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Bacterial Inactivation of Transforming Deoxyribonucleate

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Competent and noncompetent bacteria are able to bind deoxyribonucleate (DNA). The consequence of this binding is different for the two types of bacteria. Competent bacteria are able to utilize the DNA for transformation. DNA exposed to noncompetent bacteria loses its biological activity with a coincident reduction in the double-stranded molecular weight. The reduction in molecular weight does not appear to be sufficient to account for the loss of biological activity observed.

Techniques for separating competent from noncompetent bacteria in competent cultures of *Bacillus subtilis* were developed simultaneously by Cahn and Fox (3), by Hadden and Nester (6), and by Singh and Pitale (14). Such fractionation has allowed us to examine some of the effects of the presence of noncompetent bacteria on transformation.

Exposure of a competent culture to transforming deoxyribonucleate (DNA) results in an increase in the number of transformants for only a limited time. The frequency of transformants appears to reach a saturation value, even though the bacteria remain competent and very little of the DNA has been removed from solution.

We will describe a series of experiments showing that this "saturation" phenomenon results from an interaction between noncompetent bacteria and DNA that does not lead to fixation or transformation, but yields DNA which has lost its biological activity and suffered a limited number of endonucleolytic double-strand breaks. The reduction in molecular weight of the DNA is not sufficient to account for the extent of loss of biological activity. These endonucleolytic breaks may be caused by the same enzyme that McCarthy and Nester have previously described (13).

MATERIALS AND METHODS

Strains of bacteria. *B. subtilis* strain SB25 (*his2 trp2*, linked markers) was used as a recipient for all transformations. DNA was isolated from SB25 H$^-$ (*trp2*$^+$ *his2*$^-$), SB291 (*trp2*$^-$*sul*^{r}), SH113 (*his2*$^+$*trp2*$^+$) and SHE-1 (*sul*^{r}, *his2*$^-$*trp2*$^-$). DNA labeled with ^{32}P was isolated from 168 Sa. (*trp2*$^+$*his2*$^+$*sul*^{r}), *Salmonella typhimurium* strain 18, and *S. typhimurium* phage P22 *c1*^{7}*h21* (9). The latter two strains were obtained from D. Botstein (Massachusetts Institute of Technology).

Media and competent bacteria. LS medium has been previously described (3) and contains 0.08% NH_4Cl, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% sodium citrate-water, 0.033% $MgCl_2 \cdot 6H_2O$, 0.0014% Na_2SO_4. Competent cultures were grown and transformed according to methods already described (3). Transformants for *his*$^+$*trp*$^+$ markers were scored as previously described, but *trp*$^+$ and *sul*^{r} transformants were scored in selective media containing 0.002% vitamin-free Casamino Acids (Difco).

DNA isolation. Transforming DNA was isolated by the method described by Marmur (11).

B. subtilis DNA containing ^{32}P was isolated from bacteria grown in medium containing 1% tryptone broth (Difco 1) and prepared as follows. The 1% tryptone broth was mixed with 0.1 volume of concentrated NH_4OH, made 10^{-2} M in $MgSO_4$, and insoluble material was removed by filtration. The filtrate is adjusted to *p*H 7.2 with HCl and then autoclaved (method from R. Yuan, Harvard Univ.).

To avoid extensive handling of radioactive preparations of (168 *sul*^{r}) DNA, all extraction procedures through the first chloroform-isoamyl alcohol extraction were carried out in a 250-ml Nalge polypropylene centrifuge bottle (Servall). The extract was treated with 400 μg of bovine pancreatic (ribonuclease) Code RASE per ml (Worthington Biochemical Corp., Freehold, N.J.) and 200 units of T1 ribonuclease (Worthington Biochemical Corp.) at 37 C for 30 min. Pronase B (600 μg/ml) was added and the mixture was dialyzed against 2 changes of 0.15 M NaCl plus 0.015 M sodium citrate (SSC) overnight at 37 C. This procedure resulted in DNA preparations that had less than 10% of the label resistant to hot 5% (w/v) trichloroacetic acid treatment. The ^{32}P-labeled material precipitated in 5% cold trichloroacetic acid and was not rendered soluble to cold trichloroacetic acid when treated with 0.5 M NaOH at 45 C for 2 hr, indicating that 90 to 100% of the ^{32}P label was in DNA.

P22 phage labeled with ^{14}C-thymine was obtained

from G. Sonenshein. The DNA from the P22 phage was extracted by diluting 0.1 ml of labeled phage with 0.9 ml of SSC, adding 2% sarcosyl, heating to 65 C and cooling. A 25-μliter amount contained 2,500 counts per min and 9×10^9 phage equivalent of DNA per ml.

P22 DNA labeled with ^{32}P was prepared according to the procedure of D. Botstein (1). The phage preparations used had a final specific activity of approximately 6×10^{-6} counts per min per phage.

Fractionation of bacteria. Discontinuous Renografin gradients were prepared, centrifuged, and collected by a modification of the method previously described (3). In place of the SW25.1 rotor, the SW27 rotor was used routinely, fractionating 10 to 15 ml of competent culture in each tube. In addition, the separation gradient was made by first putting 20 ml of the floating Renografin solution in the tube and then injecting the cushion underneath this layer. The gradients were run at 25,000 rev per min for 20 min at 20 C. The fractionated bacteria were washed free of the Renografin on a HA WP membrane filter (0.45-μm pore size, Millipore Corp., Bedford, Mass.) with LS medium, then washed with 0.2 M $MgCl_2$, washed again with LS medium, and resuspended in growth medium. The lower density bacterial fraction (T-band) contains all of the competent bacteria in the culture, and the more dense fraction (B-band) contains virtually no competent bacteria (3).

After fractionation the bacteria were transformed by exposing competent cultures—unfractionated, fractionated, and reconstructed (T band plus B band)—to DNA for varying lengths of time. Transformation was terminated by diluting 0.2 ml of a culture into 0.2 ml of LS medium containing 20 μg per ml of electrophoretically purified deoxyribonuclease I (Worthington Biochemical Corp.). DNA concentration, the number of T- (competent fraction) and B-band (non-competent) bacteria, and the time of exposure to DNA are stated for each experiment.

Pulse transformation was carried out by first mixing 5 ml of culture with 0.8 μg of DNA per ml containing *trp*$^+$*Sul*^{s} markers. At timed intervals after starting, 0.5 ml of the transforming culture was removed and added to a test tube containing 0.2 μg of *trp*$^-$*Sul*^{r} DNA (0.4 μg/ml). After 11.5-min exposure to the second DNA, transformation was terminated with 10 μg of deoxyribonuclease per ml and the samples were scored for the number of *Sul*^{r} transformants.

Sucrose gradients. Neutral and alkaline sucrose gradients were made with a concentration range from 20 (w/v) to 5%. The 20% sucrose solution contained 29.2 g of NaCl, 100 g of sucrose, 790 mg of tris(hydroxymethyl) aminomethane (Tris)-chloride, 186 mg of ethylenediaminetetraacetic acid (EDTA), 400 ml of water. The ingredients were dissolved and the *p*H was adjusted to 8.0 by addition of NaOH. The volume was brought to 500 ml. The 5% sucrose (w/v) in neutral solution contained 25 g of sucrose and was otherwise the same. The 20% sucrose (w/v) in alkaline solution contained 26.3 g of NaCl, 100 g of sucrose, 372 mg of EDTA, 2 g of NaOH, and 400 ml of water. The *p*H was adjusted to 12.0 with NaOH and the volume was brought to 500 ml with water. The 5% sucrose (w/v) in alkaline solution contained 25 g of sucrose and was otherwise the same. The solutions were sterilized by filtration. The 5-ml linear alkaline and neutral sucrose gradients were layered with 0.1 ml of solution containing DNA (less than 5 μg/ml) which had been exposed to various treatments. Sucrose gradients were run at 17.5 C for 110 min at 39,000 rev/min in the Spinco model SW50 rotor with ^{14}C-labeled P22 DNA as a marker. The tubes were punctured and three-drop fractions were collected. Samples were analyzed for radioactivity by drying samples on filter discs (21 mm, Schleicher & Schuell, Keene, N.H.), washing the papers three times in cold 5% trichloroacetic acid and three times in ethanol, and counting the filters in toluene liquifluor scintillator fluid. When fractions were to be tested for transforming activity, they were collected in clean, sterile Wasserman tubes, a fraction of each (approximately 1/3 of the volume) was counted, and the remainder was used directly for transformation by adding 0.5 ml of concentrated T-band bacteria.

The sedimentation values were calculated by determining the distance from the top of the gradient that the DNA had moved relative to the distance the reference DNA had moved (2). The molecular weight of the DNA was calculated from neutral and alkali gradient sedimentation values by using the equations of Studier (15).

The DNA sedimented on alkaline gradients was denatured by first overlaying the alkaline gradient with 0.1 ml of 0.25 M NaOH, overlaying 0.1 ml of the DNA solution, and allowing the gradient to stand at room temperature for 30 min before centrifugation.

Inactivation of the DNA by the B-band bacteria was carried out by incubating DNA in the presence of bacteria at 30 C. In general, 0.5 ml of DNA in LS medium plus 0.5 ml of bacteria were mixed. The DNA was incubated in the presence of bacteria for varying lengths of time and the bacteria were removed by centrifugation at $1,000 \times g$ for 5 min before the DNA was tested for residual transforming activity.

To inactivate by shearing, DNA in 1 ml of LS medium containing 30% glycerol was sheared for 10 min at 0 C in a fluted glass flask (1.5 cm diameter and 3 cm in length) with a Virtis homogenizer (The VirTis Co., Inc., Gardiner, N.Y.) by using a 1-cm diameter steel blade. The speed of the homogenizer from 4,000 to 16,000 rev/min was monitored with a stroboscopic tachometer (General Radio Corp., Concord, Mass.). Solutions were directly tested for residual transforming activity and dialyzed against SSC for sedimentation on sucrose gradients.

Determination of radioactivity. Scintillation counting was done in Wheaton vials (Milville, N.J.) on a Mark I (Nuclear-Chicago Corp.) or models LS250 and LS233 scintillation counters (Beckman Instruments, Inc.). Toluene-Liquifluor containing 42 ml of Liquifluor (New England Nuclear Corp., Boston, Mass.) per liter of toluene was used as the scintillation solution.

RESULTS

By using fractionated competent cultures, it was possible to study the kinetics of transformation of purified competent bacteria. It was found

that with T-band bacteria the kinetics were consistent with a bisubstrate model (F. P. Haseltine, Ph.D. thesis, Massachusetts Institute of Technology, 1969) similar to that proposed by Fox and Hotchkiss (4) for pneumococcus. However, if B-band bacteria were added to the culture, there was a reduction in the rate of formation of transformants. Figure 1 illustrates an experiment showing this effect. The presence of B-band bacteria also results in a reduction of the final yield of transformants.

Although B-band bacteria are incapable of incorporating DNA (3), it is possible that they are capable of binding DNA in some reversible form. Such binding would result in a reduction of the effective concentration of DNA in solution and account for a reduced rate of formation of transformants among the T-band bacteria.

The linear rate of increase of transformants was determined in the presence of a fixed concentration of DNA, varying the concentration of T-band bacteria in the presence and absence of a fixed concentration of B-band bacteria. The data are displayed on a Lineweaver-Burk plot where the reciprocal of the rate versus the reciprocal of the concentration of T-band bacteria is plotted (Fig. 2). It would appear that B-band bacteria compete with T-band bacteria for DNA in solution. The binding constant K_s (concentration for 0.5 maximum saturation) for T-band bacteria is 3×10^7 bacteria per ml. The binding constant K_i, the concentration for 0.5 maximum inhibition, is also about 3×10^7 bacteria per ml (F. P. Haseltine, Ph.D. thesis, Massachusetts Institute of Technology, 1969).

In addition to the reduction in the rate of formation of transformants in the presence of B-band bacteria, there is a reduction in the observed maximum yield of transformants. The accumulation of transformants appears to terminate at an earlier time, suggesting that the presence of B-band bacteria results in either inactivation of the added DNA or destruction of the competence of the T-band bacteria.

To examine the latter alternative, T-band bacteria and a mixture of T-band and B-band bacteria were exposed to DNA for various lengths of time before adding a second DNA, carrying a detectable marker, for a fixed interval. Any loss of transformability would be reflected in the reduction of the yield of transformants determined by the second DNA added. One culture contained only T-band bacteria (3.2×10^7/ml) and the other an equal density of T-band bacteria to which had been added 5×10^7 per ml of B-band bacteria as described above. The first DNA (Sul^s 0.8 μg per ml) was added, followed at various

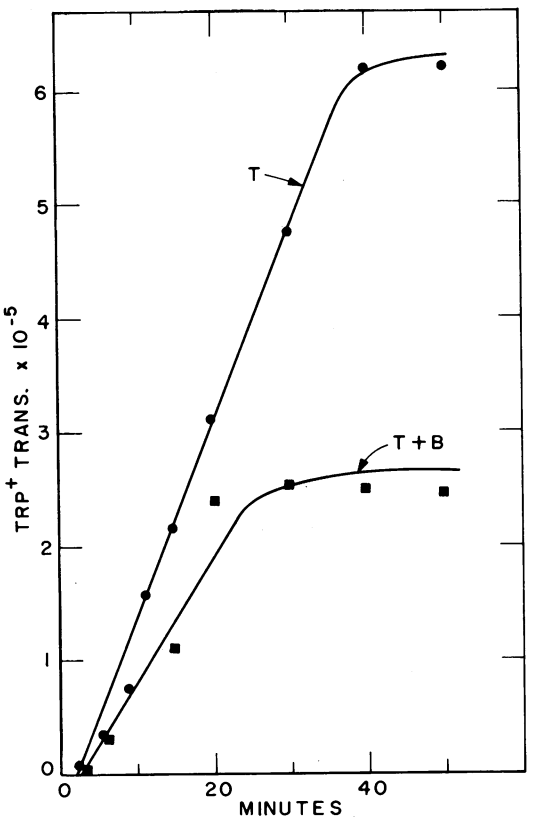

FIG. 1. *Number of transformants found as a function of time. An 0.8-μg amount of DNA per ml of SB25 trp$^+$Sul^s. Symbols: ●, T-band only (3.2×10^7 bacteria); ■, 3.2×10^7 T- and 5×10^7 B-band bacteria.*

times with the second DNA (Sul^r 0.4 μg/ml). Transformation was terminated with deoxyribonuclease after 11.5-min exposure to the second DNA. The yields of Sul^r transformants formed during these pulses are given in Fig. 3. A parallel control showed that the competence of these cultures remained unchanged for the duration of this experiment. Note that when only T-band bacteria were present, the number of Sul^r transformants formed during the exposure to Sul^r DNA remained constant. Since the DNA concentration exceeds saturation, the number of Sul^r transformants was not only dependent on the concentration of Sul^r DNA but also on the concentration of Sul^s DNA. The effective concentration of Sul^r DNA was proportional to $Sul^r/(Sul^r + Sul^s)$ under these conditions.

Transformation with Sul^r DNA alone yielded three times as many Sul^r transformants as in the mixture described. If the number of Sul^r transformants formed during a pulse remained con-

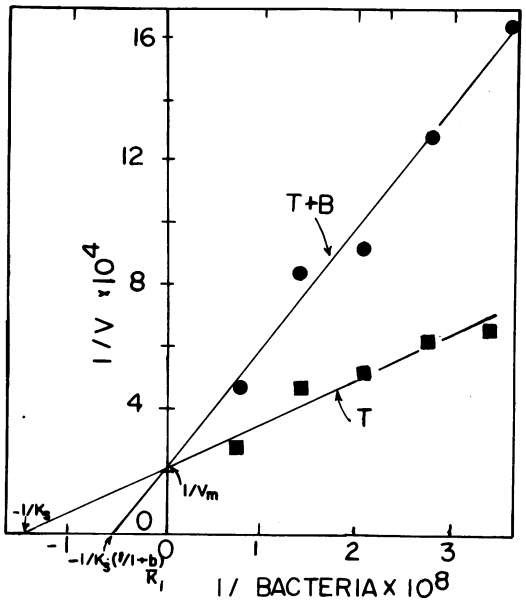

FIG. 2. *Plot of 1/V (trans per milliliter per minute) versus 1/C, where C is concentration of T-band bacteria with and without B-band. Plot used to calculate K_s, V_m, and K_i at 0.009 μg of SHE-1 DNA per ml. B-band concentration was 8.7 × 10⁷ per ml.*

stant throughout the experiment, the ratio of $Sul^r/(Sul^r + Sul^s)$ could be assumed to remain constant, i.e., the effective concentration of Sul^s was maintained (7). Such an observation would also indicate that the competence of the bacteria remained constant. These two conditions were satisfied when T-band bacteria were used. However, in the presence of B-band bacteria, the yield of Sul^r transformed bacteria increases at later pulse times. This increase suggests that the Sul^s DNA loses its ability to compete with the Sul^r DNA in transformation, i.e., the effective concentration of the Sul^r DNA increases with time. It appears that B-band bacteria are capable of acting on DNA so as to destroy its capacity to act as a competitor in the transformation reaction. These observations further show that B-band bacteria do not reduce the competence of the T-band bacteria.

Inactivation of transforming DNA by exposure to B-band bacteria was tested directly. After various times of exposure, the bacteria were removed and the DNA was tested for residual transforming activity (Table 1). Incubation of DNA in the supernatant medium in which B-band bacteria had been suspended (for at least 30 min at 30 C) showed no effect on the DNA, whereas a similar exposure to a suspension of B-band bacteria results in the loss of more than 95% of the transforming activity. This experiment demonstrates that the B-band bacteria are capable of inactivating the DNA. Inactivation of DNA by an exonuclease did not appear to be important, since less than 5% of the radioactivity in the ^{32}P-labeled DNA was rendered soluble in cold perchloric acid (0.5 M) after a 1-hr exposure to B-band bacteria (more than 95% of the transforming activity was lost). Inactivation could be the result of many single-strand breaks (as occur when DNA is inactivated by pancreatic endonuclease I), or by double-strand breaks (crudely similar to those resulting from shear).
from shear).

To investigate the product of inactivation, ^{32}P-labeled transforming DNA was exposed to B-band bacteria for varying lengths of time, and its sedimentation coefficient ($S_{20,w}$) was determined by zone sedimentation in sucrose gradients. Figure 4 shows a series of neutral sucrose gradients of the DNA exposed for various lengths of time to B-band bacteria. As can be seen, the exposure to B-band bacteria results in a reduction of the sedimentation rate of the DNA. Figure 5 shows alkaline gradients of the same deoxyribonucleates. From sedimentation coefficients, it was possible to calculate the molecular

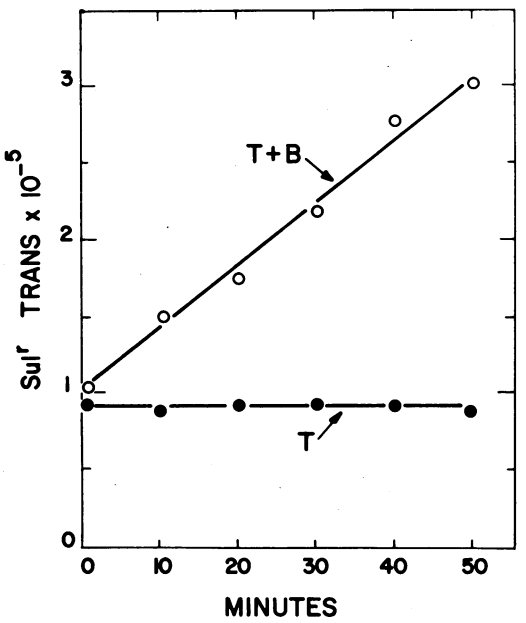

FIG. 3. *Sul^r transformants formed during an 11.5-min exposure to 0.4 μg of SB291 trp^-Sul^r DNA per ml in the presence of 0.8 μg of SB25 trp^+Sul^s DNA per ml. The time scale is that of the initiation of the pulse. Symbols: O, T-band; ■, T- plus B-band bacteria. (Same experiment as Fig. 1.)*

TABLE 1. *Inactivation of transforming deoxyribonucleate (DNA) by exposure to B-band bacteria*

Time exposed to B-band cells before transformation	No. of *trp*$^+$ transductants/ml
No treatment	7.0×10^4
0 min[b]	4.5
5	3.3
10	1.0
23	0.5
30	0.2

[a] B-band cells at 2.4×10^8 bacteria per ml in LS medium plus 0.02 M $MgCl_2$. Deoxyribonucleate (DNA) concentration (0.5 μg per ml of SHE-1) was exposed for times indicated and centrifuged at 1,000 $\times$ *g* for 5 min and the supernatant fluid was used to transform T-band bacteria. Dilution of supernatant fluid 0.1 into T-band culture (2.0×10^7 bacteria per ml) gave a final DNA concentration in transforming mixture of 0.05 μg.

weight of double-stranded and single-stranded DNA. Molecular weights and sedimentation coefficients were calculated by using ^{14}C-labeled P22 DNA molecular weight = 27×10^6 as reference DNA and calculating $S_{20,w}{}^0$ values by comparing the distance that the reference DNA had moved. $S_{20,w} = S_{ref,20,w}(D_2/D_{ref})$ according to Burgi and Hershey (2). From Studier's equations, native DNA in neutral sucrose has $S_{20,w} = 0.0822\ M^{0.346}$ and in alkaline sucrose, single-stranded DNA has an $S_{20,w} = 0.0528\ M^{0.400}$ (15).

From Table 2 it can be seen that the molecular weight of double-stranded DNA was reduced from 30×10^6 to 10×10^6 after 60 min of exposure to B-band bacteria. At the same time, single-stranded DNA was reduced from 8×10^6 to 2×10^6. Exposure to B-band bacteria results in a reduction of the molecular weight of double-stranded DNA with rather few additional single-strand breaks, suggesting the action of a double-strand endonuclease.

The effect of exposure to B-band bacteria on DNA from another source was examined. DNA from *Salmonella* phage P22 was convenient because of its size and uniform molecular weight of 27×10^6.

Figure 6 shows that the $S_{20,w}$ of ^{32}P-labeled P22 phage DNA exposed to B-band bacteria fell as a function of time. Here, there was a reduction of the molecular weight of native DNA from 27×10^6 to 3.2×10^6, after a 30-min exposure to B-band bacteria—and the single strand (alkaline gradient) molecular weight was reduced from 13×10^6 to 1.6×10^6 (Fig. 7). After a 1-hr exposure, we found a molecular weight of native DNA of 2.0×10^6 and a single-strand molecular weight of 6.4×10^5. Less than 1% of ^{32}P was released as acid-soluble material. These observations show that the B-band bacteria exert a double-strand endonucleolytic action on *B. subtilis* as well as P22 DNA.

Reduction of the molecular weight of transforming DNA by shearing results in the loss of biological activity (5, 10). Could the reduction of molecular weight after exposure to B-band bacteria account for the loss of biological activity?

As can be seen in Table 3, shearing resulted in both a reduction of molecular weight and of transforming activity. Exposure to B-band bacteria, however, causes a much greater loss in transforming activity for a given reduction in molecular weight. For example, a reduction in molecular weight from 26×10^6 to 7 to 8×10^6 by shearing resulted in survival of 22% transforming activity, whereas a reduction in molecular weight to 9×10^6 by exposure to B-band bacteria resulted in a survival of only 1.6% of the original transforming activity.

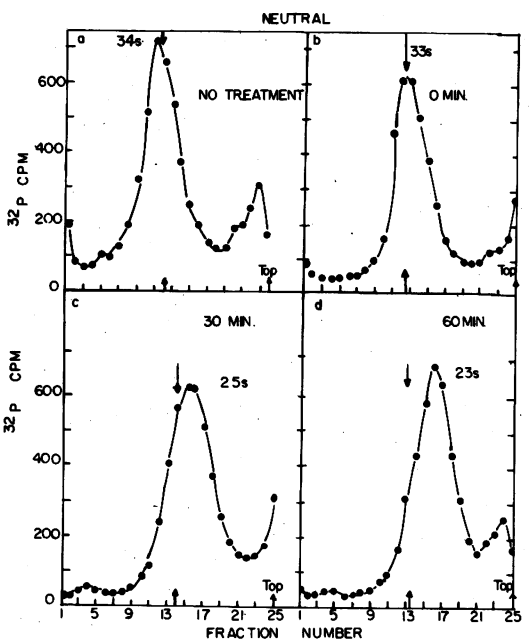

FIG. 4. *Neutral sucrose gradients of B. subtilis DNA exposed to B-band bacteria. A 0.05-μg transforming DNA sedimented through in a linear 5 to 20% sucrose gradient (see Materials and Methods). ^{14}C-labeled P22 DNA is used as a marker. B-band bacteria = 4×10^8 per ml and DNA concentration at 0.2 μg per ml. (a) Not exposed to B-band bacteria, (b) mixed with B-band bacteria and centrifuged immediately, (c) exposed to B-band bacteria for 30 min, (d) exposed 1 hr to B-band bacteria. Arrow designates position of the marker whose sedimentation coefficient is 33S.*

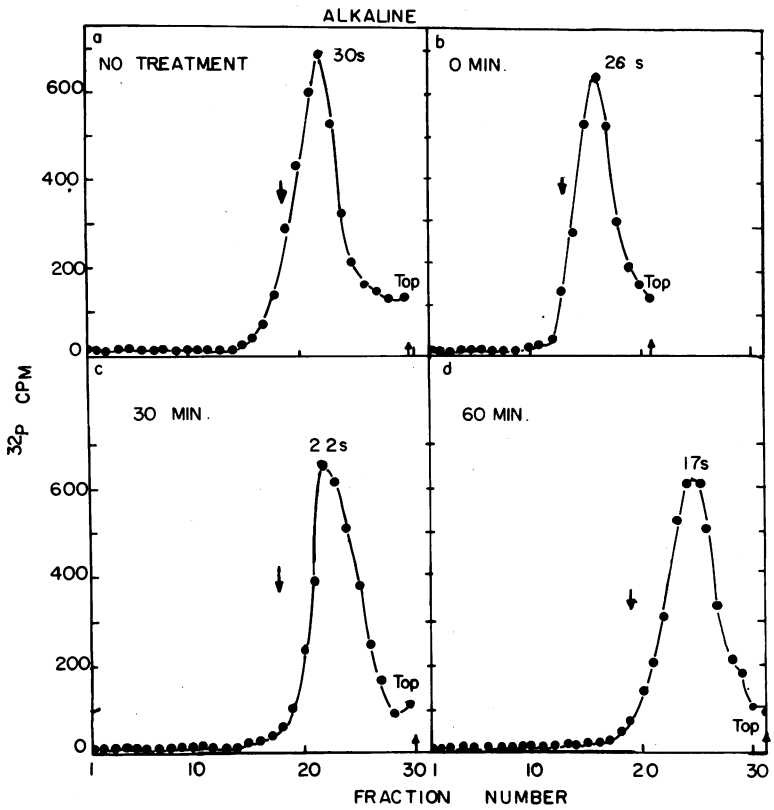

FIG. 5. *Alkaline sucrose gradients of ^{32}P-labeled B. subtilis DNA exposed to B-band bacteria. Same as Fig. 4 but sedimented on alkaline sucrose gradients, pH 12.1. Arrow designates position of marker having a sedimentation coefficient of 37S.*

TABLE 2. *Reduction of molecular weight of Bacillus subtilis deoxyribonucleate exposed to B-band bacteria*[a]

Time exposed to B-band (min)	Native $s_{20,w}$	Mol wt of double strand	Denatured $s_{20,w}$	Mol wt of single strand
Unexposed	34	30	30	8
0	33	27	26	5
30	25	12	22	3.5
60	23	10	17	2

[a] Values from Figures 4 and 5.

The action of B-band bacteria on DNA does not mimic the effect of shearing. Far less biological activity remains in DNA that has been exposed to B-band bacteria than can be accounted for on the basis of its reduction in molecular weight. It was therefore of interest to examine the molecular weight distribution of the material in which biological activity had survived. To do this, DNA, inactivated by shearing or exposure to B-band bacteria, was sedimented on sucrose gradients. Fractions of the gradient were collected and their transforming activities were determined. The presence of ^{32}P label in the transforming DNA permitted independent determination of the sedimentation behavior of the transforming activity and the total DNA. From Fig. 8 it is evident that in the case of unsheared and sheared DNA, the transforming activity sedimented at a rate similar to that of the total DNA. In the gradient (Fig. 8b) containing DNA inactivated by B-band bacteria, the transforming activity sedimented faster than did the total DNA, suggesting that the residual activity might be associated with DNA which has not been acted upon by the B-band bacteria.

Ganesan and Lederberg (5) reported that shearing DNA results in a greater loss of transforming activity of a pair of linked markers than either of the markers singly. It would be interesting to examine the inactivation of a pair of linked markers in DNA exposed to B-band bacteria. In this study, the linked markers *trp2his2* were used. They co-transform with a frequency of 55% (55% *trp2* transformants are also *his2* transformants).

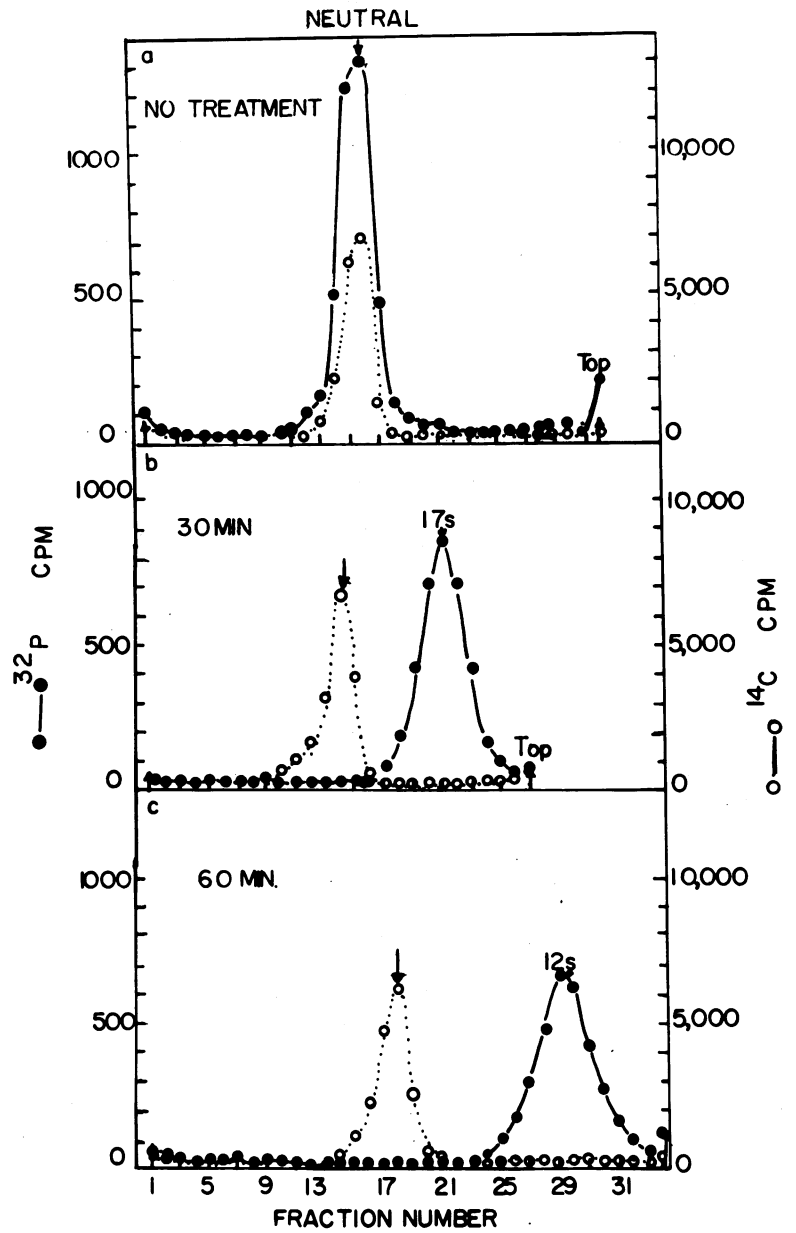

FIG. 6. *Neutral sucrose gradients of ^{32}P-labeled P22 DNA treated with B-band bacteria. ^{14}C-labeled P22 DNA serves as a reference. Less than 10^9 phage equivalents are treated and sedimented. (a) No exposure to B-band bacteria, (b) 30-min exposure to 2×10^8 B-band bacteria, (c) 1-hr exposure to 2×10^8 B-band bacteria. Arrow indicates location of marker 33S.*

In the case of shearing, each marker behaves, at least in part, as a single target and can to some extent be inactivated independently of the other. This independent inactivation as well as physical separation of markers account for the exaggerated loss of linked-marker activity (5). DNA was exposed to B-band bacteria and at timed intervals the bacteria were removed and the DNA was tested to determine the amount of *trp2*$^+$ and linked *trp2*$^+$*his2*$^+$ activity surviving in the DNA.

It can be seen that exposure to B-band bacteria (Fig. 9) destroyed the activity of both *trp2*$^+$ and *his2*$^+$*trp2*$^+$ markers at the same rate. This could be accounted for if exposure to the B-band bacteria resulted in inactivation of the whole

remaining fragment or fragments of DNA, thus no longer permitting transformation of single or linked markers with product DNA fragments. Here the activity of the marker pair and the single marker behaved as if they were contained in a single "target."

Exposure of DNA to B-band bacteria results in reduction in molecular weight, the loss of transforming activity, and loss of the capacity of this DNA to compete in transformation (*see* Fig. 3). Can the loss of capacity to compete be accounted for by the reduction in molecular weight? The competitive ability of the sheared $Sul^s trp^+$ DNA was tested against Sul^r DNA (Table 4). The presence of Sul^s DNA resulted in a reduction by a factor of three in the number of Sul^r transformants, whether or not the Sul^s DNA had been sheared. The data described in Table 4 show that, despite very substantial loss of transforming activity, sheared DNA retains its ability to compete.

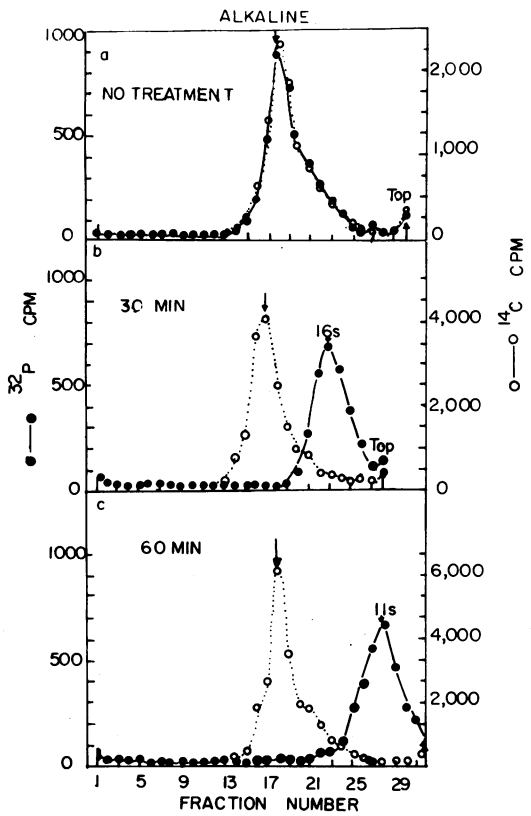

FIG. 7. *Alkaline sucrose gradients of ^{32}P-labeled P22 DNA exposed to B-band bacteria. Same DNA as in Fig. 6 but sedimented in alkaline sucrose, pH 12.1. Arrow indicates location of marker 37S.*

It should be mentioned that the endonuclease activity and the inactivation property of B-band bacteria, fractionated at competence, were also found in noncompetent cultures, as well as in T-band cultures which had been heated to 50 C for 10 min. The bacteria survive this heat treatment although competence is lost. This observation suggests a similarity to the *B. subtilis* enzyme described by Nester and McCarthy (13), which causes endonucleolytic breaks in DNA.

Little activity has as yet been demonstrated in the T-band competent cultures, which is surprising, since the temporal accumulation of transformants at high concentrations of T-band bacteria (greater than 5×10^7) terminates in a manner qualitatively similar to that observed with cultures containing B-band bacteria (Fig. 1).

From Table 5, one can see that there was little loss in transforming activity of DNA exposed to competent bacteria (35% loss for T-band versus 98% with noncompetent C-1 and heat-treated T-band bacteria). The concentration of bacteria in these experiments was sufficiently high to permit detection of nuclease activity at a similar density to B-band bacteria. So far, we have been able to detect little nuclease activity in competent T-band bacteria except after heating to 50 C.

DISCUSSION

Noncompetent B-band bacteria are unable to incorporate DNA in a deoxyribonuclease-re-

TABLE 3. *Molecular weights and transforming activities of deoxyribonucleate (DNA) broken by shearing and exposure to B-band bacteria*

^{32}P-labeled *Bacillus subtilis* DNA	$S_{20,w}$°	Mol wt	*trp*$^+$ Transformant	Per cent of original
0 rev/min	30	23×10^6	1.5×10^5	100
4,000 rev/min[a]	21	8×10^6	3.3×10^4	22
12,000 rev/min[a]	16	3.2×10^6	9.2×10^3	6.2
DNA[a] exposed for 30 min to B-band bacteria	22	9×10^6	2.5×10^3	1.6

[a] Compared to 22*S* (9×10^6 molecular weight of *Escherichia coli* DNA from P. Rosenthal) on neutral sucrose. B-band bacteria (10^8) were added to DNA and spun out after 30 min. Transformation is done at approximately 0.1 μg/ml.

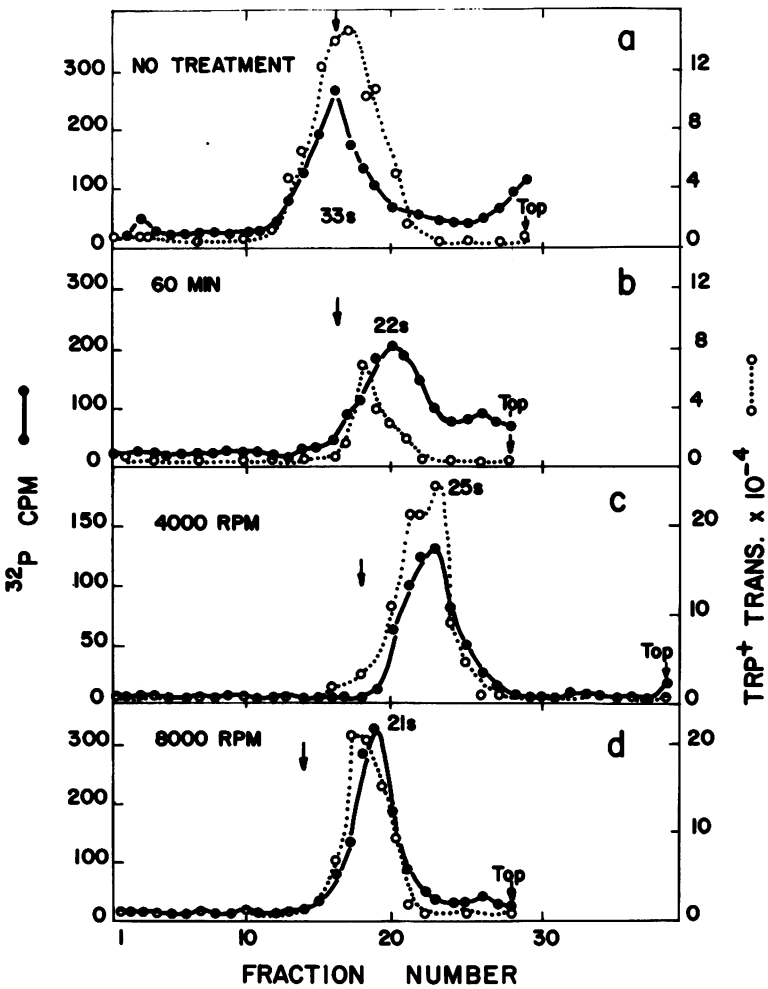

FIG. 8. *A 0.05-μg amount of ^{32}P-labeled DNA run on neutral sucrose after treatment with B-band bacteria or shearing. Fractions were collected, samples were counted, and transforming activity was determined. ^{14}C-labeled P22 phage was used as marker. (a) No treatment of DNA, (b) 30-minute exposure to 4 × 10^8 B-band bacteria, (c, d) shearing at 4,000 and 8,000 rev/min, respectively. Arrow indicates location of marker at 33S; ■, trp+ transformants; ●, counts per min ^{32}P.*

sistant form. Nevertheless, their presence in a competent culture reduces the effective DNA concentration. Examination of the kinetics of the reaction suggests that they are capable of forming some kind of unstable complex with DNA. There are two demonstrable consequences of exposure of DNA to B-band bacteria which seem likely to be the result of this binding. The DNA suffers double-strand endonucleolytic cleavage and also loses biological activity. Comparison with shear-broken DNA indicates that the reduction in molecular weight alone is not sufficient to account for the loss of biological activity. In fact, the inactivated material appears to have lost its capacity to interact at all with competent bacteria, as indicated here by the loss of its capacity to compete with biologically active transforming DNA. Consistent with the loss of capacity to compete, earlier observations (3) have shown that, under a wide variety of transformation conditions, there is little variability in the amount of DNA taken up per bacterium transformed.

Although shear, modest deoxyribonuclease I treatment and ultraviolet exposure (5, 8, 10) reduce transforming activity of DNA, these treatments result in little loss of the capacity of the inactivated DNA to compete or become incorporated in the transformation reaction. By all of these criteria the inactivation resulting from

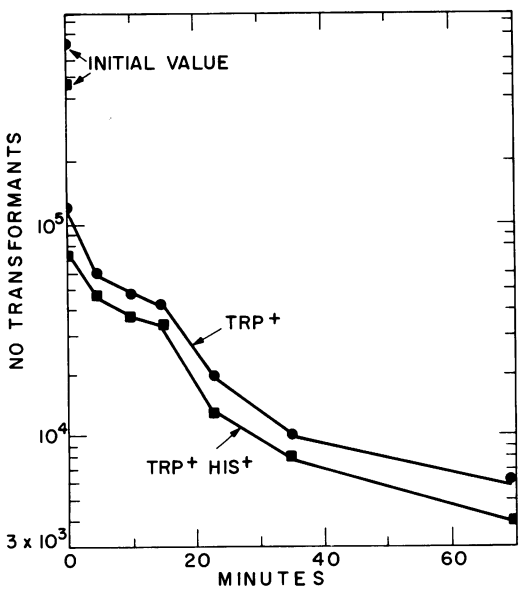

FIG. 9. *Surviving activity of trp+ and trp+his+ markers as a function of time of exposure to B-band at 0.2 μg per ml. SH113.*

TABLE 4. *Bacteria transformed with a mixture of 0.4 μg of Sul^{r}trp$^-$ deoxyribonucleate (DNA) per ml and 1.3 μg of Sul^{s}trp$^+$ DNA per ml sheared at varying speeds*

Mixture at rev/min	Per cent activity of original *trp*$^+$ activity[a]	No. of Sul^r transformants per ml
No Sul^s DNA		8.7×10^5
0	100	2.7×10^5
4,000	80	3.3×10^5
8,000	50	2.9×10^5
12,000	8	3.3×10^5
16,000	2	2.8×10^5

[a] Per cent of *trp*$^+$ activity for sheared DNA was determined at 0.13 μg per ml of DNA.

exposure to B-band bacteria is very different from other inactivation processes that have been described. The inactivation of DNA by noncompetent bacteria may account for the observation that the increase of transformants ceases in unfractionated cultures after approximately 20 min of DNA exposure.

Although it seems reasonable, there is no strong argument for assuming a connection between the endonuclease action and the loss of transforming activity. No attempt has yet been made to separate these activities. What does seem clear is that the reduction in molecular weight is not sufficient to account for the loss of activity. It is attractive to consider the possibility

TABLE 5. *Activities of deoxyribonucleate (DNA) exposed to a noncompetent culture and fractionated competent T-band bacteria*

Fraction.	No. of bacteria incubated with DNA prior to transformation	Per cent activity
DNA only	0	100
C-1 noncompetent[b]	10^8	3
T[a]	1.3×10^8	65
T-heat treated 50 C[a]	1.3×10^8	2

[a] Same culture; one half was heated at 50 C for 10 min and cooled, the other half was untreated. Both cultures were mixed with DNA for 30 min at 30 C. Transformations were carried out at 0.05 μg of 168 Sa, trp$^+$ DNA. Bacteria were incubated with DNA at 0.5 μg per ml (T-band plus DNA gave rise to 8.3×10^6 *trp*$^+$ transformants per ml before T-band was removed from solution by centrifugation. Heat-treated T-band bacteria plus DNA produced no transformants).

[b] C-1 cultures have been defined previously (3).

that some chemical modification of the DNA, in addition to, or as part of, the reaction that leads to the double-strand breakage, yields a product that is inert in transformation. It is not yet possible to say what such a modification might be, although a likely candidate would be an end modification that results in the loss of capacity to bind to competent bacteria. One possibly significant observation is that, although T-band bacteria appear to have little nuclease activity, heating to 50 C results in a loss of competence (12) and the appearance of activity. The endonuclease, perhaps masked in T-band bacteria, might even play a role in transformation.

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