding of hybrid fractions before and after denaturation was compared. The native hybrid DNA was found to band approximately at its original density position. Again, the hybrid DNA after denaturation moved to a denser position than would be expected for denaturation alone. The fact that the denatured hybrid did not shift all the way to the heavy position suggested that covalency existed between the recipient DNA and the integrated donor strand. It may be concluded from the density of the native hybrid DNA as well as the large increase in density as a result of denaturation, that very large segments of donor DNA were incorporated. Estimates of the size of the donor piece integrated gave a value of two-thirds of one strand of DNA of molecular weight sixteen

million or about six $\times$ 10^6 daltons. It should, however, be noted that the hybrid region is quite broad and represents material with a considerable amount of density heterogeneity. One is inclined to suggest that this represents the transfer of various sized pieces during the transformation process as suggested by Hotchkiss a number of years ago (10). It could also reflect a random breakage of DNA during extraction.

DISCUSSION

The picture of the recombination process that emerges from the foregoing results may be visualized as follows. The DNA is taken up in a double-stranded form as evidenced by the fact that the intracellular donor DNA is present in a heavy native position, and is biologically active. No appreciable amount is found in the denatured heavy position. After uptake the donor DNA undergoes some inactivation to a fixed level. This DNA then undergoes a pairing relationship with the bacterial chromosome; however, there is little direct evidence for the pairing step. In the pairing reaction there is a transfer of one strand to the recipient DNA and the breakdown of the other strand which is rather quickly incorporated into recipient DNA under normal growing conditions. Under conditions in which the cell is not synthesizing an appreciable amount of DNA this material may be solubilized and released into the medium as indicated by the experiments of Stuy (11) with cells that had taken up DNA under rather restrictive conditions of growth. The association of donor and recipient DNA can be prevented or at least considerably reduced by irradiating donor DNA or recipient cells with ultraviolet light. Integration can also be blocked by lowering the temperature to 18°C or less. The inability of ultraviolet-light treated DNA to integrate may be ascribed to the cross-linking of the two strands which precludes the opening out of the helix to affect the necessary complementary base pairing. The effect of low temperature in preventing integration may be due to either the slow rate of enzymatic activity or the requirements for energy for the recombination process. Cesium chloride density gradient centrifugation analysis of extracts of transformed cells showed that during recombination discrete pieces large enough to cause a shift in density are integrated into the recipient chromosomes. Rebanding of hybrid fractions before and after denaturation clearly showed that only one of the two donor strands was integrated. Apparently one strand paired with its complementary homologue and the other strand was excised and degraded. Although no evidence could be obtained for the presence of denatured donor DNA, some donor material was found to enter the light recipient DNA without concomitant biological activity.

Following incorporation of donor DNA into the recipient chromosome covalency was quickly established with the recipient DNA. Data showed that

the donor and recipient DNA's were already covalently linked by 10 min after DNA uptake and hybrid material present at zero minutes after DNA uptake was, also, already covalently linked.

The transfer of DNA during the integration reaction seems to involve pieces which are considerably different in size, since a considerable amount of density heterogeneity is exhibited by the hybrid DNA. It is interesting in this respect that the SC recombinants which were observed were somewhat lighter than C recombinants. This result would be expected if a large donor segment bearing both the C marker and the streptomycin marker were integrated. It has been demonstrated previously (3) that the density of S, C, and SC recombinant material was essentially the same under conditions in which no heavy isotopes were incorporated.

Preliminary evidence has been obtained to show that either strand of the DNA may be incorporated during the integration process (12).

It is believed that this model of the transformation process explains an apparent paradox which was observed earlier when it was found that the synthesis of transforming DNA was initiated relatively soon after DNA uptake, approximately 15 min, whereas replication of transforming cells did not occur until some two generations later (1). A one generation lag was ascribed to the fact that *H. influenzae* appears as chains of two in a growing culture, whereas the other generation lag could possibly be due to the need for an additional round of replication. It is now clear that this additional round of replication involved the formation of the complementary strand of transforming DNA.

Our results are essentially in agreement with those of Fox and Allen (13) and Bodmer and Ganesan (15) with respect to single strand integration. The difference between the pneumococcal, *subtilis*, and *hemophilus* transformation systems probably reflect time differences in the various steps leading to integration.

It should be noted that the integration of a single strand during the process of transformation with the concomitant loss of the other strand makes it rather unlikely that the process of recombination and transformation is the same as the process of recombination in higher organisms which involves chromatid exchange with conservation of both partners.

The authors wish to thank Mrs. Marion Schaffer Dorfman for her excellent technical assistance.
This work was aided by Grant No. A1-4557 from the United States Public Health Service.

REFERENCES

1. VOLL, M. J., and GOODGAL, S. H., Recombination during transformation in *Hemophilus influenzae*, *Proc. Nat. Acad. Sc.*, 1961, **47**, 505.
2. FOX, M. S., Fate of transforming deoxyribonucleate following fixation by transformable bacteria, *Nature*, **187**, 1004.

3. VOLL, M. J., and GOODGAL, S. H., On the stability of recombinant DNA during transformation in *Hemophilus influenzae*, *Biochim. et Biophysica Acta* 1966, **118,** 65.
4. NOTANI, N. K., and GOODGAL, S. H., On the nature of integration and genetic recombination during transformation in *Hemophilus influenzae*, *Fed. Proc.*, 1965, **24,** 468.
5. NOTANI, N. K., FRANKEL, F. R., and GOODGAL, S. H., The association of donor and recipient DNA's during transformation in *Hemophilus influenzae*, *Mendel Mem. Symp. Prague*, 1965, in press.
6. NOTANI, N. K., and GOODGAL, S. H., Decrease in integration of transforming DNA of *Hemophilus influenzae* following ultraviolet irradiation, *J. Mol. Biol.*, 1965, **13,** 611.
7. VOLL, M. J., Ph.D. Thesis, University of Pennsylvania, Philadelphia, 1965.
8. VOLL, M. J., and GOODGAL, S. H., Loss of activity of transforming DNA after uptake by *Hemophilus influenzae*, *J. Bact.*, 1965, **90,** 813.
9. NOTANI, N. K., and GOODGAL, S. H., manuscripts in preparation.
10. HOTCHKISS, R. D., Size limitations governing the incorporation of genetic material in the bacterial transformations and other non-reciprocal recombinations, *Sym. Soc. Exp. Biol.*, 1958, **12,** 49.
11. STUY, J. H., Fate of transforming DNA in the *Hemophilus influenzae* transformation system, *J. Mol. Biol.*, 1965, **13,** 554.
12. GOODGAL, S. H., and NOTANI, N. K., Evidence for transformation by either strand of DNA of *Hemophilus influenzae*, *Fed. Proc.*, 1966, **25,** 707.
13. FOX, M. S., and ALLEN, M. K., On the mechanism of deoxyribonucleate integration in pneumococcal transformation, *Proc. Nat. Acad. Sc.*, 1964, **52,** 412.
14. LACKS, S., Molecular fate of DNA in genetic transformation of pneumococcus, *J. Mol. Biol.*, 1962, **5,** 119.
15. BODMER, W., and GANESAN, H. T., Biochemical and genetic studies of integration and recombination in *B. subtilis* transformation, *Genetics*, 1964, **50,** 717.
16. MARMUR, J., A procedure for isolation of DNA from microorganisms, *J. Mol. Biol.*, 1961, **3,** 208.

Discussion

Dr. Hotchkiss: I suppose it is clear to everyone that two kinds of measure are being made, the measure of information such as markers transferred and then the matter: a mass measure of DNA in the form of P32 or heavy atoms, so that the genetic marker where it is assayed is a sampling of what the total DNA measured chemically or isotopically may be going through.

Dr. Goodgal said something about the unity of biochemistry, and I suppose I've always thought that where there's unity there's biochemistry and where there's not unity there's biology. We have two biologists trained as geneticists, on the second part of the morning's program. I think you'll find that they are operating at levels indistinguishable from those of the first two speakers. That's because they're modern

biologists. Dr. Ephrussi-Taylor was really one of the very first to find markers that were subgenic in nature when they were turning up in high organisms; she is really the first to have them and operate with them in DNA material. And, if any of us had been able to understand what was going on then, we would have known they were the beginnings of fine structure. She has chosen this part of her work rather more than the physical chemical part which she is also interested in and occupied with for her paper today and her actual title will be: "Genetic Studies of Recombining DNA in Pneumococcal Transformation" by Drs. Ephrussi-Taylor and Gray of Western Reserve University.