STUDIES ON THE INITIAL ATTACHMENT AND UPTAKE OF DNA IN *BACILLUS SUBTILIS* TRANSFORMATION

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STUDIES ON THE INITIAL ATTACHMENT AND UPTAKE

OF DNA IN BACILLUS SUBTILIS TRANSFORMATION

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Dedicated to my Father, who always stimulated my initiative and never failed to make me proud.

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CHAPTER I

INTRODUCTION

Genetic transformation in bacteria, as discovered by Griffith in 1928 (1), was originally interpreted as the recipient cells already having the capability of expressing a certain trait in several ways, but requiring the transforming principle to stimulate a particular expression (2). Avery, McLeod, and McCarty in 1944 (3) revealed that the transforming principle was deoxyribonucleic acid (DNA), which identified DNA as the molecule carrying hereditary determinants.

Bacterial transformation is now defined as the transport of exogenous DNA into a recipient bacterium, its integration into the bacterial genome, and its subsequent expression. A variety of bacterial species are capable of transformation, including Pneumococcus, Streptococcus, Diplococcus, Hemophilus, Neisseria, Rhizobium, and Bacillus. Although particulars in the transformation of each species may differ, all transformable species share common features of the process. Yasbin, et al. (4) have described seven stages in the transformation of Bacillus subtilis.

1. Initial attachment of exogenous DNA to a competent bacterium.
2. Formation of a deoxyribonuclease resistant DNA-cell complex.
3. Formation of a DNA-cell complex with the donor DNA having single strand characteristics.
4. Transport of the donor DNA across the cell membrane.

5. Formation of a donor-recipient DNA complex.

6. Integration of donor DNA into the recipient chromosome.

7. Replication of the recombinant DNA.

Since this study is concerned with the initial attachment and uptake of DNA in B. subtillis, only literature pertaining to stages 1-4 described above will be reviewed. Total attachment will be defined as that donor DNA which is bound to a recipient bacterium. Initial attachment is that DNA attached to the bacterium which has not begun to be taken up. Irreversible uptake is the process rendering attached DNA insensitive to extracellular nucleases. Literature to be discussed will be done so in terms of these definitions.

Since the premier steps in the transformation sequence are those involving the initial attachment of donor DNA to a recipient cell, and its subsequent uptake, some understanding of the ill-defined physiological state of the bacterium, called competence, which gives the bacterium these capabilities, is essential. Spizizen (5) suggested that competence may be related to sporulation. Some asporogenous mutants have impaired ability for competence development, but this does not necessitate a direct relationship. Whether an asporogenous mutant can express competence may depend on whether the block occurs before or after the competence phase of presporulation.

The state of competency is unstable, and its induction varies with the specific strain of bacteria. That competency occurs at a specific time during cell growth was demonstrated by Hotchkiss (6), using synchronized Diplococcus and observing waves of competency. Spencer and Herriott (7) found a high level of competence could be induced in Hemophilus influenza if cells, previously grown in complex medium, were

incubated in a defined medium which would limit cell divisions. A similar "step down" or unbalanced growth procedure for competence development can be used for B. licheniformis (8) and B. subtilis (9).

Once competent, B. subtilis are different from those that are non-competent in several respects. Consistent with the presporulation hypothesis, Nester (10) and Nester and Stocker (11) showed competent and newly transformed cells were neither multiplying nor synthesizing cell wall material, due to their penicillin resistance. These experiments used the nutritional step down procedure of Anagnostopoulos (9) for competence development, in which the cells are beginning to enter a stationary phase prior to resuspension in transformation medium. Singh and Pitale (12) studied the effect of penicillin on cells made competent by the nutritional step down procedure and by the spore germination method of Ephrati-Elizur (13). They found transformable cells are initially susceptible to penicillin, but if grown without interruption, penicillin resistance is obtained after 90 to 180 minutes.

B. subtilis also shows distinct alterations in macromolecular synthesis during competence development. Competent cells are heterogeneous with respect to DNA synthesis; approximately half the competent cells do not synthesize DNA and the remainder do so at a reduced rate (14). Protein synthesis is essential in developing competent cells (15), yet stable RNA synthesis does not occur at an appreciable rate. Dooley, Hadden, and Nester (15) suggest such alteration in synthesis will lead to the decreased buoyant density of these cells.

Spizizen (16) suggested that competent B. subtilis were changed in their cell wall structure, showing that after a comparable period of growth, a more transformable strain contains more galactosamine

and alanine, but less glutamic acid, diaminopimelic acid, and glucosamine than a less transformable strain. The galactosamine content in a transformable strain increases during growth and reaches a maximum at the peak of competence. Young (17) found that competent strains of B. subtilis have more N-acetyl galactosamine associated with the cell wall teichoic acid. Suspensions of competent cell walls will also lyse three to five times faster than noncompetent cell walls, due to the presence of N-acylmuramyl-L-alanine amidase (18).

Dooley, Nester, and Hadden (15) resolved five stages through which a competent cell must pass, based on the described alterations.

1. An alteration in the rates of DNA and RNA synthesis, such that a cell becomes "precompetent".
2. Irreversible commitment to the precompetent state, after which a return to normal growth is impossible.
3. Acquirement of a low buoyant density.
4. Conversion to the competent state, accompanied by biosynthetic latency, enlarged mesosomes, and altered cell wall.
5. Outgrowth from the competent state.

The described compositional changes in the cell wall occurring with competence may be reflected in physical properties of the cell surface. That competency is related to the surface charge was shown by Jensen and Haas (19,20) who found a range of surface charge values within which competent cells will undergo transformation. They found a newly competent cell appears quickly and carries the maximal negative charge. There is a kinetic flow of competent cells from the higher to lower charged fractions of the population, indicating a progressive loss of the surface charge.

Competent cells are also smaller and less dense than noncompetent ones. Singh and Pitale (21), using zonal centrifugation in linear sucrose gradients, found normal vegetative cells to have a density between 1.427 and 1.136 g/ml compared to between 1.105 and 1.115 g/ml for transformable cells. Their lower density permits the use of density gradient centrifugation for isolation of competent cells using linear sucrose gradients (22,23), and linear (24) or discontinuous (25) gradients of Renografin.

This ability for separation of the competent fraction allows measurement of its size. Fractionating transformed B. subtilis on discontinuous Renografin gradients, Cahn and Fox (24) found 90-95% of the bacteria in a heavy fraction which contained less than 2% of the transformants. A lighter band accounted for more than 98% of the transformants. Alternatively, Goodgal and Herriott (26) estimated the size of the competent fraction in H. influenza from the frequency of two single transformants (N_1 and N_2), the double transformants (N_D) for the two unlinked markers, and the total number of cells (B). The percentage of competent cells equals $(N_1N_2/N_DB) \times 100$. Using markers with frequencies of transformation between 0.5% and 0.75%, they found the competent cells to be 75% of the total population. Using this calculation for competent Pneumococcus (27), a value greater than 100% was obtained. Cahn and Fox (24) suggested that the estimate arrived at by Goodgal and Herriott's method be divided by two, because only half of the potential double transformants are seen. If only a single strand of DNA is integrated, then either of the two strands has an equal efficiency for transformation. This method also assumes the cells are uninuclear.

Vermeulen and Venema (28) compared the estimated size of the competent fraction obtained by Goodgal and Herriott's (26) method with that obtained by autoradiography of competent B. subtilis exposed to labeled donor DNA. Similar results were obtained by either method; approximately 15-17% of the culture was competent. The size of the competent fraction, as determined from the frequency of double transformants was independent of the DNA concentration and length of exposure to DNA. Using autoradiography, Vermeulen and Venema also showed increasing transformation frequencies with longer exposures to donor DNA to be linearly related to the amount of DNA taken up by individual cells.

The DNA for transformation of competent bacteria may be made available by a purification scheme (29), cell lysis (30,31), or by spontaneous release by actively growing cells (32). Molecular size and configuration of donor DNA is significant in the transformation process. Sonication of pneumococcal DNA (33) to a molecular weight of 1.2×10^6 was accomplished before complete loss of transforming activity. B. subtilis DNA has an average molecular weight of 2.1×10^7, depending on the method of isolation and the assumed molecular weight of the intact genome (34). Szybalski and Opara-Kubinska (35) found B. subtilis DNA has little transforming activity below a molecular weight of 10^7.

Barnhart and Herriott (36) found sonication of Haemophilus DNA resulted in progressive reduction in attachment, irreversible uptake, and transformation. One minute of sonication reduced transformation by 90%, total attachment by 60%, and irreversible uptake by only 20-25%, implying that competent cells can bind and take up inactive pieces

of DNA. Subsequent studies (37,38) found the reduction of transformation with sonication comparable to the reduction of integration of fragments into the recipient genome. Lacks (39), showing how DNA size affects the probability of transformation in Pneumococcus, derived an expression for this probability as a function of DNA length and the frequency of exchanges between donor and recipient strands (switching frequency). The probability of integration depends not only on the location of a marker, but on the length of the DNA on which it resides (39). Guild, et al. (40) presented a model based on pneumococcal transformation, using the expression of Lacks (39), which can be applied to other transformation systems. Randolph and Setlow (38) found the inactivation of H. influenza DNA by sonic radiation to fit this model, rather than a target theory of inactivation.

Inactivation of transforming DNA by ionizing radiation, similar to sonication, occurs with a reduction in transformation proportional to the reduction in integration. Ultraviolet irradiation reduces transformation to a greater degree than the reduction in integration. This may reflect the products of such inactivation. Sonic irradiation produces 5'-phosphoryl-terminated polynucleotides with little or no base specificity (41,42) from double strand breaks (43). Ionizing radiation produces single and double strand breaks, while ultraviolet irradiation forms pyrimidine dimers.

Heat denaturation of transforming DNA in Pneumococcus (44), B. subtilis (45), and H. influenza (46) does not remove all of the transforming activity. Guild (47) and Rownd, et al. (46) showed that the transforming activity remaining with the denatured DNA did not separate after sedimentation in a CsCl density gradient, as native DNA would.

The irreversible uptake system can accomodate DNA in a form other than native. Goodgal and Herriott (26) found heat denaturation reduced attachment and irreverisble uptake to 29% and 6% of the native control, respectively, in H. influenza. Digestion of the denatured DNA with E. coli phosphodiesterase, active only on single deoxypolynucleotide strands, did not reduce the transforming activity or irreversible uptake, implying specificity of configuration for uptake, but perhaps not for attachment.

If the number of transformants is measured as a function of DNA concentration, a linear relationship is observed until saturation is reached. Such a curve indicates that the interaction is between a competent bacterium and a single molecule of transforming DNA. Such saturation and autoradiographic data (48) support the presence of definite DNA binding sites on the bacterial surface.

As the first point of contact to the donor DNA, the cell wall of the transformable bacterium should contain the binding site or at least play a role in transformation. Young (49) found removal of the cell wall after DNA attachment did not alter the DNA absorbed to the protoplast, concluding DNase resistant DNA was firmly membrane bound. Hirokawa and Ikeda (50) and Tichy and Kohoutova (52) have both reported transformation in protoplasts, but discrepancies in their findings makes an evaluation difficult. Tichy and Landman (51) explain the results of Hirokawa and Ikeda (50), Tichy, Rytir, and Kohoutova (53), who transfected B. subtilis treated with lysozyme, and Prozorov (54), who enhanced the transformability of B. subtilis by light lysozyme treatment, in terms of the formation of "quasi spheroplasts". Tichy and Landman found no transformation in protoplasts, but did find DNA

attached to quasi spheroplasts would lead to transformation if cell wall biosynthesis was resumed. Protoplasting does not allow entry of transforming DNA, perhaps due to the expulsion of membranous structures called mesosomes. Plating protoplasts on media which lowers the number of mesosomes reduces the recovery of transformants from the bacilli obtained (51).

Wolstenholme, Vermeulen, and Venema (55) provided evidence for the involvement of mesosomes in B. subtilis transformation, using electron microscopic autoradiography. Exposure of competent cells to tritiated DNA showed the absorbed donor DNA associated with, but outside the mesosomes, and particularly those located in the cytoplasm. The total number of mesosomes increased several fold during maximal competence, compared with the number present during earlier stages of growth. One role for mesosomes is suggested to involve the production of enzymes required for the integration of donor DNA and/or for the degradation of donor DNA not to be incorporated.

Vermeulen and Venema (56), using serial section electron microscopy, distinguished two types of mesosomes; those connected only to the plasma membrane and those connected to the nuclear bodies. Besides different cellular distributions, the number of each type depends on the degree of competency of a bacterium. Nuclear mesosomes increase in number during the increase in competence of B. subtilis, with a concommitant decrease in the number of plasma membrane mesosomes. As competence decreases, the reverse is seen. Electron microscopic autoradiography of sections of B. subtilis showed ^{3}H-DNA did become associated with the mesosomes, and that with time, the DNA migrates towards the nucleoid (57). After completion of DNA uptake, transport of DNA

is complete within 15 to 60 minutes, and recombinant type activity is completed within 45 minutes after incubation. This does not support the view of Javor and Tomaz (48) that uptake and integration of DNA occurs at the same cellular location. They had autoradiographic evidence showing 90% of the grains were located at the tips or middle of B. subtilis after exposure to labeled DNA. The irreversibly absorbed DNA remained at these sites even after 90 minutes of incubation. Vermeulen and Venema presumed that during its migration the DNA is associated with the nuclear mesosomes, except in the case of DNA uptake in the tip regions, where only plasma membrane mesosomes are found. This DNA is transported peripherally to the middle zones, and then to the nucleoids.

Akrigg, Ayad, and Blamire (58) have postulated a mechanism for DNA uptake in B. subtilis in which the donor molecule passes through only one intact membrane, that of the mesosome. A cell wall localized lytic enzyme is first released and attacks a weak spot in the cell wall. The attacked area would be close to a mesosome, exposing the mesosome to the medium when the cell wall ruptures. Donor DNA associates with the mesosome and migrates to the recipient DNA at a replicating point, where it is integrated. Kohoutova and Kocourek (59) have similarly shown the competence substance in pneumococcal transformation binds to specific sugar determinants on the cell surface. They suggest its binding imposes a change in the surface charge, allowing the reversible binding of donor DNA, and might induce an autolytic enzyme to produce weakened spots in the cell wall.

Specific information on the nature of intermediates of donor origin in any transformation system will shed light upon the attachment

and uptake sequence. The structure of transforming DNA on the surface of Pneumococcus was studied by Morrison and Guild (60). The DNA taken up is single stranded and of lower molecular weight than single strands of the original donor DNA (61,62), being reduced to a median strand length of 2.2×10^6 daltons by double strand scissions, and remaining this length upon transport into the cell. They found little DNase-sensitive denatured donor DNA or DNase-resistant native donor DNA, suggesting the separation of a donor DNA strand from its complement and its uptake to a DNase resistant form are closely associated in Pneumococcus.

A similar process may be involved in the uptake of B. subtilis transforming DNA. Studies on the physical nature of re-extracted donor DNA have yielded a variety of results. The data of Bodmer and Ganesan (63) could not distinguish between the uptake of single or double strand intermediates. Pene and Romig (64) and Ayad and Barker (65) found no evidence for a single stranded intermediate.

Piechowska and Fox (66) extracted single stranded donor DNA from DNase treated transformed cultures of B. subtilis. The majority of donor DNA was not associated with the recipient DNA, but was complexed with a cellular component(s) upon extraction. Longer incubation before extraction found the labeled donor DNA disappearing from the complex and appearing in association with the recipient DNA. Piechowska and Fox compare the cellular component with T_4 gene 32 product, which specifically binds to denatured DNA (67) and may be involved in T_4 recombination (68).

Davidoff-Abelson and Dubnau (69) found isolation of high molecular weight single stranded DNA of donor origin could be accomplished by the

presence of a basic protein during lysis.

Arwert and Venema (70), studying the eclipse phase of donor marker activity, found the donor DNA in lysates to be double stranded and temporarily complexed with a cellular component. In Pneumococcus (71) and B. subtilis (72), but not Hemophilus (73), little donor marker activity is found in DNA reisolated immediately from the complex of a competent bacterium and donor DNA. The eclipsed donor marker activity reappears after a period of incubation. The difference between the single strand eclipsed DNA of Piechowska and Fox (66) and the double strand intermediate of Arwert and Venema (70) may be attributed to different conditions used in releasing the DNA from the cellular component. Three pieces of evidence are used by Arwert and Venema to support double strand eclipsed intermediates; 1) in the earliest sample of lysate, the DNA is slowly sedimenting, 2) donor marker activity is resistant to shear, and 3) the ratio of donor to resident marker activity depends on the DNA concentration used in transforming the second culture.

Dooley and Nester (74) isolated DNA-membrane complexes in transformed B. subtilis on Renografin gradients. Donor DNA binds to the membrane soon after uptake in a manner different than the binding of the recipient genome. The donor DNA is more resistant to shear than recipient DNA, and can be released from the membrane by nonionic detergents or elevated temperatures. A portion of the membrane bound donor DNA is released 10 minutes after uptake, coinciding with the first evidence of recombinant DNA. Enrichment of recombinant DNA in the membrane suggests integration occurs on or near the cell membrane.

Dubnau and Cirigliano (75) suggest donor DNA attaches to the competent cell surface with no detectable delay, and is converted to

double strand fragments (DSF) by double strand endonucleolytic cleavage (76). Still susceptible to DNase treatment, the DSF attach to and penetrate the cell membrane, after which it is DNase resistant. Acid soluble products of the donor DNA, primarily 5'-mononucleotides, are released into the medium (76), presumably due to the action of an exonuclease located externally to the cell membrane. A similar model has been proposed for Pneumococcus (77). Originally, Dubnau and Cirigliano (76) found single strand fragments (SSF) in transformed cell lysates, but of too low a molecular weight to lead to integration. A modified DNA extraction procedure (69) allowed isolation of high molecular SSF formed from DSF (76). Davidoff-Abelson and Dubnau (69) also showed that the formation of the donor recipient complex (DRC) closely followed the disappearance of SSF. The linear and progressive entry of the DNA (75) may involve a unique polarity of the strand. A linear penetration is further supported by the finding that as the DSF fraction decreases in amount, so does its average molecular weight (75).

DSF have lower transforming activity than fragments produced by pancreatic DNase (76). Acid DNase II (78) endonucleolytically degrades by double strand cleavage, and inactivates Hemophilus transforming DNA. Sonication and E. coli endonuclease I are less effective inhibitors (126). Pancreatic DNase, endonuclease I and sonication produce 5' phosphate ends (79,80), while DNase II (78) and the B. subtilis endonuclease (76) generate 3' phosphate termini. Dubnau and Cirigliano (75) suggest such differences in the strand ends, or other unique properties such as single strand segments or specific nucleotide sequences, lower the transforming activity of DSF by destroying its recognition of attachment sites on the competent cell surface. Morrison and Guild

(60) showed that double strand fragments of pneumococcal DNA have transforming activity comparable with that expected for sheared DNA of the same size. This does not support the involvement of a sequence specific nuclease, which would produce fragments with genetically identifiable termini.

Consistent with the size of DSF in B. subtilis, Scher and Dubnau (82) have described a Mn^{2+} or Ca^{2+} stimulated endonuclease that produces fragments of molecular weights from 3×10^6 to 20×10^6.

Venema, Pritchard and Venema-Schroder (72) suggest recovery from the eclipse period precedes integration. Arwert and Venema (70) argue recovery of donor activity is due to integration. Supporting the latter proposal are the two steps involved in the formation of the donor-recipient complex, as described by Dubnau and Davidoff-Abelson (83). The donor DNA is first attached to the recipient chromosome by noncovalent interactions, and has low donor marker transforming activity. A covalent association follows, with the recovery of the donor marker activity and appearance of recombinant marker transforming activity.

Erickson and Braun (84) and Erickson and Copeland (85) propose that DNA replication is required for transformation in B. subtilis. Uptake would occur concomitantly with integration at the replication point, being driven by the movement of the replication point relative to the entry sites of the donor DNA. However, DNA synthesis in competent and newly transformed cells is very low (14,15). Bodmer (86), using 5-bromouracil incorporation and buoyant density analysis, and Dubnau and Cirigliano (87), using an inhibitor of DNA replication in B. subtilis, 6-(p-hydroxyphenylazo)-uracil, provided evidence against

the involvement of DNA replication.

The initial attachment of DNA to the surface of competent B. subtilis is sensitive to shear, and Dubnau and Cirigliano (75) attribute this to the endwise attachment of transforming DNA. If excessive shear is avoided, donor DNA with a sedimentation rate higher than that of DSF can be obtained. This material decreases in amount and in sedimentation rate as the DSF appear. They assume the conversion to DSF confers the resistance to shear to the donor DNA. Transformed cells become resistant to shear before they become DNase resistant. Williams and Green (88) have described the polar entry of DNA in transfection of B. subtilis with phage SP82G, and found uptake involved an initial reversible binding of one specific end, followed by attachment of the second end. The two ends become insensitive to shear before they attain DNase resistance, and before the center of the transfecting DNA becomes shear resistant. Such a two point attachment may be involved in the B. subtilis transformation system. Harris and Barr (89) report that although some DNA at early times is DNase resistant, it may not be irreversibly bound, and can be washed away.

Studying certain parameters and liabilities of the initial attachment and irreversible uptake of DNA would provide a clue to the details of the first steps in the transformation sequence. Barnhart and Herriott (36) studied initial attachment and irreversible uptake of labeled DNA in H. influenza with respect to a variety of parameters. Reversible binding decreased gradually with increasing ionic strength and increased as the pH of the medium decreased, suggesting the initial interaction of DNA with the competent cell surface is ionic in nature. Irreversible uptake had an ionic strength optimum of 0.1 and a pH op-

timum of 6.8.

Since competence involves maintenance of a negative charge on the cell surface, ionic strength and pH changes in the medium may alter the basis on which initial attachment is dependent. Such an alteration may involve the nature of the cell wall composition. Glycerol or ribotol containing teichoic acids occur in the cell walls of most gram positive bacteria, although their function is not well understood. Kohoutova (90) has shown in Diplococcus pneumoniae that teichoic acids serve as a receptor for competence factors. If choline is absent from the teichoic acid in this organism, the autolytic system is almost completely inactive (91), and the cells are noncompetent (92). Young (93) and Glaser et al. (94) have shown in B. subtilis that glucosylated teichoic acids serve as bacteriophage receptor sites. Doyle et al. (95) have described the polyelectrolyte nature of teichoic acids in B. subtilis. Teichoic acid assumes an extended structure in distilled water or dilute buffers. Increasing the salt concentration results in a random coil configuration, caused by a loss of secondary structure and evidenced by changes in viscosity and ultraviolet spectra. Divalent cations show no effect on the teichoic acid structure. Changes in the nature of the cell wall are reflected in the inhibition of the binding of phage Φ25 to B. subtilis and the precipitation of B. subtilis cell wall digests with concanavalin A by the presence of NaCl.

If the initial attachment of DNA to a competent cell was ionic in nature, then temperature should have little effect on this reaction. Lerman and Tolmach (96) did propose an initial attachment reaction in pneumococcal transformation which was temperature and energy independent, and distinct from transport. However, Barnhart and Herriott (36)

and Stuy and Stern (97) found the reversible attachment and irreversible uptake of DNA in H. influenza to be independent and dependent, respectively, of temperature. Irreversible uptake was maximal at 36° C., agreeing with the optimum temperature of 34° C. for transformation in this organsim (26).

Exposure of competent H. influenza to DNA at reduced temperatures led to few transformants (26,36). In B. subtilis, Dubnau (75) claims all initial attachment and uptake of donor DNA ceases upon chilling to 0° C. Morrison and Guild (60) showed little binding occurs at 0° C. in Pneumococcus, but that DNase-resistant DNA increases during incubation at 0° of cells with DNA bound at 25°. Such entry involved conversion of DNA to a single strand intermediate, as at 25° C. It also showed a sensitivity to EDTA, as it would at 30° C. Strauss (98) described an early energy dependent step in the entry of donor DNA in B. subtilis, in which the DNA is DNase-resistant, yet sensitive to cold and cyanide. This implies DNase-resistant DNA is not necessarily inside the cell.

Reduction in temperature may affect the attachment of DNA to a bacterium, in addition to slowing down or stopping the irreversible uptake. Williams and Green (88) found chilling the complex B. subtilis and initially attached transfecting DNA to 4° did not release labeled DNA or recoverable genetic markers, but did prevent marker rescue. Their interpretation is chilling to 4° prevents binding of the second end of the DNA strand, a requirement for the uptake of transfecting DNA, by an alteration in the binding site. If resistance to shear is attained before chilling, indicative of a successful second point of attachment, 4° chilling no longer prevents uptake.

The elevation of temperature can also affect the attachment and

uptake of DNA in competent bacteria. Heating a competent culture of B. subtilis at 50° for 15 minutes prior to the addition of DNA reduces DNase-resistant uptake and transformation (99). McCarty and Nester (99) postulate a heat labile factor is required for uptake and/or retention of donor DNA. Once having lost their competency, heated cells did not regain this ability for at least several hours, suggesting the heat labile factor is synthesized only once during competency. Similarly, Ravin and Ma (100) have reduced the transformability of Streptococcus by heating to 48°.

The temperature dependency of the uptake of donor DNA suggests the involvement of an enzymic reaction, perhaps requiring metabolic energy. Young and Spizizen (45) have shown cyanide and 2,4-dinitrophenol exclude labeled DNA from competent B. subtilis. Barnhart and Herriott (36) observed the irreversible uptake of DNA in H. influenza to be sensitive to the metabolic inhibitors 2,4 dinitrophenol, iodoacetate, and NaN_3; total attachment being affected only by the latter two inhibitors. The cyanide sensitivity of DNase-resistant donor DNA in B. subtilis (98) implies an energy requiring reaction subsequent to the stage defined as irreversible uptake.

A variety of treatments other than metabolic inhibitors perturb the initial steps in the transformation sequence. Morrison (101) described an early intermediate state of donor DNA during uptake in B. subtilis by virtue of the termination of uptake by the addition of excess EDTA. Donor markers become EDTA resistant before acquiring DNase-resistance. Addition of excess Mg^{2+} overcomes the inhibition by EDTA. Morrison accounts for the effect of EDTA on transformation by proposing that it prevents initiation of uptake, explaining his observation that

markers bound to the cell before the addition of EDTA were not kept from entering the cell when EDTA was added. He postulates the following steps for the combination of initial attachment and uptake:

1. Cell + DNA → Cell - DNA
2. Cell - DNA $\xrightarrow{Mg^{2+}}$ Cell - DNA*
3. Cell - DNA* → Cell - (DNA)

In step 1, initial cell-DNA complexes form linearly with time and are wash resistant. Step 2 follows with the bound markers becoming EDTA-resistant. The final step involves the EDTA resistant markers becoming DNase-resistant.

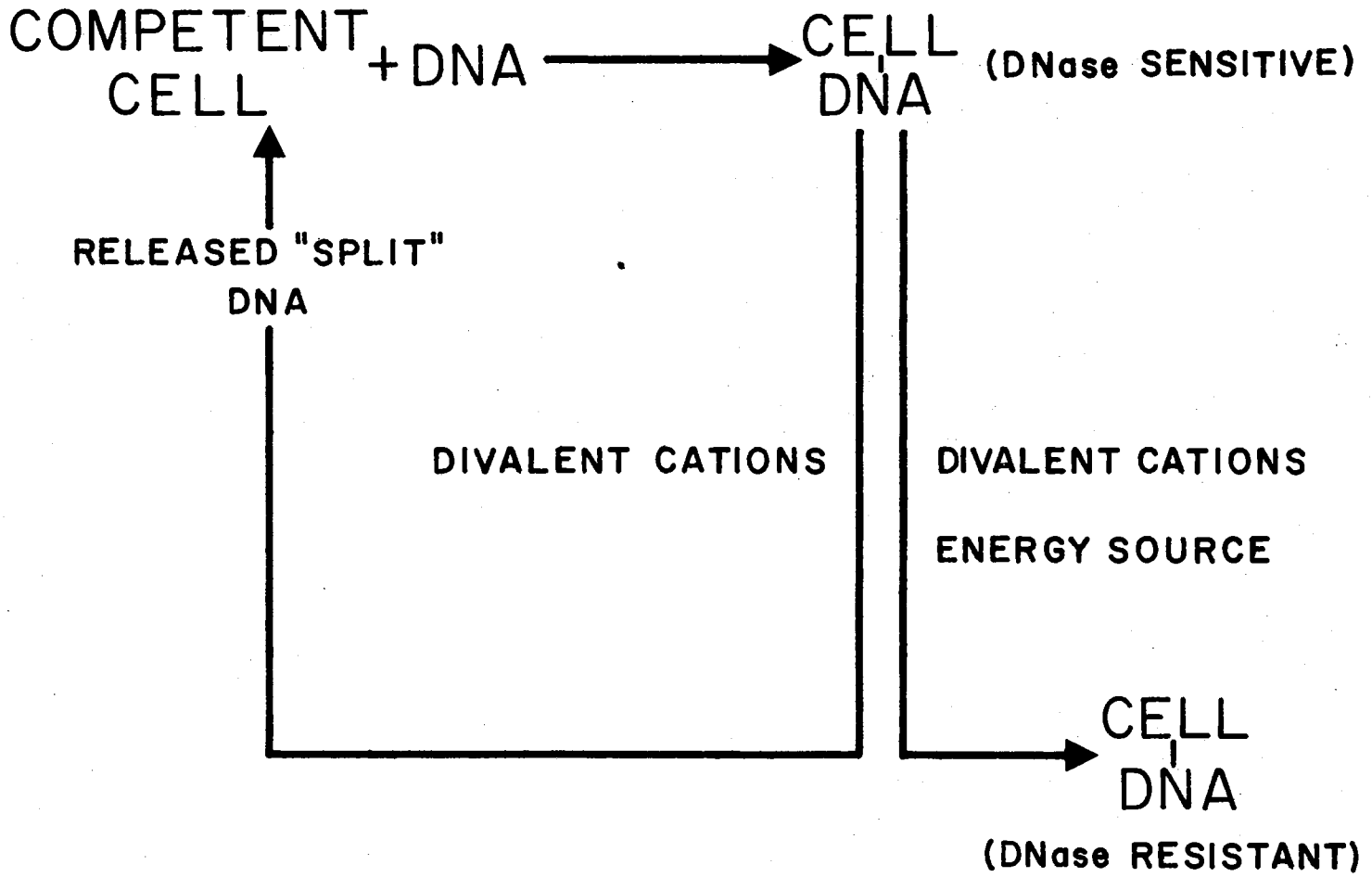
Seto and Tomaz (102) utilized EDTA to separate initial attachment from uptake in Pneumococcus, and confirmed the findings of Morrison and Guild (60) and Lacks (103) concerning the involvement of surface located nucleases in transformation. Labeled DNA loaded onto competent cells in the presence of EDTA accumulates in a DNase-sensitive form, with subsequent steps in the transformation sequence being inhibited. Completion of the transformation process in such preloaded cells was accomplished by the removal of EDTA and the readdition of divalent cations. A rapid decrease in the amount of attached donor DNA was observed, accompanied by an increase in irreversibly bound DNA, the emergence of potential genetic transformants, and the release of acid soluble fragments into the medium. Their interpretation involves Mg^{2+} dependent nucleases required for the uptake of donor molecules attached to the competence-specific sites. They suggest fragments released into the medium, although of lower molecular weight, still retain the ability to reattach to the competent cell surface. The production of such acid soluble fragments was not affected by energy deprivation,

whereas irreversible uptake and transformation were inhibited. The recycling of donor DNA in the Seto and Tomaz (102) model for pneumococcal transformation is shown in Figure 1.

Seto and Tomaz (102) also found the amount of initial attachment was greatly increased in the presence of divalent cation complexing agents, such as EDTA and EGTA.

Another inhibitor of transformation, whose effects are not as well defined as those of EDTA, is phenethyl alcohol. Richardson and Leach (104) found that treatment of competent B. subtilis with 0.05% phenethyl alcohol reduced transformation by 66% without affecting the viability or growth rate. Initial attachment, measured at 4^{o}, was not affected, but the irreversible uptake was reduced. They found no binding or association of phenethyl alcohol to transforming DNA, nor was there any effect on the biological or physical properties of the DNA. That the inhibitory effect was upon competent cells was demonstrated by varying the time of incubation and concentration of competent cells. Urban and Wyss (81), using a 0.3% concentration of phenethyl alcohol, arrived at similar conclusions. Once potential transformants acquired DNase resistance, they were also resistant to phenethyl alcohol. Richardson, Pierson, and Leach (106) found that phenethyl alcohol also inhibited the uptake of thymine, uridine, and serine. All the phenethyl alcohol could be removed by three washes, restoring the ability for thymine, uridine, and serine transport, but not affecting the inhibition of uptake of transforming DNA. Pierson, Weppner, and Leach (unpublished results) have found a protein in the cultural fluid of competent B. subtilis which reverses the phenethyl alcohol inhibition, implicating it as a component in the transport process.

Figure 1. The Seto and Tomaz Model for Pneumococcal Transformation


COMPETENT CELL
+ DNA
CELL DNA
(DNase SENSITIVE)
RELEASED "SPLIT" DNA
DIVALENT CATIONS
DIVALENT CATIONS
ENERGY SOURCE
CELL DNA
(DNase RESISTANT)

Pierson, Weppner, and Leach (107) described another protein factor released from competent B. subtilis by an osmotic shock treatment. Such an osmotic shock reduces transformation by 80-90% by a depression of irreversible uptake. A shock fluid preparation does not restore transformability to osmotically shocked cells, but will enhance the transformability of low competence strains. Only the cultural fluid from a competent culture will restore transformation and DNA uptake in shocked B. subtilis.

Physical and chemical alteration of the bacterial surface will also perturb the initial attachment and irreversible uptake of donor DNA. Oxidation by metaperiodate inhibits conjugation in E. coli (108), the mating reaction of Hansenula wingei (109), male specific phage absorption to E. coli (110), competence development and transformation in H. influenza (111), and transformation in B. subtilis (112). Posinelli and Barlati (112) found periodate treatment of B. subtilis reduced transformation without loss of cell viability. The inhibition was effective if periodate was added immediately before, or within five minutes after the addition of donor DNA. If cells had been incubated with DNA for 30 minutes, addition of periodate had no effect. The action of periodate is not upon the transforming DNA. While only the inhibition of irreversible uptake was measured, Posinelli and Barlati suggest periodate acts on either the attachment sites or the transport mechanism.

Ranhand (113) used the differences in the oxidation of streptococcal surface components at 0^{o} and 37^{o}, and the subsequent effect on binding of donor DNA to demonstrate that the binding sites contain amino acids. Metaperiodate oxidizes monomeric or polymeric carbohy-

drates at specific sites at 0^{o} and 37^{o} (114). Only at 37^{o}, however, will metaperiodate oxidize certain amino acids occurring internally or N-terminally in peptide chains (115). Five millimolar periodate at 37^{o} reduced transformation, total attachment, and irreversible uptake to a greater degree than oxidation by a similar concentration at 0^{o}. Ranhand concluded both initial attachment and irreversible uptake involved a moiety containing susceptible amino acids. Increasing the periodate concentration to 10 mM substantially increased the reduction of attachment, uptake and transformation at 0^{o}, suggesting that the protein involved in attachment of the DNA is linked to a carbohydrate.

Location of the initial attachment sites on the surface of the bacterium exposes them to somewhat the same extent as other surface located structures. Endo (116) observed that treatment of competent B. subtilis in a Waring blendor before exposure to DNA reduces transformation. Such a vigorous treatment might remove structures involved in the attachment and transport of donor DNA, much as it does to other bacterial structures. Wollman and Jacob (117) employed high speed mixing in a Waring blendor to interrupt the chromosomal transfer in conjugating E. coli. RNA bacteriophages adsorbed to the F pili of male E. coli can also be disrupted by a blendor treatment, with no loss in cell viability (118). The F pili themselves can be isolated by shearing them from the cell during blending (119,120).

Finally, antisera against surface components on competent Pneumococci (121), H. influenza (123), and Neisseria catarrhalis (122,124) have been prepared and inhibit transformation. Similar antisera against noncompetent cultures of these organisms were not inhibitory. Bingham and Barnhart (123) found antisera against competent H. influ-

enza reduced irreversible uptake of donor DNA by 80%, suggesting the antigenic determinants were related to the uptake mechanism.

Erickson et al. (125) observed antisera against single strand oligo and polynucleotides inhibited transformation, requiring interaction with the recipient bacteria for maximal inhibition. This was based on the findings that 1) inhibition was independent of the concentration of donor DNA, 2) pre-exposure of the donor DNA to the specific antibodies did not enhance the inhibition, and 3) inhibition of the antiserum could not be removed by washing cells incubated with the antibodies prior to addition of DNA. Erickson, Young, and Braun (125) found the inhibition by such antibodies occurred at a stage when the donor DNA is DNase resistant, implying, as did the observations of Strauss (98), that DNase insensitivity does not necessarily indicate the donor DNA is inside the bacterium.

The intent of this study is the characterization of the initial attachment reaction of transforming DNA to competent B. subtilis, and the relationship of this reaction with the subsequent uptake of attached DNA. Results are reported concerning the comparison of attachment of DNA at 4^{o} or 37^{o}, with respect to various parameters and sensitivities of the reaction, and the effect of treatment in a Waring blendor on the complete transformation process and the initial attachment and uptake of DNA.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

Glycine, K_2HPO_4, KH_2PO_4, ammonium sulfate, magnesium sulfate, magnesium chloride, potassium chloride, sodium citrate, Tris (hydroxymethyl) amino methane, trichloroacetic acid, and sodium meta periodate were obtained from Fisher Scientific Company. Ethylene-diamine tetra acetic acid (EDTA) and diphenylamine were obtained from Aldrich Chemical Company. Potassium cyanide was purchased from the J. T. Baker Chemical Company, while sodium azide and 2, 4 dinitrophenol and sodium dodecyl sulfate were purchased from Matheson, Coleman and Bell. L-Aspartic acid, L-histidine, and phenethyl alcohol are obtained from Eastman Organic Chemicals. L-Methionine and L-arginine were secured from Cyclo Chemical Corporation. L-Leucine, L-threonine, deoxyribonucleic acid (salmon sperm), and casein hydrosylate were obtained from Nutritional Biochmicals Corporation. L-Valine and L-lysine were obtained from California Biochemical Research. L-Tryptophan was purchased from Mann Research Laboratories, Incorporated. Thymidine was purchased from Schwarz Bioresearch. Phenol, sucrose, and glucose were purchased from Mallinckrodt Chemical Works. 3,5 Diacetamide-2,4,6 triiodobenzoic acid (Renografin) was obtained from E. R. Squibb and Sons. DNase (E.C. No. 3.1.4.5), trypsin (E.C. No. 3.4.21.4), and lysozyme (E.C. No. 3.2.1.17) were secured from Worthington Biochemicals. Pro-

nase (B grade) (E.C. No. 3.4.24.4) was purchased from Calbiochem, as was D-alanine-1-^{14}C (7.5 mC/mmole). Protease (subtilisn) (E.C. No. 3.4.21.14) was obtained from Sigma. L-Leucine-^{14}C (291 mC/mmole) and glycerol-2-^{3}H (200 mC/mmole) were purchased from New England Nuclear. Tritiated thymidine (57.8 C/mmole) was obtained from International Chemical and Nuclear Corporation. Bacto-agar, Bacto-yeast extract, Bacto-tryptose, Bacto-tryptose blood base agar, Bacto-beef extract, Bacto-nutrient agar, and Bacto-nutrient broth are all products of Difco Laboratories. Millipore filters (HA 0.45u) were purchased from Millipore Filter Corporation. Gelman Metricel GA-6 filters were purchased from the Gelman Instrument Company. B. subtilis, strains 168 C^- (ind^- $competent^-$), SB 25 (ind_2^- his_2^-) and Br 151 (ind^-) were the gift of F. E. Young, while strains WT and FH 2006 (ind^- thy^-) were given by W. C. McDonald and I. C. Felkner, respectively.

Methods

Isolation of Donor DNA

B. subtilis, strains WT and Br 151, were grown in 10-12 liters of minimal medium (131) supplemented with 0.1% yeast extract at 37° for 14 hours with aeration of 12 liters per minute. The cells were harvested using a Sharples Super Centrifuge, and the donor DNA isolated by the method of Saito and Miura (29). Determination of the DNA concentration was accomplished by the Burton modification (133) of the basic diphenylamine reaction (134).

Preparation of Tritiated DNA

B. subtilis, strain FH 2006 (ind^- thy^-), were grown overnight in

tryptose blood base broth at 37°. These cells were inoculated into minimal medium supplemented with 0.05% acid hydrolyzed casein, 50 µg/ml thymidine, and 50 µg/ml tryptophan. The cells were incubated at 37° with shaking until reaching an absorbance at 630 nm of 0.64 (1 cm light path). After sedimentation, these cells were suspended to an A_{630} of approximately 0.22 in 50 ml of minimal medium containing 0.05% acid hydrolyzed casein, 2 mC ^{3}H-thymidine, sufficient thymidine to give a final concentration of 10 µg/ml, and 50 µg/ml tryptophan. The cells were incubated at 37° with shaking for 2 hr, after which the cells were sedimented and washed twice with minimal medium plus 10 µg/ml thymidine. Tritiated DNA was extracted and purified as described by Richardson (138), with a minor modification. After the phenol extraction, labeled DNA was precipitated by the addition of cold ethanol. The DNA was redissolved in sterile 0.015 M NaCl 0.0015 M sodium citrate pH 7.0, and dialyzed against 2 liters of the described saline-citrate solution for 48 hr (3 changes). The first two changes contained 10 µg/ml cold thymidine. The specific activity of the tritiated DNA was 6 µC/mg.

Isolation of E. coli DNA

E. coli, Crookes strain, were grown in M-9 minimal medium (140) to late exponential or early stationary growth at 37°. Isolation of the DNA was accomplished by the method of Cosloy and Oishi (135). The DNA was dissolved in sterile 0.015 M NaCl 0.0015 M sodium citrate pH 7.1 and refrigerated.

Transformation Procedure

B. subtilis, strains Br 151 (ind^-), SB 25 (ind_2^- his_2^-), and

168 C^- (ind^- $competent^-$), were maintained on plates of tryptose blood base agar at room temperature. Strain Br 151 was routinely used for transformation, initial attachment, and uptake studies, while strains SB 25 and 168 C^- were cultured and used only periodically. Monthly, a fresh TBA plate was subcultured from a working culture of strain Br 151, incubated overnight at 37°, then stored at room temperature. Ten ml of tryptose blood base broth was inoculated from such a working culture, and incubated 12 hr at 37°. These cells were sedimented and an aliquot suspended to an absorbance of 0.15 at 630 nm (1 cm light path) in 30 ml of warm minimal medium plus 50 µg/ml each of histidine, tryptophan, valine, glycine, arginine, aspartic acid, lysine, threonine, and methionine, with enough magnesium sulfate to give a final concentration of 0.072%. Minimal medium supplemented with these components was designated transformation medium. The culture was incubated with shaking at 37° for 5½ hours to develop maximum competence. This is a modification of the procedure of Bott and Wilson (136).

Competent bacteria were transformed by the addition of DNA (5 µg/ml), followed by a 30 minute incubation at 37°. Serial dilutions were made in minimal medium at room temperature. Try^+ transformants were scored on minimal agar plates supplemented with 10 µg/ml histidine and 0.1% acid hydrolyzed casein. The total viable cell titer was scored on minimal agar supplemented with 10 µg/ml each of histidine and tryptophan, plus 0.1% acid hydrolyzed casein. Colonies were visible within 17 hours of growth at 37°.

Measurement of the Total Attached DNA

Competent cells were normally exposed to 3 µg/ml ^{3}H-DNA plus 20

μg/ml cold thymidine. Aliquots removed at appropriate times were pipetted into chilled sterile glass centrifuge tubes, and immediately sedimented at 10,000 x g for 10 minutes at 0°. After the supernatant solution was carefully decanted, 2 volumes of ice cold minimal medium was pipetted down the side of the centrifuge tube and the pellet carefully suspended with a sterile pipette. This washing procedure was repeated twice to remove unattached DNA (139). Care is taken to always keep samples as cold as possible. After the final wash, the cells are suspended to the original volume in ice cold minimal medium. An aliquot was filtered onto a Gelman GA-6 filter in a Bradley filtration device, and washed with 10 ml of ice cold minimal medium. Filters were dried under a heat lamp and counted in Bray's scintillation fluid (137), using a Packard Model 3320 Liquid Scintillation Spectrometer.

Measurement of the Irreversible Uptake of DNA

Aliquots of competent cells exposed to ^{3}H-DNA, as described above, were removed at appropriate times to glass centrifuge tubes. DNase (0.1 μg/ml) was added, along with enough $MgCl_2$ to raise the Mg^{2+} concentration to 10 mM, and incubated for 10 minutes at 37°. The suspension was centrifuged at 10,000 x g for 10 minutes at 25°, and the cells suspended in minimal medium plus 50 μg/ml DNase. The cells were incubated an additional 10 minutes, then sedimented and washed again with 2 volumes of minimal medium. The cells were finally suspended to their original volume in minimal medium and filtered onto Gelman GA-6 filters. The filters were dried and counted by liquid scintillation spectrometry, as previously described.

Attachment and Uptake of DNA in the Presence of EDTA

A suspension of competent bacteria was made 10 mM in EDTA one minute prior to the addition of transforming DNA. Measurement of the amount of labeled donor DNA attached in the presence of EDTA was identical to the method described previously, with the exception that the two washings were done with minimal medium plus 10 mM EDTA. Irreversible uptake was measured by chilling the cell suspension to 15° before the addition of 10 µg/ml DNase plus 15 mM $MgCl_2$. Uptake is negligible at this temperature, allowing the addition of Mg^{2+} for DNase activity. The nuclease-treated cells were incubated for 20-30 minutes at 15° before sedimentation and suspension in minimal medium. The cells were incubated an additional 10 minutes at 15° with 50 µg/ml DNase, followed by centrifugation and a final washing with 2 volumes of minimal medium. The medium previously described for measurement of irreversible uptake was continued from this point.

Measurement of Trichloroacetic Acid Soluble DNA Fragments

Competent bacteria exposed to ^3H-DNA, as previously described, were sedimented and the supernatant solution carefully decanted and saved. One hundred µg/ml bovine serum albumin was added to an aliquot of the supernatant solution. To this was added an equal volume of 10% TCA, and the solution was chilled in an ice bath for 20 minutes. The precipitated solution was centrifuged at 25,000 x g for 10 minutes at 4°. The supernatant solution was again carefully decanted and saved. A 1 ml aliquot of this supernatant solution was counted by liquid scintillation spectrometry.

Restoration of DNA Uptake and Transformation in EDTA-Treated Bacteria

A suspension of competent cells incubated with transforming DNA in the presence of 10 mM EDTA were sedimented and washed once with minimal medium plus 10 mM EDTA to remove unattached DNA. At this point, the cells were considered "preloaded" with transforming DNA. The cells were suspended in minimal media lacking Mg^{2+}. The suspension was then made 30 mM in $MgCl_2$ and incubated at 37^{o}. Transformation, total attachment, irreversible uptake, and the production of acid soluble fragments could be measured as a function of time after the addition of excess Mg^{2+}.

Total Uptake and Incorporation of Radioactive Precursors

Saturating amounts of precursor were added to a suspension of competent bacteria, followed by the immediate addition of the labeled precursor. Samples were taken at various time intervals. Total uptake was measured by collecting 1.0 ml aliquots of the bacteria on Gelman GA-6 filters, and washing with 10 ml of minimal medium. The incorporation of the precursor into macromolecules was measured by adding 1.0 ml of the cell suspension to an equal volume of cold 10% trichloroacetic acid. The mixture was chilled in ice for 20 minutes, then filtered on a Gelman GA-6 filter. A 10 ml wash with cold minimal medium followed. The filters were dried and counted by liquid scintillation spectrometry. Accumulation of radioactive precursor was calculated by subtracting the radioactivity incorporated from the total radioactivity taken up by the cells.

Osmotic Shock Procedure

The osmotic shock procedure described by Pierson (139) was used. Competent bacteria were sedimented at 25° and suspended to one half their original volume in 0.25 M sucrose - 0.033 M Tris-HCl, pH 7.2, and 10^{-4} M EDTA by vortex mixing. These cells were incubated at room temperature for 10 minutes with occasional mixing. The suspension was sedimented by centrifugation at 15,000 x g. The supernatant was discarded, and the pellet resuspended to the original volume in 5×10^{-4} M $MgCl_2$ at 4°. The suspension was vigorously mixed and incubated in an ice bath for 10 minutes, with occasional mixing. The cells were centrifuged at 4° for 10 minutes at 15,000 x g. After removal of the supernatant, the cells were resuspended to their original volume in transformation medium, and designated osmotically shocked.

Treatment of Competent Bacteria with Sodium Meta Periodate

Competent bacteria were sedimented by centrifugation, and suspended in minimal medium lacking sodium citrate and glucose. Enough KCl was added to the medium to maintain the original ionic strength. The cells were sedimented again, and suspended in this same medium. The suspension was made 0.5 mM in sodium meta periodate, and incubated at 37° or 0° for 10 minutes. The reaction was terminated by making the suspension 1% in glucose. The cells were then assayed for total attachment, irreversible uptake, or transformation as previously described.

Treatment of Bacteria in a Waring Blendor

Competent bacteria were pipetted into a sterile, covered chamber for a Waring blendor (model number 1041). A minimum volume of 15 ml

was used in this 360 ml chamber (Eberbach Corporation) to insure coverage of the blade for complete agitation. In some experiments, the speed of blending was controlled by a Powerstat (Superior Electric Company) at a setting of 55. At appropriate intervals, blending was stopped, an aliquot removed, and the treatment then continued. During long periods of blending, the base of the chamber was chilled in ice to dissipate heat.

Sedimentation of Competent Bacteria in Renografin Gradients

3,5-Diacetamido-2,4,6-triiodobenzoic acid was dissolved in water to a final concentration of 76%, requiring the addition of NaOH to maintain the pH at 7.0. Commercially available solutions, called Renografin-76 are 76% 3,5-diacetamido-2,4,6-triiodobenzoic acid, but are arbitrarily considered to be 100%. Discontinuous and linear gradients were prepared as described by Hadden and Nester (25). Competent suspensions of bacteria were sedimented and suspended in the appropriate percentage Renografin for application to the gradient. Five ml gradients were centrifuged for 30 minutes at 4^{o} in the SW 50.1 head of a Beckman model L2-65 preparative ultracentrifuge at 15,000 RPM (21,000 x g). Thirty ml gradients were similarly centrifuged in the Beckman SW 25.1 head (22,889 x g). Fractions were collected aseptically by use of a Beckman Fractionating System, for 5 ml gradients, or a Buchler gradient fractionator in the case of 30 ml gradients.

CHAPTER III

THE ATTACHMENT OF DNA TO COMPETENT B. SUBTILIS AT 4°

If the initial attraction and attachment of transforming DNA to the competent cell surface is ionic in nature, Lerman and Tolmach (44) proposed it should be independent of temperature. Such an assumption justified the measurement of initial attachment at 4°, where the metabolic energy-requiring uptake process would be inoperative. However, if subsequent to the initial adhesion of donor DNA there is a specific attachment to sites on the competent cell surface involved in the transformation process, a temperature dependency may be exhibited. This possibility can be examined by studying the effect of varying temperature on the attachment and uptake of labeled DNA, and transformation.

Establishment of the Temperature Dependency of Initial Attachment of DNA

If competent cells are exposed to transforming DNA for a short interval of time over a range of temperatures, followed by rapid removal of unattached DNA by Millipore filtration, the number of resulting transformants at 37° should reflect any temperature dependency of the initial attachment reaction. The results of such an experiment are shown in Table I. Attachment at 37° results in maximal transformation, with the lower temperatues limiting the efficiency of transformation

TABLE I

PRODUCTION OF TRANSFORMANTS FROM INITIAL ATTACHMENT OF DNA AT VARIOUS TEMPERATURE

Temperature	Minutes of Incubation with DNA	Transformants/ml x 10^{-3}
	0.5	8.3
4^{o}	1.0	4.4
	5.0	13.7
	0.5	38.5
25^{o}	1.0	58.4
	5.0	57.2
	0.5	26.1
37^{o}	1.0	47.5
	5.0	271.0
	0.5	29.9
45^{o}	1.0	31.9
	5.0	162.0

Competent Br 151 were incubated at the appropriate temperature for 10 minutes prior to the addition of 1 µg/ml DNA. After incubation for the designated time, the bacteria were serially diluted into minimal medium of the same temperature. One ml of a 10^{-2} or 10^{-3} dilution was quickly filtered onto a sterile Millipore filter (HAWG 047, 0.45µ). The filters were placed on minimal medium agar plates, as described in the Methods section, for the scoring of Try^{+} transformants. Plates were incubated at 37^{o} overnight.

most effectively. The short period of exposure to DNA approximates the initial lag period in the appearance of transformants with time when attachment is terminated by DNase treatment. It was assumed that such short exposure allowed primarily the initial attachment of DNA before its irreversible uptake began.

Table II shows the results of a similar experiment, where attachment of DNA was permitted for a 15 minute period at various temperatures and terminated by dilution. The level of transformation after the initial 15 minute incubation displays a temperature dependency, due to the involvement of enzymic reactions and requirement for metabolic energy in the sequence. If a 1:100 dilution into warm minimal medium is made after the 15 minute period, followed by an additional 30 minute incubation at 37^{o} before plating, an increase in the amount of transformation is observed in each case. Assuming the amount of DNA initially attached was temperature independent, then that attached DNA should result in comparable levels of transformation when incubated at the optimal temperature for the entire sequence.

Measurement of Total Attachment and Uptake of DNA at Various Temperatures

The difference in the level of transformation after attachment at various temperatures does not distinguish between the possibilities that the temperature dependency results from an alteration in the attachment sites and/or a decrease in the amount of DNA being attached. Table III shows the total amount of labeled donor DNA associated with competent bacteria and the irreversible uptake after a 15 minute incubation at various temperatures. The optimal temperatures for attach-

TABLE II

TRANSFORMATION AT 37° FOLLOWING INITIAL ATTACHMENT OF DNA AT VARIOUS TEMPERATURES

Temperature	Transformants/ml x 10^3 15 Minute Incubation	1:100 Dilution and Shift to 37° for 30 Minutes
0°	7	44
4°	6	64
15°	6	73
25°	40	168
37°	271	344
45°	1	1

Competent Br 151 were incubated at the appropriate temperature for 10 minutes prior to the addition of 5 µg/ml DNA. Incubation was continued for 15 minutes before a 1:100 dilution was made into warm transformation medium, which was then incubated an additional 30 minutes at 37°. Transformation was determined at the end of the initial 15 minute incubation and after the 1:100 dilution and additional incubation, as described in the Methods section.

TABLE III

ATTACHMENT AT UPTAKE OF DNA AT VARIOUS TEMPERATURES

Temperature	Total Attachment CPM/ml	Irreversible Uptake CPM/ml	Transformants/ ml x 10^3
0^o	222	82	11
4^o	227	95	16
15^o	303	82	12
25^o	2422	144	49
37^o	2228	197	680
45^o	129	63	1
50^o	55	0	0

Competent Br 151 were incubated at the appropriate temperature for 10 minutes prior to the addition of 3 µg/ml ^{3}H-DNA or 5 µg/ml DNA for the measurement of total attachment/uptake or transformation, respectively. After a 15 minute incubation, the amount of total attachment, irreversible uptake, and transformation was determined as described in the Methods section.

ment and irreversible uptake range from 25°-37°, while the complete transformation sequence is optimal at 37°. The amount of irreversible uptake measured at the temperature below 25° is most likely due to the incubation at 37° after the addition of 0.1 µg/ml DNase. The amount of irreversible uptake was negligible if this DNase treatment was altered to a 20 minute incubation at 15° with 10 µg/ml DNase.

Separation of 4° Attached DNA with Competent Bacteria

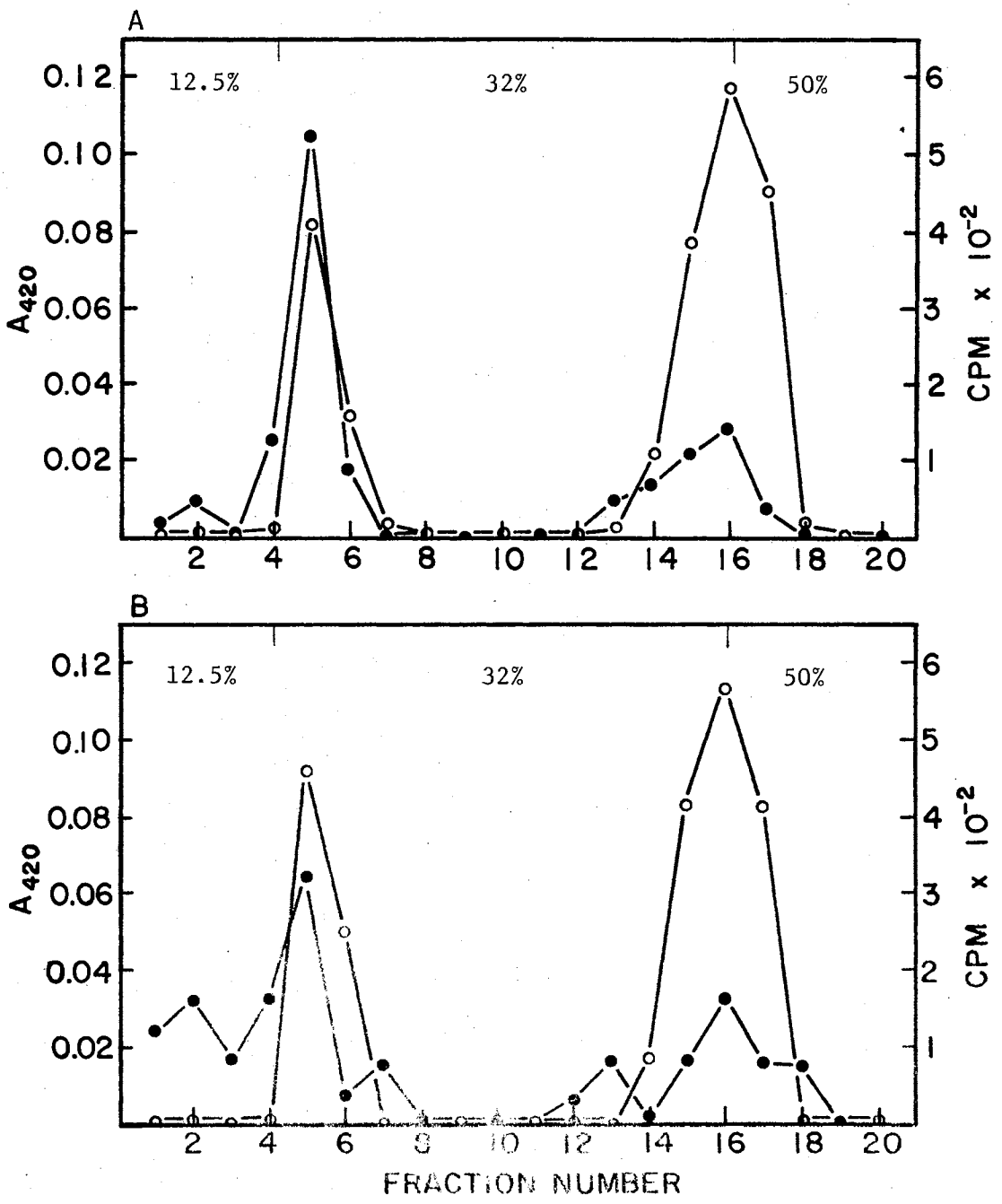
The labeled donor DNA attached to the bacteria at 4° was investigated with respect to its involvement in the transformation sequence. Cahn and Fox (24) and Hadden and Nester (25) have both described the fractionation of competent bacteria in transformed cultures of B. subtilis on Renografin gradients. Separation of the competent fraction from the noncompetent cells on a discontinuous Renografin gradient after incubation with labeled donor DNA at 4° revealed the DNA to be attached to the competent population of bacteria (Figure 2).

Participation of 4° Attached DNA in the Transformation Process

Having shown that DNA attached at 4° does so to competent bacteria, the ability of this DNA to participate in the transformation sequence was investigated. Either prototrophic (Try^+), auxotrophic (Try^-), or no DNA at all was attached to competent cells at 4°. After removal of unattached DNA, the cells were exposed to prototrophic DNA and incubated an additional 30 minutes at 37° before scoring for transformants (Table IV). Included in Table IV are the results of removal

Figure 2. Separation of 4^{o} Attached DNA with Competent Bacteria on a Renografin Gradient

Competent Br 151 were incubated at 4^{o} or 37^{o} prior to the addition of 3 µg/ml ^{3}H-DNA. Bacteria were incubated at both temperatures with ^{3}H-DNA for 30 minutes. Bacteria incubated at 37^{o} were incubated with 50 µg/ml DNase for 15 minutes, then washed once in minimal medium to label the competent fraction of the population. Bacteria incubated at 4^{o} were sedimented and washed twice with a 2X volume of cold transformation medium. Final suspension of both samples was made in 12.5% Renografin to one fifth the original volume, and 0.2 ml was layered on to a 5 ml 12.5-32-50% step gradient of Renografin. The discontinuous gradients were centrifuged in a SW 50.1 head in Beckman Model L2-65 Preparative Ultracentrifuge at 15,000 RPM at 4^{o} for 30 minutes. Gradients were fractionated in a Beckman Fractionating System, monitoring the optical density at 420 nm (○). Fractions of 4 drops were collected and the radioactivity (●) counted using liquid scintillation spectrometry, as described in the Methods section.


A
12.5%
32%
50%
0.12
0.10
0.08
0.06
0.04
0.02
A_{420}
6
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CPM x 10^{-2}
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B
12.5%
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A_{420}
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1
CPM x 10^{-2}
2 4 6 8 10 12 14 16 18 20
FRACTION NUMBER

TABLE IV

REDUCTION OF TRANSFORMATION AT 37° BY ATTACHMENT OF DNA AT 4°

4° 15 Minutes	Wash & Resuspend	37° 30 Minutes	% Transformation
None		Try^+	0.148
Try^+		None	0.005
Try^+		Try^+	0.095
Try^+		Try^-	0.002
Try^-		Try^+	0.086
4° 15 Minutes	**DNase & Wash**	**37° 30 Minutes**	**% Transformation**
Try^+		Try^+	0.171
Try^-		Try^+	0.120

Competent Br 151 were incubated at 4° for 10 minutes prior to the addition of 5 µg/ml Try^+ or Try^- DNA. Cells were incubated for 15 minutes at 4° in the presence of DNA, followed by a washing by sedimentation and suspension in a 2X volume of cold transformation medium. In the case of DNase treated cells, the bacteria were exposed to 50 µg/ml DNase for 15 minutes at 37°, then washed twice in transformation medium. In both cases, the bacteria were finally suspended in warm transformation medium and 5 µg/ml Try^+ or Try^- DNA. Incubation was continued at 37° for 30 minutes. Try^+ transformants were scored for as described in the Methods section.

of the DNA attached at 4° by DNase prior to the addition of prototrophic DNA at 37°.

The reduction in the amount of transformation at 37°, when cells were incubated at 4° with auxotrophic or prototrophic DNA, suggests the DNA attached at the reduced temperature, not able itself to lead to any significant transformation, does interfere with the subsequent transformation at 37°.

Specificity of DNA Attachment at 4°

The effect of exposure of competent bacteria at 4° to a variety of treated or heterologous DNAs prior to their transformation at 37° with homologous prototrophic DNA would define the specificity of the attachment at 4°. The results of such an experiment are shown in Table V, and reveal that of the DNAs incubated with competent bacteria at 4°, only the denatured homologous DNA failed to reduce the amount of subsequent transformation at 37°.

Evidence for the Alteration of Initial Attachment Sites by Reduction in Temperature

This reduction in transformation was further investigated by following a time course of transformation at 37°, after a 15 minute incubation with auxotrophic DNA at 4° and 37°. Figure 3 reveals a qualitative similarity between the results produced by attaching the auxotrophic DNA at either temperature. No apparent effect on the culture's transformability at 37° occurred because of the incubation at 4° prior to exposure to transforming DNA at 37°. A constant, but

TABLE V

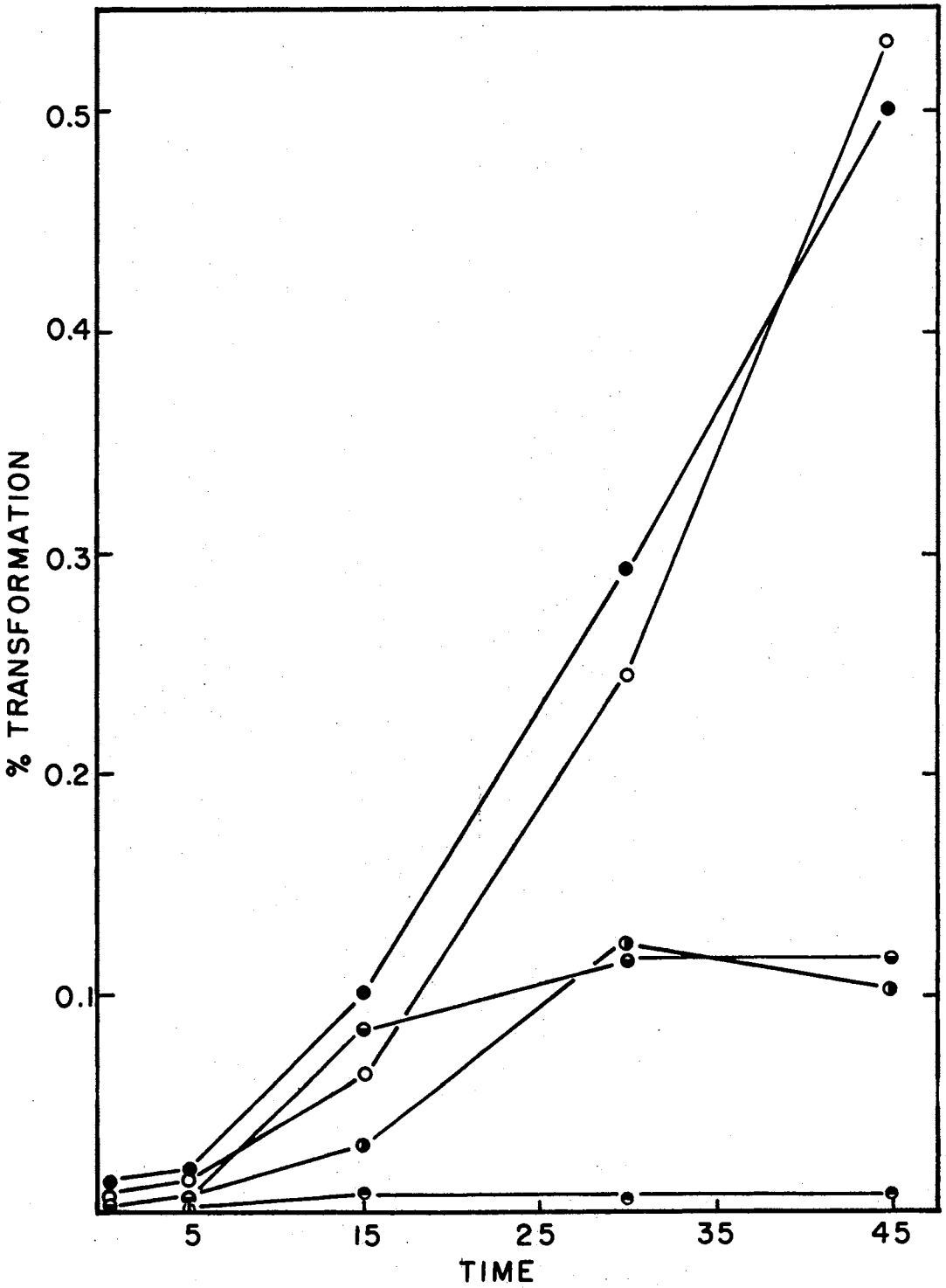
SPECIFICITY OF ATTACHMENT AT 4^{o}

DNA Attached at 4^{o}	% Control Transformation
Nothing	100%
Try^{+}	67%
Try^{-}	58%
E. coli	65%
Salmon Sperm	57%
T_2	33%
Sheared Homologous DNA	76%
Heat Denatured Homologous DNA	95%

Competent Br 151 were incubated at 4^{o} for 10 minutes prior to the addition of 5 µg/ml of a specific DNA, except in the case of salmon sperm DNA which was added to a concentration of 100 µg/ml. The bacteria were incubated for 15 minutes at 4^{o}, sedimented and washed once in chilled transformation medium, and finally suspended in warm transformation medium plus 5 µg/ml Try^{+} DNA. Each sample was incubated 30 minutes at 37^{o} before scoring for Try^{+} transformants, as described in the Methods section. Try^{+} DNA was thermally denatured by heating at 100^{o} for 6 minutes, then rapid cooling in an ice water bath. Sheared Try^{+} DNA was prepared by forcefully passing DNA through a sterile 25 gauge hypodermic needle five times.

Figure 3. A Time Course of Transformation After Attachment of Auxotrophic DNA at 4^{o} or 37^{o}

Competent Br 151 were incubated at 4^{o} or 37^{o} for 10 minutes prior to the addition of any DNA. To one suspension equilibrated at 4^{o} (◒) and one at 37^{o} (◓) was added 5 µg/ml TRY^{-}DNA, followed by a 15 minute incubation at the appropriate temperature. All samples, including those not exposed to auxotrophic DNA at 4^{o} (○) or 37^{o} (●), were sedimented and washed once with minimal medium at the appropriate temperature. All bacteria were suspended in warm transformation medium plus 5 µg/ml TRY^{+}DNA, and incubated at 37^{o}. Transformation was determined at the designated intervals, as described in the Methods section.


0.5
0.4
0.3
0.2
0.1
% TRANSFORMATION
5
15
25
35
45
TIME

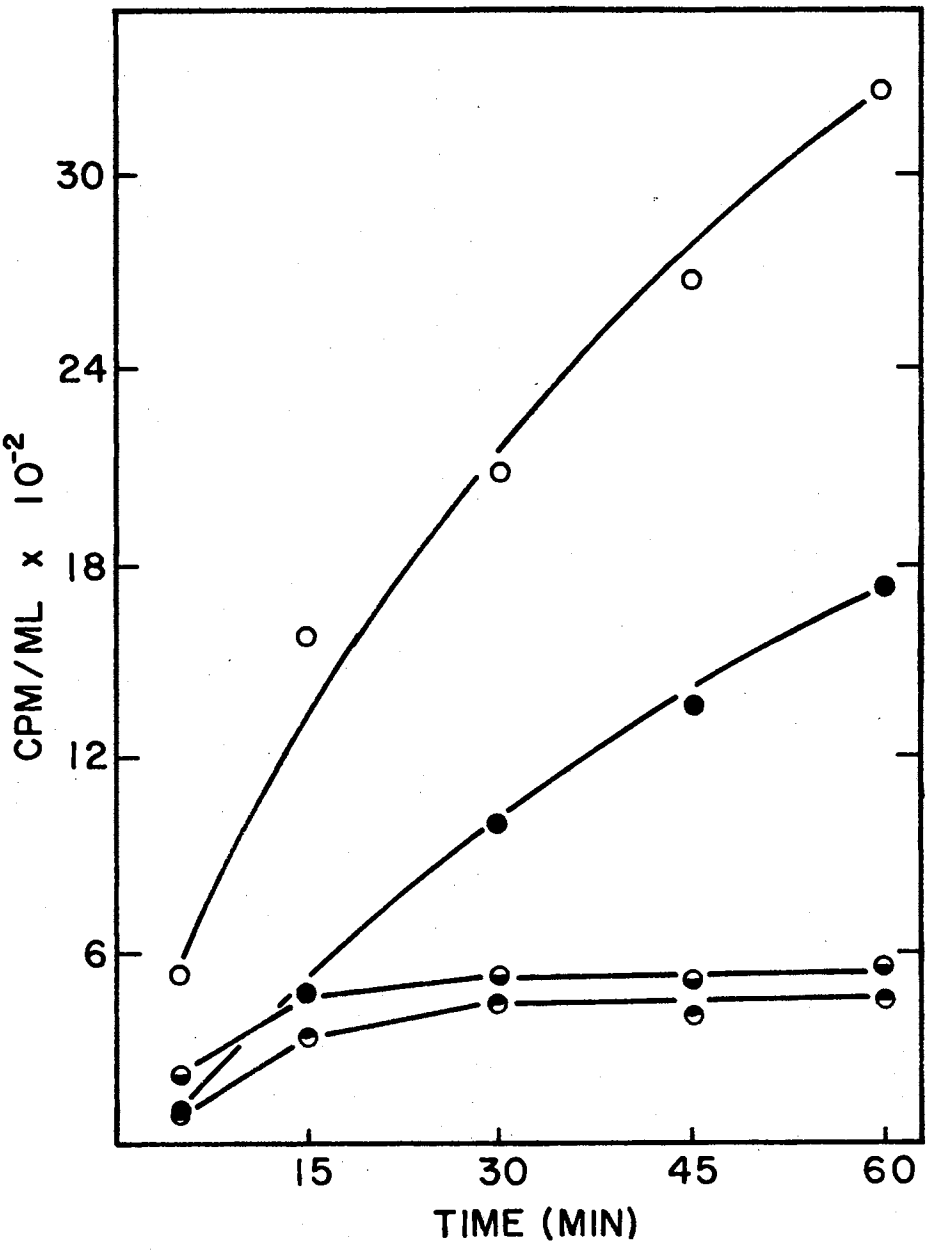
negligible level of transformation occurred if prototrophic DNA was attached at 4^{o}, then incubated at 37^{o} without introduction of any other DNA. The rates of appearance of transformants in the cases where the bacteria were first exposed to auxotrophic DNA were slower than the controls'. Also, in both cases, no increase in the amount of transformation occurred after a 30 minute incubation with donor DNA at 37^{o}.

Such results are substantiated by a similar experiment, where the total amount of attached donor DNA was followed as a function of time. Figure 4 shows the amount of DNA associated with competent bacteria increases linearly with time. A 15 minute incubation at 4^{o} prior to addition of ^{3}H-DNA and incubation at 37^{o} resulted in a reduction in the amount of ^{3}H-DNA attached to the bacteria. Considering that prior incubation at 4^{o} did not affect the appearance of transformants at 37^{o}, the difference in the amount of DNA associated with the chilled and unchilled cells may not have been involved in the transformation sequence. Incubation of the bacteria with unlabeled DNA at 4^{o} or 37^{o} prior to the addition of ^{3}H-DNA and incubation at 37^{o} substantially reduced the rate of attachment of labeled DNA to the cells. In both cases, after 30 minutes of incubation with ^{3}H-DNA, no further increase in the amount of labeled DNA associated with the bacteria was detected.

In both these time course experiments, the auxotrophic or unlabeled DNA attached at 37^{o} reduces the subsequent appearance of transformants or amount of ^{3}H-DNA associated with the bacteria, respectively, because of its ability to enter the transformation sequence and preferentially compete with the second addition of DNA. However, the reduction in the appearance of transformants or amount of ^{3}H-DNA attached to the bacteria by incubation with DNA at 4^{o} cannot be explained by the

Figure 4. A Time Course of Attachment of ^{3}H-DNA After Attachment of Unlabeled DNA at 4^{o} or 37^{o}

Competent Br 151 were incubated at 4^{o} or 37^{o} for 10 minutes prior to the addition of any DNA. To one suspension equilibrated at 4^{o} (◓) and one at 37^{o} (◒) was added 3 µg/ml unlabeled homologous DNA, followed by a 15 minute incubation at the appropriate temperature. All samples, including those not exposed to unlabeled DNA at 4^{o} (●) or 37^{o} (○), were sedimented and washed once with minimal medium at the appropriate temperature. All bacteria were suspended in warm transformation medium plus 3 µg/ml ^{3}H-DNA, and incubated at 37^{o}. Total attachment was determined at the designated intervals, as described in the Methods section.


30
24
18
12
6
CPM/ML x 10^{-2}
15
30
45
60
TIME (MIN)

entry of DNA into the transformation process at this reduced temperature. The observed results may be explained by the reduction in temperature altering or masking the normal attachment sites, such that a distinctly different type of attachment of DNA occurs at 4^{o}. DNA attached at 4^{o} could prevent the occupied sites from returning to their normal state, and thus reduce any subsequent attachment and transformation at 37^{o}.

If such an alteration in the attachment site does occur with chilling to 4^{o}, it may be reflected in a change in a particular sensitivity of the transformation process. Akrigg, Ayad, and Barker (141) reported the reduction in transformability of competent B. subtilis by repetitive washing at room temperature, suggesting that a loosely bound essential factor was being removed. Tables VI and VII show a 60% and 70% reduction in the amount of transformation and H-DNA associated with the bacteria, respectively, after washing. The effect of washing is minimized if the cells are chilled to 4^{o}, then washed at this low temperature. Washing at 4^{o} or 37^{o} failed to reduce the amount of DNA bound at 4^{o}. However, washing at 4^{o} prior to the attachment of DNA at 37^{o} only reduces the amount of attachment by 25%, compared to an unwashed control, and this may result from the temperature shock. Similarly, Table VII shows washing at 4^{o} prior to incubation of the bacteria with the transforming DNA at 37^{o} resulted in only a 7% reduction in the amount of transformation.

The ability of DNA attached at 4^{o} to lead to transformation when incubated at 37^{o} was reduced by washing at the reduced temperature. Such bacteria were transformed to only 1/3 of the amount an unwashed sample treated similarly was. This distinguishes the sequence of

TABLE VI

EFFECT OF 2X WASHING ON BINDING SITES AVAILABLE AT 37° AND 4°

Treatment	CPM Attached/ml
Control (37°)	3350
Control (4°)	319
2X Wash (37°)	976
2X Wash (4°)	306
2X Wash (4°) ⟶ 37°	2553
2X Wash (37°) ⟶ 4°	360

Competent Br 151 were incubated at either 4° or 37° for 10 minutes prior to sedimentation and a 2X washing with minimal medium of the same temperature. The bacteria were finally suspended in transformation medium of the appropriate temperature, and each sample was divided in half. One half of either sample was incubated at the opposing temperature for 10 minutes. Three μg/ml ^{3}H-DNA was added to each sample and incubated for 15 minutes. Total attachment was measured as described in the Methods section.

TABLE VII

EFFECT 2X WASHING HAS ON TRANSFORMATION EFFICIENCIES AT 4^{o} AND 37^{o}

Treatment	% Transformation	% Control
Control (30 Min. at 37^{o})	0.635	100
$Control_2$ (15 Min. at 37^{o})	0.279	44
2X Wash (37^{o})	0.264	42
Cells + DNA $\longrightarrow$ 4^{o} (15 Min. at 37^{o}) (1:100;30 Min.)	0.260	*93
Control 4^{o} (30 Min. at 4^{o})	0.002	<1
2X Wash (4^{o}) $\longrightarrow$ 37^{o} + DNA	0.589	93
2X Wash (4^{o}) $\longrightarrow$ 37^{o} + DNA (1:100;30 Min.)	0.011	*4
4^{o} + DNA $\longrightarrow$ 37^{o} (15 Min. at 4^{o}) (1:100;30 Min.)	0.033	*12

Competent Br 151 were incubated at either 4^{o} or 37^{o} for 10 minutes prior to sedimentation and a 2X wash with minimal medium of the same temperature. The bacteria were finally suspended in transformation medium of the appropriate temperature, and each sample was divided in half. One half of either sample was incubated at the opposing temperature for 10 minutes prior to the addition of 5 µg/ml DNA to all the samples. Incubation with DNA was continued for 15 minutes, followed by 1:100 dilution into warm transformation medium, 1:100 dilution into chilled transformation medium, or determination of transformation, or, continued for 30 minutes, then the determination of transformation. The 1:100 dilutions into transformation medium were incubated an additional 30 minutes at the designated temperature before determination of transformation, as described in the Methods section. Asterisked values represent percent of $Control_2$.

events leading to transformation at 37° after attachment at 4° from those occurring entirely at 37°. Prior washing at 4° apparently removes some factor(s) required for the successful transformation from DNA attached at 4°.

Once the DNA is attached at 37°, chilling the cells to 4° stops the transformation sequence and allows only that transformation which occurred before reduction in temperature to be expressed.

Separation of Initial Attachment of DNA from the Irreversible Uptake

In the previously described experiments, the total amount of ^{3}H-DNA associated with the competent bacteria was used as an assay to study the attachment of donor DNA. This total amount of DNA associated with the bacteria includes the contribution of DNA that has begun to be processed by the uptake system. A method for separating the initial attachment reaction from the rest of the sequence was sought.

An attempt to separate initial attachment by the use of various metabolic inhibitors is shown in Table VIII. All the inhibitors examined reduced the amount of irreversible uptake and transformation, but also altered the extent of attachment. The possibility that these inhibitors may indirectly reduce the amount of initial attachment made their use unattractive.

Morrison (101) proposed EDTA could inhibit the uptake of attached DNA by preventing the initiation of uptake. He described an early intermediate state for attached donor DNA in which it was sensitive to DNase, but despite the presence of EDTA, could attain DNase resistance. If such an organic chelator only prevented the initiation of uptake,

TABLE VIII

EFFECT OF METABOLIC INHIBITORS ON ATTACHMENT AND UPTAKE OF DNA

Treatment	Total Attachment	Irreversible Uptake	% Transformation
Control	6446	1339	0.148
4^{o}	265	80	0.008
- Glucose	3149	420	0.029
10 mM NaN_3	1466	359	0.018
25 mM CN^-	957	238	0.002
1 mM DNP	608	52	0.001
0.5 mM IO_4	96	8	0.004

A competent culture of Br 151 was divided into 7 equal portions, and each was sedimented by centrifugation. All except -Glucose and 0.5 mM $IO_4^=$ were suspended in transformation medium plus the designated final concentration of each inhibitor. -Glucose was suspended in transformation medium lacking glucose, and the 0.5 mM $IO_4^=$ was suspended in transformation medium minus glucose and citrate. Incubation with each inhibitor was continued for 10 minutes at 37^{o}. Periodate oxidation was terminated as described in the Methods section. Three µg/ml ^{3}H-DNA or 5 µg/ml DNA was added and incubated with the bacteria for 30 minutes for the measurement of attachment/uptake or transformation, respectively. Total attachment, irreversible uptake, and transformation were determined as described in the Methods section.

the initial attachment should remain unaffected. Table IX shows the effect of two organic chelators, EDTA and EGTA, and the absence of Mg^{2+} on a competent culture's ability to attach and irreversibly take up ^{3}H-DNA. The absence of Mg^{2+} or the presence of 10 mM EDTA in the medium effectively reduced the irreversible uptake of DNA. The Ca^{2+} specific chelator EGTA failed to prevent irreversible uptake, and consistently increased the total amount of DNA attached to the bacteria.

A time course of transformation in the absence of Mg^{2+} and/or presence of 10 mM EDTA is shown in Figure 5. The use of 10 mM EDTA added to the bacteria one minute before the addition of ^{3}H-DNA was adopted to measure initial attachment of DNA, because it effectively prevented the irreversible uptake of donor DNA without reducing the amount of DNA attached.

Comparison of the Initial Attachment of DNA at 4^{o} and 37^{o} in the Presence of 10 mM EDTA

The initial attachment of donor H-DNA in the presence of 10 mM EDTA at 4^{o} and 37^{o} is shown in Table X. The amount of attachment at 4^{o} is not substantially reduced by prior addition of EDTA.

Inability of DNA Attached at 4^{o} to Enter the Early Intermediate State

Addition of 10 mM EDTA after a period of incubation with transforming DNA prevents further uptake of attached DNA which is not "EDTA resistant". Transformation at 37^{o} from DNA attached at 4^{o} shows a definite sensitivity to EDTA, as seen in Table XI. Addition of EDTA after a 15 minute incubation at 4^{o} with donor DNA resulted in a very

TABLE IX

EFFECT OF ORGANIC CHELATORS AND ABSENCE OF Mg^{2+} ON ATTACHMENT AND UPTAKE

Treatment	Total Attachment	Irreversible Uptake	% Transformation
Control	3150	916	0.114
- Mg^{2+}	3610	225	0.011
10 mM EDTA	3220	368	0.002
10 mM EGTA	4548	860	0.082
- Mg^{2+} plus 10 mM EDTA	2292	355	0.001

Competent Br 151 were sedimented by centrifugation and resuspended in transformation medium containing the designated concentration of EDTA or EGTA, or transformation medium minus Mg^{2+} with or without EDTA. After 1 minute incubation, 3 µg/ml ^{3}H-DNA or 5 µg/ml DNA was added and incubated at 37° for 30 minutes for the measurement of attachment/uptake or transformation, respectively. Total attachment, irreversible uptake, and transformation were determined as described in the Methods section.

Figure 5. A Time Course of Transformation in the Absence of Mg^{2+} and/or Presence of 10 mM EDTA

Competent Br 151 were sedimented and suspended in transformation medium (●), transformation medium $-Mg^{2+}$ (◓), transformation medium + 10 mM EDTA (◒), or transformation medium $-Mg^{2+}$ + 10 mM EDTA (○). Five μg/ml DNA was added and incubated at 37°. Transformation was measured at the designated intervals, as described in the Methods section.

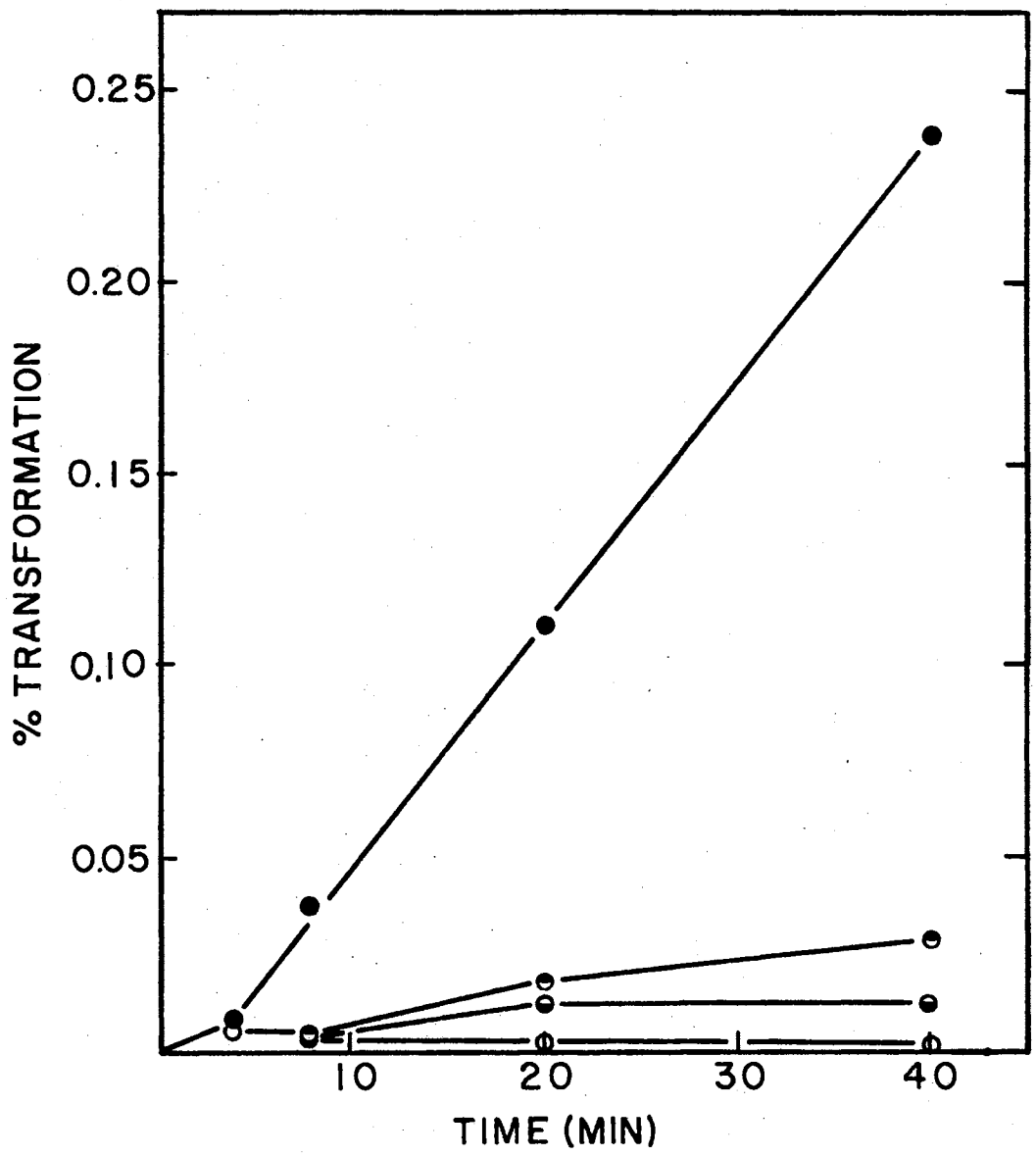

0.25
0.20
0.15
0.10
0.05
% TRANSFORMATION
10
20
30
40
TIME (MIN)

TABLE X

COMPARISON OF INITIAL ATTACHMENT OF DNA AT 4^{o} AND 37^{o} IN THE PRESENCE OF 10 mM EDTA

Temperature	Attached ^{3}H-DNA (CPM/ml)
37^{o}	4700
37^{o} plus 10 mM EDTA	4423
4^{o}	550
4^{o} plus 10 mM EDTA	475

Competent Br 151 were incubated at 4^{o} or 37^{o} for 10 minutes prior to the addition of EDTA to a final concentration of 10 mM. After 1 minute incubation in the presence of EDTA at either temperature, 3 µg/ml ^{3}H-DNA was added and incubated for 30 minutes at 4^{o} or 37^{o}. Attachment was measured as described in the Methods section.

TABLE XI

INABILITY OF DNA ATTACHED AT 4° TO ENTER THE EARLY INTERMEDIATE STATE

Temperature	% Transformation
37°	
Control	0.110
Cells + 10 mM EDTA ⟶ DNA	0.002
Cells + DNA ⟶ 10 mM EDTA	0.080
4°	
Cells + DNA (4°) ⟶ 1:100 dilution 37° for 30 minutes	0.012
Cells + 10 mM EDTA ⟶ 1:100 dilution then DNA (4°) 37° for 30 minutes	0.000
Cells + DNA (4°) 15 minutes at 4° then 10 mM EDTA ⟶ 1:100 dilution 37° for 30 minutes	0.001

Competent Br 151 were incubated at 4° or 37° for 10 minutes prior to the addition of 5 µg/ml DNA or EDTA to a final concentration of 10 mM. In samples at 4° and 37° to which DNA was first added, after a 15 minute incubation, EDTA was added to a final concentration of 10 mM and the incubation continued for an additional 15 minutes. At 4°, DNA attachment was allowed for 15 minutes, followed by 1:100 dilution in warm minimal medium and a 30 minute incubation at 37°. Transformation was measured as described in the Methods section.

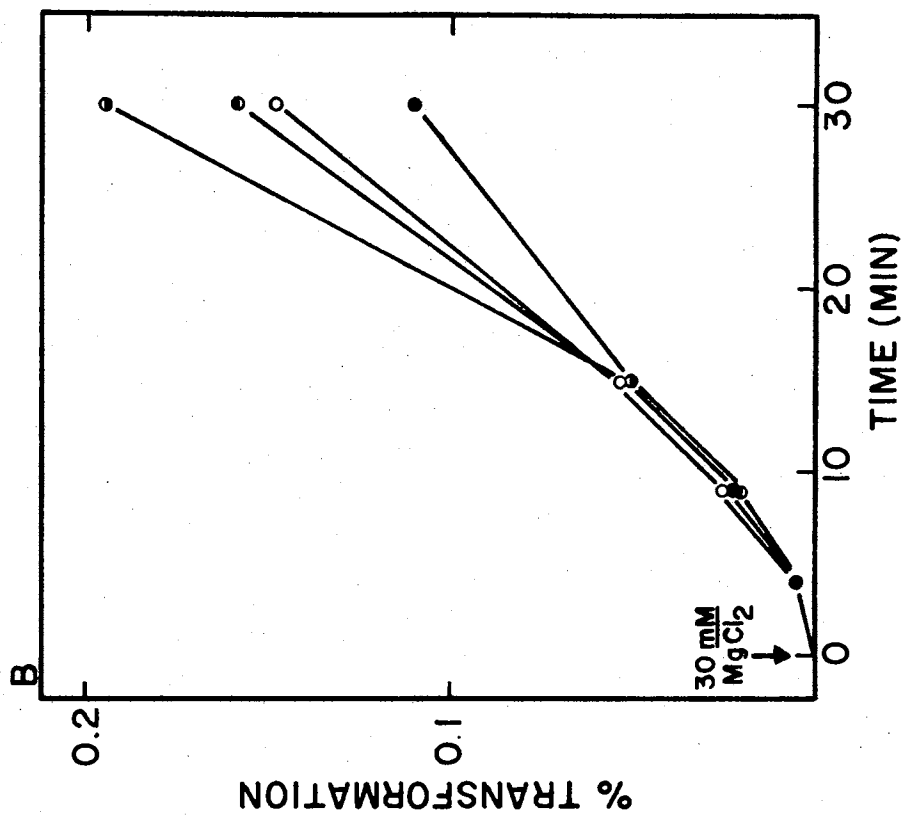
low level of transformation at 37°. Of the DNA attached at 4°, only a small portion was capable of becoming EDTA resistant at 4°, thus the negligible level of transformation when incubated at 37°. This implies that entry of attached DNA into Morrison's early intermediate state (101) is temperature dependent, or that alteration by reduction of temperature prevents any step subsequent to the initial attachment reaction. DNA attached at 4° in the presence of 10 mM EDTA was unable to lead to any transformation at 37°.

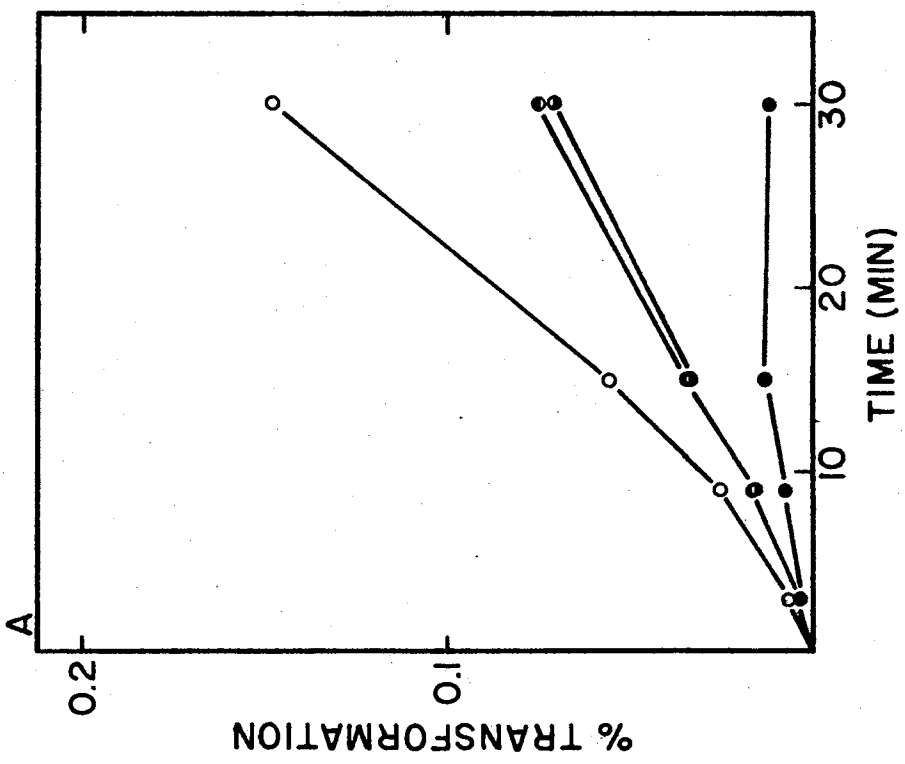
Reversibility of DNA Attached in the Presence of 10 mM EDTA

With the DNA initially attached in the presence of 10 mM EDTA prevented from entering the bacteria, the reversibility of the attachment can be measured upon the addition of 30 mM $MgCl_2$ by incubation with a second DNA. Competent bacteria were preloaded with various DNAs in the presence of 10 mM EDTA, then transformed with Try^+ DNA by reversing the inhibition of EDTA with 30 mM Mg^{2+}, as shown in Figure 6. Transformation under these circumstances is compared to the transformation of bacteria similarly incubated with the same variety of DNAs, but in the absence of 10 mM EDTA. The reduction in transformation caused by the uptake of an auxotrophic or heterologous DNA prior to the addition of prototrophic DNA does not occur if such competitive DNA is only initially attached in the presence of EDTA. This attached DNA was displaced by the excess of prototrophic DNA, which upon the addition of excess Mg^{2+} entered and transformed the bacteria.

Figure 6. Reversibility of DNA Attached in the Presence of 10 mM EDTA

A culture of competent Br 151 was divided in half. Each half was divided into 4 portions, which are represented in one panel of the figure. In the first panel, to three of the samples was added 5 μg/ml of either TRY^- homologous DNA (◓), E. coli DNA (◒), or nothing (○). The fourth sample was made 10 mM in EDTA. All samples were sedimented and washed once in warm minimal medium, except in the EDTA sample, where Mg^{2+} was deleted. The bacteria were finally suspended in minimal medium (except for the EDTA sample, where Mg^{2+} deleted) plus 5 μg/ml TRY^+ DNA, and incubated at 37°. The second panel represents samples which were all made 10 mM in EDTA before the addition of any DNA. A similar procedure, as described above was followed for these samples, with the exception that all washing was done with minimal medium minus Mg^{2+}. In these samples, zero time was denoted by the addition of 30 mM $MgCl_2$. Transformation was determined at the designated time as described in the Methods section.


B
30 mM MgCl2
0
10
20
30
TIME (MIN)
0.1
0.2
% TRANSFORMATION


A
0
10
20
30
TIME (MIN)
0.1
0.2
% TRANSFORMATION

Analysis of the Entry of Donor DNA Initially Attached at 4^{o} and 37^{o} in the Presence of 10 mM EDTA

Morrison (101) described the reversibility of the inhibition of DNA uptake by EDTA treatment in B. subtilis with addition of excess Mg^{2+}. However, in our attempts to reverse the effect of EDTA, the level of transformation was never restored to that of the control, suggesting that addition of Mg^{2+} was not singularly sufficient.

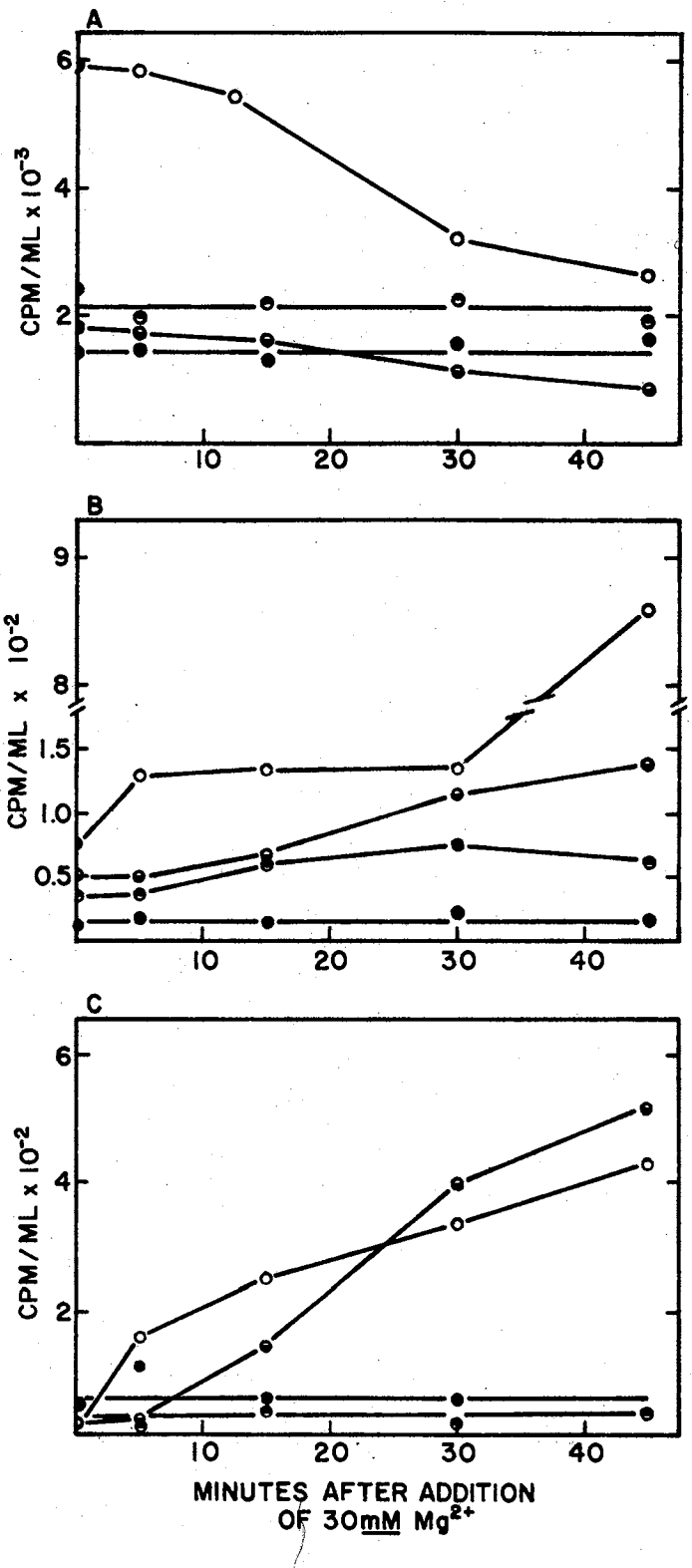
An indirect comparison can be made between the transformation at 4^{o} and 37^{o} from DNA attached at either temperature by following the events which accompany entry of donor DNA into a recipient bacterium. This was accomplished by preloading competent bacteria with ^{3}H-DNA in the presence of 10 mM EDTA at 4^{o} and 37^{o}. Unattached DNA was removed by washing, followed by addition of 30 mM $MgCl_2$ to initiate uptake at either temperature. Figure 7a illustrates the decrease in the total radioactivity occurring during the uptake of attached DNA. At 37^{o}, this decrease is observed in cell to which DNA was initially attached at 37^{o} or at 4^{o}, then shifted to 37^{o}. This decrease is accompanied, in both cases, by an increase in irreversibly bound DNA and the release of acid soluble fragments into the medium. Neither DNA attached at 4^{o} nor that attached at 37^{o}, then shifted to 4^{o} showed any indications of beginning entry into the bacteria at the reduced temperature. The amount of ^{3}H-DNA attached to the bacteria exposed to DNA at 37^{o}, then shifted to 4^{o} at zero time decreased to less than 50% of the 37^{o} control. This suggests the attachment of DNA in the presence of EDTA is sensitive to chilling, even before the addition of excess Mg^{2+}. DNA

released under these circumstances remained intact, since no increase in acid soluble fragments occurred concommitantly.

The results presented in this chapter have established the temperature dependency of the attachment of donor DNA to competent bacteria, and the separation of the initial attachment reaction from the uptake process by addition of 10 mM EDTA. The next section will compare the initial attachment of DNA at 4^{o} and 37^{o} with respect to a variety of parameters.

Figure 7. Analysis of the Entry of DNA Initially Attached at 4^{o} and 37^{o}

Competent Br 151 were incubated at 4^{o} or 37^{o} for 10 minutes prior to the addition of EDTA to a final concentration of 10 mM. After 1 minute, 3 μg/ml ^{3}H-DNA was added and incubated for 15 minutes. The bacteria were sedimented and washed once in a 2X volume of minimal medium + 10 mM EDTA at the appropriate temperature. The cells were finally suspended in minimal medium minus Mg^{2+} at the appropriate temperature, and each sample was divided in two. Samples in which ^{3}H-DNA was initially attached at 4^{o} were incubated at 4^{o} (●) or shifted to 37^{o} (◒). Samples in which ^{3}H-DNA was initially attached at 37^{o} were incubated at 37^{o} (○) or shifted to 4^{o} (◓). Zero time was designated by the addition of 30 mM $MgCl_2$. Total attachment (Panel A), irreversible uptake (Panel B), and the production of acid soluble fragments (Panel C) were measured as described in the Methods section.


A
B
C
CPM/ML x 10⁻³
CPM/ML x 10⁻²
CPM/ML x 10⁻²
6
4
2
9
8
1.5
1.0
0.5
6
4
2
10
20
30
40
MINUTES AFTER ADDITION
OF 30mM Mg²⁺

CHAPTER IV

STUDIES ON PARAMETERS AND SENSITIVITIES OF THE INITIAL ATTACHMENT AND UPTAKE OF DNA IN B. SUBTILIS

The previous section established the temperature dependency of the initial attachment of donor DNA to competent B. subtilis. An attachment of DNA to the competent bacteria at 4^{o} was identified, and suggested the alteration of normal binding sites by the reduction in temperature. In this chapter, such 4^{o} binding will be compared to the attachment of DNA at 37^{o} with respect to various parameters and liabilities.

Initial Attachment in B. Subtilis Strains of Varying Competency

The initial attachment of ^{3}H-DNA at 4^{o} and 37^{o} to strains differing in their ability to attain competence is shown in Table XII. Included in the table is the amount of transformation in each strain after a 30 minute incubation at 37^{o} with 5 µg/ml DNA.

Initial Attachment and Uptake During Competence Development

A high transforming strain, Br 151, was introduced into the competence regime, as described in the Methods section. At hourly inter-

TABLE XII

INITIAL ATTACHMENT AT 4° AND 37° TO STRAINS OF VARYING COMPETENCY

Strain	CPM/ml Attached 4°	37°	% Transformation
WT	723	225	-
C⁻168	67	332	0.017
SB25	174	572	0.053
Br151	896	2916	0.158
NONCOMPETENT Br151	119	176	0.0

Various strains of B. subtilis were grown in the competence regime, and at the end of 5½ hours growth, an aliquot of each was made 10 mM in EDTA. 3 µg/ml ^{3}H-DNA was added and incubated at 37° for 15 minutes. Initial attachment was measured as described in the Methods section.

vals, samples were removed to determine the ability to attach ^{3}H-DNA at 4° and 37°, and to take up the DNA at 37°. Included in Table XIII is the amount of transformation occurring after a 30 minute incubation at 37° with 5 µg/ml DNA to follow the development of transformability. The ability to attach donor DNA at 4° occurred as the culture attained competency.

Effect of Medium pH on Initial Attachment

After development of maximal competency, the bacteria were sedimented and suspended in transformation medium adjusted to various pH's. The initial attachment of ^{3}H-DNA at 4° and 37° was determined under these conditions (Table XIV). Attachment was maximal at pH 7 for both temperatures. At either temperature, an increase in attachment occurred at pH 4. Since DNA is a negatively charged molecule, this reduction of pH may eliminate some repulsion at the surface of the competent cell by limiting the number of similarly charged groups, or increase the number of positively charged groups.

Measurement of irreversible uptake was hampered by the varying effect of pH on the DNase treatment, and thus was omitted.

Effect of Medium Ionic Strength on Initial Attachment and Transformation

Similar to the experiment described above, competent bacteria were sedimented and suspended in transformation medium of varying ionic strength. Figure 8 compares the amount of attachment at 4° and 37° with respect to the ionic strength of the medium. The attachment of donor DNA at 37° decreases with increasing ionic strength, while attach-

TABLE XIII

INITIAL ATTACHMENT AT 4° AND 37°
DURING COMPETENCE DEVELOPMENT

Hour	CPM/ml Attached 4°	37°	Irreversible Uptake	% Transformation
1	17	145	54	0.0
2	23	476	46	0.0004
3	56	375	52	0.003
4	65	279	60	0.006
5	368	3055	151	0.091
6	430	4838	513	0.35

At hourly intervals during the development of competence in Br 151, aliquots were removed. Three µg/ml ^{3}H-DNA or 5 µg/ml DNA was added and incubated for 30 minutes for the determination attachment/ uptake of transformation, respectively. EDTA was added to a final concentration of 10 mM prior to DNA addition for measurement of initial attachment.

TABLE XIV

EFFECT OF pH ON ATTACHMENT
AT 4° AND 37°

pH of Medium	CPM Attached/ml 4°	37°
4	1908	1144
5	417	324
6	287	494
7	1124	3651
8	286	456
9	314	320

Competent Br 151 were sedimented and resuspended in transformation media of varying pH plus EDTA at a concentration of 10 mM. 3 µg/ml ^{3}H-DNA was added and incubated at either temperature for 30 minutes. Initial attachment was determined as described in the Methods section. Transformation media was adjusted in pH by the addition of 1 N HCl or 1 N NaOH and monitering the pH change on a Radiometer pH meter.

ment at 4° increases under the same conditions.

Measurements of the uptake of attached ^{3}H-DNA resulted in irreproducible results, due to the effect of high ionic strength on DNase activity. However, the extent of uptake at 37° was indirectly measured by the amount of transformation occurring in media of increasing ionic strength. Figure 9 illustrates transformation at 37° rapidly declines with increasing ionic strength. This decline in transformation parallels the decline in initial attachment.

Comparison of the transformation resulting from the increase in attached DNA at 4° with increasing ionic strength was difficult at 4° because of the negligible levels of transformation obtained. Therefore, transforming DNA was attached at various ionic strengths at 4°, followed by a 1:100 dilution in warm minimal medium of identical ionic strength and an additional 30 minute incubation at 37°. Figure 10 reveals that the increase in attachment at 4° resulted in an increase in subsequent transformation at 37° as the ionic strength increased to 0.5. Increasing the ionic strength above this value progressively reduced transformation, indicating a high ionic strength sensitivity of DNA uptake at 37°. That DNA attached at elevated ionic strength at 4° could lead to transformation at 37° implies that this is not a non-specific binding.

Inhibition of Initial Attachment and Uptake of DNA by a Cold Osmotic Shock

Pierson (139) described a cold osmotic shock procedure which reduced the irreversible uptake of DNA and subsequent transformation by 70-90%. Table XV shows the attachment, irreversible uptake, and trans-

Figure 8. Effect of Ionic Strength on Initial Attachment

Competent Br 151 were sedimented and resuspended in transformation media of varying ionic strength. The ionic strength was increased by the addition of KCl to the medium. Suspensions at each ionic strength were divided in half, one half being incubated at 4^{o} for 10 minutes (●), the other at 37^{o} for 10 minutes (○). Three μg/ml ^{3}H-DNA was added and incubated at the appropriate temperature for 15 minutes. Total attachment was determined as described in the Methods section, with the exception that all washings were done with minimal medium of the appropriate ionic strength.

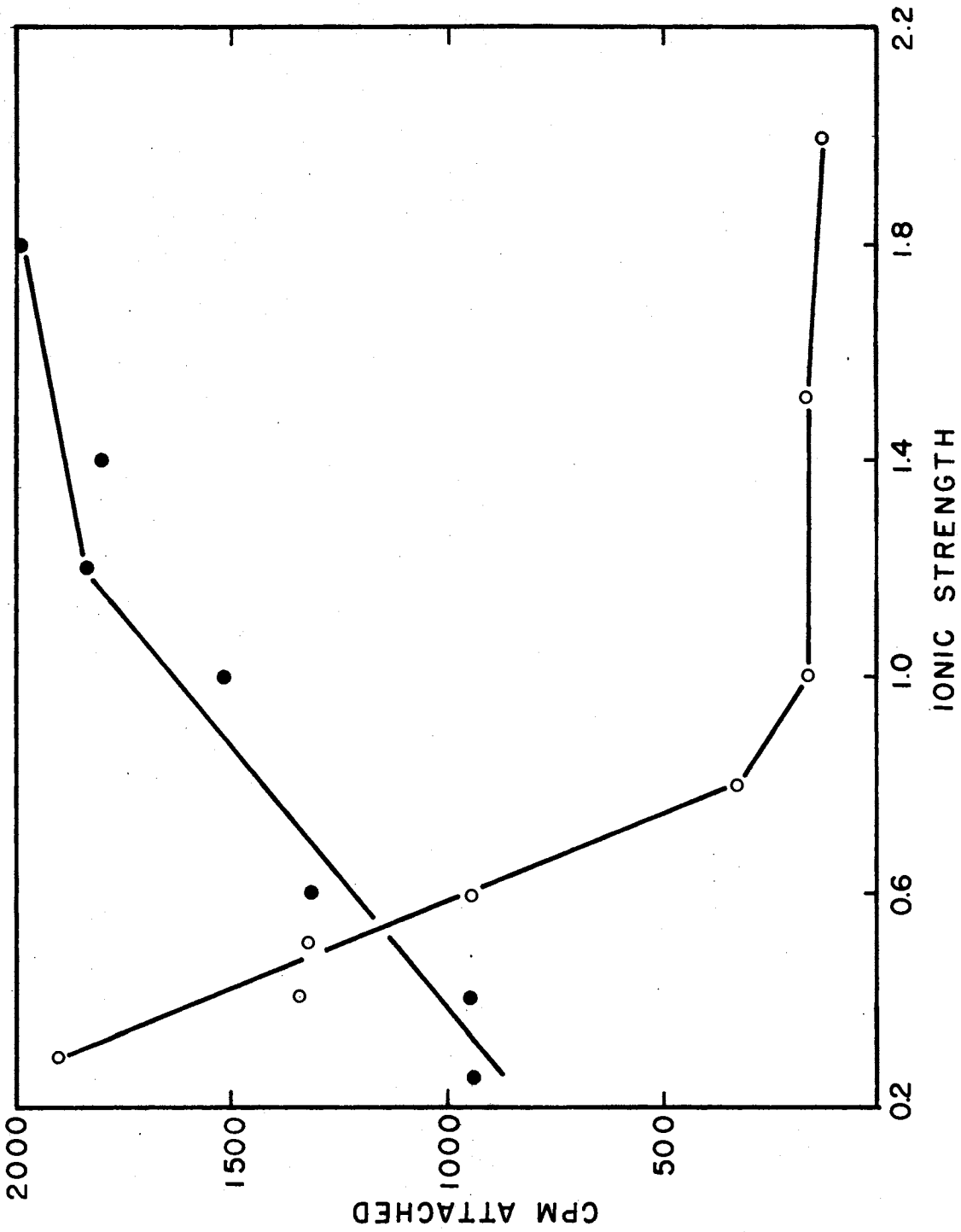

CPM ATTACHED
2000
1500
1000
500
0.2
0.6
1.0
1.4
1.8
2.2
IONIC STRENGTH

Figure 9. Transformation at 37^{o} with Increasing Ionic Strength

Competent Br 151 were sedimented and suspended in media adjusted to various ionic strength by the addition of KCl. After 5 minutes incubation at 37^{o}, 5 µg/ml DNA was added and incubated for 30 minutes. Transformation was determined as described in the Methods section, with the exception that all serial dilutions were made in media of the appropriate ionic strength.

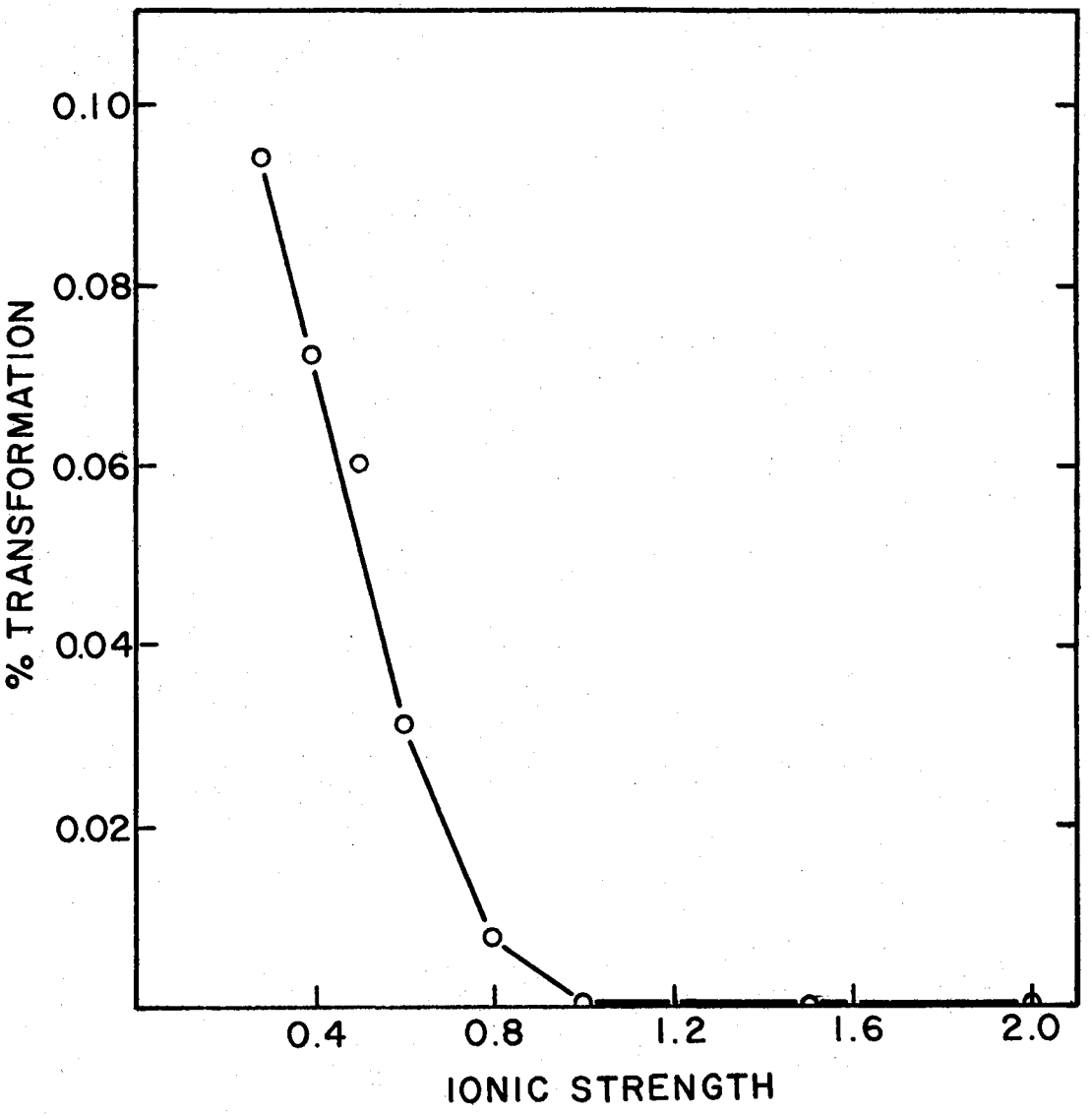
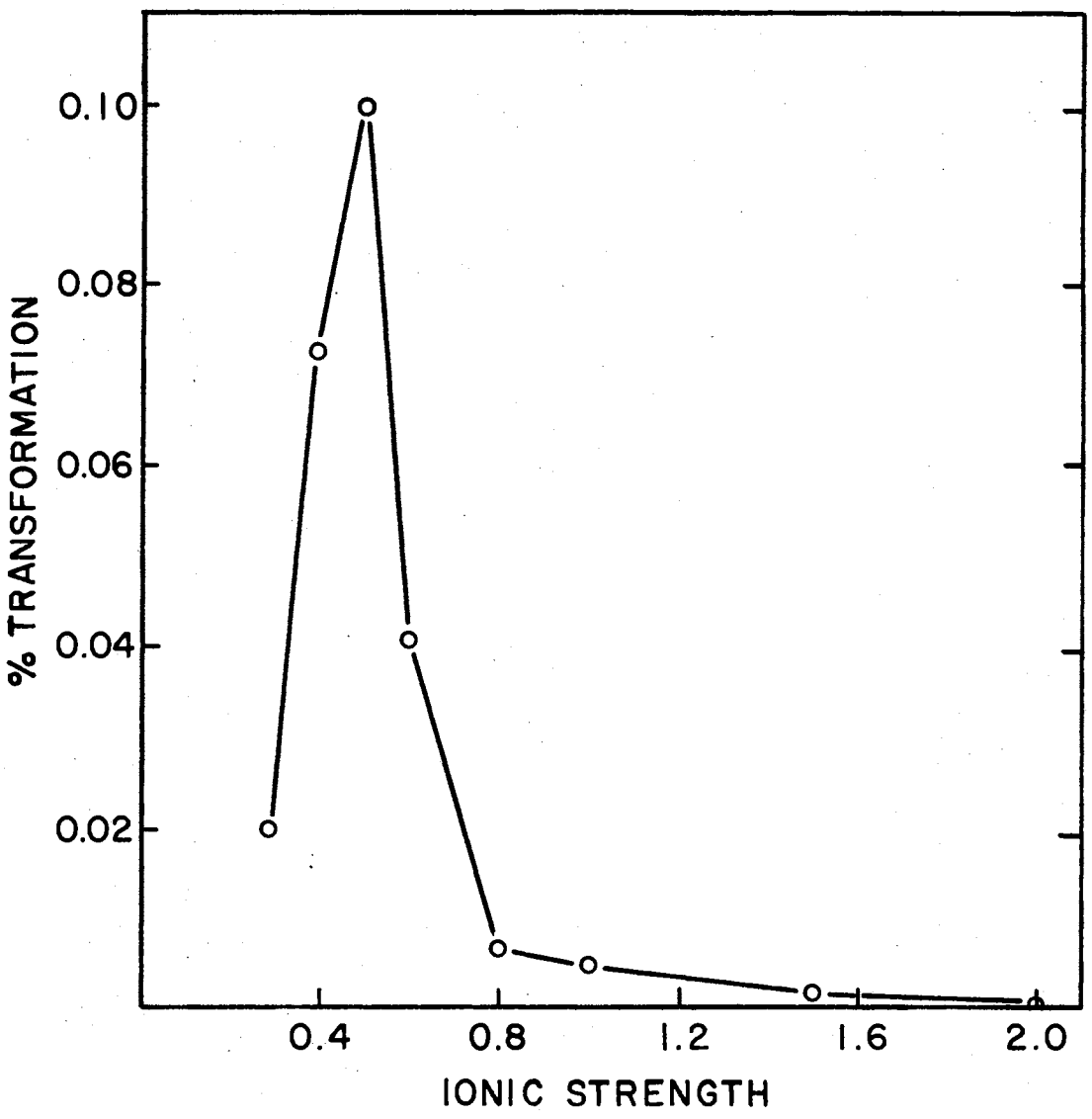

0.10
0.08
0.06
0.04
0.02
% TRANSFORMATION
0.4
0.8
1.2
1.6
2.0
IONIC STRENGTH

Figure 10. Transformation at 37^{o} from DNA Initially Attached at 4^{o} in Media of Increasing Ionic Strength

Competent Br 151 were sedimented and suspended in media adjusted to various ionic strength by the addition of KCl. The suspensions were incubated at 4^{o} for 10 minutes prior to the addition of 5 µg/ml DNA. After 15 minutes of incubation at 4^{o}, a 1:100 dilution into warm transformation medium of the appropriate ionic strength was made and incubated at 37^{o} for 30 minutes. Transformation was determined as described in the Methods section with the exception that all serial dilutions were made in media of the appropriate ionic strength.


0.10
0.08
0.06
0.04
0.02
% TRANSFORMATION
0.4
0.8
1.2
1.6
2.0
IONIC STRENGTH

formation at 4^{o} and 37^{o} after an osmotic shocking at 37^{o}. Such a treatment reduced the attachment and uptake of DNA by 75% and 66%, respectively, and the amount of transformation by 70% at 37^{o}. The effect on transformation and uptake in shocked cells chilled to 4^{o} was more severe. Attachment was not reduced, while the irreversible uptake and transformation were too low to measure.

Attachment at the reduced temperature is not as labile to osmotic shocking as the attachment at 37^{o} is, supporting the assumption that the sites to which attachment occurs at these two temperatures are different. Since the osmotic shock treatment must in part occur at 25^{o}, no information on the susceptibility of these measurements to osmotic shocking at 4^{o} was obtained.

Initial Attachment and Uptake of DNA After Phenethyl Alcohol Treatment

Addition of PEA one hour before maximal competence to a final concentration of 0.05% reduces transformation by 50-70% (138). The attachment and uptake of ^{3}H-DNA at 4^{o} and 37^{o} in PEA-treated bacteria is shown in Table XVI. PEA reduced both the attachment and uptake of ^{3}H-DNA at 37^{o} by 88% and 66%, respectively. However, the amount of initial attachment at 4^{o} was unaffected. The irreversible uptake at 4^{o} was reduced to a negligible level.

Effect of Incubation at 50^{o} on the Initial Attachment and Uptake of DNA

McCarty and Nester (99) described a reduction in the transformation of *B. subtilis* by brief incubation of the bacteria at 50^{o} prior

TABLE XV

EFFECT OF COLD OSMOTIC SHOCK ON ATTACHMENT AND UPTAKE OF DNA

Treatment	Initial Attachment	Irreversible Uptake	% Transformation
37°			
Control	8815	1324	0.800
Shocked	1286	181	0.239
4°			
Control	476	94	0.003
Shocked	408	60	0.000

Competent Br 151 were osmotically shocked as described in the Methods section. Shocked cells were suspended in transformation medium and incubated at 4° and 37° for 10 minutes. EDTA was added to a concentration of 10 mM in samples from each temperature for the measurement of initial attachment. 3 µg/ml ^{3}H-DNA or 5 µg/ml DNA was added and incubated for 30 minutes for the determination of initial attachment/uptake or transformation, respectively.

TABLE XVI

INITIAL ATTACHMENT AND UPTAKE OF DNA
AFTER PHENETHYL ALCOHOL TREATMENT

Treatment	Initial Attachment	Irreversible Uptake	% Transformation
37°			
Control	2742	327	0.14
PEA-Treated	600	113	0.04
4°			
Control	468	68	0.002
PEA-Treated	512	3	0.001

Phenethyl alcohol was added to competent cells at a final concentration of 0.05% one hour prior to maximum competence. After one hour incubation, the bacteria were washed twice with minimal medium, sedimented and suspended in transformation medium. The suspension was divided in half, one half being incubated at 4° or 37° for 10 minutes as were the controls prior to the addition of 3 µg/ml ^{3}H-DNA or 5 µg/ml DNA for the measurement of attachment/uptake or transformation respectively. Aliquots for the measurement of initial attachment were made 10 mM in EDTA before addition of ^{3}H-DNA. Incubation was continued for 30 minutes, after which initial attachment, irreversible uptake, and transformation were determined as described in the Methods section.

to the addition of transforming DNA at 37°. Figure 11 illustrates the progressive decrease in attachment, irreversible uptake, and transformation with increasing incubation at 50°. The results indicate a heat labile factor(s) involved in both the attachment and uptake of DNA at 37°. Exposure of 50° incubated bacteria to ^{3}H-DNA at 4° resulted in the same amount of attachment as the 4° control, but no measurable uptake or subsequent transformation. Attachment at the reduced temperature is not heat labile, either because this reaction does not involve any heat labile components, or because any such components are not exposed until the temperature is reduced to 4°.

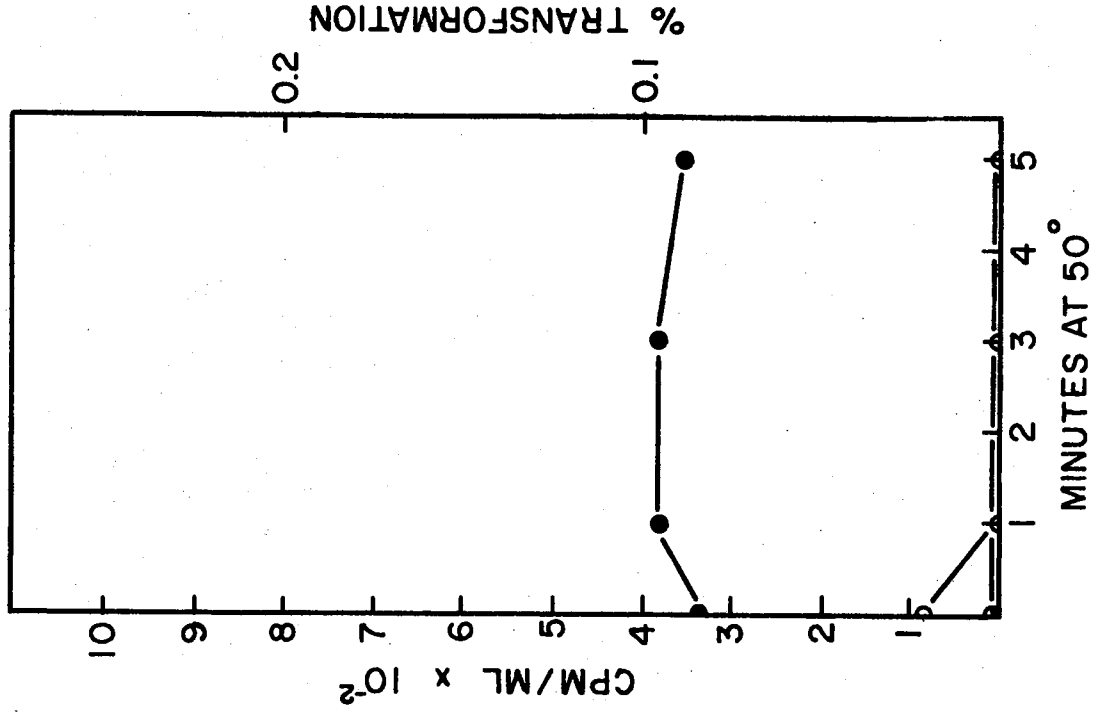
Effect of Sodium Meta Periodate on the Initial Attachment and Uptake of DNA

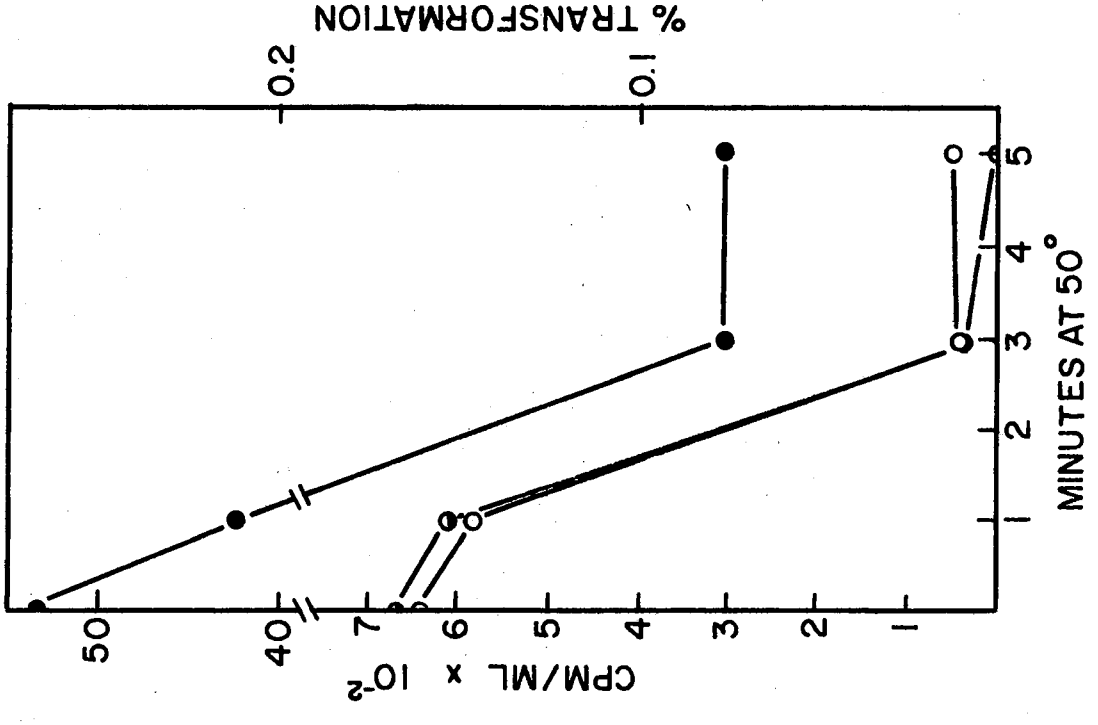
Exposure of competent B. subtilis to 5 x 10^{-4} M sodium meta periodate for 10 minutes prior to incubation with transforming DNA decreases the subsequent amount of transformation (112). Ranhand (113) has compared the results of periodate oxidation at 0° and 37° in Streptococcus to demonstrate that the DNA binding sites contain amino acids. At 37° and 0°, meta periodate oxidizes both carbohydrates and certain amino acids occurring internally in peptide chains (115), while at 0°, only the oxidation of carbohydrates occurs (114).

The results of periodate oxidation of competent B. subtilis, with respect to initial attachment, irreversible uptake, and transformation at 4° and 37° is shown in Table XVII. The irreversible uptake of DNA at 37° was reduced by periodate treatment, regardless of the temperature at which it occurred. This is supported by the inhibition of transformation at both temperatures, and in the case of DNA attached

Figure 11. Effect of Incubation at 50^{o} on Initial Attachment and Uptake of ^{3}H-DNA

Competent BR 151 were heated in a 50^{o} water bath for the designated lengths of time, then incubated at 4^{o} or 37^{o} for 10 minutes. Three µg/ml ^{3}H-DNA or 5 µg/ml DNA was added and incubated for 30 minutes for the measurement of initial attachment/uptake or transformation, respectively. Aliquots of bacteria used for the measurement of initial attachment were made 10 mM in EDTA before the addition of ^{3}H-DNA. Initial attachment (●) irreversible uptake (◒), and transformation (○) were measured as described in the Methods section.


% TRANSFORMATION
0.2
0.1
CPM/ML x 10^{-2}
10
9
8
7
6
5
4
3
2
1
MINUTES AT 50°
1
2
3
4
5


% TRANSFORMATION
0.2
0.1
CPM/ML x 10^{-2}
50
40
7
6
5
4
3
2
1
MINUTES AT 50°
1
2
3
4
5

at 4^{o}, but shifted to 37^{o} to complete the transformation sequence. Initial attachment at 37^{o} was reduced by periodate oxidation at 37^{o}, but not to such an extent by treatment at 0^{o}. At 4^{o}, attachment was not as affected by prior periodate oxidation at 37^{o} as it was by the same treatment at 0^{o}.

The observations on the susceptibility of 37^{o} attachment support the findings of Ranhand (113). However, the reduction of 4^{o} binding only by periodate treatment at 0^{o} supports an explanation of the differential effect of periodate oxidation by the alteration of attachment sites. Since 0^{o} periodate treatment oxidizes only carbohydrates, the resulting reduction of 4^{o} attachment should also be produced by a 37^{o} oxidation, unless the periodate sensitive components are not available at 37^{o}. A similar logic could be used to explain the 37^{o} results.

To investigate the effect of periodate oxidation subsequent to the attachment of DNA, the experiment described in Figure 12 was performed. This would assay for the effect of periodate on transformation in bacteria to which DNA had only become initially attached, and in bacteria in which the uptake of DNA was actively occurring. Figure 13 illustrates that periodate oxidation during the intact transformation process did reduce transformation. If only initial attachment of DNA was permitted before the periodate treatment by the addition of EDTA, no subsequent transformation was observed after the addition of excess Mg^{2+}. Either the oxidation released the attached DNA, or it destroyed the bacteria's capacity for irreversible uptake.

TABLE XVII

EFFECT OF PERIODATE OXIDATION AT 0° OR 37° ON INITIAL ATTACHMENT, UPTAKE, AND TRANSFORMATION AT 4° AND 37°

Treatment and Temperature	Initial Attachment CPM/ml	Irreversible Uptake CPM/ml	% Transformation
37°			
Control	10342	594	0.311
$IO_4^=$ at 0° for 10 minutes	6323	191	0.014
$IO_4^=$ at 37° for 10 minutes	1513	102	0.000
4°			
Control	770	73	0.002
$IO_4^=$ at 0° for 10 minutes	254	71	0.002
$IO_4^=$ at 37° for 10 minutes	643	84	0.002
4° + DNA 15 minutes ⟶	1:100 dilution 30 minute incubation at 37°		0.036
$IO_4^=$ at 0° for 10 minutes, then 4° + DNA 15 minutes ⟶	1:100 dilution 30 minute incubation at 37°		0.003
$IO_4^=$ at 37° for 10 minutes, then 4° + DNA 15 minutes ⟶	1:100 dilution 30 minute incubation at 37°		0.000

Figure 12. Experimental Sequence for the Effect of Periodate Treatment on Transformation Subsequent to Initial Attachment of DNA

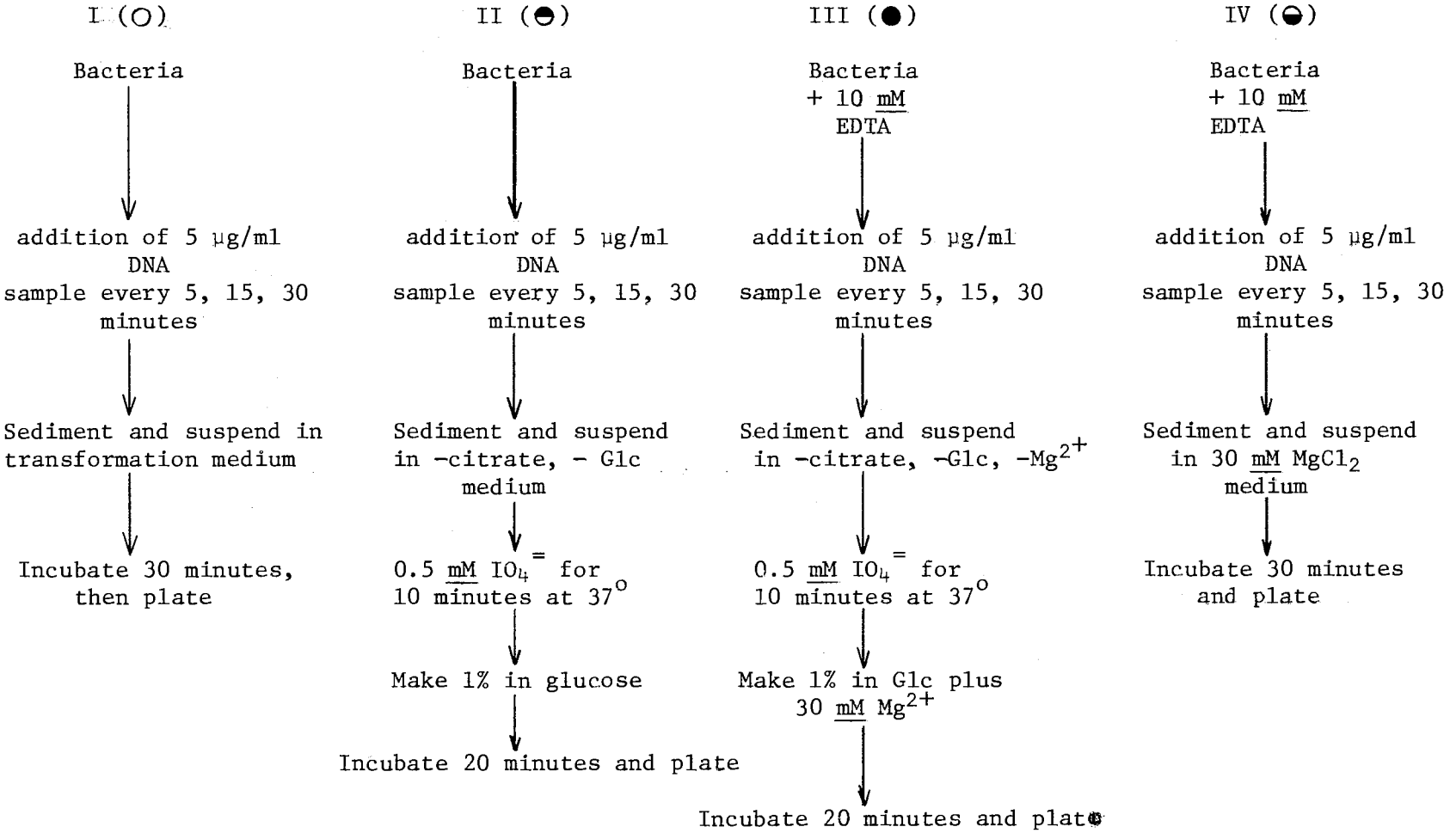
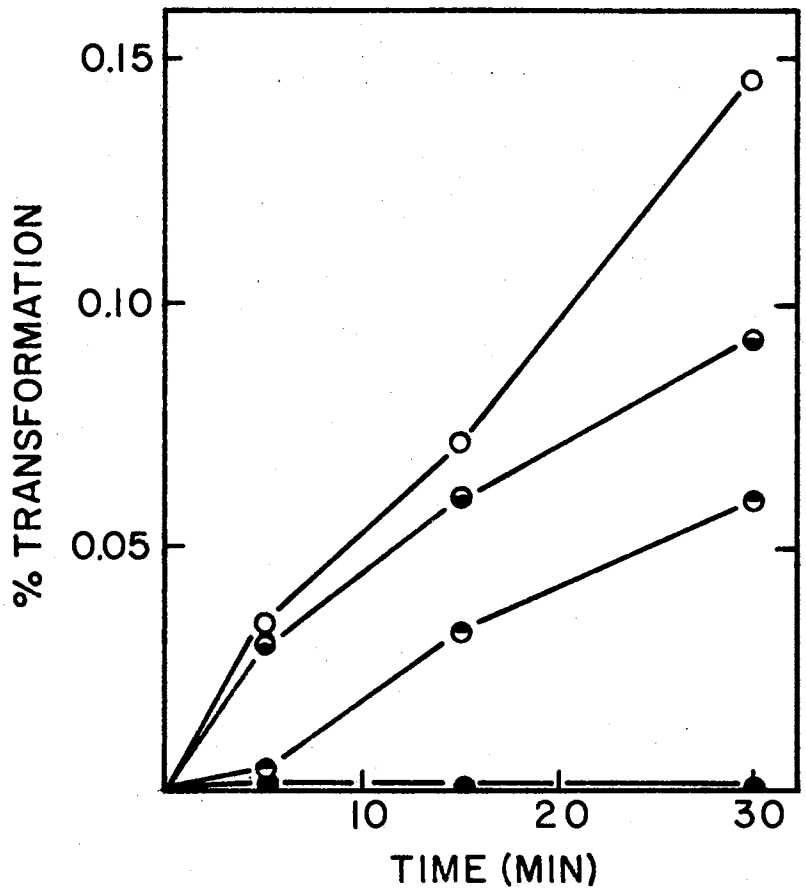

I (○)
Bacteria
addition of 5 μg/ml DNA
sample every 5, 15, 30 minutes
Sediment and suspend in transformation medium
Incubate 30 minutes, then plate
II (◒)
Bacteria
addition of 5 μg/ml DNA
sample every 5, 15, 30 minutes
Sediment and suspend in -citrate, - Glc medium
0.5 mM $IO_4^=$ for 10 minutes at 37°
Make 1% in glucose
Incubate 20 minutes and plate
III (●)
Bacteria + 10 mM EDTA
addition of 5 μg/ml DNA
sample every 5, 15, 30 minutes
Sediment and suspend in -citrate, -Glc, $-Mg^{2+}$
0.5 mM $IO_4^=$ for 10 minutes at 37°
Make 1% in Glc plus 30 mM Mg^{2+}
Incubate 20 minutes and plate
IV (◒)
Bacteria + 10 mM EDTA
addition of 5 μg/ml DNA
sample every 5, 15, 30 minutes
Sediment and suspend in 30 mM $MgCl_2$ medium
Incubate 30 minutes and plate

Figure 13. Effect of Periodate Treatment Subsequent to Initial Attachment

The experimental procedure was described in Figure 12.


0.15
0.10
0.05
% TRANSFORMATION
10
20
30
TIME (MIN)

CHAPTER V

THE EFFECT OF TREATMENT IN A WARING BLENDOR ON THE TRANSFORMABILITY OF *B. SUBTILIS*

Little information is available on the surface structure of competent bacteria. The sensitivity of competent bacteria to various treatments and the antigenicity of the intact competent bacterium, antisera against which will effectively inhibit transformation, suggests the presence of specific structures on the cell surface. The identity of such structures remains obscure. However, the ability of three bacterial species, *Moraxella nonliquefaciens*, *M. bovis*, and *M. kingi*, to undergo genetic transformation is dependent upon colony type and the amount of fimbriation (132). Highly fimbriated Moraxellae formed spreading, agar corroding colonies, the cells of which were several orders of magnitude more competent than weakly or non-fimbriated cells from non-corroding colonies. Non-fimbriated recipient Moraxellae may exhibit a low level of competence, and the presence of fimbriae does not necessarily result in a high level of competency. Thus, the specific role of fimbriae in the transformation of these species has not been resolved. The presence of fimbriae on the cell surface may confer unique properties to the bacterium, or may only reflect the presence of a competence factor(s).

Endo (116) reported the removal of flagella and reduction in transformability in *B. subtilis* by treatment in a Waring blendor. Al-

though gram positive bacteria, such as B. subtilis, do not possess fimbriae, the agitation by blending may remove or disorient surface structures on the competent bacterium, affecting their transformability. Investigation of the effect of blending may aid in the identification of the structure and function of such surface moieties.

Effect of Treatment in a Waring Blendor on Transformation

The sensitivity of a competent culture of B. subtilis to treatment in a Waring blendor was determined by measuring the level of transformation and the total amount of labeled DNA associated with cells blended for various lengths of time prior to the addition of DNA. Figure 14 reveals that contrary to the results of Endo (116) brief blending enhanced the culture's transformability, while drastically reducing the amount of labeled DNA associated with the cells. More extensive blending failed to further reduce the amount of DNA bound, but did progressively decrease the amount of transformation to a negligible level. The cell titer was not substantially reduced until the blending continued for 2 minutes or longer.

Figure 15 is a plot of the ratio of the number of transformants per amount of bound DNA as a function of the extent of blending. This indicates the efficiency of the transformation process. The data shows an immediate increase for the first 30 seconds of blending, followed by a rapid decline to below the control value. Different competent cultures showed slight variations in the sensitivity of transformability and cell viability to the duration of blending. However, a 10 second treatment consistently enahnced transformation and a 45 second blending

Figure 14. Effect of Treatment in a Waring Blendor on Attachment of ^{3}H-DNA and Transformation

Competent Br 151 were treated in a Waring blendor for the indicated lengths of time. Three µg/ml ^{3}H-DNA or 5 µg/ml DNA was added and incubated for 30 minutes at 37^{o} for measurement of total attachment (●) or transformation (○), respectively. Total attachment and transformation were determined as described in the Methods section. The viable titer (◒) was obtained from scoring bacteria on minimal medium agar plates supplemented with tryptophan.

Figure 15. Efficiency of DNA attached to Blended Cells to Produce Transformation

The number of transformants per ml produced after various lengths of blending was divided by the amount of ^{3}H-DNA associated with a ml of the competent culture after the identical length of blending. This number was plotted as a function of the extent of blendor treatment.

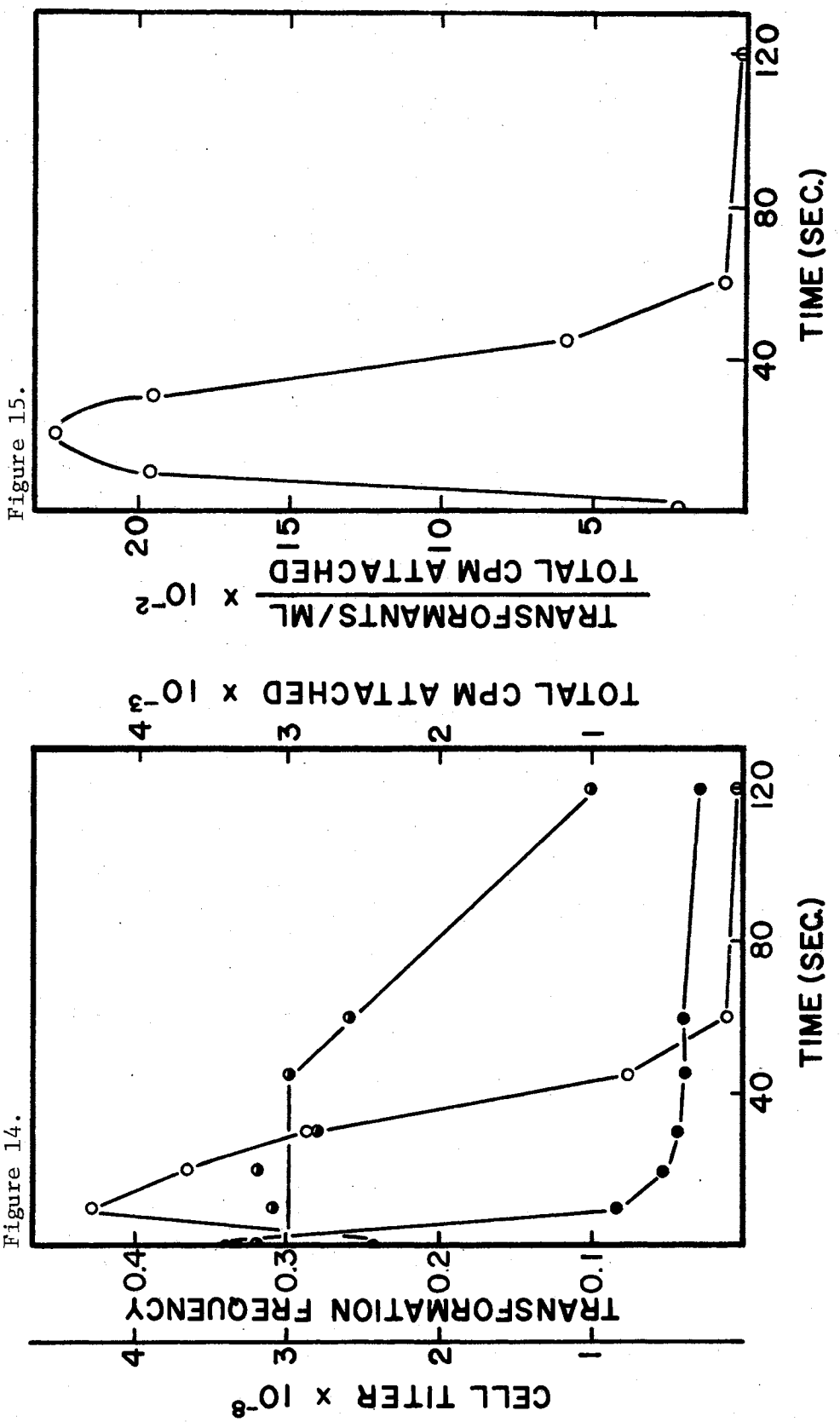


Figure 14.

Figure 15.

inhibited transformation.

The effect of blending specifically on the initial attachment of donor DNA and its irreversible uptake is shown in Figure 16. Initial attachment of DNA is immediately reduced by only 10 seconds of blending to a level which remains approximately constant, regardless of further blending. However, the irreversible uptake of DNA appears to be affected only after blending for 30 seconds or more.

Resolution of the effect of blending on transformation is complicated by its variation with the degree of treatment. The reduction in the amount of donor DNA attached to the bacteria with no effect on irreversible uptake and the same or increased level of transformation suggests a portion of the DNA associated with untreated competent cells may not necessarily be involved in the transformation sequence.

Blending cells exposed to transforming DNA prior to treatment should delineate the sensitive portion of the transformation sequence. Table XVIII shows the results of bacteria incubated with DNA for 30 minutes, followed by a 10 minute exposure to 50 μg/ml DNase to terminate further transformation, then blended. No effect on the level of transformability is observed if the blending occurs after the DNase resistant uptake of DNA. The amount of transformation remained constant with increasing length of blending until a reduction in cell viability occurred. Thus, the described reduction in transformation with extensive blending is not due to any specific susceptibility of the competent cells to the treatment.

Table XIX shows that the brief blending of strains differing in their ability for competence development did increase all their transformabilities. The difference in the transformability between strains

Figure 16. Effect of Blending on Initial Attachment and Irreversible Uptake of H-DNA

Competent Br 151 were treated in a Waring blendor for the indicated lengths of time. At each time, an aliquot was removed and made 10 mM in EDTA for the measurement of initial attachment (○). A second aliquot was removed for the measurement of irreversible uptake (●). Three μg/ml ^{3}H-DNA was added and incubated for 30 minutes. Initial attachment and irreversible uptake were determined as described in the Methods section.

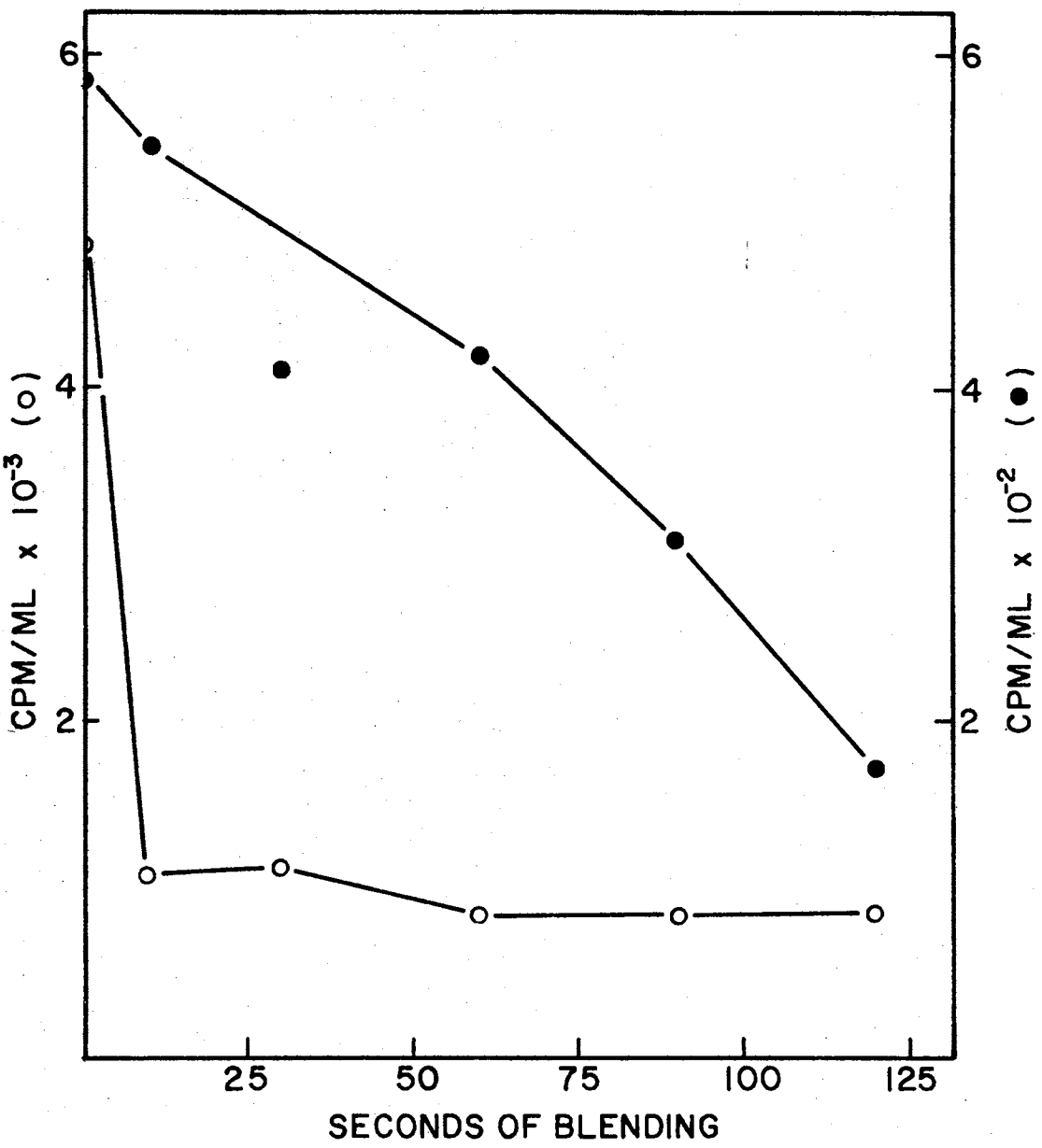

6
4
2
CPM/ML x 10^{-3} (o)
6
4
2
CPM/ML x 10^{-2} (●)
25
50
75
100
125
SECONDS OF BLENDING

TABLE XVIII

THE EFFECT OF BLENDING ON A PREVIOUSLY TRANSFORMED CULTURE

Length of Blending	% Transformation	Cell Titer X 10^{-8}
Control	0.427	2.6
15 Seconds	0.467	2.4
30 Seconds	0.444	2.2
45 Seconds	0.452	2.5
1 Minute	0.484	1.9
2 Minutes	0.376	1.3

Competent Br 151 were exposed to 5 µg/ml DNA for 30 minutes at 37°, after which time 50 µg/ml DNase was added and incubated for 15 minutes at 37°. The bacteria were sedimented and suspended in warm transformation medium. The suspension was blended in a Waring blendor for the designated periods of time. Aliquots were removed and scored for TRY^+ transformants, as described in the Methods section.

TABLE XIX

THE EFFECT OF BRIEF BLENDING ON THE TRANSFORMABILITY OF SEVERAL STRAINS OF *BACILLUS SUBTILIS*

Strains	% Transformation	
	Untreated	Blended
Br 151	0.145	0.267
SB 25	0.030	0.064
$C^{-}168$	0.011	0.019

Competent *B. subtilis* strains Br 151, SB 25, and $C^{-}168$ were individually blended for 10 seconds in a Waring blendor. To samples of each, and also to those of untreated controls for each strain, was added 5 μg/ml DNA. Incubation at 37° for 30 minutes was allowed before scoring for TRY^{+} transformants as described in the Methods section.

remained the same after blending.

Cellular Alterations Due to Blending

The primary effect of treatment in a Waring blendor on B. subtilis was investigated with respect to the susceptibility of various cellular activities other than transformation. Figure 17 is a comparison of the growth rate of untreated bacteria and bacteria blended to an extent which increased their transformability. Included in the figure are growth curves after a 1:10 dilution of bacteria into fresh transformation medium. The cells blended for 10 or as long as 60 seconds grew faster with or without the dilution into fresh medium, compared to the control. The increased growth rate with blending may result from a physical alteration in the cells, but more likely represents the effect of a thorough aeration.

Since transformation involves the active transport of DNA through the cell wall and membrane, the sensitivity to blending may result from an alteration in properties of the cell wall, membrane, or the transport process itself. The sensitivity of another active transport process to blending may substantiate one or more of these possibilities. The uptake, accumulation and incorporation of ^{14}C-L-leucine in bacteria blended for lengths of time short of reducing cell viability is shown in Figure 18. No change is apparent, implying the integrity of this transport system and subsequent ability to incorporate L-leucine were not altered. If blending is continued until reduction of the cell titer occurs, the uptake, accumulation and incorporation are sharply reduced.

Figure 17. Effect of Blending on the Growth Rate of Competent Bacteria

Competent Br 151 were treated in a Waring blendor for 10 seconds. The blended suspension (●) and an untreated control (○) were incubated at 37°, or diluted 1:10 in fresh transformation medium supplemented with transformation enhancing amino acids {untreated control (◒), blended (◓)}. Bacterial growth was monitored by following the increase in turbidity at 630 nm on a Beckman DU spectrophotometer.

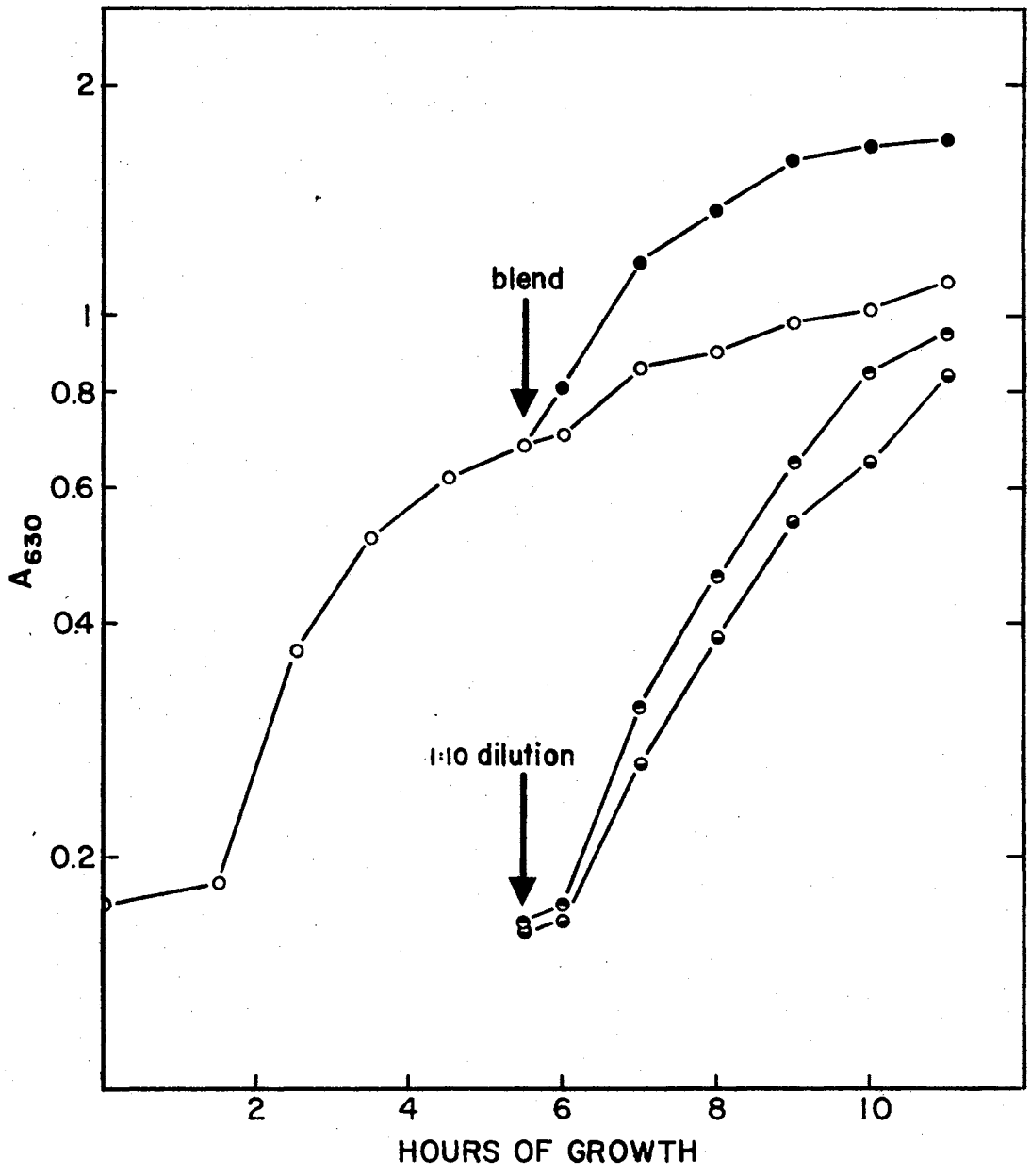
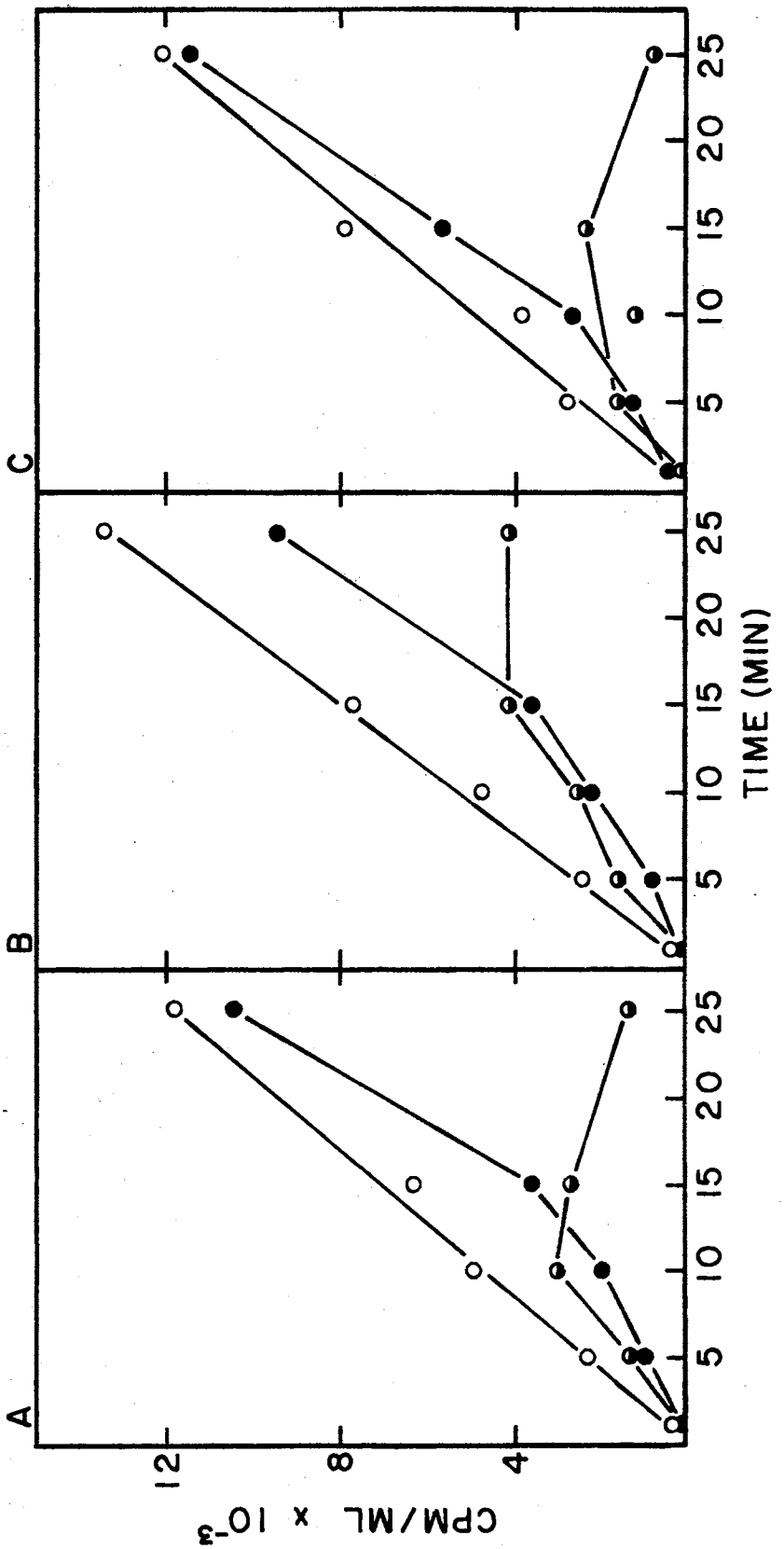

2
1
0.8
0.6
0.4
0.2
A_{630}
blend
1:10 dilution
2
4
6
8
10
HOURS OF GROWTH

Figure 18. Uptake, Incorporation, and Accumulation of ^{14}C-L-Leucine in Blended Cells

Competent Br 151 were untreated (Panel A) or treated in a Waring Blendor for 10 seconds (Panel B) or 60 seconds (Panel C). To the cell suspensions was added 0.5 μC/ml ^{14}C-L-leucine plus a 250 fold excess of cold leucine. Uptake (○), incorporation (●), and accumulation (◒) of ^{14}C-L-leucine were determined as described in the Methods section.


A
B
C
CPM/ML x 10⁻³
4
8
12
5
10
15
20
25
TIME (MIN)

Cell Wall Integrity After Blending

An apparent subtle alteration of the cell wall by blending was detected by an increase in the amount of ^{14}C-D-alanine incorporated by treated bacteria. The extent of this increase varied with the length of the blending treatment (Figure 19). Cells blended for only 10 seconds showed a 43% increase in the incorporation of D-alanine after a 35 minute incubation. As the length of blending increased, the increase in incorporation progressively lessened. This decrease of incorporation may be the result of a combination of factors. Included in Figure 19 are the rates of uptake of D-alanine in blended bacteria. The variation in uptake parallels that of incorporation, suggesting that extensive blending upsets either the uptake of D-alanine or the incorporation of newly synthesized peptidoglycan into the injured wall. Since the uptake of D-alanine in the most extensively blended cells is still increased over the control, it is more likely such blending disrupts the wall repair mechanism, with the result of decreasing the uptake and incorporation of D-alanine.

The possibility that blending weakens the cell wall was not supported by an increase in the sensitivity of blended cells to a lysozyme treatment (Table XX). The rate of decrease in turbidity was unaffected in cultures blended up to 2.5 minutes. That briefly blended cells do not exhibit an increased sensitivity to lysozyme does not rule out a weakening in the cell wall.

A function of the cell wall is to protect cytoplasmic entities from osmotic damage. Thus, damage to the cell wall may be reflected in the susceptibility of blended cells to an osmotic shock. Figure

Figure 19. Uptake and Incorporation of ^{14}C-D-Alanine in Blended Cells

Competent Br 151 were treated in a Waring blendor for the designated lengths of time; untreated control (○), 10 seconds (●), 25 seconds (◒), and 45 seconds (◒). Samples were made 2 X 10^{-5} M in ^{14}C-D-alanine and incubated at 37°. At designated intervals, 1 ml aliquots were removed, filtered onto Gelman GA-6 filters and washed immediately with minimal medium to measure uptake (A). A similar aliquot was added to 10% TCA, chilled for 15 minutes at 0°, and filtered to determine incorporation (B).

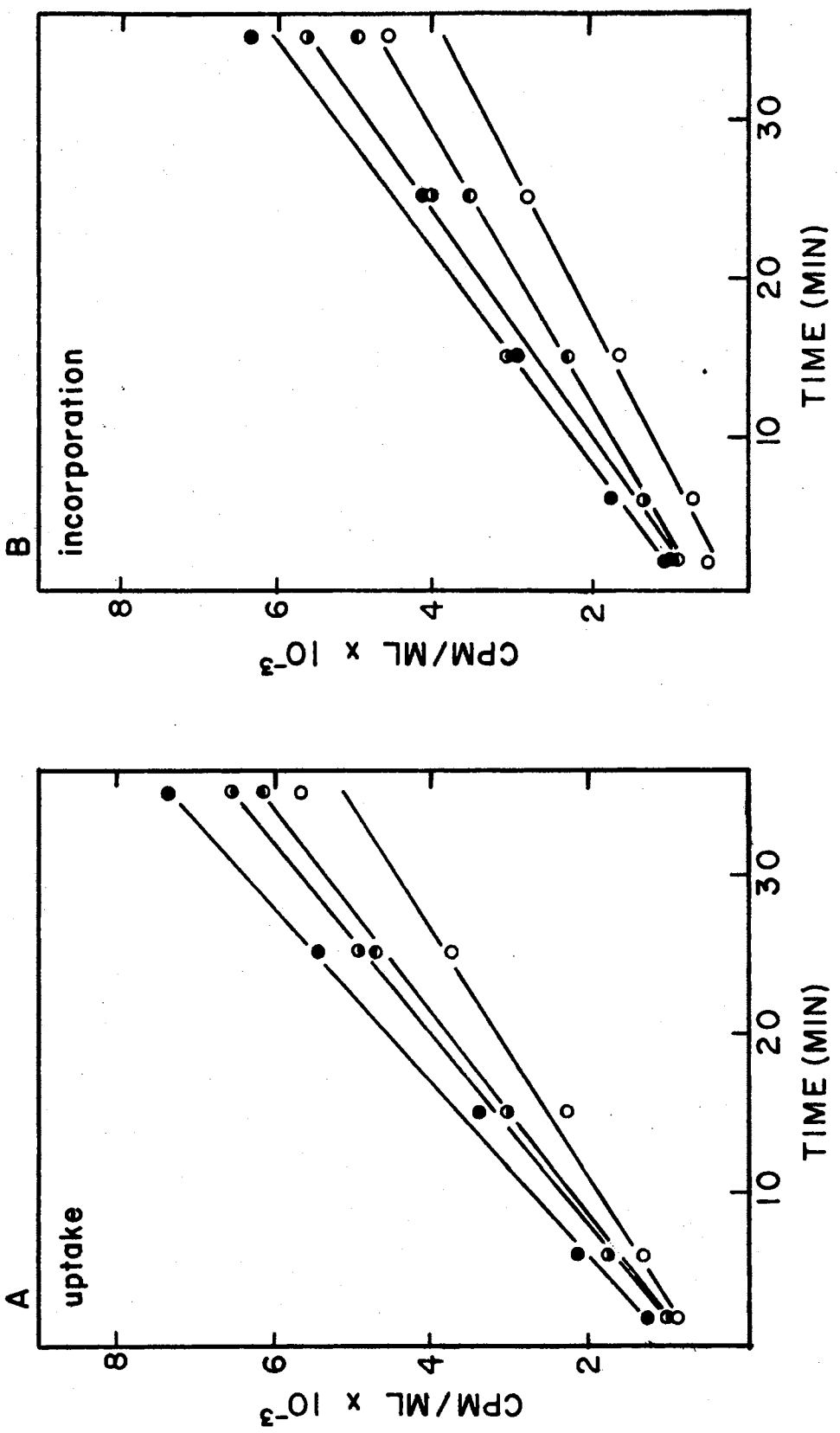

A
uptake
B
incorporation
CPM/ML x 10⁻³
2
4
6
8
10
20
30
TIME (MIN)

TABLE XX

SENSITIVITY OF BLENDED CELLS TO LYSOZYME

Seconds of Blending	A_{540}/minute
Control	0.058
10	0.058
20	0.048
30	0.044
45	0.048
60	0.052
90	0.052
120	0.056
150	0.050
180	0.060
240	0.062

Competent Br 151 were treated in a Waring blendor for the designated lengths of time. 500 µg/ml of lysozyme was added to each sample and the rate of decrease in turbidity, as measured at 540mm, was determined on a Hitachi Perkin-Elmer double beam spectrophotometer.

20 reveals such an osmotic shock treatment did not reduce viability in blended cells, until the blending itself did. The osmotic shock procedure used was that described by Pierson (139) for the removal of a competence factor from the "periplasmic space" of B. subtilis.

With evidence for the injury of the cell wall by the blendor treatment, the release of periplasmic or cytoplasmic material was determined by measuring for the release of labeled DNA, protein, and phospholipid in the cultural fluid (Table XXI). No detectable DNA or phospholipid was released, while only 0.5% of the total labeled protein was released by blending. This can be accounted for by the removal of flagella by the treatment.

Phase Contrast and Electron Microscopy of Blended Cells

Observation of blended bacteria by phase contrast or electron microscopy revealed only one predominant change in their morphology, that being the removal of flagella. Untreated cells were rod shaped and very motile under phase contrast microscopy. Blending for 10 seconds, an amount which enhanced transformability, caused no change in the bacillus shape, but substantially decreased their motility. Increasing the length of blending in 30 second intervals to a total of 4 minutes did not alter the cells' shape, nor did it break up chains of several bacilli in length which were frequently seen.

Electron microscopy of negatively stained blended bacteria shows the complete removal of flagella (Figure 21). Broken flagella are seen in the background, with the blended cells often appearing rounded. Since this rounding was not observed under phase contrast microscopy, it may be the result of the uranyl acetate negative staining. This

Figure 20. Susceptibility of Blended Cells to Osmotic Shocking

Competent Br 151 were blended in a Waring blendor for various lengths of time. At each time, 0.1 ml was removed and serially diluted for determining the cell titer (O) and a 2 ml aliquot was removed for osmotic shocking (●), as described in the Methods section. Osmotically shocked blended cells were serially diluted and plated for determination of the viable cell titer.

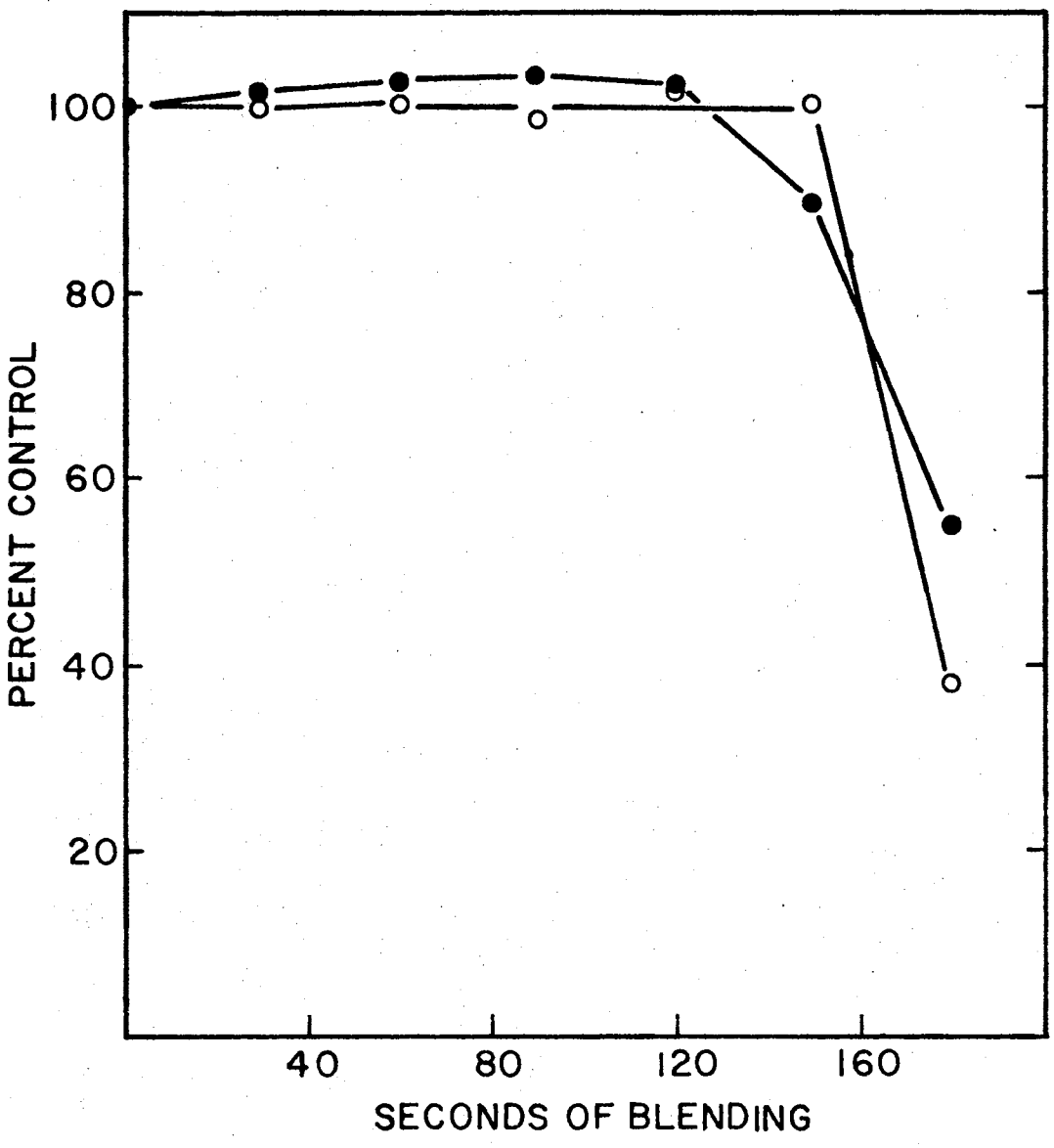

100
80
60
40
20
PERCENT CONTROL
40
80
120
160
SECONDS OF BLENDING

TABLE XXI

RELEASE OF DNA, PHOSPHOLIPID, AND PROTEIN FROM BLENDED CELLS

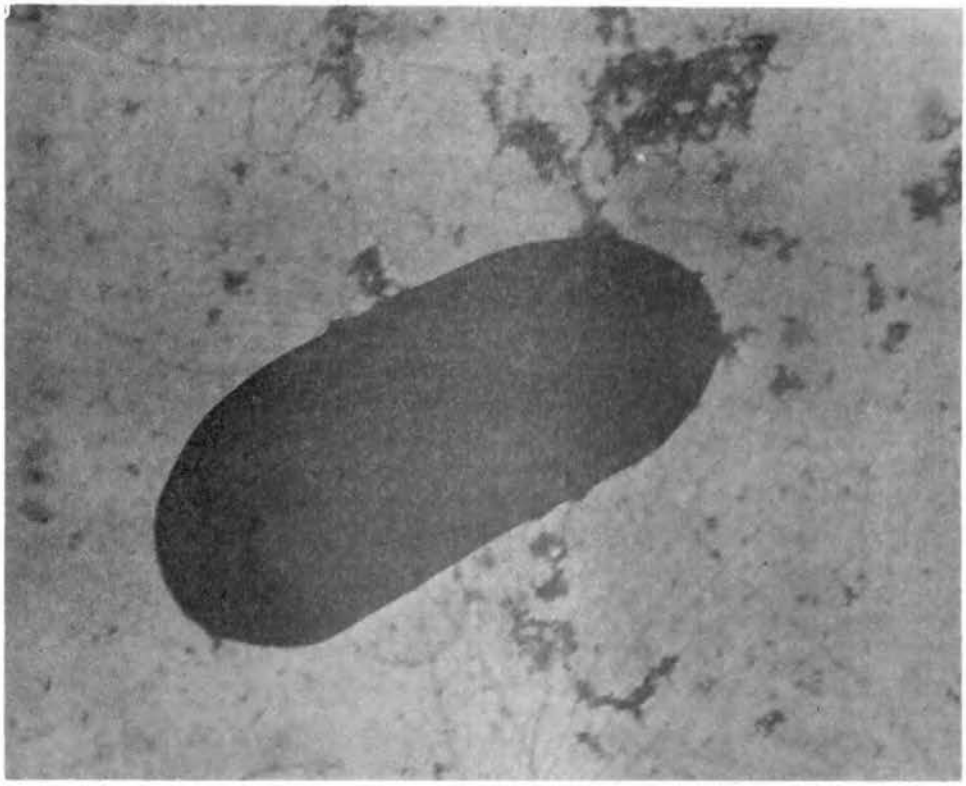
	Total Incorporation CPM/ml	% Total Incorporation Released From Blending Various Lengths of Time		
		15 sec.	60 sec.	90 sec.
Protein (^{14}C-leu)	20299	0.5%	0.48%	0.5%
Phospholipid (^{3}H-l-glycerol)	17240	0.0%	0.0%	0.0%
DNA (^{3}H-thymidine)	12080	0.0%	0.0%	0.0%

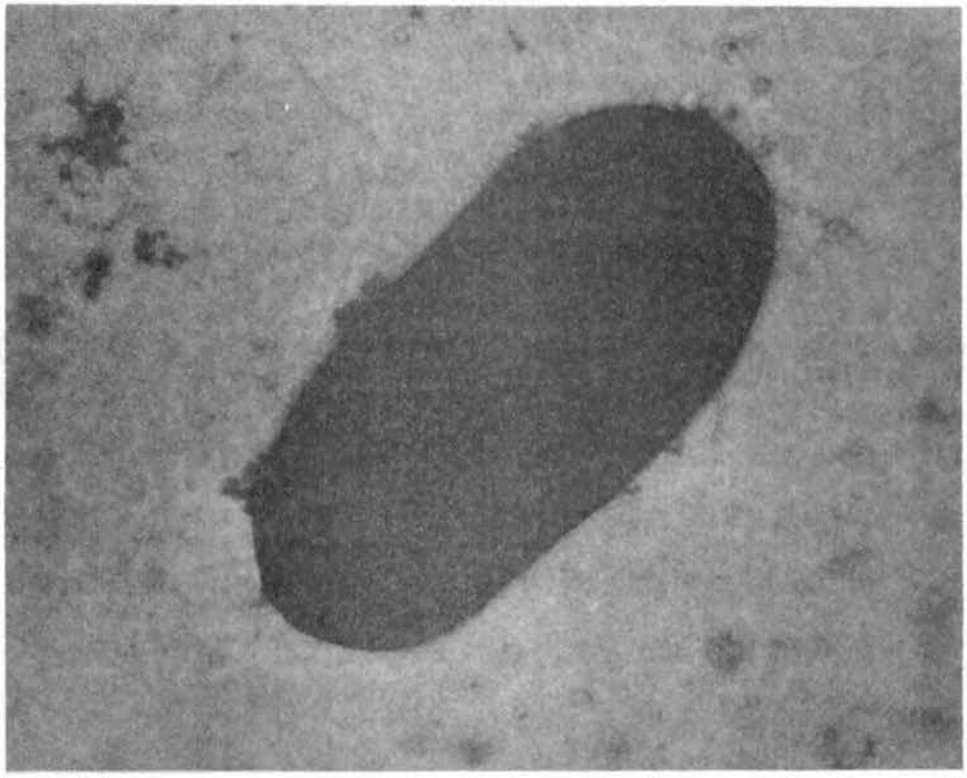
A 90 ml culture of Br 151 was grown in the competence regime. To label DNA, 2 hr before maximal competency, 30 ml of the culture was removed and made 1 µC/ml in ^{3}H-thymidine and 75 µg/ml in 2-deoxyadenosine. To label phospholipid, a second 30 ml of the culture was removed at the same time and made 1 µC/ml in glycerol-2-^{3}H and 50 µg/ml in unlabeled glycerol. To label protein, the remaining 30 ml of culture was made 0.5 µC/ml in ^{14}C-L-leucine with the addition of a 250 fold excess of unlabeled leucine after 4½ hours of growth. This suspension was incubated for 30 minutes, then sedimented and resuspended in transformation medium plus 60 µg/ml unlabeled leucine. Incubation was continued to 5½ hours total growth in all cases. Prior to blending, cells were washed in minimal medium plus 60 µg/ml of the respective unlabeled precursor until the supernatant solution showed negligible levels of radioactivity. The cells were blended the designated lengths of time, and aliquots were sedimented and the supernatant solution saved.

An aliquot of the supernatant solution was directly counted for the estimation of released DNA. The supernatant was precipitated by 2 volumes of cold 10% TCA for 30 minutes at 0° for estimation of phospholipid. Protein was estimated by the addition of 50 µg/ml BSA, then an equal volume of cold 10% TCA. Both precipitated phospholipid and protein were filtered onto Gelman GA-6 filters and counted by liquid scintillation spectrometry.

Figure 21. Electron Micrograph of Negatively Stained Blended *B. Subtilis*

Competent Br 151, either untreated or blended for 10 seconds, were mounted on Formvar coated grids (100 mesh) and negatively stained with 2% uranyl acetate. The preparations were observed in a Phillips EM-200 electron microscope. An untreated control is pictured in the upper photograph. A bacterium from a suspension blended for 10 seconds is shown in the lower photograph. Photographs are magnified approximately 12,000X.





also implies some structural weakening in the blended cells.

Characteristics of the Transformability of Blended B. Subtilis

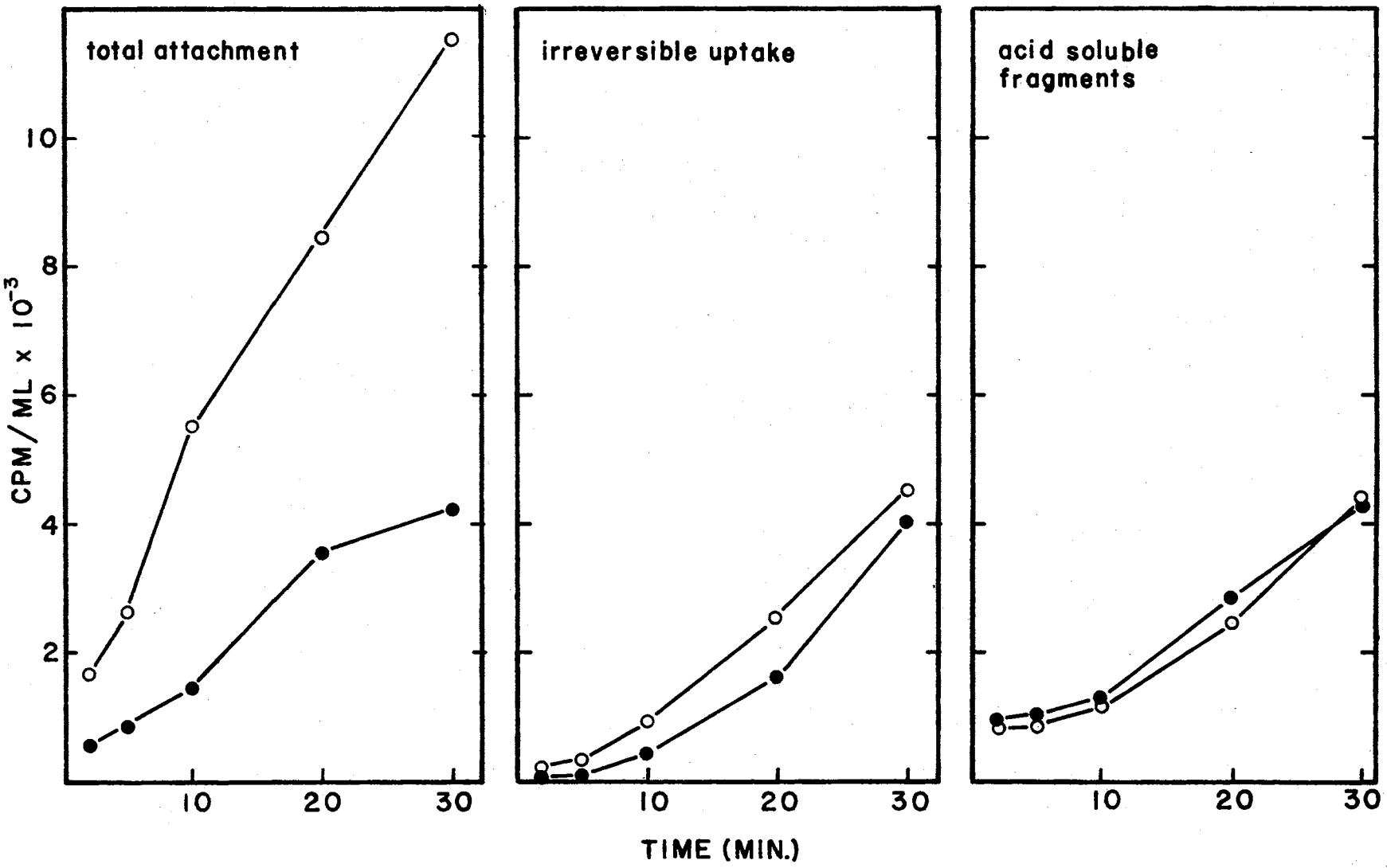
Blended cultures of competent B. subtilis vary in transformability depending upon the length of treatment. Previously described experiments indicated several cellular activities other than transformation were not as susceptible to blending. This establishes a specific sensitivity of the transformation process in competent cells to the blending treatment. Identification of the alterations and the cellular component(s) being altered would help to explain the resulting enhancement or reduction in transformation. The general weakening of the cell wall, as the only identifiable alteration, may lead to part of the observed changes in transformability. More specific alterations within the transformation system itself were investigated by comparing certain characteristics of the transformation systems in blended and untreated cultures.

Figure 22 is a kinetic analysis of the processing of attached donor DNA in a control and briefly blended cultures. Figure 22a shows a reduction by blending in the total amount of ^{3}H-DNA attached. A comparison of the irreversible uptake of attached DNA (Figure 22b) reveals little difference, as would be expected from the results presented in Figure 16. The production of acid soluble fragments accompanying the irreversible uptake of DNA, as shown in Figure 22c, failed to reveal any difference.

A time course of the appearance of transformants in blended and untreated cells terminated at appropriate times by the addition of DNase is shown in Figure 23. In each case, there is an initial 3-5

Figure 22. A Kinetic Analysis of the Processing of ^{3}H-DNA by Blended Bacteria

Competent Br 151 were treated in a Waring blendor for 10 seconds. Three μg/ml ^{3}H-DNA was added to both the briefly blended cells (●), and an untreated control (○), then incubated at 37°. At designated intervals, total attachment, irreversible uptake, and the production of acid soluble fragments was determined, as described in the Methods section.


total attachment
irreversible uptake
acid soluble fragments
CPM/ML x 10^{-3}
2
4
6
8
10
10
20
30
TIME (MIN.)

minute sensitivity to DNase before the irreversible DNase resistant uptake of DNA. After attainment of a resistance to DNase, the blended culture eventually showed a 50% increase in transformability.

Two possible explanations of these results are that blending causes an increase in the rate of uptake of donor DNA, or that the blending increases the number of competent bacteria, by altering "pre-competent" cells.

The second possibility was investigated by following the ability of a 10 second blending treatment to enhance transformability at various times during the competence regime. Table XXII shows the blendor treatment was effective at enhancing transformation not only before and during the period of maximal competence, but also after this period, when the culture's competency was waning. This would imply the blendor treatment alters only those bacteria which are or were competent.

If the blending did increase the number of competent bacteria, the distribution of bacteria between noncompetent and competent fractions should change when sedimented through gradients of Renografin. As a control on the effect of blending on the sedimentation of B. subtilis through a 25-50% linear Renografin gradient, blended and untreated samples from a culture not grown in the competence regime were sedimented through such a gradient. Figure 24 reveals the blending produced a population of bacteria heterogeneous in density. The sedimentation patterns of competent cultures blended for various lengths of time are shown in Figure 25. Two fractions of cells are found in the gradient, the lighter fraction being composed of competent cells labeled by their DNase resistant uptake of ^{3}H-DNA. Blending alters the

Figure 23. A Time Course of the Appearance of Transformants in a Blended Culture

Competent Br 151 were blended for 10 seconds in a Waring blendor. Five μg/ml TRY^+ DNA was added to both the briefly blended cells (●) and untreated control (○), followed by incubation at 37°. At designated times, 1 ml aliquots were removed from each suspension, 50 μg/ml DNase was added, and an additional 10 minute incuation at 37° was made before serial dilution and scoring for transformants, as described in the Methods section.

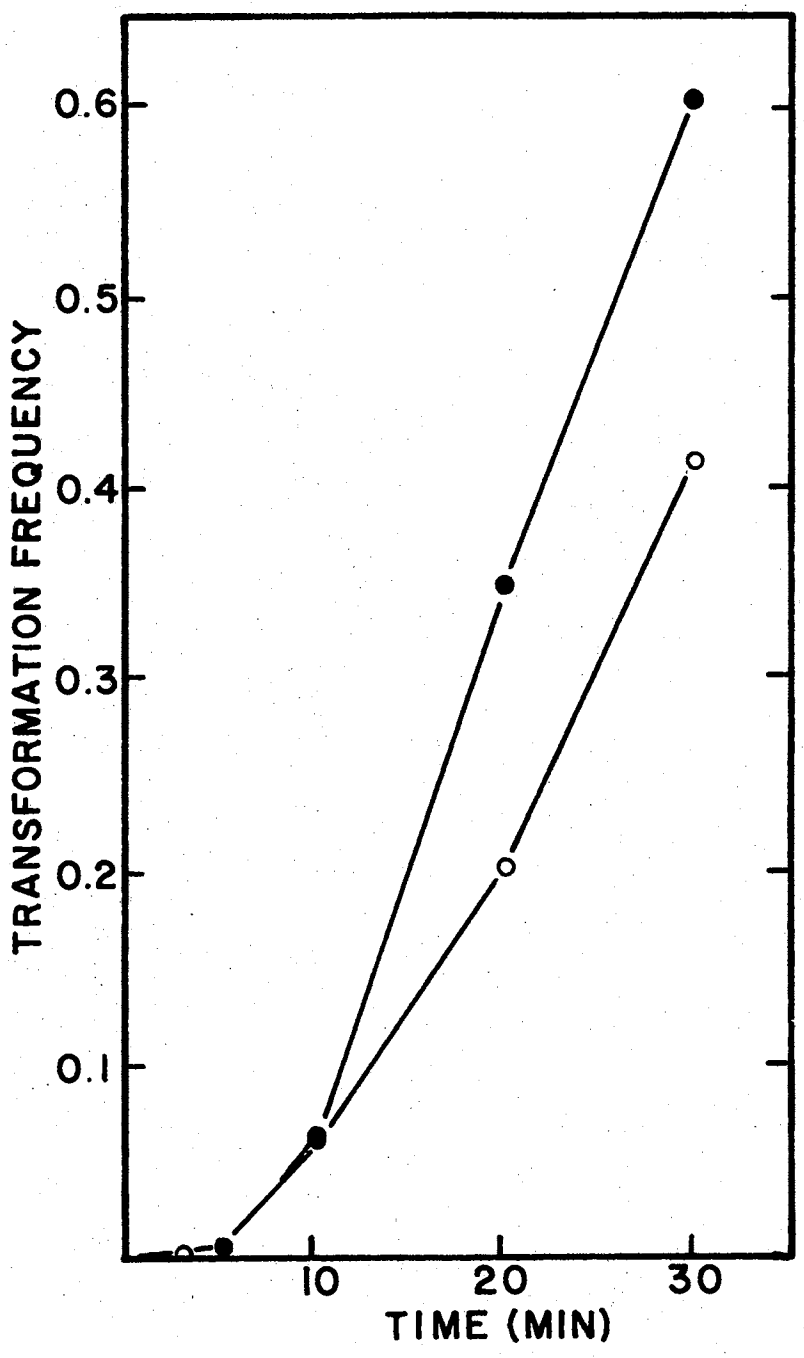

TRANSFORMATION FREQUENCY
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
TIME (MIN)

TABLE XXII

THE EFFECT OF BLENDING ON TRANSFORMATION AT VARIOUS TIMES DURING COMPETENCE DEVELOPMENT

Time in Competence Regime	% Transformation	
	Untreated	Blended
2½ Hours	0.020	0.030
4 Hours	0.016	0.014
5½ Hours	0.360	0.857
9 Hours	0.120	0.298

B. subtilis, strain Br 151, were grown in the competence regime, as described in the Methods section. At designated times, 15 ml aliquots were removed for treatment in a Waring blendor for 10 seconds. Five µg/ml TRY^{+}DNA was added and incubated for 30 minutes for the measurement of transformation in both untreated and blended bacteria.

Figure 24. Sedimentation of Blended Noncompetent B. Subtilis in a 25-50% Renografin Gradient

B. subtilis Br 151 were grown 5½ hours in minimal medium plus 0.5% hydrolyzed casein and 100 µg/ml L-tryptophan. The bacteria were blended for 10 seconds in a Waring blendor, then sedimented and resuspended to one fifth their original volume in 25% Renografin. A 0.2 ml aliquot of cells, either untreated or blended, were applied to a 5 ml 25-40% linear Renografin gradient, and centrifuged in an SW 50.1 head of a Beckman model L2-65 preparative ultracentrifuge, as described in the Methods section. The tube on the left contains untreated bacteria, and the tube on the right contains the blended bacteria.

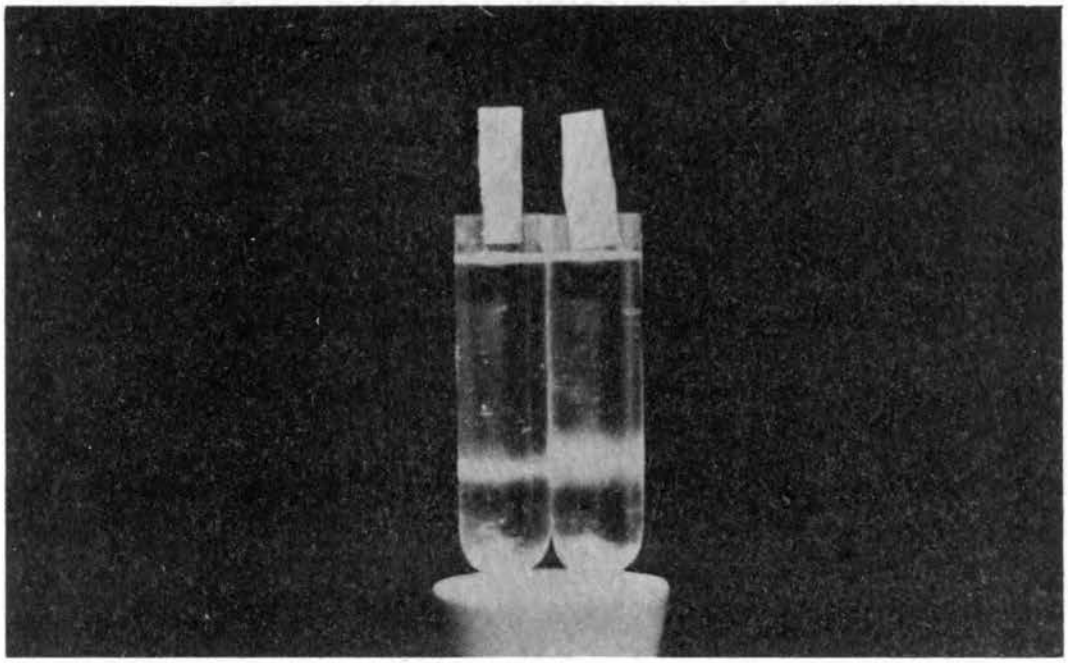
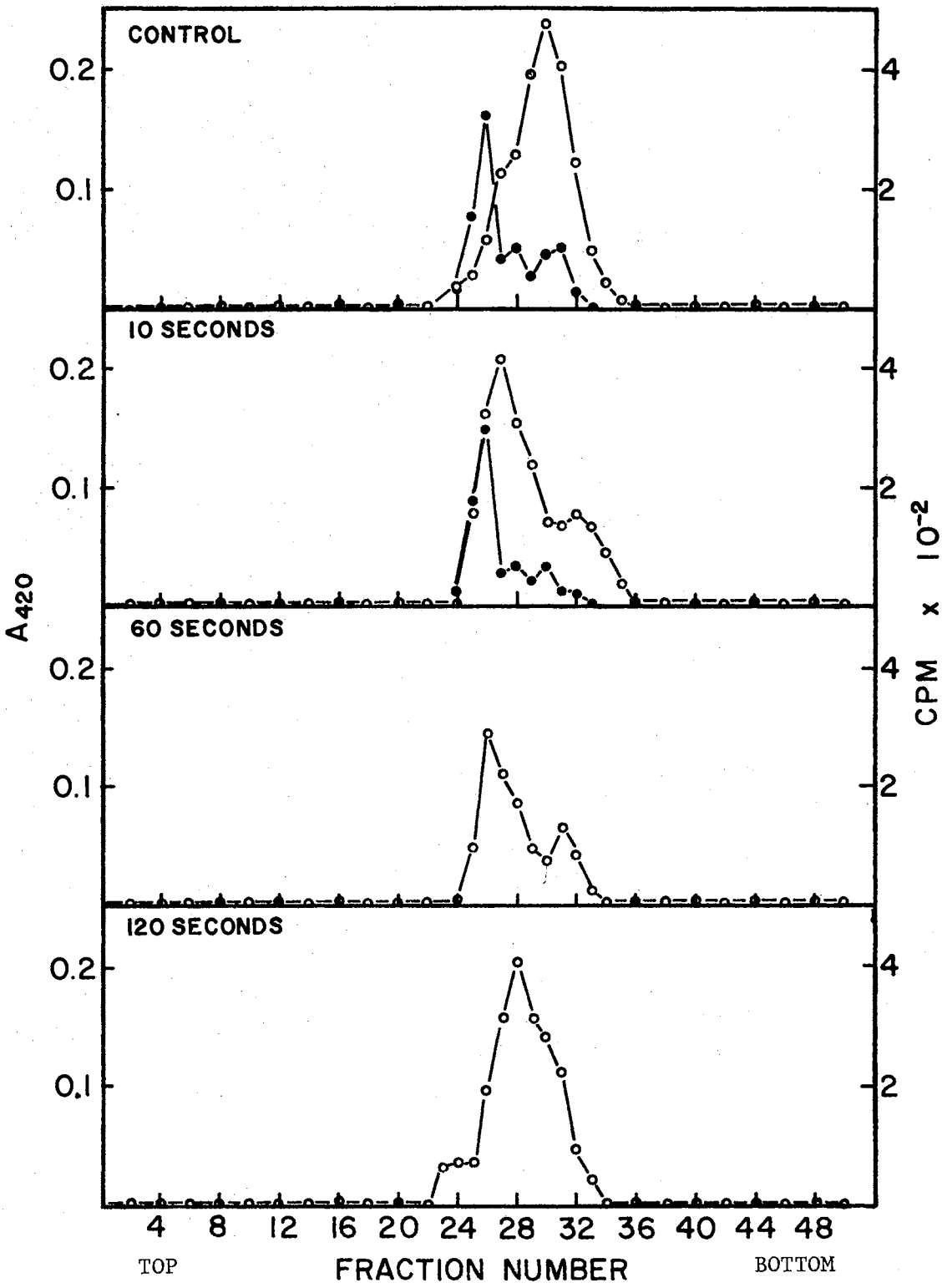


Figure 25. Sedimentation of Blended Competent B. Subtilis in a 20-40% Renografin Gradient

Competent Br 151 were treated in a Waring blendor for various lengths of time. Three μg/ml ^{3}H-DNA was added to each suspension, including an untreated control, and incubated at 37^{o} for 30 minutes, followed by the addition of 50 μg/ml DNase and an additional 15 minute incubation at 37^{o}. The bacteria were sedimented and washed once in a 2 X volume of minimal medium. The cells, were finally suspended in 20% Renografin to one fifth their original volume, and 0.2 ml was applied to a 5 ml 20-40% linear Renografin gradient, and centrifuged in an SW 50.1 head of a Beckman model L2-65 preparative ultracentrifuge, as described in the Methods section. Four drop fractions were collected, using a Beckman Fractionating system. Optical density was determined at 420 nm (O). Samples were counted by liquid scintillation spectrometry to determine the location of radioactivity (●).


CONTROL
10 SECONDS
60 SECONDS
120 SECONDS
0.2
0.1
4
2
A420
CPM x 10-2
4 8 12 16 20 24 28 32 36 40 44 48
TOP
FRACTION NUMBER
BOTTOM

distribution of cells between the two fractions with the concentration of bacteria increasing in the lighter band. The position and amount of ^{3}H-DNA labeled bacteria remained approximately the same in a culture blended for 10 seconds. Blending for 60 and 120 seconds completely inhibited uptake of ^{3}H-DNA. Interpretation of this experiment is difficult, since the control experiment revealed blending produced bacteria of heterogeneous density.

The explanation of the enhanced transformability by brief blending as being due to an increase in the population of competent cells appears unlikely. The alternative explanation of an increased rate of uptake of attached DNA is not supported by the data presented thus far. Such an increase in the rate of uptake should result in an earlier attainment of DNase resistance in the appearance of transformants (Figure 23). Neither is there an earlier resistance to DNase, nor an increased amount of irreversible uptake at any time during the kinetic analysis (Figure 22b) in briefly blended cells.

Reduction of Transformability in Blended Bacteria by Osmotic Shock

The transformation process in blended cells was compared to a control with respect to their response to an osmotic shocking (Table XXIII). The osmotically shocked control culture was reduced by 70% in transformability and irreversible uptake, and 80% in total attachment. Blending resulted in an approximate doubling in transformation, while reducing the total attached DNA by 36%. The irreversible uptake in blended cells remained the same. Osmotic shocking after the blendor treatment reduced the level of transformation to that comparable to the

TABLE XXIII

THE EFFECT OF OSMOTIC SHOCK ON BLENDED AND UNTREATED COMPETENT BACTERIA

Treatment	% Control Transformation	Total Attachment	Irreversible Uptake
Control	100	3181	591
Osmotically Shocked	32	611 (19%)	183 (31%)
Blend (10 seconds)	188	2037 (64%)	520 (88%)
Blend (10 seconds) Osmotically Shocked	31 (16%)	803 (25%) (39%)	217 (37%) (42%)

A culture of competent Br 151 was divided, half was untreated and the other half was treated for 10 seconds in a Waring blendor. Aliquots of each were removed as unshocked controls. The remainder of each suspension was osmotically shocked, as described in the Methods section. Shocked cells were suspended in transformation medium. Separate aliquots used to measure initial attachments were made 10 mM in EDTA prior to addition of ^{3}H-DNA. Initial attachment, irreversible uptake, and transformation were determined as described in the Methods section. Three μg/ml ^{3}H-DNA or 5 μg/ml DNA was added to both control and shocked cells to measure initial attachment/uptake or transformation, respectively. Values in parentheses denote percent of the control values, and, in the case of shocked blended cells the percent of the blended control.

shocked control, but to only 16% of the blended cells. The total attachment and irreversible uptake of DNA were both reduced to 60% of the blended control, and to 25% and 37%, respectively, of the untreated control. The results of osmotically shocking blended cells appear neither more nor less drastic than shocking of a control.

Reversibility of the Effect of Osmotic Shocking in Blended Cells

A concentrated solution of cultural fluid from competent B. subtilis can restore the transformability of osmotically shocked or PEA-treated cells to a level of 75-100% and 60%, respectively, of the original value. However, a concentrated supernatant solution did not restore the level of transformation in the osmotically shocked blended cells to the same extent as it did in the shocked control (Table XXIV). The blendor treatment, while not making the competent bacteria any more or less labile to osmotic shock, does disturb some part of the transformation process, making the bacteria unresponsive to the concentrated supernatant treatment.

Ability of Concentrated Cultural Fluid on Reversing Various Inhibitions

That a brief blending treatment may release a competence factor(s) into the medium was investigated by determining the ability of concentrated cultural fluid from blended cells to reverse various inhibitions. Table XXV reveals the effect of the cultural fluids on cells reduced in transformation by osmotic shock, PEA treatment, or excessive blending. Substantial restoration in osmotically shocked and PEA treated

bacteria, 43% and 59% respectively, was accomplished by the cultural fluid of untreated cells. However, this treatment failed to reverse the inhibition of extensively blended bacteria. Concentrated cultural fluid from blended B. subtilis was unable to restore transformability in any instance. The thorough aeration during blending may oxidize the factor(s) present in the cultural fluid which enables restoration of transformability.

Similarly, the ability of the supernatant solution from a blended culture to increase transformation in a low transforming strain was studied (Table XXVI). Concentrated cultural fluid from untreated bacteria produced a 2 fold increase in the transformation of the poorly competent bacteria. The blended supernatant did not increase such transformation.

Duration of the Inhibition by Extensive Blending

To determine the duration of the reduction of transformability by extensive blending, the ability of such a blended culture to regain competency was followed. At maximal competency, a portion of the culture was removed and blended for 45 seconds. Transformation in blended and untreated samples was measured as a function of length in the competence regime. At 9 hours, a 1:10 dilution of both samples into fresh transformation medium, plus transformation enhancing amino acids was made. After another 5 hours of growth, the control culture passed through a second, but less intense wave of competency. Blended cells also passed through a second period of competency 5-6 hours after dilution, but it was negligible compared to the control (Figure 24).

TABLE XXIV

REVERSAL OF OSMOTIC SHOCK INHIBITION BY CONCENTRATED CULTURAL FLUID

Treatment	% Transformation
Untreated Cells	
Control	0.500
Osmotically Shocked	0.134
Osmotically Shocked, then addition of concentrated supernatant	0.314
Blended Cells	
Control	0.776
Osmotically Shocked	0.127
Osmotically Shocked, then addition of concentrated supernatant	0.108

A culture of competent Br 151 was divided in two portions, half was untreated and the other half was blended for 10 seconds in a Waring blendor. Aliquots of each were removed as unshocked controls. The remainder of each suspension was osmotically shocked as described in the Methods section. Half of each shocked suspensions was suspended in transformation medium. The remainder was suspended in a solution of concentration supernatant solution was continued for 30 minutes before addition of 3 μg/ml ^{3}H-DNA or 5 μg/ml DNA for the measurement of initial attachment uptake or transformation respectively. Aliquots of bacteria used for the measurement of initial attachment were made 10 mM in EDTA prior to the addition of ^{3}H-DNA. Initial attachment, irreversible uptake, and transformation were determined as described in the Methods section. Cultural fluid was concentrated 10 X by rotary evaporation, and 0.3 ml was used per ml of bacteria.

TABLE XXV

THE ABILITY OF CONCENTRATED CULTURAL FLUID FROM BLENDED AND UNTREATED CELLS TO REVERSE VARIOUS INHIBITIONS

Treatment	% Control Transformation
Control	100
Blend (45 seconds)	13
Blend + Blend Supernatant	12
Blend + Untreated Supernatant	16
Osmotically Shocked	35
Shocked + Untreated Supernatant	78
Shocked + Blended Supernatant	22
0.05% Phenethyl Alcohol	1
Phenethyl Alcohol + Untreated Supernatant	60
Phenethyl Alcohol + Blended Supernatant	6

The cultural fluid from untreated competent bacteria and that from competent bacteria treated in a Waring blendor for 10 seconds was obtained by centrifugation. Such supernatant solution was concentrated 10 fold by rotary evaporation. Competent bacteria were inhibited in transformability by a 0.05% phenethyl alcohol treatment, osmotic shocking, or extensive blending. One ml aliquots of each were suspended in transformation media plus 0.3 ml of either concentrated supernatant. Cells were incubated in the supernatant solutions for 30 minutes prior to the addition of 5 μg/ml DNA, followed by an additional 30 minutes incubation. TRY^+ transformants were scored for as described in the Methods section.

TABLE XXVI

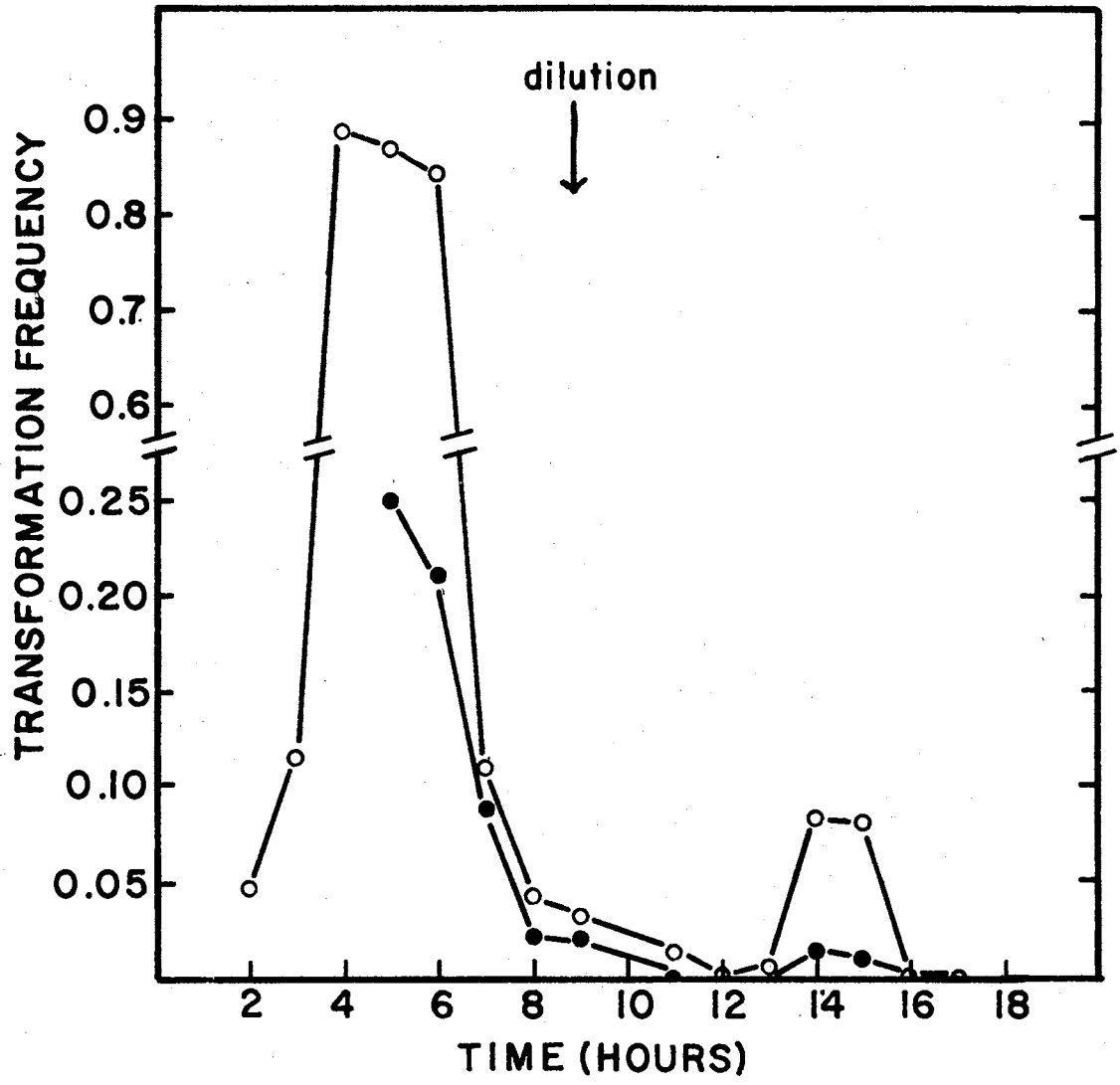
THE ABILITY OF CULTURAL FLUIDS TO INCREASE TRANSFORMABILITY IN STRAINS OF LOW COMPETENCY

Strain of *B. subtilis*	% Control Transformation	Enhancement of Transformability by	
		Untreated Supernatant	Blended Supernatant
SB 25	0.065	0.102	0.074
C^- 168	0.011	0.045	0.009

The cultural fluid from untreated competent bacteria and that from competent bacteria treated in a Waring blendor for 10 seconds was obtained by centrifugation. Such supernatant solution was concentrated 10 fold by rotary evaporation. Competent SB 25 and C^- 168 cells were sedimented and resuspended in transformation medium plus 0.3 ml of either concentrated supernatant. Cells were incubated in the supernatant solutions for 30 minutes prior to the addition of 5 μg/ml DNA, followed by an additional 30 minutes incubation. TRY^+ transformants were scored for as described in the Methods section.

Figure 26. Duration of the Reduction in Transformation by Extensive Blending

B. subtilis Br 151 were grown in the competence regime, as described in the Methods section. At the peak of competence, a portion of the culture was blended in a Waring blendor for 45 seconds. Transformation in both untreated (○) and blended (●) cells was determined at the designated intervals. After 9 hr, a 1:10 dilution into fresh transformation medium plus transformation enhancing amino acids was made in both cases. Transformation was followed for an additional 7 hours.


dilution
TRANSFORMATION FREQUENCY
0.9
0.8
0.7
0.6
0.25
0.20
0.15
0.10
0.05
2
4
6
8
10
12
14
16
18
TIME (HOURS)

CHAPTER VI

DISCUSSION

Initial Attachment and Uptake of DNA in Competent B. Subtilis

A transformable strain of B. subtilis acquires the ability to bind and take up DNA through specific alterations in the cell wall and membrane during competence development. These alterations establish in competent bacteria a sequence of reactions which takes attached DNA through various intermediate forms before the final integration into the recipient genome. As the premier step in this process and a distinguishing feature of competent bacteria, the initial attachment of donor DNA and its relation to the uptake of DNA are still poorly understood.

Similar to the findings of Lerman and Tolmach (44) with Pneumococcus, Table III shows the temperature dependency of irreversible uptake and transformation in B. subtilis. However, it also reveals a quantitative difference in the total amount of ^{3}H-DNA associated with the bacteria at temperatures below 25^{o}. This is contrary to the postulation of Lerman and Tolmach (44) that the attachment of DNA should be independent of temperature because of its ionic nature. The uptake of DNA and appearance of transformants should display a temperature dependency, due to the involvement of enzymatic and energy requiring reactions. Although the initial adhesion of DNA to the binding site

may be ionic, the availability of this specific site for attachment may be temperature dependent.

Young (17) found removal of the cell wall after DNA attachment did not alter the DNA absorbed to the protoplast, concluding that the membrane was the site of initial attachment. However, the cell wall must play a role in the transformation process. Tichy and Landman (51) observed no transformation in protoplasts, but did find DNA attached to quasi-spheroplasts would lead to transformation if cell wall biosynthesis was resumed. The lack of transformation in protoplasts was explained by the expulsion of mesosomes. That mesosomes are required for at least the entry of DNA was shown by the reduction of transformation in "mesosomeless" bacilli produced by plating protoplasts on 25% gelatin media (51), and the electron autoradiographic evidence of Wolstenholme et al. (55) and Vermeulen and Venema (56,57).

Under anaerabic conditions, mesosomes in B. subtilis will be released from the cell after 2 hours of incubation at 0^{o} (142). Brief chilling to 4^{o} may result in an alteration in the integrity of the mesosome, affecting the attachment and uptake of DNA.

Successful transformation requires at least the partial integrity of a combination of the cell wall and membrane. Initial attachment to a specific binding site which will ultimately lead to transformation may be the step requiring this integrity. Reduction of temperature could disturb such organization, resulting in a reduction of both attachment and uptake.

In B. subtilis, Dubnau (75) failed to find either the initial attachment or irreversible uptake of H-DNA upon chilling the bacteria to 0^{o}. Employing a different technique for the removal of unattached

^{3}H-DNA than Dubnau did, Table III does show a measurable amount of DNA was attached at 0^{o}, with only a negligible amount of irreversible uptake. If only the initial attachment of ^{3}H-DNA in the presence of 10 mM EDTA was measured, binding at a reduced temperature was still observed (Table X).

If the period for attachment of donor DNA at various temperatures was brief enough to exclude extensive irreversible uptake, the appearance of transformants at 37^{o} should depend only upon the extent of initial attachment. Table I reveals the amount of transformation does vary with the temperature of initial binding. Table II shows not only the differing efficiency of transformation over a range of temperatures, but the capacity of increasing the efficiency of that DNA which is attached at a suboptimal temperature to lead to transformation at 37^{o}. This difference in the level of transformation at 37^{o} after attachment at various temperatures does not distinguish between the possibilities that the temperature dependency results from an alteration in the attachment sites and/or a decrease in the amount of DNA being attached.

The attachment of DNA to competent bacteria at 4^{o} was investigated to determine its significance in the transformation sequence in comparison with attachment at 37^{o}. Sedimentation of a competent culture exposed to ^{3}H-DNA at 4^{o} on a discontinuous Renografin gradient showed the radioactivity to be associated with the competent fraction (Figure 2). That this attachment occurs at least in part to sites which will lead to transforamtion at a permissive temperature was shown in Table II. This does not exclude the possibility that DNA bound at 4^{o} may migrate to another site when the temperature is raised.

The 4^{o} attached DNA can interfere with subsequent transformation

with a distinguishable DNA at 37° (Table IV). Attachment of either an auxotrophic (Try^-) or prototrophic (Try^+) DNA at 4° decreased by 36-42% transformation with only the Try^+ DNA at 37°. A DNase treatment after the 4° incubation, but before exposure to DNA at 37°, removed the interference. The reduction in transformation with the second addition of DNA at 37° suggests the attachment of DNA at 4° is irreversible, to the extent that it cannot be displaced by the addition of an excess of a second DNA. It has not attained DNase resistance by any uptake which would lead to transformation.

A broad specificity in the type of DNA capable of attachment at 4° is implied in Table IV. All the heterologous types of DNA bound at 4° succeeded in interfering with transformation with the homologous DNA at 37°. The ability of a competent bacterium to bind and take up a strand of heterologous DNA depends on the particular strain of bacteria. Varying results have been obtained in H. influenza, with Newman and Stuy (127) showing that the competent bacteria could take up but not integrate lambda DNA, but Scocca, Poland, and Zoon (128) showing no uptake of DNA from either E. coli or Xenopus laevis. The latter authors found the heterologous DNA neither interfered with uptake of homologous DNA nor was degraded by the bacteria, and thus proposed that a surface located mechanism for recognition of homologous DNA exists in H. influenza. B. subtilis, however, is capable of taking up and incorporating heterologous DNA (64). Dubnau (75) reported that the addition of excess salmon sperm DNA prevented further initiation of DNA-cell attachments.

Least effective of the DNAs attached at 4° on subsequent transformation at 37° was heat denatured homologous DNA. Goodgal and Her-

riott (26) had shown in H. influenza a specificity of configuration for uptake, but not to as great an extent for attachment of DNA. The results in Table V imply DNA of native configuration, but not of extensive size, is required for interference at 37^{o} from attachment at 4^{o}. It is possible that denatured DNA attaches at 4^{o}, but is released upon elevation of temperature.

Analysis of a time course of the total attachment of DNA and production of transformants at 37^{o} in bacteria previously incubated at 4^{o} for 10 minutes, shows the prior reduction in temperature had no effect on the transformability of the bacteria, but did reduce the extent of attachment of DNA (Figures 3 and 4). This reduction in attachment of DNA cannot be used as an explanation for the results in Table IV, since a concommitant reduction in transformation was not observed. The appearance of transformants at 37^{o} in a competent culture exposed first to auxotrophic DNA at 4^{o} was qualitatively similar to that of a culture first exposed to auxotrophic DNA at 37^{o} (Figure 3). In either case, there was a reduced rate in the appearance of transformants, which, after 30 minutes of incubation at 37^{o}, ceased to increase. Figure 4 illustrated similar results for the total attachment of ^{3}H-DNA at 37^{o} after exposure to unlabeled DNA at 4^{o} or 37^{o}.

Exposure of competent B. subtilis to transforming DNA results in an increase in the appearance of transformants for only a limited time. Erickson (143) suggests this may be due to the release of bound DNA or the selective destruction of potential transformants. However, Haseltine and Fox (130) described an inactivation of transforming DNA by the noncompetent population of a transformable culture. The inactivation is due to an endonucleolytic double strand cleavage, but the reduction

in molecular weight alone is not enough to explain the loss of biological activity. The inactivated donor DNA also loses its ability to attach to the competent bacteria, as indicated by the loss of ability to compete with biologically active DNA. Thus, the plateauing of the extent of initial attachment and the appearance of transformants in cells first exposed to DNA at 37° may be due to a combination of competition by DNA attached in the first exposure and inactivation by the noncompetent bacteria. The similar results obtained by incubation at 4° first with un unlabeled, prototrophic, or auxotrophic DNA may be due to a similar set of circumstances, or that DNA attached at 4° is to an altered binding site. This attachment does not permit the return of the site to its normal state when the temperature is raised, and subsequent attachment and transformation is limited by the number of unoccupied sites.

Evidence for such an alteration with the reduction of temperature is implied in the comparison of attachment at 4° and 37°, with respect to a variety of parameters. Initial attachment at 4° is rapid and does not increase with time. The formation of DNA-cell complexes at 37° occurs linearly, agreeing with the findings of Morrison (101). The appearance of attachment at 4° in a competent culture parallels that of attachment at 47°, suggesting that competency is a requirement for the ability to bind DNA at 4° (Table XIII). This agrees with the previous observation that the radioactivity bound at 4° does so to the competent fraction of bacteria. Also, strains capable of attaining varying degrees of competency exhibited varying abilities to bind DNA at 4° (Table XII).

A significant difference between attachment at 4° and 37° occurs

with respect to increasing the ionic strength of the medium (Figure 9). That the 4^{o} attachment increases with increasing ionic strength, in contrast to the decrease in attachment at 37^{o}, suggests that it is a characteristic of a different or altered binding site. The ability of the attached DNA at 4^{o} to lead to some transformation at 37^{o}, or to compete with a second DNA addition at 37^{o} implies that 4^{o} attachment site is an alteration of the site normally present. Such an alteration could result from a disorganization of the cell wall and plasma membrane at the attachment site. Increasing the ionic strength under these conditions may affect the configuration of cell wall teichoic acid, due to its polyelectrolyte nature (95), and produce another altered attachment site with increasing affinity for DNA. Under normal conditions, the configurational change in teichoic acid with increasing ionic strength reduces attachment, assuming the ionic strength changes do induce such a change in the intact cell. This assumption is supported by the observation that increasing the ionic strength of the medium inhibits the binding of phage ϕ 25, which binds to glucosylated teichoic acids in B. subtilis (95).

Additional support for the alteration in the attachment site at 4^{o} is provided by changes in several liabilities of the transformation process. Repetitive washing, a treatment which at 37^{o} reduces both attachment of DNA and transformation by 70%, was minimized in effect if it occurred at 4^{o} (Table VI). Washing at 4^{o} or 37^{o} did not reduce the amount of DNA bound at 4^{o}. Since the washing presumably removes a loosely bound factor involved in attachment, the reduction of temperature does not make this factor as available for removal. Apparently the factor is not involved in attachment at 4^{o}, since its presence or

absence does not affect the 4^{o} binding. Washing at 4^{o} failed to reduce subsequent transformation at 37^{o} (Table VII).

Other treatments which inhibited both the attachment and uptake of donor DNA at 37^{o}, such as osmotic shocking, 0.05% PEA treatment, and incubation at 50^{o} for several minutes, failed to affect the attachment of DNA at 4^{o} (Tables XV, XVI, and Figure 11). Administration of these inhibitory treatments, however, had to occur at temperatures other than 4^{o}, so any interpretation of their direct effect on 4^{o} attachment is impossible. Only the assumption that the alteration producing the 4^{o} binding site had not yet occurred can explain their resistance to the treatment.

Periodate oxidation of surface material on the competent bacteria at 0^{o} did not reduce the initial attachment of DNA to the same extent as oxidation at 37^{o} did. However, in either case, the uptake of DNA was reduced by 80% or more. The effect of periodate oxidation on attachment at 4^{o} produced the opposite results. Oxidation at 37^{o} did not reduce the extent of attachment to the degree that oxidation at 0^{o} did. Using Ranhand's (113) logic, this implies that amino acids are contained in the binding sites available at 37^{o}. However, the results of the 4^{o} attachment after periodate oxidation do not support such a general conclusion. The reduction in 4^{o} attachment by periodate treatment at 0^{o} should have also been observed by treatment at 37^{o}, since carbohydrates are susceptible at either temperature. The data suggests that the oxidized cellular components at 0^{o}, where the altered binding site would exist, are not available to oxidation at 37^{o}. Similarly, the effect of periodate oxidation at 0^{o} on 37^{o} attachment could be interpreted as the normal binding site components not being as available

to oxidation at 0^{o} as they would be at 37^{o}. Periodate treatment at 37^{o} after the initial attachment of DNA in the presence of EDTA prevented the occurrence of any transformation after the initiation of uptake by the addition of Mg^{2+}. Either the oxidation released the attached DNA, or it destroyed the bacteria's capacity for irreversible uptake. This distinguishes a separation between the initial attachment of DNA and its uptake.

Analysis of the entry of attached donor DNA upon the addition of excess Mg^{2+} was shown in Figure 7. A decrease in the total attached DNA is accompanied by an increase in the irreversible uptake of donor DNA and production of TCA soluble fragments in the case of the entry at 37^{o} of ^{3}H-DNA initially attached at 37^{o}. If DNA initially attached at 37^{o} was incubated at 4^{o} before addition of Mg^{2+}, the amount of ^{3}H-DNA attached decreased to less than 50% of the 37^{o} control, suggesting that the attachment of DNA in the presence of EDTA is sensitive to chilling. This sensitivity may result from the implied alteration in the attachment site. Subsequent addition of Mg^{2+} in this case did not initiate any entry of attached DNA into the bacterium, as would be expected from the established temperature dependency of the uptake system. DNA attached at 4^{o} showed no indication of beginning entry at 4^{o}, but if shifted to 37^{o} prior to addition of Mg^{2+}, there was a detectable increase in irreversible uptake with time, accompanied by a decrease in total attachment and the production of TCA soluble fragments.

The observation that DNA attached at 37^{o} does not enter the cell at 4^{o} was supported by the data shown in Table VII. The amount of transformation produced from a 15 minute incubation at 37^{o} was not increased by an additional 30 minute incubation at 4^{o}.

The Effect of Treatment in a Waring Blendor on the Transformability of B. Subtilis

The unique composition of the cell wall in competent B. subtilis has been established (5). The state of competency may also confer a unique surface architecture to the bacterium for the initial attachment and uptake of donor DNA. Three species of Moraxella, a gram negative organism, undergo genetic transformation, depending upon the colony type and amount of fimbriation (132). The specific role of the fimbriae in transformation has not been identified, but may confer specific properties to the surface of the bacterium or may only reflect the presence of a competence factor. No information is available on the effect of removal or alteration of the fimbriae on this transformation system.

Endo (116) employed a Waring blendor treatment of competent B. subtilis to reduce transformation. A period of incubation after the blending treatment restored the transformability to the inhibited bacteria. The only alteration reported by Endo was the complete removal of flagella by blending.

Figure 14 is a measurement of the level of transformation, the total attachment of ^{3}H-DNA, and the cell titer of a competent culture blended for various lengths of time. A brief blending was sufficient to reduce the amount of attachment of DNA to an essentially constant level. However, the transformability of the culture initially increased, reaching a maximum after 10 seconds of blending, then progressively decreased to a minimum value after 60 seconds of blending. The effect of blending on the cell viability could vary with each culture,

but seldom was reduced before two minutes of blending.

An increase in the transformability of a culture with a concomitant reduction in the amount of donor DNA attached suggests an increase in the efficiency of that DNA which is attached to produce transformants (Figure 15). It also suggests all of the DNA attached to the surface of a competent cell surface is not necessarily involved in the transformation process. Supporting this suggestion is the observation that the blending treatment does not reduce the amount of irreversible uptake until the length of the treatment begins to inhibit transformation (Figure 16). A brief blending was capable of increasing the transformability of even a poorly competent strain (Table XIX), implying that the effect of the treatment was general and not based on the release and distribution of a competence factor from highly transformable cells.

That the effect of blending only increases the transformability of bacteria that have not yet irreversibly taken up donor DNA is described in Table XVIII. Blending of cells transformed prior to the treatment for various lengths of time had no effect on the amount of transformation. Thus, the stage in the transformation sequence which is affected by blending occurs at or before the irreversible uptake of DNA. These results also illustrate that the reduction of transformation is not due to the specific destruction of competent bacteria by the blending.

The appearance of transformants in briefly blended and untreated cultures, whose transformation was terminated at intervals by the addition of DNAse, revealed the treated cells eventually produced 50% more transformants than the control, after the initial lag period (Figure

23). Such a result could be explained by an increase in the rate of uptake of transforming DNA, or by the production of more competent bacteria by the alteration of "precompetent" cells. Both possibilities were investigated.

A kinetic analysis of the DNA attachment and events accompanying the entry of donor DNA into the recipient bacteria were shown in Figure 22. Only a reduction in the total attachment of DNA to blended cells was apparent. The irreversible uptake of DNA and production of acid soluble fragments were identical. If blending were to increase the uptake of DNA, it should be reflected in an increased amount of DNase resistant DNA. Similarly, an increased rate of uptake should show a briefer lag in the attainment of DNase resistance with respect to the appearance of transformants in Figure 23. Thus, none of the data supports the interpretation that the effect of blending on transformation is due to an increase in the rate of DNA uptake.

The alternative, that blending enlarges the competent fraction of the population, was investigated by following the ability of a brief blending treatment to enhance transformation at various times during competence regime (Table XXII). Assuming that "precompetent" bacteria would be required for such enhancement, then no effect should be observed in bacteria which are growing out of competency. However, blending successfully increased transformation at any time tested. A change in the distribution of bacteria between noncompetent and competent fractions, when separated on linear Renografin gradients, was observed (Figure 25). A control revealed the blending of a culture not exposed to the competence regime, which normally sedimented as a uniform population in the gradient, resulted in a heterogeneous and dif-

fuse band (Figure 24). Thus, the increase in the lighter band of competent cells in Figure 12 may be due to this heterogeneity of density caused by blending.

The explanation of the enhanced transformability by brief blending as being due either to an increase in the rate of uptake of DNA or to an increase in the competent population seems unlikely. A third, and speculative possibility for the enhancement is a disruption of the extracellular nuclease (75) involved in the uptake of DNA, such that larger segments of single strand donor DNA are entering the bacteria. Since only a single strand of DNA enters the bacterium, the majority of the acid soluble fragments produced is from degradation of the complementary strand. Increasing the length of the single strand taken up would not substantially reduce the production of acid soluble fragments. Although more donor DNA would enter the bacteria, the radio assay used to measure irreversible uptake may not be sensitive enough to measure the increased amount. Considering the probability of integration depends not only on the location of a marker, but also on the length of the DNA on which it resides (39), the increased length of DNA entering the cell would definitely enhance the transformability.

The blending of competent bacteria did not cause the release of detectable amounts of recipient DNA or phospholipid, however, 0.5% of the total cellular protein labeled was released into the cultural fluid (TAble XXI). This can be accounted for by the removal of flagella (Figure 21) from the surface of the bacteria. The blendor treatment had no effect on the uptake and incorporation of ^{14}C-L-leucine (Figure 18), but did increase the uptake and incorporation of ^{14}C-D-alanine (Figure 19). This suggests the blending weakened the cell

wall repair. Both uptake and incorporation of D-alanine were maximally stimulated by only 10 seconds of blending. More extensive treatment reduced both, but never to a level below the control. The flagellin protein of B. subtilis contains 15% alanine (105), presumably of the L configuration. It also contains 7% leucine (105). Even if the increased uptake and incorporation of ^{14}C-D-alanine was possibly due to flagella regeneration, an increase in ^{14}C-L-leucine uptake and incorporation would also be expected. The implied weakening of the cell wall was not supported by an increased sensitivity to depolymerization by lysozyme (Table XX) or reduction in viability by osmotic shock (Figure 20).

Kohoutova (90) suggested a function of a competence factor may be the selective weakening of the cell wall to provide substrate for an autolytic enzyme. The lesion in the cell wall thus produced would be the site of penetration for transforming DNA. Prozorov (54) and Tichy and Lanman (51) enhanced the transformability of B. subtilis by a light lysozyme treatment which formed quasi spheroplasts. Although blending did not alter the cell wall to the extent of forming quasi spheraplasts, the enhancement of transformability may be related.

Extensive blending caused an alteration severe enough to suppress the development of a second wave of competency (Figure 24). The blending of cells was also shown to increase the growth rate of the bacteria even after a dilution into fresh medium. This increased growth rate may not permit the bacteria to enter the presporulation cycle (5) involved in competence development.

The osmotic shocking of briefly blended cells reduced their transformability to the same level as the shocking of untreated cells

(Table XXIII). Thus, the alteration by which blending enhances transformation does not reduce the susceptibility of competent cells to osmotic shocking. More significantly, reversal of this inhibition by a factor(s) in the cultural fluid of competent bacteria was not as effective in blended cells as in the control (Table XXIV). The osmotic shock procedure is assumed to remove a surface located factor required for the irreversible uptake of donor DNA (139). Its replacement by the cultural fluid component(s) must require a specific surface architecture or organization which is partially destroyed by the blending treatment.

The irreversibility of the reduction in transformation by extensive blending, alluded to by its duration (Figure 24), suggests a structural alteration, rather than the removal of a competence factor. Neither the supernatant solution from untreated competent cells nor that of briefly blended cells was able to reverse the inhibition (Table XXV). The cultural fluid from briefly blended cells was also incapable of reversing the inhibition of osmotic shocking or 0.05% PEA treatment (Table XXV). It was also unable to stimulate transformation in less competent strains of B. subtilis (Table XVI). The thorough aeration during blending may oxidize those competence factors normally present. If this were the case, those factors in the supernatant would not normally be involved in the transformation process, since their destruction by brief blending did not affect transformation.

CHAPTER VII

SUMMARY

The extent of transformation at 37^{o} is dependent upon the temperature at which the DNA is initially attached. Both the initial attachment and irreversible uptake of donor DNA are quantitatively dependent upon the temperature, reflecting the requirement for a specific binding site and involvement of enzymic or energy requiring reactions in the transport of DNA.

Evidence supporting the alteration of attachment sites by reduction of temperature was presented. That the DNA attached at reduced temperatures, while not resulting in efficient transformation at those temperatures, will increase in efficiency when incubated at 37^{o} suggests such attachment is to normal binding sites. DNA attached at 4^{o} does so to the competent fraction of the bacterial population. Attachment of an unlabeled or auxotrophic DNA at 4^{o} reduced the subsequent attachment of ^{3}H-DNA and production of transformants, respectively, at 37^{o}. A variety of heterologous and sheared DNA had this ability to attach at 4^{o} and interfere with transformation at 37^{o}. Only heat denatured homologous DNA had no effect.

Entry of DNA attached in the presence of 10 mM EDTA was achieved only upon addition of Mg^{2+} at 37^{o}, regardless of the temperature at which the DNA was initially attached. No entry of DNA initially attached at 4^{o} or 37^{o} occurred at 4^{o}. DNA initially attached at 37^{o}

in the presence of EDTA was sensitive to chilling, resulting in the release of at least 50% of the originally attached DNA.

Alteration of the attachment site by reduction in temperature was supported by comparison of several parameters and sensitivities of initial attachment at 4^{o} and 37^{o}. Increasing the medium ionic strength resulted in decreasing the initial attachment and transformability of bacteria at 37^{o}, but increased the initial attachment of DNA at 4^{o}. The increased amount of DNA attached at high ionic strength could lead to increased transformation at 37^{o}. Repetitive washing of bacteria at 37^{o}, prior to exposure to DNA, reduced transformation and attachment of DNA at 37^{o}, but not at 4^{o}. The same procedure performed at 4^{o} did not affect attachment at 4^{o}, nor did it severely reduce initial attachment or transformation at 37^{o}. Similar results were obtained for the sensitivity of 4^{o} and 37^{o} attachment to osmotic shock, 0.05% PEA treatment, heating bacteria to 50^{o}, and differential periodate oxidation at 0^{o} and 37^{o}. Reduction of temperature to 4^{o} may result in a disorientation of the DNA attachment sites, such that their affinity for DNA and susceptibility towards inhibitory treatments directed against them are reduced.

The sensitivity of competent B. subtilis to treatment in a Waring blendor was determined by measuring the level of transformation and amount of DNA attached to cells blended for various lengths of time. Brief blending was capable of enhanceing a culture's transformability, while reducing the amount of DNA initially attached by 50-80%. Blending did not affect the irreversible uptake of DNA until its duration was longer than 30 seconds. Extensive blending failed to further reduce the amount of DNA bound, but did progressively decrease the amount of transformation to a negligible level. The treatment had no effect

on cells transformed prior to blending, identifying the sensitive portion of the sequence as occurring before the irreversible uptake of DNA. The reduction of transformation was not due to the selective destruction of competent bacteria.

Blending had no effect on the uptake and incorporation of ^{14}C-L-leucine. An increase in the uptake and incorporation of ^{14}C-D-alanine was observed in blended cells, suggesting a weakening of the cell wall. The possibility of a weakened cell wall in blended bacteria, however, was not evidenced by an increased susceptibility to lysozyme treatment or osmotic shock. No detectable amounts of recipient DNA or phospholipid were released into the medium during blending. 0.5% of the total labeled cellular protein was released by blending, but can be accounted for by the removal of flagella.

A kinetic analysis of events accompanying the entry of attached DNA into recipient bacteria showed no difference between control and briefly blended cells. The appearance of transformants in briefly blended cells exhibited a brief lag, due to attainment of DNase resistance, similar to an untreated control. Eventually, the blended culture produced 50% more transformants. Explanation of these results by the blending treatment increasing the rate of uptake of donor DNA, or increasing the number of competent bacteria is unlikely. One possibility is that brief blending disrupts the action of a surface located endonuclease involved in the uptake process, such that larger single strands of donor origin are being taken up. The increased length would increase the probability of a markers integration, and thus increase transformability.

The enhanced transformability of briefly blended cells was as

susceptible to osmotic shock as a control. The reversal of the inhibition by osmotic shocking by treatment with a concentrated cultural fluid from a competent culture was not as effective in blended cells as in the control. Such a supernatant treatment could not reverse the inhibition of transformation by extensive blending. The cultural fluid from briefly blended cells was incapable of reversing the inhibition of transformation by osmotic shock and 0.05% PEA treatment of competent cells. This rules out the possibility of blending releasing and distributing a competence factor throughout the culture.

The alteration caused by extensive blending was severe enough to prevent a treated culture from passing through a second wave of competency. Since blending reduces attachment of DNA to a constant level immediately, but progressively affects the irreversible uptake, a primary effect of it would be directed against the initiation of uptake from initially attached DNA.

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