JOURNAL OF BACTERIOLOGY, Feb., 1965
Vol. 89, No. 2

Printed in U.S.A.

Lag Period Characterizing the Entry of Transforming Deoxyribonucleic Acid into *Bacillus subtilis*[1]

JAMES S. LEVINE[2] AND NORMAN STRAUSS[3]

Department of Microbiology, Yale University School of Medicine, New Haven, Connecticut

Received for publication 24 August 1964

ABSTRACT

LEVINE, JAMES S. (Yale University, New Haven, Conn.), AND NORMAN STRAUSS. Lag period characterizing the entry of transforming deoxyribonucleic acid into *Bacillus subtilis*. J. Bacteriol. **89**:281-287. 1965.—The kinetics of appearance of transformants as a function of time of exposure to deoxyribonucleic acid (DNA) has been studied in *Bacillus subtilis*. A short lag period of approximately 1 min at 37 C is evident when the transformation is terminated with deoxyribonuclease. The length of this lag is independent of the genetic trait transferred. Moreover, the lag is unaffected by transforming DNA concentration, by the presence of homologous unmarked DNA, and by shearing and cross-linking of the transforming DNA. The lag shows a strong inverse temperature dependence. The energy of activation is 13.9 kcal. The lag is abolished when the transformation is terminated by washing instead of by addition of deoxyribonuclease. These results are taken to indicate an immediate adsorption of DNA to cells, followed by a deoxyribonuclease-sensitive period of 1 min, during which time the genetic trait is entering the cell. These results make feasible an investigation of the configuration assumed by the DNA molecule during entry into the cell.

Cellular uptake of macromolecules is a widely occurring phenomenon, the mechanism of which, at least for the case of bacteria, remains obscure. Bacterial transformation represents a useful system for the study of this phenomenon. This system guarantees that the macromolecule taken up by the cell, namely deoxyribonucleic acid (DNA), must retain a high degree of structural integrity, and also permits the measurement of uptake, which is reflected in the subsequent phenotypic expression of the genetic characters carried by the DNA.

Previous work involving investigations of the mechanism of entry of DNA has been carried out primarily with the pneumococcus (Fox and Hotchkiss, 1957; Lerman and Tolmach, 1957) and with *Haemophilus influenzae* (Barnhart and Herriott, 1963), and has indicated the presence of an initial reversible binding of DNA, followed by a deoxyribonuclease-resistant (irreversible) phase. Evidence also was obtained that, after entry, DNA undergoes a single-stranded phase during which the donor genetic information is undetectable (Lacks, 1962).

[1] Submitted in partial fulfillment of the requirements for the M.D. degree by James S. Levine.

[2] Present address: Department of Pediatrics, Bronx Municipal Hospital Center, Bronx, N.Y.

[3] Present address: Department of Biology, State University of New York, Buffalo.

Studies on *Bacillus subtilis* have been less extensive. Anagnostopoulos and Spizizen (1961) and Nester (1964) studied the development of competence in *B. subtilis*, and Young and Spizizen (1961, 1963) studied the incorporation of P^{32}-labeled DNA during transformation. Finally, Kohiyama and Saito (1960) detected the presence of a lag, 2 to 4 min, in the appearance of transformants as a function of time. They suggested that this period was the time necessary for the synthesis of an inducible permease.

Although some information was obtained in these various studies concerning the adsorption of DNA to the cell and the final disposition of the donor material in the host cell, a notable lack of information is evident concerning the events which take place between the time of adsorption and the reappearance of the donor material as part of the host genome.

The purpose of this investigation has been to study the kinetics of DNA uptake in the *B. subtilis* transformation system to obtain information regarding the mechanism of DNA entry into the cell, as well as the factors which control this process. More specifically, the following questions can be posed: (i) what configuration does the DNA assume during transport into the cell; (ii) what structures have been developed by the cell for the transport of DNA into the cell? The work to be presented in this and subsequent

reports attempts to answer the first of these two questions.

MATERIALS AND METHODS

Organisms. All strains of *B. subtilis* used in these experiments were derived from the tryptophan-requiring strain 168 (try_2^-) of Burkholder and Giles (1947). Strain SB25 requiring tryptophan and histidine ($try_2^- his_2^-$) was isolated by E. Nester, University of Washington, Seattle, by ultraviolet irradiation of strain 168. An arginine-requiring strain, designated arg^-77, was derived from strain 168 by ultraviolet irradiation; the tryptophan requirement was relieved by introduction of wild-type marker by transformation. This arginine lesion was found to be unlinked to either of the SB25 markers (Strauss, *unpublished data*). Strain 168 wild type was obtained by spontaneous reversion of strain 168 try_2^- to prototrophy.

Media. All media were based on the minimal medium of Anagnostopoulos and Spizizen (1961): $(NH_4)_2SO_4$, 0.2%; K_2HPO_4, 1.4%; KH_2PO_4, 0.6%; sodium citrate·$2H_2O$, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.02%; glucose, 0.5%. The following variations were used: S-2-H, minimal medium to which were added L-tryptophan (50 μg/ml), L-histidine (50 μg/ml), and casein hydrolysate (acid; 0.02%); S-3-H, minimal medium to which were added L-tryptophan (5 μg/ml), L-histidine (50 μg/ml), casein acid hydrolysate (0.01%), and α,α′-dipyridyl (20 μg/ml); minimal-yeast extract-lactate medium, minimal medium to which were added yeast extract (0.25%) and sodium lactate (1.5%).

Agar medium for assay of transformants consisted of minimal medium to which were added sodium lactate (0.024%) and 50 μg/ml of each of the following: L-alanine, L-ammonium aspartate, L-cysteine, glycine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-proline, L-serine, L-threonine, and agar (Difco, Noble), 1.8%.

Millipore filters were purchased from the Millipore Filter Corp., Bedford, Mass. Filters had a pore size of 0.45 μ and a diameter of 47 mm.

Deoxyribonuclease. Deoxyribonuclease (2× crystallized) was obtained from Worthington Biochemical Corp., Freehold, N.J. Enzyme solutions were kept at 4 C for not more than 1 week after preparation. After this period, the deoxyribonuclease deteriorated sufficiently to give erratic results.

Extraction of DNA. B. subtilis strain 168 wild type was grown in minimal-yeast extract-lactate medium for 10 hr at 37 C, harvested, and DNA was isolated according to the method of Marmur (1961), except lysozyme treatment was carried out in the presence of 20% sucrose, and lysis was accomplished by the addition of sodium lauryl sulfate (1%).

DNA concentration was determined by the method of Burton (1956).

Preparation of cross-linked DNA. Wild-type DNA was treated with HNO_2 as described by Geiduschek (1961). That this DNA (HNO_2-DNA) was cross-linked was indicated by the following: a 25% retention of transforming activity after thermal denaturation (Marmur and Lane, 1960) as opposed to 5% for native DNA; a hyperchromic shift of 7% after thermal denaturation as opposed to 31% for native DNA.

Transformation and assay procedures. Transformation was carried out by a modification of the method of Anagnostopoulos and Spizizen (1961). The recipient strain was grown overnight (12 to 18 hr) at 37 C on a Tryptose Blood Agar Base slant, suspended in S-2-H medium, inoculated into S-2-H medium to an optical density of 17 to 18 (Klett, 540 mμ), and placed on a rotary shaker at 37 C. After a Klett reading of 170 to 190 had been attained (4 to 5 hr), the culture was diluted 10-fold with S-3-H medium and was replaced on the rotary shaker (37 C) for 105 min, at which time the cells were maximally competent. DNA was added to the culture, and samples were removed at intervals. The transformation was terminated as described below, and was plated for the assay of transformants. For those cases in which low frequencies of transformation were desired, cells were transferred from S-2-H to S-3-H at a Klett reading of 120. This procedure decreased the rate of appearance of transformants with time by approximately 10-fold.

Temperature studies were carried out by allowing the cells to attain competence in the usual manner. After 100 min in S-3-H medium, the culture flask was transferred to a bath of the desired temperature and was allowed to equilibrate for 5 min. DNA was added to the culture, and samples were removed for assay of transformants.

All samples were plated in duplicate, and the results are given as the average of the duplicate determinations. The standard deviation of such platings is 12%.

A final concentration of 1.5 μg/ml of DNA was used in all transformation experiments, except when otherwise indicated. This concentration was found experimentally to be the minimal saturating level of DNA under the conditions employed in these experiments.

Termination of transformation was accomplished either by the addition of deoxyribonuclease (50 μg/ml, final concentration) and $MgSO_4$ (5 μmole/ml, final concentration), or by rapid dilution and washing of the cells with minimal medium after their retention on Millipore filters. The former procedure will be referred to in the text as the "deoxyribonuclease method"; the latter will be referred to as the "washing method."

Deoxyribonuclease-treated transformants were plated directly on agar medium, and transformants were counted in 24 to 48 hr. Washed cells were plated by placing the Millipore filters directly on agar plates face down. Transformants were counted in 48 hr. Comparison of the two techniques indicated that no adverse effects resulted from the filtration method. Reversion and contamination controls were routine.

RESULTS

These experiments were designed to study the appearance of *B. subtilis* transformants as a function of the duration of contact of recipient cells and DNA. In each experiment, competent recipient cells were mixed with DNA, samples were removed at intervals, the transformation was terminated by one of the procedures described in Materials and Methods, and suitable dilutions of the transformed culture were plated for the assay of transformants.

Kinetics of transformation to try$^+$ at 37 C by the deoxyribonuclease method. The transformation of SB25 to tryptophan independence was carried out at 37 C (Fig. 1 and 2). Deoxyribonuclease was used to terminate the transformation. We observed that: (i) there was a definite lag of 1 to 1.5 min before the appearance of any transformants, and (ii) the kinetics curve is not linear between 1.5 and 4 min, but indicates a gradually increasing rate of appearance of transformants.

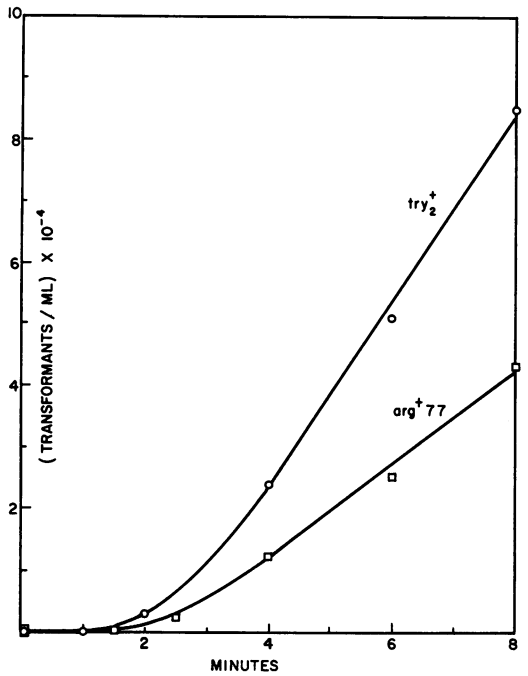

FIG. 1. *Early appearance of transformants. DNA from a wild-type organism was added to competent SB25 cells (try$_2^-$his$_2^-$) at zero time. Samples were removed at intervals to a tube containing deoxyribonuclease. Cells were then diluted appropriately and plated on minimal medium supplemented with histidine to determine the numbers of try$_2^+$ transformants. For the case of the arginine locus, strain arg$^-$77 was used as recipient and was plated on minimal medium to determine the numbers of arg$^+$ transformants.*

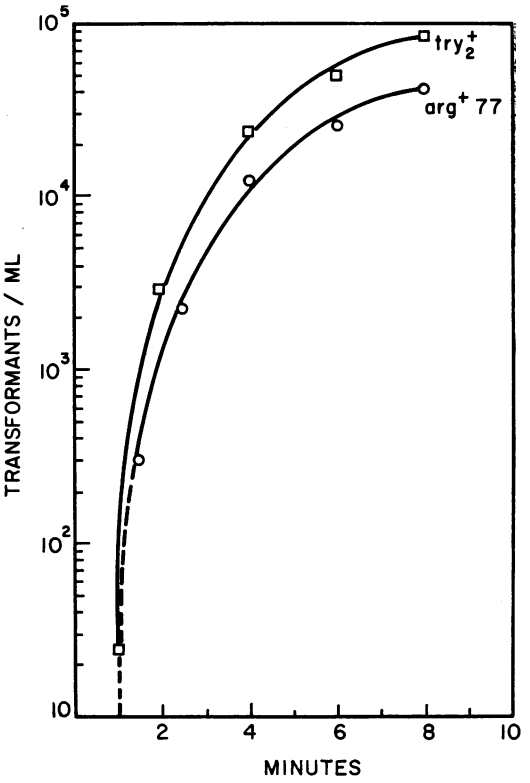

FIG. 2. *Semilogarithmic plot of the data of Fig. 1.*

That the observed lag is an absolute one and not an artifact deriving from the assay of too small a sample is indicated by the semilogarithmic plot of the data. The curve resulting from such a depiction of the data originates at approximately 1.0 min with virtually infinite slope. Moreover, experimentally induced 10-fold reductions in transformation frequency (see Materials and Methods) had no effect on the length of the lag period as determined in this manner.

Kinetics of transformation to arg$^+$ at 37 C by the deoxyribonuclease method. The same procedure was repeated by using as recipient an arginine-requiring mutant (*arg*$^-$77) whose genetic lesion is unlinked to *try$_2$*, and transforming it to arginine independence (Fig. 1 and 2). Deoxyribonuclease was used to terminate the transformation. The lag and the general shape of the kinetics curves are similar to those obtained for the tryptophan marker. The same experiment was carried out with SB25 as recipient cell, and assaying for transfer of the double marker try$_2^+$his$_2^+$ (linked). The results were similar to

those of the previously described experiments with respect to the duration of the lag and the shape of the kinetics curve.

It was concluded from these experiments that the lag and the nonlinearity in the appearance of transformants are independent of any particular genetic trait, but seem to be characteristic of the *B. subtilis* transformation process in general when carried out under these conditions.

Effect of temperature on the lag period. Figure 3 depicts the kinetics curve obtained at 26 C. The shape of the curve appears similar to that obtained at 37 C. Below 23 C, the total yield of transformants began to decrease drastically, and the curve obtained at 18 C assumed a sigmoidal appearance. It was evident, moreover, that the lag period was increased at 26 C. An Arrhenius plot of the relationship of the length of the lag period to the temperature is shown in Fig. 4. The activation energy was calculated to be 13.9 kcal.

Kinetics of transformation of try$_2^+$ at 37 C by the washing method. The possibility that the kinetics are a function of the method used to terminate the transformation prompted the execution of the same experiment with a washing procedure to prevent further transformation. Transformation of SB25 to tryptophan independence was carried out at 37 C with the washing method to terminate the transformation. The results are shown in Fig. 5 with a kinetics curve obtained simultaneously by the deoxyribonuclease method from the same culture. The kinetics curve obtained by the washing method differed from that obtained by the deoxyribonuclease method in two important respects: (i) there was no lag; (ii) the kinetics were linear.

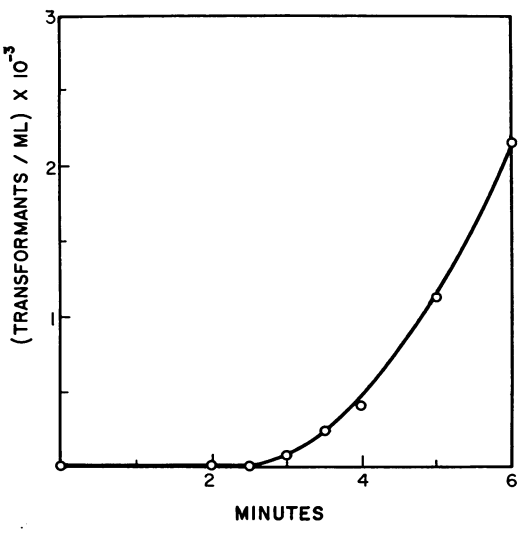

FIG. 3. *Effect of temperature on the appearance of try$_2^+$ transformants. Strain SB25 was brought to competence and transferred to a 26 C water bath for 5 min. DNA was added to the cells, and samples were removed at intervals to tubes containing deoxyribonuclease. Cells were diluted appropriately and were plated for the assay of try$_2^+$ transformants.*

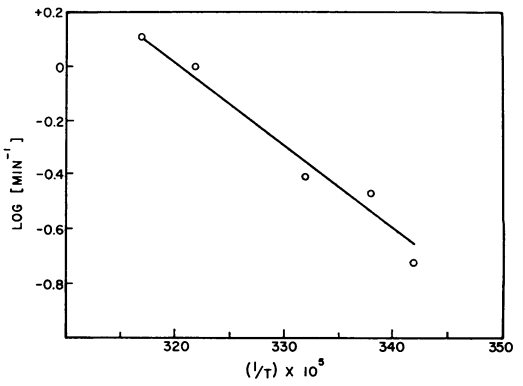

FIG. 4. *Arrhenius plot of the relation between the length of the lag period and the temperature. The reciprocal of the lag was used as a measure of rate. Each point represents the average obtained from two or more separate experiments. The position and slope of the line were determined statistically.*

This experiment indicates that, although transformation was not detectable by the deoxyribonuclease method for 1 to 1.5 min after the addition of DNA, effective binding of DNA to recipient cells began to take place as soon as the two were mixed.

Kinetics of transformation to try$^+$ at 37 C by the delayed deoxyribonuclease method. Transformation of SB25 to tryptophan independence was carried out at 37 C. Samples of the transformation mixture were removed at intervals and washed as in the washing method. Instead of removing and plating the filter membrane containing the transformants, 10 ml of minimal medium were gently pipetted into the filter tower, the contents of which were allowed to stand at room temperature for 4 min (slightly longer than the lag period at 26 C). Deoxyribonuclease and $MgSO_4$ were then added in final concentrations of 50 μg/ml and 5 μmole/ml, respectively. After 1 min, the liquid was filtered through, and the filter membrane was removed and plated as described above. The results are shown in Fig. 6. This experiment demonstrates both the immediate binding of DNA to recipient cells, as well as the subsequent acquisition of deoxyribonuclease resistance by these molecules during the 4-min waiting period. It further indicates that the lag

shown in Fig. 1 is not an inevitable result of the use of deoxyribonuclease.

Effect of various agents on the kinetics of transformation to try+ at 37 C by the deoxyribonuclease method. Chloramphenicol was added in varying amounts to a competent culture of SB25 10 min before the addition of DNA to give final concentrations between 20 and 160 μg/ml. These concentrations are well in excess of those known to be completely inhibitory for protein synthesis in vegetatively growing cells of *B. subtilis* (Strauss, *unpublished data*). After the addition of DNA, samples were taken at various time intervals; these were treated with deoxyribonuclease and diluted in the absence of chloramphenicol prior to plating on an appropriate selective medium. Controls were run which were identical, except for the omission of chloramphenicol. The addition of chloramphenicol had no effect on the lag or on any aspect of the kinetics curve. These results indicate that protein synthesis does not play an important role in the entry process.

Variations in DNA concentration over a 50-fold range had no effect on the length of the lag or the shape of the kinetics curve for the appearance of transformants as a function of time, although the overall rate of appearance of transformants varied directly with the DNA concentrations which were less than saturating.

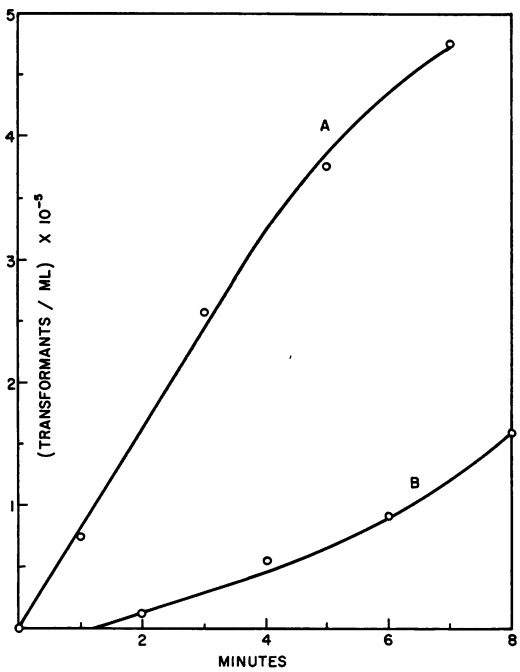

FIG. 5. *Termination of transformation by washing instead of deoxyribonuclease. Strain SB25 was brought to competence and exposed to wild-type DNA; samples were subjected at intervals to the washing procedure as described in Materials and Methods (curve A). The same culture was treated with deoxyribonuclease in the usual manner (curve B).*

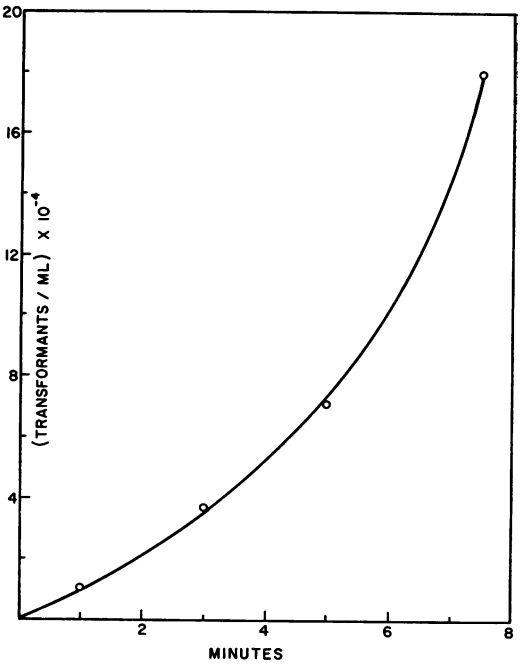

FIG. 6. *Effect of the delayed use of deoxyribonuclease on the kinetics of appearance of transformants. Strain SB25 was brought to competence and exposed to wild-type DNA; samples of the complex were transferred to a Millipore filter apparatus and washed. Minimal medium was added to the filter tower with the suction off, and the cells were permitted to incubate for 4 min at 29 C. Deoxyribonuclease was added; after 5 min, the supernatant fluid was filtered off, and the Millipore filter pad containing the cells was plated for try+ transformants as described in the text.*

An excess of homologous unmarked DNA (15 to 30 μg/ml) was added to a competent culture of SB25 simultaneously with the transforming DNA. There was no effect on the lag or the shape of the kinetics curve, although the rate of appearance of transformants varied inversely with the amount of unmarked DNA added.

The double stranded helix of DNA treated with HNO_2 was reported to be cross-linked by means of covalent bonds (Geiduschek, 1961). HNO_2-DNA was prepared from *B. subtilis* wild-type DNA by the method of Geiduschek (1961). The kinetics curve obtained for transformation by HNO_2-DNA showed no change in

the lag compared with that obtained with untreated DNA when either his_2^+ or try_2^+ transformants were measured, although the overall rate of appearance of transformants was substantially reduced. These results indicated that cross-linking has no effect on the delay in the appearance of transformants.

DISCUSSION

The data presented above serve to characterize to some extent the nature of the events which occur early after the addition of transforming DNA to a competent culture of *B. subtilis*. It is readily apparent that the manner in which transformants appeared as a function of time depended upon the method used to terminate the transformation. If deoxyribonuclease was used to prevent further transformation, the kinetics were characterized by a lag period lasting 1 to 1.5 min, with subsequent appearance of transformants at an increasing rate (Fig. 1 and 2). In contrast, the separation of competent cells from transforming DNA by a washing procedure resulted in kinetics which showed no lag and which were linear for nearly 6 min (Fig. 5).

These findings lead to the following conclusions. Effective attachment of DNA to the cell occurs with unmeasurable velocity upon mixing the cells with the DNA. This conclusion is based on the finding that the washing procedure permits transformation to proceed from zero time. The linearity of the plot from zero time can also be construed as an indication that only the DNA which is in the process of obtaining entry into the cell resists the washing procedure. Were this not so, it would appear that transformants were present at zero time. This procedure, therefore, provides a measure of the rate of transformation of the population, and it also implies that the rate-limiting step is not that of adsorption. Despite the rapid attachment of DNA, deoxyribonuclease can prevent the expression of the marker for 1 min, indicating that some relatively slow process takes place after attachment which renders the potential transformant insensitive to deoxyribonuclease, and which requires a definite period of time.

The strong inverse dependency of the length of the lag period on the temperature, as indicated in Fig. 4, and the magnitude of the energy of activation, suggest that the period of time necessary to render the transformation deoxyribonuclease-insensitive involves an enzymatic process. The possibility that cellular entry of DNA is accomplished by an energy-requiring system has been suggested by Young and Spizizen (1963). Lacks (1962) suggested the involvement of deoxyribonuclease in the process.

Neither the concentration of DNA nor the presence of homologous unmarked DNA had any effect on the length of the lag, again indicating that the lag represents a process occurring subsequent to attachment of the DNA to the cell. Cross-linkages introduced into the double helix had no effect on the length of the lag period. Finally, the delay in the appearance of transformants bore no relationship to the genetic character which was transferred.

Kohiyama and Saito (1960) suggested that the lag in the appearance of transformants with time might be due to the induction of a DNA permease, but offered little in support of this concept. This possibility now seems unlikely in view of the observation that preincubation of competent cells with high concentrations of chloramphenicol affects neither the lag nor any aspect of the kinetics curve. This observation is in agreement with the recently reported results of Nester (1964).

The results described above are compatible with two modes of entry of DNA into the cell. The entry might occur in a linear manner (Lacks, 1962) or, alternatively, the cell might in some way coil the DNA molecule so that the entire length of the molecule becomes deoxyribonuclease-insensitive simultaneously. The characterization of this lag now provides a means for obtaining an answer to the question of which of these two models is the correct one. Evidence will be presented in a subsequent publication (Strauss, *in preparation*) that DNA attains insensitivity to deoxyribonuclease in a linear fashion during the process of transformation

ADDENDUM IN PROOF

Stuy and Stern (1964) recently reported the characterization of a lag period occurring in the *Haemophilus* system. They concluded that the lag period is related to the time of entry for a DNA molecule. The relationship was not clarified. The authors also suggested that DNA enters the cell in linear fashion.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants 2R-37-C1 to J.S.L. and GM 08994 to N.S. from the National Institutes of Health.

LITERATURE CITED

ANAGNOSTOPOULOS, C., AND J. SPIZIZEN. 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.

BARNHART, B. J., AND R. M. HERRIOTT. 1963. Penetration of deoxyribonucleic acid into *Hemophilus influenzae*. Biochim. Biophys. Acta **76**:25–39.

BURKHOLDER, P. R., AND N. H. GILES. 1947. Induced biochemical mutations in *Bacillus subtilis*. Amer. J. Botany **34**:345–349.

BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.

FOX, M. S., AND R. D. HOTCHKISS. 1957. Initiation of bacterial transformation. Nature **179**:1322–1325.

GEIDUSCHEK, E. P. 1961. "Reversible" DNA. Proc. Nat. Acad. Sci. U.S. **47**:950–955.

KOHIYAMA, M., AND H. SAITO. 1960. Stimulation of genetic transformation in *Bacillus subtilis* by biologically inactive DNA and polyphosphate. Biochim. Biophys. Acta **41**:180.

LACKS, S. 1962. Molecular fate of DNA in genetic transformation in pneumococcus. J. Mol. Biol. **5**:119–131.

LERMAN, L. S., AND L. J. TOLMACH. 1957. Genetic transformation. I. Cellular incorporation of DNA accompanying transformation in *pneumococcus*. Biochim. Biophys. Acta **26**:68–82.

MARMUR, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.

MARMUR, J., AND D. LANE. 1960. Strand separation and specific recombination in deoxyribonucleic acids: biological studies. Proc. Nat. Acad. Sci. U.S. **46**:453–461.

NESTER, E. W. 1964. Penicillin resistance of competent cells in deoxyribonucleic acid transformation of *Bacillus subtilis*. J. Bacteriol. **87**: 867–875.

STUY, J. H., AND D. STERN. 1964. The kinetics of DNA uptake by *Hemophilus influenzae*. J. Gen. Microbiol. **35**:391–400.

YOUNG, F. E., AND J. SPIZIZEN. 1961. Physiological and genetic factors affecting transformation of *Bacillus subtilis*. J. Bacteriol. **81**:823–829.

YOUNG, F. E., AND J. SPIZIZEN. 1963. Incorporation of deoxyribonucleic acid in the *Bacillus subtilis* transformation system. J. Bacteriol. **86**:392–400.