THE EFFECT OF PHENETHYL ALCOHOL UPON

BACILLUS SUBTILIS TRANSFORMATION

By

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CHAPTER I

REVIEW OF THE LITERATURE

In 1953 Lilley and Brewer (1) demonstrated that phenethyl alcohol (PEA) was a means for selecting gram-positive bacteria from mixed flora. The growth of seven gram-negative enteric organisms was completely inhibited on trypticase soy agar supplemented with 0.25% PEA (v/v) while Staphylococcus aureus, Streptococcus faecalis, and Diplococcus pneumoniae formed colonies. A more extensive investigation into this selective inhibition by Berrah and Konetzka (2) revealed that 0.25% PEA inhibited growth of gram-negative organisms with the exception of Pseudomonas fluorescens which was not inhibited at 0.35%. On the other hand, gram-positive organisms were not inhibited by 0.35% PEA, but all bacteria tested except the streptococci were inhibited by 0.5% PEA.

Berrah and Konetzka (2) investigated the mechanism of inhibition of PEA in Escherichia coli since PEA was more inhibitory to gram-negative bacteria. The action of PEA (0.25%) was bacteriostatic and resulted in filamentous bacterial forms similar to those observed by Barner and Cohen (3) in thymineless death of a thymine-requiring strain of E. coli. In the presence of PEA (0.32%) there was no net synthesis of deoxyribonucleic acid (DNA) whereas protein and ribonucleic acid (RNA) synthesis were unaffected as determined by the diphenylamine reaction (4), protein determination (5), and orcinol reaction (6)

respectively.

The replication of T2 phage in E. coli was inhibited in the presence of PEA (7). Folsome (8) found that 0.34% PEA inhibited recombination and heterozygote formation in T4 phage infected E. coli.

Treich and Konetzka (9) studying the incorporation of ^{14}C-thymine into acid insoluble material in E. coli demonstrated that the replication of DNA, occurring at the time of PEA (0.32%) addition, was completed, but the initiation of a second cycle of DNA replication was inhibited. Similar findings were reported by Lark and Lark (10), e.g., DNA replication stopped in the same region of the chromosome as it did in the absence of required amino acids. This region was postulated to be the beginning of the chromosome--the point from which the normal cycle of replication is initiated (11, 12). Lark and Lark (10) envisioned that this inhibition occurred by PEA's inhibiting the synthesis or modifying the structure of one of the proteins required for chromosome replication. Treich and Konetzka (9) suggested that the inhibition by PEA of DNA replication was mediated by the alteration of the bacterial membrane.

Conjugation in E. coli was inhibited by 0.31% PEA (13). This indicated that DNA synthesis was necessary for chromosome transfer (13), and, on removal of PEA, the kinetics of chromosome transfer began by the initiation of a new cycle of replication (14, 15).

The speed of reversal of inhibition of DNA synthesis and the fact that supplementation of the medium with DNA precursors could not reverse the inhibition of PEA suggested that PEA was physically interacting with DNA rendering it unacceptable to the DNA polymerase. Mendelson and Fraser (16) found no effect by PEA (0.35%-0.6%) on the

physical properties of DNA such as thermal denaturation, renaturation, and viscosity. The physicochemical properties of DNA isolated from 0.5% PEA-treated and non-treated E. coli were found to be the same (17); therefore PEA was not affecting the physical properties of DNA. Studies by Zahn et al. (18) of the in vitro synthesis of DNA by DNA-nucleotidyl-transferase (E. C. No. 2.7.7.7. [polymerase]) from calf thymus, revealed that 0.5% PEA was inhibitory.

Additional effects of PEA on E. coli have been described and "the mode" of action of PEA is currently disputed. Studies by Nonoyama and Ikeda (19) with the RNA phages β and MS-2 of E. coli showed PEA (0.3%) inhibited replication of viral RNA. Rosenkranz et al. (17) determined the concentrations of PEA required for the inhibition of various processes in E. coli and found the process most sensitive to PEA was that of the induction of alkaline phosphatase (50% inhibition with 0.07% PEA in resting cells and 0.04% PEA in growing cells). RNA and DNA synthesis studies (determined by the incorporation of ^{32}P into RNA and DNA) required 0.17% for 50% inhibition of DNA synthesis and 0.09% for RNA synthesis. Protein synthesis as determined by ^{14}C-amino acid incorporation required 0.17% for 50% inhibition. Since enzyme induction requires the specific messenger ribonucleic acid (mRNA) and synthesis of the specific enzyme (protein), it was concluded that mRNA was the point of inhibition of PEA. Prevost and Moses (20) investigated the effect of PEA on the synthesis of RNA, DNA, protein, and β-galactosidase induction in E. coli and did not find a preferential inhibition of mRNA synthesis over general RNA synthesis.

A third site of PEA action has been suggested by the finding that PEA alters the permeability of germinated conidia of Neurospora

crassa (21). PEA (0.1% - 0.3%) inhibited RNA, DNA, and protein synthesis to an equal extent and the primary effect noted in the presence of PEA (0.25%) was the severe restriction of the accumulation of L-leucine, L-tryptophan, and α-amino-isobutyric acid. Silver and Wendt (22) investigated the action of PEA on acriflavin (10-methyl-2, 8-diamino acridine) uptake and potassium leakage in E. coli. There was a rapid and reversible breakdown in the permeability barrier when cells were treated with 0.25% PEA or 0.25% toluene. The authors suggested two possible mechanisms for the inhibitory effect of PEA on the many varied processes: (1) there may be a direct structural coupling of the process to the bacterial membrane or (2) the inhibition may be indirect and results by leakage from the cytoplasm of essential small molecules.

Several other systems have been examined for sensitivity to PEA, including mammalian cells. Leach et al. (23) found that Ehrlich II B and L cells treated with PEA (0.05% and 0.5%) showed a decrease in DNA synthesis, and the DNA became soluble. In BHK 21 cells PEA (0.04%) may interfere primarily with RNA synthesis (determined by the incorporation of radioactive uracil into acid insoluble material) and its effect on DNA and protein synthesis is due to the primary effect on RNA (24). Roizman (25) found that PEA (0.1% - 0.2%) inhibited the formation of infectious progeny in HEp-2 cells infected with herpes simplex virus (DNA virus). In chicken liver cells Higgins et al. (26) have shown that 0.5% PEA acts on the membrane of the lysosome causing a partial breakdown of the membrane and subsequent release of degradative enzymes.

Slepecky (27) found that 0.25% PEA inhibited germination and sporulation of Bacillus megaterium under conditions where no synthesis

of DNA could be demonstrated and suggested that PEA was preventing the formation of stable informational RNA. Remsen et al. (28) later demonstrated that the inhibition of sporulation by PEA (0.35%) in Bacillus cereus was due to its effect on the early fore-spore membrane formation. PEA (0.25%) has also been shown to affect the reaggregation of Micrococcus lysodeikticus membranes (29).

White and White (30) found that PEA (0.25%) was synergistic with mitomycin C, streptonigrin, and porfiromycin (inhibitors of DNA metabolism) and suggested that PEA was not a specific inhibitor of DNA synthesis. Sodium cyanide and 2,4-dinitrophenol were also synergistic with PEA leading to the suggestion that PEA might be acting as an inhibitor of electron transport. Using glucose as the substrate, Wolgamott (29) has shown that 0.25% PEA increases the rate and amount of oxygen uptake in E. coli. This indicated the possible uncoupling of oxidative phosphorylation by PEA.

The effect of PEA on the cell-free synthesis of polyphenylalanine was studied by Rosenkranz et al. (17). Using synthetic messenger (poly U) in the cell-free system of Nirenberg and Matthaei (31), polyphenylalanine synthesis was insensitive to inhibition by 0.1% PEA. A cell-free extract derived from PEA treated cells demonstrated that neither ribosomes, transfer RNA (tRNA), or the enzymes necessary were affected by exposure of E. coli to PEA.

When PEA was added to the B. subtilis transformation system, an inhibition was noted at concentrations (0.05%) which had no effect upon viability or growth rate (32, 33, 34). The inhibitory effect of PEA is apparently upon competent cells and their ability to take up DNA.

Each of the three possible sites of action of PEA (DNA synthesis,

RNA synthesis, and the cell membrane) is involved in DNA-mediated transformation, and procedures to differentiate each of these processes in transformation may be devised to study the mechanism of action of PEA. Transforming activity permits the evaluation of *in vitro* treatment of DNA with PEA which is more sensitive than the study of the physicochemical properties of the DNA.

Bacterial transformation involves the functioning of DNA with incumbent configurational requirements for synapsis, integration, and replication of the donated segment of DNA. The expression of the genetic information introduced by transformation requires the synthesis of mRNA and the protein corresponding to the new information. The first step of transformation requires the passage of DNA across the permeability barrier (membrane) by an active process which can be measured by the uptake of radioactive DNA. Since formation of the protein corresponding to the new information incorporated does not occur during the first 3 to 4 hours following uptake (35), exploitation of time differences will differentiate between uptake and expression. Therefore, the transformation system enables one to study the effect of PEA upon numerous processes involved in a common end.

Bacterial genetics has been investigated primarily by three different processes of genetic transfer: conjugation, transduction, and transformation. Transformation involves uptake and incorporation of an exogenous piece of DNA from a genetically different strain of bacteria into the genome of the cell. The characteristics coded for in this newly integrated piece of DNA are phenotypically expressed.

The process of transformation was first observed by Griffith in 1928 in a virulent strain of pneumococci (36). Avery, MacLeod, and

McCarty (37) later showed the transforming factor was DNA, thus laying the foundation for the recognition of the hereditary material as DNA.

In addition to pneumococcus, transformation has been found to take place in certain species of the genera: Streptococcus, Hemophilus, Neisseria, Bacillus, and Rhizobium. The majority of studies have been conducted with pneumococcus, Hemophilus influenzae, and B. subtilis. The existence of transformable B. subtilis was discovered by Spizizen (38) and this organism has been widely used in transformation studies because it may be grown on a defined medium and exists in the form of mutants requiring various amino acids.

For genetic transformation to occur, the cells must be in a poorly defined physiological state termed competence. The state of competence in B. subtilis cells persists for 3 or 4 hours (39, 50). Competence is usually associated with the initial phase of transformation because uptake of heterologous DNA (i.e. from a genetically unrelated species) or transfection with viral DNA (where no recombination is involved) is maximal when competence is maximum. On the other hand, competent and noncompetent cells are transduced with equal frequencies for genetic markers which require recombination. This indicates that both competent and noncompetent cells are able to carry on the process of integration and recombination but not that of the uptake of exogenous DNA (41). However, the process with which competence is involved cannot be specified but is under genetic control. Stable, noncompetent mutants, which have lost the ability to bind DNA at any stage of growth, have been isolated from competent strains (42, 43). The development of competence requires the chelation of cupric ions, the auxotrophic requirements, and magnesium. The amino acids tryptophan,

histidine, glycine, valine, arginine, methionine, threonine, lysine, and aspartic acid have been found to stimulate transformation (44).

Protein synthesis is required for the development of competence (39, 45), but inhibition of DNA synthesis by thymine starvation had no effect upon the development of competence in a thymine-requiring *B. subtilis* strain (46). This is in agreement with the findings by Bodmer (47) that competent cells showed very little DNA synthesis. Nester (39) suggested that competent cells were non-growing since the competent cells were more resistant to penicillin which preferentially kills growing cells (48).

The capacity for sporulation has been associated with competence in *B. subtilis* (43, 49), and both Spizizen *et al*. (50) and Young (51) suggest the involvement of the fore-spore membrane in the process of transformation. However, sporulation mutants of *B. subtilis* which are still transformable have been isolated (52).

Spizizen (53) has suggested that an altered cell wall structure resulting from unbalanced growth conditions was the chemical basis for competence in *B. subtilis*. Transformable strains of *B. subtilis* have a higher content of N-acetyl galactosamine associated with the teichoic acid of cell walls than do poorly transformable strains (54). The N-acetyl galactosamine content of the cell wall reached a maximum at the time of maximum competence (49). An increased substitution of galactosamine on the terminal phosphorous groups could decrease the net negative charge of the cell wall and facilitate penetration of DNA. Jensen and Haas (55) studied the absorption of methylene blue (a basic dye containing an auxochromophore cationic group) to *B. subtilis* cells and found that the point of minimal dye-binding correlated with the

point of optimal competence. Fractionation of cells on membrane filters and elution with media of varying ionic strength revealed that newly competent *B. subtilis* carried a maximal negative charge and underwent transformation within a range of surface-charge values (56). The mutation to actinomycin resistance in *B. subtilis* was usually correlated with the loss of the ability to develop competence (57) and probably was due to permeability changes (58). Investigations into the inhibition of transformation by periodate by Polsinelli and Barlati (59) revealed that the site of action was a component of the cell wall involved in the binding and/or in the penetration of donor DNA in *B. subtilis*.

Thomas (60) advanced the theory of partial protoplast formation during competence to account for the penetration of large macromolecules such as DNA through the mesh work of the cell wall. This theory was strengthened by the discovery of an autolytic N-acyl-muramyl-*L*-alanine amidase associated with cell walls of *B. subtilis* and displaying a higher activity in competent cells than in noncompetent cells (53, 61). Maximal activity of this autolytic enzyme in the growth cycle was found just prior to the onset of competence. Through the action of this autolytic enzyme, focal relaxation and gaps might be produced in the cell wall thereby facilitating the penetration of the DNA molecule.

Extracellular proteins which have the ability of inducing competence in noncompetent cells have been identified in several transforming systems: *Streptococcus* (62, 63), pneumococcus (64, 65), and *B. cereus* (66); however, studies with *H. influenzae* do not indicate the transfer of competence. Charpak and Dedoner (67) showed that addition

of phosphate-extracted acetone powders of the supernatant solution from competent B. subtilis strain 168 allowed transformation of B. subtilis var. niger. This competence factor was destroyed by trypsin (E. C. No. 3.4.4.4) or chymotrypsin (E. C. No. 3.4.4.8) treatment and heating for five minutes at 100°. Akrigg, Ayad, and Barker (68) separated a competence-inducing activity by DEAE-cellulose chromatography of aqueous extracts of competent cells. Extracts of noncompetent cells and cultures treated with chloramphenicol to prevent competence development showed a significant decrease in the peak containing the competence-inducing factor as determined by the absorbance at 260 mμ and 280 mμ.

The process of transformation has been divided into five steps (69):

(1) initial attachment of donor DNA to the competent recipient cell,

(2) entry of the DNA into the cell by means of a specific transport system,

(3) synapsis between the donor DNA and the recipient's genome,

(4) recombination between the donor DNA and the resident genome,

(5) expression of the newly introduced genetic information.

One of the processes that distinguishes a competent cell from a noncompetent one is the ability to take up exogenous DNA, and this uptake is proportional to the number of transformants obtained (43). Two steps are believed to be involved in DNA uptake--the first a reversible attachment of the DNA molecule to the cell and the second an irreversible transport of the DNA molecule to the interior of the cell. Studies with pneumococcus and Hemophilus first suggested the

reversible attachment. Lerman and Tolmach (70) demonstrated a reversible association between DNA (^{32}P labeled) and pneumococcal cells at low temperatures. This DNA could be removed by warming or treating with deoxyribonuclease (DNase). In Hemophilus the reversible and irreversible uptake of DNA was distinguished by ionic strength variations suggesting an ionic interaction between DNA and the cells (71). The reversible uptake was thought to precede the irreversible uptake of DNA (44, 45). When all the DNA was removed from solution, except that reversibly bound to the Hemophilus cells, only 3% of the usual number of transformants were obtained (72). Investigations into the kinetics of the uptake of DNA in B. subtilis by Levine and Strauss (73) indicated an immediate adsorption of DNA to the cells followed by the irreversible uptake of DNA. Young (personal communication), working with B. subtilis, is not able to demonstrate the existence of two steps in DNA uptake.

The irreversible uptake of DNA is probably the penetration of the DNA into the cell or the attachment of the DNA to the cell in such a way that it is insensitive to DNase. The requirement for energy has been shown by the inhibition of uptake by inhibitors of oxidative phosphorylation such as 2,4-dinitrophenol and cyanide (43). In B. subtilis an energy of activation of 13.4 kcal. was required for the uptake of DNA (73). The energy requirement, speed, and kinetics of DNA uptake indicate an active transport mechanism (72).

The process by which DNA penetrates the cell has been studied by following the effect of DNase treatment upon the appearance of transformants. A 2 to 4 minute lag period, presumably in DNA uptake, was noted as measured by the appearance of transformants in B. subtilis

exposed to DNA for varying time periods. This lag was suggested as the time required to synthesize an inducible permease. Further studies with *B. subtilis* demonstrated that this lag period was 2.5 seconds for any marker (73, 74, 75). A correlation between the map distance of two markers and the appearance of double transformants strongly suggested that the DNA molecule penetrated the cell in a lengthwise fashion. Conformation of longitudinal entry was made by Strauss (75), who showed a rapid increase of the co-transfer frequency of an unselected marker after the lag period. The rate of DNA uptake was found to be about 55 nucleotide pairs per second (75).

Shortly after the uptake of DNA, recombination of the donor DNA with the genome of the cell occurs. This is envisioned to occur first by synapsis, which involves the recognition of the complement of donor DNA by hydrogen bonding (76). Synapsis is followed by integration through which the recipient cell obtains the information contained in the piece of donor DNA. There are two possibilities whereby the cell could acquire the new heritable traits: (1) the material could be physically inserted into the resident genome (breakage and reunion) or (2) the message could be transmitted without physical transfer (copy choice) (77).

Integration and recombination in *B. subtilis* appear to occur by breakage and reunion because donor DNA has been found to be physically associated with the recipient's genome (78, 79). Also, actinomycin, which prevented the breakage of exogenous DNA, has been shown to prevent the formation of recombinate type DNA in *B. subtilis* protoplasts (80).

The process of recombination can occur during a time when DNA

synthesis is 0.15% that of the normal cell (47). Action of inhibitors indicates that recombination does not require protein synthesis or a supply of energy (80). Bodmer (47) has proposed a model in which donor DNA is integrated at the stationary replicating point of the competent cell which is in a state of suspended DNA synthesis. The integration is predominantly in the old strand rather than in the newly synthesized strand (47). The replicating point has been shown to be associated with the cell membrane and would be readily accessible for contact with donor DNA (81).

Although all information for B. subtilis indicates that integration and recombination occur by breakage and reunion, information on steps prior to integration and recombination does not agree as well. In pneumococcus, Lacks et al. (82) demonstrated that the donor DNA was converted into single strands and small fragments followed by the integration of the single strand into the recipient cell's genome. Pene and Romig (78), investigating the fate of transforming DNA in B. subtilis, were not able to detect any single stranded DNA in cells after uptake had occurred. Using radioactive labeling of DNA, they were able to show that the donor DNA was physically inserted into the cell's genome. Bodmer and Ganesan (70) also demonstrated that portions of the intact donor DNA were incorporated into the recipient genome; however, these data suggested that a single strand was inserted into the recipient cell's genome. Donor DNA, when introduced into competent B. subtilis cells, showed a transient loss of transforming activity shortly after uptake; transforming activity reappeared as a function of time of incubation (83). This finding indicated the formation of a single stranded intermediate. Data obtained by using DNA from

genetically different parents before and after melting and reannealing indicated that the transforming activity was not changed by reannealing, therefore, suggesting the formation of a single stranded intermediate (83). Elegant experiments by Chilton (84) were consistent with the single strand integration model demonstrating that either of the strands have equal probability of being integrated. Most of the data (50) are consistent with the integration of a single strand of donor DNA.

After uptake of donor DNA by the competent cell, "potential transformants" are formed. Studies with B. subtilis (79) indicated that in less than 30 minutes after DNA uptake, the donor DNA was incorporated into the recipient cell's genome and held there by bonds resistant to shearing or denaturation. However, there was a delay in the expression of markers. The enzyme tryptophan synthetase was not synthesized by newly transformed cells until 3 to 4 hours after the uptake of DNA even though linkage of the markers try_2 his_2 had occurred in 30 minutes (35). Kammen, Wojnar, and Cannelakis (40) have also shown with B. subtilis the existence of intracellular competition with homologous or heterologous DNA that had been previously taken up. Sequential introduction into the same cells of homologous, but genetically distinguishable, DNAs showed that at least 50 to 60% of the potential transformants had not irreversibly fixed the first type of DNA for 2.5 hours after its introduction. This finding contravenes previous assumptions that the linkage of donor DNA to the genome by phosphodiester bonds occurred during this time period. Another investigation (85) demonstrated that re-extracted DNA showing joint activity of donor and recipient markers had abnormal physical properties with

regard to heat sensitivity and renaturation indicating that despite the appearance of recombinant-type activity, integration was not complete. Thus, donor DNA is not as quickly inserted into the recipient cell's genome in B. subtilis as indicated by pycnographic fractionation.

Numerous investigations into the metabolic processes taking place in the potential transformants have been made. Potential transformants are neither multiplying nor synthesizing cell wall material (36, 86). McCarthy and Nester (87) studied the synthesis of various macromolecules in newly transformed B. subtilis cells by following the kinetics of suicide after exposure to tritiated precursors of the various macromolecules. There was a reduced rate of DNA synthesis in the potential transformants. However, they were actively synthesizing protein. Long-lived mRNA was not involved in this synthesis. This latter finding was further substantiated by an investigation which showed that the survival of potential transformants was dependent upon synthesis of a polypeptide material not mediated by mRNA (88). This polypeptide might function by stabilization of DNA after entry into the cell.

The metabolic state of the newly transformed B. subtilis cell is unique for approximately 3 hours after DNA uptake. Three to four hours after DNA uptake, transformants begin to multiply and carry on the normal metabolic processes. Since a competent culture is comprised chiefly of noncompetent cells whose activities may obscure those of the competent or newly transformed cells, the study of these cells is considerably more difficult and the findings less clear. Therefore, a procedure for the separation of competent or newly transformed cells would be advantageous. Singh and Pitale's (89) findings indicate the separation of newly transformed B. subtilis cells from bulk population

by sucrose gradient centrifugation. Refinement of this technique could add considerably to the understanding of bacterial transformation.

The present study was undertaken to determine the effect of PEA upon transformation in *B. subtilis*. This dissertation presents evidence that PEA inhibits transformation. The elucidation of the step or steps affected by PEA are covered in the first part of the dissertation. The second part is concerned with the effect of concentrations of PEA that inhibit transformation on various aspects of cellular metabolism.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

Bacto-agar, Bacto-yeast extract, Bacto-tryptose blood agar base, Bacto-brain-heart infusion, Bacto-tryptose, and Bacto-beef extract were obtained from Difco Laboratories. Deoxyribonuclease (E. C. No. 3.1.4.5) and lysozyme (E. C. No. 3.2.1.17) were Worthington Biochemical Corporation products. L-histidine, L-threonine, L-aspartic acid, glycine, L-proline, casein hydrolysate (acid), and desoxyribonucleic acid (from salmon sperm) were purchased from Nutritional Biochemicals Corporation. L-lysine and L-valine came from California Biochemical Research while L-tryptophan was from Mann Research Laboratories. Pronase (Grade B) and uracil were purchased from Calbiochem. Phenethyl alcohol and (ethylenediamine)tetraacetic acid were obtained from Eastman Organic Chemicals, and lithium aluminum hydride and (hydroxymethyl)-aminomethane from Fischer Scientific Company. Dodecyl sodium sulfate was purchased from Matheson, Coleman, and Bell while dimethyl sulfoxide was from Aldrich Chemical Company. Thymidine, tritiated thymidine (14.0 c/mmole), uracil-5-^{3}H (7.2 c/mmole), uracil-2-^{14}C (45 mc/mmole), and L-leucine-1-^{14}C (130 mc/mmole) were obtained from Schwarz Bioresearch Incorporated. L-proline-^{14}C (250 mc/mmole), L-serine-^{14}C (127 mc/mmole), and phenyl-acetic-1-^{14}C acid (8.4 mc/mmole) were purchased from New England Nuclear. Millipore filters (HA 0.45 µ) were from

Millipore Filter Corporation, and glass filters were from W. and R. Balston (Whatman GF/A). Gifts of B. subtilis, strains 168 C^- (ind^- $competent^-$) and SB 25 (ind_2^- his_2^-) were received from F. E. Young. Strains WT and FH 2006 (ind^-thy^-) were given by W. C. McDonald and I. C. Felkner respectively. The B. subtilis bacteriophage SP-10 was supplied by W. P. Romig.

Methods

Isolation of Donor DNA

B. subtilis, strain WT, was grown in minimal medium (38) supplemented with 0.1% yeast extract at 37° with forced aeration for 12 to 14 hours. The cells were harvested in a refrigerated Sharples Super Centrifuge, and donor DNA was prepared by the procedure of Saito and Miura (90). DNA concentrations were determined by absorbance measurements at 260 mμ.

Preparation of Tritiated DNA

B. subtilis, strain FH 2006 (ind^-thy^-), was grown overnight (16 hours) on a brain-heart infusion agar plate at 37°. Bacteria from this plate were used to inoculate warm minimal medium supplemented with 0.05% acid hydrolyzed casein and 50 μg/ml of thymidine and incubated with shaking at 37° until an absorbance at 630 mμ (A_{630}) of 0.64 (½ inch light path) was reached. The cells were finally suspended to an A_{630} of less than 0.22 in 50 ml of minimal medium plus 0.05% acid hydrolyzed casein, ^3H-thymidine (2 mc per 20 to 50 ml), and enough thymidine to give a final concentration of 10 μg/ml. The cells were grown with shaking at 37° for three generations. The resulting

suspension was centrifuged and washed twice with minimal medium. The cells were then taken up in 3 ml of 0.15 M NaCl, 0.1 M (ethylenediamino)tetraacetic acid (EDTA) (pH 8.0), and 12 mg of lysozyme (E. C. No. 3.2.1.17) was added and incubated with the cells for 10 to 20 minutes at 37°. The cells were then frozen in dry ice, thawed, and taken up in 7 ml of 0.1 M (hydroxymethyl)aminomethane, 1% dodecyl sodium sulfate, 0.1 M NaCl (pH 9.0). The suspension was frozen and thawed two more times and then incubated with 8 mg/ml of pronase at 37° overnight (at least 7 hours). An equal volume of cold phenol (saturated with 0.1 M (hydroxymethyl)aminomethane, 1% dodecyl sodium sulfate, 0.1 M NaCl) was added after pronase treatment and the resulting emulsion shaken in the cold (4°) for 20 minutes. The solution was then centrifuged for 10 minutes at 18,000 x g at 4°. The aqueous layer (top layer) was removed and dialyzed against two liters of 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0) for 48 hours with three changes. The DNA concentration was determined by the method of Burton (4). The tritiated DNA was precipitated by adding trichloroacetic acid to a final concentration of 5% and allowing the mixture to stand in the cold (4°) for 10 minutes. The precipitate was collected on Millipore Filters (HA 0.45μ), dried, and counted in a Packard Scintillation Spectrometer using Bray's scintillation fluid (91). The specific activity of the tritiated DNA was 50 μc/mg and 100% of the radioactivity was precipitated with 5% trichloroacetic acid.

Transformation Procedures

Two different transformation procedures were used which are designated A and B.

B. subtilis SB 25 (ind_2^- his_2^-) was grown on a tryptose blood base agar plate at room temperature. An inoculum obtained from this plate was added to tryptose blood base broth which was grown overnight (24 to 16 hours). These were grown with shaking at 37° as were the following subsequent cell preparations.

Procedure A was a modification of the procedure of Young and Spizizen (43). The overnight culture was diluted 30-fold into fresh tryptose blood base broth and grown for an additional 8 hours after which the culture was diluted 10-fold into fresh minimal medium supplemented with 10 μg/ml each of tryptophan and histidine plus 0.01% acid hydrolyzed casein. After 3 hours in the supplemented minimal medium, maximum competence was obtained.

Procedure B was a modification of the procedure of Bott and Wilson (42). The overnight culture was centrifuged and the cells resuspended in fresh minimal medium supplemented with 50 μg/ml each of histidine, tryptophan, valine, glycine, aspartic acid, threonine, methionine, lysine, and arginine plus enough magnesium sulfate to give a final concentration of 0.072%. The cells were adjusted to an absorbance of 0.1 at 630 mμ (½ inch light path) in this medium. The culture was incubated for 5 hours at which time maximum competence occurs.

At the time maximum competence occurred, in either procedure, DNA (5 μg/ml) was added and incubated with the cells for 30 to 45 minutes. Dilutions were made at room temperature in minimal medium. Double transformants bearing the linked ind_2^+ and his_2^+ markers (try^+ his^+) were scored on minimal agar; total his_2^+ or ind_2^+ transformants were scored on minimal agar supplemented with 10 μg/ml of tryptophan (his^+) or histidine(try^+), respectively. The total viable cells were scored on

minimal agar supplemented with 10 μg/ml of both tryptophan and histidine. Frequencies of transformation of 0.2% to 1.6% were obtained by both procedures. The concentrations (percent) of PEA added to the transformation system were calculated on a volume to volume basis.

Uptake of Tritiated DNA

Competent *B. subtilis* SB 25 cells were obtained by either procedure A or B. Tritiated DNA was added to 3 ml of the cell suspension and incubated with the cells for 45 minutes at 37° with shaking; 0.1 μg/ml of DNase was then added to the cell suspension and incubated for 10 minutes at room temperature. At this time aliquots of each suspension were diluted to determine the degree of transformation. The cells were collected by centrifugation and resuspended in minimal medium plus 50 μg/ml DNase. The cells were again collected by centrifugation and washed a third and final time with minimal medium. The cells were finally suspended in 1 ml of minimal medium and counted using a Packard Scintillation Spectrometer with Bray's scintillation fluid (91).

Storage of Competent Cells in Liquid Nitrogen

Competent cells were obtained as described by procedure B by centrifuging cells one hour before maximum competence (after 4 hours of growth) and concentrated 10-fold in minimal medium which was supplemented as described in procedure B. Dimethyl sulfoxide was added to a final concentration of 15%. The concentrated cell suspension was placed in ampules and then sealed and stored in liquid nitrogen until needed.

When cells were to be used, they were thawed at 37° and immediately diluted 10-fold in fresh, warm minimal medium supplemented as described in procedure B. The cells were then incubated at 37° with shaking for 1.5 hours at which time maximum competence occurred. DNA (5 μg/ml) was then added and incubated with the cells for 30 to 40 minutes after which dilutions and plating were carried out as described previously.

Synthesis of Phenethyl-1-^{14}C Alcohol

Phenethyl-1-^{14}C alcohol (^{14}C-PEA) was prepared by lithium aluminum hydride reduction of the methyl ester of phenylacetic-1-^{14}C acid. The methyl ester of phenylacetic-1-^{14}C acid was formed by the action of diazomethane upon the acid. Methyl phenylacetate (111 mg) dissolved in 1.3 ml of absolute ether was added dropwise to 22 mg of lithium aluminum hydride in 4.2 ml of absolute ether at room temperature. The resulting mixture was refluxed for 45 minutes and the excess lithium aluminum hydride hydrolyzed by addition of water. The ^{14}C-PEA was extracted with ether and the ether removed by evaporation. The ^{14}C-PEA was shown to be at least 99.5% radiochemically pure by gas chromatography and had a specific activity of 50 μc/mmole.

Uptake of ^{14}C-*L*-Leucine, ^{3}H-Thymidine, and ^{14}C-Uracil

B. Subtilis, strain SB 25 or FH 2006 (for thymidine uptake) from a tryptose blood agar plate was used to inoculate 5 ml of minimal medium supplemented with 50 μg/ml of histidine and tryptophan (plus 50 μg/ml of thymidine when strain FH 2006 was used) and grown at 37° with shaking overnight. Two ml of the overnight growth were diluted 10-fold

into fresh medium supplemented in the same way as the overnight growth. After 4 hours of growth, 4 ml of the culture were diluted 5-fold into fresh minimal medium with and without PEA (0.05%) containing 50 µg/ml of tryptophan and histidine plus the radioactive compound studied.

The incorporation of L-leucine-1-^{14}C, tritiated thymidine, and uracil-5-^{14}C into protein or nucleic acids was measured by precipitation with cold trichloroacetic acid (final concentration 5%). The precipitate was collected on glass filters (Whatman GF/A) after standing 5 to 10 minutes in the cold (4°). The filters were washed with 5 ml of cold trichloroacetic acid, dried, and counted in a Packard Scintillation Spectrometer using Bray's scintillation fluid (91).

Total Uptake and Incorporation of Radioactive Precursors

B. subtilis (SB 25 or FH 2006) from a tryptose blood agar plate was used to inoculate tryptose blood broth (or minimal medium plus 50 µg/ml of tryptophan, histidine, and thymidine for strain FH 2006). After overnight growth at 37° with shaking, the cells were collected by centrifugation and suspended in minimal medium supplemented with 50 µg/ml each of tryptophan and histidine (plus 50 µg/ml of thymidine for the FH 2006 strain) to an absorbance at 630 mµ of 0.1 (½ inch light path). The cells were grown at 37° with shaking for 5 hours and diluted 5-fold into minimal medium with and without PEA supplemented with 50 µg/ml each of tryptophan and histidine plus the precursor (at a saturating concentration). After growth for 15 minutes, the radioactive precursor was added to the cells. Total uptake was measured by collecting 0.5 ml of the cell suspension with a syringe and washing

with 0.5 ml of cold minimal medium on a Millipore Filter (HA 0.45 μ). The filters were then further washed with 5 ml of cold minimal medium. The incorporation of the radioactive compound into macromolecules was measured by adding 0.5 ml of the cell suspension and 0.5 ml of minimal medium to 1 ml of cold 10% trichloroacetic acid. The mixture was allowed to stand in the cold (4°) for 10 minutes and then filtered on a Millipore Filter (HA 0.45 μ) washing with 5 ml of cold 5% trichloroacetic acid. The filters were dried and counted as described previously. The accumulation was calculated by subtracting the radioactivity incorporated from the total radioactivity taken up by the cells.

Sucrose Density Gradient Centrifugation

About 10 ml of a linear sucrose gradient (8% w/v to 30% w/v sucrose supplemented with minimal medium plus 10 μg/ml each of tryptophan and histidine) was layered into a 1.5 cm x 9.5 cm cellulose nitrate centrifuge tube by means of a gradient apparatus (92). Competent SB 25 cells were obtained and collected by Millipore (HA 0.45 μ) filtration and concentrated 5-fold in 8% w/v sucrose. Approximately 5×10^{8} cells in 0.1 ml of the concentrated cell suspension were layered carefully on the gradient and centrifuged at 4,000 rpm for 5 minutes in a Sorvall Refrigerated Centrifuge (RC-2) using a swinging bucket head (HB-4). Two or three drop fractions were collected with the refractive index of the odd-numbered fractions being measured. The even-numbered fractions were then incubated for 30 minutes at 37° with shaking with 5 μg/ml DNA. The dilution and plating were conducted as described previously.

CHAPTER III

SUCROSE DENSITY GRADIENT CENTRIFUGATION OF COMPETENT *B. subtilis* CELLS

Singh and Pitale (89) separated *B. subtilis* potential transformants from the bulk population by sucrose density gradient centrifugation. The cell population after incubation with DNA formed two distinct bands--one containing the majority of the cells and a lighter band of transformants. This experimental approach was used to study the separation of competent cells from the bulk population.

Effect of Various Media Supplemented with 15% Sucrose upon Viability and Transformability

To determine the sucrose gradient composition which had no effect upon the viability or transformability of *B. subtilis*, five different media consisting primarily of sucrose (15%) with different supplements were tested (Table I). The viability and transformability obtained in minimal medium supplemented with 10 µg/ml each of tryptophan and histidine (line 6) were the controls. Both viability and transformability decreased when the cells were suspended in the 15% sucrose solution (line 1). Medium containing sucrose (15%) supplemented with NaCl and socium citrate (line 2) also caused a decrease in viability and transformability. When sucrose was supplemented with minimal medium (line 3), a decrease in cell titer was observed (due to the

TABLE I

EFFECT OF VARIOUS MEDIA ON CELL VIABILITY AND TRANSFORMABILITY

Suspending Medium	Experiment A: Minutes of Incubation, Colonies per ml (10^6) — 0	Experiment A: Minutes of Incubation, Colonies per ml (10^6) — 60	Experiment B: Try^+ Transformants per ml (10^4)
1. 15% Sucrose	58	30	39
2. 15% Sucrose + 0.1 M NaCl + 0.05 M NaCitrate	64	52	159
3. 15% Sucrose + Minimal Medium	70	52	440
4. 15% Sucrose + Minimal Medium (- glucose - NaCitrate + try + his)	70	99	435
5. 15% Sucrose + Minimal Medium (+ try + his)	78	109	540
6. Minimal Medium (+ try + his)	71	102	550

The composition of minimal medium is described in the Experimental section. The concentration of the amino acids tryptophan and histidine was 10 µg/ml.

Experiment A. Competent cells obtained by procedure A were concentrated 5-fold in 15% sucrose (w/v) by centrifugation and then diluted 5-fold into the various media. Samples were taken at the times indicated. The 60 minute incubation was at room temperature without shaking. The number of viable cells was determined by plating on nutrient agar.

Experiment B. Competent cells were obtained by procedure A. The cells were collected by centrifugation and suspended in the various media. DNA (5 µg/ml) was added and incubated with the cells for 30 minutes at 37° with shaking after which dilution and plating were conducted.

absence of the required amino acids, tryptophan and histidine), but the transformability was only slightly decreased. Sucrose (15%) supplemented with minimal medium which did not contain glucose or sodium citrate but contained 10 μg/ml of both tryptophan and histidine (line 4) decreased the transformability slightly and had little effect upon viability or growth. When sucrose (15%) was supplemented with minimal medium (plus 10 μg/ml of tryptophan and histidine), as is shown in line 5, there was no effect upon the number of cells present or transformability after incubation for 60 minutes. Therefore, the sucrose gradients used for the centrifugation of competent *B. subtilis* were supplemented with minimal medium plus 10 μg/ml of tryptophan and histidine.

Sucrose Density Gradient Centrifugation of Competent Cells

Linear sucrose gradients of 8% to 30% were used in centrifuging maximally competent *B. subtilis*. Figures 1 and 2 demonstrate the distribution of competent cells and total cells throughout the centrifuged gradient. The competent cells were detected by adding transforming DNA and determining the number of transformants in the various fractions. The fractions, collected where the bulk of the cells banded, were diluted before addition of DNA in order that the cell concentration in the fractions would be approximately the same, thus eliminating any variation in the frequency of transformation due to cell concentration. The refractive index of the various fractions demonstrates that the gradient was linear (Figure 1). Figure 1 also shows that the bulk population bands in a single peak. The frequency

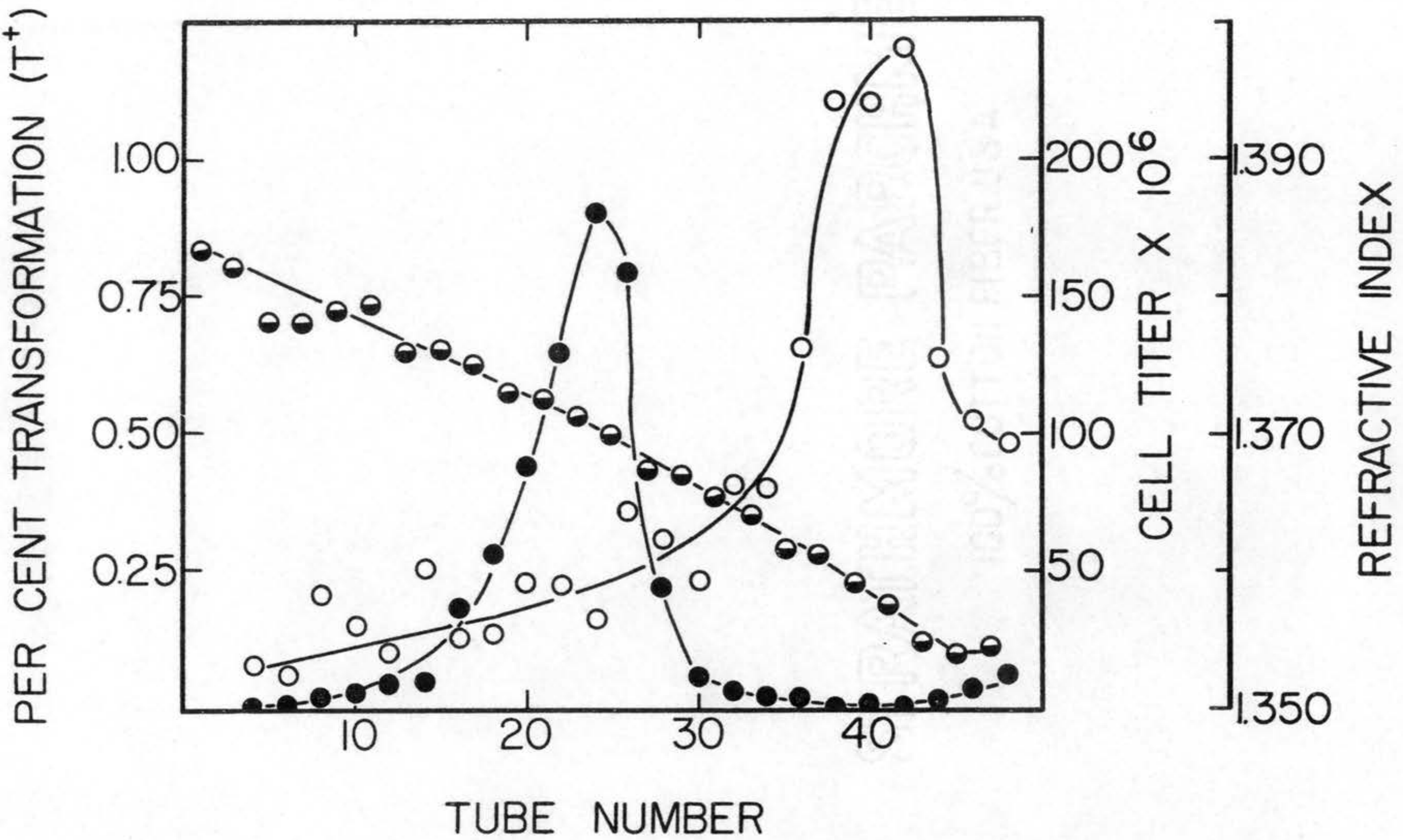
Figure 1. Sucrose Density Gradient Centrifugation of Competent _B_. _subtilis_ Cells I

The procedure given in Chapter II was followed in determining the number of try^+ transformants and total number of cells of the even numbered fractions and the refractive index of the odd numbered fractions.

◐ Refractive Index

● Cell Titer

0 Percent Transformation (try^+)



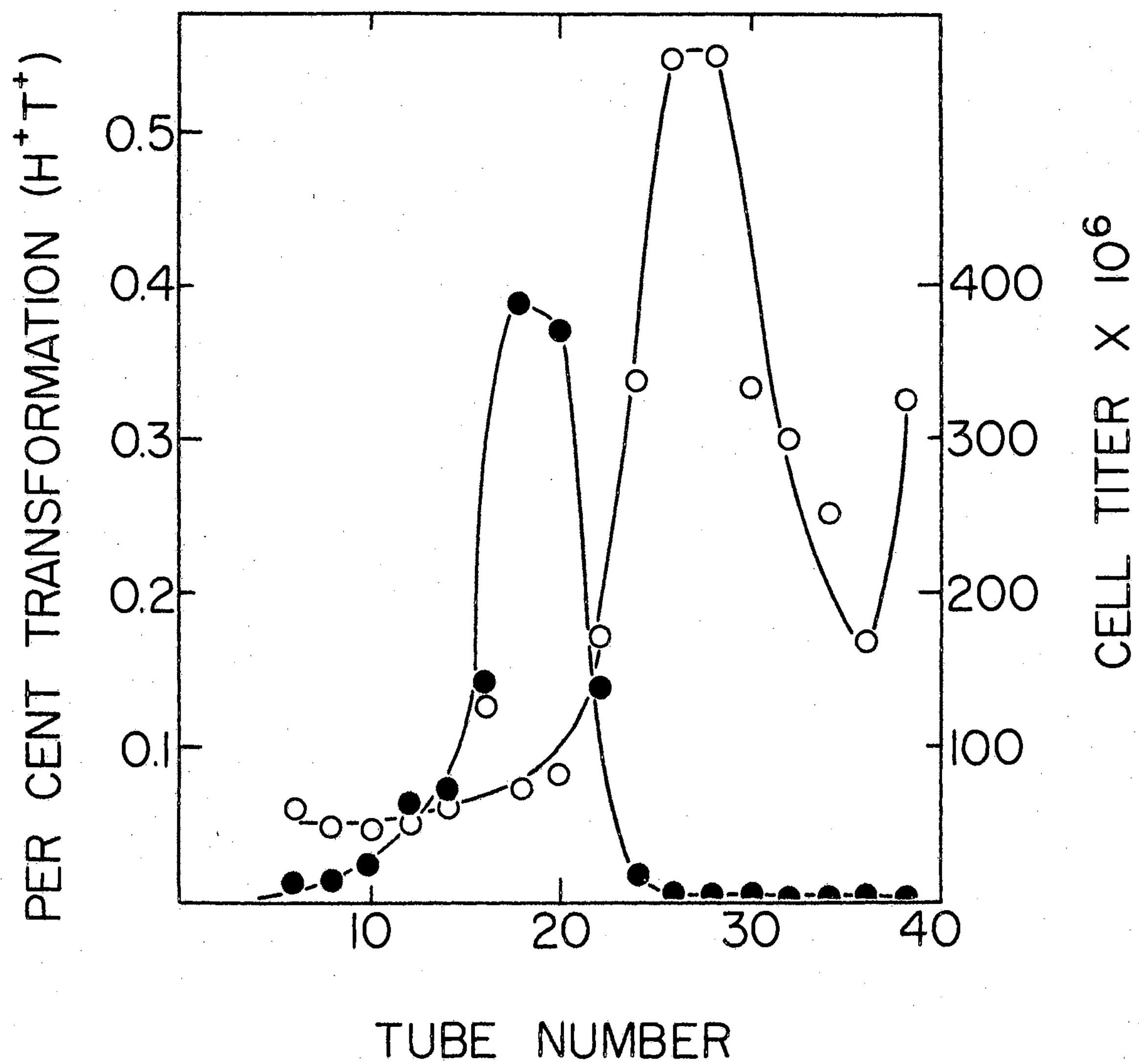
PER CENT TRANSFORMATION (T+)
1.00
0.75
0.50
0.25
TUBE NUMBER
10
20
30
40
CELL TITER X 10^6
200
150
100
50
REFRACTIVE INDEX
1.390
1.370
1.350

Figure 2. Sucrose Density Gradient Centrifugation of Competent *B. subtilis* Cells II

The procedure given in Chapter II was followed determining the number of try^+ his^+ transformants and total number of cells of the various fractions.

O Percent Transformation (try^+ his^+)

● Cell Titer



PER CENT TRANSFORMATION (H^+T^+)
0.5
0.4
0.3
0.2
0.1
CELL TITER X 10^6
400
300
200
100
10
20
30
40
TUBE NUMBER

of transformation of the tryptophan marker also forms a single peak which was less dense than that of the cell population. The frequency of transformation at the peak is about 1.2%. A fraction of the competent cells, which was not concentrated or centrifuged, gave a transformation frequency for the tryptophan marker of 0.31%. Competent cells which were filtered and concentrated gave a transformation frequency for the tryptophan marker of 0.1%. Thus, there is a 4- to 10-fold increase in the frequency of transformation.

Figure 2 shows this is not limited to the tryptophan marker but also occurs with the linked markers of tryptophan and histidine. The frequency of transformation obtained at the peak was 0.55%. Competent cells treated normally gave a transformation frequency for the linked histidine and tryptophan markers of 0.17% while cells that were filtered and concentrated gave a frequency of transformation of 0.08%. With the linked tryptophan and histidine markers, a 3- to 7-fold increase in the frequency of transformation was obtained.

Thus, sucrose density gradient centrifugation of competent cells results in the concentration of competent cells having a higher frequency of transformation than those obtained in parallel control experiments. The competent cells banded at a position on the gradient higher than the bulk of the cells demonstrating that competent cells sediment at a slower rate and consequently are less dense than the bulk population.

Recovery of Cells (Competent and Noncompetent) from Sucrose Gradients

Although Table I demonstrated that the medium used in the

gradients had no effect upon cell viability, the possibility that competent or noncompetent cells were being selectively killed or lost on the gradient during the process of centrifugation has not been eliminated. The sum of the competent cells and total number of cells obtained in each of the fractions of the gradient was determined and compared to the input (Table II). There was no detectable loss of either competent or noncompetent cells after centrifugation.

These findings indicate that there is a physical difference between competent cells and the normal bulk of cells which allows a partial separation of competent cells with a 3- to 4-fold increase in the transformation frequency.

TABLE II

RECOVERY OF CELLS FROM GRADIENT

Experiment Number	Total No. Input	(10^7) Cells Recovery	No. Competent Input	(10^4) Cells Recovery
1	63	63	12	12
2	17	15	30	35
3	80	80	18	16
Ave.	53	53	20	20

Three independent sucrose gradient (8 - 30% w/v) centrifugations were run determining the number of competent and noncompetent cells placed on the gradient (input) and recovered from the gradient (recovery). The number of competent cells was determined by transformation. The number of competent and noncompetent cells recovered from the gradient after centrifugation, was calculated by determining the number of competent and noncompetent cells obtained in each fraction, and summing all the fractions together.

CHAPTER IV

THE EFFECT OF PEA UPON *B.* *SUBTILIS* TRANSFORMATION

Effect of PEA upon Growth Rate

Since different effects of various concentrations of PEA on the growth of various bacteria have been observed (1, 2), the effect of various concentrations of PEA upon the growth of *B. subtilis* was determined. The result of a typical experiment measuring the growth by absorbance at 630 mμ is shown in Figure 3. Essentially complete inhibition of growth was obtained at concentrations of PEA of 0.4%, while 0.2% PEA produced a slight inhibition of the growth rate. PEA at concentrations below 0.1% had no significant effect upon the growth rate.

Effect of PEA upon Viability

The effect of PEA upon the viability of *B. subtilis* was studied by exposing competent cells to varying PEA concentrations for 60 minutes followed by dilution and plating to determine the number of viable cells. PEA treatment at concentrations greater than 0.15% (Figure 4) caused significant killing of cells. Concentrations below 0.1% had no noticeable effect upon viability.

Figure 3. Growth of *B. subtilis* in the Presence of PEA

B. subtilis 168 was grown overnight in minimal medium supplemented with 50 μg/ml of tryptophan and then diluted in fresh medium to a final A_{630} of 0.1. The indicated concentrations of PEA were added, and growth was followed by measuring the A_{630} at the indicated time intervals.

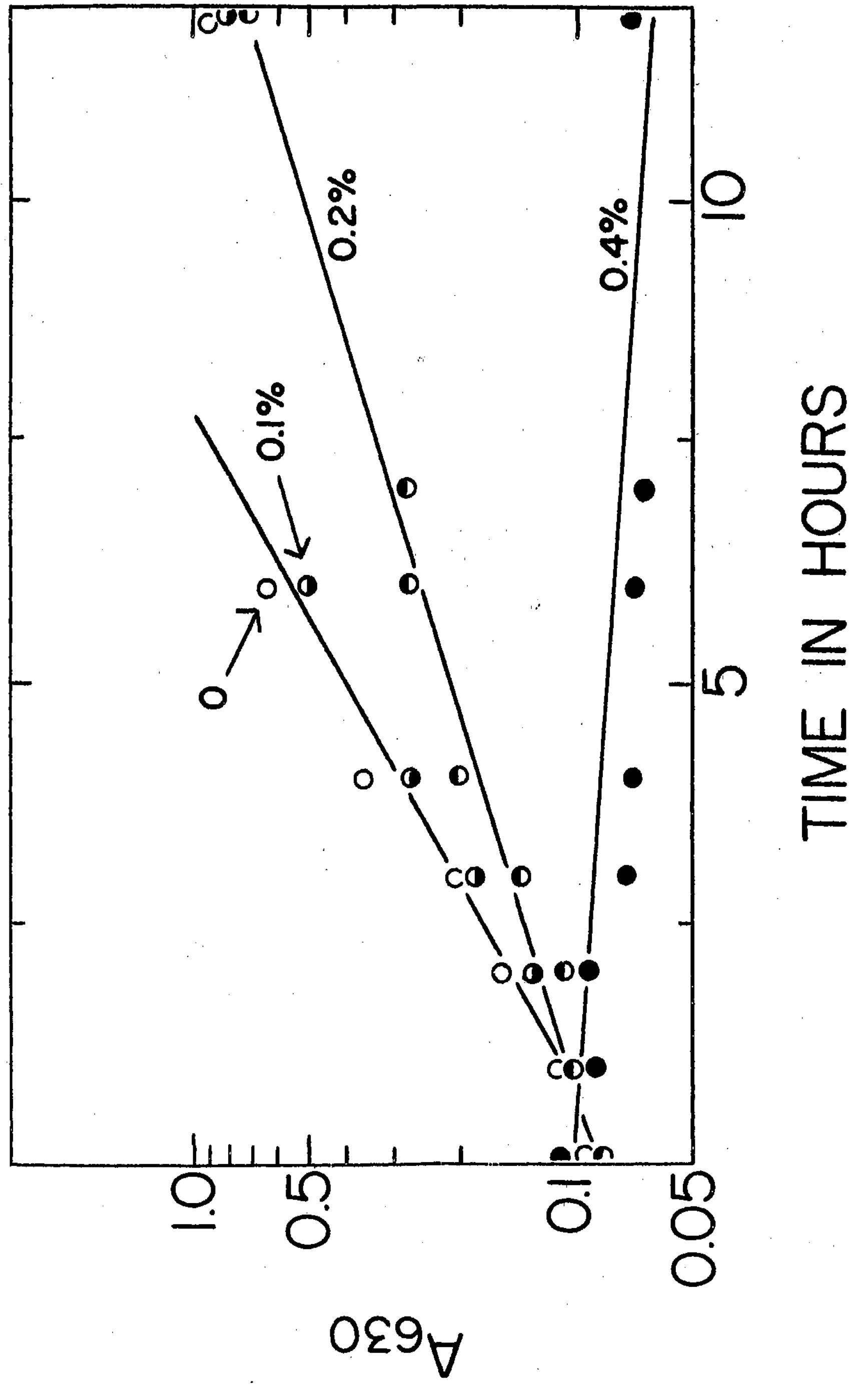
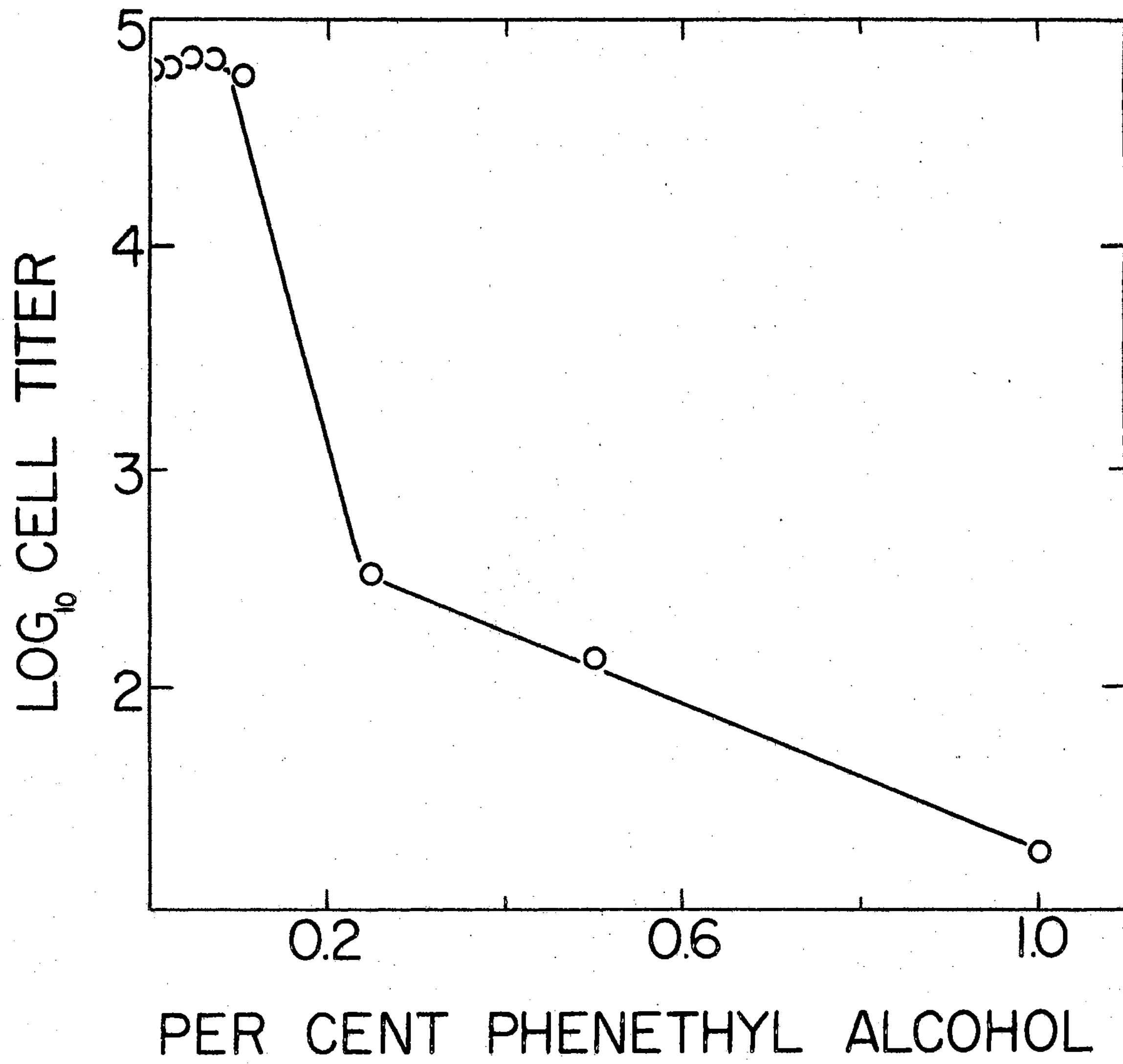

A630
1.0
0.5
0.1
0.05
TIME IN HOURS
5
10
0.1%
0.2%
0.4%

Figure 4. Effect of PEA upon Viability of *B. subtilis*

Competent *B. subtilis* 168 were obtained by procedure A (in Materials and Methods) and exposed to the various concentrations of PEA for 60 minutes. Samples were then diluted and the number of viable cells determined by plating on nutrient agar.


5
4
3
2
LOG_{10} CELL TITER
0.2
0.6
1.0
PER CENT PHENETHYL ALCOHOL

Effect of 10 Hour Exposure to 0.05% PEA upon Growth

A concentration of 0.05% PEA was selected for further experiments. The effect of inclusion of this concentration of PEA with growing *B. subtilis* for times up to 10 hours was determined. Two cultures of *B. subtilis* were treated identically except that 0.05% PEA was added to one of them. Figure 5 demonstrates that 0.05% PEA had no significant effect upon the growth or viability of *B. subtilis*.

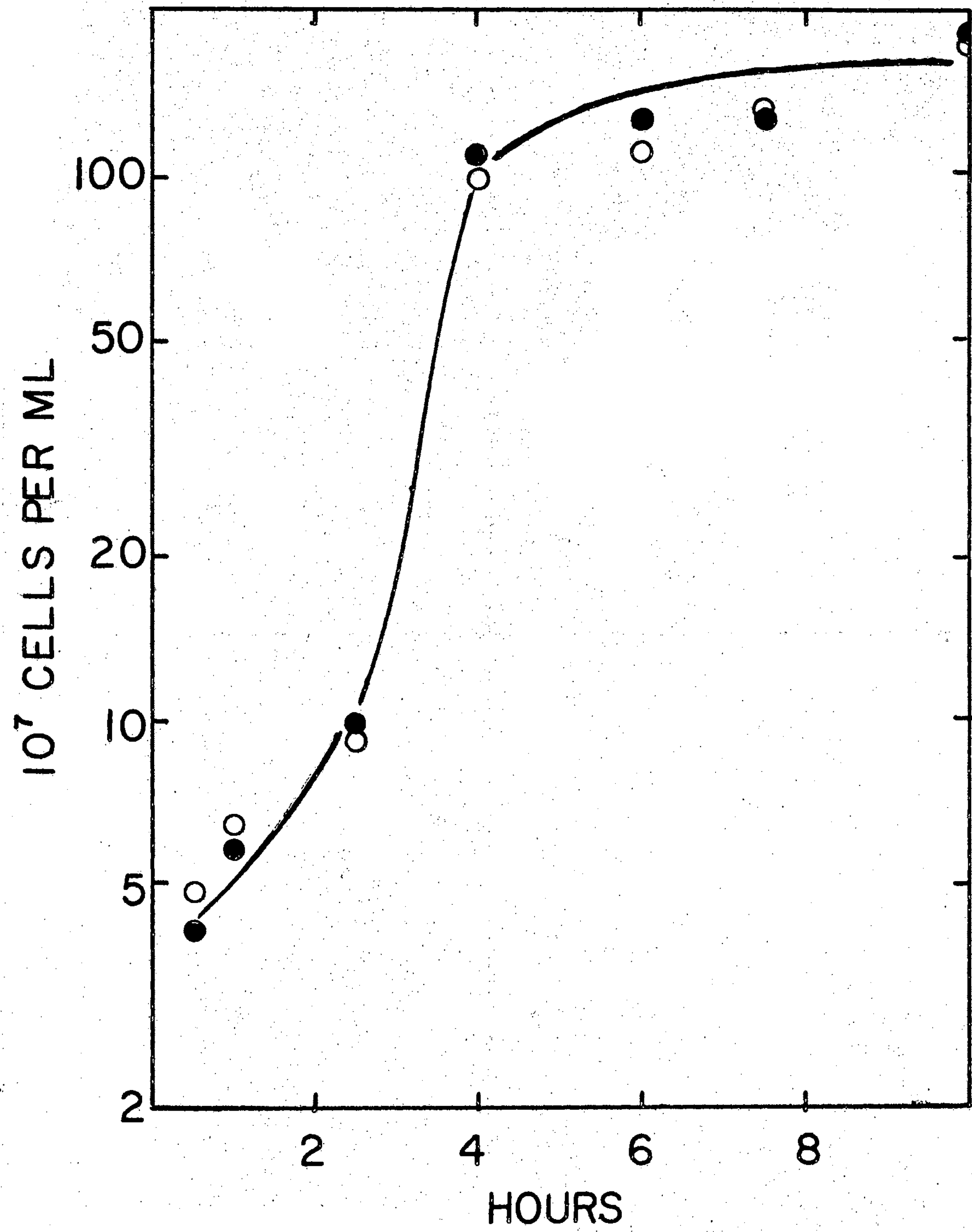
Effect of PEA on the *B. subtilis* Transforming System

The previous findings indicated that PEA at a concentration of 0.05% had no effect upon growth of *B. subtilis*. The effect of this PEA concentration upon the *B. subtilis* transformation system was determined by adding PEA to competent cells concomitantly with the DNA. Table III shows the results of a representative experiment demonstrating (line 4) that PEA caused a six- to seven-fold decrease in the frequency (percent) of transformation. PEA added after incubation of competent cells and DNA, but prior to dilution, failed to inhibit transformation (line 5). Controls for reversion or mutagenesis caused by PEA are shown in lines 1 and 2 respectively. Similar results were obtained using the histidine marker. With 0.05% PEA there was no decrease in the recipient cell population during the experiment (also see Figure 5).

The inhibition of transformation by PEA could be due to the action of PEA upon transforming DNA or competent cells since these are the components of the transformation system. This inhibition of transformation was further explored in order to determine the site of action

Figure 5. Effect of 0.05% PEA upon Cell Viability and Growth

B. subtilis SB 25 cells were grown overnight in tryptose blood base broth and used to inoculate fresh minimal medium supplemented with tryptophan and histidine (10 µg/ml each and 0.01% acid hydrolyzed casein to an A_{630} of 0.1). To one sample 0.05% PEA was added (●). Samples were taken at the indicated times, diluted, and plated on nutrient agar plates to determine the number of viable cells.



10^7 CELLS PER ML
100
50
20
10
5
2
2
4
6
8
HOURS

of PEA.

TABLE III

EFFECT OF PEA UPON TRANSFORMATION

Procedure	Percent Transformation (Try$^+$)
1. No DNA	0
2. No DNA + PEA	0
3. DNA	0.94
4. DNA + PEA	0.13
5. DNA then PEA	0.91

Competent *B. subtilis* SB 25 cells, obtained by procedure A, were exposed to 5 μg/ml of DNA for 30 minutes. PEA (0.05%) was added simultaneously with the DNA when indicated, except in line 5 where the PEA was added after the 30 minute transformation period, but prior to dilution and plating. The percent transformation is the number of tryptophan$^+$ cells/ total number of recipient cells, the whole term multiplied by 100.

Effect of PEA on Transforming DNA

Berrah and Konetzka (2) postulated that PEA selectively inhibited DNA synthesis. The possibility of a functional modification of DNA

structure was investigated by determining the effect of PEA treatment upon the transforming activity of DNA. Increased sensitivity is obtained by using biological measurements rather than physiochemical measurements (see Chapter IV). DNA was treated with 0.5% PEA for 60 minutes, and then the DNA recovered by ethanol precipitation. Figure 6 shows the dose response curve for PEA treated and untreated DNA. There was no apparent difference in the ability of PEA treated and untreated DNA to transform either in regions where transformation was proportional to DNA concentration or at saturating concentrations. Thus, PEA treatment of DNA did not irreversibly modify tryptophan transforming activity. However, the above experiment does not rule out an alcohol reversible interaction between DNA and PEA. The experiments described below used radioactive PEA to search for a DNA-PEA complex. ^{14}C-PEA was synthesized (as described in Materials and Methods), and the binding of PEA to DNA was sought as a coincidence of radioactivity (PEA) and A_{260} (DNA) peaks using molecular sieve chromatography, methylated albumin-coated kieselguhr (MAK) chromatography, or CsCl density gradient centrifugation.

Molecular Sieve Chromatography of ^{14}C-PEA DNA Mixture

Molecular sieve chromatography on Sephadex G 25 was used to separate DNA from PEA. The large molecules of B. subtilis DNA were eluted rapidly from the column since they could not enter the Sephadex particles. The small molecules of PEA eluted later. Figure 7 shows a separation of a sample of DNA which had been incubated with PEA. Only background radioactivity eluted with the DNA indicating no binding

Figure 6. Effect of PEA upon Transforming Activity of DNA

One sample of DNA, ●, was treated for one hour with 0.5% PEA at room temperature, precipitated with 95% ethanol, washed twice, and dissolved in 0.15 M sodium chloride-0.015 M sodium citrate. Another sample of DNA, 0, received equivalent treatment except PEA was omitted. DNA concentration was determined by the method of Burton (4). Recovery of DNA was 95% for PEA treated DNA and 88% for nontreated DNA. The effect of several concentrations of the two DNA preparations on transformation was determined using competent *B. subtilis* SB 25 cells obtained by procedure B.

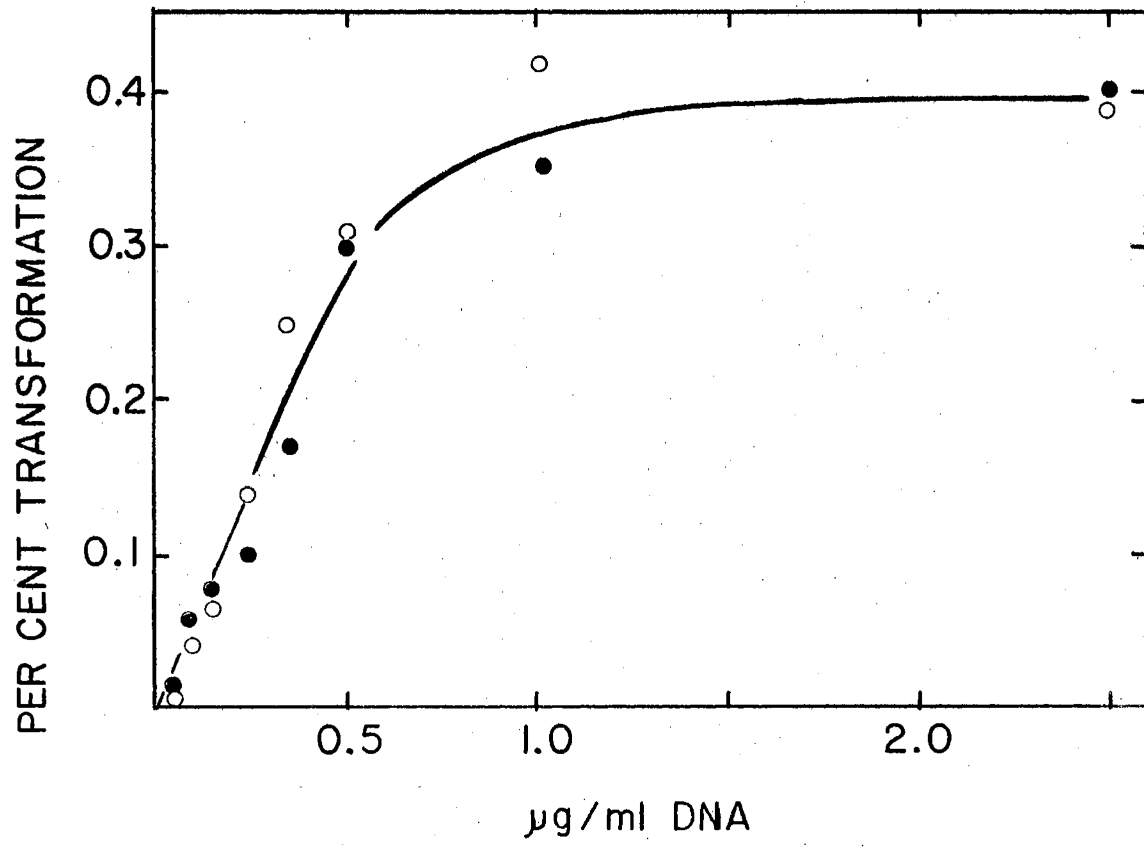
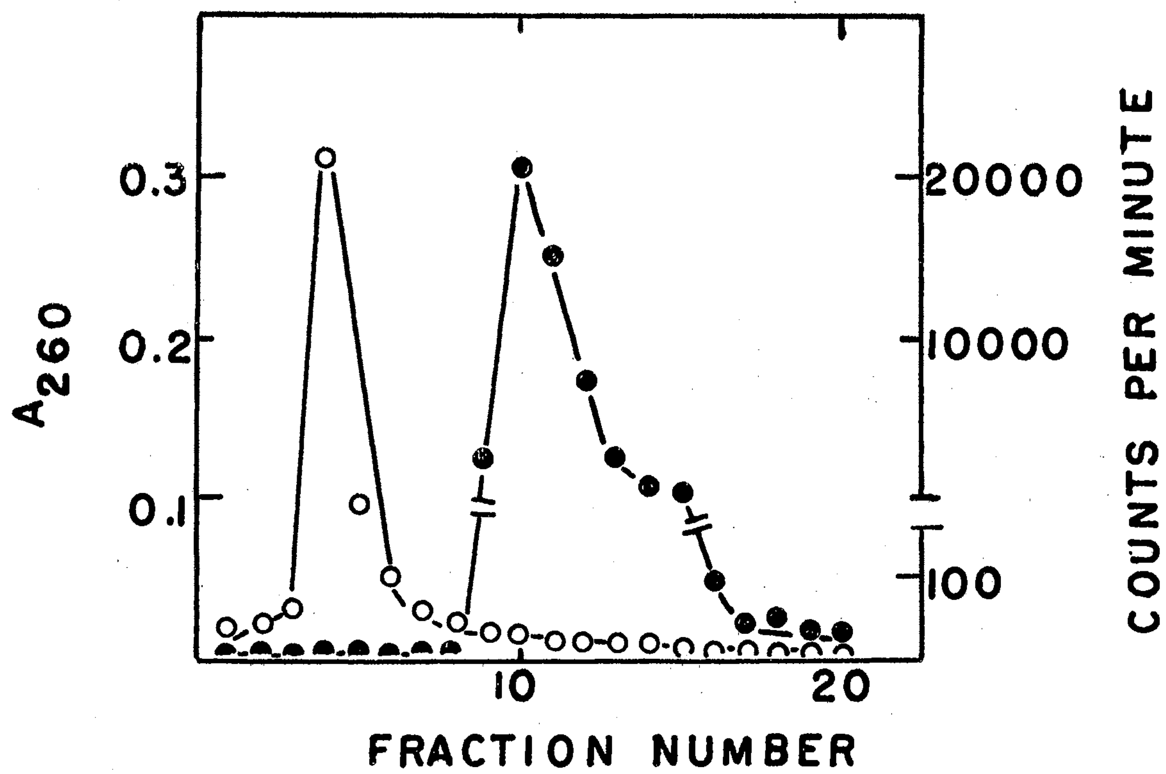

0.4
0.3
0.2
0.1
PER CENT TRANSFORMATION
0.5
1.0
2.0
μg/ml DNA

Figure 7. Sephadex Chromatography of PEA Treated DNA

A sample of DNA (160 μg) was incubated for 60 minutes at 37° with ^{14}C-PEA (250,000 CPM) in 1 ml volume and then chromatographed on a Sephadex G-25 column (20 mm x 150 mm). Elution was with 0.015 M NaCl, 0.00015 M sodium citrate, and 5 ml fractions were collected. The A_{260} (0) and the radioactivity (●) were determined for 1 ml of each fraction. Note the break in the counts per minute scale.



0.3
0.2
0.1
A_{260}
20000
10000
100
COUNTS PER MINUTE
10
20
FRACTION NUMBER

to DNA.

MAK Chromatography of ^{14}C-PEA DNA Mixture

Figure 8 displays the results of chromatographing on a MAK column a mixture of DNA and PEA which had been incubated together for one hour. The acidic DNA was retained by the basic protein-coated support and was eluted with buffer of increasing ionic strength. The radioactive PEA eluted first with the washing solvent. A small amount of the DNA which was not retained by the MAK column was eluted next. The delay in elution was presumably due to the larger molecular size of DNA. When the ionic strength was increased, the major peak of DNA eluted. There was no coincidence of the radioactive and A_{260} peaks.

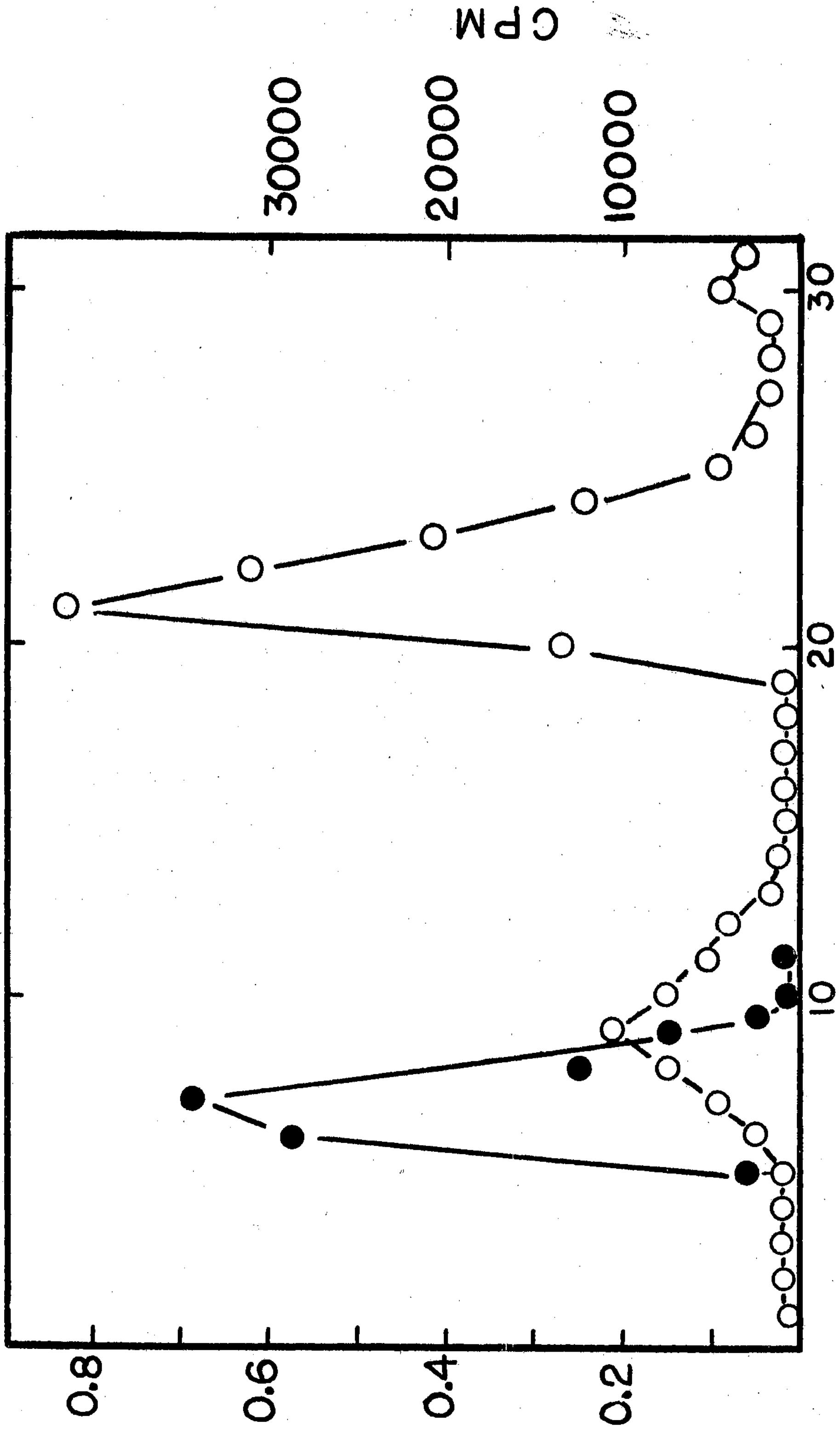
CsCl Centrifugation of ^{14}C-PEA DNA Mixture

Figure 9 reveals the results of CsCl density gradient centrifugation of an incubated mixture of DNA and PEA. The DNA peak was close to the bottom of the tube, and little radioactivity was found at this point. The remaining radioactivity due to PEA was distributed throughout the remainder of the gradient increasing as the density decreased.

Thus, three relatively mild methods for separation have been applied and good separations of incubated mixtures of PEA and DNA have been obtained. In no case was there coincidence of peaks of radioactive PEA and DNA. Evidence for the interaction of PEA and DNA has not been obtained by these experimental approaches.

Figure 8. MAK Chromatography of PEA Treated DNA

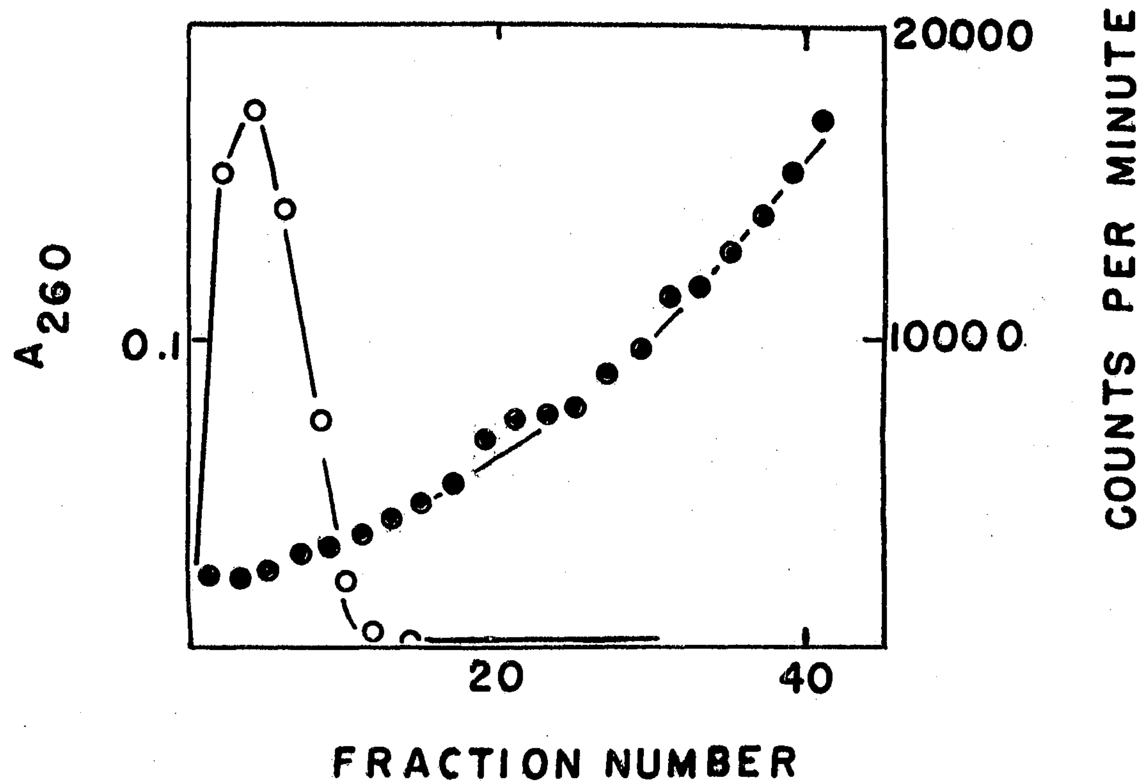
A 250 μg sample of DNA was incubated with ^{14}C-PEA (450,000 CPM) in a volume of 1 ml for 60 minutes at 37° then chromatographed on a MAK column as described by Kano-Sueoka and Sueoka (93). The column was washed with 75 ml of 0.4 M NaCl, 0.05 M sodium phosphate buffer pH 6.7 after application of the DNA. The column was then eluted with 50 ml of 0.8 M NaCl, 0.05 M sodium phosphate buffer pH 6.7 and then 30 ml of 2 M NaCl, 0.05 M sodium phosphate buffer pH 6.7. Five ml fractions were collected and analyzed for A_{260}, O, and radioactivity, ●.



CPM
30000
20000
10000
0.8
0.6
0.4
0.2
A 260
10
20
30
FRACTION NUMBER

Figure 9. Cesium Chloride Density Gradient Centrifugation of PEA Treated DNA

A sample of 500 µg of DNA was incubated for 60 minutes at 37° with ^{14}C-PEA (320,000 CPM). One ml was layered on top of 3 ml of 60% cesium chloride and centrifuged for 40 hours at 35,000 rpm in the SW 39 rotor of the Spinco L 2 centrifuge. Two drop fractions were collected and alternate fractions were examined for A_{260} (0) after dilution to 3 ml and radioactivity (●) by counting in a Packard scintillation counter using Bray's scintillation fluid (91).



A260
0.1
20000
10000
COUNTS PER MINUTE
20
40
FRACTION NUMBER

Effect of PEA upon the Thermal Denaturation of DNA

Changes (variation) in the structure of DNA molecules can be detected by differences in the thermal transition curves; therefore, the effect of PEA treatment on the thermal denaturation of DNA was determined. Figure 10 shows that the thermal denaturation curves for DNA in the presence and absence of PEA (0.1%) were identical; thus, there is no change in DNA structure detectable by this measurement. This finding is in agreement with previous reports (16, 17). PEA apparently has no physical effect upon isolated *B. subtilis* DNA.

Effect of Various PEA Concentrations upon Transformation

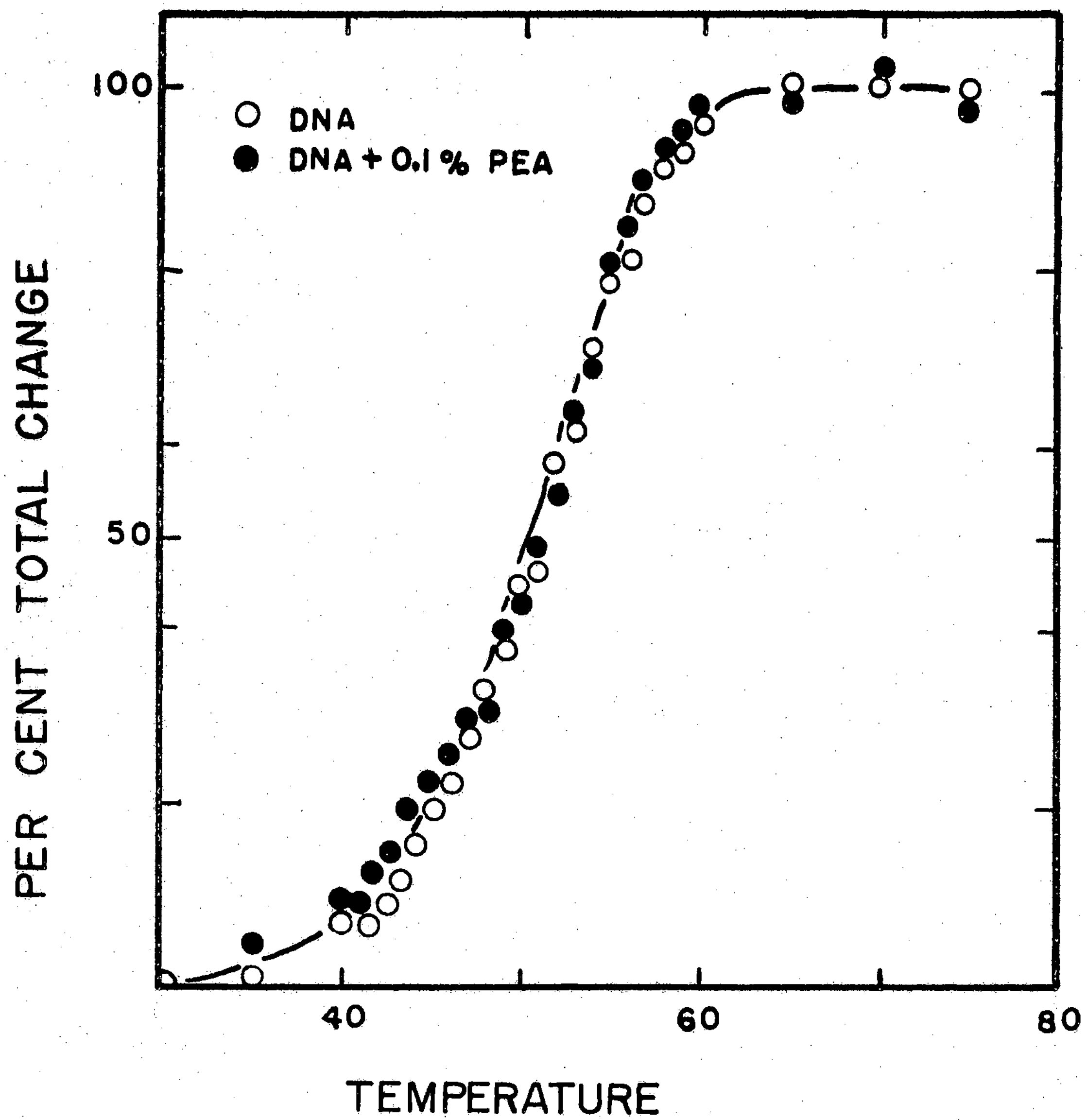
Figure 11 shows the effect of concentrations of PEA up to 0.15% upon transformation. Concentrations of PEA below 0.01% did not inhibit transformation while concentrations between 0.02% and 0.05% produced a linear inhibition of transformation. Concentrations of PEA above 0.15% killed the recipient population as well as inhibiting transformation.

Effect of the Time of PEA Addition

In the previous experiments, PEA was added to the transformation system simultaneously with DNA. Figure 12 shows the effect of adding PEA (0.05%) at various times relative to the addition of DNA. When PEA was added 15 to 20 minutes after addition of DNA, no inhibition was observed. The inhibition was maximal when PEA was incubated for at least an hour with the cells prior to the addition of DNA. In none

Figure 10. Effect of PEA on the Tm of DNA

The blank sample contained 0.1% PEA (v/v) to correct for any change in the extinction coefficient of PEA with temperature. The melting profiles of *B. subtilis* WT DNA, 0, and DNA in the presence of 0.1% PEA, ●, were determined using a Gilford Model 2000 system. The solvent used was composed of 50% absolute methanol and 50% 0.015 *M* NaCl 0.00015 *M* sodium citrate.



100
50
DNA
DNA + 0.1 % PEA
PER CENT TOTAL CHANGE
40
60
80
TEMPERATURE

Figure 11. Effect of PEA Concentrations upon Transformation

PEA at the indicated concentrations was added concomitantly with DNA (5 μg/ml) to B. subtilis SB 25 cells obtained by transformation procedure A. The DNA and PEA were incubated with the competent cells for 30 minutes at 37° with shaking before dilution and plating. The percent of tryptophan^{+} transformants is shown.

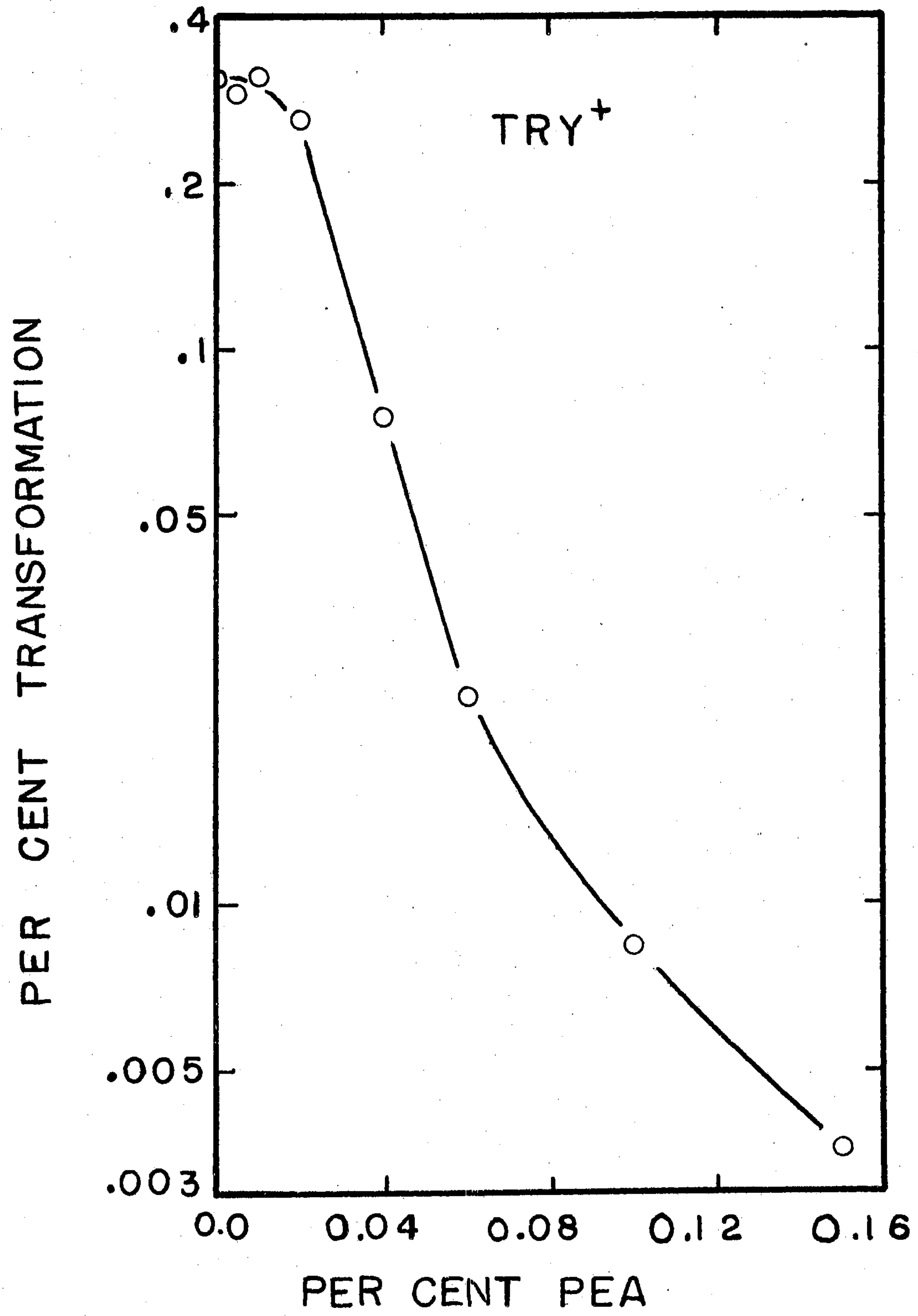
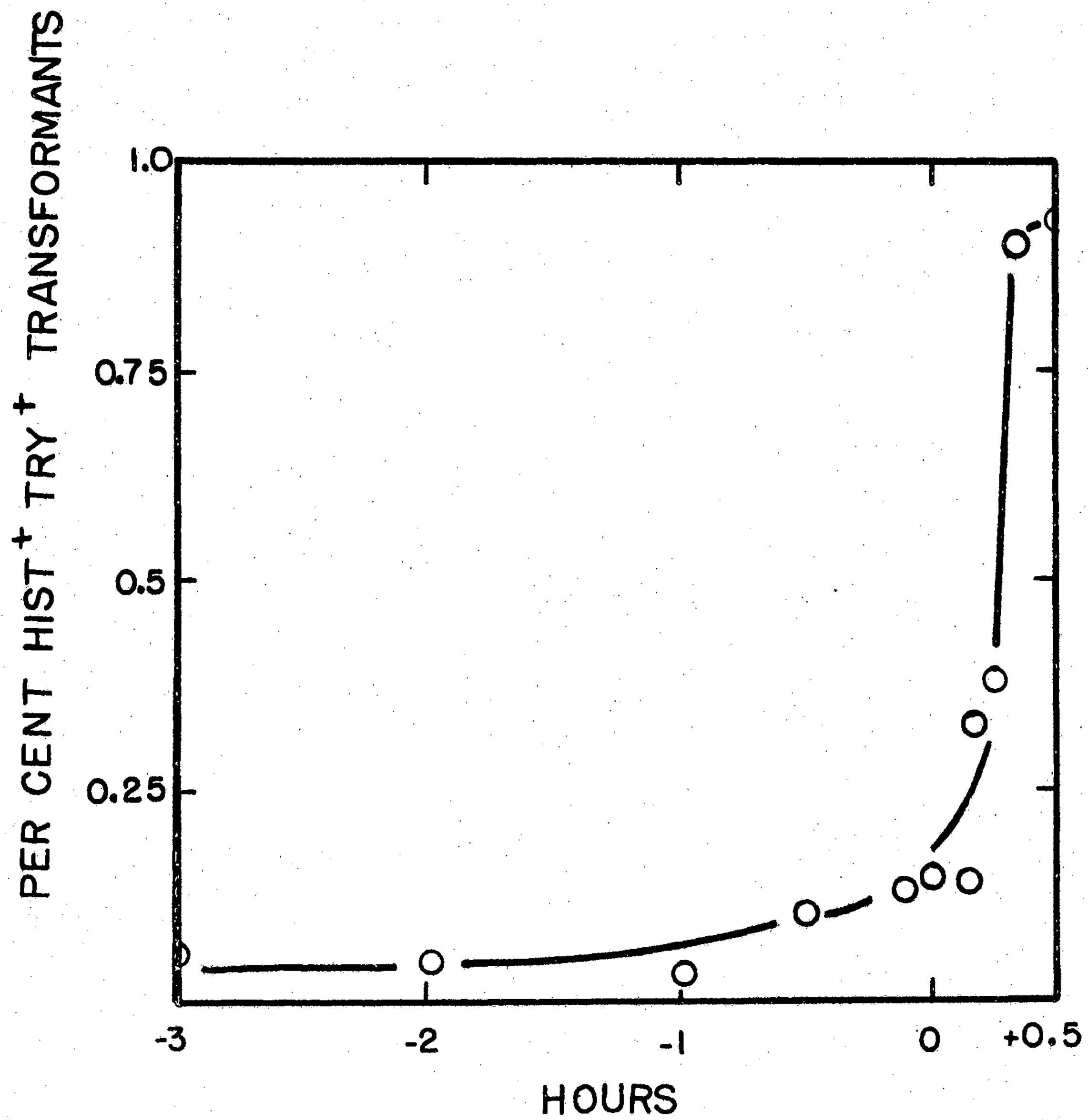

TRY+
PER CENT TRANSFORMATION
.4
.2
.1
.05
.01
.005
.003
0.0
0.04
0.08
0.12
0.16
PER CENT PEA

Figure 12. Effect of Time of Addition of PEA upon Transformation

PEA (0.05%) was added at the indicated times to the transformation system and incubated with cells until dilution and plating. DNA (5 μg/ml) was added at time zero and incubated for 30 minutes at 37° with shaking before dilution and plating. The negative times indicate addition of PEA to competent cells prior to DNA addition. Competent cells were obtained by procedure A. The percent of tryptophan^{+} histidine^{+} transformants is shown.


PER CENT HIST + TRY + TRANSFORMANTS
1.0
0.75
0.5
0.25
-3
-2
-1
0
+0.5
HOURS

of these cases did the PEA reduce the recipient (total) titer. The reaction of PEA which produced maximum inhibition could have been the result of one of two effects: (1) the reaction of PEA with competent cells is slow, or (2) at early times the maximum number of competent cells has not developed, and PEA prevents their development.

Table IV also shows the effect of addition of PEA (0.05%) at different times during competence development upon the number of tryptophan transformants. For the experiment shown in line 1 no PEA was present, and for line 2 PEA (0.05%) was added at the time of dilution of the overnight culture which was allowed to grow for eight hours. Then the culture was diluted into the transformation medium and incubated for 3 hours at which time maximum competence was obtained. PEA was added at this dilution (lines 2 and 3) and remained during the 30 minute exposure to DNA. For line 4 PEA was added concomitantly with the DNA. There were no differences in the total viable cell titers under the four experimental conditions, but Table IV shows a marked difference in the number of transformants obtained. These results are consistent with the previous findings (Figure 12) in that the presence of PEA appears to have prevented the development of competence.

Reversal of PEA Inhibition by Addition of Excess Competent Cells

To gain further insight into the mechanism of the inhibition of transformation by PEA, factors which could reverse the inhibition were studied. Excess amounts of DNA and competent cells were added to a mixture of competent cells, DNA, and PEA which had a defined level of inhibition. Table V demonstrates that the addition of a two-fold

TABLE IV

EFFECT OF INCUBATION OF PEA WITH COMPETENT CELLS FOR VARIOUS TIME PERIODS DURING THE DEVELOPMENT OF COMPETENCE AND TRANSFORMATION

Time	Try^+ (10^4/ml) Transformants	Percent Transformation
1. No PEA	4230	0.3
2. 8 hr + 3 hr + 30 min	51	0.003
3. 3 hr + 30 min	122	0.008
4. 30 min	1560	0.1

PEA (0.05%) was present for the indicated times. The times correspond to those found optimum using transformation procedure A for the development of competence of the recipient culture. DNA (5 μg/ml) was added at the start of the 30 minute period and incubated with the cells at 37° with shaking prior to dilution and plating.

TABLE V

EFFECT OF EXCESS COMPETENT CELLS AND DNA UPON REVERSAL OF PEA INHIBITION OF TRANSFORMATION

Additions	Try^+ His^+ (10^4/ml) Transformants	Percent Transformation
No PEA	430	0.70
PEA	230	0.34
2 x DNA + PEA	238	0.30
7 x Cells + PEA	2540	0.58

PEA (0.05%) and DNA (5 μg/ml) were added as indicated except in line 3 where 10 μg/ml of DNA were added to competent cells (7 x 10^8 cells/ml) and incubated for 30 minutes at 37° with shaking. Excess competent cells were obtained by centrifugation and this concentrated competent cell suspension was added to the normal suspension simultaneously with DNA and PEA. The competent cells were obtained by procedure A. The percent and total number of $tryptophan^+$ $histidine^+$ transformants are shown.

excess of DNA failed to reverse the inhibition of 0.05% PEA. However, when a 7-fold excess of competent cells was added, the inhibition was reversed. Not only were more transformants obtained, but the frequency (percent) of transformation was increased. Since the defined amount of inhibition obtained in the system was not complete with 0.05% PEA, the addition of more of the substance with which PEA reacts would result in greater numbers of transformants. Since competent cells could reverse the inhibition of 0.05% PEA while DNA had no effect, the effect of PEA appears to be upon the cells rather than upon the transforming DNA.

Reversal of PEA Inhibition by Removal of PEA

Since the preceding experiments indicated that PEA was acting upon competent cells, PEA was incubated with competent cells for varying lengths of time and the cells were removed by centrifugation prior to the addition of DNA. Table VI reveals that inhibition of transformation by incubating competent cells with PEA was time-dependent and that at least one hour incubation was required for inhibition when the PEA was removed by centrifugation prior to DNA addition. The inhibition could be reversed by removal of PEA prior to DNA addition if the PEA had been incubated with the cells 30 minutes or less.

Time of Regain of Competence after Centrifugation

Since treatment of competent cells with 0.05% PEA for 60 minutes inhibited transformation even when the PEA was removed prior to the addition of DNA, the time course of the return of competence after PEA (0.05%) treatment for 75 minutes was followed. In Figure 13 the wave

TABLE VI

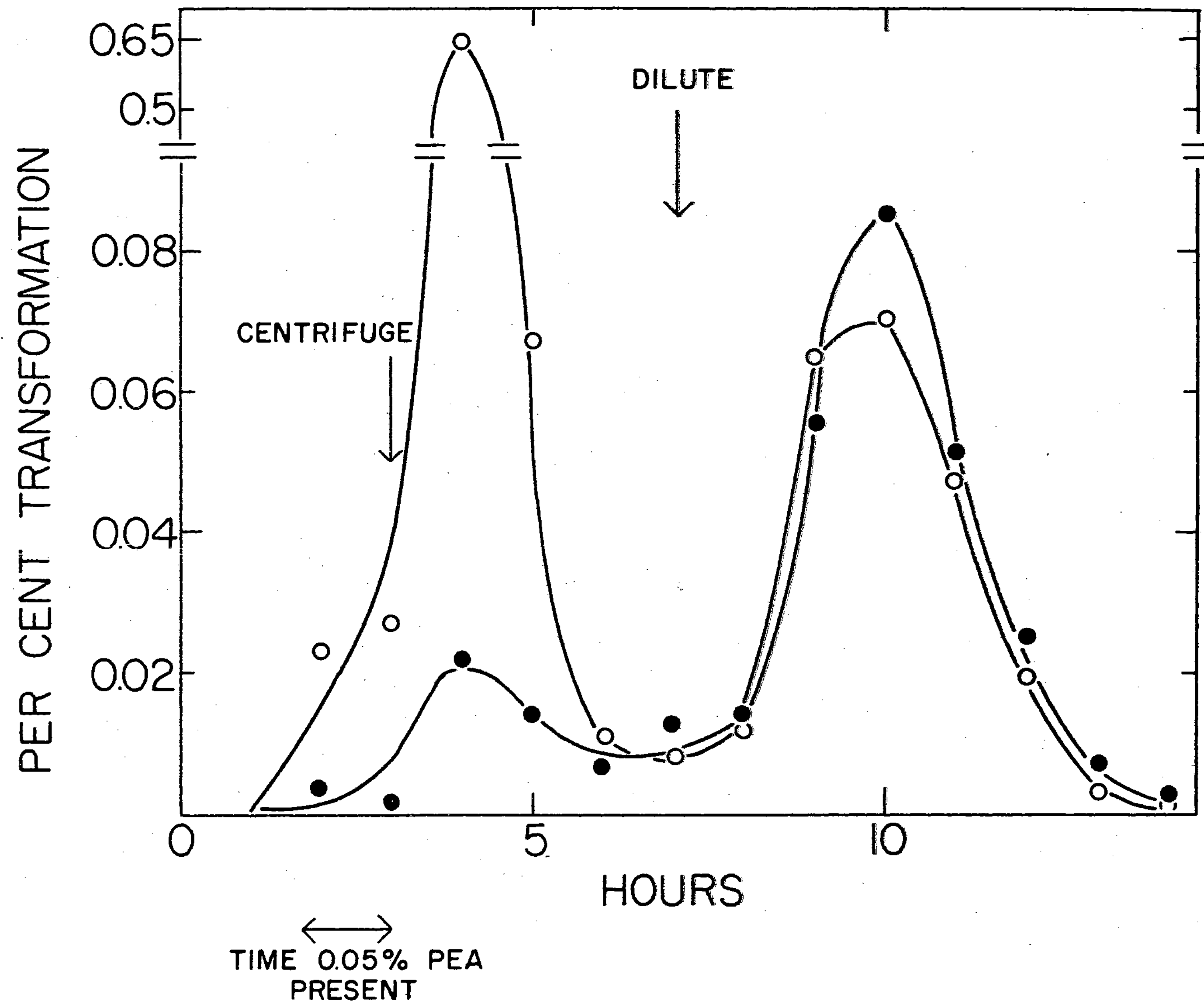
EFFECT OF REMOVAL OF PEA BY CENTRIFUGATION
UPON INHIBITION OF TRANSFORMATION

Time (min.) PEA Incubated With Cells Prior to Removal	(10^3) Transformants per ml		
	try^+his^+	his^+	try^+
180	62	94	99
120	100	86	94
60	104	174	186
30	247	305	435
5	295	434	395
None	315	359	457

PEA (0.05%) was added at various times during the 3 hour incubation in transformation medium (procedure A). PEA was removed prior to the addition of DNA by centrifugation. The cells were suspended in fresh minimal medium supplemented with 10 µg/ml of tryptophan and histidine and 0.01% acid hydrolyzed casein. Five µg/ml of DNA were then added and incubated for 30 minutes at which time dilutions and platings were done. No significant variation in cell titer was noted, the average cell titer being 8×10^8 cells per ml.

Figure 13. Regain of Competence Following Centrifugation after PEA Treatment

A competent culture of B. subtilis SB 25 was obtained by procedure A. The culture was divided, and half of the cells were treated with PEA (0.05%) for 75 minutes (time period 1.75 to 3 hours), and then the PEA was removed by centrifugation. Both the PEA treated, ●, and untreated, O, cells were taken up in fresh minimal medium supplemented with 10 µg/ml of tryptophan and histidine and 0.01% acid hydrolyzed casein and incubated with shaking at 37°. Samples were taken every hour and tested for the level of competence by adding 5 µg/ml of DNA and incubating for 30 minutes at 37° followed by dilution and plating to determine the number of tryptophan^{+} transformants.


0.65
0.5
0.08
0.06
0.04
0.02
PER CENT TRANSFORMATION
DILUTE
CENTRIFUGE
0
5
10
HOURS
TIME 0.05% PEA
PRESENT

of competence developing after 3 hours of incubation in transformation medium was inhibited in the PEA-treated culture even though the PEA had been removed from the cells by centrifugation. The second wave of competence which developed after dilution into fresh medium was not influenced by prior PEA treatment. PEA-treated competent cells do not regain competence without going through the same physiological conditions as a normal culture. Whatever effect PEA has on competent cells cannot be reversed by removal of PEA if PEA has been incubated 60 minutes or longer with the competent cells.

Interaction of ^{14}C-PEA with Cells

The preceding experiments indicated that the PEA acted upon the competent cell; therefore the interaction of ^{14}C-PEA and competent cells was studied. When cells were incubated with ^{14}C-PEA and then centrifuged and washed (with minimal medium), all the radioactivity was removed by the third centrifugation (Table VII). Almost 99% of the ^{14}C-PEA was removed by the first centrifugation indicating a rapid removal of PEA by washing.

The one percent of ^{14}C-PEA that was retained by the cells after one washing was analyzed by the Park-Hancock fractionation procedure (94). Cells were labeled by incubating ^{14}C-PEA with competent cells for 2 hours. The cells were then removed by Millipore filtration, washed, and filtered again. Table VIII shows the distribution of the ^{14}C-PEA in the various cell fractions. The radioactivity present in the cold trichloroacetic acid extract was probably that which would be removed by further washing. A significant amount of the radioactivity was found associated with the alcohol soluble fraction which contains

TABLE VII

REMOVAL OF ^{14}C-PEA BY CENTRIFUGATION AND WASHING

Incubation Time	CPM per ml of Cell Suspension Number of Times Centrifuged			
	0	1	2	3
3 Hours	573,020	7,795	265	0
10 Minutes	548,250	7,530	180	0

Competent B. subtilis SB 25 cells (obtained by procedure A) were incubated for 3 hours or 10 minutes with ^{14}C-PEA (76 μg/ml). The cells were centrifuged and suspended in fresh minimal medium 3 times. One ml of each of the resulting cell suspensions was suspended in Bray's scintillation fluid (91) and counted on a Packard scintillation counter.

TABLE VIII

PARK-HANCOCK FRACTIONATION OF ^{14}C-PEA LABELED B. SUBTILIS CELLS

Fraction	CPM	% of Total Radioactivity	Components of Fraction
Cold Trichloroacetic Acid (5%)	695	80.0	All low molecular weight compounds soluble in 5% TCA
Ethanol - H_2O (75%)	137	16.0	Ethanol soluble lipids and proteins
Hot Trichloroacetic Acid	20	2.3	Breakdown products of nucleic acids and teichoic acid
Trypsin Soluble	7	0.7	Trypsin-degraded proteins
Residue	0	0.0	Mucopeptide of wall

Competent B. subtilis, SB 25 cells were obtained by procedure A and labeled with ^{14}C-PEA (45,000 CPM/ml of cell suspension) by incubation for 2 hours at 37°. The cells were filtered on Millipore membrane filters and suspended in 5 ml of minimal medium. The cells were filtered again onto a Millipore filter and then suspended in water for fractionation by the Park-Hancock procedure (94).

mainly cell membrane components. Sanders and Leach (95) found that substances interacting with lipid material gave equivocal distributions when fractionated by the Park and Hancock procedure (94). Boiling the cells prior to fractionation prevented artifacts in distribution. When this procedure (95) was applied, there was no significant difference in the results obtained.

Effect of PEA upon Potential Transformants

Since PEA interacts with the component cells to render them incapable of transformation, an investigation was conducted to determine which step of the transformation process was being inhibited by PEA. The first experiments were designed to separate initial attachment and transport of the DNA from the intracellular processes of synapsis, integration, and expression. Nester and Stocker (35) have demonstrated a biosynthetic latency of 3 to 4 hours before the enzyme tryptophan synthetase is formed. Association of incoming markers with the resident genome does not occur until after a 3 to 4 hour incubation period (40). Table IX demonstrates that when PEA was added after the 30 minute exposure to DNA and incubated for either 5 or 30 minutes prior to dilution, there was no effect of PEA upon transformation. When the potential transformants were plated in the presence of 0.05% PEA, no inhibition was observed (Table X). These experiments eliminate the possibility of preferential killing of transformants and action of PEA during the latter processes of transformation such as recombination and expression. If PEA inhibits the first reactions of transformation when the DNA is extracellular, then transformation should become resistant to PEA inhibition at the same time that DNase

TABLE IX

EFFECT OF PEA UPON POTENTIAL TRANSFORMANTS
WHEN INCUBATED WITH CELLS PRIOR TO PLATING

Time of PEA Addition	% Try^+ Transformants
No PEA	0.47
5 Minutes	0.49
30 Minutes	0.50

Competent B. subtilis SB 25 cells were obtained by procedure B (Chapter II) and incubated with DNA (5 µg/ml) for 30 minutes and then treated with PEA (0.05%) for the indicated time intervals before dilution and plating.

TABLE X

EFFECT OF PEA UPON POTENTIAL TRANSFORMANTS

(10^4) Transformants per ml	No PEA	PEA
Try^+His^+	111	105
His^+	156	150
Try^+	215	225
Total No. of (10^7) Cells per ml	147	163

Competent B. subtilis SB 25 cells were obtained by procedure B and incubated with DNA (5 μg/ml) for 30 minutes. The cells were then diluted and plated on medium with and without 0.05% PEA. Table X gives the average of three independent experiments.

sensitivity is lost.

Kinetics of Stopping Transformation

The above possibility was tested by comparing the kinetics of inhibiting transformation by PEA and DNase treatments (Figure 14). The kinetics of the action of PEA is similar to that of DNase; that is, once the DNA is within the cells, it is no longer destroyed by DNase, and the cells are no longer sensitive to PEA.

Effect of PEA upon Recovery from Ultraviolet Irradiation

Since the process of repair of ultraviolet irradiation damage and the recombinational portion of transformation are related (96), the effect of PEA on ultraviolet irradiation damage repair was determined as an additional check for the interference of PEA with the recombination required in transformation. When 0.05% PEA was incubated with ultraviolet irradiated cells, no effect upon the recovery of the cells in the presence of light was noted (Figure 15). Identical results were obtained when recovery in the presence of PEA occurred in the dark (Figure 16). PEA, therefore, has no effect upon the ability of cells to recover from ultraviolet irradiation at concentrations which inhibit transformation, and the shared recombinational processes between transformation and irradiation damage repair are eliminated from consideration as the site of action of PEA.

Effect of PEA upon the Uptake of ^{3}H-DNA

The experiments that have been detailed above suggest that PEA

Figure 14. Comparison of the Action of DNase and PEA upon Transformation

DNase (10 µg/ml) and PEA (0.05%) were added at the times indicated to a competent culture of B. subtilis SB 25 obtained by procedure A. The number of transformants obtained with PEA treatment were corrected so that the number of tryptophan^{+} transformants obtained at zero time were the same as with DNase treatment. This number of tryptophan^{+} transformants was subtracted from all the following values of PEA treatment.

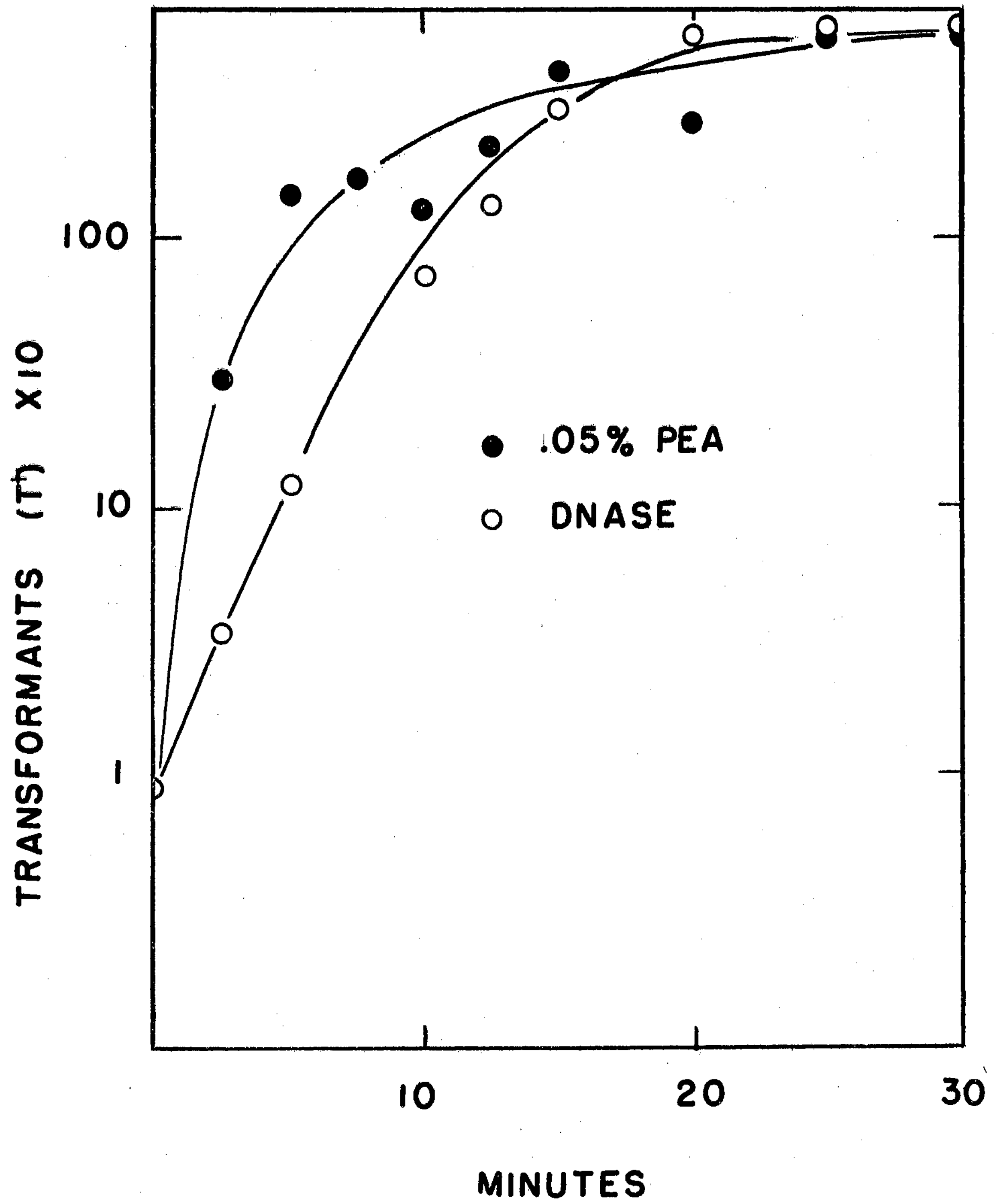

100
10
1
TRANSFORMANTS (T⁺) X10
.05% PEA
DNASE
10
20
30
MINUTES

Figure 15. Effect of PEA upon Ultraviolet Irradiation Recovery in the Light

B. subtilis SB 25 competent cells (5 x 10^8 cells/ml) were obtained by procedure A. Twenty ml of the cell suspension were placed in a petri dish and irradiated with an ultraviolet light four inches above the cell suspension. Samples of 0.1 ml were taken at the indicated times, diluted, and plated on nutrient agar with, 0, and without, ●, 0.05% PEA. All processes were carried out in the light.

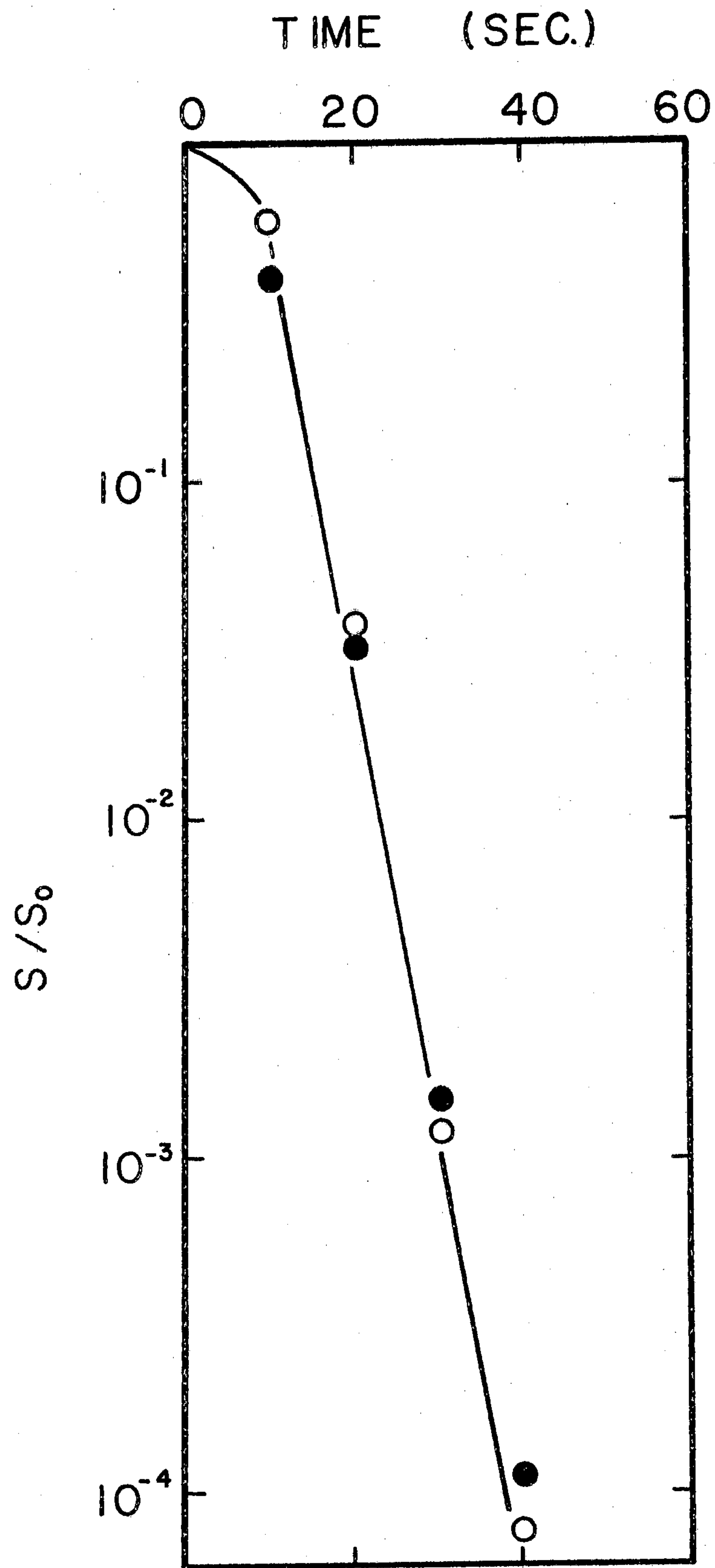
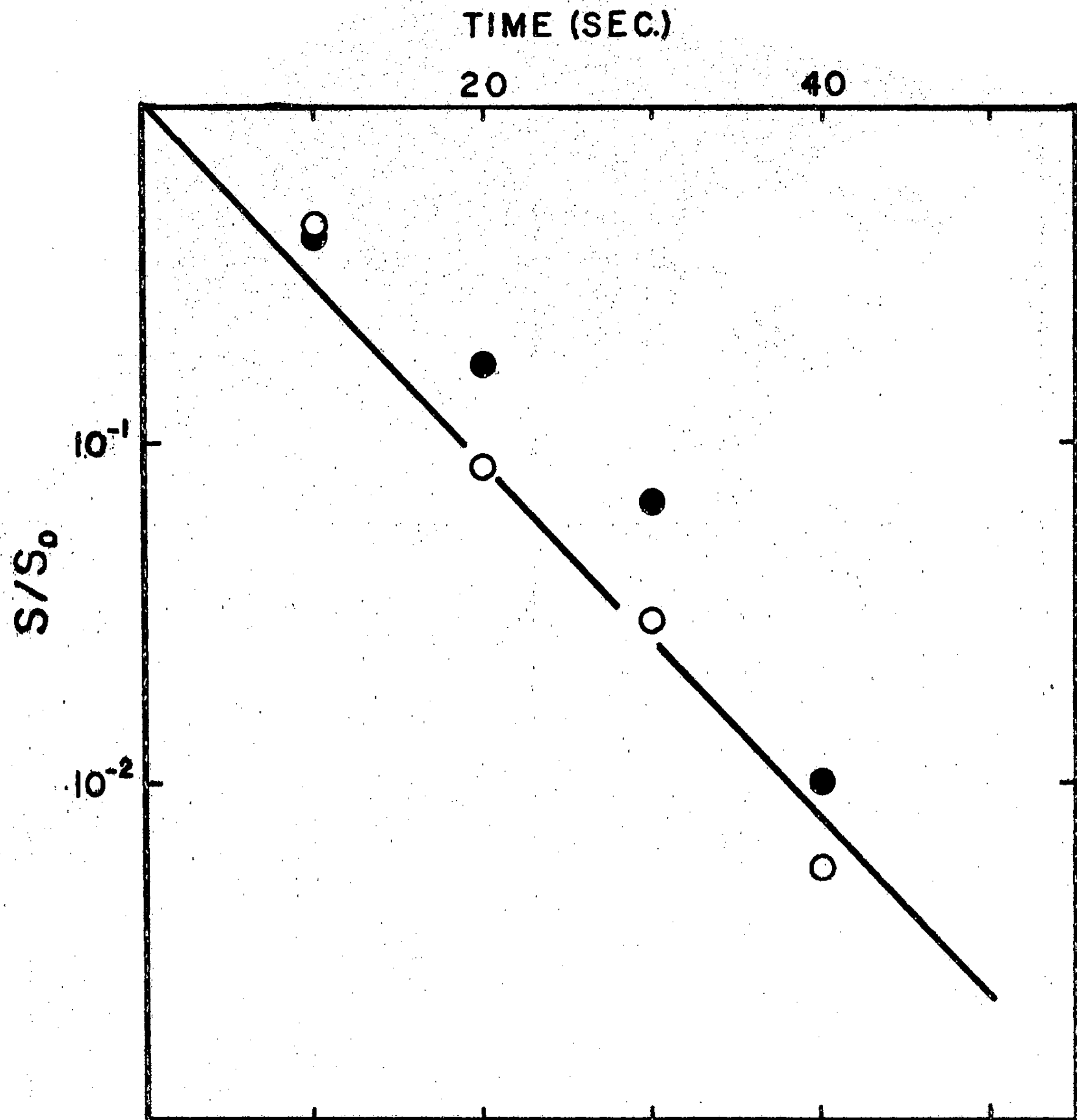

TIME (SEC.)
0
20
40
60
10^{-1}
10^{-2}
10^{-3}
10^{-4}
S/S_o

Figure 16. Effect of PEA upon Dark Recovery from Ultraviolet Irradiation

Competent *B. subtilis* cells (4 x 10^8 cell/ml) were obtained by procedure A. Twenty ml of the cell suspension were placed in a petri dish and irradiated with an ultraviolet light 4 inches above the cells suspension. Samples of 0.1 ml were taken at the indicated times, diluted, and plated on nutrient agar with, O, and without, ●, 0.05% PEA. All processes (including overnight incubation of plates) were carried out in the dark.



TIME (SEC.)
20
40
10^{-1}
10^{-2}
S/S_0

might act by inhibiting the uptake of DNA, and this hypothesis is subject to direct test using radioactive transforming DNA. Table XI shows that the incorporation of ^{3}H-DNA into the cells after removal of exogenous DNA by DNase treatment was decreased by PEA (0.05%) treatment. A control in which cells were exposed to ^{3}H-DNA previously incubated with DNase, indicated the radioactivity found associated with the cells was due to the uptake of intact ^{3}H-DNA and not to contaminating radioactivity nor thymidine produced by degradation of the DNA. The frequency of transformation was also decreased proportionally by PEA treatment in these experiments.

Effect of Removal of PEA upon Uptake of ^{3}H-DNA

Previous experiments demonstrated that removal of PEA after a 60 minute treatment did not reverse the inhibition of transformation, and the results shown in Table XII reveal that the uptake of radioactive DNA is still inhibited under these conditions. DNase treatment of ^{3}H-DNA prior to addition of competent cells revealed that the radioactivity associated with the cells was due to intact DNA. PEA treatment of competent cells gave a 57% inhibition of transformation while a 51% inhibition of uptake of ^{3}H-DNA was observed. Thus, removal of PEA after an incubation period of 60 minutes does not reverse the inhibition of DNA uptake just as it did not reverse the inhibition of transformation.

Effect of PEA upon the Reversible Attachment of ^{3}H-DNA to Competent Cells

There are two stages involved in the uptake of DNA during

TABLE XI

EFFECT OF PEA UPON THE UPTAKE OF ^{3}H-DNA

Addition	CPM	His^{+} (10^{3}/ml) Transformants
No PEA	317	300
PEA	34	36

Competent B. subtilis SB 25 cells obtained by procedure A were treated for 45 minutes with ^{3}H-DNA (0.1 μg/3ml of cells) and with or without 0.05% PEA. At the end of the transformation period, the procedure described in Chapter II was followed. This experiment was done in duplicate and the figures given are an average with the CPM obtained with DNase treated ^{3}H-DNA subtracted.

TABLE XII

EFFECT OF REMOVAL OF PEA UPON
THE UPTAKE OF ^{3}H-DNA

	CPM per 3 ml	No. His$^+$ (10^3) Transformants per ml	Total No. (10^7) Cells per ml
Prior DNase Treatment of the ^{3}H-DNA	104	0.1	59
No PEA	1740	309	69
0.05% PEA	866	134	72

Competent B. subtilis SB 25 cells were obtained by procedure B. PEA (0.05%) was incubated with the competent cells during the final hour of incubation. The PEA was removed from the cells by centrifugation and suspended in fresh medium and incubated for 30 minutes at which time 0.1 µg ^{3}H-DNA/3 ml of cells was added for 45 minutes, and the procedure described in Chapter II was followed. A control was run where no PEA was added. This experiment was done in triplicate, and the figures given are the average.

transformation, and experiments discussed in this section differentiate between these two steps. Since the irreversible uptake of DNA is preceded by a reversible binding of the DNA to the cell (69, 70), the effect of PEA upon the reversible binding (initial attachment) of DNA was investigated. The experimental approach of Lerman and Tolmach (69) for studying the reversible attachment of ^{32}P-DNA to pneumococcus cells was applied to the B. subtilis transformation system (Figures 17 and 18). Figure 17 demonstrates that a residual amount of ^{3}H-DNA remains associated with the cells after washing four times with cold medium. All of the radioactivity that is not associated with the cells was removed by one washing. Figure 18 demonstrates that, when the cells are incubated at 0°, 75% of the radioactivity remains associated with the cells after 45 minutes. At 37° half of the radioactivity is lost after an incubation of 45 minutes. Cells treated with DNase at 37° retain only 10% of the radioactivity after incubation for 30 minutes. These experiments are comparable with those of Lerman and Tolmach (69) with pneumococcus demonstrating a reversible and DNase sensitive attachment of ^{3}H-DNA to cells.

The effect of PEA upon the reversible binding of ^{3}H-DNA to chilled competent cells is shown in Table XIII. The radioactivity associated with the cells after one washing was the same in the treated or untreated cells. Treatment with DNase removed 95% of the radioactivity associated with the cells demonstrating that the ^{3}H-DNA was reversibly (DNase sensitive) associated with the cells. Thus, PEA (0.05%) is not inhibiting the initial attachment stage of transformation.

Figure 17. Washing of Competent *B. subtilis* Cells Exposed to ^{3}H-DNA

Competent *B. subtilis* SB 25 cells obtained by procedure B were placed in ice for ten minutes. ^{3}H-DNA (0.1 µg/3ml) was then added to the cells and incubated for one hour in the cold. The cells were then centrifuged and washed with cold minimal medium the indicated number of times. The radioactivity associated with the cells was determined by counting in a Packard scintillation spectrometer counter using Bray's scintillation fluid (91).

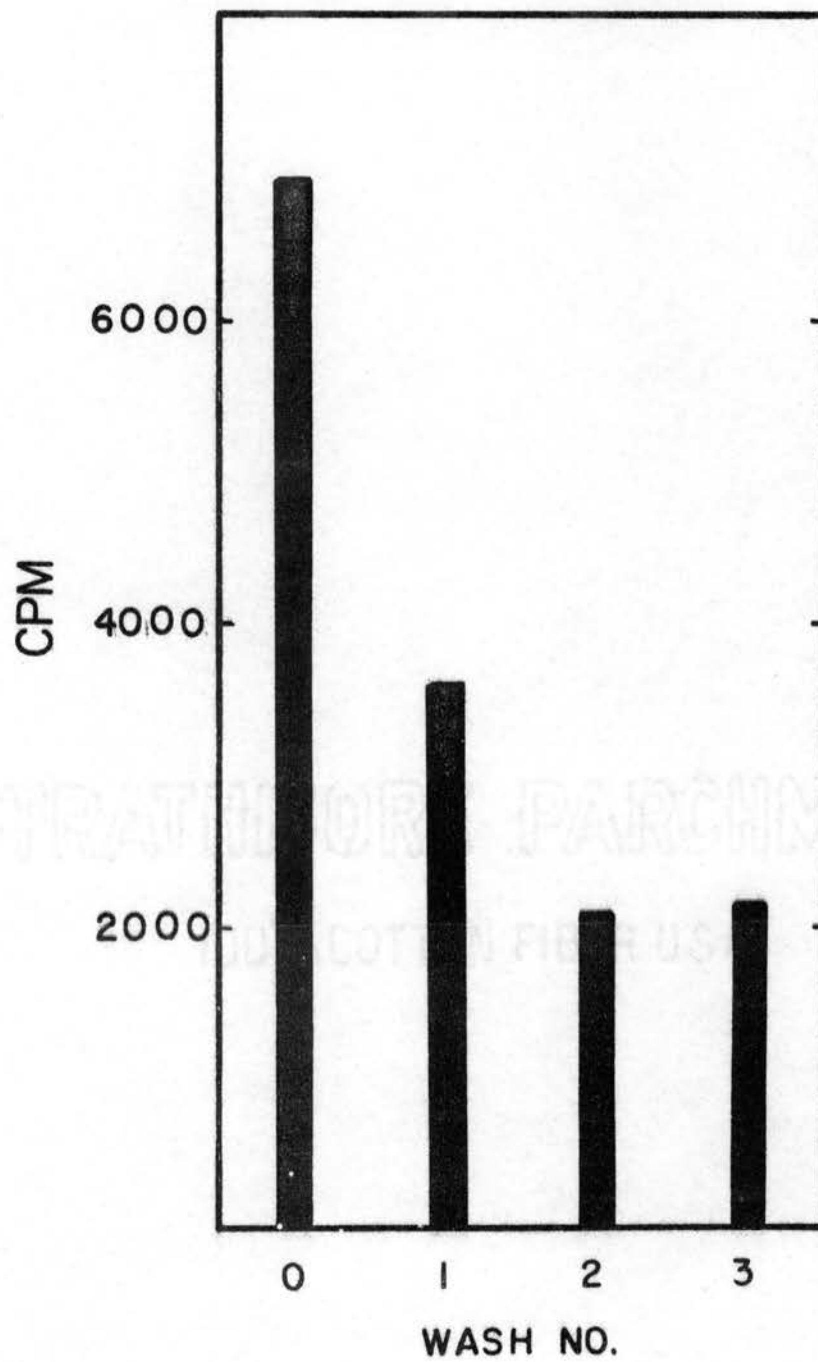
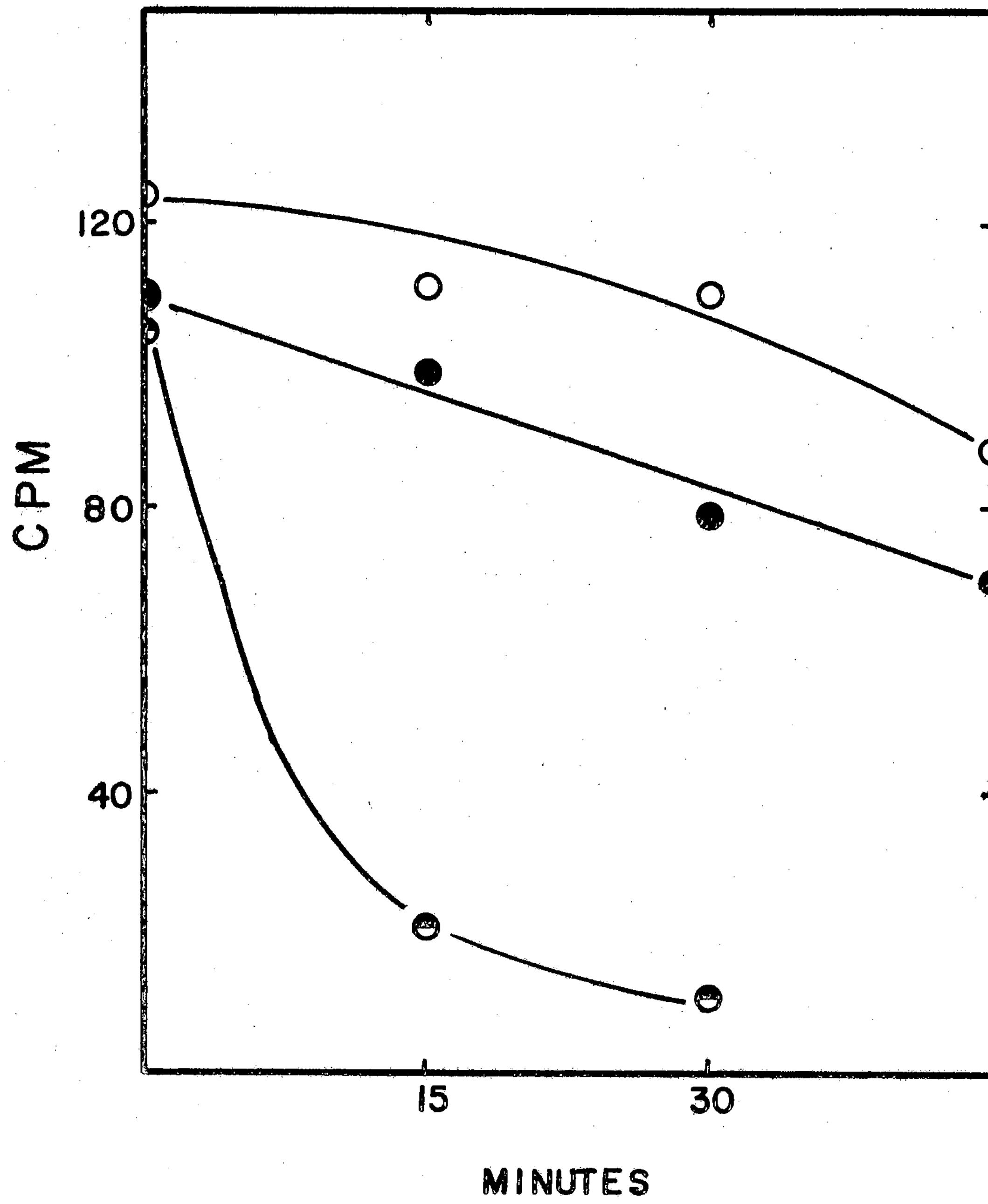

CPM
6000
4000
2000
0
1
2
3
WASH NO.

Figure 18. Effect of Various Temperature and DNase Treatment upon the Reversible Binding of DNA to B. subtilis Cells

Competent B. subtilis SB 25 cells obtained by procedure B were placed in ice for 10 minutes. ^{3}H-DNA (0.1 μg/3ml) was then added to the cells and incubated for one hour in the cold. The cells were then centrifuged and washed once with cold minimal medium and suspended in minimal medium (supplemented as described in procedure B) and incubated at 0°, O, 37° C, ●, and 37° with 0.1 mg/ml DNase, ●. Samples of 1 ml were taken at the indicated times and the cells centrifuged, suspended in water, and the radioactivity associated with the cells determined.



120
80
40
CPM
15
30
MINUTES

TABLE XIII

EFFECT OF PEA UPON THE REVERSIBLE BINDING OF DNA TO B. SUBTILIS

Addition	CPM Associated with Chilled Cells	CPM Associated with Cells after DNase Treatment
No PEA	1290	80
0.05% PEA	1260	89

Competent B. subtilis SB 25 cells were obtained by procedure B (6 ml) and chilled in an ice bath for 10 minutes. PEA (0.05%) was added at this time. ^{3}H-DNA (0.1 μg/3 ml of cells) was then added and incubated with the chilled cells for 60 minutes. The cells were centrifuged down, washed with cold minimal medium, and centrifuged again. The cells were then suspended in 2 ml of minimal medium and a 1 ml sample was taken to determine the radioactivity associated with the cells after one washing. The other sample was diluted to 3 ml with warm minimal medium supplemented with 0.1 mg/ml DNase. The cells were incubated with the DNase for 20 minutes at 37° and then removed by centrifugation and suspended in 1 ml of minimal medium and the radioactivity was determined. The results given are an average of two experiments.

Effect of PEA upon Permeability

An effect of PEA on the transport of transforming DNA into competent cells has been demonstrated; however, questions concerning the specificity of the inhibition of transport were not investigated.

The effect of 0.05% PEA on the biosynthesis of the three macromolecular species was determined by measuring the incorporation of radioactive precursors into trichloracetic acid insoluble material. Figure 19 shows that the incorporation of radioactive thymidine into DNA in a thymine requiring strain of *B. subtilis* was not affected by the presence of PEA. Likewise, Figure 20 shows that there is no effect by PEA upon the incorporation of leucine into protein. Figure 21 shows that PEA reduced the extent to which uracil was incorporated, while the rate of incorporation remained roughly equivalent in the presence and absence of PEA. Thus, under the conditions of these experiments there was a reduction in the amount of RNA synthesis while the synthesis of protein and DNA was not affected. Experiments done in this manner measure the overall result of several reactions, and the information obtained depends upon which reaction is rate limiting. These experiments are included here since previous investigations (9, 10, 17, 20) have made use of such experiments in the development of the proposed site of action of PEA. Therefore, it is important to establish if errors exist in the interpretation of such experiments.

At least two series of reactions are involved in the measurement: (1) transport (rate of accumulation) of radioactive precursors into the free, small molecule cell pool and (2) the series of reactions bringing about the incorporation of the precursors into the macromolecular species. To differentiate between the two reactions, uptake of the

Figure 19. Effect of PEA upon ^{3}H-Thymidine Uptake

B. *subtilis* FH 2006 cells were incubated with, ●, and without, O, PEA (0.05%) and with ^{3}H-thymidine (0.025 μc/ml). At the indicated times 1 ml samples were treated as described in Chapter II.

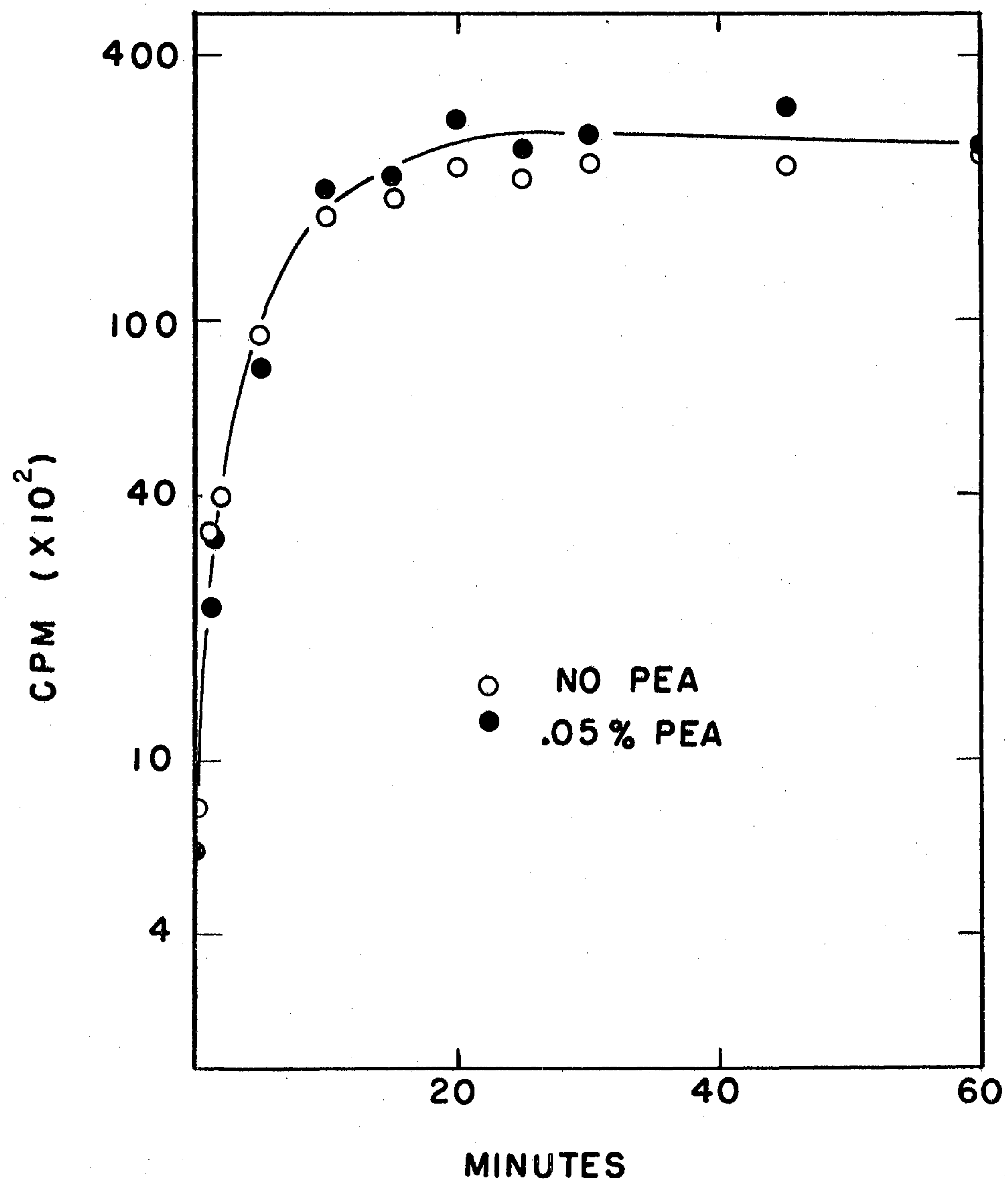

400
100
40
10
4
CPM (X10^2)
20
40
60
MINUTES
NO PEA
.05 % PEA

Figure 20. Effect of PEA upon ^{14}C-L-Leucine Uptake

B. *subtilis* (SB 25) cells with, ●, and without, O, PEA (0.05%) were incubated with ^{14}C-L-leucine (0.025 μc/ml). At the indicated times, 1 ml samples were treated as described in Chapter II.

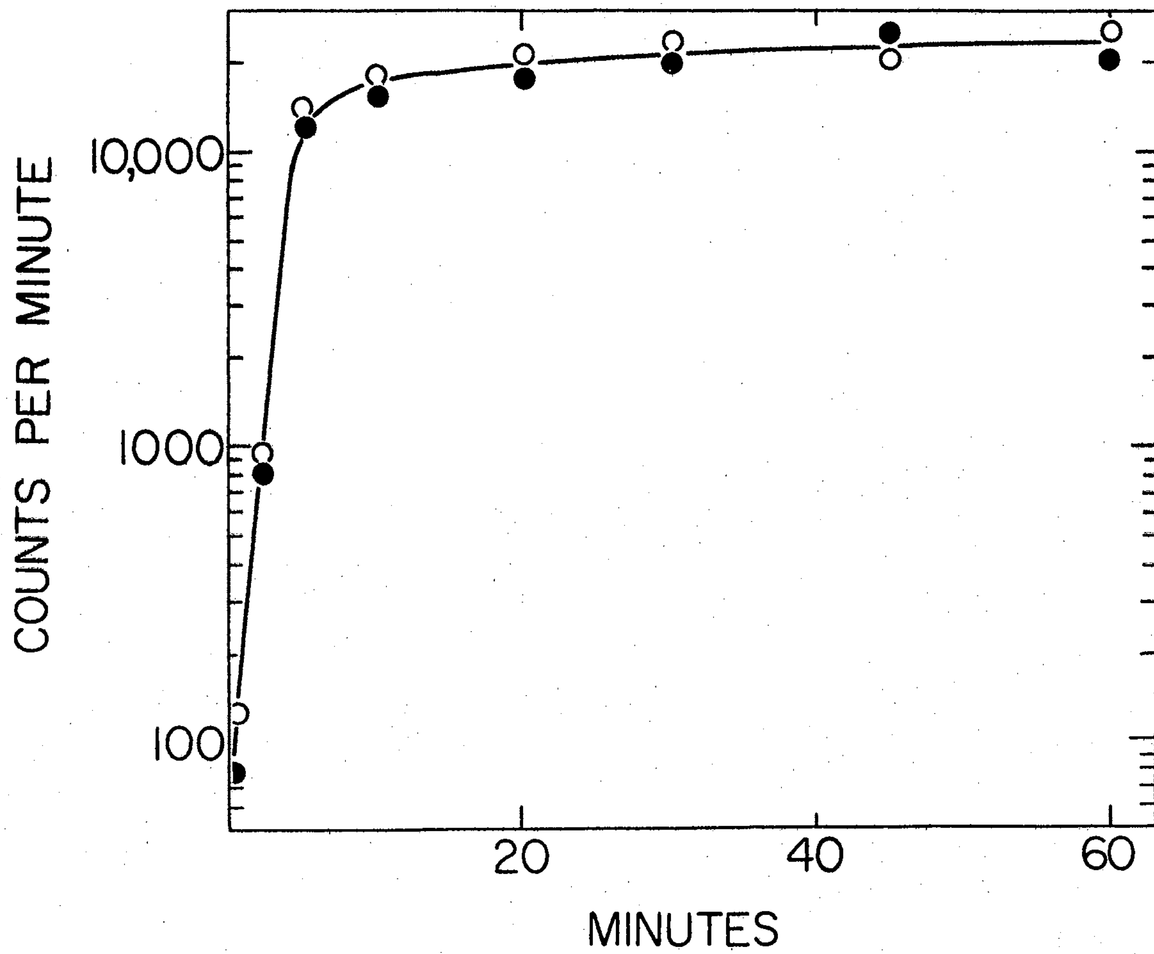
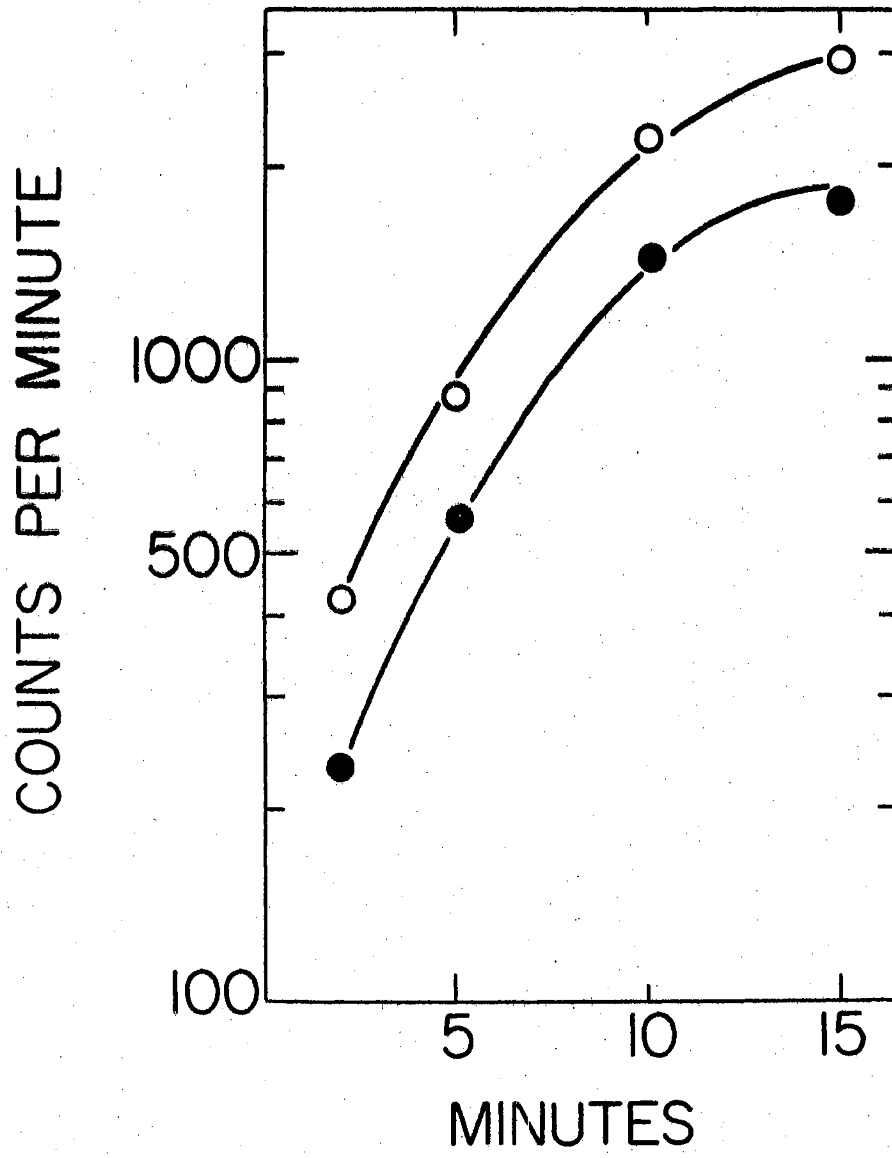

COUNTS PER MINUTE
10,000
1000
100
20
40
60
MINUTES

Figure 21. Effect of PEA upon ^{14}C-Uracil Uptake

B. subtilis (SB 25) cells with, ●, and without, O, PEA (0.05%) were incubated with ^{14}C-uracil (0.025 μc/ml). At the indicated times 1 ml samples were treated as described in Chapter II.


COUNTS PER MINUTE
1000
500
100
5
10
15
MINUTES

radioactive precursors into the bacterial cell was determined by measuring the radioactivity associated with cells collected by Millipore filtration. Incorporation was measured by the radioactivity in the trichloroacetic acid precipitate collected by Millipore filtration. The accumulation was calculated by the difference between the total radioactivity taken up by the cells and that incorporated into trichloroacetic acid insoluble material.

Figures 22, 23, and 24 show the uptake, accumulation, and incorporation of ^{14}C-L-serine, ^{3}H-uracil, and ^{3}H-thymidine in the presence and absence of 0.05% PEA and in Table XIV the initial rates of uptake, accumulation, and incorporation of the precursors are recorded. PEA reduces the rate of uptake, accumulation, and incorporation of all three radioactive precursors. Since the amount and rate of incorporation of radioactivity depends upon the amount of precursor in the pool, correction must be made for the reduced rate of transport of the precursor into the pool. When such corrections are made, the reduced rate of L-serine, uracil, and thymidine incorporation is approximately equivalent to the reduction of accumulation rate by PEA. Therefore, the effects of PEA upon RNA synthesis measured by the incorporation of radioactive uracil into trichloroacetic acid precipitable material observed by other workers (17, 20) is probably due to inhibition of transport rather than a decrease in RNA synthesis. A higher concentration of PEA (0.2%) shows greater inhibition of the incorporation of uracil and thymidine into acid precipitable material, but this inhibition is associated with a proportional increase in the inhibition of the uptake of these compounds into the cell (Figures 23 and 24).

Figure 22. Uptake, Accumulation, and Incorporation of ^{14}C-L-Serine in the Presence and Absence of PEA

The procedure in Chapter II was followed using B. subtilis SB 25 cells. Cells were incubated in minimal medium supplemented with 50 μg/ml of tryptophan and histidine plus ^{14}C-L-serine (0.5 μc/ml) and a 250-fold excess of L-serine (100 mμmole/ml) in the presence and absence of 0.05% PEA. Samples of 0.5 ml were removed at the indicated times and the uptake, O, accumulation, θ, and incorporation, ●, determined as described in Chapter II.

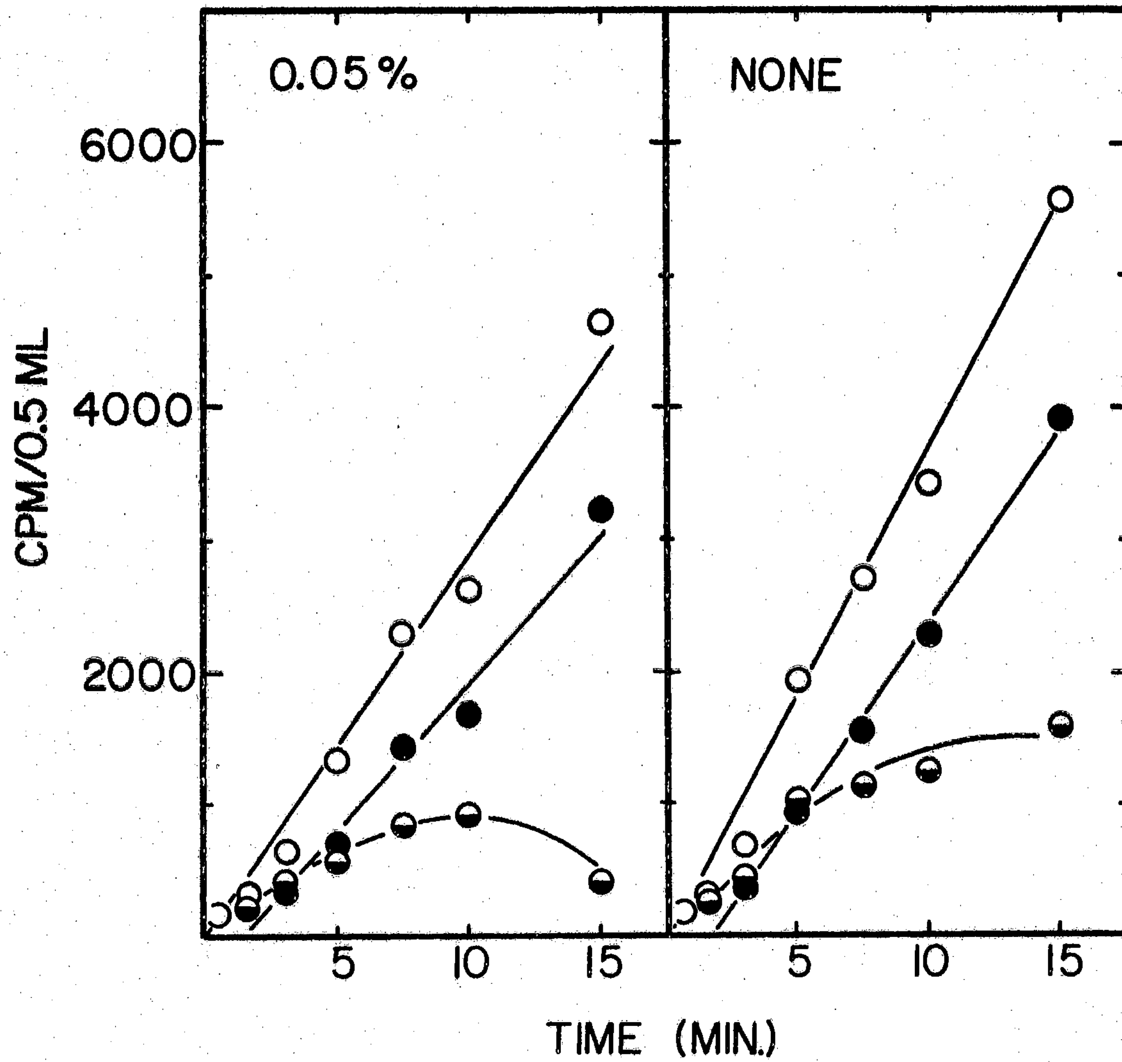

0.05 %
NONE
6000
4000
2000
CPM/0.5 ML
5
10
15
5
10
15
TIME (MIN.)

Figure 23. Uptake, Accumulation, and Incorporation of ^{3}H-Uracil in the Presence and Absence of PEA

B. *subtilis* SB 25 cells were diluted 5-fold into minimal medium supplemented with 50 μg/ml of tryptophan and histidine and 50 mμmole/ml of uracil in the presence (0.2% and 0.05%) and absence of PEA. After incubation at 37° for 15 minutes, 1.0 μc/ml of ^{3}H-uracil was added to the cells and this is indicated as time zero. Samples of 0.5 ml were removed at the indicated times and the uptake, 0, accumulation, ◐, and incorporation, ●, determined as described in Chapter II.

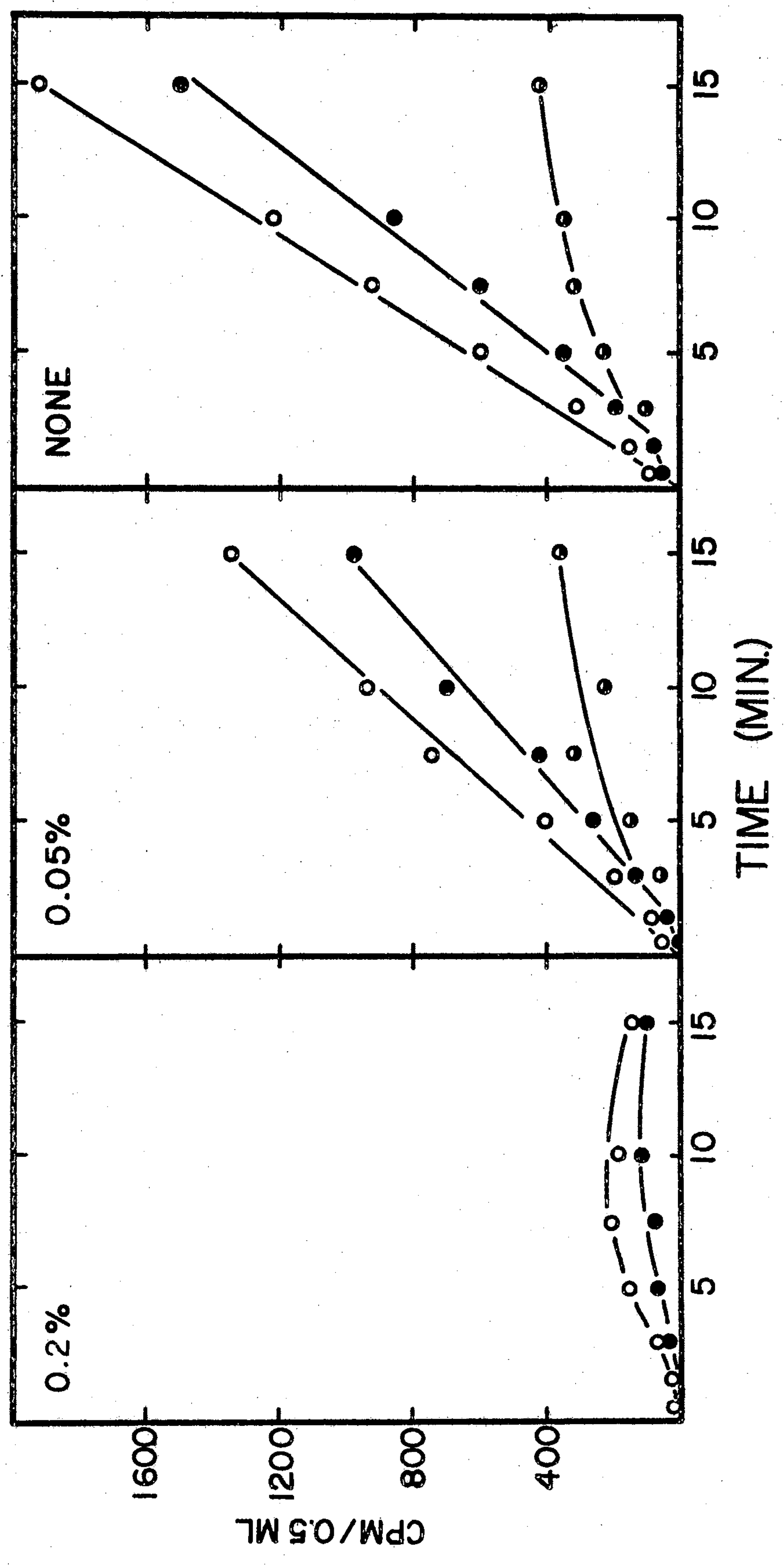
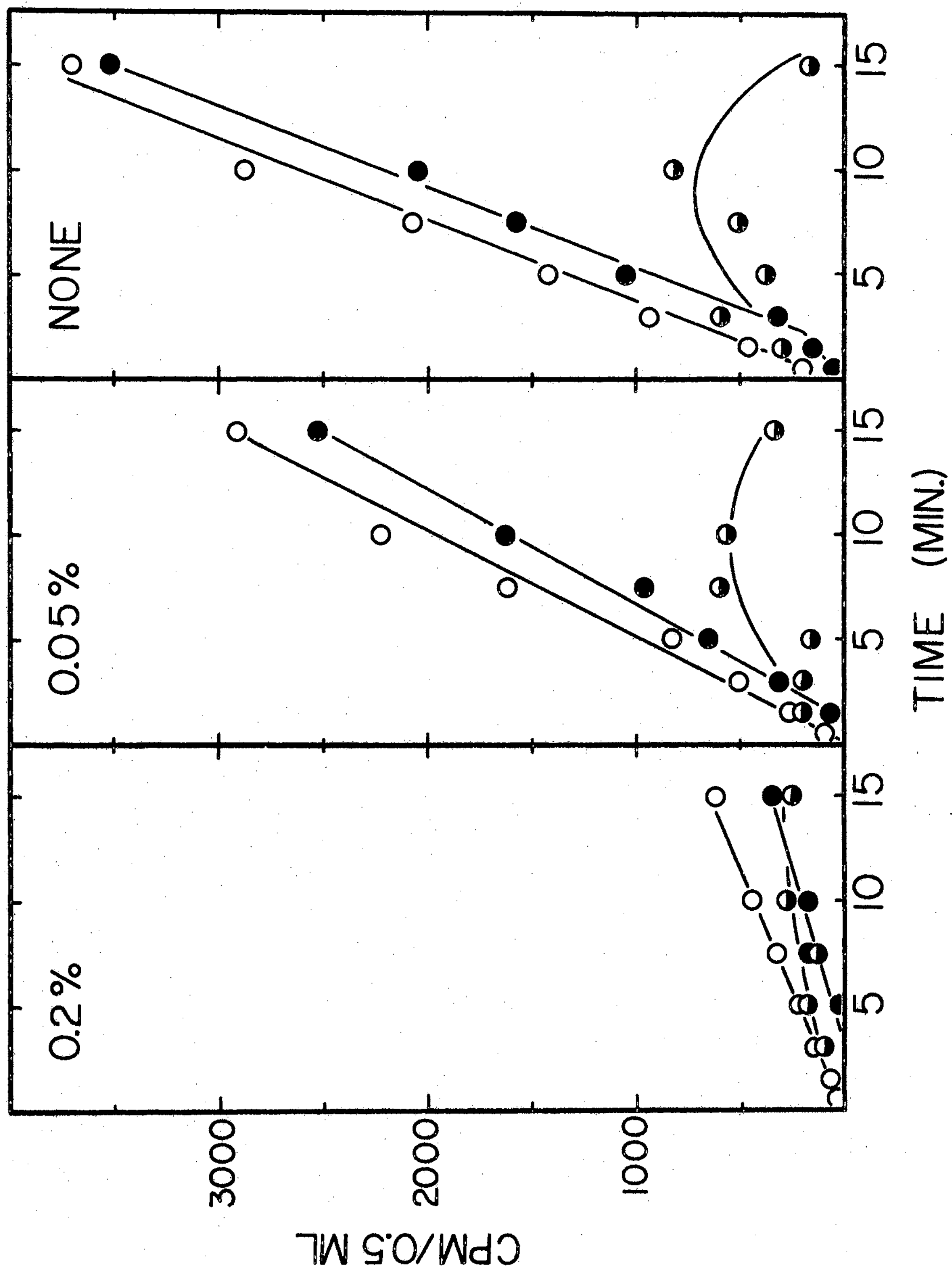

NONE
0.05%
0.2%
TIME (MIN.)
CPM/0.5 ML
1600
1200
800
400
5
10
15

Figure 24. Uptake, Accumulation, and Incorporation of ^{3}H-Thymidine in the Presence and Absence of PEA

B. subtilis FH 2006 cells were obtained as described in Chapter II and were diluted 5-fold into fresh minimal medium containing 50 μg/ml of tryptophan and histidine supplemented with 5 mμmole/ml of thymidine in the presence and absence of 0.05% PEA. After incubation for 15 minutes, 1.0 μc/ml of ^{3}H-thymidine was added and this is shown as time zero. Samples of 0.5 ml were removed at the indicated times and the uptake, O, accumulation, ⊖, and incorporation, ●, determined as described in Chapter II.



0.2%
0.05%
NONE
TIME (MIN.)
CPM/0.5 ML
3000
2000
1000
5
10
15

TABLE XIV

RATES OF UPTAKE, ACCUMULATION, AND INCORPORATION OF L-SERINE, URACIL, AND THYMIDINE

	Rate (mμmoles per hour per 10^9 cells)					
	Uptake	% of Control	Accumulation	% of Control	Incorporation	% of Control
L-Serine						
Addition						
No PEA	138.0		117.3		108.1	
PEA	104.0	75	87.4	76	85.1	79
Treatment						
No PEA	609.5		431.2		172.5	
PEA	609.5	100	431.2	100	172.5	100
Uracil						
Addition						
No PEA	62.7		31.4		28.5	
PEA	38.5	61	21.4	68	18.5	65
Treatment						
No PEA	119.7		74.1		58.4	
PEA	119.7	100	74.1	100	58.4	100
Thymidine						
Addition						
No PEA	10.98		7.38		3.96	
PEA	7.18	66	5.22	71	2.79	70
Treatment						
No PEA	7.56		6.48		1.17	
PEA	7.56	100	6.48	100	1.17	100

Rates of uptake, accumulation, and incorporation of L-serine, uracil, and thymidine were calculated from Figures 22, 23, 24, 25, 26, and 27. These rates are the initial rates of uptake, accumulation, and incorporation determined during the initial time periods.

Figures 25, 26, 27, and Table XIV demonstrate that PEA (0.05%) treatment (60 minutes), subsequent removal by centrifugation, and incubation for 30 minutes in PEA-free medium has no effect upon the uptake, accumulation, and incorporation of ^{14}C-L-serine, ^{3}H-uracil, ^{3}H-thymidine. However, removal of PEA and incubation for 30 minutes failed to reverse the inhibition of transformation or uptake of ^{3}H-DNA (Table XII) suggesting that the inhibition by PEA of transformation was more specific than an inhibition of uptake in general.

Effect of PEA upon Leakage

Other measurements of the effect of PEA on permeability are shown in Table XV. Experiment A shows only a slightly greater amount of leakage of 260 mμ absorbing material from *B. subtilis* cells treated with 0.05% PEA for 60 minutes. In experiment B, proteins were labeled by growth of cells in the presence of ^{14}C-leucine. Addition of 0.05% PEA to the incubation medium resulted in only a slightly greater release of radioactivity than was observed with the control (no PEA). The slight leakage caused by the presence of 0.05% PEA does not appear to be significant enough to be responsible for the inhibition of transformation.

Figure 25. Effect of PEA Treatment and Removal upon the Uptake, Accumulation, and Incorporation of ^{14}C-L-Serine

B. subtilis SB 25 cells were incubated as described in Chapter II except that PEA (0.05%) was added to half of the cells during the final hour of incubation. After 5 hours, the cells were centrifuged down and suspended in fresh minimal medium without PEA and supplemented with tryptophan and histidine (50 µg/ml). The cells were incubated 30 minutes and then diluted 5-fold into medium containing ^{14}C-L-serine (1.0 µc/ml) and a 250-fold excess of L-serine (100 mµmole/ml). Samples of 0.5 ml were removed at the indicated times of PEA treated and untreated cells. Uptake, O, accumulation, ◐, and incorporation, ●, were determined as described in Chapter II.

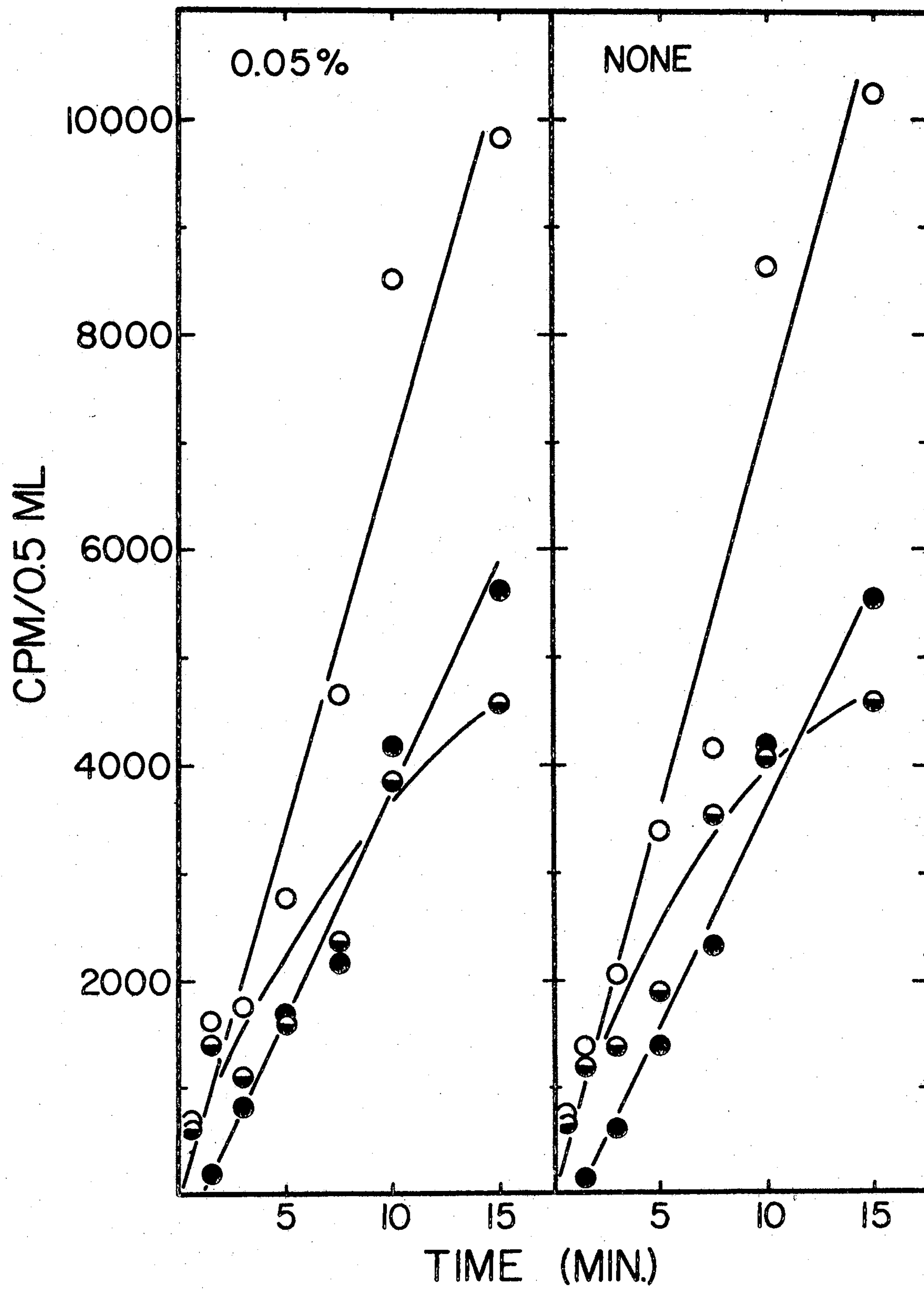

0.05%
NONE
10000
8000
6000
4000
2000
CPM/0.5 ML
5
10
15
5
10
15
TIME (MIN.)

Figure 26. Effect of PEA Treatment and Removal upon the Uptake, Accumulation, and Incorporation of ^{3}H-Uracil

B. subtilis SB 25 cells were obtained as described in Chapter II and incubated with and without PEA (0.05%) the final hour of incubation. The cells were then centrifuged down and suspended in fresh minimal medium (plus 50 μg/ml of tryptophan and histidine) and incubated for an additional 15 minutes before dilution (5-fold) into medium containing 50 mμmole/ml uracil. After incubation for 15 minutes at 37°, 1.0 μc/ml ^{3}H-uracil was added and samples of 0.5 ml were taken at the indicated times from PEA treated and untreated cells. Uptake, O, accumulation, ◐, and incorporation, ●, were determined as described in Chapter II.

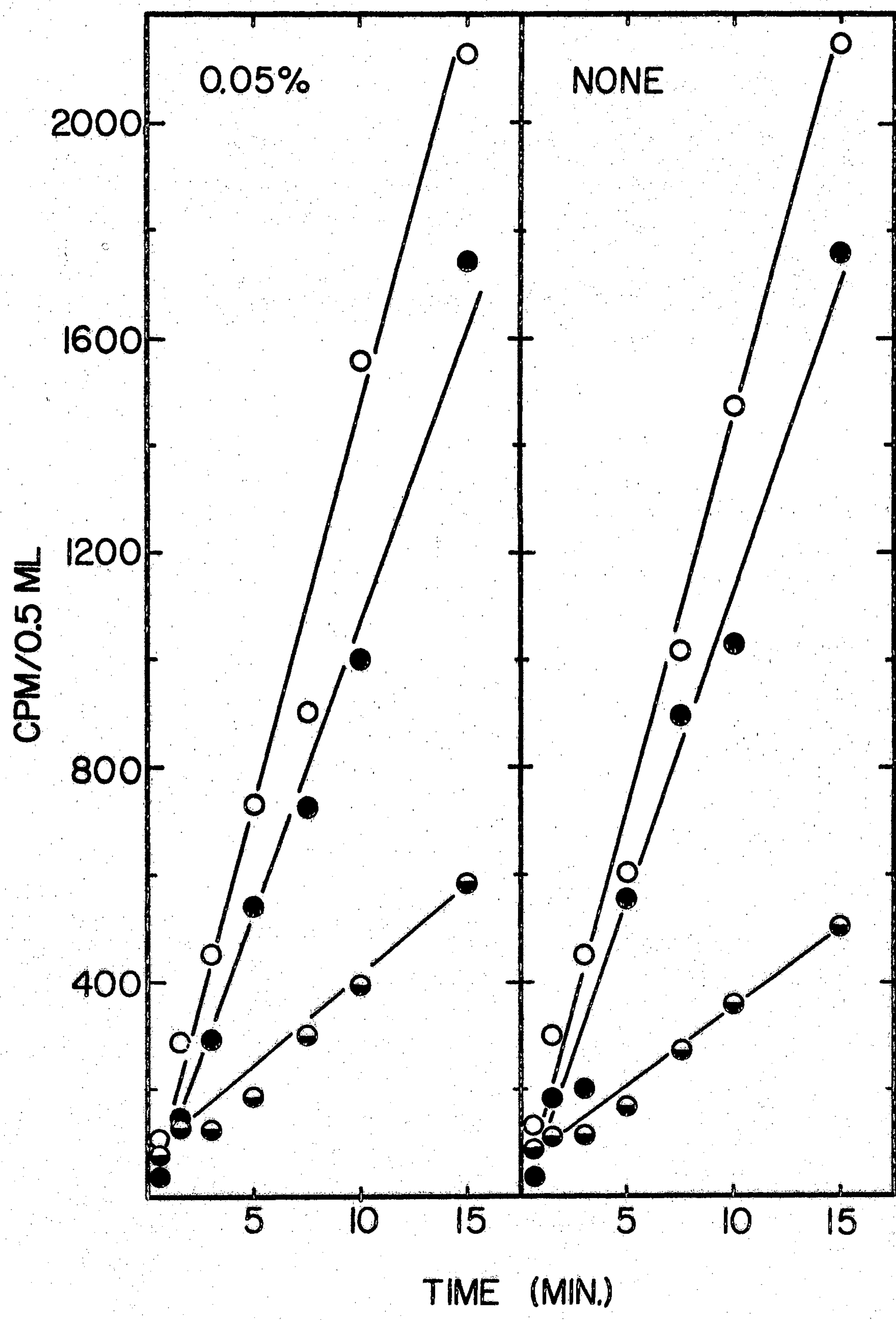

0.05%
NONE
2000
1600
1200
800
400
CPM/0.5 ML
5
10
15
5
10
15
TIME (MIN.)

Figure 27. Effect of PEA Treatment and Removal upon the Uptake, Accumulation, and Incorporation of ^{3}H-Thymidine

B. subtilis FH 2006 cells were obtained as described in Chapter II and incubated with and without PEA (0.05%) during the final hour of incubation. The cells were then centrifuged down and suspended in fresh minimal medium (plus 50 μg/ml of tryptophan and histidine and 5 mμmole/ml of thymidine) and incubated for an additional 30 minutes. The cells were then diluted 5-fold into minimal medium (plus 50 μg/ml of tryptophan and histidine) supplemented with ^{3}H-thymidine (1.0 μc/ml) and a 72-fold excess of thymidine (5 mμmole/ml) and samples of 0.5 ml were removed at the indicated time from PEA treated and untreated cells. Uptake, O, accumulation, ◒, and incorporation, ●, were determined as described in Chapter II.

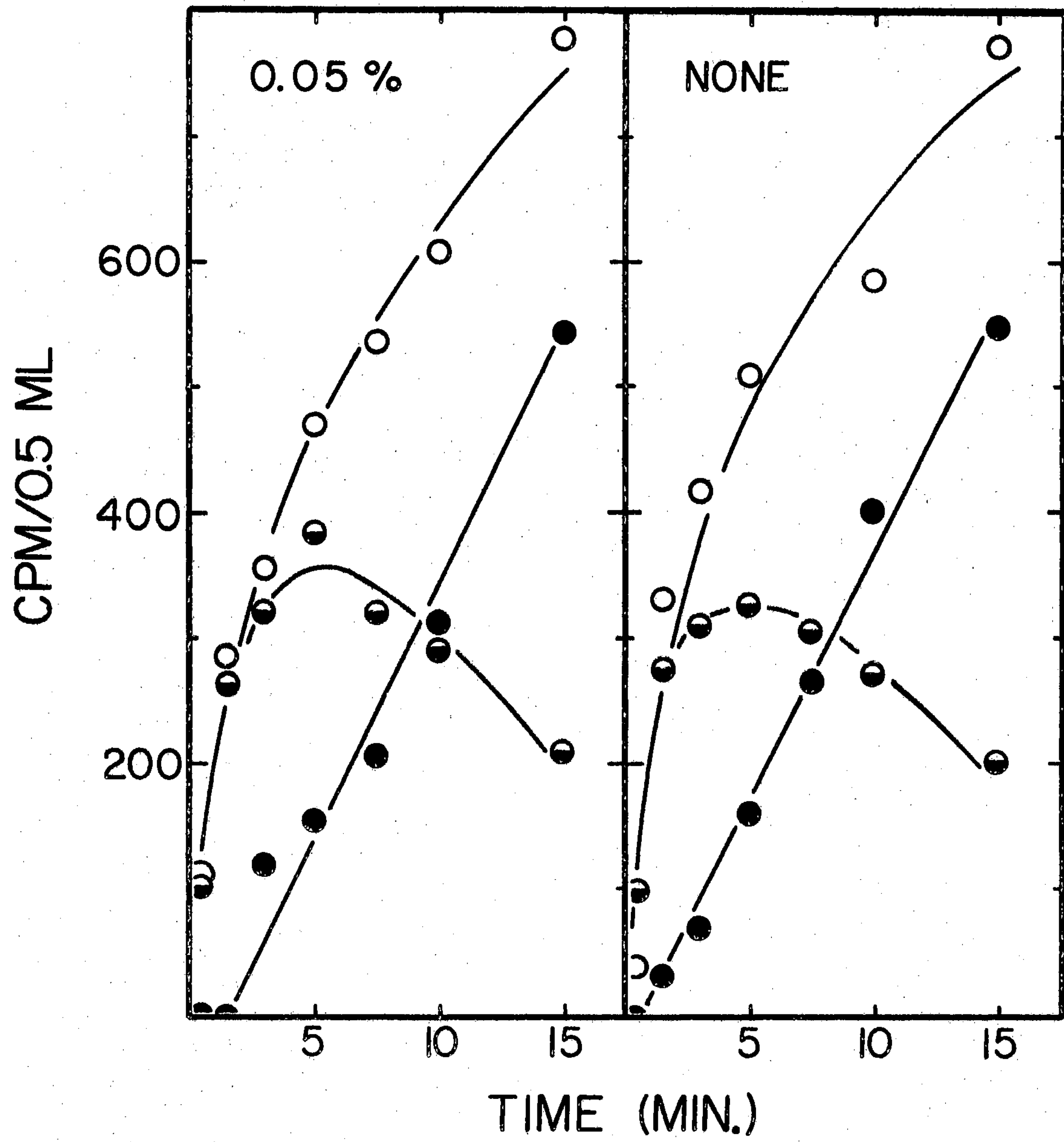

0.05 %
NONE
600
400
200
CPM/0.5 ML
5
10
15
5
10
15
TIME (MIN.)

TABLE XV

EFFECT OF PEA UPON LEAKAGE OF A_{260} MATERIAL AND PROTEIN

Experiment A	A_{260}
Cells	0.211
Cells + PEA	0.285

Experiment B	CPM/ml
Cells	7,250
Cells + PEA	7,956

Experiment A: B. subtilis cells were grown 1 hour with or without PEA (0.05%), removed and harvested by centrifugation. The PEA was extracted from the supernatant solution with ether. The absorbance of the supernatant solution was then measured at 260 mμ.

Experiment B: B. subtilis cells were grown in minimal medium (plus 50 μg/ml of both tryptophan and histidine), supplemented with ^{14}C-L-leucine for 5 hours. The cells were then centrifuged, washed, and suspended in fresh minimal medium (supplemented with 50 μg/ml of tryptophan and histidine) with and without PEA (0.05%). The cells were incubated at 37° for 1 hour, then removed by centrifugation. The radioactivity of 1 ml of the supernatant solution was determined. The cells contained 170,000 CPM/ml, thus the leakage was 4.4% of the total radioactivity.

CHAPTER V

DISCUSSION

Sucrose Density Gradient Centrifugation of Competent *B. subtilis* Cells

Competence is a physiological state required of bacterial cells before they can undergo transformation; however, many facets of this process are unclear. The procedures for obtaining maximum competence are empirical and vary with such factors as changing laboratories, water, supplies, etc. The only way to detect a competent cell is through the process of transformation or transfection after which the cell has been modified. The absence of a suitable method for separation of competent cells from the bulk population and the fact that competent cells make up only 1% to 5% of the cell population (35) are the primary reasons for the lag in the understanding of competence.

In *B. subtilis*, competence appears optimally in minimal medium toward the end of exponential growth (42, 97) while complex media yield populations of low competence (43). The essential requirements for the development of competence are glucose-salts, the auxotrophic requirements, additional small quantities of amino acids which are not required for growth but stimulate competence (histidine, tryptophan, valine, glycine, aspartic acid, threonine, methionine, lysine, and arginine), chelation of cupric ions, and magnesium ions (38). Spizizen and co-workers (43, 97) obtained competent cultures by "step-down" to

growth limiting medium after 4 or 5 hours of normal growth, which suggests that competence is associated with unbalanced growth; however, with transfection (42),"step-down" conditions are not necessary, and the onset of competence varies with the growth rate but consistently occurs 3 hours after cessation of logarithmic growth. Therefore, competence in B. subtilis is associated with a specific phase of growth.

Evidence has accumulated which suggests that there are biochemical and physical differences between competent and noncompetent cells which might make possible their separation. Nester and Stocker (35) found that competent cells were more resistant to penicillin than noncompetent cells. Competent B. subtilis cells contain more N-acyl muramyl-L-alanine amidase than cells from poorly transformable strains (61). Jensen and Haas (55, 56) have shown differences in the surface charge of competent and noncompetent cells. A study (87) of macromolecular syntheses in competent and newly transformed cells demonstrated that competent cells were latent in synthesis of DNA and stable RNA, but were extensively synthesizing protein.

A genetically homogeneous population of bacteria is heterogeneous with respect to the individual cellular processes occurring. Thus, although each cell independently traces the same growth cycle within small statistical fluctuations (98), at any instant a culture consists of cells of all ages. Since competence is related to the growth cycle at the end of exponential growth, the more homogeneous the cell population the higher the level of competence. Therefore, the synchronization of the growth of a bacterial population is of importance in the development of competence. Most procedures for the attaining of

competence result in synchrony by controlling growth conditions. Other methods for obtaining a homogeneous population are based on the fact that the cell size varies with the phase of growth. Gillis *et al*. (99) used a filtration technique for synchronizing cells of the vegetative phase just prior to the inception of sporulation. However, the time required for sporulation permits asynchrony to develop limiting the effectiveness of such a procedure. Since the size distribution of cell populations can be correlated with their age distribution (98), Manor and Haselkorn (100) developed a CsCl zonal centrifugation technique for size separation of an exponentially growing culture of *E. coli*. Church and Halvorson (101) used sucrose as the supporting medium for spore isolation, but, because of the high density of the spores, most of the preparation was recovered as a pellet. Later Tamir and Gilvarg (102) separated and characterized vegetative cells and spores of *B. megaterium* on a gradient formed with Renografin (methyl-glucamine N,N'-diacetyl-3,5-diamino-2,4,6-triiodobenzoate). The gradient pattern also provided a measure of the efficiency of sporulation and degree of synchrony of a sporulating culture.

Singh and Pitale (89) used sucrose density gradient centrifugation to separate potential transformants from the bulk population. The sucrose medium was supplemented with 0.1 *M* NaCl and 0.05 *M* sodium citrate. The data presented in Table I revealed that suspension of competent cells in this medium resulted in the loss of viability and transformability. When sucrose was supplemented with minimal medium plus 10 μg/ml each of tryptophan and histidine, no decrease in viability or transformability of *B. subtilis* was found.

Sucrose density gradient centrifugation of cells on a linear 8% to

30% (w/v) sucrose gradient resulted in procuring a peak of cells which had a higher frequency of transformation than the bulk of the population (Figures 1 and 2). The fraction of cells which contained a higher frequency of competent cells sedimented slower than the bulk population. The fractions at the peak of the band of competent cells had a frequency of transformation 3- to 4-fold higher than control cells.

The possibility that the peak of cells with a higher frequency was an artifact due to different cell concentrations in the various fractions was eliminated by adjusting the cell concentrations by dilution. Bulk or competent cells were not selectively lost or destroyed by the process of sucrose gradient centrifugation (Table II).

While this dissertation was in preparation and after this material had been presented at professional meetings[1], three independent reports on the separation of competent cells using density gradient centrifugation have appeared (103, 104, 105). Singh and Pitale (103) found that the transformed cells of a population had taken up radioactive DNA while the bulk population had not. The cells were centrifuged again using sucrose supplemented with 0.1 M NaCl and 0.05 M sodium citrate and the centrifugation was performed after exposure of the culture to DNA. Cahn and Fox (104) used gradients composed of Renografin for the separation of the *B. subtilis* culture. Two components were obtained on the basis of buoyant density with competent cells being lighter.

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[1]Based on papers presented at the Tetrasectional Meeting of the American Chemical Society, Tulsa, Oklahoma, March 18, 1967; Transformation Meeting, Estes Park, Colorado, June 14, 1967; and West Central States Biochemistry Conference, Lincoln, Nebraska, November 4, 1967.

The cells were recovered by Millipore filtration of the fractions, and the only cells capable of incorporating transforming DNA were the lighter ones. Similar results were also obtained by Hadden and Nester (105) with the additional finding that transformation was inhibited in the presence of 2.5% (or higher) Renografin.

The method of density gradient centrifugation described in Chapter II has at least two significant advantages over the methods previously reported (103, 104, 105), one of which is that techniques reported herein involve only a 5 minute centrifugation. The other methods involved a centrifugation time of 20 to 40 minutes. Since competence is a transient state, during a 30 minute period after maximum competence has been obtained, there can be a 50% loss in competence (42). Therefore, the importance of employing a technique requiring less time is readily evident. Another advantage of the method described herein is the fact that the medium used for the gradient has no effect upon the viability or transformability of *B. subtilis*. The medium used by Singh and Pitale (89, 103) decreased both the viability and transformability while the presence of Renografin (105) inhibited transformation. Therefore, methods using Renografin (104, 105) required the removal of cells from the Renografin before transformation was carried out. Since the suspending medium described in Chapter III had no effect upon transformation, the fractions collected can be assayed directly for the number of competent cells present by transformation.

Effect of PEA upon Transformation

Three possible sites for the action of PEA upon bacterial cells

have been suggested: 1) inhibition of DNA replication at the chromosome level, 2) inhibition of the synthesis of RNA (possibly messenger RNA), or 3) effects upon cellular permeability (by action on the cell membrane). These three sites are involved in the process of bacterial transformation; therefore, establishment of the effect of PEA upon B. subtilis transformation would add important information concerning the mode of the action of PEA.

When 0.25% PEA was added to Trypticase soy agar and various bacterial species were streaked out, most gram-positive bacteria including B. subtilis grew while the growth of gram-negative bacteria was inhibited (1, 2). Exceptions were the gram-negative bacterium Pseudomonas fluorescens, which grew at a PEA concentration of 0.35%, and the inhibition of gram-positive Mycobacterium phlei and Micrococcus smegmatis at 0.25% PEA. At a concentration of 0.5%, all organisms tested were inhibited except the streptococci. Since the B. subtilis transformation system was used in the present study, the effect of varying concentrations of PEA upon growth was determined by more quantitative techniques. Concentrations of 0.1% PEA or less had no effect as determined by turbidity measurements (Figure 3). When the effect of a 60 minute incubation of PEA upon the viability of B. subtilis was tested by plating, similar results were noted (Figure 4). In searching for the primary site of PEA action the stratagem was adopted that a concentration of PEA which does not inhibit general cellular processes would allow the study of the effect of PEA upon bacterial transformation in a relatively uncomplicated fashion. Since concentrations of PEA below 0.1% appeared to have no effect upon growth or viability, PEA at a concentration of 0.05% was incubated with B.

subtilis for periods as long as 10 hours (Figure 5). There was no effect on viability, and this concentration was used for most of the experiments.

When 0.05% PEA (4×10^{-3} M) was added concomitantly with DNA to competent B. subtilis cells, a 50% to 70% decrease in transformation was observed. Concentrations of PEA between 0.01% and 0.15% inhibit transformation without decreasing the total cell titer (Figure 11). Maximum inhibition of transformation is observed when competent cells are incubated with PEA an hour or more prior to the addition of DNA (Figure 12).

Zahn et al. (18) demonstrated the inhibition of in vitro DNA synthesis by 4×10^{-2} M PEA. Since initiation of DNA replication and RNA synthesis involve the proper functioning of DNA in a particular configuration, the effects of PEA could be due to the physical interaction of PEA with DNA. PEA at concentrations of 10^{-2} to 10^{-3} M had no effect upon the physicochemical properties of DNA such as thermal denaturation, renaturation, and viscosity (16, 17). There were no studies pertaining to the effect of PEA upon the biological activity of DNA. Since a study of the biological activity of DNA would give greater sensitivity for detection of the effect of PEA upon DNA than would physicochemical studies (106), the assay of transforming activity of DNA treated with PEA was performed. PEA at a concentration of 10^{-2} M did not irreversibly alter the biological activity of DNA (Figure 6). The possibility that DNA reversibly interacted with only a few PEA molecules could not, however, be eliminated. Use of ^{14}C-PEA allowed the detection of PEA at concentrations as low as 10^{-6} to 10^{-7} M and, therefore, increased the sensitivity of detection of complex

formation between PEA and DNA. Using three different systems (Sephadex and MAK column chromatography and CsCl density gradient centrifugation) for the detection of complex formation, no evidence for the interaction of PEA and DNA was observed. Using the values for molecular weight and gene size for B. subtilis DNA obtained by Nester, Schafer, and Lederberg (107), calculations revealed that the level for detection of complex formation between radioactive PEA and DNA was of the order of 10 PEA binding sites per gene.

Mahler and Mehrotra (108) found that detection of the interaction of cadaverine and putrescine with DNA by alteration of the melting temperature required concentrations of the diamines of 10^{-3} M. Use of radioactive PEA as described in Chapter IV increased the sensitivity of detection of PEA-DNA complex formation more than 1000-fold over the physicochemical methods. Investigations which demonstrated the interaction of acridine orange (109) and actinomycin (110, 111) with DNA used concentrations of 10^{-5} M. Since the sensitivity of detection of complex formation between PEA and DNA reported herein is greater than that previously reported for the detection of complex formation between DNA and diamines, acridine orange, and actinomycin, PEA does not form a complex with DNA at the same order of magnitude as the other agents.

Further information was gained about the nature of the inhibition of transformation by PEA by the addition of excess DNA and competent cells with PEA to the transformation system. An excess of DNA did not reverse the inhibition of transformation while an excess of competent cells reversed the inhibition both by increasing the number of transformants and the transformation frequency (Table V). These results indicate that PEA affects competent cells rather than DNA.

The inhibition of transformation was observed even when PEA was removed prior to the transformation period if the competent cells had been incubated with PEA an hour or more (Table VI). PEA treated cells do not regain competence (in the absence of PEA) until a new wave of competence is initiated (Figure 13).

To determine PEA's site of action it was necessary to establish which step of transformation PEA was influencing. Nester and Stocker (35) demonstrated a biosynthetic latency of 3 to 4 hours in the synthesis (expression) of the enzyme for which genetic information had been introduced. There is also a 3 to 4 hour lag after uptake of exogenous DNA before covalent association of the introduced markers with the recipient genome (40). Therefore, it is possible to divide transformation into steps associated with extracellular processes of reversible and irreversible uptake and those dealing with the intracellular processes of synapsis, integration, and expression. Inhibition of transformation after removal of PEA and the similarity in the kinetics of inhibition of transformation by PEA and DNase (Figure 14) suggest PEA is inhibiting the extracellular processes which occur after DNA uptake. Addition of PEA and incubation with cells 30 minutes after addition of DNA (Table X) had no effect upon transformation.

Direct measurements of the uptake of DNA were made using ^{3}H-DNA. PEA inhibited the transport of ^{3}H-DNA (Table XI) into the bacteria to the same extent as transformation while there was no effect upon the reversible (DNase sensitive) attachment of ^{3}H-DNA to the cell (Table XIII). Thus, PEA, at a concentration which has no effect upon the growth or viability, inhibits *B. subtilis* transformation by inhibiting the transport of exogenous DNA without affecting the other steps of

transformation. Since the steps of transformation which involve expression of the newly acquired information require the synthesis of messenger RNA and specific protein, the absence of inhibition of expression by PEA under these experimental conditions suggests that the main effect on transformation is on the transport process.

PEA (0.05%) did not affect the synthesis of protein, DNA, or RNA but inhibited the uptake into the cell of the radioactive precursors of each of the macromolecules. This inhibition of uptake could possibly be partially responsible for the conflicting views concerning PEA's mode of action. Inhibition of DNA and RNA synthesis previously reported (9, 17, 20) was measured by determining the incorporation of radioactive precursors into acid precipitable material without regard to the pool sizes. The data reported herein indicates that the permeability effects and pool sizes cannot be ignored.

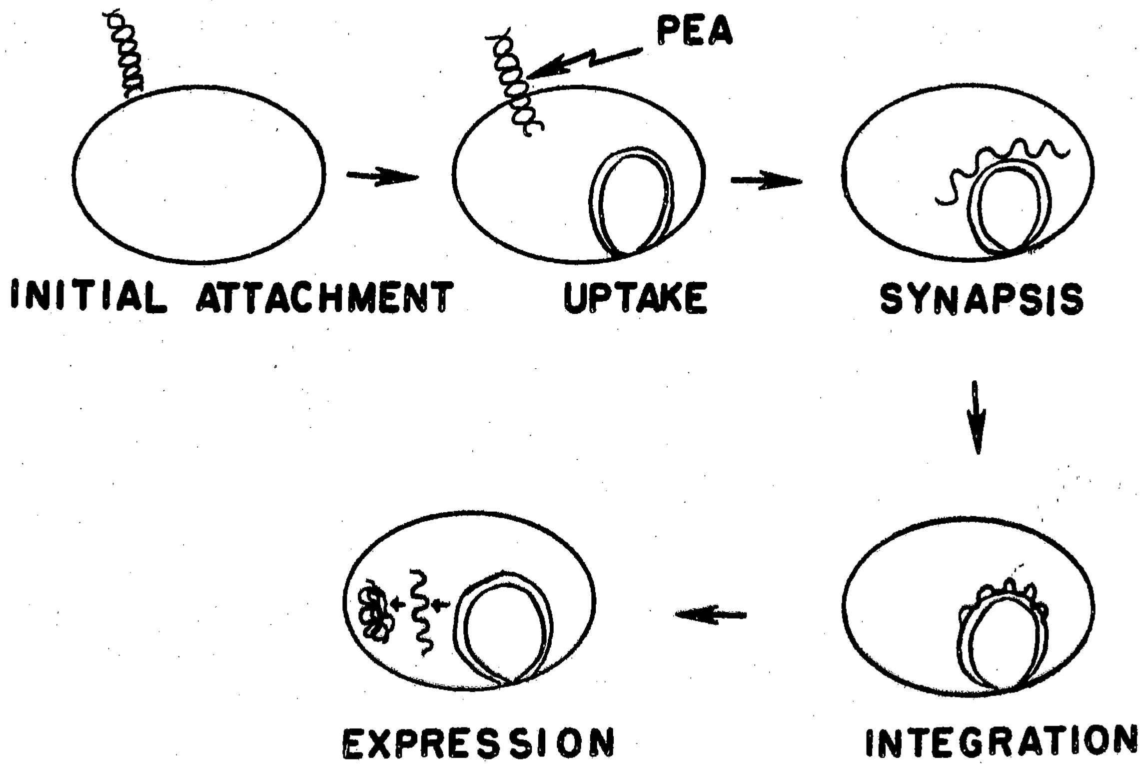
Although PEA inhibits the transport of radioactive precursors, Figures 25, 26, and 27 reveal that removal of PEA reverses the inhibition of uptake of radioactive precursors but not the inhibition of ^{3}H-DNA uptake or transformation (Table XII). Therefore, the inhibition of the uptake of DNA into competent cells is more specific and protracted than the inhibition of uptake in general.

The inhibitory action of PEA on the transforming system is on the competent cells since: 1) greater inhibition resulted when competent cells were treated with PEA prior to the addition of DNA; 2) inclusion of PEA in the incubation medium during the development of competence prevented the appearance of competence while the cell titer increased; 3) PEA inhibited transformation even though it had been removed from the competent cells by centrifugation and washing prior to the addition

of DNA if the treatment of competent cells with PEA had been longer than an hour; and 4) the inhibition of the transformation system was reversed by the addition of excess competent cells but not by the addition of excess DNA. PEA acts on competent cells rendering them noncompetent so that they are unable to take up DNA. This inhibition of DNA uptake by competent cells occurs at a concentration of PEA which has no effect upon the general cellular processes.

Figure 28 shows a schematic representation of transformation indicating the process or step affected by PEA.

Figure 28. Site of Action of PEA upon the *B. subtilis* Transformation System


PEA
INITIAL ATTACHMENT
UPTAKE
SYNAPSIS
INTEGRATION
EXPRESSION

SUMMARY

Competent cells can be separated from the bulk population by sucrose density gradient centrifugation. The competent cells sediment slower than the bulk of the cells indicating that they are less dense than noncompetent cells. The density gradient centrifugation procedure described herein requires less time than previously reported procedures and the supporting medium has no effect upon viability or transformability.

PEA at a concentration of 0.05% does not reduce the growth rate of B. subtilis as determined by A_{630} measurements nor does a 60 minute exposure to this concentration reduce the viable titer. When 0.05% PEA was added concomitantly with DNA to competent B. subtilis, there was a 50% to 70% inhibition of transformation without any effect on the viable titer of the cells. Treatment of isolated transforming DNA with 0.5% PEA was without any effect on the specific biological activity, and no evidence for binding or interaction of ^{14}C-PEA and DNA was obtained by Sephadex chromatography, MAK chromatography, or CsCl density gradient centrifugation. Addition of PEA after the uptake of DNA was completed did not inhibit transformation, and the potential transformants became resistant to DNase and PEA inhibition at the same time. Incubation of competent cells with PEA prior to the addition of DNA resulted in a greater inhibition of transformation, and, if this incubation was longer than an hour, removal of the PEA by centrifugation and washing did not restore competence. An excess of competent

cells reversed the PEA inhibition of transformation, but an excess of DNA did not. PEA inhibits the transport of radioactive DNA but not the initial attachment of DNA to competent cells. Concentrations of PEA (0.05%) which inhibited transformation do not affect the synthesis of DNA, RNA, or protein. PEA (0.05%) does inhibit the uptake of radioactive precursors of macromolecules but this inhibition is reversed by the removal of PEA while the inhibition of uptake of radioactive DNA is not. Thus, PEA acts upon the competent cell in some manner rendering it unable to take up DNA.

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