BIOSYNTHETIC LATENCY IN EARLY STAGES OF DEOXYRIBONUCLEIC ACID TRANSFORMATION IN *BACILLUS SUBTILIS*[1]

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ABSTRACT

NESTER, E. W. (University of Washington, Seattle) AND B. A. D. STOCKER. Biosynthetic latency in early stages of deoxyribonucleic acid transformation in *Bacillus subtilis*. J. Bacteriol. **86**:785–796. 1963—In the *Bacillus subtilis* deoxyribonucleic acid (DNA) transformation system, transformants do not increase in number for 3 to 5 hr after the addition of DNA. During most of this period, the transformants are resistant to the bactericidal action of penicillin under conditions which result in the killing of over 90% of the recipient population. This lag in growth and nonmultiplication of the transformants (inferred from penicillin resistance) is also reflected in a lag in the synthesis of an enzyme specified by the donor DNA. Thus, when a cell population deficient in the enzyme tryptophan synthetase is transformed to tryptophan independence, activity of this enzyme cannot be detected in whole cells until 3 to 4 hr after the cells have been exposed to the DNA. Recombination between donor and recipient DNA occurs long before this. Even 30 min after exposure of a competent population of try_2^- his_2^+ cells to try_2^+ his_2^- DNA, 20% of the total try_2^+ activity found in re-extracted DNA exists as recombinant DNA, try_2^+ his_2^+. This value, the maximal linkage obtained, remains constant during incubation of the DNA-treated culture for an additional 5 hr. In addition to the heterogeneous response of a DNA-treated competent culture to penicillin killing, the recipient culture appears to be heterogeneous in ability to undergo transformation. Thus, the frequency of joint transformation of two unlinked markers is much higher than would be expected on the basis of the random coincidence of more than one DNA molecule entering the same cell in a uniformly competent recipient population. A possible relationship between these two aspects of heterogeneity of a DNA-treated recipient population is discussed.

In the pneumococcus deoxyribonucleic acid (DNA)-transformation system, integration of donor DNA occurs within 10 min after its uptake by the recipient cells (Fox, 1960). Immediately upon integration, but apparently not before, this DNA functions in enzyme synthesis (Lacks and Hotchkiss, 1960), and replicates in concert with the DNA of the recipient population (Fox and Hotchkiss, 1960). Under the usual conditions of transformation in pneumococcus, the majority of the bacteria in a competent culture appears to be susceptible to transformation, since the proportion of bacteria simultaneously transformed for two unlinked loci is approximately equal to, or often even less than, the product of the proportions of transformants for each of the single loci (Ephrussi-Taylor, 1959). Competency appears to be gained and lost in a cyclical fashion, the duration of the cycle being dependent on the temperature of incubation (Hotchkiss, 1954). Although the transformation system in *Haemophilus* has not been so extensively studied, as far as is known the same general picture holds true (Voll and Goodgal, 1961; Goodgal and Herriott, 1961).

In studies on the kinetics of genetic segregation of markers introduced on DNA molecules in the *Bacillus subtilis* transformation system, we made several observations which were difficult to reconcile with the general sequence of events described for the pneumococcus and *Haemophilus* transformation systems. Specifically, a lag of 3 to 5 hr was consistently observed before transformants increased in number. Phenotypic expression of fla^+ (motile) and try^+ transformants occurred

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TABLE 1. *Strains of Bacillus subtilis*

Strain no.	Genotype*
168	try_2^-
SB 1	$his_1^- try_2^-$ (unlinked)
SB 19	prototroph
SB 25	$try_2^- his_2^-$ (linked)
SB 32	his_2^-
SB 69	$his_2^- tyr_2^-$ (linked)
SB 455	mtr^r
WB 492	$try_3^- mtr^r$ (linked)

* Symbols: *his* = histidine; *try* = tryptophan; mtr^r = 5-methyl-tryptophan resistance; *tyr* = tyrosine.

only after a lag of 2.5 to 4 hr (Stocker, 1963). In addition, the number of double transformations of unlinked markers was consistently higher than would be expected on the basis of random coincidence of single markers entering the same cell in a uniformly competent population. The present paper reports studies aimed at elucidating the significance of these observations.

MATERIALS AND METHODS

The procedure described previously (Nester and Lederberg, 1961) for preparing competent cells was modified in the following manner. After overnight incubation in Penassay Broth (Antibiotic Medium No. 3), the culture was washed once, and was diluted into the minimal medium described by Spizizen (1958), supplemented with 0.04% acid-hydrolyzed casein (Nutritional Biochemicals Corp., Cleveland, Ohio) and 50 μg per ml of L-tryptophan. The turbidity of the culture was adjusted to give a reading of 60 on a Klett-Summerson colorimeter, with a 66 filter. After incubation at 37 C for 4 hr, the culture was diluted 1:10 into the minimal medium, supplemented with 0.1% acid-hydrolyzed casein and 50 μg per ml of DL-tryptophan [CHT-10 medium of Nester and Lederberg (1961)]. Appropriate reversion and contamination controls were run with each experiment.

Chemicals. Chemicals were generally purchased from Calbiochem, and were used without further purification. The C^{14} indole was chromatographically pure and was not further purified. Penicillin G (E. R. Squibb & Sons) and penicillinase (Neutrapen, Schenley Laboratories) were both stored at −20 C after dilution into modified Davis minimal medium. DNA was prepared by the procedure of Marmur (1961) or of Nester and Lederberg (1961). In all cases, it was deproteinized but was not treated to remove ribonucleic acid (RNA).

Enzyme assay. The assay for tryptophan synthetase was performed on whole cells, using a modification of the procedure of Rachmeler (1960). A washed-cell suspension was incubated with C^{14} indole and serine at 37 C, the cells were toluenized, and the tryptophan was quantitatively precipitated as a mercury-sulfate-tryptophan complex. A constant amount of unlabeled carrier L-tryptophan was added to all reaction mixtures at the completion of the reaction, and no correction was made for self-absorption.

Bacterial strains. The bacterial strains used are listed in Table 1. All strains were derived from the *B. subtilis* try_2^- mutant (168) and thr^- mutant (23).

RESULTS

Lag in the multiplication of transformants. Studies on the kinetics of multiplication of try_2^+ transformants revealed that after DNA addition a remarkably long lag occurred before transformants increased in number. At 32 C, try_2^+ transformants do not increase in number even when incubated for 5 hr after DNA addition. This corresponds to an increase of eight generations in the recipient population which is still in the logarithmic growth phase (Fig. 1). This lag prevails at 32 C irrespective of whether the culture is incubated in a medium containing tryptophan (CHT-10), or in an otherwise identical medium lacking tryptophan (CH). A significant decrease in the absolute number of transformants is consistently observed under conditions in which the nontransformed recipient population can multiply. This decrease, which often begins within 30 min after the addition of deoxyribonuclease but generally reaches its maximum 3 to 4 hr later, often obscures the exact time at which the number of transformants does increase. The reduction may reach 10% but generally is of the order of 50 to 75% of the original number of transformants. This decrease can be markedly reduced or eliminated if the recipient culture is washed and then resuspended in a medium free from tryptophan. Further studies are necessary to determine the basis of this killing.

At 37 and 40 C, the growth rate of *B. subtilis* is greater than at 32 C, and the number of transformants increases at an earlier time as well (Fig. 1). At 37 C, in both CH and CHT-10 medium this

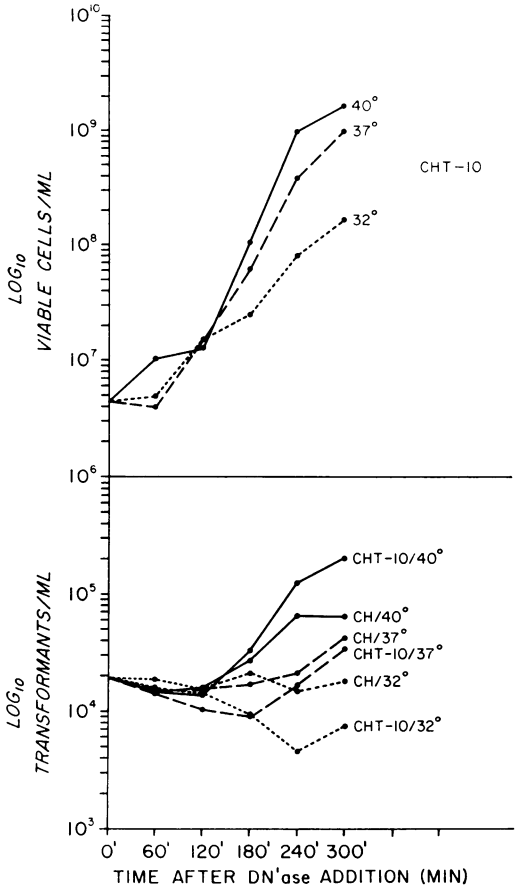

FIG. 1. *Kinetics of transformant try+ and viable count increase at various temperatures, in the presence and absence of tryptophan. Strain 168 was prepared for competency by the usual procedure. SB 19 DNA (2.4 μg/ml, final concentration) was added, and 30 min later deoxyribonuclease (20 μg/ml + 0.01 M $MgSO_4$, final concentration) was added. The culture was incubated for an additional 2 min, centrifuged, diluted 1:50 into 5 ml of preheated CHT-10 (containing tryptophan) or CH (no tryptophan), and incubation was continued with aeration at the indicated temperature. Samples were removed and assayed for transformants and viable count at the indicated times.*

increase begins between 180 and 240 min after DNA treatment, by which time the viable count of the total culture has increased about 16-fold. At 40 C, the lag is even shorter, between 120 and 180 min, by which time the whole population has increased about eightfold. Thus, the duration of the lag appears to be dependent on the growth rate of the recipient population and not on the temperature per se.

The delay in the increase of transformants is not limited to the try_2 locus, nor is it demonstrable only in a liquid medium. Thus, when transformants from the cross SB 19 (his_2^+ tyr_1^+) —× SB 69 (his_2^- tyr_1^-) were plated on media supplemented with either histidine or tyrosine, and the cells were respread after various periods of incubation at 37 C, there was no increase in the number of transformants after 5 hr, although the recipient population, plated on minimal medium supplemented with both histidine and tyrosine, underwent nearly five divisions in the same interval (Fig. 2). The lag of 180 min in the replication of the viable cells is a consequence, most likely, of transferring the cells from a casein hydrolysate-supplemented liquid medium (CHT-

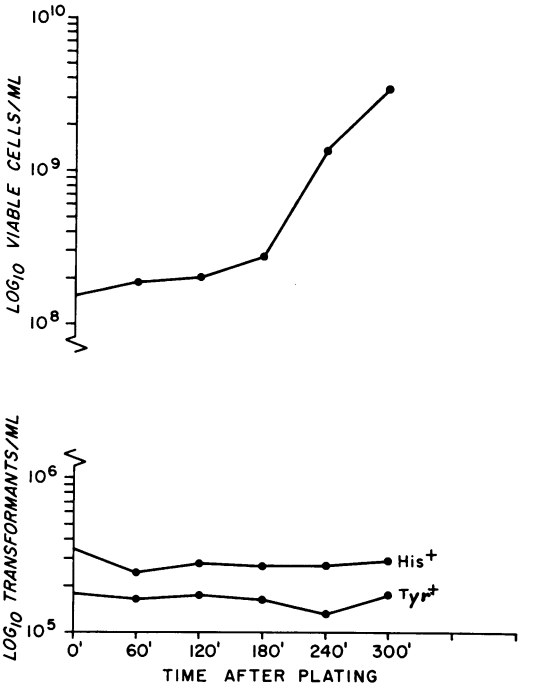

FIG. 2. *Kinetics of increase of viable cells and transformants on solid media. SB 69 (his_2^- tyr_2^-) was prepared for competency as usual, with the 4-hr incubation medium supplemented with L-tyrosine and L-histidine (10 μg/ml, final concentration). After incubation with SB 19 DNA (3 μg/ml, final concentration) for 30 min, the culture was plated on tyrosine, histidine, and tyrosine + histidine supplemented minimal agar plates. These plates were incubated at 37 C, and, at the indicated times, 0.1 ml of sterile minimal medium was added and spread over the entire surface of a plate with a bent glass rod; the plates were reincubated at 37 C.*

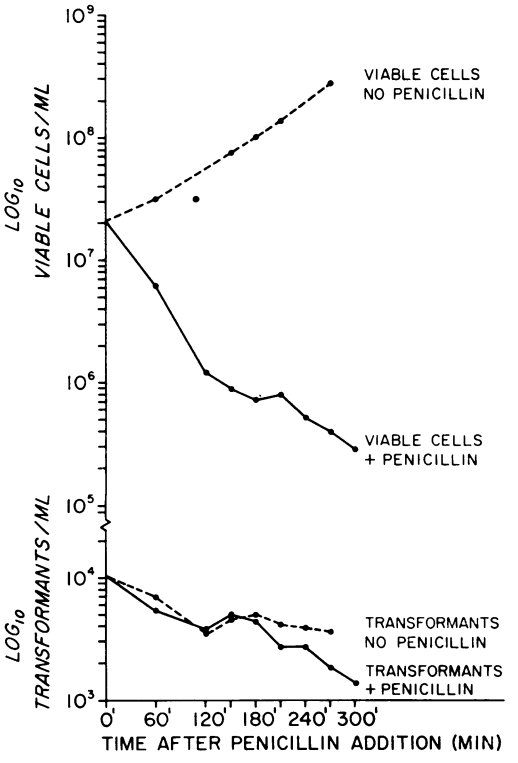

FIG. 3. *Kinetics of penicillin killing of viable cells and transformants. SB 19 DNA (3 μg/ml, final concentration) was added to competent 168, and 30 min later deoxyribonuclease (4 μg/ml in 5 × 10^{-3} M $MgSO_4$, final concentration) was added. Incubation was continued for an additional 5 min, the culture was then diluted 1:5 into CHT-10 medium prewarmed to 32 C, containing 200 units/ml of penicillin G where indicated, and 0.1-ml samples were removed at the specified times for assay of total viable cells and transformants. Whenever penicillin was used, the initial dilution was made into a dilution blank, supplemented with 400 μg/ml of penicillinase. This was incubated for 5 min before further dilutions were made.*

10) to a solid minimal agar supplemented only with histidine and tyrosine.

Thus, regardless of whether DNA-treated auxotrophic cells are incubated in a complete medium permitting the multiplication of untransformed bacteria, or in a deficient medium, permitting multiplication only of transformants, the number of bacteria able to produce transformant colonies does not detectably increase until after a lag of 2 to 5 hr.

The time at which the multiplication of transformants can first be demonstrated is markedly dependent on the temperature of incubation. Temperatures which approach the optimal temperature for growth of the recipient population (37 or 40 C) give a shorter lag time than does 32 C.

Effect of penicillin on viability of transformants. Several explanations can be proposed for the lag observed before an increase in transformants can be demonstrated. The three most likely possibilities are (i) lag in nuclear segregation in the transformants, (ii) transmission without replication of the exogenous DNA among the progeny of a multiplying recipient, or (iii) a lag before onset of growth or division of the transformants. These possibilities involve either a growing and multiplying transformant (i and ii) or one which is not multiplying (iii). Since our strain 168, unlike many *B. subtilis* strains, produces no detectable penicillinase (D. A. Dubnau, *personal communication*) and is highly susceptible to penicillin, a distinction can be made in theory between hypothesis iii and the other two with respect to resistance of the transformants to penicillin. Delay in growth and multiplication of the transformants should be manifest as a resistance to penicillin, which kills only growing bacteria. To test this possibility, strain 168 was prepared for competency by the standard procedure and exposed to DNA for 30 min. At this time, deoxyribonuclease was added, and the culture was diluted fivefold into two samples of CHT-10, prewarmed to 30 C. Penicillin (200 units/ml) was added to one sample, and the treated and control cultures were incubated at 30 C with aeration. Samples were removed periodically (into diluent containing 400 units per ml of penicillinase in the case of the treated culture), and were assayed on nutrient agar for a total viable count and on minimal medium supplemented with casein hydrolysate for transformants. The results of this experiment (Fig. 3) demonstrate that in the presence of penicillin the total number of viable cells decreases with approximately first-order kinetics for the first 120 min, then remains almost constant for approximately 90 min before decreasing again at a somewhat slower rate. On the other hand, the absolute number of transformants both in the presence and absence of penicillin is parallel until approximately 210 to 240 min after deoxyribonuclease and penicillin addition. At this time, the transformants become susceptible to penicillin killing. These data illustrate that transformants are totally resistant

to the killing action of penicillin during an interval in which over 90% of the total population is killed. The kinetics of penicillin killing (Fig. 3) are generally quite reproducible, in particular, a nearly exponential killing of the total population during the first 2 hr (±30 min), and the significantly decreased rate of killing lasting 1 to 2 hr. Transformants are routinely resistant to penicillin killing for at least 3 to 5 hr under controlled conditions of transformation. The reduction in the number of transformants in control cultures without penicillin may be so variable that the exact time at which transformants become sensitive to penicillin may be obscured.

The above evidence clearly shows that bacteria of a DNA-treated competent culture behave heterogeneously on incubation in a complete medium. Most cells are killed if exposed to penicillin; a minority, the transformants, do not multiply and are not killed by exposure to penicillin.

Additional evidence for heterogeneity in the recipient population. A different line of investigation indicates that the "competent" culture is heterogeneous even before exposure to DNA, a majority of the bacteria being insusceptible and a minority being highly susceptible to transformation. If the culture exposed to DNA consisted of uninucleate cells of uniform susceptibility to transformation and if transformation of one nucleus by the uptake and integration of one molecule of DNA did not affect the probability of this nucleus taking up and being transformed by another DNA molecule, then the proportion of bacteria doubly transformed for two unlinked loci, by the uptake of two different molecules of DNA, would be the product of the proportions of bacteria transformed for each single locus. This relation would hold whether the culture was exposed to a saturating or to a limiting concentration of DNA. In the *B. subtilis* transformation system, however, the observed frequency *per bacterium* of double transformations for any pair of unlinked loci is consistently much higher than the product of the frequency for the two single transformations (Anagnostopoulos and Crawford, 1961; Nester, Schafer, and Lederberg, 1963). For instance, the transformation by wild-type DNA of strain SB 1, carrying the two unlinked loci, his_1 and try_2, yields a proportion of his^+ try^+ transformants much greater than the product of the proportions of try^+ and his^+ transformants (Table 2). The most plausible explanation for this excess frequency is a heterogeneity of the "competent" culture, a majority of the bacteria being insusceptible or poorly susceptible and a minority highly susceptible to transformation.

If the competent minority, uniformly susceptible to transformation and uninucleate, forms a fraction $1/x$ of the whole population, the proportion of competent bacteria theoretically transformed for a single character will be x times greater than the actually observed proportion of single transformations *per bacterium* in the whole population. Therefore, the theoretically predicted rate of double transformations *per competent bacterium* will be x^2 times greater than the product of the observed proportions of single transformations *per bacterium*. The theoretical rate of double transformations calculated *per bacterium* instead of *per competent bacterium* will be x times the product of the observed rates of single transformations *per bacterium*. On this oversimplified model, the expected proportion of double transformants *per bacterium* for any two unlinked characters divided by the observed proportion of double transformations for these characters then gives the value $1/x$, the fraction of competent bacteria in the culture. Experimentally, mutant cultures vary greatly in their ability to undergo DNA transformation (Nester et al., 1963). With cultures of high competence, exposure to excess DNA results in 0.001 to 0.005 of single transformations in the total population, and the size of the competent fraction calculated as above is generally about 0.01 to 0.05 (Table 2). As theoretically predicted, subsaturating as well as saturating DNA concentrations on the same culture yield similar estimates by calculation from the frequency of single and double transformations (Table 2). If the standard procedure for obtaining competence as measured by the proportion of single transformations resulting from exposure to saturating DNA concentrations is deliberately varied or, for unknown reasons, is suboptimal, the fraction of competent bacteria, calculated as above, may be as low as 0.0002, or even lower (Table 2).

Other factors possibly involved in the lag in the increase of transformants. Although the lack of growth and multiplication of transformants, inferred from their penicillin resistance, suffices to account for part of the lag in the multiplication of transformants, other factors may be involved. Transformation of a bacterium results in only a part of the entire genetic complement of the

TABLE 2. *Calculation of competency of recipient cultures**

Expt no.	DNA concn	Frequency of transformants			Product of single frequencies (= 3 × 4)	Proportion of competent cells† (= 6/5 × 100)
		try$^+$	*his*$^+$	*try*$^+$ *his*$^+$		
	μg/ml					%
1‡	15	8.8×10^{-6}	11×10^{-6}	4×10^{-7}	9.7×10^{-11}	0.024
2	23.1	1.3×10^{-3}	1.5×10^{-3}	8.3×10^{-5}	2.0×10^{-6}	2.4
	2.3	1.2×10^{-3}	9.0×10^{-4}	2.1×10^{-5}	1.1×10^{-6}	5.2
	0.23	3.5×10^{-4}	4.9×10^{-4}	3.8×10^{-6}	1.7×10^{-7}	4.5
	0.023	4.0×10^{-5}	4.3×10^{-5}	6.7×10^{-8}	1.7×10^{-9}	2.6

* Recipient culture, SB 1 (*try*$_2^-$ *his*$_1^-$); DNA, SB 19 (*try*$_2^+$ *his*$_1^+$). The cultures were prepared for competency by the procedure of Nester and Lederberg (1961) with a supplementation of L-histidine (10 μg/ml) in CHT-2. After 4 hr of growth, the culture was diluted with 2 parts of CHT-10. After contact with DNA for 30 min, the cells were centrifuged and washed once prior to plating on supplemented and unsupplemented media.

† Percentage of competent cells = $\frac{(\text{frequency } try^+)\ (\text{frequency } his^+)}{\text{frequency } his^+\ try^+} \times 100$. (See text for discussion.)

The data in this paper give a much smaller value for the proportion of competent cells in the population than can be calculated from data presented in a previous paper (Nester and Lederberg, 1961; Table 3). This may be explained in part at least by a misleadingly high proportion of single transformants having been recovered in the earlier work (approximately two- to threefold) as a result of using chilled diluent blanks in the assay for total viable and transformed cells. This procedure was subsequently shown to reduce the viable count more than the count of transformants because of the longer exposure of the former sample to the cold diluent. No significant differential killing effect of the low temperature on the total viable cells and the transformants was detected when the time in the cold blanks was equal. A misplaced decimal point in the value of the column entitled "calculated as random coincidence" accounts for the high calculated value for the cotransfer of the unlinked markers, *his*$_3$ and *try*$_2$ (ind). On the data presented, the value 0.02 should be changed to 0.002.

‡ The explanation for the poor competence of the recipient culture of experiment 1 is not known.

bacterium being genetically altered. Findings of Nester (*unpublished data*) as well as those of Stocker (1963) are consistent with the hypothesis that a competent cell contains more than one copy of each locus, but that only one becomes transformed. Thus, a transformed cell (either to streptomycin resistance or tryptophan independence) plated on nutrient agar 30 min after DNA addition produces a colony consisting of 75 to 90% cells of the recipient genotype. If the transformed bacterium contains two nuclei, only one of which is transformed, at least two generations would be required for an observable doubling in the number of transformants.

Further, at 37 C, transformants are not quantitatively resistant to the killing effect of penicillin for any appreciable time, although there is a delay of approximately 3 hr before any increase in number can be detected. Transformants are killed by penicillin at a significant rate shortly after transformation, although the rate of killing is generally less during the first hour than the rate of killing of the total population. One possible interpretation is that under some conditions transformants may be growing and multiplying more slowly than the total population (Fig. 4). A second possibility to account for the penicillin sensitivity without any detectable increase in transformants would be a growth without division of transformants.

Time of enzyme synthesis in newly transformed cells. The data of Stocker (1963) indicate that transformed cells do not express their transformed character until several hours after DNA addition. He observed a delay of 2.5 to 4 hr before the expression of two transformed characters, motility and tryptophan independence, could be detected by microscopy. Although this technique does not detect the first expression of transformant protein synthesis, the observations nevertheless indicate that the biosynthetic processes specified by donor DNA are greatly delayed, for flagella and other protein syntheses are normally geared to a generation time of approximately 40 min under these environmental conditions.

Since enzyme formation represents a more

sensitive manifestation of the biological activity of the newly introduced DNA, this phenotypic delay was evaluated by determining the kinetics of the appearance of tryptophan synthetase activity after transformation of *try*$^-$ to *try*$^+$. In the pneumococcus transformation system, enzyme synthesis could be detected within minutes after uptake of the DNA (Lacks and Hotchkiss, 1960).

For the present study, DNA was isolated from a prototroph (SB455) which has a gene mutation, linked to the tryptophan synthetase locus, manifested as resistance to 5-methyl tryptophan (*mtr*^{r}). This mutation maintains the enzyme tryptophan synthetase in a derepressed state independent of the level of exogenous tryptophan and has been tentatively identified as a regulator gene mutation (Nester et al., 1963). The recipient strain (WB 492) has the same regulator gene mutation, as well as a deficiency for the synthesis of tryptophan synthetase. The use of derepressed mutants was essential to assay the initial expression of protein synthesis and not the state of derepression of enzyme synthesis. The genetically derepressed state of both donor and recipient insures that tryptophan synthetase activity will appear as soon as the transformants become able to express the genetic information of the exogenous *try*$^+$ gene. There should be no delay resulting from an initially repressed state of the *try* genes of either the donor or the recipient. Strains carrying the *mtr*^{r} locus manifest approximately 10 to 20 times as much tryptophan synthetase activity in the presence of 20 μg per ml of tryptophan as do wild-type strains in the absence of exogenous tryptophan.

A control experiment demonstrated that 10^7 viable cells of strain SB 455 (*try*$^+$, *mtr*^{r}) donor cells could convert a measurable amount of C^{14} indole into tryptophan (Fig. 5), approximately four times the background radioactivity given by a boiled-cell suspension (250 counts/min vs. a background of 65). There is a reasonable linearity of conversion of indole to tryptophan with increasing cell concentration.

A series of competent WB 492 cultures (100 ml per flask) were exposed to DNA for 30 min. The cultures were then centrifuged, and the bacteria were resuspended in CH (no tryptophan) and aerated at 32 C. At periodic intervals, the cultures were centrifuged, and the whole cell pellet was assayed for tryptophan synthetase activity. The kinetics of detection of tryptophan

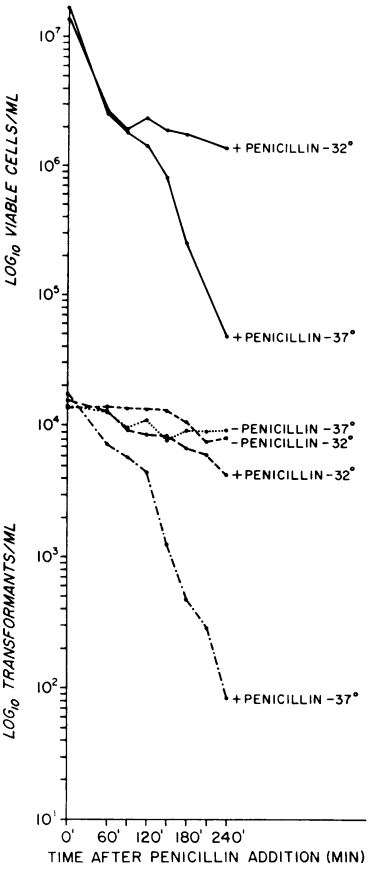

FIG. 4. *Effect of temperature on the resistance of transformants to penicillin. SB 19 DNA (2.4 μg/ml, final concentration) was added to competent 168 cells, followed 30 min later by 20 μg/ml of deoxyribonuclease + 0.01 M $MgSO_4$ (final concentration) for 5 min. The cells were then diluted 1:5 into CHT-10 prewarmed to either 32 or 37 C with or without penicillin (200 units/ml). Samples were removed and assayed for transformants and viable count at the indicated times.*

synthetase activity resulting from the cross SB 455— × WB 492 is illustrated in Fig. 6. The results indicate that there is a lag of at least 150 min after the end of the DNA treatment before enzymatic activity can be detected. Activity rises sharply from 250 min to reach a level approximately 100-fold greater than the activity measured up to 150 min. During this same time interval of 390 min, there is only a twofold increase in the observed number of transformants.

These data, in concert with those of Stocker (1963), suggest that at 32 C, not only is the transformed cell unable to grow and multiply

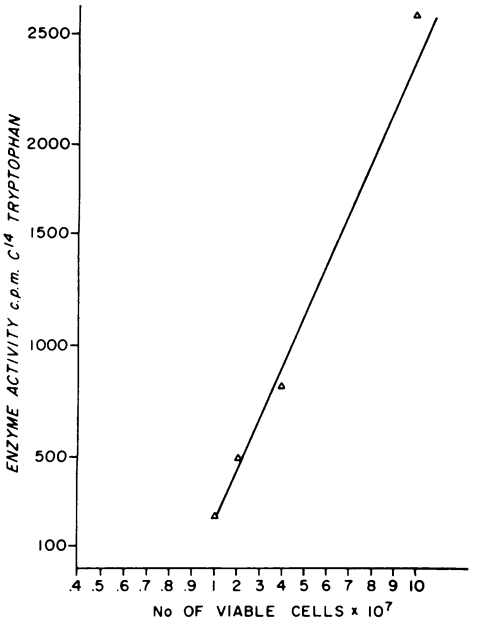

FIG. 5. *Viable cell concentration vs. tryptophan synthetase activity. SB 455 ($try_3^+ mtr^r$) was prepared for competency by the usual procedure. At the usual time for DNA addition, the culture was centrifuged, washed once in minimal medium, and resuspended in the original cell concentration in a reaction mixture containing the following (μM/ml): DL-serine, 60; pyridoxal HCl, 0.2; tris (pH 7.8), 30. This cell suspension was assayed for total viable cells and then diluted into individual tubes of reaction mixture to give cell concentration noted; 0.17 μmole of indole-2-(ring)-C-14 (specific activity, 0.6 mc/mmole) was added to each tube, and the culture was incubated at 37 C with vigorous aeration for 60 min. The tryptophan was precipitated as a $HgSO_4$-tryptophan complex, plated on Whatman no. 42 filter paper, and counted in a very thin window Nuclear-Chicago gas-flow counter. The counts/min plotted represent the radioactivity after the subtraction of the counts of a reaction mixture containing a boiled-cell suspension. The background counts were not significantly higher when unboiled WB 492 ($try_3^- mtr^r$) was incubated with C^{14} indole.*

for several hours after DNA addition, but that during this time transforming DNA molecules introduced into the recipient cell may not be functional in protein synthesis.

Integration of donor DNA into the recipient genome. Although this lag in the synthesis of tryptophan synthetase may be a result of a general biosynthetic latency of the newly transformed cell, as manifested by its penicillin resistance, it may also be due to the fact that the exogenous DNA has not yet been integrated into the recipient genome because of this metabolic latency or for some other reason. In the pneumococcus system, unintegrated DNA apparently does not function to direct enzyme synthesis (Lacks and Hotchkiss, 1960). This possibility was studied by determining the kinetics of the establishment of linkage between a marker, try^+, located on the donor DNA molecule and another marker, his_2^+, resident in the recipient genome. DNA was extracted at intervals from samples of a competent culture of try_2^- his_2^+ (linked) cells treated with DNA from a try_2^+ his_2^- strain. After partial purification, this DNA was assayed at a limiting dilution on a competent culture of a try_2^- his_2^- (SB 25) strain for the presence of his^+ (endogenous), try^+ (exogenous), and try^+ his^+ (recombinant) transforming activities. The appearance of try^+ his^+ (recombinant) transforming activity establishes that the exogenous try^+ gene has become associated with the endogenous his^+ gene, presumably by completion of the integration of the relevant part of the foreign

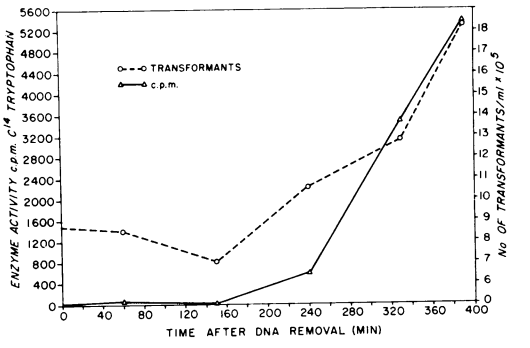

FIG. 6. *Kinetics of tryptophan synthetase formation. WB 492 ($try_3^- mtr^r$) was prepared for competency in 100-ml samples of CHT-10 in the second incubation period. The culture was in contact with SB 455 DNA (1.8 μg/ml, final concentration) for 30 min, after which the culture was centrifuged, and the cells were resuspended in the same volume of CH medium (no tryptophan). Incubation was continued at 32 C and, at the indicated times, a 100-ml sample was assayed for transformants and viable count, and centrifuged and assayed for tryptophan synthetase activity as described in Fig. 5. The counts/min in tryptophan represent the counts after subtraction of the radioactivity (75 counts/min) in the tryptophan precipitate given by a nontransformed culture of WB 492 taken through all steps in the transformation procedure and subsequent assay.*

TABLE 3. *Kinetics of integration of donor DNA into recipient genomes**

Time after DNA removal	Transformation frequency	Transformants (per ml) of re-extracted DNA— × SB 25		$\frac{his^+ try^+}{try^+}$
		try^+ (× 10^2)	$his^+ try^+$ (× 10)	(=3/2)
min				
30	0.0035	2.5	5.8	0.23
60	0.0014	1.5	3.0	0.20
90	0.00082	1.5	2.5	0.17
150	0.00039	2.1	6.6	0.31
330	0.00035	0.62	1.3	0.21
SB 19— × SB 25		8.8×10^5	7.12×10^5	0.81

* Strain 168 was prepared for competency by the usual procedure. The incubation in CHT-10 was carried out in 150-ml samples. SB 32 DNA (1.0 μg/ml, final concentration) was added to 168. After 30 min of contact, all cells were centrifuged, and the supernatant was discarded. The remaining supernatant and cell pellet were treated with deoxyribonuclease (20 μg) in 10^{-2} M $MgSO_4$ for 5 min. The cells were then resuspended to the original volume (150 ml) of CHT-10, and incubation was continued for various periods of time. This initial time is 0. After the specified intervals, the culture was centrifuged at 4 C, washed once with 0.14 M NaCl + 0.10 M Na citrate. The DNA was then isolated after lysozyme lysis at 44 C, essentially by the technique of Marmur (1961), through the first deproteinization (with the exception that Duponol and the heating to 60 C were not included). The re-extracted DNA was assayed at a limiting concentration against SB 25, using pour plates. The data of SB 19— × SB 25, done as a part of this experiment, are included for comparison. The try^+ include the try^+ his^+ transformants.

DNA molecule. The results of one such experiment are presented in Table 3. The linkage value of the DNA extracted from the treated culture, i.e., its try^+ his^+ activity divided by its try^+ activity, 1 hr from the beginning of contact of the try_2^- his_2^+ culture with try^+ his^- DNA was 0.23, at a time when tryptophan synthetase activity was undetectable (Fig. 5). This value did not change significantly during the next 300 min, in which time the tryptophan synthetase activity of the treated culture became well established (Fig. 5).

These results indicate that the inability to detect the activity of an enzyme specified by the donor DNA 150 min after termination of DNA treatment probably does not result from the nonincorporation of the DNA into the genome. Control experiments, designed to determine whether the try_2^+ his_2^- and try_2^- his_2^+ markers might undergo a nonspecific association with each other, were performed. Try_2^+ his_2^- and try_2^- his_2^+ DNA did not produce a significant number of try_2^+ his_2^+ transformants when assayed on SB 25, even when the DNA mixture had been heated and slowly cooled to permit strand separation and reformation.

DISCUSSION

The present studies indicate that a newly transformed cell of *B. subtilis* is unable to carry out several different biosynthetic reactions. The penicillin resistance of new transformants suggests that these reactions include at least one or more concerned with the growth and multiplication of the cell. Further, they appear to include all of the synthetic reactions specified by the donor DNA thus far studied. This is suggested by the fact that tryptophan synthetase enzyme activity is undetectable in whole cells until approximately 3 hr after transformation and that a similar lag occurs before motility and prototrophy are expressed (Stocker, 1963). It also appears that DNA synthesis in newly transformed cells does not parallel DNA synthesis in the remainder of the population. Thus, when DNA is added to a "competent" population, samples are removed after various periods of incubation, and the DNA is isolated, the ratio of the transforming activity of the newly introduced DNA to the recipient DNA does not remain constant when the total DNA is assayed against a strain capable of detecting both the original donor and recipient activities. Instead, the ratio of donor to recipient activity steadily decreases, suggesting that, on incubation after addition of the donor DNA, the recipient population is synthesizing a proportionately greater amount of recipient DNA compared with the donor (Nester, *unpublished results*). For these reasons, we tentatively consider the newly transformed cell to be latent in certain biosynthetic functions. Since our observations have been limited to relatively few biosynthetic functions, a more detailed analysis of the biosynthesis of additional macromoles and structural components will be required to define this unusual latency of competent cells.

The pneumococcus transformation literature is

dotted with observations of a lag in the multiplication of transformants. Thus, Hotchkiss (1953) noted that the number of streptomycin-resistant transformants did not increase for several hours after DNA addition. He later stated that transformants multiply more slowly than the remainder of the recipient population (Hotchkiss, 1957). Ravin (1960) suggested that cells which have just absorbed transforming DNA are set back in their growth. Ephrussi-Taylor (1959) observed a lag in the replication of the newly introduced genes, which she interpreted as transmission of the marker without replication for two generations. These data appear discrepant with the definitive studies of Fox and Hotchkiss (1960), who showed that the donor DNA is rapidly integrated into the recipient genome and thereafter multiplies in concert with it. However, in view of the marked effect that temperature plays in the penicillin resistance of the newly transformed *B. subtilis* cells, different procedures for preparing competent cells and subsequent treatment of transformed pneumococcus cultures might yield strikingly different results in the growth and multiplication of transformants. The frequency of the reports from different investigators on the slow growth of transformants compared with the remainder of the recipient cells suggests that, under appropriate conditions, newly transformed cells of pneumococcus may also display a biosynthetic latency similar to that demonstrated for *B. subtilis*. On the other hand, the results may reflect the presence of multiple nuclei or clumping of cells, or both, in the pneumococcus system, resulting in a nuclear segregation lag.

The relationship of the penicillin-resistant population to the transformation process is currently under investigation. The resistance of the newly transformed cell to penicillin, contrasted to the marked sensitivity of the majority of the population to killing by this antibiotic, strongly suggests that penicillin specifically differentiates transformants from nontransformants. Presumably, transformants would include all those cells which had integrated DNA and not only those specifically selected and scored as transformants. Whether this latency is a property of the competent cell (one capable of undergoing irreversible transformation), *ipso facto*, or whether it is a consequence of the uptake or integration of DNA by a competent cell is of prime importance. Recent studies (Nester, 1963) strongly indicate that the competent cell is penicillin-resistant. Thus, the addition of penicillin to a "competent" culture and its subsequent removal prior to DNA addition leads to a five- to tenfold increase in the transformation frequency, compared with an identical culture not treated with penicillin. This is most economically explained by assuming that the noncompetent cells are readily killed by the penicillin, whereas the competent cells are resistant. In addition, the kinetics of killing of a recipient population by penicillin are identical whether or not DNA has been added (Nester, 1963). All these data are consistent with the observations reported in this paper that the recipient population is heterogenous with only a minority of cells capable of undergoing transformation.

Studies are also in progress to clarify precisely why we are not able to detect tryptophan synthetase activity until several hours after DNA addition. The kinetics of the appearance of linkage between markers in the donor and recipient DNA indicate that all of the biologically active exogenotic DNA isolatable by our procedure has been integrated at a time when tryptophan synthetase is not detectable. These same studies indicate that this early recombinant DNA does represent a major fraction of the total exogenotic DNA which eventually becomes integrated into the recipient genome, as calculated from the number of try_2^+ transformants obtained with the isolated DNA compared with the number expected from the transformation frequency of the try_2 marker. Since no attempt was made to quantitatively isolate the DNA, this calculation assumes that the exogenotic and endogenotic markers are equally isolatable and equally capable of transforming the try_2^- his_2^- strain. Further studies are necessary to validate these assumptions. Nevertheless, it is unlikely that the lack of enzyme activity can be explained merely by a lack of integration of the donor DNA.

Since it was conceivable that the enzyme was synthesized very shortly after DNA addition but could not be detected because of the impermeability of the cell to the C^{14} indole or serine, similar kinetic studies were performed on lysozyme lysates of the DNA-treated population (Schwartz and Bonner, 1963). The results on cell-free extracts parallel the results gained with whole cells, namely, no significant enzyme ac-

tivity detectable until several hours after DNA addition. Thus, it appears likely that the enzyme is not being synthesized until several hours after DNA addition. Since the duration of penicillin resistance of transformants and the time during which tryptophan synthetase cannot be detected are approximately the same, it is likely that the two phenomena are related to the same basic cause—the biosynthetic latency of the newly transformed cell.

In contrast to these data, in the pneumococcus system new enzyme formation begins almost as soon as DNA integration occurs, about 6 min after DNA addition. The apparent discrepancy between *B. subtilis* and pneumococcus probably does not reflect any basic differences between the two genera in the functioning and replication of the DNA after its uptake and integration by the competent cell. If, indeed, the pneumococcus and *B. subtilis* transformants both undergo a period of biosynthetic latency, and if this latency is related to competency, the time at which enzyme synthesis occurs may reflect the time at which the transformed cell emerges from the resting state and begins actively multiplying. In pneumococcus, Hotchkiss (1954) showed that under certain conditions competence is related to a part of the normal life cycle of a cell, and lasts for approximately 15 min. In *B. subtilis*, a transformed cell (and therefore a cell which must have been competent) does not appear to multiply for several hours, as shown by its resistance to penicillin killing. Thus, while the cell remains in this nonmultiplying state, it is incapable of expressing certain metabolic functions. In pneumococcus this state is transient; in *B. subtilis*, under certain conditions, it may last for several hours.

Although our data indicate that the cell population is heterogenous with respect to competency, the quantitative calculation of the fraction of competent cells is questionable. Our model, the simplest one possible, assumes only two classes of cells, the competent minority and the noncompetent majority. However, there may be intermediate degrees of competence in individual cells. It is also possible that under some conditions the uptake or integration, or both, of one molecule of DNA facilitates the subsequent uptake or integration, or both, of a second molecule. Thus, in occasional experiments unusually high values of joint incorporation of markers, either linked or unlinked, have been observed at high DNA concentrations (Nester et al., 1963). In these experiments, the greater than theoretical incidence of double transformants might reflect the greater competence of cells which, by chance, are first transformed, in addition to a heterogeneity of the recipient population prior to the addition of the DNA. The calculated competent fraction of the recipient population in *B. subtilis*, in which the percentage of transformation for single markers is 0.1 to 0.5% at saturating levels of DNA, is less than 10% of that in the pneumococcus and *Haemophilus* transformation systems, where the frequency of single transformants may reach 5%. In these latter systems, calculations similar to those employed with the *B. subtilis* system indicate that 70 to 100% of the population is competent (Goodgal and Herriott, 1960; Fox and Hotchkiss, 1957). However, all calculations based on this model probably overestimate the proportion of competent cells in a population, since the model assumes that the transformable cells are uninucleate. If the bacteria were binucleate, then the proportion of *nuclei* transformed for each single character would be one-half the observed proportion of single transformations per bacterium. The predicted rate for double transformation, calculated per bacterium, would be one-half the product of the observed proportions of the single transformations per bacterium. Thus, the presence of more than one nucleus per bacterium would result in the observed rate of double transformations for a pair of unlinked loci being lower than the product of the rates observed for single transformations. Accordingly, if the bacteria are multinucleate or contain more than one nucleus per colony-forming unit, the size of the competent fraction calculated in this way will be an overestimate.

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