antibodies to dinucleoside phosphate react to a lesser extent with denatured DNA than do anti-mononucleosides. This decreased reaction could be caused by a difference in structure between the dinucleotide hapten and the dinucleotide linked in the denatured DNA molecule, or may reflect the more frequent occurrence of a mononucleoside than of a specific dinucleotide. We are attempting to determine if the anti-dinucleotide sera show more specificity in their reactions with DNAs of varying base composition than do anti-mononucleoside sera.

Acknowledgment

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References

Beiser, S. M., and Erlanger, B. F. (1966), *Cancer Res. 26*, 2012.
Butler, Jr., V. P., Beiser, S. M., Erlanger, B. F., Tanenbaum, S. W., Cohen, S., and Bendich, A. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 1597.
Butler, Jr., V. P., Tanenbaum, S. W., and Beiser, S. M. (1965), *J. Exp. Med. 121*, 19.
Erlanger, B. F., and Beiser, S. M. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 68.
Halloran, M. J., and Parker, W. W. (1966), *J. Immunol. 96*, 279.
Kabat, E. A., and Mayer, M. M. (1961), Kabat and Mayer's Experimental Immunochemistry, 2nd ed, Springfield, Ill., C. C Thomas.
Klein, W., Beiser, S. M., and Erlanger, B. F. (1966), *Bacteriol. Proc.*, 56.
Lacour, F., Harel, J., Harel, L., and Nahon, E. (1962), *C. R. Acad. Sci. 225*, 2322.
Plescia, O., and Braun, W. (1967), *Advan. Immunol. 6*, 231.
Reichlin, M., Hay, M., and Levine, L. (1964), *Immunochemistry 1*, 21.
Sela, M., Ungar-Waron, H., and Schechter, Y. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 285.
Tanenbaum, S. W., and Beiser, S. M. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 662.
Ungar-Waron, H., Hurwitz, E., Jaton, J. C., and Sela, M. (1967), *Biochim. Biophys. Acta 138*, 513.
Van Vunakis, H., Seaman, E., Setlow, P., and Levine, L. (1968), *Biochemistry 7*, 1265.
Wallace, S., Erlanger, B. F., and Beiser, S. M. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 376.
Wasserman, E., and Levine, L. (1961), *J. Immunol. 87*, 290.

Fate of *Bacillus subtilis* Transforming Deoxyribonucleic Acid Incorporated into Transformable *Diplococcus pneumoniae**

Emanuel Silverstein and Bipin M. Mehta†

ABSTRACT: The fate of foreign DNA within a bacterial cell was examined by incorporation of biologically and isotopically labeled *Bacillus subtilis* 168$^+$ DNA (donor) into genetically transformable and biologically labeled *Diplococcus pneumoniae* R1 cells (recipient). DNA was subsequently extracted from recipient cells and studied physically and biologically. Portions of DNA extracted from recipient cells after exposure to donor DNA were denatured by heat or alkali, sonicated, sonicated and denatured, or untreated, and then subjected to CsCl equilibrium density gradient centrifugation. Gradient fractions were analyzed for physical (radioactivity and heavy density of donor DNA) and biological (transforming activity of recipient DNA) markers. The results indicated that elements of donor DNA too small to manifest the heavy density label were incorporated by covalent linkage into resident DNA. It was not possible to isolate from recipient cells as early as 1 min after exposure to native donor DNA heavy density native or denatured donor DNA, or DNA which contained the biological activity of donor DNA or was specifically hybridizable with donor DNA. Recipient cells were not transformed for the donor biological markers sulfanilamide and erythromycin resistance, nor were their growth rates altered over a 3-hr period. Presence in the transformation medium of deoxynucleosides and deoxynucleotides in about 160 times greater abundance than present in the deoxynucleotide components of donor DNA failed to decrease and usually increased incorporation of donor DNA into recipient DNA despite isotopic evidence for simultaneous incorporation of the deoxynucleosides and deoxynucleotides into recipient DNA. Inhibition of DNA synthesis by 50 μg/ml of fluorodeoxyuridine also failed to decrease incorporation of elements of donor DNA into resident DNA. These results suggest the possibility that small oligodeoxynucleotides derived from native DNA of a disparate species may be incorporated into the DNA of *Diplococcus pneumoniae* cells under transforming conditions.

Results of genetic recombination experiments by transformation with *Diplococcus pneumoniae*, *Bacillus subtilis*, and *Hemophilus influenzae* systems have been consistent with a model of covalent linkage of a single stranded segment of transforming DNA to recipient DNA presumably at the homologous region as dictated by base-pair recognition

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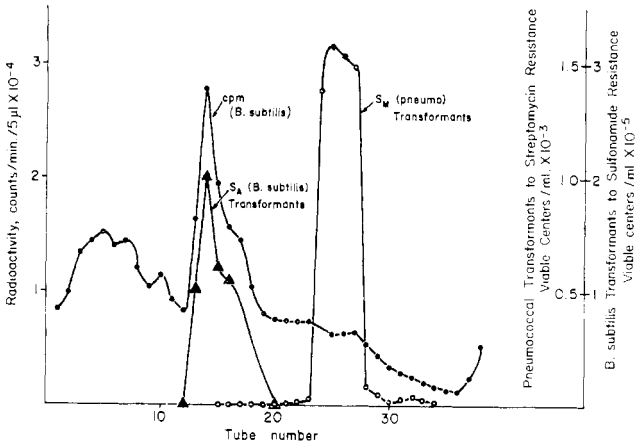

FIGURE 1: CsCl equilibrium density gradient analysis of a mixture of *B. subtilis* and *D. pneumoniae* DNA. *B. subtilis* DNA was labeled with the isotopes ^{32}P, ^{2}H, and ^{15}N as well as with the biological markers of resistance to sulfanilamide and erythromycin. Pneumococcal DNA was carrying a streptomycin-resistance marker: ●, cpm (*B. subtilis* DNA); ▲, transformants to sulfanilamide resistance (*B. subtilis* DNA marker); ○, transformants to streptomycin resistance (*D. pneumoniae* DNA marker).

(Szybalski, 1961; Bodmer, 1966; Ephrussi-Taylor and Gray, 1966; Fox, 1966; Notani and Goodgal, 1966). While transformation between related strains of *Streptococcus* and *D. pneumoniae* (Bracco *et al.*, 1957; Krauss and MacLeod, 1963) and various interspecific transformations have been shown (Marmur *et al.*, 1963), transformation between disparate genera of bacteria has not been demonstrated. On the other hand, it is known that nonspecific DNA does compete with transforming DNA in transformation, and in some cases, it has been shown that nonspecific DNA is taken up by transformable bacteria (Lerman and Tolmach, 1957), and apparently initially handled similarly to homologous DNA (Lacks *et al.*, 1967).

This work was undertaken to elucidate the physical and functional fate of transforming DNA exposed to a disparate species of transformable bacteria. The system chosen was *B. subtilis* (DNA donor carrying biological, heavy density, and radioactivity markers) and *D. pneumoniae* (recipient bearing biological markers) since the biological character of both could be tested by transformation and the additional physical markers enabled separation of the two DNA species and recognition of biologically inactive segments of DNA.

Materials and Methods

Chemicals and Bacterial Strains. Chemicals used and their sources were: deuterated acetic acid (d_4, 99% isotopic purity) and deuterium oxide (99.8%) (Bio-Rad Laboratories, Richmond, Calif.); D-glucose-*C*-d_7, 98% (Merck Sharp & Dohme of Canada, Montreal); carrier-free Na-[^{32}P]phosphate (various commercial sources; when preservatives were present they were removed by chromatography on Dowex-1 with HCl elution of phosphoric acid); [*methyl*-^{3}H]thymidine (6–14 Ci/mmole), [8-^{3}H]deoxyadenosine (4.3 Ci/mmole), [^{3}H]deoxycytidine (5.0 Ci/mmole), and [^{3}H]thymidine 5′-monophosphate (1.8 Ci/mmole) (Schwartz BioResearch, Orangeburg, N. Y.); recrystallized pancreatic deoxyribonuclease I and ribonuclease (Worthington Biochemical Corp., Freehold, N. J.).

R1, R6, RF6S7, and RF6-7 cultures of *D. pneumoniae* were obtained from Dr. Rollin Hotchkiss; *D. pneumoniae* RF4S7, *B. subtilis* 168$^+$ (sulfanilamide and erythromycin resistant), and SB29 *tyr*$^-$ *trp*$^-$ from Dr. Maurice S. Fox; and *B. subtilis* SB1 *his*$^-$ *trp*$^-$ from Dr. D. M. Green.

Preparation of Isotopically Labeled DNA. 1. *B. subtilis 168*$^+$ *DNA.* Since addition of 0.2% acetate to minimal media reduced the requirement of glucose as carbon source for maximum growth of *B. subtilis* 168$^+$ from 0.16% to 0.05–0.08%, [^{2}H]acetate was usually used to spare [^{2}H]glucose. Further increase in acetate inhibited growth despite simultaneous increase in glucose concentration. [^{32}P,^{2}H,^{15}N]*B. subtilis* 168$^+$ (sulfanilamide and erythromycin resistant) was grown in sterile [^{2}H]H_2O at pH 7.5 containing (mg/0.1l.): Tris, 606; K_2HPO_4, 11.3; $MgSO_4$, 20; $CaCl_2$, 0.1; $FeCl_3$, 0.1; NaCl, 50; KCl, 150; yeast extract, 2; [^{15}N]NH_4Cl, 50; [^{2}H]glucose and mannose, 160; or [^{2}H]glucose, 50 plus [^{2}H]acetic acid, 146.2; and 1–2 mCi of carrier-free Na-[^{32}P]phosphate. Cultures were first adapted from ^{1}H medium by several passages in 0.5 or 1.0 ml of ^{2}H medium and then inoculated with 25- to 100-fold dilution and grown for 20–46 hr at 37° in a shaking water bath. Generation time was estimated at 5 hr. [^{2}H,^{3}H,^{15}N]*B. subtilis* 168$^+$ DNA was prepared from organisms grown in the same medium lacking ^{32}P, to which 250 mCi of [^{3}H]thymidine in [^{2}H]H_2O was added during the last 8 hr of growth. [^{32}P,^{1}H]-*B. subtilis* 168$^+$ DNA was prepared in an otherwise identical ^{1}H medium which contained only one isotope, ^{32}P. Cells were harvested and washed twice by centrifugation in 20 ml of 0.15 M NaCl–0.1 M EDTA, pH 8, and the DNA isolated by a scaled-down adaptation of the method of Saito and Miura (1963). Such heavy-density DNA preparations were well separated from light DNA by CsCl density gradient centrifugation (Meselson *et al.*, 1957) (Figure 1) which was used for purification of ^{32}P-labeled preparations. Regions of the gradient which contained transforming activity as well as ^{32}P radioactivity were pooled for the DNA preparation.

2. *D. pneumoniae DNA.* [^{32}P,^{2}H,^{15}N]*D. pneumoniae* RF4S7 DNA and CsCl gradients were prepared as described by Fox and Allen (1964). Centrifugation was for 40 hr at 37,000 rpm and 20°.

^{32}P radioactivity was determined by gas-flow counting with a background of 0.5–1 cpm and ^{3}H by scintillation counting in glass vials containing 9.1 or 24.2% ethanol in toluene, 0.5% 2,5-diphenyloxazole, and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene. Correction was made for any ^{32}P spill into the ^{3}H channel as determined with a sample of high-activity ^{32}P.

Genetic Transformation Assays. B. subtilis transformation was done essentially as described by Spizizen (1959) using an SB29 or SB1 recipient and plating on media containing 200 µg/ml of sulfanilamide, 1 µg/ml of erythromycin, or missing an essential amino acid. *D. pneumoniae* transformation was done according to the methods used by Fox and Allen (1964). DNA of *D. pneumoniae* R1 or R6 was assayed by transformation of strain RF6-7 to *p*-nitrobenzoate resistance (25 µg/ml) and DNA of strain RF4S7, RF6S7, or R6 (streptomycin resistant) by transformation of RF6-7 or R1 to streptomycin resistance (150 µg/ml).

Preparation of DNA. B. subtilis DNA was prepared by the method of Saito and Miura (1963) from cultures grown overnight in Pen Assay broth with vigorous shaking at 37°. *D. pneumoniae* DNA was prepared according to the methods used by Fox and Allen (1964) from 2-l. cultures grown at 37° for 15–20 hr.

Isolation of DNA from Transformable D. pneumoniae Exposed to Isotopically Labeled B. substilis Transforming DNA.

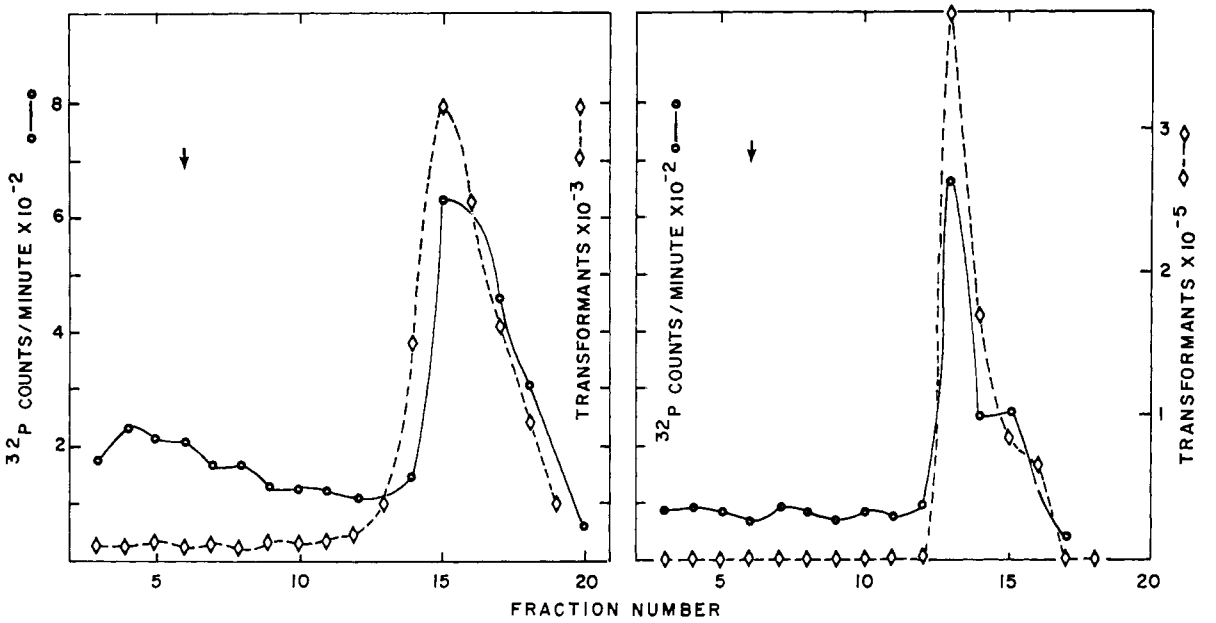

FIGURE 2: CsCl equilibrium density gradient analysis of extracts of *D. pneumoniae* R6 exposed to density and radioactivity labeled homologous DNA. DNA was extracted after incubation of the R6 culture under transforming conditions with [^{32}P,^{2}H,^{15}N]*D. pneumoniae* RF4S7 DNA for 20 min at 30°, and then for 3 min (left) or 20 min (right) at 37°: ○, ^{32}P radioactivity in 0.15 ml (derived from donor DNA); ◇, transformants to *p*-nitrobenzoate resistance (assay for recipient DNA) in 0.01 ml (left) and in 1 ml (right). Arrows indicate position of [^{32}P,^{2}H,^{15}N]-*D. pneumoniae* RF4S7 DNA run in an identical gradient.

A transformable culture of *D. pneumoniae* R6 (usually 200 ml) was thawed at 0°, placed at 30° for 15 min, collected by centrifugation at 0°, and resuspended in 20 ml of CH medium (0°) (Fox and Allen, 1964) containing 0.5 mM $CaCl_2$. A 0.2-ml sample of the culture was checked for transformability to streptomycin resistance with 2 μl of RF6S7 or R6 (streptomycin marked) DNA. The remaining culture was incubated for 6 min at 30° and 4–5 × 10^6 cpm of *B. subtilis* ^{32}P-labeled heavy-density or normal light-density DNA was then added and hydrolyzed with 0.1 mg/ml of deoxyribonulcease–0.1% $MgSO_4$, usually 20 min after addition. Samples were brought to 0°, the cells centrifuged and resuspended in the same volume of CH medium containing deoxyribonuclease and $MgSO_4$, incubated at 37° for 10 min and then washed by centrifugation (0°) five times in 150 mM NaCl–100 mM EDTA, pH 8.0–0.02% bovine serum albumin in which they were finally suspended (usually in 0.6 ml). Cells were then lysed at 37° in 5 or 10 min by addition of $^1/_{30}$ volume of 5% sodium dodecyl sulfate–0.05% deoxycholate. The lysate was deproteinized by vigorous shaking for 15 min at room temperature with an equal volume of chloroform–5% isoamyl alcohol and the aqueous layer collected after low-speed centrifugation and clarified by 15-min centrifugation at 30,000*g* at 0°. Sonication was performed in a small plastic tube after gassing with nitrogen for 5 min either in (1) a Raytheon 9 KC sonic oscillator at a setting of 80 for a total of 5 min at 30-sec intervals/min starting with the sample at 0° and circulating coolant at 10–12°, or (2) in a Branson Sonifier with a microtip attachment for a total of 5 min at 30 sec/2 min in a 0° circulating water bath. Half the sonicated sample was then heated at 100° for 10–20 min and quickly cooled to 0° in ice water. Alkali denaturation was done in a manner similar to that used by Fox and Allen (1964). All samples containing 3–6 μg of DNA were then subjected to equilibrium density gradient centrifugation with heavy ([^{2}H,^{3}H,^{15}N]*B. subtilis* 168$^+$) and sometimes light (*D. pneumoniae* RF6S7 or R6, streptomycin resistant) DNA position markers. ^{3}H- and ^{32}P-labeled heavy DNA had similar density. DNA was quantified by the method of Burton (1956).

More than 90% of the irreversibly fixed DNA label was present in the deproteinized aqueous fraction, and between 85 and 100% of the radioactivity added to the CsCl solution was recovered in the density gradient fractions. When the bacteria had been exposed for 20 min to radioactively labeled homologous or heterologous DNA between 57 and 76% of the radioactivity recovered in the gradient was in the light-density peak (characteristic of the native resident DNA), while the rest was equally divided among the other fractions. About 30–40% of the radioactivity was trichloroacetic acid soluble. There was no evidence of trapping of donor material in excess pneumococcal DNA since heavy ^{3}H-labeled *B. subtilis* DNA marker which comprised less than 10% of the total DNA in the gradients was always sharply separated from the pneumococcal DNA.

Results

CsCl Density Gradient Study of the Fate of Homologous Transforming DNA Incorporated into D. pneumoniae. The fate of transforming DNA was followed by incorporating density and radioactivity labeled homologous donor DNA into a transformable *D. pneumoniae* recipient, extracting DNA from the recipient cells at various time intervals, and assaying the fractions of a CsCl equilibrium density gradient for donor and recipient DNA by radioactivity and transforming activity markers. ^{32}P and heavy labeled homologous *D. pneumoniae* transforming DNA became associated with normal density recipient DNA within the transformed cell (Figure 2). The masking of the density label of donor DNA indicated that in the donor–recipient associated molecule the size of the donor component was small compared to that of the recipient. A minor fraction banding mainly in the region of denatured

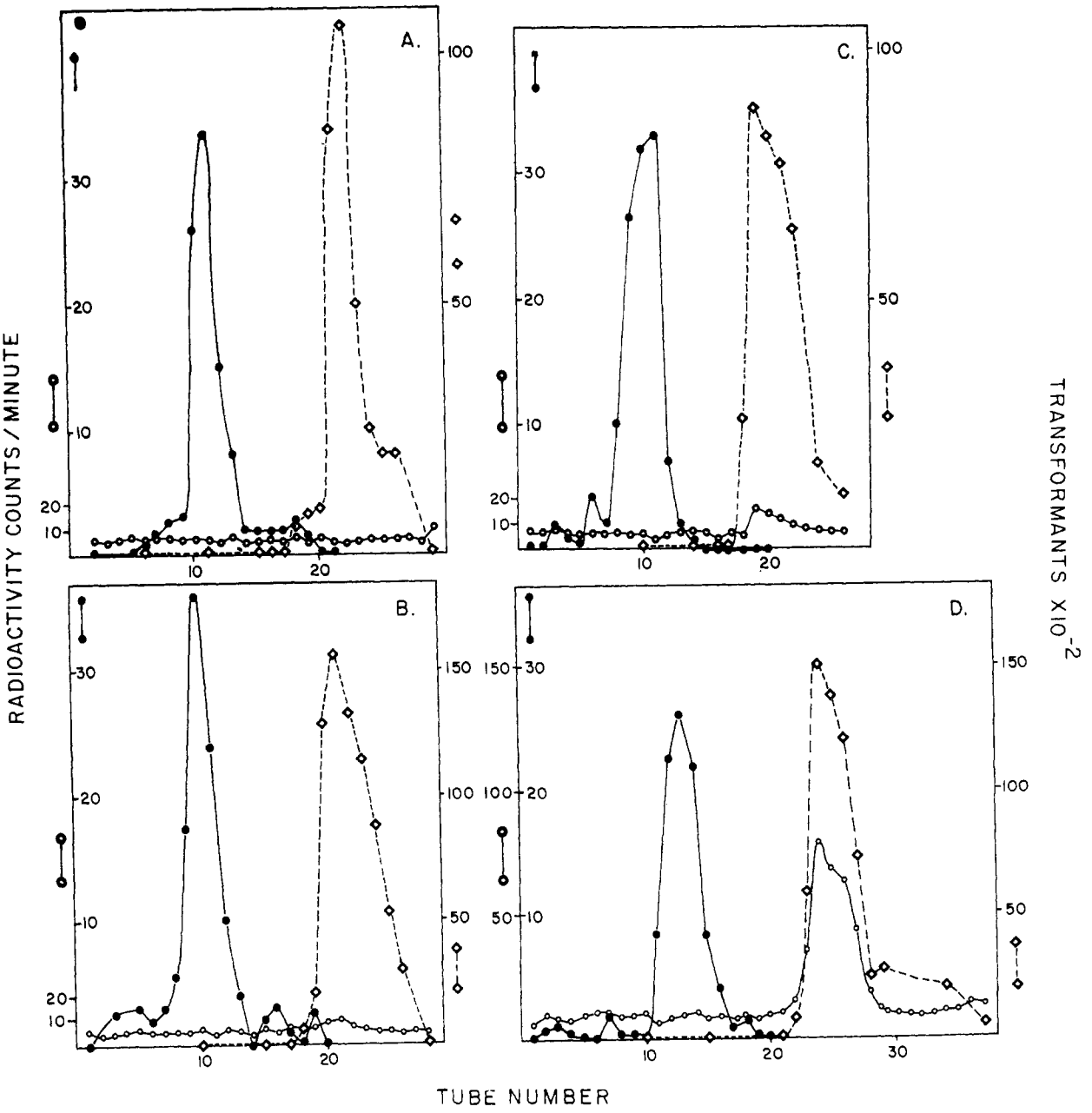

FIGURE 3: CsCl equilibrium density gradient analysis of *D. pneumoniae* R1 extracts obtained after exposure of the bacteria under transforming conditions at 30° to [^{32}P,^{2}H,^{15}N]*B. subtilis* 168^{+} transforming DNA (density and radioactivity labeled): A, deoxyribonuclease present prior to *B. subtilis* DNA addition; B, 1 min; C, 3 min; D, 20-min exposure to *B. subtilis* DNA prior to deoxyribonuclease addition; ○, ^{32}P radioactivity, cpm/50 μl; ◇, transformants/ml to *p*-nitrobenzoate resistance (*D. pneumoniae* R1 marker); ●, ^{3}H radioactivity, cpm/50 μl ([^{2}H,^{3}H,^{15}N]*B. subtilis* 168^{+} DNA heavy position marker).

heavy DNA which was present after 3-min incubation at 37° was no longer apparent after 20 min (Figure 2). Two possible explanations for disappearance of the heavy denatured material are: (a) hydrolysis to nucleotides and (b) incorporation into recipient DNA. These results are compatible with similar experiments reported by Fox and Allen (1964), as well as those of Lacks (1962) which suggested that 1–2 × 10^{6} dalton segments of single-stranded transforming DNA of either strand become covalently linked to recipient DNA with concomitant hydrolysis of single stranded DNA and covalent incorporation of some resulting small elements.

CsCl Density Gradient Study of the Fate of Heterologous Transforming DNA Incorporated into D. pneumoniae. Initial experiments indicated that ^{32}P-labeled *B. subtilis* DNA is incorporated into R1 *D. pneumoniae* cells under transforming conditions. Native triply labeled heavy-density *B. subtilis* and light *D. pneumoniae* DNA were distinctly separable in a CsCl density gradient (Figure 1). A time-dependent incorporation of ^{32}P derived from heavy-density *B. subtilis* DNA into recipient light-density *D. pneumoniae* R1 DNA was found by CsCl density gradient analysis of DNA extracts of transformable R1 cultures exposed for varying times under transforming conditions to [^{32}P,^{2}H,^{15}N]*B. subtilis* 168^{+} transforming DNA (Figure 3B,C,D). Such incorporation did not occur when denatured or deoxyribonuclease-treated donor DNA was used (Figure 3A). In contrast to results with homologous *D. pneumoniae* transforming DNA (Figure 2; Fox and Allen, 1964) no peak of denatured (or native) donor DNA was found

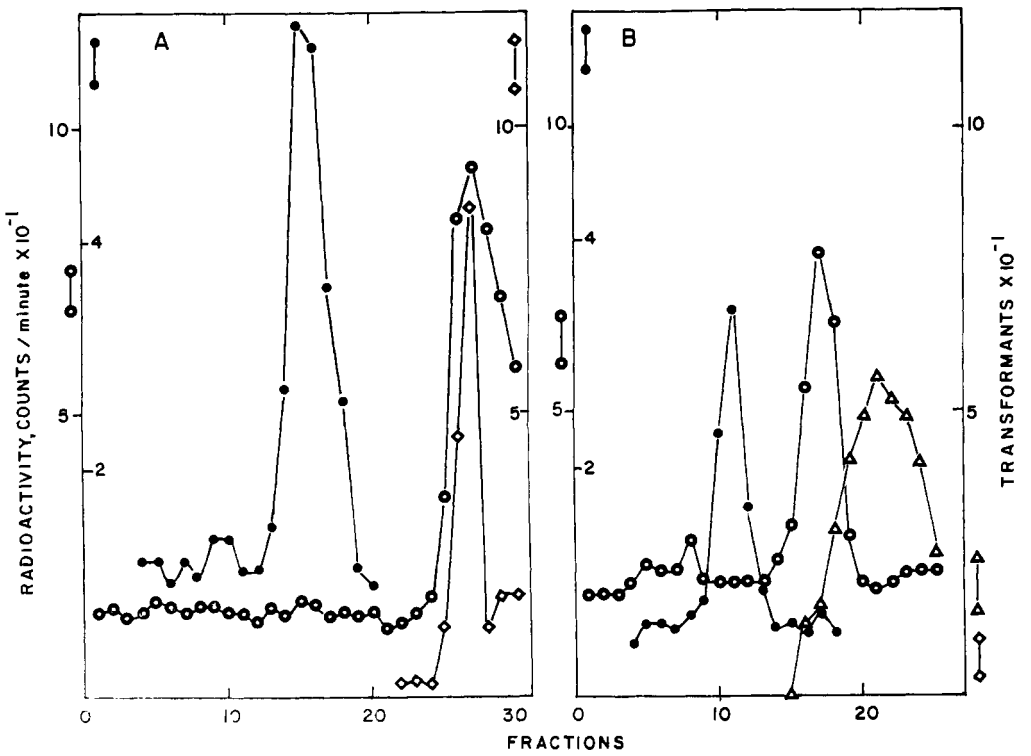

FIGURE 4: Effect of heat denaturation on the CsCl equilibrium density gradient distribution of DNA extracts of *D. pneumoniae* R1. The extracts of the pneumococcal culture were obtained following 20-min exposure of the bacteria under transforming conditions to [^{32}P,^{2}H,^{15}N]-*B. subtilis* 168^{+} transforming DNA as described in Materials and Methods: (A) no heating; (B) heat denatured; ○, ^{32}P, cpm/50 μl; ◇, transformants/10^{-2} ml to *p*-nitrobenzoate resistance (*D. pneumoniae* resident DNA); △, transformants/10^{-2} ml to streptomycin resistance (R6 light DNA position marker); ●, ^{3}H cpm/50 μl ([^{2}H,^{3}H,^{15}N]*B. subtilis* 168^{+} DNA heavy position marker).

in recipient cell extracts when recipient cells were adequately washed with a solution containing deoxyribonuclease. This result was obtained regardless of the length of time of incubation at 37° (0–40 min). Approximately 0.5% of donor radiolabel was incorporated into recipient bacteria after 20 min of uptake at 30°.

B. subtilis transforming activity was not found in the whole extract or in the CsCl density gradient fractions from recipient *D. pneumoniae*, in contrast to recovery of donor pneumococcal transforming activity banding with the recipient DNA peak in similar homologous transformation experiments (Fox and Allen, 1964), and of donor *B. subtilis* DNA transforming activity recovered in CsCl gradients of recipient *B. subtilis* cell extracts (Bodmer and Ganesan, 1964). The sensitivity of detection was estimated to be at least 30 times less for *B. subtilis* as compared to *D. pneumoniae* transformation under these conditions. The transforming activity of 0.03 μg of *B. subtilis* 168^{+} DNA added to the CsCl solutions prior to centrifugation was not detectable in the gradient fractions.

The nature of the association between the radioactively labeled donor *B. subtilis* DNA element and recipient cell *D. pneumoniae* DNA was tested by heat denaturation of the *D. pneumoniae* extract prior to equilibrium density gradient centrifugation (Figure 4). The radioactivity marker of the heavy *B. subtilis* DNA moved from a position coincident with light *D. pneumoniae* DNA to a position intermediate between light and heavy DNA at which denatured light DNA bands. This finding suggests that the association of donor with recipient cell DNA was by covalent rather than hydrogen bonding since it remained after denaturation. If the association were by hydrogen bonding the donor DNA strand would have been expected to have become free of the recipient light DNA strand and to have manifested its density label by banding in a region heavier than the native heavy DNA.

Since the density label of donor *B. subtilis* DNA was not apparent in *D. pneumoniae* cell extracts, suggesting linkage of input DNA ^{32}P marker with a much larger molecule of recipient DNA, the extracts were subjected to sonication to shear the DNA molecules to a smaller size so that the heavy-labeled piece would make up a larger part of the total molecule. The molecular weight was decreased by sonication from about 20 to 1–2 $\times$ 10^{6} daltons as estimated by sedimentation in a sucrose gradient (Burgi and Hershey, 1963). The results of sonication varied from (a) a marked shift in input ^{32}P away from resident DNA to a position almost midway between heavy and light native DNA (Figure 5A) to (b) very little shift to heavy density (Figure 5C). Resident and input DNA markers were not separated by CsCl equilibrium density gradient centrifugation when the DNA extracts were first subjected to repeated forceful extrusion through a 27-gauge needle in order to shear the DNA to smaller size.

Denaturation of the sonicated samples by heat or alkali prior to equilibrium density gradient centrifugation resulted in banding of the peak of the input DNA radioactivity marker in the intermediate position expected for light denatured DNA, with some spread of radioactivity from light native to heavy denatured regions (Figure 6). Thus, despite some degree of shift of the input radioactivity marker away from the resident DNA on sonication, the bulk of the material on denaturation assumed a density expected for denatured light DNA. This result contrasts with the findings in similar homologous transformation experiments (Fox and Allen, 1964) where sonication resulted in moderate shift of input DNA away from resi-

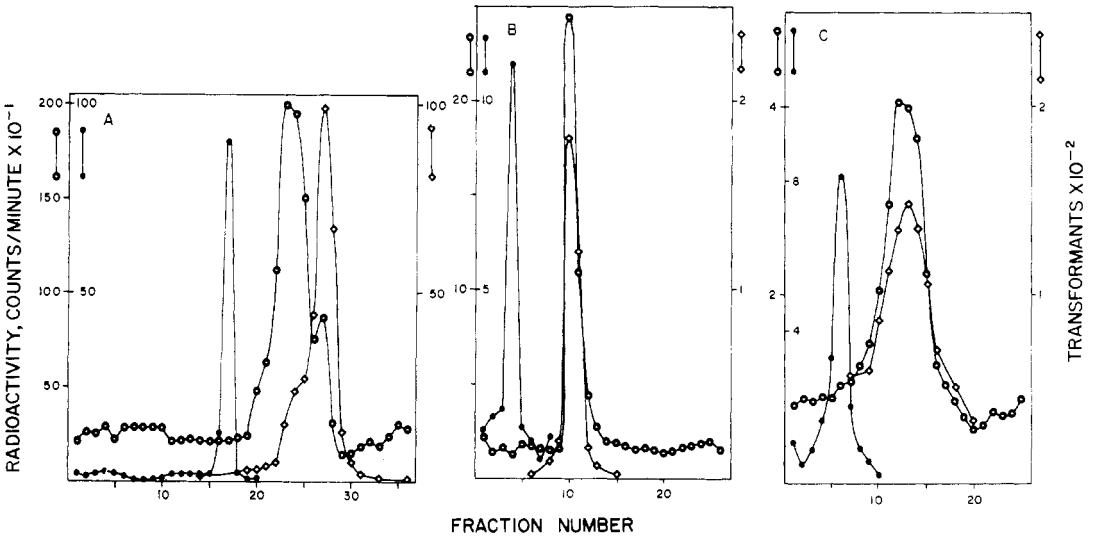

FIGURE 5: Effect of sonication on the CsCl equilibrium density gradient distribution of DNA extracts of *D. pneumoniae* R1. The extracts of the pneumococcal culture were obtained following 20-min incubation of the bacteria under transforming conditions with [^{32}P,^{2}H,^{15}N]*B. subtilis* 168^{+} transforming DNA, as described in Materials and Methods. A, B, and C represent two different experiments; A and C after sonication; B, before sonication. The gradient distribution of the unsonicated extract corresponding to A is given in Figure 3D: ○, ^{32}P, cpm/50 μl; ◇, transformants/10^{-2} ml to *p*-nitrobenzoate resistance (*D. pneumoniae* resident DNA marker); ●, ^{3}H cpm/50 μl ([^{2}H,^{3}H,^{15}N]*B. subtilis* 168^{+} DNA heavy position marker).

dent DNA toward the heavy region, but denaturation of the sonicate resulted in a significant shift of the input radioactivity marker to the heavy denatured as well as light denatured regions.

The varying shift to increased density of input marker following sonication could be accounted for by partial denaturation or by the manifestation of the heavy-density label in DNA segments of greatly decreased size. Partial denaturation on sonication would seem more likely, since the bulk of input DNA marker failed to move to a region heavier than denatured light DNA following denaturation of the sonicated sample. Such movement would have been expected if the density label were manifest in most of the short molecules bearing input DNA. However, it is not possible from these data rigorously to exclude the presence in the heavy and denatured heavy regions, where a minor amount of the radioactivity of the input DNA banded, of some DNA molecules with segments of input heavy DNA forming a sufficiently large portion of the shortened molecules to manifest the density label.

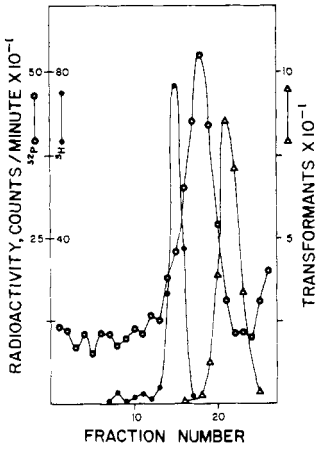

FIGURE 6: Effect of heat denaturation on the CsCl equilibrium density gradient distribution of a sonicated DNA extract of *D. pneumoniae* R1. The extract of the pneumococcal culture was obtained following 20-min exposure of the bacteria to [^{32}P, ^{2}H, ^{15}N]*B. subtilis* 168^{+} transforming DNA, as described in Materials and Methods. ^{32}P (input DNA) and resident DNA markers coincided in the same peak in the CsCl gradient of the undenatured sonicated sample: ○, ^{32}P, cpm/50 μl; △, transformants/10^{-2} ml to streptomycin resistance (*D. pneumoniae* R6 light DNA position marker); ●, ^{3}H, cpm/50 μl ([^{2}H, ^{3}H, ^{15}N]*B. subtilis* 168^{+} heavy DNA position marker).

In order to ascertain further the significance of peak shifts in the density gradients with relation to the density label of input DNA, experiments were done utilizing light ^{32}P-labeled *B. subtilis* 168^{+} DNA. There was either slight or no shift of input ^{32}P marker relative to resident DNA biological marker on sonication, while input marker moved to the position of light denatured DNA on heat or alkali denaturation. These results are similar to those obtained with heavy-density labeled input DNA except for failure to observe more marked shift of input DNA marker on sonication in any experiments. These findings suggest that the shift of input DNA marker seen after sonication may be due at least in part to partial denaturation and that the density label is largely not manifest.

Attempt was made to determine whether hydrolysis of input DNA to monodeoxynucleotides followed by incorporation of these monodeoxynucleotides into newly synthesized host DNA could account for the results. It was first ascertained in studies which will be reported more fully elsewhere that [^{3}H]thymidine was incorporated into pneumococcal DNA when added to transformable *D. pneumoniae* cells in the absence of ^{32}P-labeled *B. subtilis* DNA and that the thymidine and *B. subtilis* DNA labels incorporated into the pneumococcal DNA behaved similarly with respect to sonication and sonication followed by heat denaturation. This result clearly demonstrated that monomer units are covalently added to the DNA under the transforming conditions of these experiments and suggested that incorporation of labeled deoxynucleoside precursor and label from heterologous transforming DNA may

be related events. Whether this monomer incorporation represents DNA synthesis unrelated to recombination or repair systhesis related to recombination is not clear, but it would seem reasonable that both processes may be involved.

Since it was demonstrated that monomer units are added to resident DNA under the transforming conditions used, input DNA was incorporated into *D. pneumoniae* in the presence of a mixture of nonradioactive deoxynucleosides or deoxynucleotides estimated to be in 160-fold greater abundance than that contained in the input DNA. Simultaneous cellular uptake of a large pool of nonradioactive deoxynucleotides or deoxynucleosides convertible intracellularly into deoxynucleotides would be expected to diminish the specific activity of the radioactive pool and thus the radioactivity incorporated into resident DNA due to DNA synthesis. CsCl density gradient analyses of experiments performed in the presence and absence of deoxynucleotides indicated that no decrease occurred in incorporation of ^{32}P from input heavy DNA into the resident light DNA peak (Table I). An increase in incorporation of less than 50% was usually found, the reason for which is not known. On the basis of the 0.036% incorporation of [^{3}H]thymidylic acid found in similar experiments, a decrease of 90% of radioactivity derived from *B. subtilis* DNA incorporated into *D. pneumoniae* DNA would have been expected if monodeoxynucleotides from *B. subtilis* DNA were being incorporated and the two sources of precursors were miscible.

Similarly a mixture of deoxynucleosides 160 times greater than input DNA failed to cause any decrease in incorporation of the ^{32}P of *B. subtilis* DNA into resident DNA. Curiously, there was a marked stimulation of incorporation (Table I). Use of ^{3}H-labeled thymidine, deoxycytidine, and deoxyadenosine showed conclusively that these nucleosides were simultaneously incorporated into *D. pneumoniae* DNA under the transforming conditions used (1.7% for deoxycytidine and deoxyadenosine; 1.2% for thymidine). Attempt at increasing incorporation by use of 5% dimethyl sulfoxide during transformation was only slightly successful (Table I). A decrease of the order of 99% in the ^{32}P counts incorporated would have been expected if these represent *B. subtilis* DNA derived monodeoxynucleotides utilized for *D. pneumoniae* DNA synthesis. In similar experiments inhibition of DNA synthesis by fluorodeoxyuridine (50 μg/ml) had no significant effect on ^{32}P incorporation from [^{32}P]*B. subtilis* DNA. These data thus suggest that *B. subtilis* DNA-derived material other than mononucleotides may be incorporated into recipient *D. pneumoniae* DNA.

Hybridization Experiments. Attempt was made to discern *B. subtilis* DNA sequences by DNA–DNA hybridization on Millipore filters in DNA extracted from *D. pneumoniae* following exposure to ^{32}P-labeled *B. subtilis* DNA and then increasingly degraded to lower molecular weight with pancreatic deoxyribonuclease I. It was anticipated that small ^{32}P-labeled *B. subtilis* sequences inserted in far larger molecules of *D. pneumoniae* DNA would hybridize increasingly well to *B. subtilis* DNA with progressive decrease in size of the *D. pneumoniae* component of the DNA molecules concomitant with decrease in total molecular size. This result was not obtained (Table II). It was thus not possible to detect by hybridization the presence of *B. subtilis* sequences in the DNA extracted from *D. pneumoniae*.

Attempt at Transformation of D. pneumoniae with B. subtilis DNA. Numerous attempts were made to transform R1 or R6 *D. pneumoniae* for the sulfanilamide or erythromycin resistance markers of the *B. subtilis* 168^{+} DNA used, without success, despite their mapping in the region of ribosomal RNA which tends to be conserved among different species (Dubnau *et al.*, 1965).

TABLE I: Effect of Deoxynucleosides and Deoxynucleotides on the Incorporation of *B. subtilis* 168^{+} DNA into *D. pneumoniae* R1 DNA.[a]

Expt	Transforming DNA Label	Additions to Transformation Incubation	Total Radioactivity Incorporated into DNA Fraction[b] (cpm × 10^{-3}) ^{32}P	^{3}H
			5.0	
	^{32}P		4.5	
I		[^{3}H]Thymidine, deoxynucleosides	138	614
		[^{3}H]Deoxycytidine, deoxynucleosides	170	853
		[^{3}H]Deoxyadenosine, deoxynucleosides	160	832
		[^{3}H]Thymidine, deoxynucleosides, 5% dimethyl sulfoxide	138	794
		5% Dimethyl sulfoxide	5.2	
II	^{32}P	[^{3}H]Thymidine 5′-monophosphate, deoxynucleotides, 5% dimethyl sulfoxide	6.7[c]	54[c]
		5% Dimethyl sulfoxide	7.8[c]	
III	^{2}H,^{3}H,^{15}N			19

[a] [^{32}P]*B. subtilis* 168^{+} DNA prepared from 200 ml of culture with 3 mCi of Na-[^{32}P]phosphate was incorporated for 20 min into 0.1-ml aliquot (5 ml) of *D. pneumoniae* R1 derived from 500 ml of transformable culture in the presence and absence of the indicated deoxynucleosides and deoxynucleotides (monophosphate) (400 μg/ml, 50 μCi of ^{3}H) and DNA prepared from the recipient culture with ethanol precipitation as described in Materials and Methods. [b] Fraction precipitated by two volumes of ethanol. [c] Total radioactivity present in lysate.

Effect of B. subtilis DNA on R6 Growth Rate. Since the physical data suggested incorporation of small elements of *B. subtilis* DNA into *D. pneumoniae* DNA, search was made for alterations of recipient gene sequences by replacement insertion of different donor sequences which might have had lethal or less drastic effects manifested as a change in growth. R1 *D. pneumoniae* cultures were exposed to *B. subtilis* DNA under standard transforming conditions, then diluted tenfold in B medium and incubated at 37°. No significant difference in plate counts after 0, 20, 40, 60, 120, and 180 min of incubation was found as compared to two cultures exposed to the same amount of R1 DNA or no DNA. This result suggests that no widespread permanent alteration in DNA sequence by *B. subtilis* DNA insertion into the *D. pneumoniae* genome occurred. Replacements by identical DNA segments, a minor number of permanent mutational segments or mutational segments which undergo excision and repair are not excluded, however. Ephrussi-Taylor and Gray (1966) have suggested the

TABLE II: Hybridization to *D. pneumoniae* R6 and *B. subtilis* 168$^+$ DNA of DNA from *D. pneumoniae* R6 Exposed to [^{32}P]*B. subtilis* 168$^+$ DNA.[a]

Time of DNase Action (min)	DNA on Millipore Filter		
	D. pneumoniae R6[b]	*B. subtilis 168*$^+$[b]	Blank[b]
0	1.8	0.99	1.6
1	6.6	1.8	
2	6.1	2.4	
10	14	2.0	
60	19	2.7	
120	14	3.1	
23 hr	8.2	1.3	2.0

[a] DNA extracted from R6 *D. pneumoniae* exposed under transforming conditions to [^{32}P]*B. subtilis* 168$^+$ DNA as described in Methods and Materials was hydrolyzed at 25° for the indicated lengths of time by addition of 90 μg/ml of pancreatic deoxyribonuclease and 15 μM $MgSO_4$ to 1 ml of 0.85% NaCl–40 mM K-phosphate, pH 7.5, containing the DNA. Enzyme was inactivated and the DNA denatured by exposure to 100° for 10 min followed by rapid cooling to 0°. Hybridization was according to Denhardt (1966) with the exception of the use of 3 mM Tris base for washing of filters which were loaded with 25 μg of *D. pneumoniae* R6 or *B. subtilis* 168$^+$ DNA. [b] Percentage hybridization

frequent occurrence of random insertions of donor DNA no larger than 3×10^5 daltons in recipient DNA in pneumococcal transformation accompanied by frequent excision and repair. In contrast to the present findings is a recent report of decreased viability of recipient *Hemophilus influenzae* cells genetically transformed with DNA from a closely related species (Steinhart and Herriott, 1968). Perhaps the greater points of homology between closely related but not identical deoxyribonucleic acids would allow for more frequent recombination of significantly large segments of DNA which contain one or more base differences, which in some instances would alter critical enzyme functions resulting in lethality or altered growth.

Discussion

It has been known for some time that heterologous as well as homologous DNA may be taken up apparently equally well by *D. pneumoniae* in the transformable state (Lerman and Tolmach, 1957). The present experiments indicate that after uptake small elements of *B. subtilis* DNA (oligonucleotides and/or mononucleotides) which lack genetic transforming activity become covalently linked to resident DNA. On the other hand, both biologically active cistronic size single strands as well as small elements of pneumococcal DNA became covalently linked to resident *D. pneumoniae* DNA in genetic transformation with homologous DNA (Fox and Allen, 1964). Insertion of cistronic size segments of DNA is thus correlated with the appearance of new hereditable biological markers in genetic transformation.

In contrast to the report of Lacks *et al.* (1967) we have not found single-stranded heterologous DNA in R1 pneumococcal extracts after as little as 1-min exposure of the bacteria to *B. subtilis* DNA. The reason for this difference is not clear, although such factors as differences in washing of recipient cells, deproteinization of extracts, type of DNA, and recipient cells might be involved. A time-dependent incorporation of donor radiolabel into recipient double-stranded DNA was found in both studies. The exact correspondence in buoyant density of radiolabel derived from donor *Escherichia coli* DNA and native pneumococcal recipient DNA despite difference in density of the two deoxyribonucleic acids (Lacks *et al.*, 1967) could be accounted for equally well by *de novo* synthesis from monodeoxynucleotides as interpreted by those authors or by covalent insertion of oligodeoxynucleotides.

Covalent incorporation of oligodeoxynucleotides has been demonstrated in *in vitro* DNA synthesis catalyzed by DNA polymerase in which template and oligodeoxynucleotides could be from different organisms (Goulian, 1968). Such incorporation stimulated DNA synthesis and did not occur in the absence of nucleoside triphosphates. The phenomenon of enhanced input DNA incorporation in the presence of a large pool of DNA monomer precursors observed in the present experiments may be related to this nucleoside triphosphate requirement as well as to the increased DNA and protein synthesis in *D. pneumoniae* in the presence of deoxynucleosides and deoxynucleotides (Firshein, 1961).

The present experiments have bearing on the fundamental problem of the informational integrity of species and of cells in general. At least with regard to *B. subtilis* and *D. pneumoniae*, it is clear that *D. pneumoniae* fails to exclude the uptake of foreign DNA, but once taken up clearly recognizes the DNA as foreign and proceeds to reduce it to small fragments which lack cistronic and hence biological integrity. The basis for this recognition is not clear, but it is reasonable to assume that base pairing may be involved. In addition, a specific host restriction endonuclease (Meselson and Yuan, 1968) may recognize *B. subtilis* DNA as nonpneumococcal and introduce a few double-strand breaks in the molecule resulting in several large fragments. However, additional enzymes apparently are required for the further conversion of donor DNA into small elements and their covalent incorporation into resident *D. pneumoniae* DNA. Further, it is clear that homologous DNA, which presumably would be unaffected by resident *D. pneumoniae* restriction endonuclease, is also converted into small and large fragments which are covalently incorporated into the resident *D. pneumoniae* genome.

A possible sequence of events might involve (1) penetration of DNA through the cell wall and membrane by a metabolic process; (2) conversion of the incoming DNA into the single-stranded state, perhaps by an enzyme situated at the cell surface which separates the two strands or hydrolyzes one strand of the duplex (Lacks and Greenberg, 1967); breakage of donor DNA into several large double-stranded fragments by host restriction endonuclease hydrolysis of several phosphodiester bonds might occur prior to this step; (3) base pairing of input DNA single strands with resident DNA, possibly coordinated and simultaneous with donor single-strand formation and occurring near the cell surface at a replication point where a significant stretch of single-stranded resident DNA would exist. Tight base pairing may protect against a deoxyribonuclease anatomically situated at the region of entrance of input DNA which might attack only single-stranded DNA. Stretches of DNA not strongly base paired would be subject to this deoxyribonuclease hydrolysis. Since long stretches of tight base pairing would occur with homologous, but not

heterologous DNA, heterologous single-stranded DNA would be hydrolyzed to oligomers while homologous DNA would have long stretches preserved and then enzymatically recombined at the homologous base-paired region. Minute regions of homology between heterologous and resident DNA might also result in recombination of the small donor DNA segments. Recombination of such oligomers might also occur with homologous DNA base paired over only a small region of a differing cistron. An alternative or additional mechanism for oligomer incorporation might be in DNA synthesis (Goulian, 1968; Okazaki and Okazaki, 1969).

Whatever mechanisms are found in addition to possible host restriction endonucleases to protect the identity of the cell against intrusion of foreign DNA, it is clear that they operate efficiently to prevent biological expression of dissimilar DNA in transforming systems where such DNA is indiscriminately absorbed into the cell. Recombination in effective genetic transformation thus probably requires a large cistronic sized region containing numerous points of homology, presumably to avoid destruction of its integrity by hydrolytic enzymatic activity at some vulnerable point in the process. The intracellular preservation of the biological integrity of foreign lysogenic viral DNA is an interesting apparent exception which may be due to sufficient regions of homology and mode of entrance.

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References

Bodmer, W. F. (1966), *J. Gen. Physiol. 49, Suppl. 6*, 233.
Bodmer, W. F., and Ganesan, A. T. (1964), *Genetics 50*, 717.
Bracco, R. M., Krauss, M. R., Roe, A. S., and MacLeod, C. M. (1957), *J. Exp. Med. 106*, 247.
Burgi, E., and Hershey, A. D. (1963), *Biophys. J. 3*, 309.
Burton, K. (1956), *Biochem. J. 62*, 315.
Denhardt, D. T. (1966), *Biochem. Biophys. Res. Commun. 23*, 641.
Dubnau, D., Smith, I., Morell, P., and Marmur, J. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 491.
Ephrussi-Taylor, H., and Gray, T. C. (1966), *J. Gen. Physiol. 49, Suppl. 6*, 211.
Firshein, W. (1961), *J. Bacteriol. 82*, 169.
Fox, M. S. (1966), *J. Gen. Physiol. 49, Suppl. 6*, 183.
Fox, M. S., and Allen, M. K. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 412.
Goulian, M. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 284.
Krauss, M. R., and MacLeod, C. M. (1963), *J. Gen. Physiol. 46*, 1141.
Lacks, S. (1962), *J. Mol. Biol. 5*, 119.
Lacks, S., and Greenberg, B. (1967), *J. Biol. Chem. 242*, 3108.
Lacks, S., Greenberg, B., and Carlson, K. (1967), *J. Mol. Biol. 29*, 327.
Lerman, L. S., and Tolmach, L. V. (1957), *Biochim. Biophys. Acta 26*, 68.
Marmur, J., Falkow, S., and Mandel, M. (1963), *Annu. Rev. Microbiol. 17*, 329.
Meselson, M., Stahl, F. W., and Vinograd, J. (1957), *Proc. Nat. Acad. Sci. U. S. 43*, 581.
Meselson, M., and Yuan, R. (1968), *Nature (London) 217*, 1110.
Notani, N., and Goodgal, S. H. (1966), *J. Gen. Physiol. 49, Suppl. 6*, 197.
Okazaki, T., and Okazaki, R. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 1242.
Saito, H., and Miura, K.-I. (1963), *Biochim. Biophys. Acta 72*, 619.
Silverstein, E. (1968), *Biophys. J. 8*, A79.
Spizizen, J. (1959), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 18*, 957.
Steinhart, W. L., and Herriott, R. M. (1968), *J. Bacteriol. 96*, 1725.
Szybalski, W. (1961), *J. Chim. Phys. Physicochim. Biol. 58*, 1098.