Integration of Deoxyribonuclease-Treated DNA in *Bacillus subtilis* Transformation

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ABSTRACT Normal preparations of *B. subtilis* DNA have weight average native molecular weights of 10 to 30 $\times$ 10^6. For any given preparation the upper and lower 95% size limits may differ by a factor of ten or more. Single-stranded molecular weights indicate an average of 1 to 4 breaks per single strand of the native DNA. The reduction in transforming activity and viscosity following DNAase I digestion can be accounted for by a direct relationship between the transforming activity of a DNA and its single-stranded molecular weight. Uptake studies with DNAase I treated heavy ($^2H^{15}N$ ^3H) DNA show that single strand breaks inhibit integration less than transformation. A provisional estimate of the size of the integrated region based on correlating the single strand size of the donor-recipient complex with the donor-recipient density differences following alkali denaturation came to 1530 nucleotides. Using a competent, nonleaky thymine-requiring strain of *B. subtilis* grown in 5-BU medium before and after transformation, it was shown that (*a*) No detectable amount of DNA synthesis is necessary for the initial stages of integration. (*b*) Cells which have recently been replicating DNA are not competent. (*c*) Cells containing donor DNA show a lag in DNA replication following transformation. (*d*) When donor DNA is replicated it initially appears in a density region between light and hybrid. This indicates that it includes the transition point formed at the time of reinitiation of DNA synthesis in the presence of 5-BU following transformation. A model is proposed in which donor DNA is integrated at the stationary growing point of the competent cell, which is in a state of suspended DNA synthesis.

INTRODUCTION

There are two major reasons for a general interest in the elucidation of the molecular mechanisms of integration and recombination in DNA-mediated bacterial transformation. First, there is the hope that these mechanisms will suggest studies directed toward an understanding of the molecular basis for recombination in higher organisms. Second, the unique opportunity a transforming system provides for the study of the physical and chemical properties of DNA molecules in relation to their biological activity cannot fully be exploited without an understanding of the molecular mechanisms involved.

The current state of understanding of the *Bacillus subtilis* transformation system will be briefly reviewed in this paper. Studies on the integration of DNA treated with pancreatic DNAase underline the significance of single strand breaks and single-stranded molecular weight for the transforming activity of a DNA preparation. Precise measurements of the number of preexisting single strand breaks lead to a clearer interpretation of experiments on the integration of density labeled donor DNA (Bodmer and Ganesan, 4). Recent evidence on the involvement of DNA synthesis in transformation (Bodmer, 3) suggests that donor DNA is integrated at a stationary growing point of DNA replication. This model has profound implications for a more complete understanding of the transformation process.

Inactivation of Transforming Activity by DNAase

The fact that the transforming activity of a DNA preparation could be destroyed within 30 min by as little as 10^{-4} to 10^{-5} μg/ml of purified pancreatic DNAase (DNAase I) was one of the major initial lines of evidence that bacterial transformation was mediated by DNA. In an early paper on the effects of various physical and chemical agents on transforming DNA, Zamenhof, Alexander, and Leidy (46) showed that a major part of this loss of transforming activity as a result of DNAase I treatment occurred before there was any appreciable drop in the viscosity of the DNA. This is presumably before there is any significant reduction in the molecular weight of the DNA. The kinetics of molecular weight decline, on digestion by DNAase I, as a function of the number of bonds broken, were interpreted by Thomas (42) and Schumaker, Richards, and Schachman (37) in terms of single strand (as opposed to double strand) breakage by the enzyme. In this case no molecular weight decline is expected until breaks occur sufficiently near to each other on opposite strands of the DNA molecule that the number of hydrogen bonds between the breaks is inadequate to hold the molecule together. Measurement by titration with alkali of the number of phosphodiester bonds broken shows that molecular weight decline starts only after up to 200 bonds per molecule have already been broken (Thomas, 42). The inactivation of transforming activity before there has been any appreciable drop in viscosity, must therefore result from these single strand breaks.

Typical data for an assay in which transforming activity and viscosity were measured simultaneously are shown in Fig. 1. At a time when only 20% of the initial transforming activity remains, the viscosity is still at 85% of its initial value. Fig. 1 also shows the much more rapid loss of transformation for four linked markers as compared with the rate for only two of the markers (Bodmer, 2). The relative rates of loss of different linked activities correspond roughly to what is expected from the number of markers and their linkage relationships. The interpretation of these kinetics is, however, complicated by the very rapid over-all loss in transforming activity.

A characteristic difference between endonucleases, such as DNAase I, and exonucleases is that the latter release acid-soluble products at a much faster initial rate relative to the number of phosphodiester bonds broken. They may

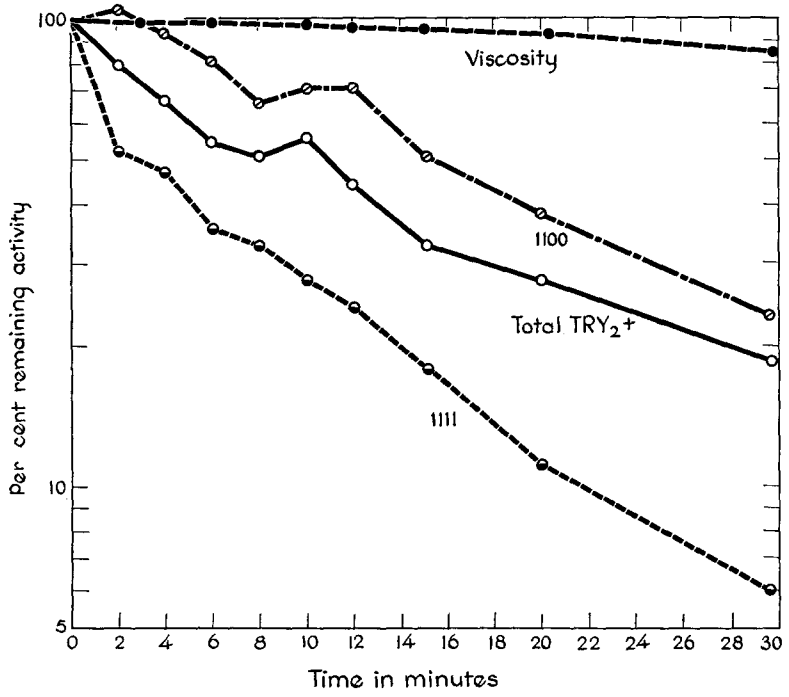

FIGURE 1. Inactivation of transforming activity by DNAase I. DNA prepared from the prototoph SB19 was incubated at 37°C at a concentration of 12 μg/ml in an assay mixture containing 0.01 M Tris buffer, pH 7, 0.01 M $MgCl_2$, and 10^{-5} μg/ml DNAase I (once crystallized, Worthington Biochemical Corporation). The enzyme was made up into 1 mg or 100 μg/ml solutions, divided into several aliquots, and frozen. A fresh aliquot was thawed for each new enzyme assay. The reaction was started by addition of the enzyme to a prewarmed assay mixture containing the DNA. At various times, as indicated, samples of 0.1 ml for transformation were added to 0.1 ml of 0.2 M citrate on ice, to which was subsequently added 1 ml of competent SB202 (*aro*$_2$, *try*$_2$, *his*$_2$, *tyr*$_1$) containing 2.2 × 10^8 cells. All samples were transformed simultaneously. Transformants were selected by plating on media lacking one of the supplements required by SB202 and were assayed for their genetic content by replication to other appropriate selective media. Donor markers are designated by a "1" and recipient markers by a "0" so that 1111 refers to the prototoph and 1100 to the genotype *his*$_2$ *tyr*$_1$, where the genetic order of the markers is as given in the designation of the strain SB202 (see Nester, Schafer, and Lederberg (27) for details of these procedures). The decrease in viscosity was measured at the same time as the reduction in transforming activity, using the same 37°C water bath and an aliquot of the same assay solution. The measurements were made using an Ostwald single bulb viscometer with a capacity of 1.2 ml and a flow time, for the assay mixture, of 106 sec. For further experimental details concerning preparation of DNA, competent cells, etc., see Bodmer (3) and Bodmer and Ganesan (4).

also cause a relatively rapid initial drop in viscosity, presumably due to the collapse of secondary structure. (Lehman and Richardson, 18) As might be expected, therefore, exonucleases result in an initial rate of loss of transforming activity relative to the release of acid-soluble products or the drop in viscosity which is much less than that for endonucleases (Bodmer, unpublished data;

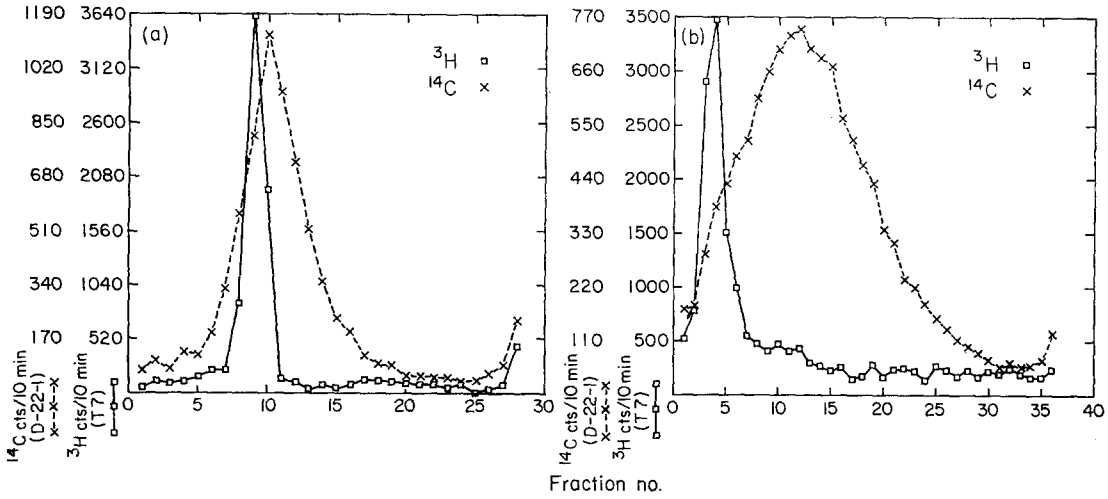

FIGURE 2. Distributions of T7 and *B. subtilis* DNA in preparative sucrose gradients at neutral (*a*) and alkali (*b*) pH. T7 DNA (^{3}H-labeled) prepared by phenol extraction following Mandel and Hershey (22), was the gift of Dr. Lucas. D-22-1 DNA (^{14}C-labeled) was prepared by C. Stewart, according to Marmur (23), from SB565 (*thy*, *try*$_2$). A mixture of the DNA's (containing a total of less than 5 μg) was layered on a 5 to 20% 5 ml linear sucrose gradient and centrifuged for 3 hr at 38,000 RPM in a Spinco Model L ultracentrifuge using the SW39 rotor. Generally from 20 to 40 fractions per tube were collected. Neutral gradients contained, in addition to sucrose, 1 M NaCl, 0.05 M Tris pH 8, and 0.001 M EDTA, whereas alkali gradients contained 0.1 N NaOH, 0.9 M NaCl, and 0.001 M EDTA. Counting was done using a Packard scintillation counter as described by Bodmer (3) and Bodmer and Ganesan (4).

see Lehman and Richardson (18) and Richardson, Inman, and Kornberg, 35). This difference provides a simple and sensitive method for distinguishing exonucleolytic from endonucleolytic attack. Thus, for example, when 22 % of the DNA has been rendered acid-soluble by *E. coli* exonuclease III, 18 % of the original biological activity still remains (Richardson et al., 35). No detectable acid-soluble products are, however, released ($<5\%$) by DNAase I even when less than 10% of the original transforming activity remains.

Correlation of Single- and Double-Stranded Molecular Weight with Transforming Activity

A comparison of the sedimentation rates of a DNA in neutral and alkaline sucrose gradients provides a sensitive measure of the number of single strand

breaks as determined by the relative double- and single-stranded molecular weights (Young and Sinsheimer (45); Studier, 40). Using a uniform DNA preparation, such as can be obtained from phages λ or T7, an average of only 1 break per three strands can easily be detected. Bacterial DNA preparations are, however, not uniform. Standard methods of preparation (e.g. Marmur, 23) fragment the bacterial chromosome into 50 to 200 pieces, presumably at random. Moreover, very slight internal degradation by nucleases during lysis of the cells is probably almost impossible to avoid even in the presence of a strong chelating agent (usually 0.1 M EDTA).

The size heterogeneity of a typical *B. subtilis* DNA, using T7 DNA as a reference, is illustrated in Fig. 2. This shows the zone sedimentation pattern of the two DNA's in preparative neutral and alkaline sucrose gradients, using the conditions given by Studier (40). Assuming a molecular weight of 26.5×10^6 for native T7, the weight average molecular weight of the *B. subtilis* DNA is 21.1×10^6 in the double-stranded (neutral) and 5.8×10^6 in the single-stranded (alkali) form, reflecting an average of one or two preexisting single strand breaks per strand of the molecules as isolated. If we assume that the variance of the distribution of the T7 DNA corresponds to that expected for a uniform preparation, then the difference between the variance of the *B. subtilis* DNA distribution and that of the T7 is an approximate measure of the molecular weight heterogeneity of the former. Assuming that the distribution of the material in the gradient is approximately normal, the mean position ± twice the square root of this corrected variance will give the molecular weight limits which include approximately 95% of the *B. subtilis* preparation. For the DNA preparation illustrated in Fig. 2 these limits are 48.7×10^6 and 5.9×10^6 for the double-stranded form and 17.4×10^6 and 0.6×10^6 for the single-stranded form. There is, thus, a more than tenfold range in the size limits of this preparation, which is by no means uncharacteristic.

In spite of this size heterogeneity of bacterial DNA, sedimentation in neutral and alkaline sucrose is still a very sensitive technique for the detection of a small number of breaks caused by low levels of DNAase treatment. The weight average molecular weights can be accurately determined from the mean position of material in the gradient. The weight average molecular weight of a polydisperse DNA preparation decreases by a factor

$$\frac{2n}{(n+1)R^2}\left[\frac{1}{(1+R/n)^n} - 1 + R\right]$$

for a mean of R breaks per initial number average molecular weight, where the initial size distribution is a gamma distribution with single parameter n (Bodmer, unpublished result). When $n = 1$, for an initial exponential distribution, this ratio is $\frac{1}{1+R}$, and when $n \to \infty$, for an initially uniform preparation, it is

$\frac{2}{R^2}[e^{-R} - 1 + R]$ as given by Charlesby (7). Since the initial ratio of weight average to number average molecular weights is $n + 1/n$, the mean number of breaks per initial weight average molecular weight is $(n + 1)\ R/n$. Thus

TABLE I

DOUBLE- AND SINGLE-STRANDED MOLECULAR WEIGHTS AND RELATIVE TRANSFORMING ACTIVITIES OF A SERIES OF DNA'S

Preparation	Molecular weight $\times 10^{-6}$		Relative transforming activity
	Double Strand	Single strand	
D-22-1	21.2	5.8	0.76
36	19.5	7.9	1
47	15.0	2.9	0.21
55A	10.9	3.9	0.43
55B	7.2	1.3	0.066
55C	6.6	0.5	0.0048
58A	17.0	2.4	0.11
58B	14.0	2.1	0.041
58C	12.8	1.3	0.024

Molecular weights were determined by preparative sucrose gradient sedimentation at neutral and alkaline pH, following Burgi and Hershey (6) and Studier (40) as described in the legend to Fig. 2. T7 DNA or *B. subtilis* D-22-1 DNA calibrated against T7 DNA, was used as a standard either in the same tube or in a parallel tube in the same centrifuge run. Molecular weights were calculated from the formula:

$$\frac{D_1}{D_2} = \left(\frac{M_1}{M_2}\right)^{\alpha}$$

where $\alpha = 0.35$ at neutral pH and 0.4 at alkaline pH, D_1, D_2 are the distances sedimented by sample and standard, and M_1, M_2 the respective molecular weights. The distances were based on the mean position of the material in the gradient and so give weight average molecular weights. Values of 26.5×10^6 and 13.25×10^6 for whole molecules of T7 DNA at neutral and alkaline pH respectively were used as basic reference molecular weights. Preparations 55B and 55C refer to aliquots of 55A treated for 20 and 75 min at 37°C, respectively, at a DNAase concentration of 3×10^{-5} μg/ml and a DNA concentration of 13 μg/ml. 58B and C are similarly treated aliquots of 58A (see Table II for further details). All the DNA's were assayed for their transforming activity simultaneously at a linear dose response concentration, using the strain SB202 (aro_2 try_2 , his_2 tyr_1) as a recipient (27).

even when $n = 1$, an average of 1 break per initial weight average molecular weight results in a 30% reduction in the weight average molecular weight.

The double- and single-stranded weight average molecular weights of a series of DNA preparations are given in Table I together with their relative transforming activities. These weights were calculated using T7 DNA, or a

DNA calibrated directly against T7 (mainly D-22-1), as a standard, following Burgi and Hershey (6) and Studier (40). The relative transforming activities were determined in a single transformation experiment in which all the DNA's were assayed simultaneously. Preparations 55B, 55C and 58B, 58C are successive mildly DNAase I–treated samples of DNA's 55 and 58 respectively. As expected the DNAase treatment causes a much more marked reduction in the single- than in the double-stranded molecular weight. Thus, for DNA 55, the double-stranded weights of samples B and C relative to A are 0.66 and 0.61 respectively whereas the corresponding relative single-stranded weights are 0.33 and 0.13. These reductions in single-stranded molecular weight correspond to a maximum of 4 and 13 breaks respectively per single strand of a molecule with molecular weight 3.9×10^6.

It seems worth noting that with the concentrations of DNA and DNAase I used, 19 μg/ml and 3×10^{-5} μg/ml respectively, there is only 1 DNAase I molecule per 1.15×10^8 nucleotides, assuming DNAase I has a molecular weight of 6×10^4. The observed rates of bond breakage, namely 4 and 13 per 3.9×10^6 single strand molecular weight in 20 and 75 min respectively, correspond to an average of 1 break per 6.4×10^4 nucleotides per min. The average rate per molecule of DNAase is, therefore, about 1.8×10^3 bonds broken per min.

The transforming activity of DNA 55C is less than one-tenth that of 55B although the double-stranded molecular weights hardly differ. This reduction must be due to the increased number of single strand breaks or equivalently the reduction in the average single-stranded size. The distributions of ^{3}H counts and transforming activities of DNA's 55A, B, and C in a preparative neutral sucrose gradient are shown in Fig. 3. The radioactivity identifies the bulk of the DNA and the arrow indicates the expected position of T7 DNA. We have, so far, found no evidence for any breakdown of *B. subtilis* DNA due to the incorporated ^{3}H at the levels used, namely 1 to 5×10^7 counts per minute per μmole. Thus, for example, DNA 36, which has the longest single-stranded molecular weight of the series shown in Table I (7.9×10^6) had been stored for a period of approximately 3 yr at the time the measurements were made. During this time it suffered no detectable loss in transforming activity in spite of an average of about 6 ^{3}H decays per molecule. The transforming activity of the untreated sample 55A follows the distribution of ^{3}H on the heavy side of the gradient, whereas the smaller DNA on the lighter side has a markedly lower specific transforming activity. This discrepancy between the distributions of transforming activity and ^{3}H is further accentuated in the two successive DNAase-treated samples. The weight average molecular weight of the transforming activity changed somewhat less than that of the ^{3}H.

The data of Table I are illustrated in Fig. 4 in the form of a plot of the logarithm of the relative transforming activity against the double- and single-

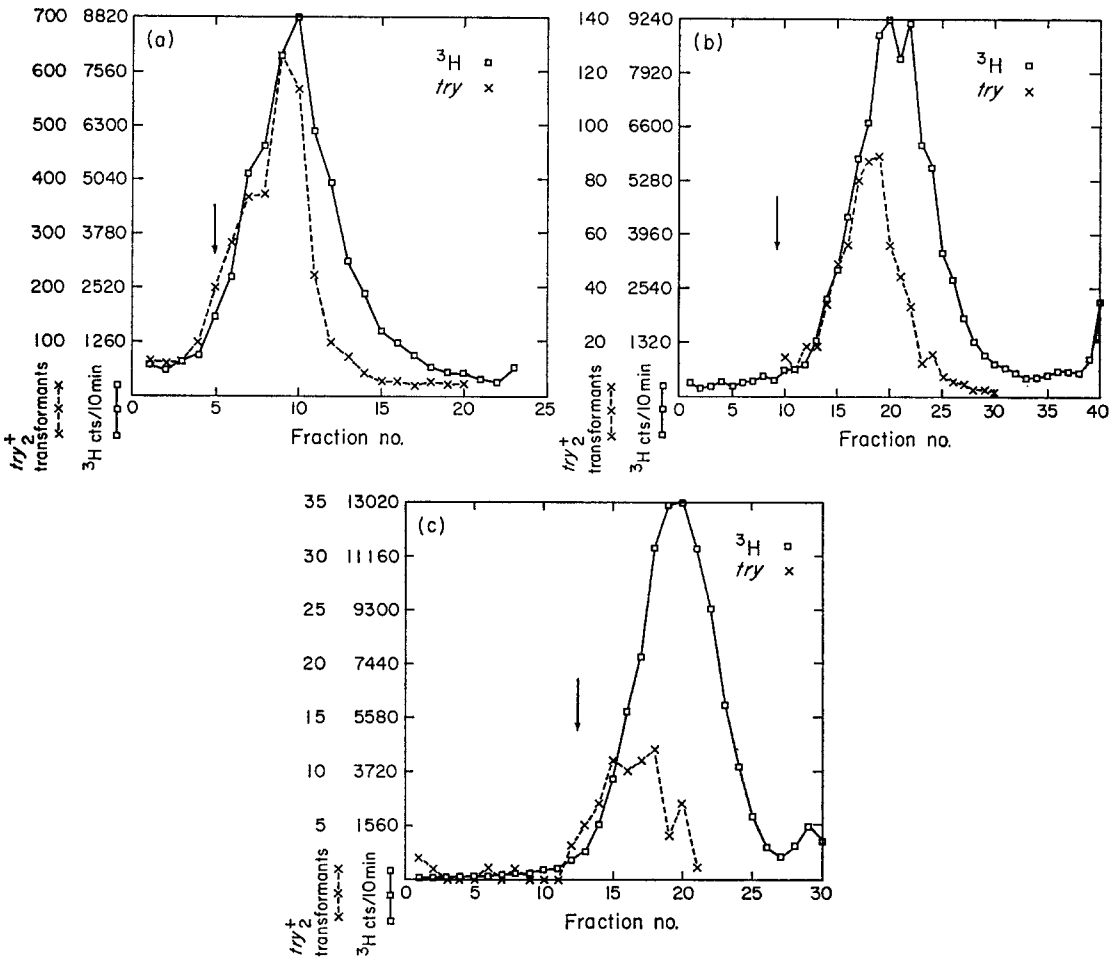

FIGURE 3. Distribution of radioactive counts and transforming activity of control and DNAase-treated *B. subtilis* DNA in neutral preparative sucrose gradients. Samples of ^{3}H-labeled DNA from SB532 (*aro*$_2$ *tyr*$_1$) were incubated at a concentration of 13 μg/ml at 37°C in an assay mixture containing 0.01 M $MgCl_2$, 0.01 M Tris pH 8, 50 μg/ml bovine serum albumin, and 3×10^{-5} μg/ml DNAase I. The reaction was terminated by addition of 0.1 M citrate and immediate chilling. Figs. 3 *a*, *b*, *c* refer respectively to a control and 20 and 75 min incubations. These are preparations 55A, B, C of Table I. The residual transforming activities of 55B and C were 15.5% and 1.1%, respectively. Transformation was assayed using SB25 (*try*$_2$ *his*$_2$) as a recipient. Sucrose gradient centrifugation and radioactivity counting were done as described in the legend to Fig. 2. The arrows indicate the expected positions for T7 DNA. Samples 55A and B were run together at a different time from 55C.

stranded molecular weights. There is an approximately linear relationship between the log transforming activity and the single-stranded molecular weight up to a value of about 4×10^6. Following this the curve bends toward a horizontal plateau or saturating level. There is no obvious relationship be-

tween transforming activity and double-stranded molecular weight, though there is clearly some correlation between size and activity. This is, of course, expected even if single strand size is the major determinant, for the double strand size sets an upper limit to the single strand size. This correlation presumably also accounts for the much lower specific activity of the smaller DNA

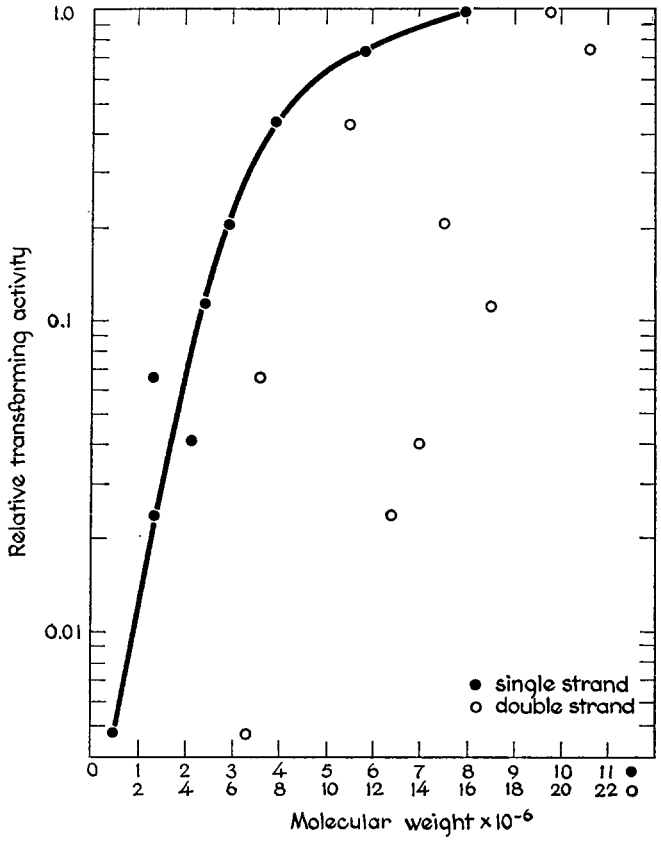

FIGURE 4. Dependence of transforming activity on single- and double-stranded molecular weight. The data plotted are given in Table I. See the legend to this table for experimental details.

in the gradients illustrated in Fig. 3. It seems clear from these data that the mean single strand size, or interval between single strand breaks, is a major factor in determining the biological activity of a DNA preparation, at least for single strand weight average molecular weights less than about 4×10^6. In view of the heterogeneity of single strand molecular weights it is possible that a major fraction of the transforming activity might be attributable to a minority of molecules containing longer than average uninterrrupted single strand regions. Assuming random attack on the DNA by DNAase, these more

active molecules will, of course, have more than the average number of bond ruptures for a given extent of treatment. However, a relationship of single strand weight average molecular weight and transforming activity also reflects a similar relationship for the single strand molecular weight of those molecules with a high specific transforming activity. It has been found that when individual fractions from an alkaline sucrose gradient are renatured with an excess of a standard denatured DNA, the majority of the recovered transforming activity is found well to the heavy side of the bulk of the DNA. Thus, for example, the transforming activity recovered from a denatured sample of DNA 58 had a weight average molecular weight which was more than 2.3 times that of the bulk of the DNA, as represented by ^{3}H counts. It has, however, been shown by Marmur and Doty (23 *a*) and Lanyi (17 *b*) that the rate and extent of renaturation of a DNA are dependent on its molecular weight. While their data do suggest that this effect is not sufficient to account for the molecular weight of the transforming activity recovered from denatured DNA, a more careful study of the correlation of size with ability to renature and to transform is needed to clarify the direct relation between single-stranded size and transforming activity.

Clearly the rate of inactivation by DNAase I of the transforming activity of a given DNA preparation will depend on its weight average single strand molecular weight. A DNA in the linear range of single-stranded molecular weights should be a more sensitive indicator of DNAase activity than one in the saturating range. Young and Sinsheimer (45) found that an average of 4 DNAase I induced single strand breaks per whole molecule of λ-DNA was needed for an average of one lethal biological activity hit as measured in the λ-transformation system (Kaiser and Hogness, 17). Kaiser (personal communication) has obtained similar data from studies on the effects of ^{32}P decay. In spite of the possibly very different requirements of this transforming system, these data are compatible with those presented in Table I and Fig. 4. Whole λ-DNA molecules have a single-stranded molecular weight of about 17.5×10^6 which after an average of 4 breaks would be reduced to a weight average molecular weight of 6.6×10^6. This value is comparable to the weight average single strand molecular weight corresponding to the shoulder of the curve plotted in Fig. 4. Starting with a λ-DNA already containing some single strand breaks one should obtain kinetics of inactivation by DNAase I similar to those observed with the *B. subtilis* DNA preparations.

A tendency for spleen DNAase (DNAase II) to cleave both strands of DNA was first noted by Oth, Fredericq, and Hacha (30). Evidence was later provided by Bernardi and Sadron (1) that this enzyme causes both double and single strand cleavage, but that in the initial stages of digestion the double strand cleavage dominates the kinetics of the decline of native molecular weight. More recent studies on uniform preparations of λ-DNA by Young and

Sinsheimer (45) implicated only double strand cleavage in the early stages of digestion by DNAase II. Similar results have also been obtained by Studier (40) for *E. coli* endonuclease I. The kinetics of inactivation of the transforming activity of *B. subtilis* DNA in relation to viscosity have been studied for both these enzymes. There was no significant departure from the results obtained with DNAase I. This is contrary to what might be expected if these enzymes caused only double strand cleavage. It thus seems likely that these enzymes cause both types of cleavage, as suggested by Bernardi and Sadron (1), but that different preparations vary in the relative rates of double and single strand cleavage.

Inactivation kinetics by DNAase and other agents have generally been interpreted in terms of target theory (see Lerman (19) for review). It is assumed that there is a "critical" region surrounding the genetic marker on the DNA molecule. A "hit" in this region inactivates the molecule with respect to transformation for the marker in question. A more satisfactory interpretation, at least for DNAase inactivation, would seem to be in terms of a distribution for the probability that a marker on the molecule will give rise to a transformant as a function of the size of the uninterrupted single-stranded region surrounding the marker. This probability distribution is presumably reflected in the shape of the curve of log transforming activity against single-stranded molecular weight shown in Fig. 4. The size of the critical region is given approximately by the molecular weight corresponding to a relative inactivation of $1/e$. This is about 3.5×10^6 or 1.05×10^4 nucleotides, which is somewhat larger than the value obtained by Lerman (19).

Uptake and Integration of DNAase-Treated DNA

Direct evidence for physical incorporation of donor DNA into the recipient genome comes from studies on the fate of heavy density labeled donor DNA following its entry into competent recipient cells (Bodmer and Ganesan (4), Fox and Allen (10), Szybalski (41), Pene and Romig, (31)). Thus Bodmer and Ganesan (4) used ^2H ^3H ^{15}N (heavy) labeled donor DNA (5) with ^{32}P ^1H ^{14}N (light) recipient cells. The donor and recipient DNA are physically separable by CsCl preparative density gradient centrifugation and their atoms can be identified by differential counting of ^3H and ^{32}P respectively. In addition a marker system which allows selective identification of the transforming activities of donor, recipient, and recombinant DNA is generally used. Integrated donor DNA (radioactive counts and biological activity) was generally found predominantly in the light recipient region. Denaturation and shearing of material from this region caused an increased density separation between ^3H and ^{32}P, but produced no material of predominantly donor density. These results clearly indicate that portions of the intact donor DNA are built into the recipient genome and held there by covalent bonds. In both *B.*

subtilis and *Pneumococcus* (Fox and Allen, 10) there is some direct physical evidence for single-stranded integration, but, as will be discussed below, the interpretation of these data is complicated by the size heterogeneity of the bacterial DNA preparations and the existence of single strand breaks.

The uptake and integration of a control heavy DNA and two successive, mildly DNAase I–treated samples of the same DNA (DNA's 58A, B, C of Table I) were studied as outlined above. After 30 min contact between the DNA and ^{14}C-labeled competent cells, an excess of DNAase was added, the cells were carefully washed to remove all traces of unincorporated DNA, and aliquots of the cell lysates were fractionated by preparative CsCl density gradient centrifugation. As in most previous experiments all donor activity was associated with the recipient material. Fractions from the light regions of these gradients were separately pooled, aliquots alkali-denatured, and then refractionated in CsCl. The denaturation caused a mean density separation between ^{3}H and ^{14}C counts which was 8 to 10% of the density difference between control light and heavy DNA's, but no donor material appeared in the heavy region. Thus by these criteria virtually all detectable donor activity in the lysates was fully integrated. A summary of the quantitation of uptake and integration for this experiment is given in Table II. Samples B and C had residual transforming activities of 0.54 and 0.19 that of sample A, whereas the corresponding relative proportions of integrated donor DNA were 0.58 and 0.42, respectively. There is a twofold excess of integrated 58C donor DNA in comparison with the expressed frequency of transformation. This excess is also reflected by the twofold lower transforming efficiency of the integrated DNA 58C (3.3 cell equivalents of donor DNA integrated per transformant) as compared with samples 58A and B. It is interesting to note that even for the control sample some 30% of the integrated DNA does not lead to the production of transformants. This may reflect, in part, the fact that recipient cells with donor integrated into the "nonsense" strand of the recipient DNA may not be able to replicate if plated immediately on selective media and so will not give rise to transformants identified in this way. However, the higher proportion of integrated DNA 58C which does not give rise to transformants (about 70%) cannot be explained in this way. It is presumably due either to a failure in the expression of the integrated DNA, perhaps because of its excision during growth after transformation, or a "suicidal" (17 *a*) failure in the ability of the cell containing integrated donor (or the first division product containing integrated DNA) to undergo subsequent division. Such effects may be found, more generally, with DNA which has been subjected to physical and chemical treatments. Lerman and Tolmach (20, 21) showed in pneumococcus, that the rate of decrease of uptake of donor ^{32}P label as a function of DNAase I treatment was approximately fivefold less than the rate of loss of transforming activity. However, it is probable that not all the counts they

found irreversibly bound to the recipient cells represented integrated DNA, as we have defined it.

The Size of the Integrated Region

The density displacements between donor and recipient counts after shearing and denaturation were used by Bodmer and Ganesan (4) in an attempt to estimate the size of the integrated region. However, their initial density

TABLE II

QUANTITATION OF UPTAKE OF DNASE-TREATED $^{2}H^{15}N^{3}H$ DNA

	58A	58B	58C
Time of DNAse treatment, *min*	0	20	50
Proportion transformants $\times 10^{-4}$	2.2	1.2	0.4
Proportion added donor DNA integrated $\times 10^{-4}$	10.5	6.1	4.4
Cell equivalents of donor DNA integrated per transformant	1.4	1.5	3.3
Proportion donor/recipient DNA $\times 10^{-4}$	5.1	4.2	2.8
Proportion donor/recipient transformants $\times 10^{-4}$	4.1	1.6	0.75

An $^{2}H^{3}H^{15}N$-labeled SB532 (*aro*$_2$ *tyr*$_1$) DNA (specific activity 2.5×10^5 counts per minute per microgram) was prepared as described by Bodmer and Schildkraut (5). Samples of this DNA were incubated at 37°C for 20 and 50 min at a concentration of 10 μg/ml with 3×10^{-5} μg/ml DNAase I under the conditions described in the legend for Fig. 3. 30 μg of control or treated DNA were incubated with 20 ml of competent ^{14}C-labeled SB25 (*try*$_2$ *his*$_2$) cells at a concentration of 1.05×10^9 per ml, with added 0.05 M $MgCl_2$. Following 30 min incubation at 30°C the cells were spun down, resuspended in 10 ml 0.1 M Tris buffer pH 8, 0.01 M $MgCl_2$, and incubated at 37°C for 10 min with 15 μg/ml DNAase. This DNAase treatment was repeated once more, following which the cells were washed twice with minimal medium, lysed, and fractionated pycnographically (on a preparative CsCl density gradient) at neutral pH. The proportions of donor to recipient DNA and the ratio of donor to recipient transformants were determined from the assays of the CsCl fractions. Transformation was assayed using SB202 (*aro*$_2$ *try*$_2$ *his*$_2$ *tyr*$_1$) as a recipient. One cell equivalent of DNA is about 5×10^{-9} μg for *B. subtilis* (Ganesan and Lederberg, 12). For further experimental details see Bodmer (3) and Bodmer and Ganesan (4).

difference, before any treatment, was much smaller by comparison with that after shearing or denaturation than expected on the simplest hypotheses. Single strand breaks and the size heterogeneity of bacterial DNA greatly complicate the interpretation of the density separations after either denaturation or shearing. Shearing may break the helix preferentially in the neighborhood of a preexisting single strand break. The final interpretation of physical evidence for single-stranded integration (Bodmer and Ganesan (4), Fox and Allen, (10)) must await a more careful analysis of the density differences in relation to the single- and double-stranded molecular weights of the materials being studied.

A direct estimate of the amount of donor DNA contained by the single-stranded pieces released on alkali denaturation of the donor-recipient DNA

complex can be obtained from a knowledge of the donor-recipient density difference in alkaline CsCl and the single-stranded size of the denatured material. The relevant fractions from the CsCl preparative density gradient centrifugations of the alkali-denatured material from the uptake experiments with DNA's 58A and 58C were pooled, dialyzed, and rerun in a preparative alkaline sucrose gradient. T7 DNA was used as a size marker in a parallel tube in the same centrifugation. The resulting weight average molecular weights were 6.8×10^6 for 58A and 13×10^6 for 58C. The corresponding double-stranded molecular weights were 52×10^6 and 61×10^6 respectively. These DNA's therefore had a maximum of 5 to 6 single strand breaks per strand of molecular weight 25 to 30×10^6. The actual number depends on the initial size heterogeneity. The maximum numbers quoted were obtained assuming an initial exponential size distribution, for which the number average molecular weight is one-half the weight average molecular weight. The density separation of denatured donor and recipient counts, ∂ and the size (number average molecular weight) of the included donor region x are related by the formula

$$\partial = \frac{\Delta x}{y}$$

where Δ is the density difference between heavy and light DNA and y the number average molecular weight of the denatured molecules. As mentioned above, ∂/Δ was 0.14 for 58A and 0.08 for 58C. Assuming an initial exponential size distribution the corresponding values for y are 3.4 and 6.5×10^6, giving estimates for x of 4.8×10^5 and 5.2×10^5, respectively.

A further study on the DNA 58B uptake material was carried out in order to confirm the relationship between the donor-recipient denatured density displacements and the amount of included heavy donor DNA. The pooled fractions from the original CsCl gradient for the uptake experiment with DNA 58B were divided into two aliquots, one of which was mildly treated with DNAase I and both were then run, concurrently in alkaline sucrose and alkaline CsCl gradients. The single-stranded weight average molecular weight of the treated aliquot was 2×10^6 as compared to 6.2×10^6 for the untreated aliquot. This corresponds to an average of just over 2 breaks per initial number average molecular weight of 3.1×10^6. The native material had an initial weight average molecular weight of 64×10^6. The relative density displacements, ∂/Δ, in alkaline CsCl were 0.32 and 0.18 for the treated and untreated samples, respectively. The untreated sample gives, therefore, an estimate of $0.18 \times 3.1 \times 10^6$ or 5.6×10^5 for the molecular weight of the included donor segment. Bodmer and Ganesan (4) give the formula

$$\frac{\partial}{\Delta} = \frac{(\kappa + 1)x^1}{\kappa x^1 + 1}$$

relating the relative density displacement, ∂/Δ, to the proportion of the molecule which is donor material, x^1, and the mean number of breaks, κ, per initial unit number average molecular weight. On rearranging we obtain

$$x = x^1 y = y \frac{\partial}{\Delta} \frac{1}{\kappa + 1 - \kappa \frac{\partial}{\Delta}},$$

for the mean size of the included donor segment, where y is the initial number average molecular weight. Substituting $y = 3.1 \times 10^6$, $\partial/\Delta = 0.32$, and $\kappa = 2.1$ gives $x = 4.1 \times 10^5$, as compared with the estimate of 5.6×10^5 obtained from the untreated sample. These four estimates of the size of the donor region give a mean single strand molecular weight of $4.9 \pm 0.3 \times 10^5$ corresponding to 1530 nucleotide pairs.

This estimate is much smaller than that given by Bodmer and Ganesan (4) who were not in a position to determine accurately the number of preexisting single strand breaks in their preparation. It could be an underestimate if there were more than one such integrated region within the double-stranded molecule of size 50 to 60 $\times 10^6$. In this case, however, shearing would not cause a density separation of donor from recipient counts until an average size was reached which had a small probability of containing two integrated regions. Such a small size for the integrated DNA could account for the observed very small density separation in untreated donor-recipient complexes. The cotransfer index obtained with the heavy DNA 58 used in these experiments was rather low; about 50% single transformants using a system of four linked markers following Nester, Schafer, and Lederberg (27). This might, in part, account for the small size of the integrated region obtained with this DNA.

The Involvement of DNA Synthesis in Uptake and Integration

Preliminary evidence that integration can occur in the absence of appreciable (i.e. less than 5 to 10%) DNA synthesis comes from the work of Fox and Hotchkiss (11) and Farmer and Rothman (9). A more detailed study of the involvement of DNA synthesis during uptake and integration has recently been published by Bodmer (3) and will be described briefly here. This study has made use of a competent thymine-requiring mutant of *B. subtilis* (9). Its inability to incorporate significant amounts of ^{32}P in the absence of exogenous thymine shows that the mutant has a firm block in thymine synthesis. Transformants are relatively resistant to thymineless death, in a manner analogous to their resistance to the killing action of penicillin (28).

Transfer of competent thymine-requiring cells to a medium containing 5-bromouracil (5-BU) at the time of addition of donor DNA allows the selective identification, by preparative pycnography (CsCl density gradient

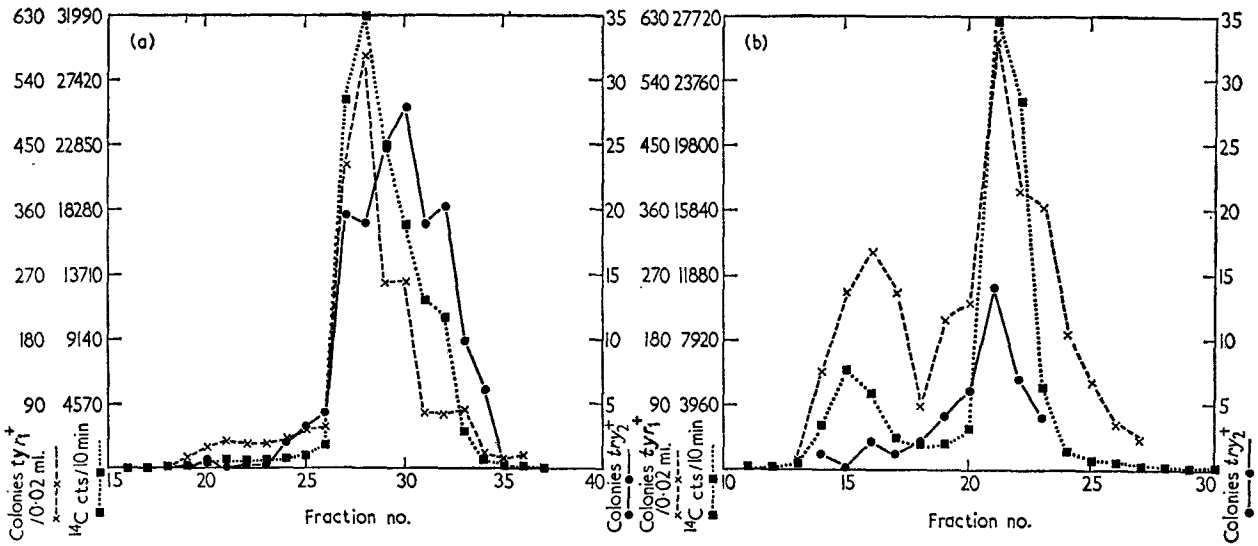

FIGURE 5. Pycnographic fractionation of the donor-recipient complex following transformation at various times after growth in a 5-BU containing medium. A competent ^{14}C-labeled culture of SB566 (*try_2 thy*) was washed, resuspended in complete medium without thymine, and incubated for 15 min at 37°C before the addition of 20 μg/ml of 5-BU. The culture was then divided into three aliquots. The first (*a*) was immediately transferred to 30°C with the addition of 1.1 μg/ml SB532 (*aro_2 tyr_1*) DNA and incubated for 15 min before harvesting and lysis. The second (*b*) and third (*c*) were similarly transformed after 30 and 75 min further incubation at 37°C, respectively. The lysates were fractionated pycnographically and the fractions analyzed for counts and transforming activity as described by Bodmer (3). *Try_2* + transformants measure donor (and recombinant) activity while ^{14}C counts and *tyr_1* + transformants measure recipient activity. The right-hand peak in each case represents unreplicated DNA. *Figs. 5 to 8 reprinted by permission from J. Mol. Biol., 1965, 14, 534.*

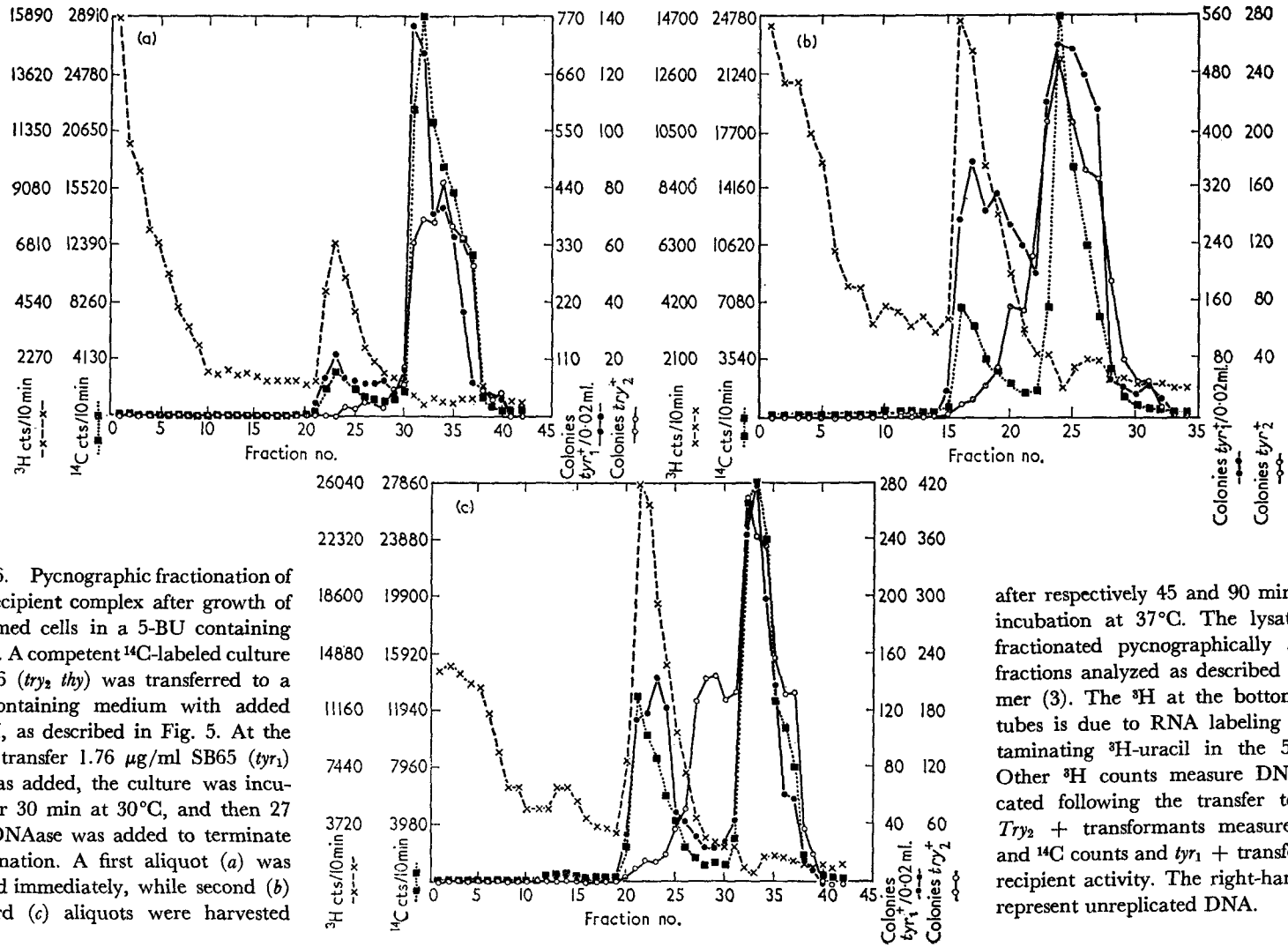

FIGURE 6. Pycnographic fractionation of donor-recipient complex after growth of transformed cells in a 5-BU containing medium. A competent ^{14}C-labeled culture of SB566 (*try*$_2$ *thy*) was transferred to a 5-BU containing medium with added 5-BU-^{3}H, as described in Fig. 5. At the time of transfer 1.76 μg/ml SB65 (*tyr*$_1$) DNA was added, the culture was incubated for 30 min at 30°C, and then 27 μg/ml DNAase was added to terminate transformation. A first aliquot (*a*) was harvested immediately, while second (*b*) and third (*c*) aliquots were harvested after respectively 45 and 90 min further incubation at 37°C. The lysates were fractionated pycnographically and the fractions analyzed as described by Bodmer (3). The ^{3}H at the bottom of the tubes is due to RNA labeling by contaminating ^{3}H-uracil in the 5-BU-^{3}H. Other ^{3}H counts measure DNA replicated following the transfer to 5-BU. *Try*$_2$ + transformants measure donor, and ^{14}C counts and *tyr*$_1$ + transformants, recipient activity. The right-hand peaks represent unreplicated DNA.

centrifugation), of DNA which has been synthesized during the transformation process. The density of donor and recombinant DNA, isolated from cells transformed under these conditions, is then a direct measure of its association with DNA newly synthesized during transformation. In such an experiment, the donor-integrated activity (try_2+) remained predominantly in the light (unreplicated) region (Fig. 5 *a*). No more than 5 to 6% overlapped into the hybrid region, representing DNA synthesis during the transformation process. This provides direct evidence that extensive DNA synthesis is not needed for the initial stages of the integration process, though an amount corresponding to about 10% of the length of the isolated molecules cannot be excluded. When the thymine-requiring competent culture was allowed to grow for some time in a 5-BU medium before the addition of donor DNA, donor activity was still not found in hybrid (replicated) regions (Figs. 5 *b* and 5 *c*). Thus donor DNA is not integrated into regions which were replicated after the transfer of the competent culture to a medium containing 5-BU. This suggests that the competent cell is not actively synthesizing DNA, which would explain its resistance to thymineless death. It had previously been shown by Nester (26) that the competent cell is resistant to the killing action of penicillin, as would be expected for a nongrowing cell.

The replication of integrated donor DNA after the termination of transformation was studied by allowing the transformed culture to grow in a 5-BU containing medium after the termination of transformation. The pycnographic distributions of donor and recipient activities for lysates from cultures left to grow for 0, 45, and 90 min in 5-BU after termination of transformation are shown in Fig. 6. The transformants are relatively resistant to the lethal effects of growth in 5-BU as they are, also, to thymineless death. The proportions of donor (try_2+) and recipient (^{14}C and tyr_1+) activities found in density regions corresponding to replicated (^{3}H counts in 5-BU) and unreplicated DNA show that there is a very significant lag in the replication of donor DNA relative to that of the recipient. Moreover, when the donor DNA is replicated, it moves to a density region intermediate between that of hybrid and light material. Pycnographic refractionation of the heavier fractions shown in Figs. 6 *b* and 6 *c*, as illustrated in Fig. 7, verifies the intermediate density of this novel donor-integrated DNA component. Molecules with this density have, on the average, about one-half of one of their strands synthesized with 5-BU.

The lag in the replication of donor DNA was confirmed by experiments in which competent cells were transferred to a D_2O -^{15}N containing medium at the time of addition of donor DNA. Donor transforming activity and ^{3}H counts showed a very significant lag in their transition to hybrid and fully heavy densities. A final time point, however, did show appreciable donor activity at the density of heavy DNA. The relatively small density separation

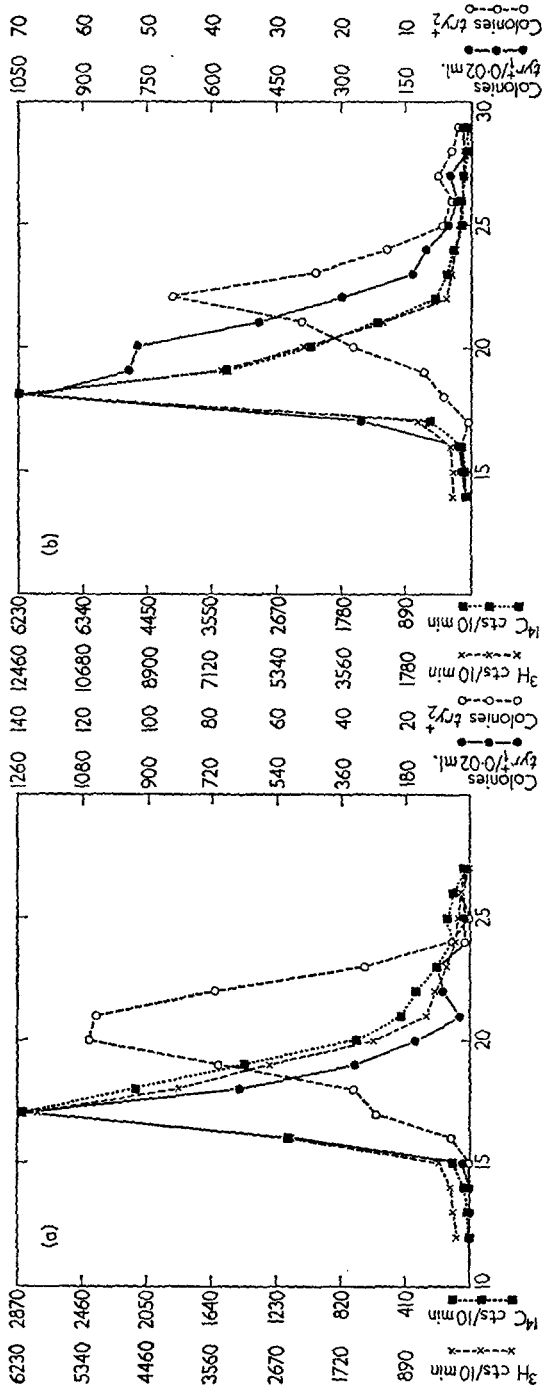

FIGURE 7. Refractionation of pooled hybrid and heavy fractions from the donor-recipient complex extracted after growth in 5-BU. Fraction Nos. 14–22 and 18–30 from the gradients shown in Figs. 6 *b* and 6 *c* respectively, were pooled and refractionated exactly as described before. "*a*" refers to the fractions from the 45 min aliquot (Fig. 6 *b*) and *b* to those from the 90 min aliquot (Fig. 6 *c*). The left-hand peaks, including ^{3}H and ^{14}C counts and tyr_1 + transformants, represent hybrid material. The density of the donor material (try_2 + transformants) is approximately 1.724 g cm^{-3}, as determined from its relative position in the gradient.

between light and heavy DNA's prevented the detection of the intermediate density donor DNA in these experiments. Significant amounts of donor ^3H counts remained in the light unreplicated positions, presumably representing integrated donor DNA in cells which did not reinitiate DNA synthesis. This suggests, as already discussed in relation to integration of DNAase-treated DNA, that incorporation of donor DNA may sometimes be a lethal event for the recipient cell.

There are three basic explanations for the occurrence of the intermediate density donor material. It is either: (*a*) the result of random repair synthesis as described by Pettijohn and Hanawalt (32), or (*b*) the replication point of normal or newly initiated DNA synthesis (14), or (*c*) a transition point, that is, the region of the DNA which was at the replicating point at the time of initiation of DNA synthesis with 5-BU (33). As described by Pettijohn and Hanawalt (32) in *E. coli*, repair synthesis involves only very short regions of the DNA. This type of repair seems an unlikely explanation for the intermediate density donor material since it would have to be very extensive (covering up to 4.5×10^4 nucleotides), particularly in the region of the integrated donor material. The intermediate density material was found to accumulate during growth of the transformed culture in 5-BU, which makes it unlikely that it is either a preexisting or newly initiated growing point, for then once synthesis had proceeded beyond the region of integration, donor DNA would be found in fully hybrid material. We are left, therefore, with the implication that the donor-integrated material of intermediate density includes the transition point formed at the time of reinitiation of DNA synthesis in the transformed cell.

A general biosynthetic latency of the transformed cell has previously been described by Nester and Stocker (28). The lag in DNA synthesis of transformed cells is consonant with their relative resistance to the lethal effects of extensive incubation in the presence of 5-BU and to thymineless death.

These observations can readily be unified by a model in which donor DNA is integrated at a stationary replicating point of the competent recipient cell, which is in a state of suspended DNA synthesis. Ganesan and Lederberg (13) have recently shown that the replicating point in *B. subtilis* is associated with a membrane fraction of the cell, suggesting that the membrane cell wall is the site of DNA synthesis. It seems very likely, therefore, that the replicating point of the chromosome may be in a position, in association with the bacterial surface, which makes it readily accessible for contact with donor DNA in solution around the cell. A schematic representation of the various stages of the integration process on the basis of such a model is shown in Fig. 8. A working model of synapsis and primary integration, before reinitiation of replication, was outlined by Bodmer and Ganesan (4). As suggested by them for *B. subtilis*, and by others (8, 15, 16, 24, 43) the initial integration may

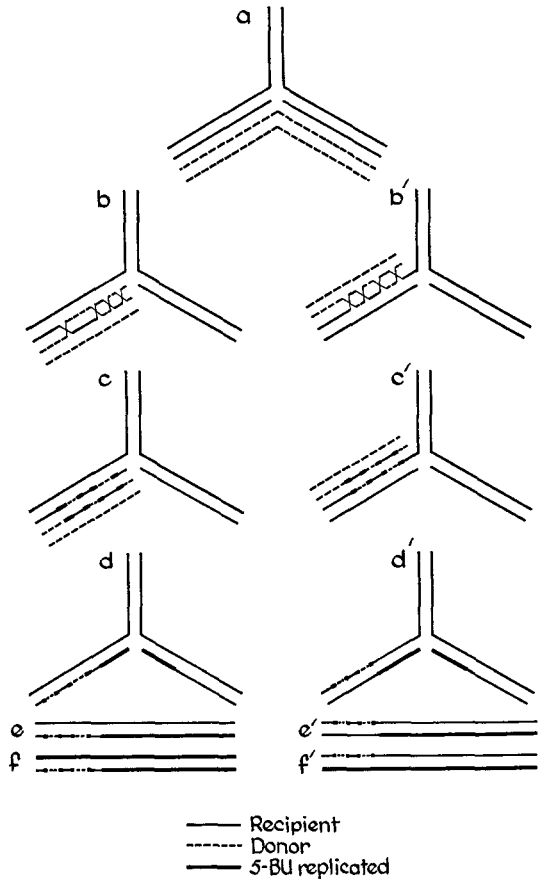

FIGURE 8. Schematic model for integration at the replicating point.

a. Stationary replicating point and incoming donor DNA.

b, *b'*. Synapsis of donor and recipient by segmental interchange of base-pairing partners.

c, *c'*. Integration by breakage of strands followed by repair DNA synthesis in unpaired regions and covalent linkage of donor to recipient material. (Note the possibility of producing "reciprocal" recombinant molecules.)

d, *d'*. Reinitiation of replication.

e, *e'*. Recombinant molecules following reinitiation of DNA synthesis.

f, *f'*. Recombinant molecules after the next round of DNA synthesis.

b, *c*, *d*, *e*, *f*. Refer to integration in new (growing) strands.

b', *c'*, *d'*, *e'*, *f'*. Refer to integration in old strands.

Note that subsequent replication produces donor material with a density between hybrid and heavy if integration is in new strands, whereas only donor with a normal hybrid density is produced if integration is only in old strands. Preliminary evidence suggests integration into old strands.

The first three steps are the same as those previously postulated by Bodmer and Ganesan (4).

involve repair DNA synthesis along gaps left uncovered by the exchange process, prior to the formation of phosphodiester linkages to join donor and recipient strands. Two different species of molecules carrying donor DNA will be formed according to whether integration is in new (*b-f*) or in old (*b'-f'*) strands. Only in the first case should donor DNA be found in the 5-BU containing strand.

Preliminary evidence that the donor activity is genuinely hybrid after a further round of replication suggests that donor DNA is predominantly integrated into old strands. This makes it even more unlikely that repair synthesis is the explanation for the initial intermediate density donor material.

DISCUSSION

There are two basic problems that must be surmounted by the "growing point" model for transformation. They arise from the prediction that each competent cell can only be transformed for markers in the neighborhood of its stationary replicating point.

The first problem is that observed frequencies of transformation cannot be accounted for unless a competent cell has a high probability of integrating a donor molecule appropriate for the region in the neighborhood of its replicating point. This implies that there must be a considerable flow of molecules through the membranous region associated with the growing point. Only those molecules homologous for the appropriate segment of the recipient genome will be permanently integrated. This molecular traffic might be responsible for the transient uptake reported for pneumococcus by Lerman and Tolmach (20). It might also account for the excess of native DNA found "inside" the cell in some of the experiments reported by Bodmer and Ganesan (4). The transiently bound DNA should be found in association with the membrane, so that its unequivocal detection will probably require careful isolation procedures analogous to those used by Ganesan and Lederberg (13). Mesosomes have been implicated in electron microscope studies in relation to the initiation and/or replication points of DNA synthesis (36). As suggested by Miller and Landman (25) the initial association of donor DNA with the recipient cell may be mediated by the mesosomes. It is of some interest that Mg^{++} is required in *B. subtilis* for DNA uptake, suggesting its possible role in some stage of the binding of donor DNA to structures of the recipient cell.

The second problem is the occurrence of multiple transformants for unlinked markers. There are two basic possible explanations for the cotransfer of two or more unlinked markers. Either (*a*) there are 2 or more growing points, or (*b*) the growing point moves slowly, allowing integration in more than one region. Both these explanations predict that some relationship should exist between the frequency of cotransfer for two unlinked markers and their relative positions on the bacterial chromosome. In the first case this frequency

should be lower for markers sufficiently close together, since the probability that two growing points will be close together is, presumably, small. In the second case there may be a polarity of the cotransfer frequency according to the orientation of the markers in relation to the direction of replication. The cotransfer frequency should now be higher for markers close together. The length of time that a nonhomologous DNA molecule can remain associated with the recipient cell before being rejected (or degraded) will be an important factor in the second explanation. Multiple growing points have been reported in *B. subtilis* (29, 44) and in *E. coli* (34). It is not inconceivable that the competent state favors the precocious initiation of DNA synthesis.

Single strand breaks caused by in vivo treatment of *B. subtilis* with alkylating agents have been shown by Strauss (38, 39) to reduce the transforming activity of the DNA extracted from treated cells in a manner closely analogous to that resulting from DNAase I digestion. This work provides preliminary extra confirmation for the correlation between the transforming activity of a DNA and its single-stranded molecular weight. In order to interpret the data on the integration of DNAase I treated DNA in terms of the growing point model, one must explain both the lower efficiency with which such DNA is integrated and the higher probability that cells containing integrated treated donor DNA will not express themselves as transformants. One possibility for the lower efficiency of integration is that the single strand breaks inhibit the unwinding process that must be involved in some way in the mediation of synapsis between donor and recipient DNA's by interchange of base-pairing partners. Integration of a DNA strand containing a break may be lethal, through the inhibition of the subsequent replication of the strand containing the break. Alternatively, integration of smaller pieces of DNA may interfere with the repair process, again leaving gaps in the integrity of the DNA which may be lethal for its subsequent replication.

The growing point model can be extended to cover recombination in higher organisms on the assumption that homologous chromosomes replicate synchronously through common growing points during the meiotic division (3). Such growing points provide natural structures at which crossing-over can occur by a combination of breaking and rejoining followed by repair DNA synthesis.

Much of the above discussion is, of course, still speculative. Speculation is, however, valuable if it leads to the formulation of concrete experiments to test proposed hypotheses, whether or not the hypotheses survive the tests.

I am grateful to Professor Joshua Lederberg for first suggesting a further investigation of the effects of DNAases on transformation. I am also indebted to him and to Dr. A. T. Ganesan and Mr. C. Laird for many stimulating and helpful discussions.

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