BIOCHEMICAL AND GENETIC STUDIES OF INTEGRATION AND RECOMBINATION IN BACILLUS SUBTILIS TRANSFORMATION

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THE formal analysis of recombination in higher organisms has suggested models for the exchange of parental material which are the background for the interpretation of the mechanisms of recombination in phages (MESELSON and WEIGLE 1961; KELLENBERGER, ZICHICHI and WEIGLE 1961; KOZINSKI and KOZINSKI 1963), and in bacteria (LEDERBERG 1955; FOX 1960; VOLL and GOODGAL 1961; EPHRUSSI-TAYLOR 1962), e.g., in transformation. It is difficult, if not impossible, to dissociate replication and recombination. The key problem is to distinguish or arrive at a compromise between the two classical hypotheses, namely copy choice *vs.* breakage and reunion. These are distinguished according to what each parental strand contributes to the recombinant—a part of its substance, or merely the information in it. The kinetics of recombinant formation in the pneumococcus and Hemophilus transformation suggests that segments of the donor DNA are rapidly integrated into the recipient genome (FOX 1960; VOLL and GOODGAL 1961; EPHRUSSI-TAYLOR 1962). LACKS (1962) has studied the incorporation of ^{32}P-labeled DNA: the DNA becomes "single-stranded" upon entry into the cells and is integrated concurrently with the phenotypic expression (streptomycin resistance) of the donor DNA. He did not explicitly separate donor and recipient material.

The following experiments on the chemistry of the *Bacillus subtilis* transforming system were aimed at the fate of donor DNA after its entry into the recipient cell, and at the mechanism of its integration. A preliminary account has been presented (BODMER and GANESAN 1963). Some preliminary results of similar experiments have also been reported by SZYBALSKI (1961).

MATERIALS AND METHODS

Preparation of ^{3}H ^{15}N ^{2}H labeled DNA: The method of preparation was based on MARMUR and SCHILDKRAUT (1961) and is described in detail by BODMER and SCHILDKRAUT (1964). The growth medium contains, per liter, 1.5 g KCl, 5 g NaCl, 50 mg Na_2SO_4, 100 mg $MgCl_2 \cdot 6H_2O$, 2 mg $CaCl_2$, 2 mg $FeCl_3$, 6.1 g K_2HPO_4, 1.9 g KH_2PO_4, mg $^{15}NH_4Cl$ (BioRad Corporation, Richmond, California), 147 mg Na_3 citrate, 2 g deuterated sugars, 1.5 g deuterated ionic syrup (containing mainly amino acids). The deuterated extracts are prepared from algae grown in 99.6 percent D_2O (BLAKE, CRESPI, MOHAN and KATZ 1961), and were generously given by DR. J. J. KATZ of Argonne National Laboratory. Care was taken in the preparation of the medium to avoid contamination with H_2O; all solutions were sterilized by filtration. A small inoculum of the required strain was first grown for about 24 to 36 hours at 37°C in 10 ml of the above medium, and then transferred to 45 to 100 ml of this same medium and grown for a further

16 to 24 hours at 37°C. This latter was used as a 10 percent inoculum for the final growth of the cells at 37°C. 5 mc thymidine-methyl ^3H (New England Nuclear) per liter of medium was added at a time allowing at least one further generation of the exponential growth, after which the cells were harvested for purification of DNA. For growing the strain SB $32his_2$ (see NESTER, SCHAFER and LEDERBERG 1963, for designation of strains), the medium was supplemented with 66 mg of L-histidine per liter. This procedure yields DNA with a specific activity of 2 to 10×10^6 cpm/μmole and a density of 1.752 to 1.755, whereas the normal native DNA has a density of 1.703. BODMER and SCHILDKRAUT (1964) have shown that such an ^3H ^{15}N ^2H labeled DNA is indistinguishable in its physical (other than density), and biological properties from an unlabeled DNA. It is also normal in its behavior as a substrate for several nucleases and for DNA polymerase from *E. coli* (RICHARDSON, SCHILDKRAUT and KORNBERG 1963).

The pycnographic separation of light native (NL), heavy native (NH), and heavy heat-denatured (DH) DNA is illustrated in Figure 1. (These designations, as described in Figure 1, are used throughout the paper.) In this experiment, 5μg of an ^3H ^{15}N ^2H labeled heat denatured DNA and 1 μg of a ^{15}N ^2H native DNA were added to a ^{32}P labeled lysate of competent cells prepared as described below. The mixture was purified and an aliquot containing a total of about 150 μg of DNA was pycnographed following the procedures outlined below. The heavy heat denatured DNA is identified by ^3H, heavy native DNA by try_2^+ transformants, and native light DNA, from the lysate, by ^{32}P and his_2^+ transformants. Only 2 percent of the try_2^+ transformants are in fractions 30 to 44, corresponding to the NL region, and less than 12 percent of the DH material is trapped in this region. There is, thus, little or no detectable aggregation of either material with the large excess of DNA from the lysate. The small amount of ^3H in the NH region represents that fraction of the labeled heavy DNA which was not fully denatured by the heat treatment.

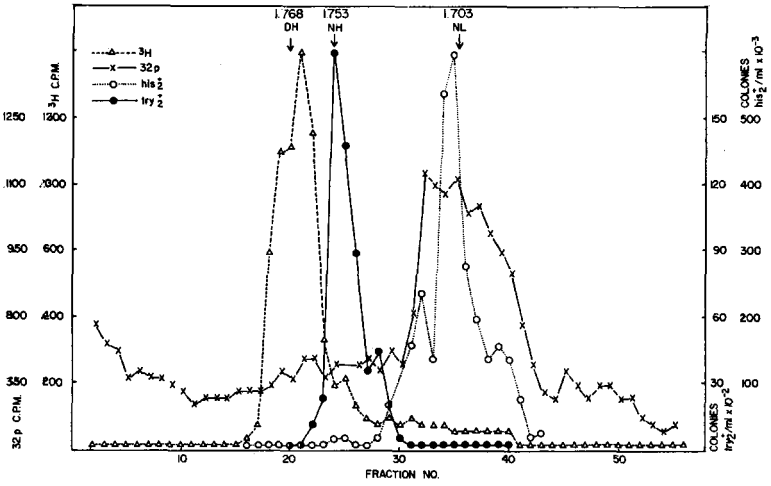


FIGURE 1.—Control pycnographic fractionation of denatured heavy, native heavy and light DNA. A lysate was prepared from 80 ml of ^{32}P labeled competent SB 168 (try_2) cells with a titer of 1.38×10^9 per ml. To this was added 5μg of heat denatured (10 min at 100°C, in SSC) SB 19 ^{15}N ^2H ^3H DNA (specific activity 1×10^7 cpm/μmole) and 1 μg of native SB 19 ^{15}N ^2H ^1H DNA. One third of the resulting purified DNA, containing about 150 μg total DNA, was pycnographed as described in MATERIALS AND METHODS. Fractions were assayed for transformation on SB 202 (try_2 tyr_1 his_2 aro_2).

The following letters are used as abbreviations in all figures and in the text: NL = Native, Light ($^{14}N^1H$); NH = Native, Heavy ($^{15}N^2H$); DH = Denatured, Heavy; HY = Hybrid. The figures above the letters NL, etc., are the corresponding densities in g cm^{-3}.

Radioactivity counting procedures: Samples up to .05 ml were placed on a Whatman GFC/1 Glass filter paper in Packard glass or polyethylene vials and dried under an infrared lamp. 8 ml (10 ml for polyethylene vials) of counting fluid, containing 4 g of 2, 5-diphenyloxazole and 50 mg 1-4 bis-2-(4-methyl-5 phenyloxazole) benzene in 1 liter toluene, was added and samples were counted on a Packard Tri-Carb Liquid Scintillation Spectrometer. From a mixture of ^{3}H and ^{32}P no ^{3}H counts spill into the ^{32}P channel, but a small proportion of ^{32}P counts spill into the ^{3}H channel. The amount of spillage varied from 0.5 to 2.5 percent, according to the instrument used and was determined independently for each series of counts. When counting mixtures of ^{14}C and ^{3}H, a similar procedure was followed. CsCl quenches ^{3}H and ^{14}C but not ^{32}P. At the concentration used for pycnography, in counting volumes of up to .03 ml, 30 percent of the ^{3}H counts were quenched. Where larger volumes have been counted, quenching has been estimated from the effect of CsCl on the ratio of ^{3}H to ^{32}P counts. For low ^{3}H counts (comparable to the background) samples were counted three to four times for 10 minutes. The polyethylene vials yield, for ^{3}H, a background count about one third that for the glass vials. However, the polyethylene vials cannot be used with the toluene-based counting fluid for more than 24 to 36 hours. In all cases we calculated the variance of the repeat counts for each sample and the ratio of this to the mean count, which gives a χ^2 for agreement of the counts with the expected Poisson distribution. In no case did the variance of either the ^{3}H or ^{32}P counts differ significantly from the mean. Repeat counts of small controlled amounts of ^{3}H verify that inaccuracies in pipetting contribute only marginally to the variance of the counts and that very small amounts of ^{3}H can be assayed provided samples are counted long enough. This variance is the basis of the statistical assessment of any given difference in counts. Thus it is possible to assess how many counts above background are needed to validate a difference at the 95 percent significance level. For example, with four repeat counts and a background of 169 counts (also determined by four repeat counts at the same time), this value is approximately 32. In general, the value is approximately $2\sqrt{2}\sqrt{B/r}$, where B is the mean background count and r the number of repeat counts, and is less than 20 counts per 10 minutes (cp10m) for all the results given here.

Purification of DNA: The cells were washed without lysis in Spizizens' Minimal medium (NESTER, SCHAFER and LEDERBERG 1963), supplemented with glucose. Isolation, purification, and analysis of the DNA was carried out as described before (GANESAN and LEDERBERG 1964).

Preparative density gradient centrifugation: Preparative runs and collection of fractions were performed as mentioned in GANESAN and LEDERBERG (1964).

Shear degradation of DNA: Shearing of DNA was performed according to NESTER, GANESAN and LEDERBERG (1963). Half molecules of about 14 million average mol. wt. were produced by shearing at 6000 rpm in a Virtis homogenizer. Increasing the speed to 12,000 rpm results in the production of approximately quarter molecules of DNA having a mol. wt. of about 7×10^6. The molecular weights were determined by sucrose gradient centrifugation, using phage T7 DNA as a standard, following BURGI and HERSHEY (1963), and by sedimentation analysis in the analytical ultracentrifuge. Details of these results will be published elsewhere.

Denaturation and renaturation of DNA samples: Heat denaturation: Heating DNA at 100°C for 10 min in SSC (0.15M NaCl + 0.015 M Na Citrate, PH 7.0) (MARMUR 1961) followed by fast cooling resulted in denaturation of the DNA as observed by a decrease in transforming activity (residual activity less than 0.1 percent) (GANESAN and LEDERBERG 1964). Denaturation is also reflected by the increase in buoyant density and hyperchromicity at 260 mμ.

Alkaline denaturation: DNA was denatured at pH 12.2 using .4 M K_2HPO_4 adjusted with 50 percent KOH solution (VINOGRAD, MORRIS, DAVIDSON and DOVE 1963). DNA denatured in this buffer was added to a buffered CsCl solution (pH 12.1). The pH was checked before and after the density gradient centrifugation. Heat renaturation of DNA was achieved by keeping it 3 hr at 69°C at a concentration of 20 μg/ml (MARMUR and DOTY 1961).

Analytical ultracentrifugation: This was performed as described by GANESAN and LEDERBERG (1964). *B. subtilis* DNA, labeled and unlabeled, and dAT polymer ($p = 1.683$) were used as standards to calculate the densities of the samples. In a few cases, the approximate molecular weight was determined by the band width method (MESELSON, STAHL and VINOGRAD 1957). Sedimentation runs were done, using 20 μg/ml DNA in 1 M NaCl for native material and in

.9 M NaCl + .1 M NaOH for alkaline denatured material, at 37,020 rpm, for about 1 hr. Ultraviolet absorption photographs were taken at 4-min intervals.

Uptake experiments: Competent cells were prepared as in NESTER, SCHAFER and LEDERBERG (1963). In order to label competent cells with ^{32}P, about 1 mc carrier-free ^{32}P (as orthophosphate purchased from Oak Ridge) was added per 100 ml of media during the 4-hr growth period. Phosphate deficiency in the growth medium prevents the cells from becoming competent. Since the ^{32}P itself does not affect competence, reasonable labeling is achieved by adding fairly high amounts of the label. Typically, only about 0.5 percent of the added ^{32}P is taken up by the cells and of this, less than 10 percent is incorporated in the DNA. The DNA isolated in this way has a specific activity of 2×10^5 cpm/μmole. Donor $^3H^2H^{15}N$ DNA was added to 250 ml of competent cells prepared in this way at a saturating concentration of .75 to .9 mμmoles/ml of 1 to 3×10^8 recipient cells or 0.2 cell equivalents of DNA per recipient cell. The mixture was incubated at 30°C for varying lengths of time. Transformation was terminated by the addition of crystalline pancreatic desoxyribonuclease (DNAase) (Worthington) at a final concentration of 20 μg per ml in M/100 $MgCl_2$. After a further 5 min at 30°C, the cells were packed hard and washed four times in minimal medium and the DNA was then extracted in the usual way. An alternative procedure was followed when the timing of the termination of transformation was not critical. Cells were packed hard before DNAase treatment and then resuspended in 20 ml of 0.1 M Tris buffer (Sigma 121) at pH 8.5 and 0.015M $MgCl_2$. DNAase was added to a concentration of 25 μg/ml, the cells were incubated for 10 min at 37°C and then washed three times in minimal medium before extracting the DNA. This procedure was found to be considerably more efficient in removing extraneous acid soluble degraded DNA which may be taken up by the cells under normal conditions. Each wash supernatant was checked for biological activity, total and acid insoluble counts. About 95 percent of the DNA was degraded and made acid soluble. The supernatants had no transforming activity. The final supernatant had acid soluble counts less than 10 percent of the total donor DNA recovered in the extracted material. Care was taken to achieve maximum extraction of the DNA from the cells: yields of 80 to 90 percent were obtained. A control experiment involving short pulse labelling of DNA for 45 seconds with ^3H thymidine followed by extraction as described above insured that there was no preferential extraction of the total cellular DNA relative to newly synthesized DNA.

RESULTS

Quantitation of DNA uptake: Only a small fraction of the donor DNA gets into the cell, and of this, again only a fraction may effect transformation. Data on the amount of DNA added per cell, the amount of incorporation and the amount effective in producing transformants are given for a series of our experiments in Table 1. From 0.5 to 4 percent of the DNA added to the suspension enters the bacteria. The ratio of the donor to the recipient DNA in the reisolated material is the number of cell equivalents of DNA taken up per transformant $\times$ proportion of transformants. Assuming that one cell equivalent of DNA can result in, at most, one transformant for a given marker, the number of cell equivalents of DNA taken up per transformant is a measure of the amount of DNA taken up which is not effective in producing transformants. This will be a minimum value since the occurrence of cells with more than one nucleus ensures at least some duplication of genetic material in a cell-equivalent of DNA. The values obtained vary from 0.89 to 35 for the *B. subtilis* data. The wide range of effectiveness of the donor DNA reflects a combination of variation in the competence of the recipient cells and differences in activity between different DNA preparations. A standard DNA is included with all transformations so that, by

TABLE 1

Quantitation of DNA uptake

			Experiment number				
			121	138	144	174	179
T/n	Cell equivalents of donor DNA per recipient cell.		0.22	0.147	0.2	0.167	.251
n	Recipient cells per ml.	$10^9\times$	5.5	9.3	2.1	0.31	0.25
u	Proportion donor DNA taken up.		0.037	0.0086	0.0052	0.0045	0.0193
p	Porportion transformants/total recipient cells.	$10^{-4}\times$	6.2	0.36	0.6	8.4	42.5
Tu/pn	Cell equivalents of donor DNA taken up per transformant.		12.9	35.	17.3	0.89	1.14
Tu/n	Proportion donor/recipient DNA.	$10^{-3}\times$	7.3	1.6	1.1	0.75	4.8
	Proportion donor/recipient transformants.	$10^{-3}\times$	0.28	0.018	0.18	0.11	1.0

T=Total DNA added per ml, in cell equivalents, assuming one cell-equivalent of DNA is about 5×10^{-9} μg for *B. subtilis* (GANESAN and LEDERBERG 1964).

comparison with this standard, some objective measure of the competence of the recipient cells and the transforming activity of a DNA is made possible. Both may vary by a factor of 5 to 10 under "normal" conditions of preparation. The maximum transformation frequency achieved with the standard was a little over 1 percent. Almost complete efficiency of donor DNA in transformation, as exhibited in the data for experiments 174 and 179, has also been reported for Hemophilus (GOODGAL and HERRIOTT 1957). In fact, since *B. subtilis* has on the average 3.0 nuclei per cell (GANESAN and LEDERBERG 1964), fully efficient utilization of donor DNA in transformation should correspond to one nuclear equivalent of DNA taken up per transformant, or 0.3 cell equivalents per transformant. Our results with *B. subtilis* do not show any direct correlation between uptake of DNA and frequency of transformation. Competence requires both the ability to take up DNA and subsequently to integrate the DNA into the recipient genome. These two processes are, apparently, separable. This has previously been emphasized by IYER and RAVIN (1962).

A marker system is always used which can selectively distinguish the transforming activities of donor and recipient DNA. A measure of the activity of the incorporated donor DNA is given by the ratio of (Donor/Recipient DNA) / (Donor/Recipient transformants). This ratio varies from 1.1 percent to 71.4 percent.

The fate of donor DNA in the cell: A preliminary uptake experiment (121) was done with labeled donor DNA from the prototroph SB 19, and with the quadruple mutant SB 202 as recipient (see Table 2 for details of the strains used). Transformation was terminated after 30 minutes and the DNA from the resulting complex was extracted and pycnographed as described in MATERIALS AND METHOD. The distributions of ^3H, ^{32}P and try_2^+, ura^+ transformants in the various fractions are shown in Figure 2. Donor and recipient DNA were separated by their difference in buoyant density and are traceable by differential counting of

TABLE 2

List of strains of Bacillus subtilis

Strain	Genotype	Growth response to
SB 19	Reference prototroph	
SB 202	*try*$_2$ *his*$_2$ *tyr*$_1$ *aro*$_2$	Shikimic acid + tryptophan + histidine + tyrosine
SB 32	*his*$_2$	Histidine
*SB 504	*try*$_2$ *tyr*$_1$ *aro*$_2$	Shikimic acid + tryptophan + tyrosine
†SB 5	*try*$_2$ *his*$_1$ *ura*$_1$	Tryptophan + histidine + uracil
‡SB 168	*try*$_2$	Tryptophan

try$_2$, *his*$_2$, *tyr*$_1$, and *aro*$_2$ are all in the same linkage group (NESTER, SCHAFER and LEDERBERG, 1963).

* Derived from SB 19—XSB 202.

† *ura* derived by ultraviolet irradiation from SB 1, *try*$_2$, *his*$_1$ (GANESAN and LEDERBERG 1964). *his*$_1$ and *his*$_2$ are unlinked (NESTER and LEDERBERG 1961).

‡ Original BURKHOLDER and GILES (1947) strain.

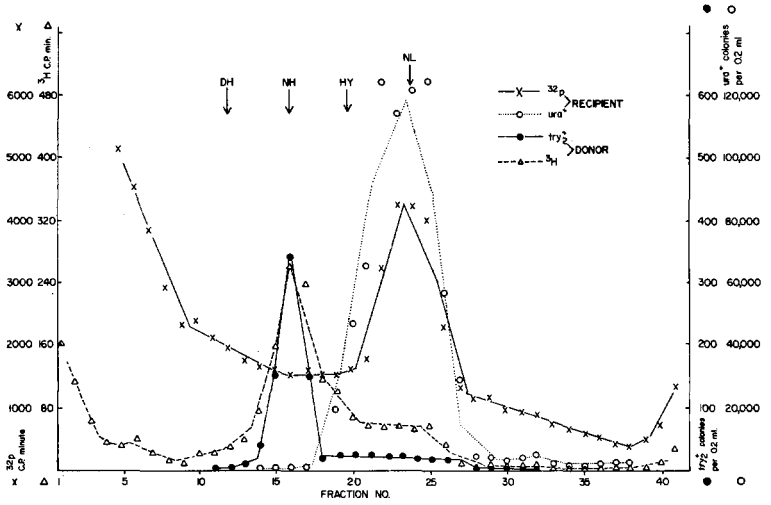
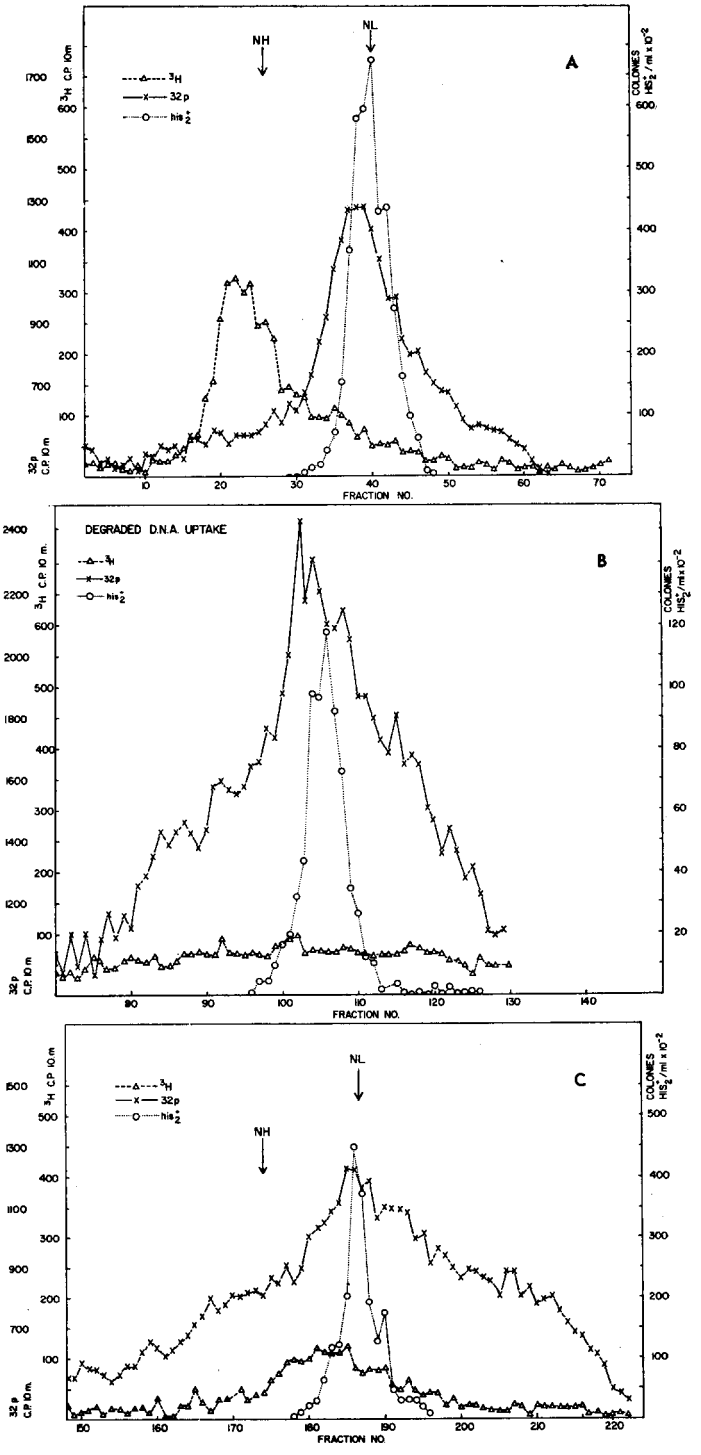


FIGURE 2.—Pycnographic fractionation of donor recipient complex after 30 minutes transformation (Experiment 121). 66 μg of SB 19 prototroph $^{15}N^2H^3H$ DNA was added to 11 ml of ^{32}P-labelled competent SB 202 (*try*$_2$ *his*$_2$ *tyr*$_1$ *aro*$_2$) cells at a titer of 5.5 × 10^9 per ml. Transformation was terminated after 30 minutes and the resultant partially purified donor recipient DNA complex was fractionated pycnographically. Transforming activity of the fractions was assayed on SB 5 (*try*$_2$ *his*$_1$ *ura*$_1$). Other designations are as in Figure 1. The expected positions of native heavy and denatured heavy DNA were calculated from the density gradient established in the control run, using the native light recipient DNA as a density marker. The control gradient is calculated from the observed separations of the three species of DNA used in the control run, whose densities were established by analytical ultracentrifugation. See text for further details. *ura*$_1$+ transformants measure total biological activity. However, because of the large excess of recipient versus donor DNA, the contribution of the donor DNA to this activity is negligible.

^{3}H and ^{32}P. In this preparation no attempt was made to remove the RNA, which accounts for the high ^{32}P counts at the bottom of the tube. The try_2^+ transformants are a measure of the biological activity of the donor DNA. This marker is very stable so that there is no problem with a background of reversions, even when assaying DNA with very little activity and at high cell concentrations. The *ura*$^+$ transformants measure overall biological activity of donor and recipient DNAs. Transformations are done in a single assay using SB 5 as recipient. The expected position of NH, hybrid (HY), and DH are calculated from the positions found in the control run as shown in Figure 1 (see below). NL serves as a reference marker. While absolute measurements of density in preparative centrifuge gradients are difficult, the relative separation of these materials has been consistent over a number of control runs. There is a major peak of donor DNA in the NH density stratum with a shoulder trailing all the way across the NL recipient stratum. The donor biological activity matches closely the ^{3}H counts for all these fractions. There is, however, a very significant amount of ^{3}H labeled material in strata of higher density than NH. Of the recovered donor ^{3}H atoms, .31 are in fractions 1 to 13, which have a try_2^+ specific activity 1/30th of the native heavy peak. .20 of the donor ^{3}H atoms are in fractions 21 to 41, corresponding to the recipient density stratum. The total recovery of ^{3}H was better than .8. Thus, donor material with the density and biological activity of native DNA can be recovered from "inside the cell" (The term "inside the cell" is used with caution. We mean the DNA that is bound in some way to the cell such that it can not be removed by DNAase treatment and subsequent washings.) This probably excludes, at least in *B. subtilis*, the suggestion of LACKS (1962) that the DNA is rendered single-stranded as it enters the cell, unless this can "renature" when inside the cell.

The specificity of the association of donor and recipient DNA, indicated by the shoulder of ^{3}H counts trailing across the NL stratum, was investigated by comparing the uptake of native donor DNA from *E. coli* and *B. subtilis* and of DNAase degraded DNA from *B. subtilis*. A ^{32}P-labeled culture of competent SB 168 was divided into three aliquots. ^{3}H ^{15}N ^{2}H DNA from *E. coli* (K-12) was added to the first aliquot, a mixture of unlabeled heavy DNA and DNAase degraded light ^{3}H DNA from *B. subtilis* (SB 19) was added to the second, and ^{3}H ^{15}N ^{2}H DNA from *B. subtilis* (SB 19) was added to the third. The cells were harvested after 30-minutes contact with the DNA, and the resulting complexes extracted and pycnographed as usual. The distributions of ^{3}H, ^{32}P and his_2^+ transformants (representing predominantly recipient biological activity) are shown in Figures 3a, 3b, and 3c respectively. The broad distribution of ^{32}P is unexplained.

About 59 percent of the *E. coli* DNA which was taken up, is in fractions 14 to 27 corresponding to the expected position for native heavy *E. coli* DNA. About 22 percent of the *E. coli* DNA is associated with the NL recipient stratum in fractions 31 to 48. In contrast to this only 21 percent of the *B. subtilis* DNA is in the NH stratum (fractions 162 to 175) and 49 percent is in the NL stratum corresponding to fractions 179 to 196. There is no evidence for any incorporation


A
NH
NL
^{3}H
32p
his$_2^+$
^{3}H C.P. 10m
32p C.P. 10 m
COLONIES HIS$_2^+$/ml x 10^{-2}
FRACTION NO.
B
DEGRADED D.N.A. UPTAKE
^{3}H C.P. 10 m.
32p C.P. 10 m
COLONIES HIS$_2^+$/ml x 10^{-2}
FRACTION NO.
C
NH
NL
^{3}H C.P. 10 m
32p C.P. 10 m
COLONIES HIS$_2^+$/ml x 10^{-2}
FRACTION NO.

of the degraded material into recipient DNA. The difference in the behavior of the *E. coli* and the *B. subtilis* DNAs is a clear indication of the relative specificity of the association between donor and recipient material. However, the possibility that some of the donor *E. coli* DNA may become associated with the recipient *B. subtilis* DNA is by no means excluded.

The appearance of native donor DNA and higher density donor material "inside the cell" seems to depend on the conditions of transformation, in particular the length of time between the addition of DNA and harvesting the cells, and on the extent to which the extracts have been purified, in particular the removal of RNA. Preliminary experiments suggest that when transformation is terminated after 10 minutes by the addition of DNAase and the cells are then incubated further at 37°C, the unintegrated donor material is degraded within the next 30 to 60 minutes. Further work aimed at clarifying the nature of the unintegrated material and its subsequent fate inside the cell is now in progress.

In the experiments briefly described in the preceding paragraph donor DNA from SB 32 (*his*$_2$) was used with SB 504 (*try*$_2$, *tyr*$_1$, *aro*$_2$) as the recipient: a combination of strains which allows the selective identification of recombinant genotypes. The recombinants are *his*$_2^+$ which are also transformed to prototrophy for one or more of the markers *try*$_2$, *tyr*$_1$, *aro*$_2$. In all transformation assays to detect donor activity in the extracted DNA the recipient strain SB 202 (*aro*$_2^-$ *his*$_2^-$ *try*$_2^-$ *tyr*$_1^-$) was plated on a medium supplemented with shikimic acid, histidine and tyrosine. This selects for *try*$_2^+$ transformants identifying donor activity, and allows segregation of the other linked markers, which are scored as in NESTER, SCHAFER and LEDERBERG (1963). At the assay levels of DNA, *try*$_2^+$ *his*$_2^+$ transformants are not found in simple mixtures of *try*$_2^+$ *his*$_2^-$ and *try*$_2^-$ *his*$_2^+$ DNA, and so can only come from recombinant molecules. Recombinant molecules are found after 10 minutes contact of cells with DNA, at which time the association between donor and recipient DNA is also observed. The proportion of recombinants does not change significantly during 60 minutes subsequent growth, the mean proportion being 0.38. Thus, as in Pneumococcus (Fox 1960), recombinant molecules are formed promptly after the addition of DNA to the cells. Recombination data from this and also a further experiment pooled with respect to four density ranges, are shown in Figure 4. There is no significant change in the pro-

FIGURE 3.—Comparison of the uptake of *E. coli* DNA and degraded *B. subtilis* DNA with native *B. subtilis* DNA. A 60 ml culture of ^{32}P-labeled competent SB 168 (*try*$_2$) cells at a titer of 1.2×10^9 per ml, was split into three aliquots. DNA was added to these as follows: (a) 45 μg of ^{15}H ^{2}H ^{3}H DNA from *E. coli* (K-12), specific activity 6.8×10^6 cpm/μmole; (b) 1 μg of ^{15}N ^{2}H ^{1}H DNA from SB 19 and 84 μg of ^{3}H DNA from SB 19 (specific activity 1×10^6 cpm/μmole) treated 5 min at 30°C with DNAase at a concentration of 15 μg per ml in 0.05M $MgCl_2$ (this makes more than 85 percent of the DNA acid-soluble); (c) 22μg of ^{15}N ^{2}H ^{3}H DNA from SB 19 (specific activity 1×10^7 cpm/μmole). Cells were harvested after 30 min at 30°C, resuspended, treated with DNAase and the DNA purified as described in MATERIALS AND METHODS. Pycnographic fractionation was carried out as before and fractions were assayed for transformation on SB 202 (*try*$_2$ *tyr*$_1$ *his*$_2$ *aro*$_2$). The expected positions of native heavy (NH) and denatured heavy (DH) were calculated as described in Figure 2.

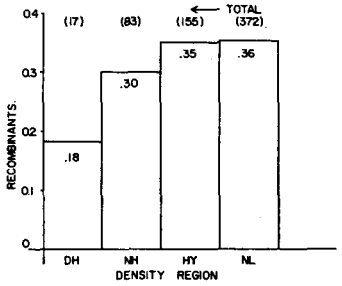


FIGURE 4.—Pooled proportions of recombinants in four successive density regions. The data were pooled from eight separate pycnographic fractionations. Column heights are proportional to the frequency of recombinants. The number of colonies scored is given at the head of each column. The four successive density ranges have boundaries at the midpoints between DH and NH, NH and HY, and HY and NL density strata, respectively. These positions are determined using the control run, as described in the text.

portion of recombinants observed in the different density strata. The proportion in the heaviest range is low, but this is based on a small number of colonies. Nevertheless, if recombination were coextensive only with the integration of donor material, fewer recombinants should appear in the NH to DH strata.

The donor-recipient complex: A further experiment was addressed to the association between the donor and the recipient DNA. Labeled donor DNA from the prototroph SB 19 was used with SB 168 as the recipient strain. Transformation was terminated after 30 minutes and the DNA was extracted and pycnographed as described above. The fractionation data from one of three separate gradients run at the same time using as nearly as possible identical conditions are shown in Figure 5. The donor biological activity (try_2^+ transformants) and

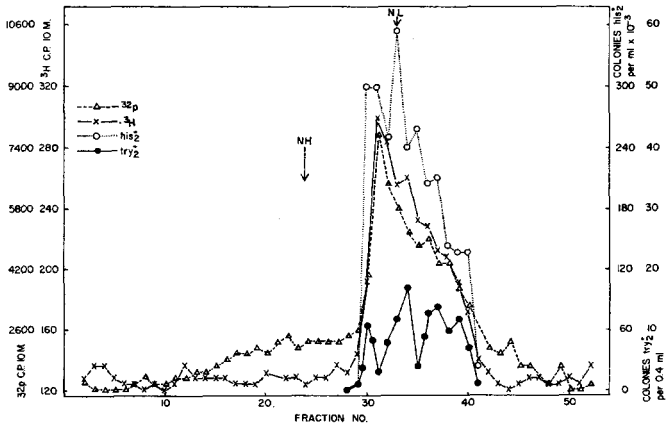


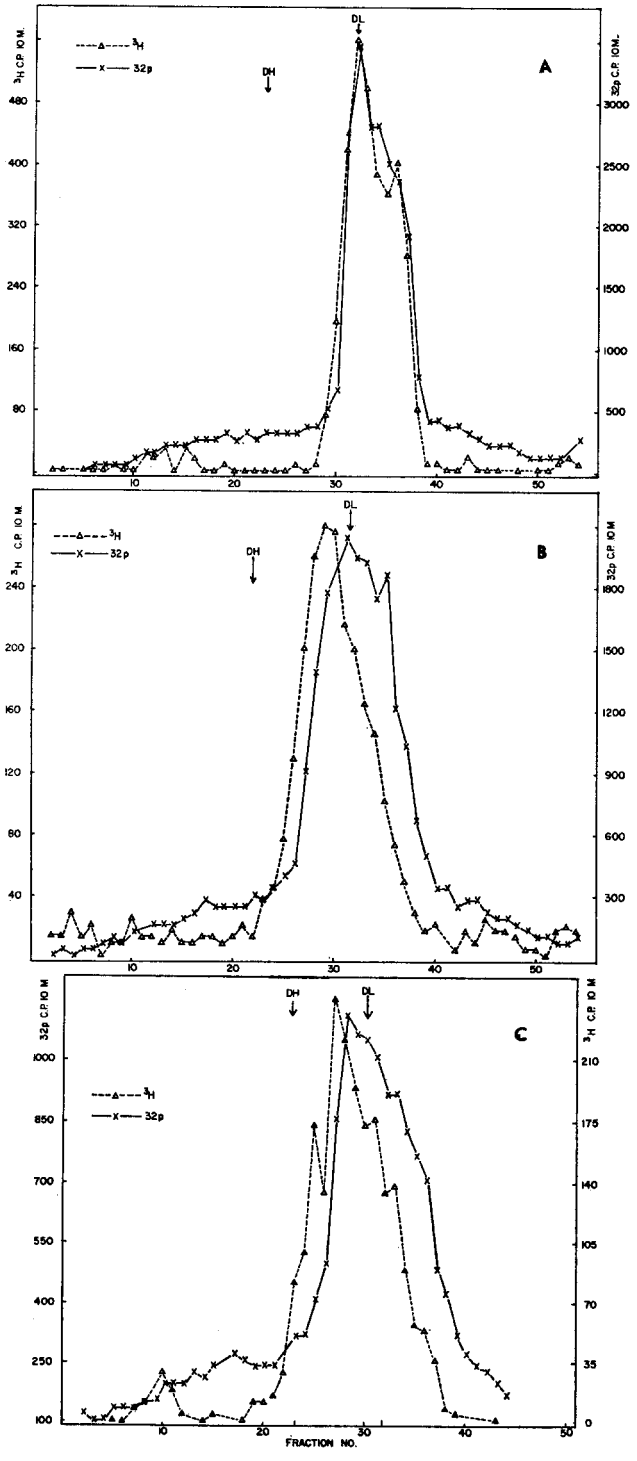
FIGURE 5.—Pycnographic fractionation of donor recipient complex after 30 minutes transformation. (Experiment 174). 66 μg of SB 19 ^{15}N ^{2}H ^{3}H DNA were added to 250 ml of ^{32}P-labeled competent SB 168 (try_2) cells at a titer of 3.1 $\times$ 10^8 per ml. Transformation was terminated after 30 min and the resultant donor-recipient complex extracted and analyzed as described in Figures 2 and 3. The figure shows the analysis of one of three identical runs.

^{3}H are found only in the recipient NL stratum. There is a shoulder of ^{32}P counts on the heavy side of the main ^{32}P peak. This was observed in all three runs and was also found to varying extents in other experiments. Rerunning a fraction well removed from the main peak in the analytical ultracentrifuge showed it to have a density consonant with its position in the preparative gradient. This material may be analogous to that described by ROLFE (1963).

The fractions from the NL strata of all three runs were pooled. One aliquot was rerun without further treatment. A second was sheared at 6000 rpm as described in MATERIALS AND METHODS, to reduce the average molecular weight by a factor of 2, and a third was sheared at 12,000 rpm to reduce the molecular weight by a factor of 4. A fourth was heated for 10 minutes in SSC (MARMUR 1961), and a fifth was run in CsCl solution at pH 12.2 (VINOGRAD, MORRIS, DAVIDSON and DOVE 1963). The results obtained on pycnographic fractionation of these five aliquots are shown in Figures 6 and 7. The expected positions of light and heavy DNA are calculated as before. The bond between donor and recipient DNA is stable to the applied shearing or alkali or thermal denaturation, for in no case is there a significant amount of DNA in the heavy region.

The four treated aliquots show a clear increase in the mean density difference between ^{3}H and ^{32}P as compared with the control. To verify that these density displacements were due only to the heavy isotope incorporated in the donor DNA, a control uptake experiment was done using as donor a light (^{14}N ^{1}H) ^{3}H labeled DNA from SB 32 (*his*$_2$) and as recipient SB 168 (*try*$_2$). The pycnogram for this experiment is shown in Figure 8. The donor ^{3}H and recipient ^{32}P have, apart from the ^{32}P shoulder on the heavy side of the main peak, almost identical distributions. As described for the experiment using heavy donor DNA, fractions from the NL strata were pooled and one aliquot was rerun without further treatment, a second sheared at 6000 rpm, and a third heat denatured. The pycnograms of these three aliquots are shown in Figure 9. In all three cases, the distributions of ^{3}H and ^{32}P are completely superimposed. This shows clearly that the only contribution to the density displacements observed with a heavy donor DNA is from the heavy isotopes incorporated in this DNA. The distributions of donor and recipient biological activities for the control run shown in Figure 9a were also completely concordant with those of the ^{3}H and ^{32}P. 12.5 percent of the donor colonies were *try*$_2^+$ and *his*$_2^+$ recombinants.

Estimates of the density differences between ^{3}H and ^{32}P are obtained from the mean differences in fraction numbers of the two distributions corrected for the density gradient established in the control runs. This gradient is almost linear over short distances and has a slope of approximately 0.06 g cm^{-3} per cm. Knowing the volume of liquid per fraction collected, and that there is a displacement 0.85 cm per ml of liquid in the tube, any given displacement in fraction number can be converted to cm and hence to density displacement. These displacements are given in Table 3. The three pycnograms, shown in Figure 9, of the material obtained using light ^{3}H labeled donor DNA, give an empirical standard error of 0.391×10^{-3} g cm^{-3} for such a density displacement. On this basis the displacement of 0.8×10^{-3} g cm^{-3} in the control differs from zero at about a 10 percent


A
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FRACTION NO.

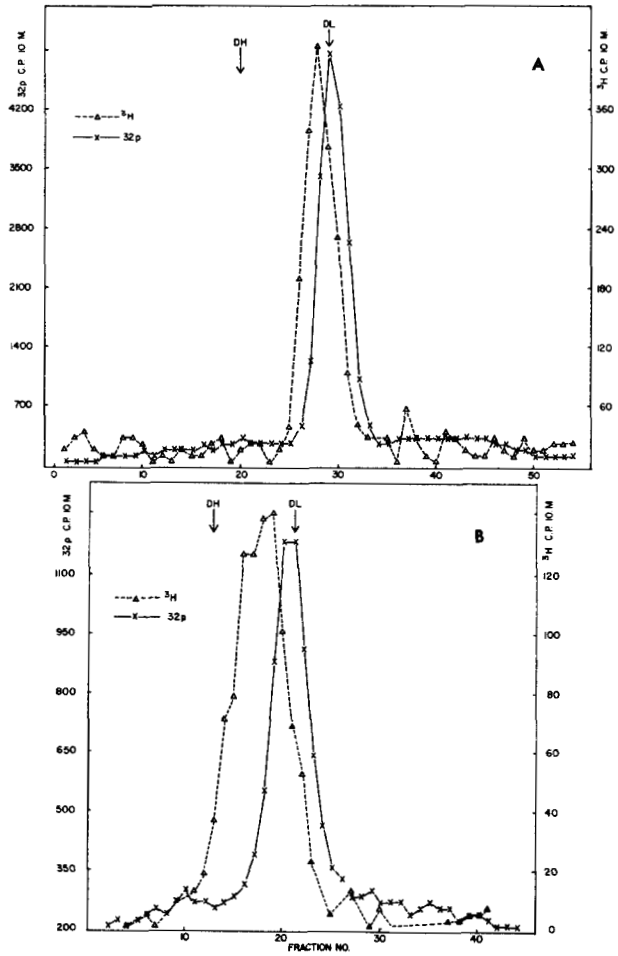

FIGURE 7.—Refractionation of pooled "Recipient" fractions from donor-recipient complex after denaturation. (Experiment 174). Two more aliquots of the material described in Figures 5 and 6 were fractionated. (a) After heating 10 min at 100°C in SSC and then fast-cooling; (b) In CsCl at pH 12.2. (See text for further details.)

significance level. A direct estimate of the mean proportion of donor material in a recipient (transformant) molecule is given by $\Delta\rho/50$, where $\Delta\rho$ is the observed density displacement, and the density difference between heavy and light DNA is .05 g cm^{-3}.

FIGURE 6.—Refractionation of pooled "Recipient" fractions from donor-recipient complex after shearing. (Experiment 174). Fractions from the native light recipient regions of the runs described in Figure 5 were pooled and split into three aliquots which were fractionated: (a) Without further treatment (control); (b) after shearing at 6,000rpm to reduce molecular weight to about 1.4×10^7; (c) after shearing at 12,000rpm to reduce molecular weight to about 7×10^6. (See text for further details.)

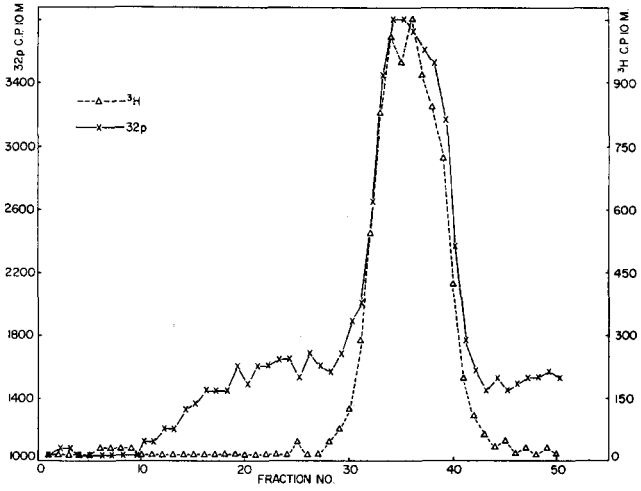

FIGURE 8.—Pycnographic fractionation of donor-recipient complex using a light (^{14}N ^{1}H ^{3}H) donor DNA. 78μg of SB 32 (*his*$_2$) ^{14}N ^{1}H ^{3}H DNA were added to 250 ml of ^{32}P-labeled competent SB 168 (*try*$_2$) cells at a titer of 2.5 $\times$ 10^8 per ml. Transformation was terminated after 30 min and the resulting donor-recipient complex extracted and analyzed as previously described. The data for one of three identical runs are shown.

Shearing into halves and quarters should, with either single or double stranded integration, increase the density displacements between ^{3}H and ^{32}P by approximately two- and three-fold, respectively. The considerably larger observed increases could be explained by appreciable denaturation on shearing. However, HERSHEY, GOLDBERG, BURGI and INGRAHAM (1963) have shown, for T5 DNA, that shearing at 5°C does not cause any detectable denaturation. They also showed that the denaturation resulting from shearing at higher temperatures (45°C) is localized near the center of the molecule and presumably does not cause any strand separation. Our preparations were sheared at 4°C, and showed

TABLE 3

Density displacement of donor (^{3}H) from recipient (^{32}P) DNA

Treatment	Mean $\Delta\rho$ (10^{-3}g cm^{-3})	Mean percent donor DNA
Control	0.8	1.6
Sheared: halves	8.0	16.0
Sheared: quarters	11.0	22.0
Heat denatured	6.5	13.0
Alkaline denatured	13.8	27.6

$\Delta\rho$ is the density displacement from the NL position (ρ=1.703g cm^{-3}). A displacement of one fraction in 50 for a gradient column of 5 ml corresponds to $\Delta\rho = 5.1 \times 10^{-3}$g cm^{-3}. The material used comes from pooling fractions in the native light density strata from three similar runs (see Figure 5). See text and MATERIALS and METHODS for details of the various treatments.

The analogous pycnograms of the material obtained using light ^{3}H donor DNA give an empirical standard error of 0.39×10^{-3}g cm^{-3} (see Figure 9).

The percent donor DNA is $\Delta\rho/50$, since the density displacement between donor and recipient is 0.05 g cm^{-3}.

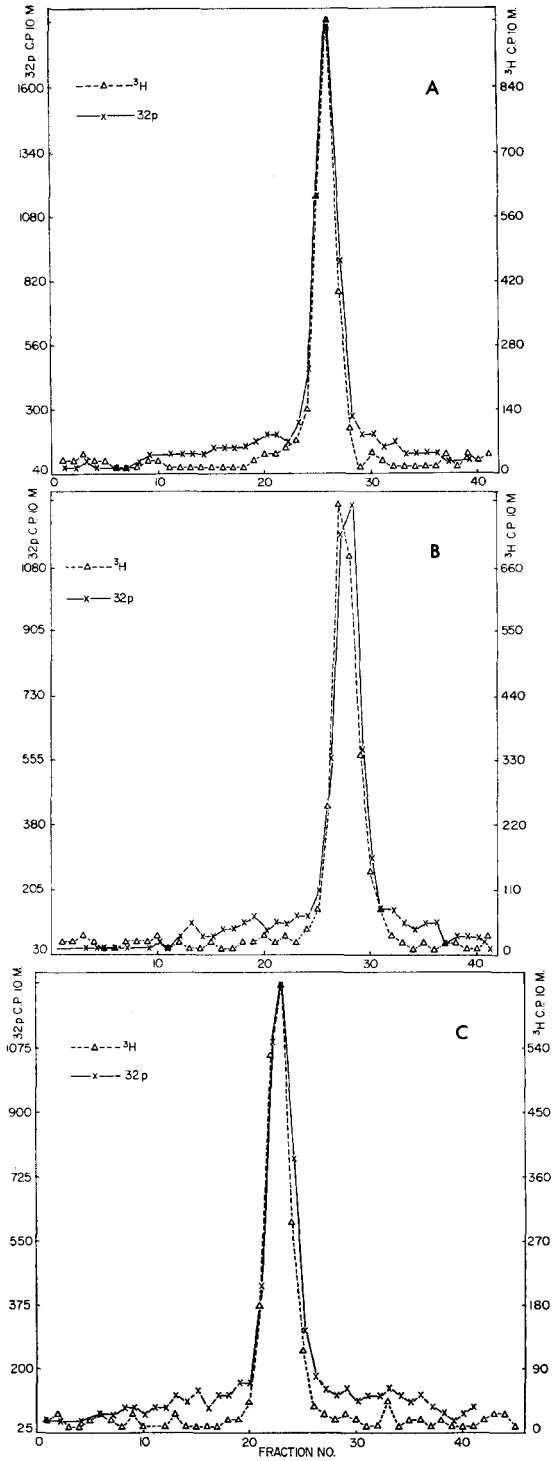

FIGURE 9.—Refractionation of pooled recipient fractions from donor-recipient complex, using a light donor DNA, after shearing and denaturation. Fractions from the recipient regions of the runs described in Figure 8 were pooled and split into three aliquots which were fractionated (a) without further treatment (control), (2) after shearing at 6,000rpm to halve molecular weight, and (c) after heating 10 min at 100°C and fast-cooling.

no increase in density as determined in the analytical ultracentrifuge. Preferential denaturation of the donor regions would have been detected in the control experiments, using light donor DNA. Assuming no fragmentation of molecules on denaturation, there should be no increase in the density displacement between ^3H and ^{32}P if integration is at the double stranded level, and an increase by a factor of 2 if integration is in a hybrid region. The observed 8- and 17-fold increases for heat and alkali denatured material respectively, are too large to be attributed to the small number of preexisting single-strand breaks that occur in our material (see below). It thus seems most likely that the control density difference has been underestimated, perhaps because of accentuated aggregation effects when the density difference between transformant and recipient molecules is very small. This effect is likely to be further accentuated for heat denatured DNA, because of nonspecific reaggregation on cooling, and may therefore account for the discrepancy between heat and alkali denaturation. It has been verified that the density gradient is unaffected by the alkaline run conditions.

The observed displacements on denaturation and shearing can be used to obtain rough estimates of the size of the integrated region and the number of preexisting single-strand breaks. Suppose that integration occurs in restricted regions of the recipient molecule such that a proportion r of such a region consists of donor material. Suppose further that x, the mean total length of such integrated regions in a molecule of unit length, is distributed at random amongst an average of l pieces. Then the mean length of an integrated segment is x/l. Let k be the mean number of breaks per unit length, then if breaks occur at random with respect to the donor material, the mean number of breaks per integrated segment is kx/l. Hence the mean length of an uninterrupted integrated segment is $\frac{x/l}{kx/l+1} = \frac{x}{kx+l}$. Now, assuming the probability of a broken fragment carrying more than one integrated segment is small, the mean density of fragments of length y, which contain an integrated segment, is approximately

$$\rho = \left[\rho_1 \left(y - \frac{x}{kx+l} \right) + \frac{((1-r)\rho_1 + r\rho_2)x}{kx+l} \right] \Big/ y,$$

where ρ_1 and ρ_2 are the densities of recipient and donor DNA, respectively. The density displacement between donor (^3H) and recipient (^{32}P) is

$$\rho - \rho_1 = \frac{1}{y} \frac{r\Delta x}{kx+l}, \text{ where } \Delta = \rho_2 - \rho_1.$$

The weight average density displacement is thus approximately $\delta = \frac{(k+1)r\Delta x}{kx+l}$, since the weight average of $1/y$ is simply the number average molecular weight, which after k breaks is approrimately $1/k + 1$. Substituting $\Delta = 50 \times 10^{-3}$ g cm^{-3}, $\delta = 8 \times 10^{-3}$ g cm^{-3} for $k = 1$ and $\delta = 11 \times 10^{-3}$ for $k = 2$ in equation (1) gives $rx = 0.088\ l$ and $x = l/10$. It seems unlikely that $l > 1$, since even $l = 2$ would give $\delta = 50 \times .088 \times 2 = 8.8 \times 10^{-3}$ g cm^{-3} for the control untreated material, which is greater than the displacement for the halves and would surely have been detected. Using these estimates and assuming $\delta = 13.8 \times 10^{-3}$ g cm^{-3} for the denatured DNA displacement, we obtain for the number of pre-existing breaks

per single strand, $k = 3.1$ for double-stranded integration and $k = 0.83$ for single-stranded integration. A direct approximate estimate of the number of single-strand breaks can be obtained by comparing mean sedimentation rates in the analytical ultracentrifuge at neutral and alkaline pH. If there are no single-strand breaks these sedimentation rates should be the same (W. STUDIER, personal communication). The derived ratio of molecular weights should lie between $\frac{1}{1+k}$ and $\frac{2}{k^2}(e^{-k}-1+k)$ depending on the dispersion of the initial DNA preparation (CHARLESBY 1954). The sedimentation patterns show a trail of about 20 to 25 percent of the total alkaline denatured material, which does not sediment. There is actually no significant difference in the overall sedimentation rates. A difference corresponding to $k = 3$ should have been detected even for an initially homogeneous preparation. Thus, our data suggest single-stranded integration and provide clear evidence that portions of the intact donor DNA are built into the recipient genome and held there by bonds which are resistant to shearing and denaturation.

To obtain further evidence for a direct correlation between the incorporated ^3H and the donor biological activity, individual fractions from the fractionation of the heat denatured donor-recipient complex, depicted in Figure 7a, were renatured with DNA from SB 202 (aro_2^- try_2^- his_2^- tyr_1^-). A summary of the data on the recovery of donor (try_2^+) and recipient (his_2^+) transforming activity is given in Table 4. There is a striking dependence of the ratio of donor over recipient activity on density, which is highly significant, in spite of the small number of donor colonies recovered. This provides, perhaps, the most direct evidence that the donor ^3H we find associated with recipient material is, in fact, the integrated DNA which gives rise to transformants.

DISCUSSION

Our data cannot clearly distinguish double-stranded from single-stranded integration. The estimated number of preexisting single-strand breaks obtained from the alkali denatured material is somewhat high if integration is assumed to

TABLE 4

Correlation of donor transforming activity with density

Fractions	his_2^+ Titer/ml $\times 10^{-3}$	try_2^+ Number of colonies	Ratio try_2^+/his_2^+
225–7	32	10	0.31
228–30	334	21	0.066
231–33	163	2	0.012

DNA from SB 202 (aro_2 try_2 his_2 tyr_1) was heated 10 min at 100° in SSC at a concentration of 28 μg per ml. 0.4 ml of this denatured material was added to each of fractions 25 to 33 from the pycnogram illustrated in Figure 7a, and the mixture was dialyzed against 2×SSC. Renaturation was carried out as described in MATERIALS AND METHODS. Renatured fractions were assayed individually for his_2^+ (recipient), and try_2^+ (donor) transforming activity, using SB 202 as a recipient.

Heterogeneity $\chi_2^2 = 30.2$, $P \ll 0.1$ percent.

χ_1^2, to test linear trend, $= 22.7$, $P \ll 0.1$ percent.

be double-stranded. Assuming double-stranded integration, we estimate that the integrated region extends over 10 percent of the average recipient molecule and consists of 88 percent donor atoms. Taking 28×10^6 as our control molecular weight, this gives a value of 2.8×10^6 for the molecular weight of the integrated region corresponding to about 4.2×10^3 nucleotide pairs.

The effects of DNAase on transformation of linked markers suggest single-stranded integration (BODMER 1963 and in preparation). Treatment of transforming DNA with DNAase I (pancreatic deoxyribonuclease) results in a loss of linkage before there has been any appreciable drop in viscosity, and hence in molecular weight. LERMAN and TOLMACH (1957) have shown, at least in Pneumococcus, that such mild treatment with DNAase does not appreciably affect the uptake of the DNA. Thus single-strand breaks in the DNA inhibit the cotransfer of linked markers. With single-stranded integration the proportion of donor material in an integrated region, r, must be less than 0.5. For $r = 0.5$, x is about 0.2. In other words, the integrated region extends over about 20 percent of the transformant strand, corresponding to a length of about 8.4×10^3 nucleotide pairs. A typical SB 19 (prototroph) donor DNA gives about 50 percent prototrophs when used with SB 202 (aro_2^- try_2^- his_2^- tyr_1^-) as a recipient. These four markers cover the major part of the aromatic linkage group, which probably contains 10 to 15 cistrons (NESTER, SCHAFER and LEDERBERG 1963). Assuming an average length of 10^3 nucleotide pairs per cistron, this corresponds to 1.0×10^4-1.5×10^4 nucleotide pairs. This length must therefore be covered at least 50 percent of the time by an integrated region, which corresponds reasonably with the estimated size of such a region based on single-stranded integration.

It seems natural to speculate that hydrogen bonding of complementary DNA strands mediates the recognition or synapsis of donor with recipient DNA. A schematic model for synapsis and integration based on hydrogen bonding, is illustrated in Figure 10. Double crossover types (1011, 1001, etc.) would originate from multiple switching of pairing partners. Integration involves strand breakage followed by repair along gaps within the integrated region which are not covered by the donor DNA and finally the formation of phospho-diester bonds to join the integrated region to the recipient strand. The repair process may be analogous to that already described for an *in vitro* system by RICHARDSON, LEHMAN and KORNBERG (1964). The region over which cotransfer of markers in transformation can be detected is at most about 1 to 2 percent of the whole genome and thus corresponds in size to the region of effective pairing postulated by PRITCHARD (1955, 1960a,b), CHASE and DOERMANN (1958), and others to explain high negative interference in genetic fine-structure analysis. Thus, if a hybrid integrated region in transformation corresponds to such a region of effective pairing, our data are not in conflict with the elegant experiments of MESELSON and WEIGLE (1961, and personal communication), and KELLENBERGER, *et al.* (1961), suggesting that in phage λ recombination involves breakage and rejoining of double-stranded molecules. These authors are observing recombination over a major portion of the λ chromosome, whereas in transformation we are studying the region corresponding to the neighborhood of a point of exchange. An inter-

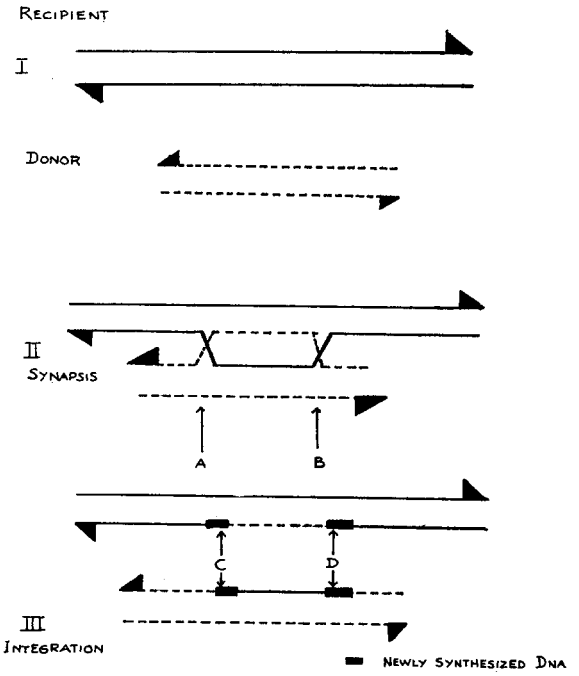

FIGURE 10.—Schematic model for synapsis and integration during transformation. (I) Intact donor and recipient molecules. (II) Synapsis by segmental interchange of hydrogen bonding between donor and recipient strands. (III) Integration by breakage of strands (A, B) followed by repair of unpaired regions (C, D) using the unbroken strand as template and covalent linkage to recipient (donor) material.

esting consequence of such a model for integration during transformation is that donor molecules should be left with some recipient material, giving rise to (reciprocal) recombinant molecules of predominantly donor density. This was in fact observed as illustrated by the data shown in Figure 4. The frequency of recombinant genotypes did not show any significant variation with density.

Models of recombination for higher organisms based on hybrid (heteroduplex) synaptic regions with repair of gaps left by imperfect reciprocal exchange of pairing partners can explain a number of features of recombination in fungi and bacteria including the association between gene conversion and crossing over. Such models have been discussed recently by WHITEHOUSE (1963) and HOLLIDAY (1964).

The models discussed above have invoked DNA synthesis only for a repair process. It has, so far, proved impossible to assess accurately the amount of DNA synthesis that occurs during transformation. Levels necessary for a repair process or even for synthesis of regions much larger than the integrated region cannot be ruled out. However, it seems unlikely that transformation can occur only in a replicating region, for with the observed almost fully efficient utilization for transformation of donor DNA taken up into the cell, this would imply that almost every transformed cell replicated that part of the genome containing the selected

marker during the 10 to 20 minutes following addition of donor DNA. An alternative hypothesis would be that replication is initiated in an integrated region. The role of replication during transformation and the kinetics of the processes leading to integration are under further investigation.

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SUMMARY

DNA was isolated from a multiply marked strain of *B. subtilis* grown on ^{15}N ^{2}H medium with added ^{3}H thymidine. Competent cells were prepared in an ^{14}N ^{1}H medium containing ^{32}P.

Using the heavy labeled DNA as a donor, transformation was terminated at 10 to 30 minutes with DNAase. The DNA purified from the donor recipient complex was fractionated pycnographically. Donor and recipient DNA differed in buoyant density and were traceable by differential counting of ^{32}P and ^{3}H. Donor atoms were found in density strata corresponding to native donor but also in native recipient, hybrid, and "denatured" donor DNA. These DNA fractions were assayed for their genotypic content by transformation. Donor and recombinant genotypes were found in all of these strata but the last, which is biologically inactive. The relative number of donor atoms found in the heavy strata decreases when the cells are allowed to grow after transformation. The association of donor atoms (i.e. ^{3}H) with a recipient stratum persisted on refractionation of the DNA, and was also found when transformation was terminated after only 10 minutes. There is an increased density separation of ^{3}H and ^{32}P counts on denaturation by heat or alkali and on shearing, but no appearance of material of predominantly donor density. The data provide clear evidence that portions of the intact donor DNA are built into the recipient genome and held there by bonds which are resistant to shearing or denaturation. They also suggest single-stranded integration. Assuming single-stranded integration, an integrated region contains about 8.4×10^3 nucleotide pairs. The implication of the results for models of recombination during transformation and for models of recombination in higher organisms are discussed.

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