THE EFFECT OF OSMOTIC SHOCKING UPON *BACILLUS SUBTILIS* TRANSFORMATION

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Griffith's (1) discovery of bacterial transformation in 1928 is a landmark in the study of genetics, for it not only showed that hereditary determinants could be transferred from one bacterium to another, but it established the foundation for the identification of DNA as the true hereditary material. In 1944 Avery, McLeod, and McCarty (2) demonstrated that the transforming principle was deoxyribonucleic acid (DNA).

Transformation is important for genetic studies and analysis of those organisms which are not amenable to transduction or conjugation and offers a method for fine mapping of bacterial chromosomes. It is the only method presently available for correlating the effects of chemical or physical alterations in the structure of DNA with biological activity. This allows for direct investigation of the structure-function relationship of DNA. For example, the effects of ultraviolet irridiation and other mutagens on DNA have been largely elucidated by such studies.

Bacillus subtilis is the most desirable organism for transformation studies because: (1) it is avirulent, (2) it can be grown in a simple, chemically defined medium, and (3) numerous auxotrophic mutants can be isolated.

The molecular mechanisms of bacterial transformation are obscure

despite intense research. Bodmer (3) has divided the process of transformation into the following five steps:

1. Initial attachment of donor DNA to the competent recipient cell.
2. Entry of the DNA into the cell by means of a specific transport system.
3. Synapsis between the donor DNA and the recipient's genome.
4. Recombination between the donor DNA and the resident genome.
5. Expression of the newly introduced genetic information.

The most basic problem in transformation is the manner of DNA entry into the recipient cell. This study will be limited to the early steps in the transformation process and will not concern the events taking place during synapsis and recombination. The early steps involve the interaction of donor DNA and recipient cells, which must be in a poorly defined physiological state known as competence.

Transforming DNA may be derived from chemical and physical purification, cell lysis (4,5), or by spontaneous release from actively growing cells (6). Szybalski and Opara-Kubinska (7) found that *B. subtilis* DNA exhibits little or no transforming activity below 10^7 molecular weight. Depending upon the molecular weight value used for the intact genome and the method of isolation, transforming DNA has an average molecular weight of about 2.1×10^7 (8), and hence, the genome may be broken into 40-160 fragments by mechanical and hydrodynamic shear forces. The depolymerization of DNA by DNase destroys transforming activity. Heating to the melting temperature followed by rapid cooling removes all but a small residual transforming activity (9). Transforming DNA should be double stranded and of native configuration even though possible degradation of one strand may follow irreversible

binding (10). Transforming DNA is irreversibly bound when it can not be removed by either DNase or washing (11).

The early stages of transformation (12,13,14,15) are kinetically similar to phage adsorption (16). That is, the interaction between DNA and a recipient cell results from random collisions, followed by reversible binding of the DNA to the cell, and irreversible binding. The collision efficiency is very high and suggests that the surface of the cell is modified in some way which holds the DNA upon collision impact (14). The sensitivity of this binding process to pH, ionic strength, and divalent ions suggest ionic interactions between the highly charged DNA molecule and charged groups on the bacterial surface (17). Young and Spizizen (18) have shown that inhibitors of oxidative phosphorylation such as cyanide and 2, 4-dinitrophenol prevent irreversible uptake of DNA by competent cells. Also, an energy source such as glucose must be present, suggesting a metabolically primed active transport system for DNA.

When the number of transformants is measured as a function of DNA concentration, a direct relationship is observed until saturation is achieved. This type of curve indicates that each transformant may arise from the interaction of a recipient bacterium with a single molecule of transforming DNA (19,20,21). The DNA saturation phenomenon and autoradiography data (22,23) are consistent with definite binding or receptor sites on the surface of the bacterium. For example Tomasz (23) has shown that radioactive DNA binds only at the terminal ends and at the equatorial axes of the organism.

Competence is a transitory state of the recipient population and usually occurs in late logarithmic growth and lasts through the early

stationary phase--a period of three to four hours (24). The per cent of the population which is competent during the peak of competence ranges from 5-100 per cent (25). The exact physiological state which the bacterium must attain is still a mystery, but the empirical optimum conditions have been established.

Singh and Pitale (26) separated competent cells from the bulk of the population by zonal centrifugation in linear sucrose gradients. Normal vegetative cells have a density between 1.427 and 1.136 g/ml while the transformed cells have a density between 1.105 and 1.115 g/ml (26). Subsequent work demonstrated separation of competent cultures on gradients of Renograffin-76 (27,28). With step gradients of Renograffin-76, 95% of all competent cells were found in the lighter fraction. This allowed for rapid isolation of highly competent B. subtilis.

Since the initial interaction between DNA and the recipient cell is ionic, research concerning the surface charge characteristics was conducted. Plummer and James (29) found that the charge density at the cell surface depends on the physiological state of the culture. Using a measure of the electrokinetic potential at the cell surface, Jensen and Haas (30,31) concluded that competence is directly related to the surface charge of the recipient cell with a sudden increase in electronegativity occurring as competence develops. The appearance of competence is asynchronous and the negative charge is slowly discharged. The bacterium also undergoes definite physiological changes regarding pathways involving glucose degradation, but these changes may be associated with sporulation (32). Spizizen (33,34) first suggested an interrelationship between sporulation and competence since mutations affecting

sporulation also affected transformability. Schaeffer (10) has minimized the relationship between sporulation and transformation since many transformable strains are non-sporulating. The blocked step or steps in sporulation might not affect transformation if the blocks were in the latter stages of sporulation. While the competent state of *B. subtilis* may require some early functions of sporulation (35,36), it probably is an alternative to sporulation.

Morphological differences between genetically competent (C^+) and non-competent (C^-) cells are not readily discernable. However, the cell walls of C^+ strains contain more alanine and galactosamine and less diaminopimelic acid and glucosamine (37,38). The galactosamine content of C^+ strains increased with growth and peaked during maximum competence. Young (39) also found that suspensions of C^+ cell walls lysed three to five times faster than C^- cell walls because of N-acylmuramyl-L-alanine amidase (40). The autolytic activity is present throughout growth and might be involved in cell division, since it reaches a maximum during logarithmic growth (39).

Removal of the cell wall after exposure to DNA does not significantly reduce the amount of membrane bound DNA (22). However, the cell wall does appear necessary for the transformation process in some capacity. Highly contradictory results regarding the affect of lysozyme treatment on transformation have been reported. Tichy and Landman (41) concluded that lysozyme treated cultures are heterogeneous and contain lysozyme resistant cells, osmotically sensitive rods, "quasi-protoplasts", and protoplasts. The cell wall must be present before transport is completed. Hence, if quasi-protoplasts are prepared, DNA added, and are allowed to synthesize a cell wall after DNA binding,

maximum transformation frequency is achieved.

When competence appears during early logarithmic growth, transformed cells are more likely to lyse than the remainder of the population. However, if competence is achieved in late stationary phase, this tendency toward preferential lysis is not observed (6). Competence achieved during different times in the growth cycle and under different growth conditions may vary in some important aspects. The competence achieved and described in one laboratory may differ considerably from the state of competence noted by another investigator.

The number of mesosomes (intracellular membranous structures) increases two fold during maximum competence (42). This increase may be connected with other processes such as sporulation and more data are needed to fix a role in transformation.

A majority of competent cells are uninucleate (43), but this has been questioned (44). However, competent cells do appear smaller than normal vegetative cells (23).

Anagnostopoulos and Spizizen (24) first defined the conditions necessary for attainment of competence. Bott and Wilson (45) developed a widely accepted method for achieving a relatively high degree of competence. The basic medium contains minimal salts and glucose. Magnesium (5mM) is required for DNA binding and uptake. Acid-hydrolyzed casein enhances competence, but Bott and Wilson found that commercial casein hydrolysate contained inhibitory amino acids (alanine, glutamic acid, leucine, isoleucine, and proline). However, a synthetic mixture of the stimulatory amino acids (arginine, histidine, threonine, glycine, valine, aspartic acid, lysine and methionine) produced maximum competence.

Copper, manganese, and iron ions are inhibitory to competence development (18,45,47). The carbon source was inconsequential while no binding or uptake of DNA occurred in Tris buffer containing glucose. The optimum pH ranged from 6.9 to 7.4 and the optimum temperature range was 34 to 37° C. In addition, agitation or aeration increased DNA uptake (18,46). Polyphosphate and L-histidine enhance transformation probably due to chelation of copper ions (18,47,48). The medium contains 0.14 M potassium phosphate, but the phosphate anion competitively inhibits transformation at DNA concentrations in the linear response region. Stewart (49) demonstrated that if phosphate was omitted, the pH was unaffected and the transformation frequency was increased five-fold in the linear response region of DNA concentration.

Competence is genetically determined, but the exact nature of the genetic control is not presently understood. Spizizen (33,34) has suggested some genetic relationship between competence and certain phases of the process leading to sporulation.

Normally the competent culture is exposed to the DNA for thirty minutes. However, if the incubation is more than forty-five minutes, the frequency of transformation drops, possibly due to the release of bound DNA or the destruction of potential transformants (50).

Protein synthesis is required for competence development. Actinomycin D and puromycin not only inhibit competence development but destroy existing competence. If puromycin was added simultaneously with the DNA, DNA uptake was not inhibited, but as the interval increased between addition of puromycin and DNA, there was a drastic reduction in DNA uptake (51). Thus messenger-RNA directed protein synthesis is required not only for development of competence but also for its

maintenance, and the protein products have a very short half-life. Ganesan and Buckman and Bodmer (52,53) found that inhibition of DNA synthesis in competent cells by 5-fluorodeoxyuridine had little effect on DNA uptake or on transformation.

If one considers the binding of DNA to a competent cell as a simple enzyme-substrate reaction, the rate constant for the dissociation of the cell-DNA complex is thirty times greater than the rate constant for irreversible incorporation (12).

The rate at which the bound DNA attains a DNase-insensitive state is temperature dependent, which indicates involvement of enzyme catalyzed steps. By using an Arrhenius plot of length of lag as a function of temperature, the activation energy required for the transition of reversibly bound DNA to irreversibly bound DNA is 13.9 K cal (54).

Strauss (15) noted that the lag period required for the joint entry of two linked markers varied directly with the map distance between them. That is, the farther apart two genetic markers are on the DNA molecule, the longer the time period required for both to achieve the DNase-insensitive state. This proves that the transforming DNA molecule enters the competent cell in a linear manner.

Autoradiography has shown that the transforming DNA remains at membrane sites even after 90 minutes (22). Thus, the DNase-insensitive location is either on or in the membrane structure. Since removal of the cell wall after previous exposure to DNA does not result in significant release of transforming DNA (22), the transforming DNA is tightly bound to the cell membrane. However, Kammen, Wojnar, and Canellakis (51) suggested that genetically active irreversibly bound DNA can be displaced by genetically inactive DNA. Erickson (55) noted that

irreversibly bound DNA exhibits single-stranded properties, since antibodies specific for single-stranded DNA inhibit transformation. But, maximum inhibition occurs when the antibodies are incubated with the recipient cells before addition of DNA.

Kammen, Beloff, and Canellakis (56) found that addition of a mixture of amino acids stabilized potential transformants. This stabilization was also achieved by high concentrations of puromycin (100-200 μg/ml). When the amino acid mixture was added to a culture containing a low concentration of puromycin, only the transformants were protected from the bacteriocidal effect of the puromycin. Thus, polypeptide synthesis is required for the retention of irreversibly bound DNA, but the synthesis of this polypeptide is not mediated by messenger RNA, and, therefore, is not the same protein required to attain competence.

If a competent culture is heated to 50° for five minutes, the transformation frequency is reduced 90% without concomitant cell death. When the DNA reacted with the cells prior to the heat step, the decrease in transformation was much less (57). Thus, a factor necessary for retention of bound DNA is destroyed by heat and cannot be synthesized for about three hours.

A succinct recapitulation will be used to focus attention on the key to understanding transformation, that is, elucidation of the competent state. The initial reversible binding, which is ionic in nature, is followed by irreversible binding of the DNA in or about the cell membrane. The linear penetration of the DNA is followed by recombination between the recipient's genome and the transforming DNA. Cells can become competent in a defined glucose minimal medium with a particular mixture of amino acids being stimulatory. In addition, competence

is under genetic control since high and low transforming strains can be isolated. The competent cells differ from other cells in physical properties such as size and density, and in many physiological and metabolic ways.

Competent cells can produce a competence factor (CF) which can induce, stimulate, or transfer competence to non-competent cells. Competence factors have been reported for Pneumococcus (58,59,60), Streptococcus (61,62,63,64), and B. subtilis (65,66).

Pakula and Walczak (62) found an extracellular factor produced by competent cells of Streptococcus Group H, strain Challis, which induced competence in the non-competent cells of strain Wicky. The Wicky cells bound some radioactive DNA, but were incapable of transformation until the addition of the CF of the Challis cells. The factor is non-dialyzable, is inactivated by heating at 115° for sixty minutes, and has a molecular weight of 5000-7000. Pakula (62) found the CF in the supernatant solutions of cultures of strain Challis growing in a rich undefined medium containing Bacto-peptone.

Dobrzanski and Osowiecki (64) prepares CF by "tearing" it loose from the surface of strain Challis cells. A culture is harvested twenty minutes before maximum competence and concentrated one hundred fold. The pH is adjusted to 2.0 by HCl. The cell suspension is then subjected to a thermal shock of 60° for 10 minutes. The cocci are removed by centrifugation and the supernatant solution is adjusted to pH 7.6 by addition of NaOH. The activation of Wicky cells by CF is concentration dependent. The optimum period of contact of CF and Wicky cells is 30 minutes--the same as with the CF prepared by Pakula (63). The CF was inactivated by trypsin, pronase, pancreatin, and papain while

lysozyme and RNase had no effect. Ten minutes exposure at 100° resulted in only a 22% loss of activity. All activity was lost only after exposure for 90 minutes at 100°. Dobrzanski (67) has improved the procedure of preparing CF by precipitating competence factor from the supernatant solution by 60% ammonium sulfate. The resulting precipitate was suspended at pH 8.0 at one-tenth the original volume and dialyzed. The non-dialyzable portion was chromatographed on a carboxymethyl cellulose column. The factor was eluted with a linear NaCl gradient (0-0.5 M). The appropriate fractions were pooled and lyophilized. This product was designated as "highly purified competence factor". The purified CF has the same general properties as described above for the crude competence factor.

Pakula and Ray (68) reported that non-dialyzable peptides and a mixture of amino acids must be present in the medium for maximum release of competence factor. Neopeptone (Difco) is the peptide source. The level of competence of the Challis cells paralleled the concentration of cell-bound CF. The release of CF into the medium resulted in a corresponding decrease in competency. The cell bound CF may be different from the cell free competence factor since protein synthesis must occur for function of the competence factor. An antibody prepared against crude competence factor will inhibit transformation if added prior to DNA addition. The same inhibition occurs if antibodies against competent cells were used.

The streptococcal competence factor(s) constitute an interesting class of polypeptides. The competence factors prepared from two closely related strains of group H streptococci, Streptococcus sanguis (69) and Challis, showed no cross-reactivity. However, the antibodies against

Challis CF inhibited the CF of Streptococcus sanguis. This suggests that the competence factors have a specific different cell binding site but a common antibody binding site (70).

Pakula and coworkers (70) also found that if competent cells from strain Challis or Wicky were suspended in 0.001 N hydrochloric acid, they immediately precipitated into large aggregates. There was an inverse correlation between the level of competence and the affinity of the cells toward methylene blue. This was in good agreement with the work of Jensen and Haas (30). A change in surface charge accompanies the conversion of non-competent cells to competent cells.

Tomasz and Hotchkiss (58) and Tomasz (59) have also presented evidence for a competence "activator" in Diplococcus. By means of a U-tube experiment in which a membrane filter separated two cultures, one competent and one incompetent, the latter became competent after 60 minutes incubation at 30°. The active material was eluted from cell debris and has maximal activity at pH 7.7. The activity was enhanced by mercaptoethanol and inhibited by proteolytic enzymes. Maleate (0.05 M) nullified the CF's activating effect on transformation, but DNA attachment was apparently unaffected. Antibodies against competent cells prevented the CF from converting non-competent α-hemolytic Streptococcus, strain D, a close relative. The factor has a molecular weight of approximately 10,000.

Pneumococcal CF is prepared by harvesting cells prior to development of maximum competence and washing them thoroughly in a saline-phosphate buffer, pH 7.5, containing mercaptoethanol. The cell suspension is heated to 60° for 10 minutes to kill the cells and solubilize the factor. After repeated extraction of the cell debris by the same

washing solvent, the supernatant solutions are pooled and quick frozen.

One outstanding difference between the B. subtilis and Pneumococcus transformation systems is the length of the competence period. B. subtilis maintains competence for three to four hours while Pneumococcus maintains high competence for about fifteen minutes. The decline of pneumococcal competence can be correlated with the production of a macromolecular inhibitor which prevents competence from spreading throughout a culture (58,59). The mechanism of the inhibitor has not been ascertained, but it is non-dialyzable, stable to 90° for 15 minutes, stable to RNase, and has no effect on cells already competent. Unlike the pneumococcal CF, the inhibitor is not inactivated by filter sterilization. Hence, it can be prepared by exhaustively dialyzing sterile filtrates of medium followed by lyophilization.

Kohoutova (60,72) obtained different results when searching for the pneumococcal competence factor. A factor was present in the supernatant solution prior to competence development, which enhanced the frequency of transformation when briefly incubated with the DNA before addition of recipient cells. If sterile culture filtrates were added to young non-competent recipient cells and incubated for ten minutes, a thousand fold increase in the number of transformants could be achieved. Filtrates from cultures in the stationary phase still contained the factor, and unlike the competence factor described by Tomasz and Mosser (73), it can withstand 100° for at least 30 minutes. These discrepancies suggest a multiple factor system.

Charpak and Dedonder (65) have demonstrated a competence factor in the supernatant solution of competent cultures of B. subtilis. The factor was also obtained from phosphate-extracted acetone powders of

competent B. subtilis. The factor allowed transformation of a non-competent B. subtilis, strain Niger, for methionine independence. The factor was destroyed by the proteolytic enzymes, trypsin and chymotrypsin, and also by 100° for five minutes. Felkner and Wyss (74) demonstrated that a factor found in a crude concentrate would restore competence to B. cereus, strain 569, after transformability has been lost by a washing procedure. This bacterium was apparently improperly identified and was probably B. subtilis instead (75).

Akrigg and coworkers (66) observed that a cold water wash drastically reduced transformation of B. subtilis. Transformability could be restored by adding back the wash fluid. The factor was purified by DEAE chromatography and had both autolytic and competence factor activity. The CF was prepared by collecting competent cells by centrifugation and extracting three times with water at 4°. The combined aqueous extracts were dialyzed against water for twenty four hours and lyophilized. The lyophilized powder was dissolved in Tris-HCl buffer, pH 7.4, and applied to a DEAE column (30 cm x 1.5 cm). The column was eluted with a convex gradient of 0.4 M NaCl and the absorbance at 280 nm determined. Of the five peaks, only peak II had CF activity. Further significant purification of peak II was not achieved by gel chromatography, hydroxyapatite chromatography, preparative polyacrylamide electrophoresis, or isoelectric focusing. Ultracentrifugation yielded a nearly homogeneous band with a sedimentation coefficient of 1.29 S which corresponded to a molecular weight of less than 10,000. The amino acid composition of the partially purified factor was determined, and a large proportion of the amino acids were acidic, particularly aspartic acid. The isoelectric point was between pH 1.5 and 3.0 (76). The CF

may be identical to the autolytic enzyme reported by Young (40).

The streptococcal, pneumococcal, and B. subtilis competence factors have several properties in common: all bind to specific surface sites; all have the ability to increase the ierreversible binding of transforming DNA; all are protein in nature or at least contain small proteins since they are inactivated by proteolytic enzymes; and all are destroyed by heating or filtration through membranes or glass powder. The competence factors of Pneumococcus and Streptococcus are positively charged at physiological pH while the B. subtilis competence factor is negatively charged. The molecular weights of the competence factors of the three genera range from 5000-7000 for Streptococcus to about 10,000 for the Pneumococcus and B. subtilis (77). Autolytic activity has only been demonstrated in the B. subtilis CF preparations. Table I summarizes characteristics of the competence factors obtained from bacteria.

Akrigg, Ayad, and Blamire (78) proposed a theory for the uptake of exogenous DNA by B. subtilis. In the first stage an autolytic enzyme is released from the cell wall and attacks the wall, probably at a region of weakness. One of these points of weakness is located next to a mesosome where normal cell division occurs. When the cell wall ruptures next to the mesosome, part of the mesosome is exposed to the outside environment. The exogenous DNA is taken up into the mesosome and finds the recipient's genome, at the replication point. If this particular connection between the recipient's genome and the mesosome is the site of the replicating point, a specialized mechanism for the transport of DNA through the membrane might also be located there.

The goal of this research is to elucidate the mechanism of transport of the transforming DNA by competent cells. As is often the case

TABLE I

CHARACTERISTICS OF REPORTED COMPETENCE FACTORS

SOURCE	METHOD OF RELEASE	MOLECULAR WEIGHT	SENSITIVE TO PROTEOLYTIC ENZYMES	HEAT LABILE	CHARGE	DIALYZABLE
Streptococcus challis	Ammonium sulfate fractionation of supernatant solution	5,000-7,000	Yes	115^{o} 60 min	+	No
Diplococcus pneumoniae	Extraction of heat-killed cells	10,000	Yes	100^{o} 20 min	+	No
Bacillus subtilis	Cold H_2O extraction	<10,000	Yes	Yes	-	No

when attempts are made to postulate theories that encompass several biological systems, confusion results since there is no necessity for a unified mechanism. The competence factor and transport of B. subtilis DNA as studied in this thesis support this view. Results are reported concerning the reduction of transformability and transport by osmotic shocking procedures, the restoration of transformability and transport by various preparations, and the use of these preparations to increase the transformability of low competence strains.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

L-Arginine and L-methionine were obtained from Cyclo Chemical Corporation. L-Aspartic acid, phenethylalcohol, and EDTA (ethylenediamine tetra acetic acid) were obtained from Eastman Organic Chemicals. Glycine, K_2HPO_4, KH_2PO_4, glucose, sodium citrate, and Tris (hydroxymethyl) amino methane were purchased from Fisher Scientific Company. L-Histidine was obtained from the H. M. Chemical Company, while L-tryptophan and ribonuclease (E.C. No. 2.7.7.16) were purchased from the Mann Research Laboratories. L-Threonine, deoxyribonucleic acid (salmon sperm), and casein hydrolysate (acid) were secured through Nutritional Biochemicals Corporation, and L-valine was purchased from California Biochemical Research. Ammonium sulfate, L-lysine and magnesium sulfate were obtained from the J. T. Baker Chemical Company. Phenol and sucrose were purchased from the Mallinckrodt Chemical Works. Deoxyribonuclease (E.C. No. 3.1.4.5), papain (E.C. No. 3.4.4.10), and lysozyme (E.C. No. 3.2.1.17) were obtained from Worthington Biochemicals. Bacto-agar, Bacto-brain-heart infusion, Bacto-yeast extract, Bacto-tryptose, Bacto-tryptose blood agar base, Bacto-beef extract, Bacto-nutrient agar, and Bacto-nutrient broth were products of Difco Laboratories. Thymidine, tritiated thymidine (14.0 C/m mole), and tritiated uridine (2.0 C/m mole) were purhcased from Schwarz Bioresearch

Incorporated. L-Leucine 1-^{14}C (6.8 mC/m mole), uracil and pronase (Grade B) were purchased from Calbiochem. Dodecyl sodium sulfate was a product of Matheson, Coleman, and Bell. Millipore filters (HA 0.45 μ) were purchased from Millipore Filter Corporation. Gifts of B. subtilis, strains 168 C$^-$ (ind$^-$ competent$^-$) and SB 25 (ind$_2^-$ his$_2^-$) were received from F. E. Young. Strains WT and FH 2006 (ind$^-$ thy$^-$) were given by W. C. McDonald and I. C. Felkner, respectively.

Methods

Isolation of Donor DNA

B. subtilis, strain WT, was grown in minimal medium (33) (10-15 liters) supplemented with 0.1% yeast extract at 37° with aeration of 12 liters per minute for 14 hours. The cells were harvested by the use of a Sharples Super Centrifuge, and donor DNA was prepared by the procedure of Saito and Miura (78). The method of Burton (79) which is a modification of the basic diphenylamine reaction (80), was used for determination of DNA concentration.

Preparation of Tritiated DNA

B. subtilis, strain FH 2006 (ind$^-$ thy$^-$), was grown 16 hours on a plate of brain-heart infusion agar at 37°. These cells were used to inoculate minimal medium supplemented with 0.05% acid hydrolyzed casein and 50 μg/ml of thymidine. The cells were incubated at 37° with shaking until an absorbance at 630 nm (A_{630}) of 0.64 (1 cm light path) was reached. The cells were suspended to an A_{630} of 0.22 in 50 ml of minimal medium plus 0.05% acid hydrolyzed casein, ^{3}H-thymidine (2 mC per 50 ml) and sufficient thymidine to give a final concentration of

10 μg/ml. The cells were incubated at 37° with shaking for three generations. The tritiated DNA was extracted and purified as described by Richardson (81).

Transformation Procedure

B. subtilis, strain SB 25 (ind_2^- his_2^-) was maintained on a tryptose blood base agar plate (TBA) at room temperature. Cells from this plate were used for several weeks. About once a month a new plate was subcultured by streaking a loopful of the working culture on a fresh TBA plate, incubating overnight at 37°, and then the plate was stored at room temperature. A loopful of cells from this plate was used to inoculate 10 ml of tryptose blood base broth which was incubated 16 hours at 37° on a shaker. The 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 supplemented with 50 μg/ml each of histidine, tryptophan, valine, glycine, arginine, aspartic acid, lysine, threonine, and methionine plus enough magnesium sulfate to give a final concentration of 0.072%. When minimal medium was supplemented with these components, it was designated transformation medium. The culture was incubated at 37° with shaking for 5 hours to develop maximum competence. This is a modification of the procedure of Bott and Wilson (45).

At the end of the competence development period, DNA (5 μg/ml) was added and the culture incubated for 30 minutes. Dilutions were made in minimal medium at room temperature. Double transformants bearing the linked ind_2^+ and his_2^+ markers (try^+ his^+) were scored on minimal agar. Total his_2^+ or ind_2^+ transformants were scored on minimal agar supplemented with 10 μg/ml of tryptophan (his^+) or histidine (try^+),

respectively. The total viable cell count was scored on minimal agar supplemented with 10 µg/ml of histidine and tryptophan. In some studies when only the tryptophan$^+$ transformants were measured, they were scored on minimal agar plates supplemented with 10 µg/ml histidine plus 0.1% casein hydrolysate (acid). With this procedure, the transformants could be scored within 17 hours as opposed to the ususal 40 hours.

Osmotic Shock Procedure

Competent B. subtilis SB 25 were prepared as described above. The competent cells (1 x 10^9 cells per ml) were sedimented by centrifugation at room temperature. Stage I of the shock procedure involved the rapid suspension of the sedimented cells in one half of their original volume in 0.25 M sucrose - 0.033 M Tris-HCl, pH 7.2, and 10^{-4} M EDTA. The cells were incubated for 10 minutes at room temperature with occasional stirring. The suspension was then centrifuged for 10 minutes at 15,000 x g. The supernatant fluid was removed. Stage II involved the instantaneous suspension of the well drained pellet in 5 x 10^{-4} M $MgCl_2$ (equal to original volume) at 4^o. The cells were vigorously stirred by using a vortex mixer and were incubated in an ice bath for 10 minutes with occasional stirring. The suspension was again centrifuged at 4^o for 10 minutes at 15,000 x g and the supernatant fluid was removed, and the cells suspended in various solutions to their original cell density for experimentation. The pelleted cells were designated as shocked cells. This was a modification of the procedure of Nev and Heppel (82).

Preparation of Shock Fluid

A large loopful of B. subtilis SB 25 cells from the TBA plate was used to inoculate 300 ml of tryptose blood base broth and incubated at 37° with shaking for 16 hours. This suspension was centrifuged to sediment the cells. The cells were washed once with minimal medium before being suspended in 20 ml of transformation medium. The suspended cells were added to 5 liters of transformation medium to give an initial absorbance at 630 nm of 0.15. The cells were grown in a New Brunswick Fermentor at 37° with a forced aeration of 12 liters per minute and stirring (2000 rpm) for 4 to 5 hours at which time maximum competence was achieved. The cells were harvested with a Sharples Super Centrifuge and suspended in 100 ml of the Stage I shock solution. After a 10 minute incubation at 37°, the cells were sedimented and suspended in 15 ml of 50 mM potassium phosphate buffer, pH 7.0, and poured into a 20 ml syringe. The cells were instantaneously chilled by injection with moderate stirring into 75 ml of cold 5×10^{-4} M $MgCl_2$. The cells were stirred at 0° for 20 minutes, and sedimented by centrifugation. The second stage of the shock treatment was repeated twice. The extractions were pooled, and lyophilized and designated as lyophilized shock fluid.

Uptake of Tritiated DNA

Competent B. subtilis SB 25 cells were prepared as described above. Tritiated DNA (1-3 µg/ml) was added to 3 ml of cells and incubated 45 minutes at 37° with shaking. DNase (0.1 µg/ml) was added and the incubation continued for 10 minutes. An aliquot was diluted and plated to determine the transformation frequency. The remainder of the suspension

was centrifuged, and the cells suspended in minimal medium plus DNase (50 µg/ml). The cells were sedimented and washed a third time in minimal medium. The cells were suspended in 1 ml of minimal medium and collected by Millipore filtration. The Millipore filters were dried under a heat lamp and counted using a Packard Model 3320 Liquid Scintillation Spectrometer with Bray's scintillation fluid (83).

Total Uptake and Incorporation of Radioactive Precursors

Competent B. subtilis SB 25 cells were prepared as described earlier. The cells were shocked by the standard procedure and then suspended in either transformation medium or supernatant solution at 37°. Saturating amounts of precursor were added followed immediately by the addition of the radioactive precursor. Samples were taken at various time intervals. Total uptake was measured by collecting 1.0 ml of the cell suspension by Millipore filtration (HA 0.45 µ) and washing with 5.0 ml of cold minimal medium. The incorporation of the precursor into macromolecules was measured by adding 1.0 ml of the cell suspension to 1.0 ml of cold 10% trichloroacetic acid. The mixture was incubated for 15 minutes at 0° and then filtered on a Millipore filter (HA 0.45 µ). After washing with 5.0 ml of cold 5% trichloroacetic acid, the filters were dried under a heat lamp and counted as previously described. The accumulation of radioactive precursor was calculated by subtracting the radioactivity incorporated from the total radioactivity taken up by the cells.

Preparation of Cell Walls

Five liters of competent B. subtilis SB 25 or 168 C^- were prepared by use of the New Brunswick Fermentor as described earlier. The cells were harvested at maximum competence by centrifugation in the Sharples Super Centrifuge. The cells were placed in the chamber of an X-press as a thick paste, and the X-press was placed in the freezer for 18 hours. After breakage of the cells in the X-press, the material was diluted to 250 ml with cold distilled water and centrifuged for 10 minutes at 1000 x g at 4° to remove intact bacteria. The supernatant solution was centrifuged in this manner two more times, each time discarding the sediment. The supernatant solution, containing cell walls, was centrifuged at 10,000 x g for 10 minutes at 4°. The cell walls were washed 12 times with cold distilled water and lyophilized for storage. This was a modification of Young's (84) procedure.

Sucrose Density Gradient Centrifugation

A linear sucrose gradient (5% w/v to 20% w/v sucrose in 50 mM phosphate buffer, pH 6.9, plus 0.1 M NaCl) was layered into 0.5 inch x 2.0 inch cellulose nitrate centrifuge tube by means of a gradient device (85). The centrifuge tubes were treated with Siliclad and dried before use. The lyophilized shock fluid was dissolved in 5% w/v sucrose and then carefully layered on the gradient. The samples were then centrifuged the appropriate time at 4° in a SW-65 rotor at 32,000 rpm. The Spinco ultracentrifuge (L-2) was used for all runs. Two or three drop fractions were collected with the refractive index of the odd-numbered fractions being measured. The even-numbered fractions were diluted with 0.5 ml of 50 mM phosphate buffer, pH 6.9, and the absorbance at

280 nm was determined using a Gilford Recording Spectrophotometer. Various amounts of peak tubes were added to C^- cells for incubation prior to addition of DNA. The cells were diluted and plated along with a control (no additions) to determine the presence or absence of competence factor activity.

Gel Chromatography Procedures

Gel filtration glass columns of desired length and diameters were treated with Siliclad. Gels were swollen, deareated, and packed into the columns according to technical manuals (86,87). Sample density was increased by addition of solid sucrose so sample would layer on top of the gel in a uniform manner. The general uniformity of packing and the void volume was determined visually with a blue dextran sample. The maximum flow rate, which did not result in local tailing or skewing of the migration of the blue dextran band, was used.

Protein Determinations

Protein determinations were made according to Lowry (88), using the Folin-Ciocalteu reagent (89). Bovine serum albumin was used for the protein standard.

Measurement of Enzyme Activities

The shock fluid and supernatant solutions were tested for nuclease activity as described by DeWaard and Lehman (90). The RNase content was determined according to Kalnitsky, Hummel, and Dierks (91).

The proteolytic activity of the supernatant solution and shock fluid was determined by the sensitive assay of RinderKnecht, Geokas,

Silverman, and Haverback (92). The shock fluid and supernatant solution were tested for autolytic activity by the method of Akrigg and Ayad (75).

Nucleic Acid Determination

Burton's (79) method for the quantitative determination of DNA was utilized because of its high sensitivity and increased specificity. The RNA content was determined by the method of Mejbaum (93).

Restoration of Shocked Cells by Supernatant Solution

Competent B. subtilis cells were shocked as described previously. An aliquot of cells was suspended in transformation medium at 37^{o} while another aliquot was suspended in the supernatant solution of a competent culture. The cells were incubated at 37^{o} with shaking for 30 minutes. DNA (5 µg/ml) was added and the incubation continued for 30 minutes. The cells were diluted, plated, and the transformants were scored. The restoration properties of the supernatant solution were expressed as the specific activity which was defined as

$$\frac{\%\ \text{Transformation of Restored Cells/100 µg Protein}}{\frac{\%\ \text{Transformation of Shocked Cells}}{2}} = \text{Specific Activity}\ .$$

Enhancement of Transformation in C^{-} Cells by Shock Fluid

B. subtilis 168 C^{-} cells were grown in transformation medium for 5 hours, which is when maximum competence occurs. They were then sedimented and then suspended to their original volume in warm transformation medium. Then the appropriate amount (giving maximum enhancement) of lyophilized shock fluid (dissolved in 50 mM phosphate buffer,

pH 6.9) was added. The cells were incubated for 30 minutes at 37° on a shaker. DNA (5 μg/ml) was added and the incubation was continued for an additional 30 minutes. The cells were diluted in minimal medium and plated. The transformants were later scored. The efficiency of shock fluid in increasing transformation frequency was expressed as specific activity which was defined as

$$\frac{\text{\% Transformation With Shock Fluid/100 μg Protein}}{\frac{\text{\% Transformation Without Shock Fluid}}{2}} = \text{Specific Activity} \quad .$$

CHAPTER III

THE EFFECT OF COLD OSMOTIC SHOCK TREATMENT ON B. SUBTILIS CELLS

Cold osmotic shock selectively releases several surface-located degradative enzymes from E. coli, and numerous proteins involved in the uptake of specific substances are also released. About 10% of the cell's total protein is released by osmotic shock (94). Pardee (95,96, 97) crystallized a sulfate binding protein; Oxender (98,99,100,101) crystallized a protein that binds branched chain amino acids, and Anraku (102,103,104,105) purified binding proteins for both amino acids and carbohydrates. All used the shock fluid as the starting material.

Since transport of DNA is involved in genetic transformation, the cold osmotic shock treatment described in the Methods Section was applied to competent B. subtilis cells. Table II shows a marked reduction in transformability but no decrease in cell titer. Akrigg (66) has independently used a washing treatment.

Establishment of Shocking Procedure

The shock procedure involved suspension of the cells in 0.25 M sucrose, 30 mM Tris·HCl, pH 7.1, and 0.1 mM EDTA, followed by a cold 0.5 mM $MgCl_2$ wash. The role of the various components in this shock procedure was examined (Table III). The presence of all components in the first stage gave the maximum reduction in transformants (line 6). The Mg^{++} was included in the wash step, for retention of cell viability.

TABLE II

EFFECT OF OSMOTIC SHOCKING ON TRANSFORMATION

Treatment	His^+ Transformants (10^{-4})	Cell Titer (10^{-7})	% Control Transformation
None	250	90	100
Shocked	80	89	32

Competent B. subtilis SB 25 cells were obtained as described in the Methods Section. Samples were subjected to osmotic shock treatment as described in the Methods Section. After shocking, the cells were exposed to 5 µg/ml DNA for 30 minutes. The per cent of control was calculated by dividing the histidine$^+$ cells of the shocked culture by the histidine$^+$ cells of the unshocked control and then multiplying by 100.

TABLE III

EFFECT OF VARIOUS COMPONENTS OF THE SHOCK PROCEDURE

First Stage	Second Stage	His^+ Transformants (10^{-4})	Cell Titer (10^{-7})	% Control
1. None	None	208	100	100
2. Sucrose	None	185	98	89
3. Sucrose+Tris	None	150	98	72
4. Sucrose+Tris+EDTA	None	92	95	44
5. Sucrose+Tris+EDTA	4^o, H_2O	57	93	27
6. Sucrose+Tris+EDTA	4^o, $5x10^{-4}$ M $MgCl_2$	60	101	28
7. None	4^o, $5x10^{-4}$ M $MgCl_2$	120	99	58
8. None	4^o, Minimal (3 times)	147	100	71

Competent B. subtilis SB 25 cells, obtained as described in the Methods Section, were collected by centrifugation and suspended in the solutions indicated for the First Stage (0.25 M Sucrose, 0.03 M Tris, pH 7.1, and 1 x 10^{-4} M EDTA). Line 1 represents the unshocked control. After 10 minutes incubation at 25^o, the cells were centrifuged and suspended in fresh transformation medium at 37^o (lines 1-4) or H_2O at 4^o (5-7). One aliquot of cells was subjected only to the first stage of the shock procedure (line 4) while in another aliquot the first stage was omitted (line 7). The cells, which were suspended in the second stage, were incubated for 10 minutes at 4^o. These cells were centrifuged and suspended in fresh transformation medium at 37^o. Then 5 µg/ml DNA was added to all tubes, and following 30 minutes incubation at 37^o on a shaker, the cells were diluted and plated.

The effect of the sucrose concentration was examined (Figure 1). A concentration of 0.2 M sucrose was required for maximum reduction. The cell viability was unaffected by the sucrose concentrations tested.

The Tris buffer concentration is important for both maximum reduction of transformation and the maintenance of viability of shocked cells (Figure 2). Optimal conditions were achieved with 30 mM Tris buffer, but as seen in Table III EDTA was required in conjunction with Tris for maximum reduction in transformation. EDTA levels in excess of 0.1 mM did not further decrease transformation (data not shown). A synergistic effect of Tris and EDTA involving surface phenomena has been reported in E. coli (106,107). If potassium phosphate buffer is substituted in the complete system for Tris, little reduction in transformation occurs (Table IV). This establishes Tris as a definite requirement in the shock procedure.

The efficiency of the shocking procedure was independent of pH between pH 6.5 and 9.0. Transformability was reduced by nearly 80% by the shock treatment within this pH range. In addition, no effect upon viability was observed under these conditions.

A temperature of 25-37^{o} for the first stage gave maximum reduction in transformation while maintaining cell viability. Temperatures above 50^{o} resulted in cell death and temperatures below 20^{o} reduced the efficiency of the shocking procedure. A temperature of 4^{o} was most efficient in the second stage (Table V).

Figure 1. Effect of Sucrose Concentration on Transformation and Viability

Competent cells were osmotically shocked as described in the Methods Section except variations in the sucrose concentration were used as indicated. After the shock treatment the cells were transformed and the % reduction of transformability, O, was calculated as described in the Methods Section and the viability, ●, was determined by plate counting.

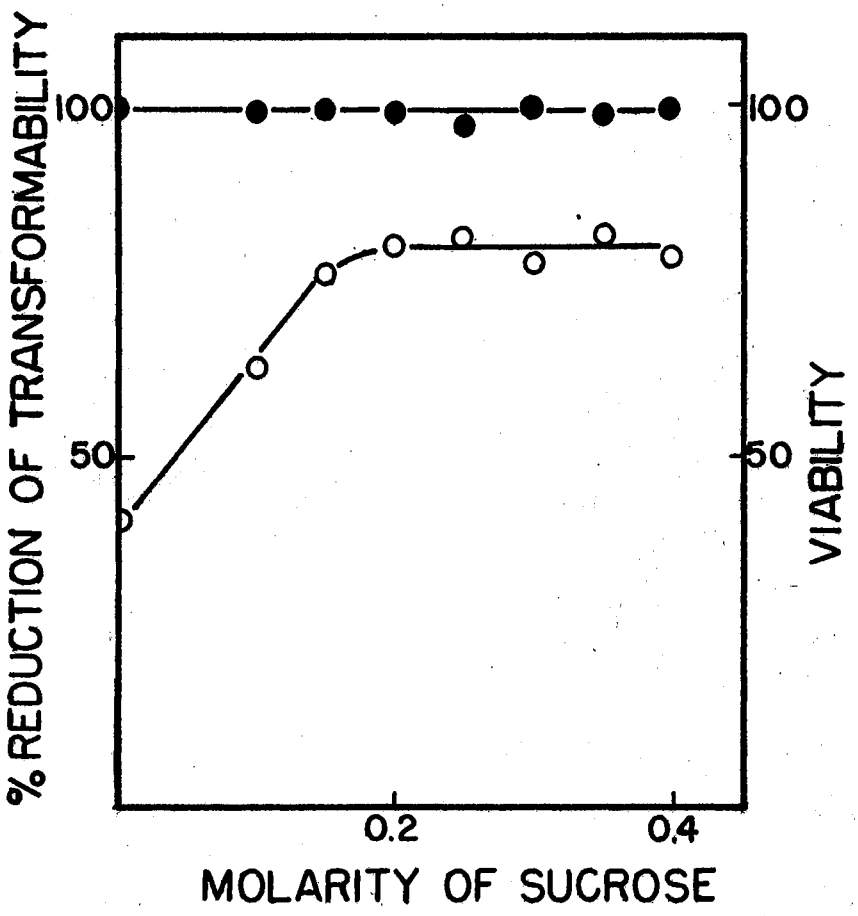

% REDUCTION OF TRANSFORMABILITY
100
50
VIABILITY
100
50
0.2
0.4
MOLARITY OF SUCROSE

Figure 2. Effect of Tris Concentration on Transformation and Viability

Competent cells were osmotically shocked utilizing different concentrations of Tris, pH 7.1, in the first stage of the shock treatment. The percent reduction of transformability, O, was calculated as described in the Methods Section. The viability, ●, was determined by plate count.

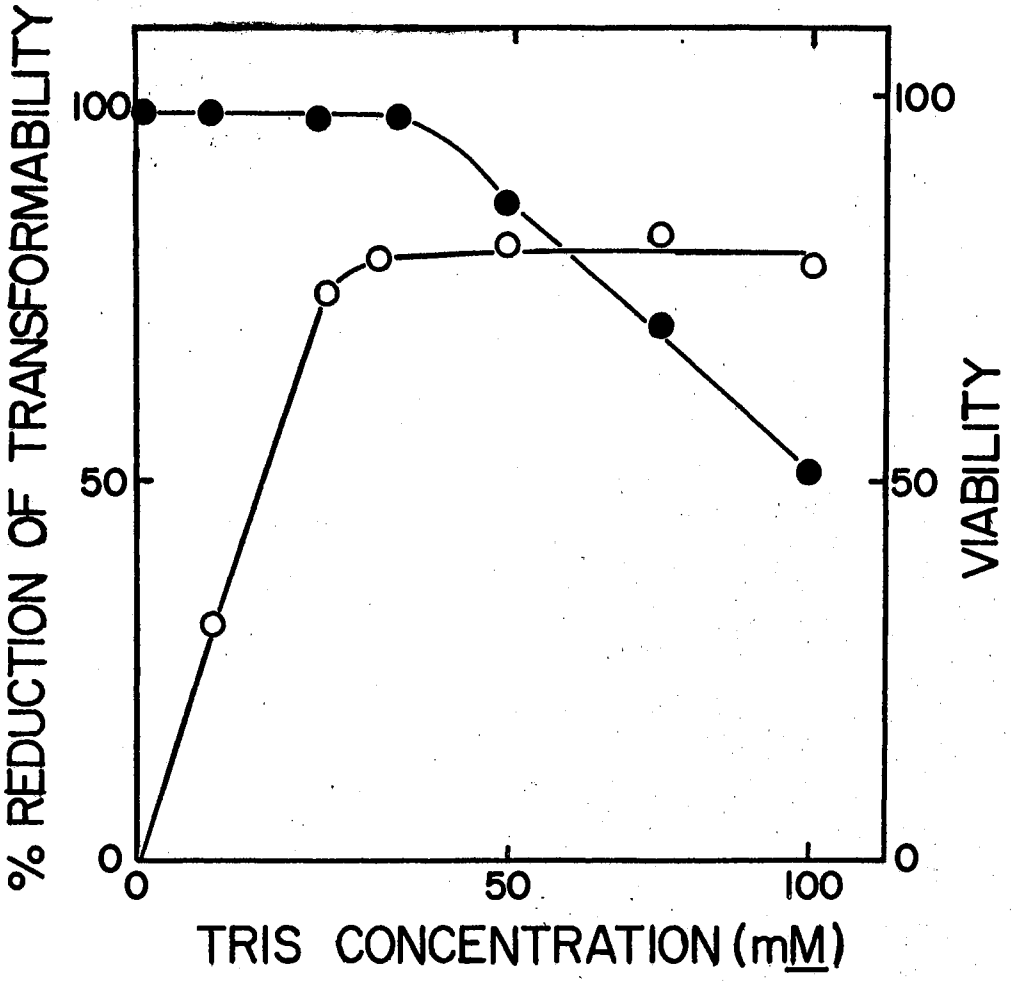

% REDUCTION OF TRANSFORMABILITY
VIABILITY
100
50
0
0
50
100
TRIS CONCENTRATION (mM)

TABLE IV

COMPARISON OF PHOSPHATE AND TRIS AS BUFFER IN SHOCK PROCEDURE

First Stage	% Reduction
Sucrose + 0.03 M Tris + EDTA	81
Sucrose + 0.1 M Phosphate + EDTA	5
Sucrose + 0.2 M Phosphate + EDTA	6
Sucrose + 0.3 M Phosphate + EDTA	4
Sucrose + 0.5 M Phosphate + EDTA	7

Competent cells were osmotically shocked using varying concentrations of potassium phosphate, pH 6.8, or Tris, pH 7.1, as indicated. All samples were incubated at 4^{o} for 10 minutes in 5×10^{-4} M $MgCl_2$, then the cells were transformed as described in the Methods Section. The per cent reduction of transformation is the number of tryptophan$^+$ cells of shocked sample/number of tryptophan$^+$ cells of unshocked culture, the whole term multiplied by 100 and then subtracted from 100.

TABLE V

DETERMINATION OF OPTIMUM TEMPERATURES FOR THE FIRST AND SECOND STAGES OF THE SHOCK PROCEDURE

Temperature		Cell Titer (10^{-7})	% Reduction
First Stage	Second Stage		
10	4	88	38
20	4	85	60
25	4	90	70
37	4	86	69
45	4	85	70
50	4	70	85
55	4	40	90
25	10	85	55
25	15	90	55
25	20	84	40
25	25	90	41

Competent cells were shocked as described in the Methods Section except for the variation in temperatures of the two solutions involved in the shock procedure. After the shock treatment the cells were transformed as described in the Methods Section. The per cent reduction is the number of tryptophan$^+$ cells of shocked culture/number of tryptophan$^+$ cells of unshocked culture, the whole term multiplied by 100 and then subtracted from 100.

Effect of DNA Uptake

There are two reactions involved in DNA uptake. First, is reversible binding of transforming DNA to the cell surface which is not energy-dependent, and the DNA can be removed either by DNase treatment or washing. The second step is an energy-dependent reaction which occurs at 30-37^{o} and, upon completion of this step, the bound DNA is not sensitive to DNase or washing.

Table VI shows that the osmotic shocking procedure exerts its effect on the second step of DNA uptake. Routinely a 70% decrease in irreversibly bound DNA occurs upon shocking. No decrease in reversible binding is observed (0^{o} values).

Effect of Osmotic Shocking of Potential Transformants

Since competent cells may have surface structural alterations, there was the possibility that potential transformants were preferentially killed by the osmotic shock treatment. The cell titer is useful only in detection of gross, non-discriminate cell killing. Preferential killing of potential transformants would go undetected due to their small number. Table VII indicates that the shock treatment does not preferentially kill potential transformants but does instead decrease transformation of competent cells.

Effect of Osmotic Shock on Permeability and Macromolecular Synthesis

Osmotic shock treatment of E. coli drastically changed the permeability to molecules normally excluded, which may be due to the presence of EDTA in the first stage (108,109,110).

The effect of osmotic shock upon the biosynthesis of the three

TABLE VI

EFFECT OF SHOCKING ON REVERSIBLE AND IRREVERSIBLE UPTAKE OF ^{3}H-DNA

Treatment	Temperature	Net CPM
Irreversible binding		
None	37	535
Shocked	37	0
Reversible binding		
None	0	298
Shocked	0	201

Cells were treated with 3 µg/ml of DNA as indicated. After 45 minutes incubation DNase (0.1 µg/ml) was added to the cell suspensions incubated at 37° and the incubation continued for 10 minutes. The cells were sedimented by centrifugation, suspended in minimal medium containing 50 µg/ml of DNase, and sedimented again. An aliquot equivalent to 2.5 ml of competent cells was counted in a liquid scintillation counter. For the cells incubated at 0° for 45 minutes the additional treatment involved sedimentation and counting. The net CPM was calculated by subtracting the counts bound by cells when the DNA was preincubated with DNase (20 µg/ml) from the counts bound by cells when DNA was not DNase treated.

TABLE VII

EFFECT OF SHOCK TREATMENT ON POTENTIAL TRANSFORMANTS

Step 1	Step 2	% Control Transformation
Transformed	None	100
None	Transformed	95
Transformed	Shocked	85
Shocked	Transformed	65

Competent cells were prepared as described in the Methods Section. Cells were exposed to DNA (5 µg/ml) for 30 minutes. The cells were sedimented and osmotically shocked as described in the Methods Section. The shocked cells were suspended in fresh transformation medium and diluted, plated, and later the transformation frequency determined and compared with the control. Concurrently, 5.0 ml of cells were osmotically shocked first as described and upon completion of the shocking procedure the cells were suspended in fresh transformation medium and exposed to DNA in the normal manner. The cells were then diluted and plated. Another aliquot of cells was utilized as an unshocked control and was transformed at step 2.

macromolecular species was determined by measuring the incorporation of radioactive precursors into cold trichloroacetic acid insoluble material. Experiments done in this manner measure the overall result of several reactions. At least two series of reactions are involved in the measurement: (1) transport (rate of accumulation) of radioactive precursors into the cell pool, and (2) the series of reactions actually resulting in the incorporation of the precursors into the respective macromolecules. Uptake of radioactive precursors was determined by measuring the radioactivity associated with cells collected by Millipore filtration. Incorporation, however, was measured by the radioactivity in the trichloroacetic acid precipitate collected in the same manner. The accumulation was calculated as the difference between the total uptake by the cells and the radioactivity incorporated into acid insoluble material.

Figures 3, 4, and 5 show the uptake, accumulation, and incorporation of ^{14}C-L-leucine, ^{3}H-uridine, and ^{3}H-thymidine in shocked and unshocked cells. Shocking had no effect upon the uptake, accumulation, or incorporation of leucine or uridine. Both the uptake and incorporation of thymidine were retarded by the shock treatment. However, this decrease could be reversed by suspending the shocked cells in supernatant solution from a competent culture.

This series of experiments was carried out to optimize the osmotic shocking conditions for reduction of transformability while retaining viability. In the next section, studies on the components of the supernatant solution and shock fluid are reported.

Figure 3. Effect of Shocking on Uptake, Accumulation and Incorporation of ^{14}C-Leucine

Competent cells were shocked and suspended in transformation medium at 37° supplemented with ^{14}C-L-leucine (0.5 μC/ml) and a 250-fold excess of L-leucine (100 mμ mole/ml). Samples of 1.0 ml were removed at the indicated times and the uptake, ●, accumulation, ⊖, and incorporation, O, was determined as described in the Methods Section.

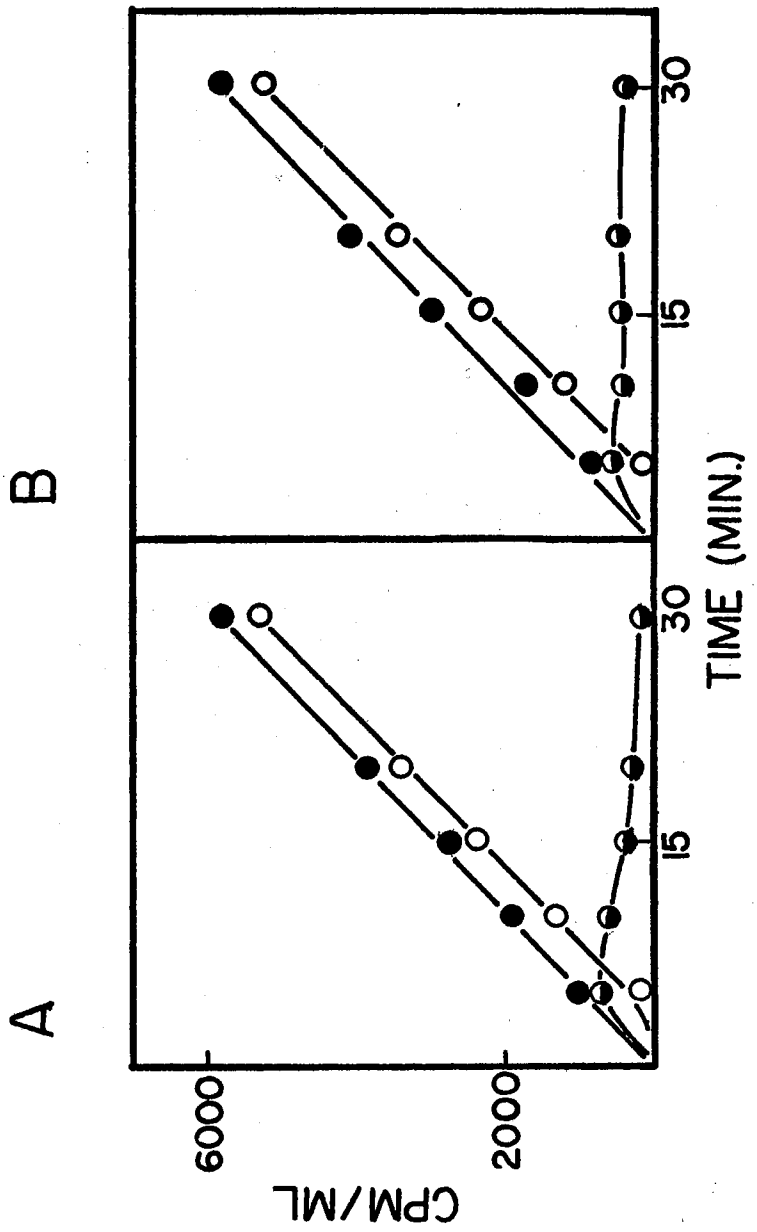

A
B
6000
2000
CPM/ML
15
30
15
30
TIME (MIN.)

Figure 4. Effect of Shocking on Uptake, Accumulation, and Incorporation of ^{3}H-Uridine

Competent cells were shocked and suspended in warm transformation medium supplemented with ^{3}H-uridine (1.0 μC/ml) plus 50 mμ mole/ml uridine. Samples of 1.0 ml were removed at the indicated times and the uptake, ●, accumulation, ⊖, and incorporation, O, were determined as described in the Methods Section for the unshocked control (A) and the shocked (B) cells.

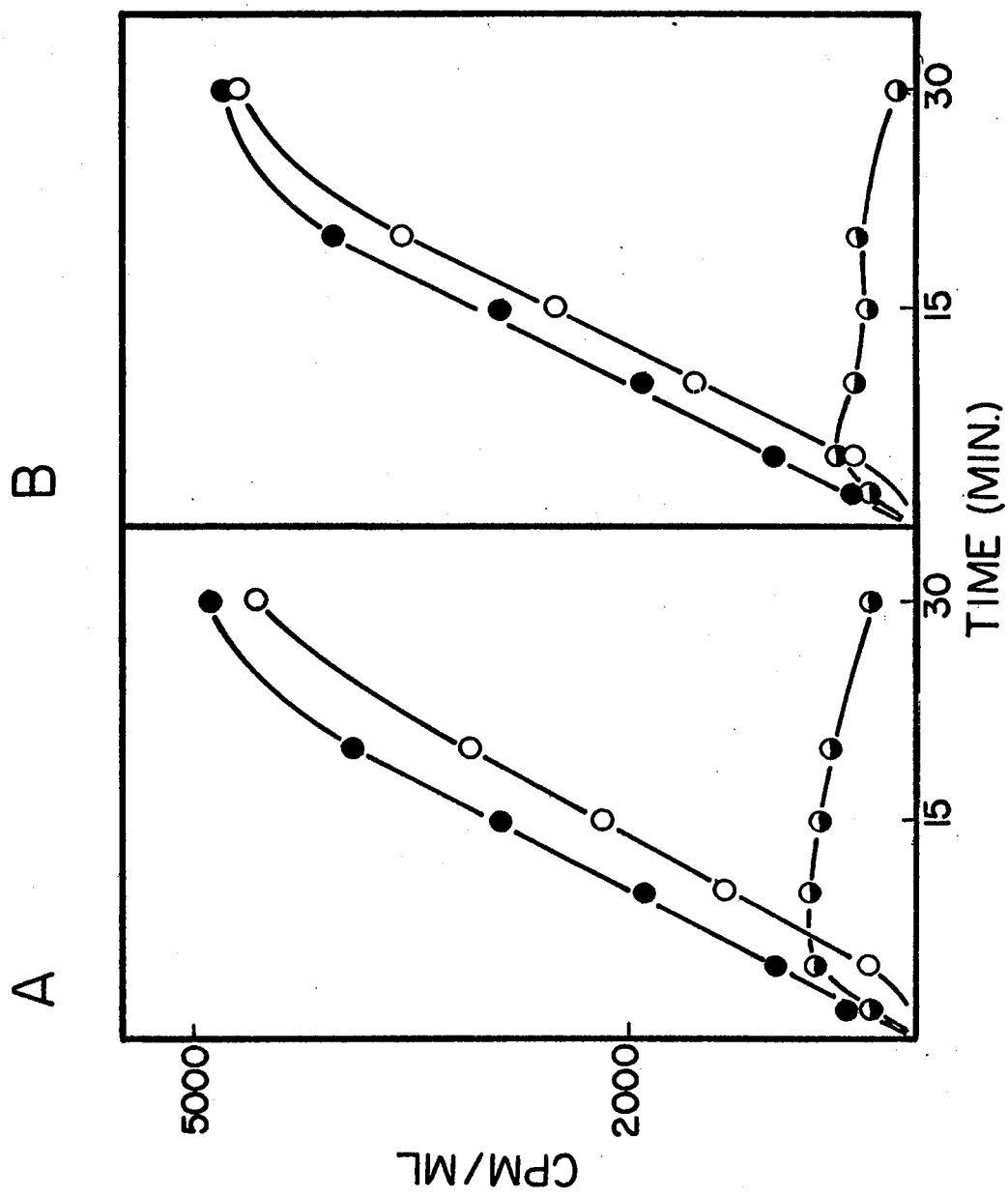
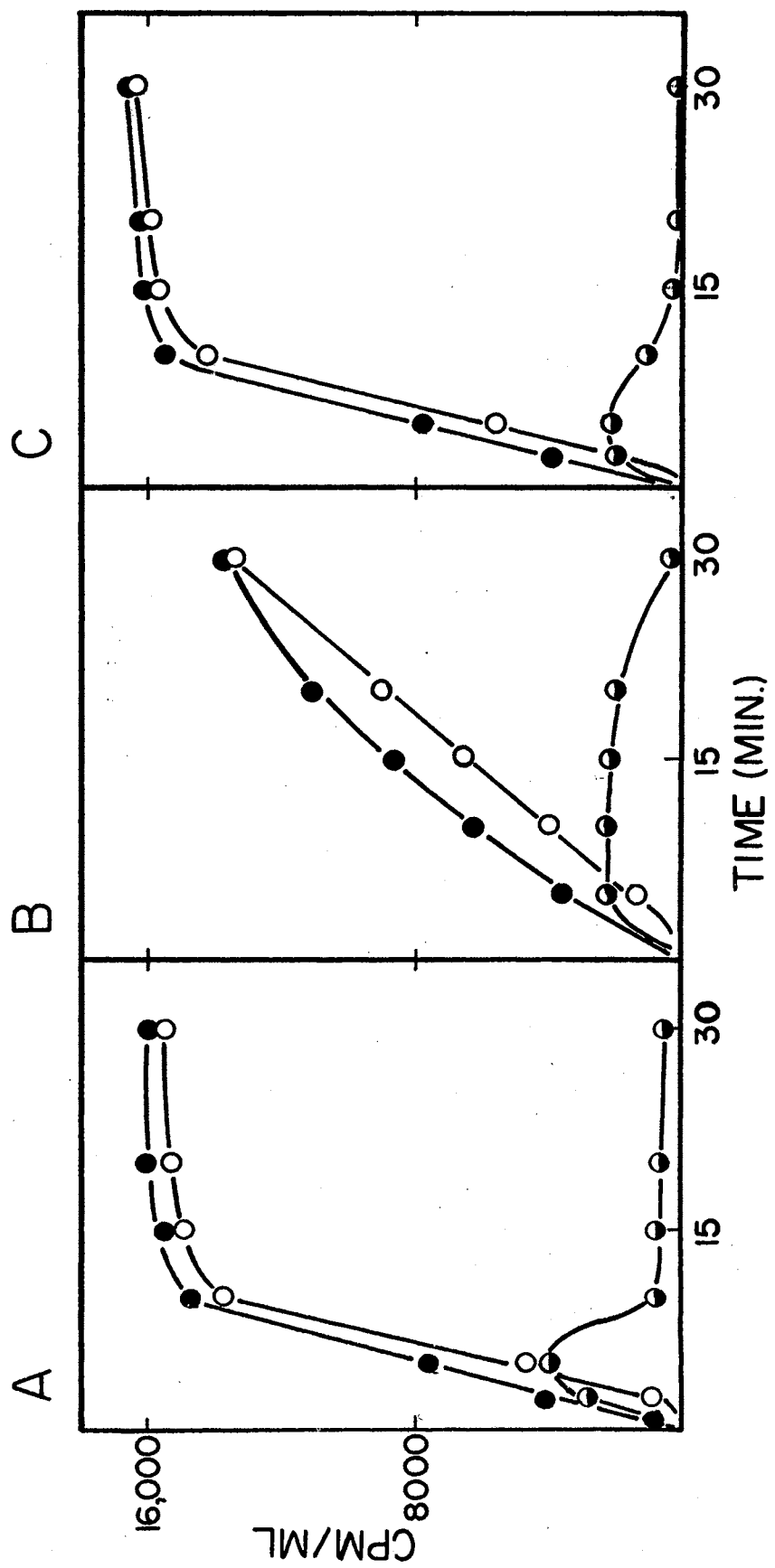

A
B
5000
2000
CPM/ML
15
30
15
30
TIME (MIN.)

Figure 5. Effect of Shocking on Uptake, Accumulation, and Incorporation of ^{3}H-Thymidine

Competent cells were shocked and suspended in either transformation medium or supernatant solution supplemented with ^{3}H-thymidine (1.0 μC/ml) and thymidine (50 mμ mole/ml). Samples of 1.0 ml were removed as indicated and the uptake, ●, accumulation, ⊖, and incorporation, O, were determined as described in the Methods Section for the unshocked control (A), shocked cells suspended in transformation medium (B), and shocked cells suspended in supernatant solution (C).


A
B
C
16,000
8000
CPM/ML
15
30
15
30
15
30
TIME (MIN.)

CHAPTER IV

STUDIES OF SUPERNATANT SOLUTION AND SHOCK FLUID OF COMPETENT CULTURES

Recovery of Transformability and DNA Uptake

Since competence factors are found in supernatant solutions of competent Streptococcus (111) and B. subtilis, the effect of supernatant solutions on osmotically shocked cells was determined.

When shocked cells are suspended in the supernatant solution and incubated for 30 minutes on a shaker at 37°, the cells recover their ability to bind DNA irreversibly and their transformability (Table VIII). The supernatant solution allows 75-100% recovery of transformability. The impaired irreversible binding of ^{3}H-DNA is almost completely reversed.

Time Course and Requirements for Recovery

Figure 6 shows the time course of recovery of transformability after shocking. When the shocked cells are incubated on a shaker at 37° under growth conditions (transformation medium), a gradual recovery of transformability proceeds after a lag of about 30 minutes with completion in three hours. However, if the shocked cells are suspended in supernatant solution, the lag period is about 15 minutes and recovery occurs more rapidly. In the presence of chloramphenicol or actinomycin D recovery does not occur. Likewise, recovery does not occur in the

TABLE VIII

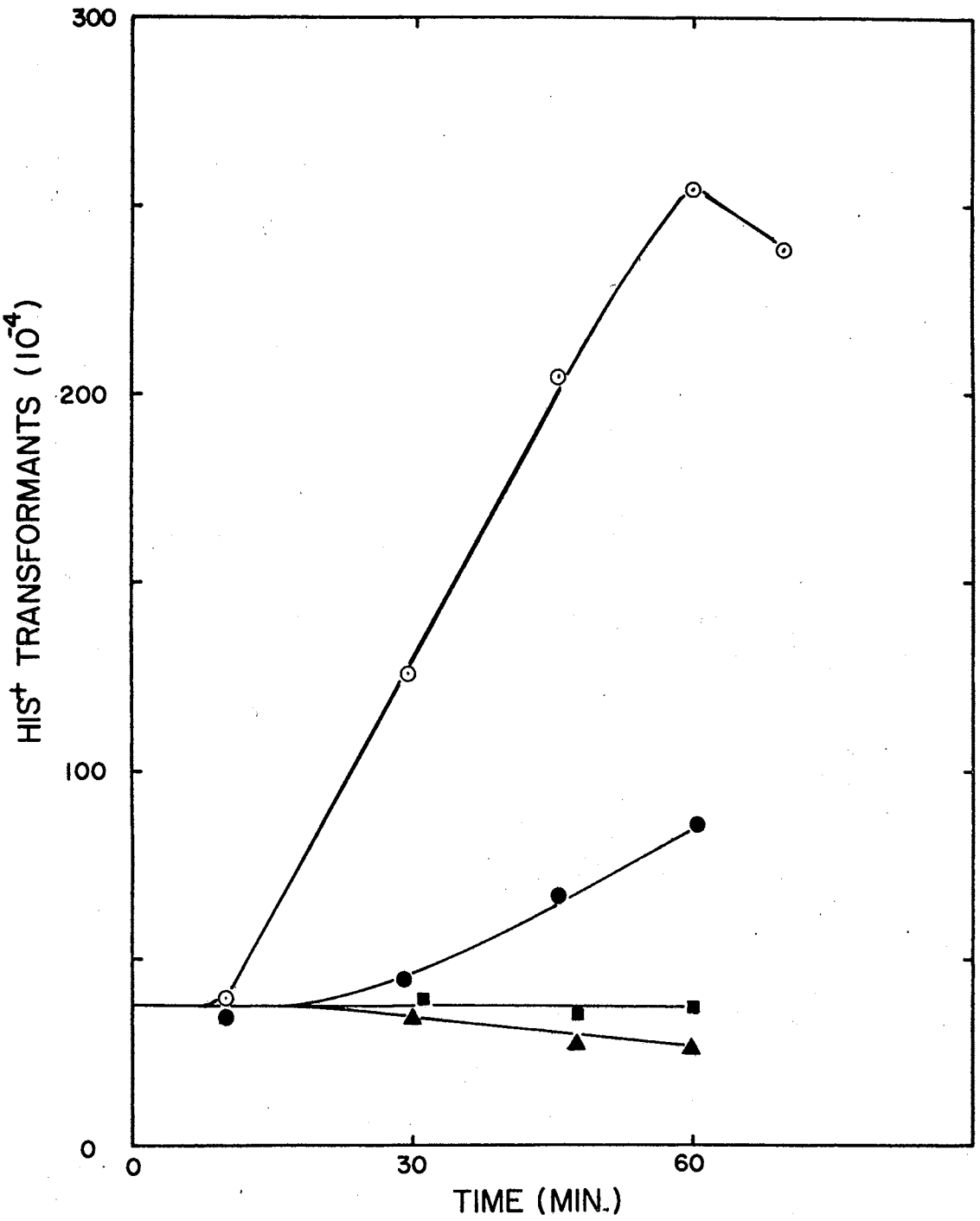
RECOVERY OF TRANSFORMABILITY OF SHOCKED CELLS AFTER SUSPENSION IN SUPERNATANT SOLUTION

Treatment	% Transformants	CPM
None	0.45	630
Shocked	0.04	302
Shocked + Supernatant Solution	0.41	510

Competent cells were osmotically shocked as described in the Methods Section. Upon completion of the shock treatment, half of the shocked cells were suspended in fresh transformation medium and the other half was suspended in the appropriate amount of supernatant solution. Unshocked cells were used as the control. An aliquot from each of the three tubes was transformed. ^{3}H-DNA (3 µg/ml) was added to 3 ml of cells from each preparation and incubated for 45 minutes. DNase (0.1 µg/ml) was added and further incubated for 10 minutes. The cells were washed twice with minimal medium containing 50 µg/ml of DNase. The cells were collected by Millipore filtration, dried, and counted in Bray's solution utilizing a liquid scintillation counter. The per cent transformation is the number of histidine^{+} cells divided by the total number of recipient cells, the whole term multiplied by 100.

Figure 6. Time Course and Requirements for Recovery of Transformability

Competent cells were shocked and suspended in either supernatant solution, O, transformation medium, ●, transformation medium plus 100 μg/ml of chloramphenicol, ■, or transformation medium plus 10 μg/ml of actinomycin D, ▲. The various samples were incubated at 37° with shaking. At the indicated times, samples were taken and transformed.


300
200
100
0
0
30
60
HIS+ TRANSFORMANTS (10-4)
TIME (MIN.)

absence of either or both essential amino acids. Shocked cells recover their transformability only under conditions which permit protein synthesis. In addition, a factor or factors present in the supernatant solution of competent cells enables rapid recovery of transformability.

Effect of Washing Competent Cells

When competent cells are washed by centrifugation at room temperature, transformability is reduced stepwise until a plateau is reached by the fourth wash (Figure 7). This suggests that a loosely bound essential factor can be removed from the cell surface by washing or more efficiently by a cold osmotic shock.

Effect of Supernatant Solution From Low Transforming Strain on Shocked Cells

Table IX shows that the supernatant solution from a 5-6 hour competent culture is much more efficient in restoring transformability in shocked cells than supernatant solution prepared from competent cells of other ages. This indicates that a surface located competence factor is released spontaneously as competence develops. In addition, when shocked cells are suspended in the supernatant solution (collected at maximum competence) of a low transforming strain, their recovery of transformability is slight (Table X). This is further evidence for the existence of a competence factor in the supernatant solution of competent B. subtilis.

Figure 7. Effect of Washing on Transformation

Competent B. subtilis SB 25 cells were sedimented by centrifugation and washed the indicated number of times with minimal medium. The cells were suspended in transformation medium and transformed as described in the Methods Section.

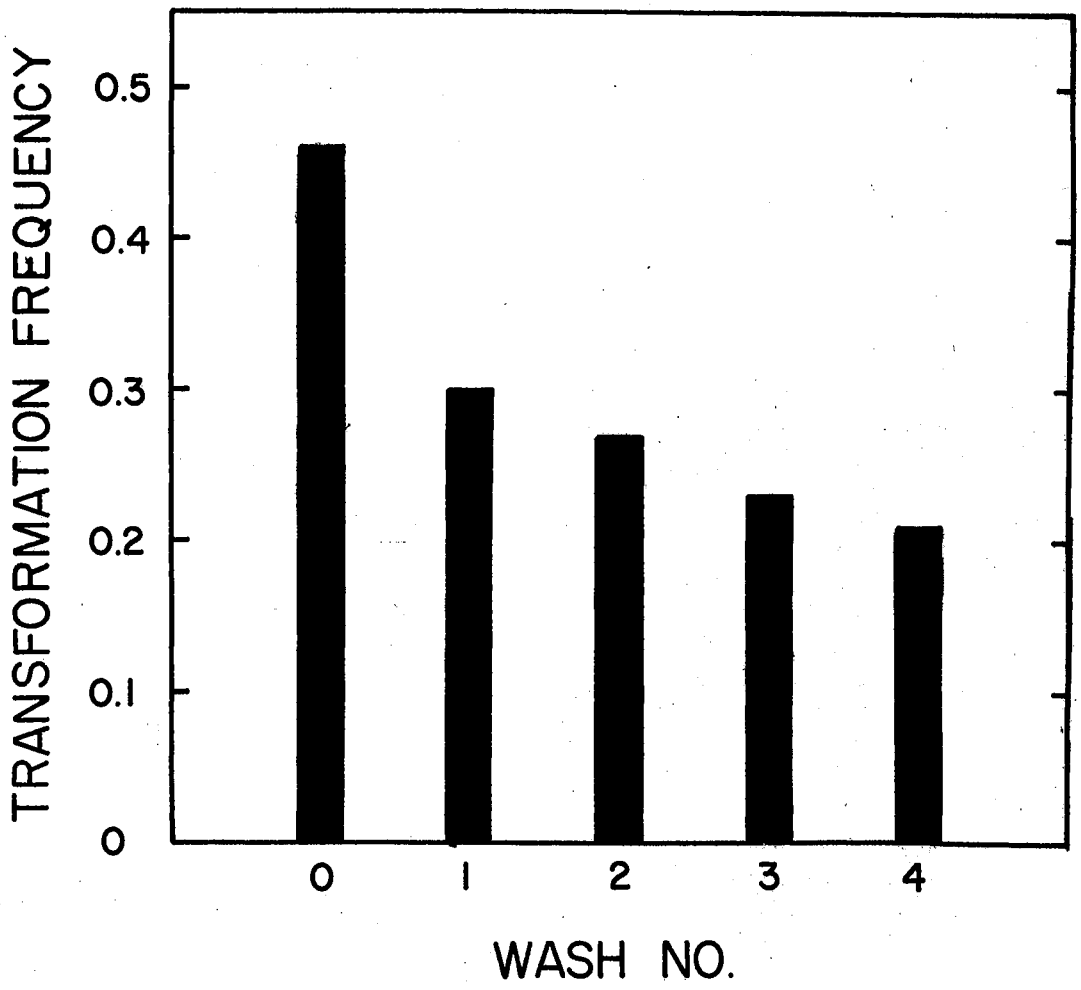

TRANSFORMATION FREQUENCY
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
WASH NO.

TABLE IX

EFFECT OF VARIOUS SUPERNATANT PREPARATIONS

Hours of Competence Development	% Unshocked Control Transformation
0 Transformation Medium	25
1	25
3	35
4	70
5	88
6	86
7	82
12	40

An overnight culture of B. subtilis SB 25 cells was used to inoculate 30 ml of transformation medium. At the indicated times of growth, aliquots were centrifuged to remove the cells and then sterilized by Millipore filtration. These various supernatant solutions were tested on shocked cells and compared to an unshocked control for restoration of transformability. One aliquot of shocked cells was suspended in transformation medium (designated 0 transformation medium) for comparison.

TABLE X

EFFECT OF SUPERNATANT SOLUTION FROM LOW TRANSFORMING STRAIN

Treatment	% Control Transformation
None	100
Shocked	24
Shocked + SB 25 supernatant solution	80
Shocked + 168 C^- supernatant solution	32

Shocked cells were suspended in either supernatant solution from B. subtilis SB 25 (high transforming strain) or supernatant solution from B. subtilis 168 C^- (low transforming strain) and incubated 30 minutes at 37^0 on a shaker and then transformed. The supernatant solution of C^- cells was obtained as described in the Methods Section.

Effect of Supernatant Solution on Phenethylalcohol (PEA) Treated Cells

Phenethylalcohol (0.05%) (112) inhibits the binding and uptake of transforming DNA by some alteration of the bacterial membrane. Table XI shows that this inhibition can be partially reversed by supernatant solution after the PEA is removed by washing.

Binding of DNA by Cell Walls of SB 25 and 168 C^-

Cell wall preparations irreversibly bind DNA. As Table XII demonstrates cell walls obtained from B. subtilis SB 25 (high transforming) bind about twice as much ^{3}H-DNA as a cell wall preparation of B. subtilis 168 C^- (low transforming). Drastic reductions in bound DNA resulted if the cell walls were treated with trypsin, lysozyme, or heated before the addition of ^{3}H-DNA.

Chemical Composition of Supernatant Solution

The supernatant solution was examined for the presence or absence of basic classes of chemical compounds. Table XIII shows that the supernatant solution contained negligible amounts of ether-extractable material (lipid). Protein (20 µg/ml) as determined by Lowry (88), and material absorbing at 260 nm were found in substantial amounts. Burton's (79) method and the orcinol reaction (93) were used to measure DNA and RNA concentrations respectively. Carbohydrate was present but could be accounted for by the glucose present in the medium. No glycoprotein was detected in polyacrylamide gel electrophoresis. The supernatant solution does not change the thermal transition curve of transforming DNA.

TABLE XI

REVERSAL OF PHENETHYLALCOHOL INHIBITION BY SUPERNATANT SOLUTION

Treatment	% Control Transformation	CPM
None	100	873
0.05% Phenethylalcohol, then transformation medium	31	406
0.05% Phenethylalcohol, then supernatant solution	60	619

Phenethylalcohol (0.05% final concentration) was added 1 hour before maximum competence to B. subtilis SB 25 cells. After 1 hour incubation at 37° the cells were washed twice with minimal medium. Then one portion was suspended in transformation medium and the other was suspended in supernatant solution. DNA was added after 30 minutes incubation at 37° and the transformation frequency was compared to an untreated control. The irreversible uptake of ^{3}H-DNA was determined as described in the Methods Section.

TABLE XII

BINDING OF ^{3}H-DNA TO CELL WALLS OF SB 25 AND 168 C^{-}

Cell Walls Source	Treatment	CPM
SB 25	None	5431
168 C^{-}	None	2653
SB 25	Trypsin (100 µg/ml)	2150
SB 25	Lysozyme (100 µg/ml)	2474
SB 25	100^{o} for 30 minutes	2363

After 5 hours growth in transformation medium 5 liters of each 168 C^{-} and SB 25, were collected by centrifugation in a Sharples Super Centrifuge. Cell walls were prepared by use of an X-press. The cell wall preparations were washed 8 times with 0.9% NaCl. The washed preparations were lyophilized for storage. The cell walls were suspended at a concentration of 25 µg/ml in 0.9% NaCl. They were incubated with ^{3}H-DNA (10 µg/ml) for 15 minutes at 37^{o} on a shaker. DNase (100 µg/ml) was added and the incubation continued for 20 minutes. Then 1.0 ml aliquots were collected by Millipore filtration and washed with 15 ml of 0.9% NaCl. The filter was dried and counted in Bray's solution in a liquid scintillation counter. As indicated by the above treatment the cell walls were incubated with the appropriate enzyme for 20 minutes prior to the addition of DNA.

TABLE XIII

SUBSTANCES DETECTED IN SUPERNATANT SOLUTION

Substance Tested for	Amount per ml
Carbohydrate other than Glucose	0
Ether-Extractable Material	2 µg
Protein	20 µg
DNA	0
RNA	0
260 nm Material	A_{260} = 0.46

Competent cells were sedimented and the supernatant solution was sterilized by Millipore filtration and tested for indicated substances. The carbohydrate content was determined by Nelson's method (111) which detects reducing sugars. Since transformation medium contains glucose (5 mg/ml), the Glucostat test (112) was also used. All the reducing sugar present was glucose. When the supernatant solution was chromatographed in isopropanol: acetic acid: H_2O (3:1:1) on Whatman No. 1, dried and then sprayed with 0.5% $NaIO_4$, and after 5 minutes at room temperature sprayed with 0.5% benzidine, only one spot corresponding to glucose was found. An aliquot of supernatant solution was extracted with ether. The ether soluble portion was transferred to a tared vial and evaporated. The protein, DNA, and RNA concentrations were determined by Lowry's method, Burton's method, and the orcinol test, respectively. The absorbance of the material at 260 nm was determined by using a Gilford recording spectrophotometer.

The supernatant solution was examined next for various enzymatic activities.

Enzymatic Composition of Supernatant Solution

Table XIV indicates that no DNase activity was detectable by the method of DeWaard and Lehman (90). Likewise, the proteolytic assay of Rinderknecht, Geokas, Silverman, and Haverback (92) demonstrated the absence of any proteolytic activity. However, proteolytic activity could be demonstrated in the supernatant solution of stationary cultures.

The supernatant solution was investigated for autolytic activity using cell walls prepared as described by Young (22) except that the cells were broken by the use of an X-press. The method of Akrigg and Ayad (75) was used for detection of autolytic activity. No discernable autolytic activity was present in the supernatant solution (Figure 8). The supernatant solution did not enhance nor inhibit the normal autolytic activity associated with the cell walls. This suggests that the factor or factors present in the supernatant solution, which is responsible for restoration of transformability in shocked cells is not the autolytic enzyme.

Characterization of Supernatant Solution

The effect of temperature on the supernatant factor is shown in Table XV. The factor loses activity rapidly at temperatures above 80^{o}.

The stability of the supernatant factor to various pH treatments is shown in Table XVI. Table XVII demonstrates how various treatments affect the supernatant factor. The supernatant factor was removed or destroyed by proteolytic enzymes, activated charcoal, calcium phosphate

TABLE XIV

DNase ACTIVITY OF SUPERNATANT SOLUTION

Incubated With DNA	% Solubilized
None	1.6
DNase	66.5
Supernatant Solution	1.6

The nuclease content of the supernatant solution from competent cells was determined by DeWaard and Lehman's method (90). A DNase concentration of 20 µg/ml and an aliquot of supernatant solution containing 20 µg/ml protein were used. After incubating for 30 minutes at 37°, the reaction was stopped by the addition of carrier DNA (5 mg/ml and $HClO_4$ (5% final concentration). After 5 minutes at 0° the suspension was centrifuged at 10,000 x g for 3 minutes. An aliquot of the supernatant was placed in a vial with 10 ml of Bray's solution. A liquid scintillation counter was used for counting the samples.

Figure 8. Effect of Supernatant Solution on Autolysis of SB 25 Cell Walls

Cell walls were prepared as described in the Method Section. The cell walls were suspended in 0.1 M phosphate buffer, pH 8.3. The native autolytic enzyme present in the cell wall was inactivated by heating at 80° for 30 minutes and used as a control, ●. Autolytic activity of the supernatant solution was determined by adding supernatant solution to the heated cell walls, O. A sample of untreated cell walls showed autolytic activity, △. Supernatant solution was added to unheated cell walls, □, to determine if the supernatant solution contained any substance which inhibited or enhanced native autolytic activity. The turbidity (A_{600}) of the samples was measured at the times indicated.

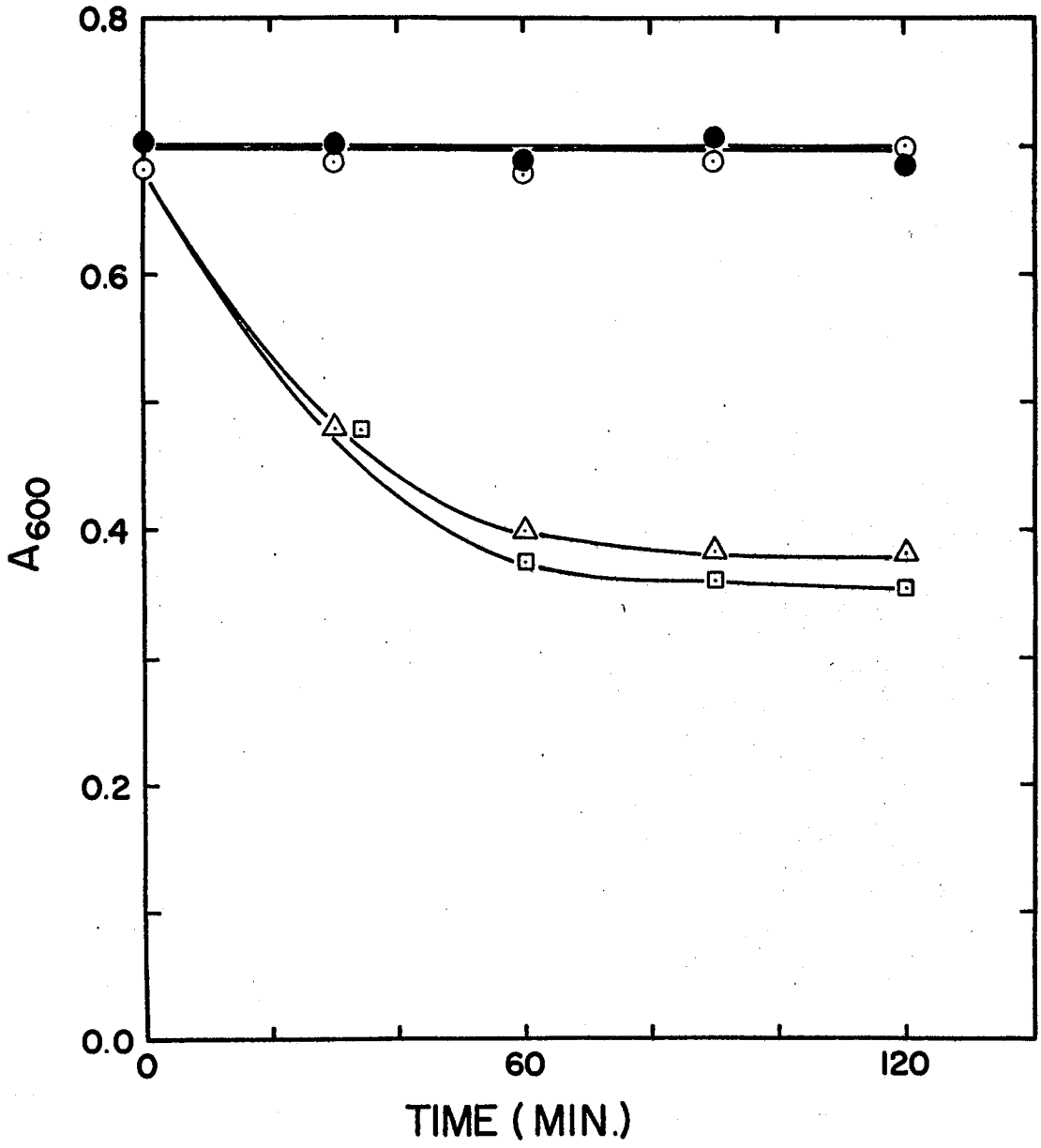

0.8
0.6
0.4
0.2
0.0
A600
0
60
120
TIME (MIN.)

TABLE XV

HEAT STABILITY OF SUPERNATANT FACTOR

	Treatment	Prior Temperature of Supernatant Solution	% Control Transformation
1.	None		100
2.	Shocked		24
3.	Shocked then Supernatant	37	80
4.	Shocked then Superantant	45	78
5.	Shocked then Supernatant	55	65
6.	Shocked then Supernatant	60	60
7.	Shocked then Supernatant	80	55
8.	Shocked then Supernatant	90	30
9.	Shocked then Supernatant	100	26

Competent cells were shocked. Aliquots of the supernatant solution were heated to the indicated temperatures for 30 minutes and equilibrated to 37^{o} before suspending the shocked cells in them. Unshocked cells were used as a control (line 1). A portion of the shocked cells was suspended in transformation medium to determine the efficiency of the shock treatment (line 2).

TABLE XVI

pH STABILITY OF SUPERNATANT SOLUTION FACTOR

pH of Treatment	% Control Transformation
8.0	40
7.5	80
6.9	90
6.5	88
6.0	60

The pH of 5 ml samples of supernatant solution was adjusted to the indicated pH by addition of HCl or NH_4OH. The pH was adjusted to pH 6.9 after 15 minutes incubation at room temperature. These treated supernatant samples were used to suspend the shocked cells. The per cent control was calculated as the number of tryptophan$^+$ transformants in the indicated samples divided by the number of tryptophan$^+$ transformants in the unshocked control, the whole term multiplied by 100.

TABLE XVII

CHARACTERIZATION OF THE SUPERNATANT SOLUTION FACTOR

Treatment	Supernatant Solution Treatment	% Control Transformation
None		100
Shocked		22
Shocked + Supernatant Solution	None	83
Shocked + Supernatant Solution	Charcoal	29
Shocked + Supernatant Solution	Pronase	21
Shocked + Supernatant Solution	Trypsin	13
Shocked + Supernatant Solution	Papain	8
Shocked + Supernatant Solution	RNase	76
Shocked + Supernatant Solution	$Ca_3(PO_4)_2$	18
Shocked + Supernatant Solution	Dowex 50	24
Shocked + Supernatant Solution	Dowex 1	75

Various aliquots of the supernatant solution were treated prior to suspension of shocked cells which had been collected by centrifugation. Charcoal (8 mg/ml) was incubated for 10 minutes at 37° with shaking. Pronase (100 µg/ml) was incubated for 10 minutes at 37°. Trypsin, papain and RNase were incubated in the same way. Calcium phosphate gel (10 mg/ml) was incubated for 10 minutes at 37° with shaking. The two Dowex resins (10 mg/ml) were washed with water until the wash water was pH 7.0 and then incubated at room temperature for 10 minutes with the supernatant solution. In each case the supernatant solution was obtained again by centrifugation, adjusted to pH 7.0, if required, and sterilized by Millipore filtration prior to testing its ability to restore transformability to shocked cells.

gel, cation exchangers, and dialysis. Anion exchangers had no effect which indicates the factor bears a net positive charge at physiological pH. Papain treatment resulted in the greatest reduction indicating that the factor is a protein. In each instance there was not a significant decrease in cell viability suggesting that the decrease in transformability is related to a specific inactivation of the supernatant factor and not merely a modification of the properties of the supernatant solution which promoted killing of the cells.

Supernatant solution can be concentrated five to twenty fold by either perevaporation or rotary evaporation but loses activity if lyophilization is employed. The recovery of transformability by the supernatant factor is concentration dependent. Table XVIII shows that maximum transformability in shocked cells is achieved by an optimum amount of concentrated supernatant. About 60 µg protein per ml gives the best result. Supernatant solution is routinely concentrated ten fold, and about 0.3 ml of this preparation is required per ml of cells for maximum restoration of transformability.

Polyacrylamide gel electrophoresis of concentrated supernatant revealed ten protein bands (Figure 9).

Purification of Supernatant Factor

The concentrated supernatant solution was subjected to fractionation on a size basis utilizing a Sephadex G-25 column (1.2 cm x 30 cm) equilibrated with 100 mM phosphate buffer, pH 7.0. The elution pattern shown in Figure 10 has two well separated peaks with two minor components and a shoulder on the first major peak. The major peak occurring at fraction 39 was L-tryptophan as determined by its ultraviolet

TABLE XVIII

VARIOUS CONCENTRATIONS OF SUPERNATANT SOLUTION

Treatment	% Control Transformation
None	100
Shocked	10
Shocked then Supernatant Solution	90
Shocked then 0.1 ml Concentrated Supernatant Solution	78
Shocked then 0.5 ml Concentrated Supernatant Solution	113
Shocked then 1.0 ml Concentrated Supernatant Solution	21
Shocked then 2.0 ml Concentrated Supernatant Solution	0
Shocked then 2.5 ml Concentrated Supernatant Solution	0

Aliquots of shocked cells were suspended in the indicated amounts of ten-fold concentrated supernatant solution and enough transformation medium was added to make the final volume 5.0 ml. The suspended cells were incubated at 37° for 30 minutes and then the DNA was added. The concentrated supernatant was prepared by rotary evaporation of crude supernatant solution followed by Millipore filtration prior to storage. An aliquot of shocked cells was suspended in unconcentrated supernatant for comparison.

Figure 9. Disc Gel Electrohporesis of Supernatant Solution

Supernatant solution was concentrated 15-fold and 0.1 ml was layered on the stacking gel. The separating gel was the standard 7% polyacrylamide gel, pH 9.5, and the electrophoresis was performed as described in the Canalco Manual (113,114,115). Amido black was the protein stain employed.

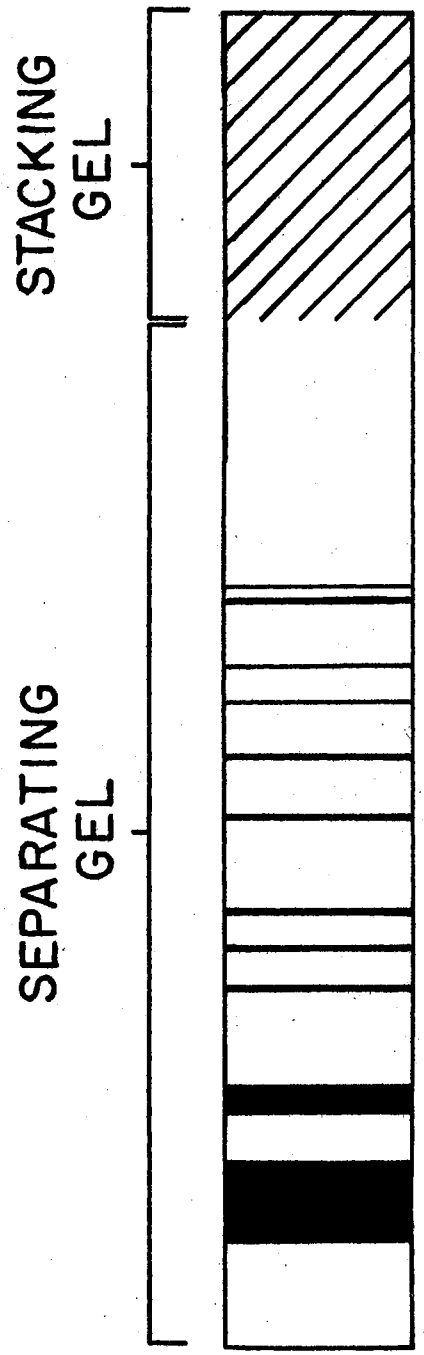
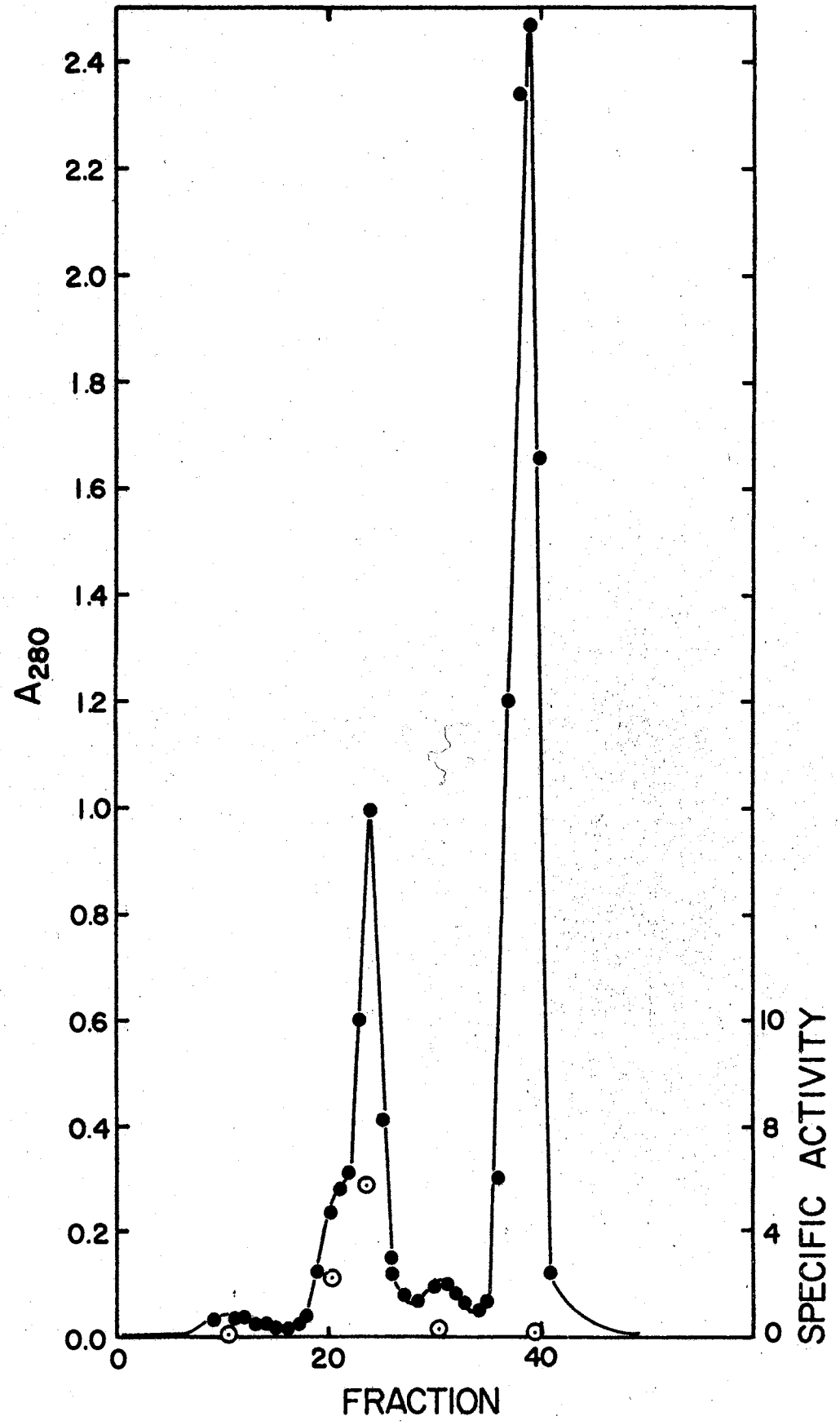

STACKING GEL
SEPARATING GEL

Figure 10. Sephadex G-25 Chromatography of Supernatant Solution

A column (1.2 x 30 cm) of Sephadex G-25 was equilibrated at 4° with 100 mM potassium phosphate buffer, pH 7.0. A 0.7 ml sample of concentrated supernatant solution (15-fold) was carefully layered on the surface of the gel. Fractions of 1.0 ml were collected by using a Gilson Fraction Collector. Various fractions were added to shocked cells. After 30 minutes incubation the cells were transformed and the specific activity was determined as described in the Methods Section. The open circles represent the specific activity of the indicated fractions determined as described in the Methods Section.


2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A_{280}
0
20
40
FRACTION
10
8
4
0
SPECIFIC ACTIVITY

spectrum, its elution pattern, and by paper chromatography. The only fractions containing competence factor activity were 23-25. Various combinations of fractions did not increase activity. There was 260 nm absorbing material at fraction 30 but little protein as determined by the Lowry method.

Even though fractions 23-25 would restore transformability to shocked cells, they did not increase transformability as well as unfractionated supernatant. Various combinations of different fractions did not result in increased transformability. In addition, the specific activity of fractions 23-25 was 6.0 as compared to supernatant solution which had a specific activity of 11.0. Various gels with different exclusion limits and properties were used, but no further fractionation occurred.

Fractionation by charge differences utilizing ion exchange materials was attempted. Carboxymethyl cellulose chromatography was utilized, because it removed the supernatant factor in batch experiments. The carboxymethyl cellulose column (1.2 cm x 30 cm) yielded three protein peaks when eluted with a linear gradient of NaCl (0-0.5 M). However, peak 2 contained the only restoring activity, but the specific activity was less than that of crude supernatant solution.

Protein precipitating with 45% ammonium sulfate had a specific activity of 7.0 while the crude supernatant factor had a specific activity of 11.0, showing that no purification was obtained. The polyacrylamide gel electrophoresis pattern was very similar to that of the unfractionated supernatant solution.

Various stabilizing agents such as mercaptoethanol and glycerol were employed during purification steps, but no difference resulted.

Effect of Shock Fluid on Cells With Reduced Transformability

Five liters of cells at maximum competence were shocked, and the shock fluid was prepared as described in the Methods Section and examined for the presence of the competence factor.

Ten milligrams of lyophilized shock fluid were dissolved per ml of 50 mM potassium phosphate buffer, pH 6.9. The amount of protein in the dissolved shock fluid varied with preparation from 2-3 mg per ml.

Various amounts of shock fluid were added to shocked cells in an attempt to restore transformability, but little increase was found. However, when shock fluid was diluted in supernatant solution, transformability of shocked cells was increased. Table XIX shows that the increased transformability was slightly higher than achieved by supernatant solution alone. The irreversible binding of DNA was also enhanced by this procedure. An optimum amount of shock fluid was required for maximum recovery, and reduction of binding of DNA and transformation occurred when shock fluid exceeded a certain level. These results suggest that at least two different factors occurring in the supernatant solution and shock fluid of competence cells are required for the most rapid recovery of transformability of shocked cells.

Effect on Phenethylalcohol Treated Cells

Shock fluid restores transformation to phenethylalcohol treated cells in a similar manner as supernatant solution (Table XX). No significant increase over that with shock fluid alone is achieved when both the supernatant solution and shock fluid are added to phenethylalcohol treated cells. Table XXI shows that neither the shock fluid nor the supernatant solution will restore transformability to cells

TABLE XIX

EFFECT OF SHOCK FLUID AND SUPERNATANT SOLUTION ON SHOCKED CELLS

Treatment	Addition	% Transformation
1. None	None	0.26
2. Shocked		0.12
3. Shocked	Supernatant Solution	0.20
4. Shocked	Shock Fluid	0.15
5. Shocked	Supernatant Solution + Shock Fluid	0.24

Competent B. subtilis SB 25 cells were shocked and suspended in either transformation medium (line 2), supernatant solution (line 3), transformation medium plus 75 µl shock fluid per ml of cells (line 4), or supernatant solution plus 75 µl shock fluid per ml of cells (line 5). The cells were incubated and transformed as described in the Methods Section.

TABLE XX

REVERSAL OF PHENETHYLALCOHOL INHIBITION

	Treatment	% Control Transformation	CPM Bound
1.	None	100	830
2.	0.05% Phenethylalcohol	35	367
3.	0.05% Phenethylalcohol, then Shock Fluid	75	640
4.	0.05% Phenethylalcohol, then Supernatant Solution	65	593
5.	0.05% Phenethylalcohol, then Shock Fluid + Supernatant Solution	77	622

Phenethylalcohol was added to competent cells at a final concentration of 0.05% one hour prior to maximum competence. After one hour incubation, the cells were then washed twice with minimal medium, sedimented and suspended in either transformation medium (lines 2, 3) or supernatant solution (lines 4, 5). To one aliquot of cells suspended in transformation (line 3) medium and to one aliquot suspended in supernatant solution (line 5) was added 50 µl of shock fluid (3 mg protein per ml). The cells were incubated for 30 minutes and then one aliquot from each reaction tube was subjected to transforming DNA while another aliquot was incubated with ^{3}H-DNA and for 45 minutes at 37°. All samples were treated with DNase (10 µg/ml) for 10 minutes at 37° and then the transformants and ^{3}H-DNA bound were determined as described in the Methods Section.

TABLE XXI

EFFECT OF TIME OF ADDITION OF PHENETHYLALCOHOL

Time of Addition (Before Maximum Competence) Hours	Treatment		
	None	Shock Fluid % Control Transformation	Supernatant Solution
3	34	33	30
2	34	36	31
1	38	80	60
0.5	53	90	75

B. subtilis SB 25 cells were inoculated into the competence developing transformation medium. At the indicated times prior to maximum competence phenethylalcohol (0.05%) was added to the individual samples. At 0 time (5 hours after inoculation) the cells were sedimented, washed twice with minimal medium, and then suspended in transformation medium, supernatant solution or shock fluid (50 µl/ml of cells). The cells were incubated 20 minutes at 37° and then transformed.

incubated with phenethylalcohol for two hours or longer.

Effect on Low Transforming Strain

To explore further the properties of the shock fluid, its effect on a low transforming strain, B. subtilis 168 C^-, was determined. Table XXII shows that a 30 minute incubation period produces a 20-fold increase in transformation frequency. Also evident is that there is an optimum amount of shock fluid that produces maximum transformation. This table demonstrates that a factor or factors is/are released in the shock fluid of a high competent strain which is active in inducing the transformability of a low transforming strain.

The lower the natural transformability of the C^- cells the more pronounced is the enhancement by shock fluid. That is, the extent of increased transformability produced by the shock fluid is proportional to the lack of competence.

Establishment of Optimum Experimental Conditions

The enhancement of transformation in 168 C^- strain by shock fluid was the assay for competence factor activity. This was the simplest and most reliable method.

Table XXIII demonstrates that maximum enhancement of transformation occurred within 30 minutes after addition of shock fluid. As mentioned earlier, to achieve maximum transformation a particular amount of shock fluid must be added. Once the optimum was determined, it remained constant for that particular shock fluid preparation, but it may vary from preparation to preparation. Usually a protein concentration of 200-300 μg per milliliter of cells yielded maximum transformation.

TABLE XXII

EFFECT OF SHOCK FLUID ON C^- CELLS

Volume of Shock Fluid Added (µl/ml of cells)	% Try^+ Transformation
None	0.01
10	0.03
25	0.16
50	0.20
75	0.13
100	0.09

Lyophilized shock fluid was diluted to a concentration of 10 mg/ml in 50 mM potassium phosphate buffer, pH 6.9. Then the indicated amount was added to 1 ml of C^- cells grown 5 hours in transformation medium. The cells were then incubated at 37° on a shaker for 30 minutes and the transformation frequency determined.

TABLE XXIII

INCUBATION TIME

Time of Incubation (minutes)	Histidine$^+$ Transformants (10^{-4})	Titer (10^{-7})
0	80	80
10	90	81
20	152	88
30	167	80
40	170	90
60	157	91

B. subtilis 168 C$^-$ cells were prepared exactly as the high transforming strain, B. subtilis SB 25. After 5 hours growth in the transformation solution the cells were centrifuged and suspended in fresh transformation medium and 50 µl of shock fluid containing 3 mg/ml of protein was added per ml of cell suspension. At the times indicated samples were removed and transformed.

Table XXIV shows the effects of varying the incubation temperature. There was a slight decrease in stimulation when shock fluid was incubated with C^- cells at 4^o. These results suggest that a biosynthetic step is not required for activation.

The effect of adding shock fluid at different times during the five hours of competence development is shown in Table XXV. No significant stimulation occurs except during a period from one hour before maximum competence is reached through 1.5 hours after the peak of competence. This represents a 2.5 hour time period during which the cells are susceptible to the shock fluid treatment. If shock fluid is incubated with the cells for the usual five hours of competence development, transformation is decreased 95 per cent.

Characterization of Shock Fluid

The shock fluid was characterized by the methods used for the supernatant solution. No reducing carbohydrate was detected by the Benedict test (116). When the shock fluid was chromatographed on paper and sprayed with benzidine-periodate, no carbohydrate was found. Again ether-extractable material (lipid) was negligible. Protein, DNA, and RNA content was determined as previously described. As Table XXVI demonstrates, only trace amounts of DNA and RNA were detected, while the protein content was 200-300 μg per milligram of lyophilized shock fluid.

Enzymatic Composition of Shock Fluid

The shock fluid had no protease activity. When DNA was incubated with shock fluid, no appreciable DNase activity was detected as followed

TABLE XXIV

TEMPERATURE OF INCUBATION

Temperature of Incubation	Histidine$^+$ Transformants (10^{-3})	Titer (10^{-7})
4°	73	75
15	126	80
20	131	77
37	130	83
37 No Shock Fluid	53	83
45	115	81
50	0	20

C^- cells were sedimented after 5 hours growth in transformation medium and suspended in transformation medium and 50 µl of shock fluid containing 3 mg/ml of protein were added per ml of cell suspension. All samples were incubated 30 minutes at the indicated temperature before 5 µg/ml of DNA was added, and the incubation was continued 30 minutes at the indicated temperatures before diluting and plating.

TABLE XXV

TIME OF ADDITION OF SHOCK FLUID TO C^- CELLS

Time Relative to Inoculation Into Medium (hours)	Histidine$^+$ Transformants (10^{-2})	Titer (10^{-7})
0	1	20
1	3	40
2	15	63
3	30	90
4	76	95
5	117	100
5 No Shock Fluid	60	100
6	112	98
7	94	102

At the times indicated C^- cells were sedimented, then suspended in transformation medium plus 50 µl shock fluid per ml of cell suspension. After 30 minutes incubation the cells were transformed.

TABLE XXVI

SUBSTANCES PRESENT IN SHOCK FLUID

Substance	Amount per ml
Carbohydrate	0
Ether-Extractable Material	0
Protein	3 mg
DNA	0
RNA	0
260 nm Material	A_{260} = 4.5

Lyophilized shock fluid, prepared as described in the Methods Section, was dissolved in 50 mM potassium phosphate buffer, pH 6.9, at a concentration of 10 mg/ml. The presence of the substances indicated was determined as described in the Methods Section.

by the release of acid precipitable material (Table XXVII). No acid-soluble oligonucleotides were released when yeast RNA was incubated in the presence of shock fluid (91).

Figure 11 demonstrates that shock fluid neither contained autolytic activity nor did it affect the normal autolytic activity associated with cell walls.

Stability of Shock Fluid

The factor in the shock fluid was stable from pH 6.0 to 7.5. Table XXVIII shows that a considerable loss of activity occurred under more acidic or basic conditions. This loss of activity due to pH alterations was irreversible.

The stability of the factor over a wide range of temperatures was determined. As Table XXIX indicates the factor was stable from 0° to 45°, but activity was lost at higher temperatures. The factor was stable in solution for at least two weeks at 4° and was stable for at least several months as the lyophilized powder.

Characterization by Enzymatic Treatments

Competence factor activity was destroyed by trypsin, pronase, and papain, but RNase had no effect (Table XXX). This suggests the factor is polypeptide in nature.

Complete loss of activity occurred upon dialysis, and reconstitution of the nondialyzable material and the dialysate was unsuccessful.

To determine if the shock fluid interacted with transforming DNA, the thermal transition curve was determined in the presence and absence of shock fluid. There was no change in the curve.

TABLE XXVII

DNase ACTIVITY OF THE SHOCK FLUID

Incubated With DNA	% Solubilized
None	1.6
DNase	66.5
Shock Fluid	1.8

The nuclease activity of the shock fluid was determined as described in the Methods Section using 66 µg of shock fluid protein per ml of the reaction mixture. The DNase treatment utilized 20 µg/ml DNase.

Figure 11. Effect of Shock Fluid on Autolysis of SB 25 Cell Walls

The autolytic content of shock fluid and its effects on native autolytic activity was determined as described in Figure 8 except that shock fluid was substituted for the supernatant solution. A sample of cell walls were heated to 80° to inactivate the native autolytic activity, ●. Autolytic activity of the shock fluid was determined by adding shock fluid to the heated cell walls, O. A sample of untreated cell walls, showed autolytic activity, Δ. Shock fluid was added to untreated cell walls, □.

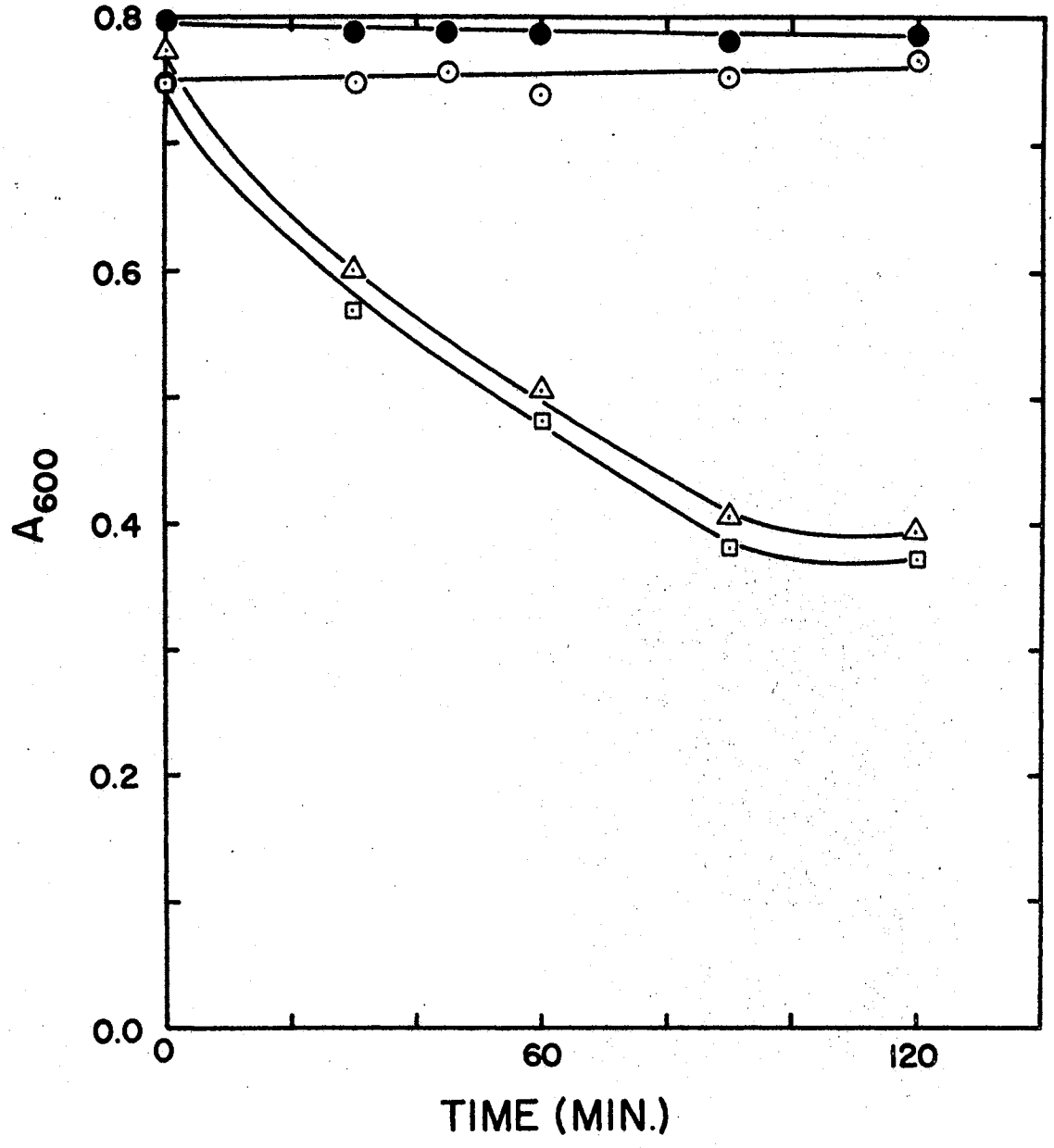

0.8
0.6
0.4
0.2
0.0
A_{600}
0
60
120
TIME (MIN.)

TABLE XXVIII

pH STABILITY OF SHOCK FLUID FACTOR

pH	His^+ Transformants (10^{-3})	Titer (10^{-7})
8.0	10	75
7.5	175	80
6.9	231	73
6.5	206	80
5.5	0	72

Samples of shock fluid were adjusted with HCl or NH_4OH to the indicated pH values. After 15 minutes incubation at room temperature, the samples were adjusted to pH 6.9. The samples of shock fluid were then used to suspend sedimented C^- cells. The normal 30 minutes incubation were followed by 30 minutes exposure to DNA.

TABLE XXIX

HEAT STABILITY OF SHOCK FLUID FACTOR

Addition to C^- Cells	Temperature of Treatment	% Control Transformation
None		100
Shock Fluid		270
Shock Fluid	0	310
Shock Fluid	37	240
Shock Fluid	45	175
Shock Fluid	50	120
Shock Fluid	60	100
Shock Fluid	80	100
Shock Fluid	100	100

Aliquots of the shock fluid were incubated at the indicated temperatures for 20 minutes. They were then equilibrated to 37° and added to sedimented C^- cells.

TABLE XXX

EFFECT OF VARIOUS ENZYMES ON SHOCK FLUID FACTOR

Enzyme Added	% Control Transformation
None	100
Trypsin	30
Pronase	28
Papain	22
RNase	88

Shock fluid was incubated 20 minutes at 37^{o} on a shaker with 50 µg/ml of the indicated enzymes. The stimulating activity of shock fluid samples was determined using C^{-} cells. The % control was calculated as the number of tryptophan^{+} transformants obtained when using the enzyme treated shock fluid/number of tryptophan^{+} transformants obtained in the presence of untreated shock fluid, the whole term multiplied by 100.

When the crude shock fluid was electrophoresed on polyacrylamide gel, sixteen bands were seen (Figure 12).

Purification of the Shock Fluid Factor

Since the shock fluid contained a factor or factors which was/were involved in competence development, various attempts were undertaken to fractionate the shock fluid and isolate the factor.

The crude shock fluid was chromatographed on a Sephadex G-25 column (1.2 cm by 30 cm) equilibrated in 100 mM potassium phosphate buffer, pH 6.9. Figure 13 shows that the elution pattern consisted of three peaks. Peak 1 was in the void volume and was the only one with competence factor activity. The protein concentration of peak 1 was 200 µg/ml and maximum transformability was achieved when only 50 µl of peak 1 eluate was added per milliliter of cells. The specific activity of crude shock fluid was 3.5 while the specific activity of peak 1 was 10.0. This represents about a 3-fold purification.

Polyacrylamide gel electrophoresis of peak 1 yielded one major band and three minor ones (Figure 14). This also demonstrated significant purification by the G-25 chromatography step since about twelve protein bands were eliminated from the gel electrophoresis pattern.

For further purification, the shock fluid was chromatographed on a G-200 column (1.2 cm x 90 cm) equilibrated in 100 mM potassium phosphate Figure 15 shows four protein peaks in the elution pattern.

The competence factor activity was located in peak 2 which was a rather broad peak. Peak 2 had a specific activity of 7.5. No other peaks or combinations of peaks contained competence factor activity unless peak 2 was present. The G-200 column was marked with catalase,

Figure 12. Disc Gel Electrophoresis of Shock Fluid

Shock Fluid was dissolved in 10% sucrose at a protein concentration of 10 mg/ml. One hundred µg of protein was layered on the standard 7% polyacrylamide gel, pH 9.5 and electrophoresed as described in Figure 9.

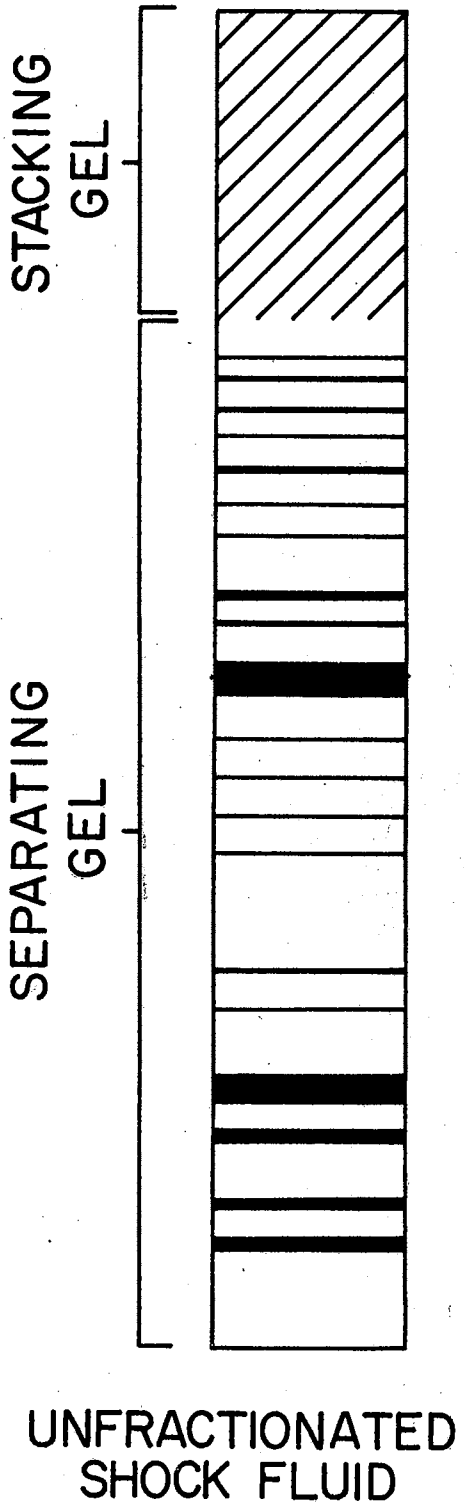

STACKING GEL
SEPARATING GEL
UNFRACTIONATED SHOCK FLUID

Figure 13. Sephadex G-25 Chromatography of Shock Fluid

A column (1.2 x 40 cm) of Sephadex G-25 was equilibrated with 100 mM potassium phosphate buffer, pH 7.0. A 0.5 ml sample of shock fluid (protein concentration of 1.6 mg/ml) was applied and 1.0 ml fractions were collected and the absorbance at 280 nm was determined. Indicated fractions were added to C^- cells as described in the Methods Section and the specific activity, ●, was determined as described in the Methods Section.

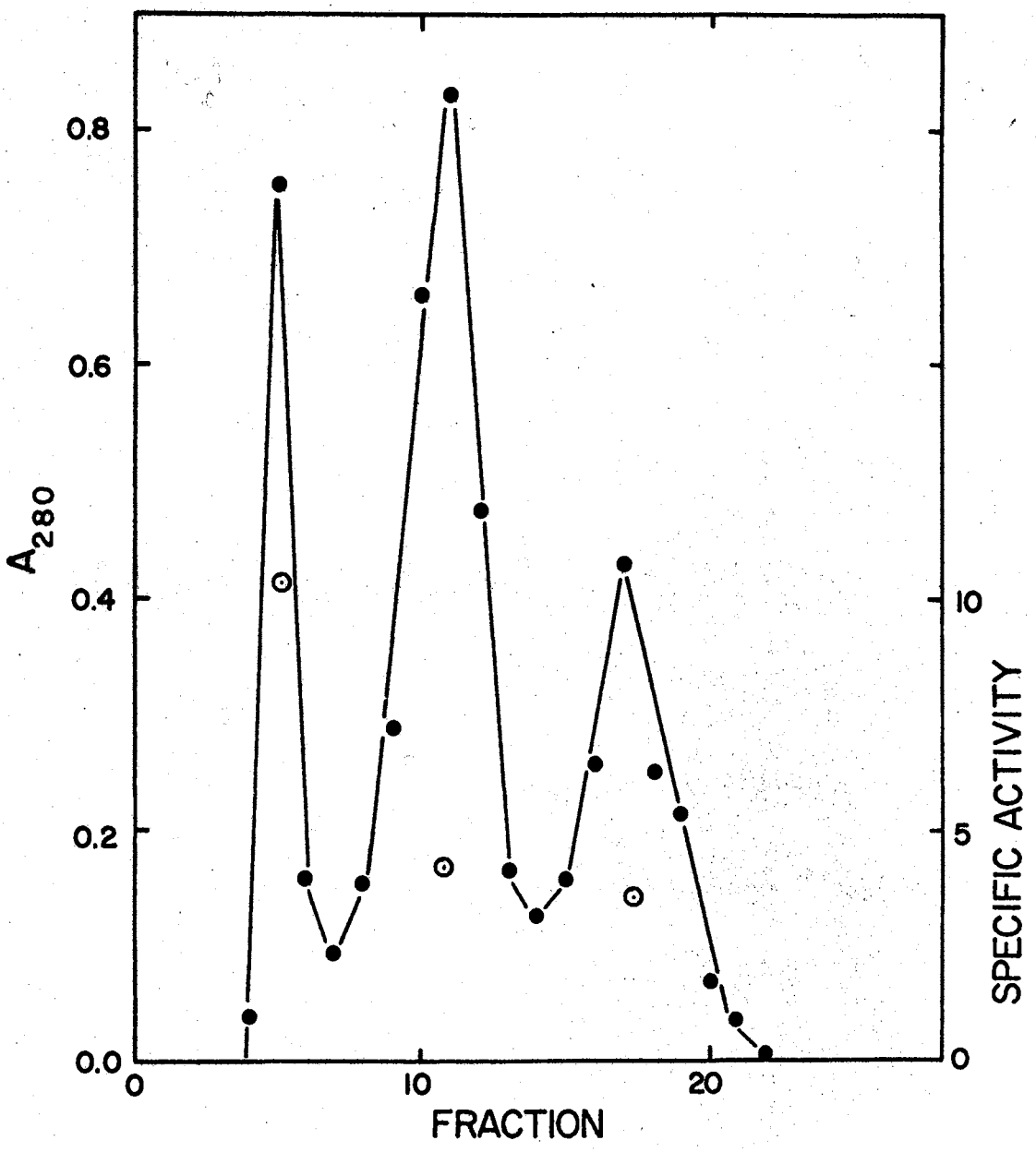

0.8
0.6
0.4
0.2
0.0
A_{280}
0
10
20
FRACTION
10
5
0
SPECIFIC ACTIVITY

Figure 14. Disc Electrophoresis of Peak 1

Peak 1 (0.05 ml) was layered on the standard 7% polyacrylamide gel, pH 9.5, and electrophoresed as described in Figure 9.

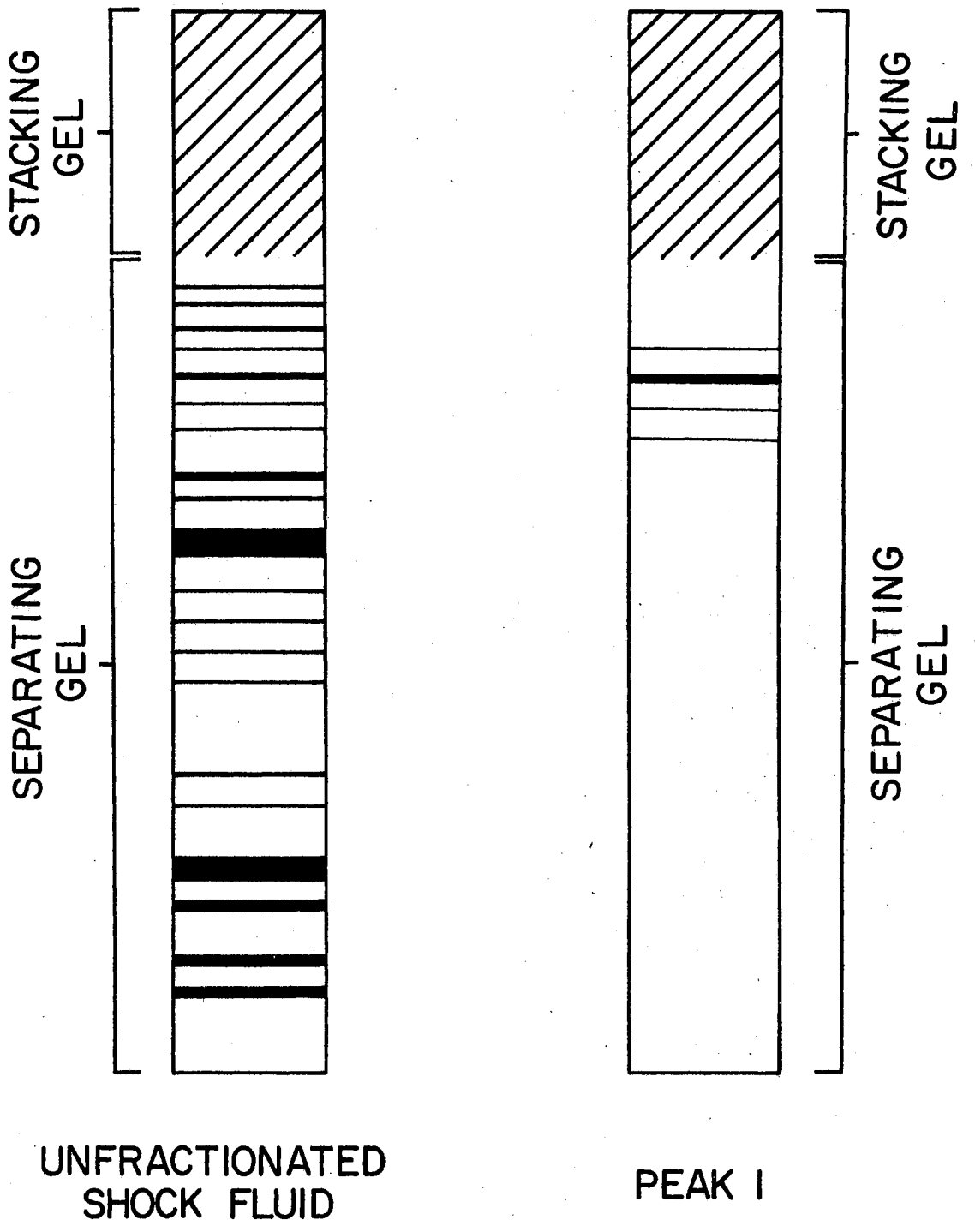
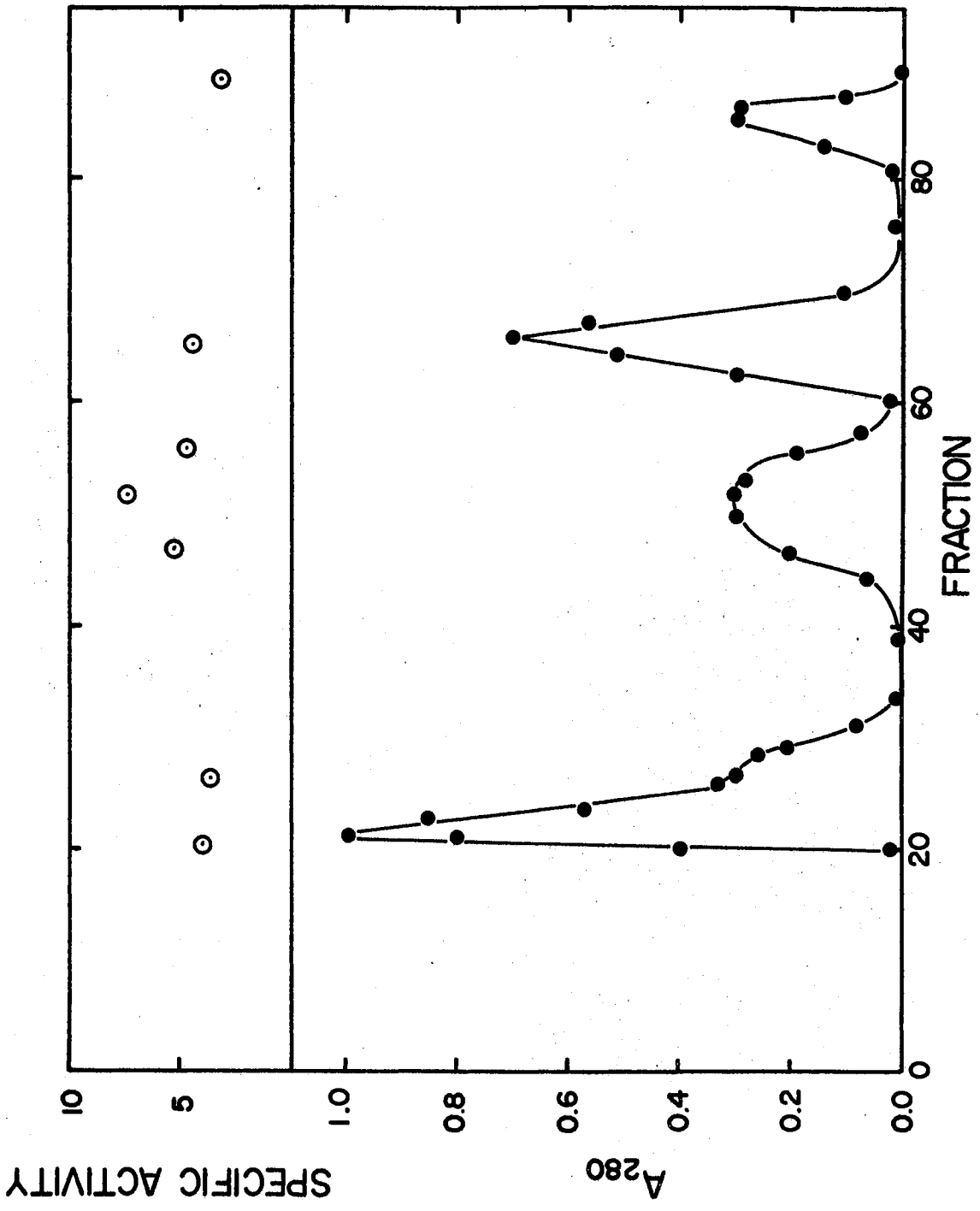

STACKING GEL
SEPARATING GEL
UNFRACTIONATED SHOCK FLUID
STACKING GEL
SEPARATING GEL
PEAK I

Figure 15. Sephadex G-200 Chromatography of Shock Fluid

A column (1.2 x 90 cm) of Sephadex G-200 was equilibrated with 100 mM potassium phosphate buffer, pH 7.0. A 0.5 ml sample of shock fluid was applied and 0.3 ml fractions were collected. Selected fractions were tested for specific activity, O, as described in Figure 13.


SPECIFIC ACTIVITY
10
5
A_{280}
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
FRACTION

lactic dehydrogenase, bovine serum albumin, and cytochrome C. Figure 16 shows that when the molecular weight was plotted versus the elution volume, the factor was eluted at a position corresponding to a molecular weight of 40,000 (117).

Even though purification resulted upon G-200 chromatography the factor was extremely labile, and attempts to improve the stability were unsuccessful. This lability seriously hampered further studies of the purified factor.

DEAE chromatography was also utilized, but no fractions stimulated transformation as much as crude shock fluid and the specific activity ranged from 2.0 to 5.0. Gradients consisting of both Tris (10-200 mM) or potassium phosphate (10-300 mM) buffers were used and pH values ranged from 6.0-8.5.

Ammonium sulfate fractionation did not yield a fraction with increased specific activity. The best specific activity achieved in this way was 3.1 as compared to 3.5 for the shock fluid.

The shock fluid was layered on a 5-20 per cent sucrose gradient as described in the Methods Section. Figure 17 shows that the shock fluid is separated into five peaks absorbing at 280 nm. Only peak 2 contained competence factor activity. This peak had a specific activity of 35 which represents the highest purification achieved. This peak, when chromatographed on a G-200 column, eluted where the competence activity was found when crude shock fluid was chromatographed.

A variety of proteins which interact with DNA have been purified by DNA-cellulose chromatography. A sample of shock fluid was layered on a DNA-cellulose column as described by Alberts, Amodio, Jenkins, Gutmann and Ferris (118). Figure 18 shows that the competence factor

Figure 16. Molecular Weight Determination of Shock Fluid Factor

The Sephadex G-200 column described in Figure 15 was used. The standard proteins used were catalase (1 mg); lactic dehydrogenase (1 mg); bovine serum albumin (3 mg); and cytochrome C (1.5 mg). All of these proteins plus 1.0 mg of competence factor were dissolved in 1.0 ml of the elution buffer (100 mM potassium phosphate buffer, pH 7.0, plus 0.1 M KCl) and layered on the column. Fractions of 0.6 ml were collected and the A_{280} determined.

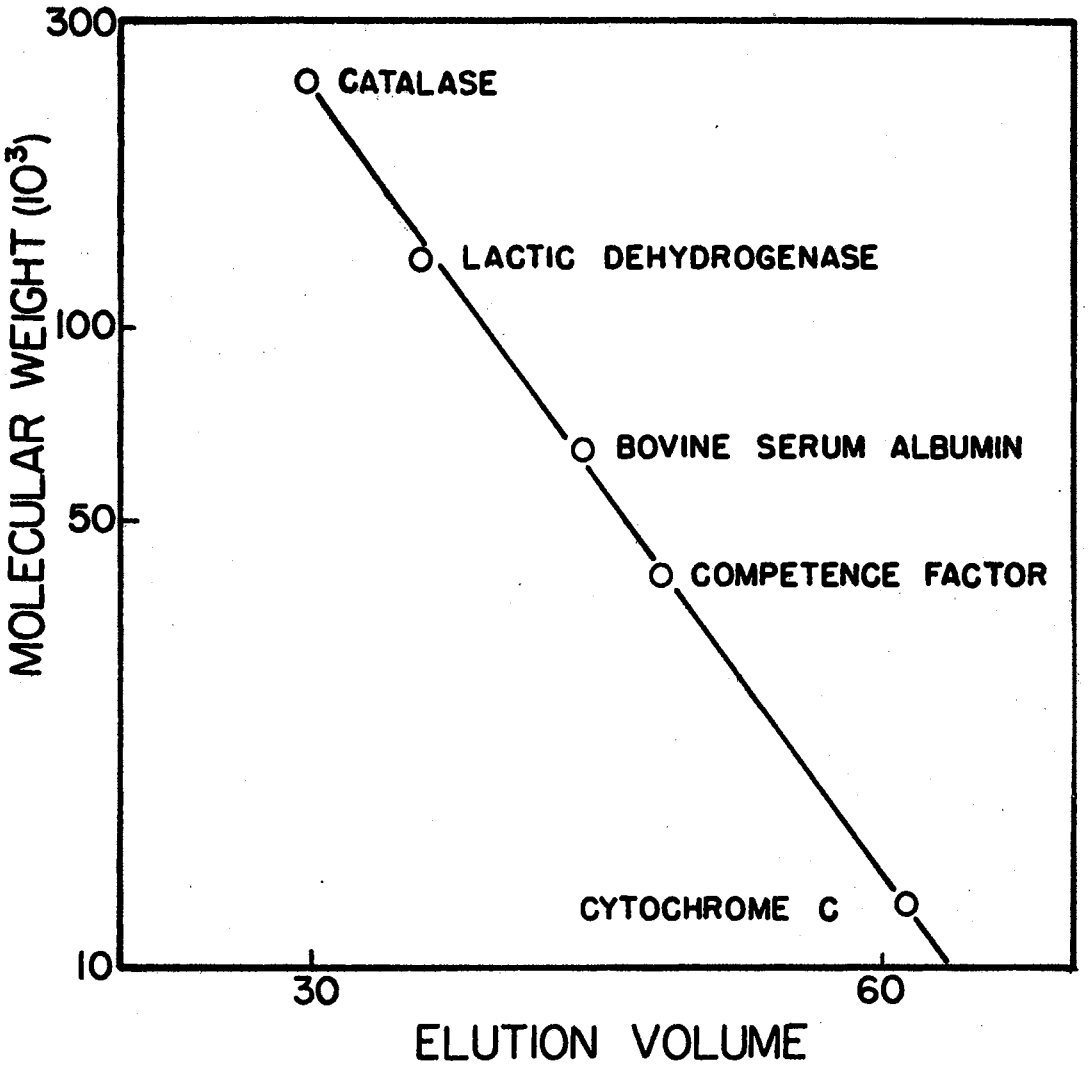

300
100
50
10
MOLECULAR WEIGHT (10^3)
CATALASE
LACTIC DEHYDROGENASE
BOVINE SERUM ALBUMIN
COMPETENCE FACTOR
CYTOCHROME C
30
60
ELUTION VOLUME

Figure 17. Sucrose Density Gradient Centrifugation of Shock Fluid

An aliquot (0.3 ml) of shock fluid was carefully layered on a 5-20% sucrose gradient prepared as described in the Methods Section. The samples were then centrifuged in a SW-65 rotor at 32,000 rpm for 150 minutes at 4°. After completion of the run, three drop fractions were collected by using a Buchler gradient collection device which punctures the bottom of the tube. The refractive index of the odd numbered fractions was determined while the A_{280} of the even fractions was determined. The specific activity of the indicated fractions was determined as described in the Methods Section.

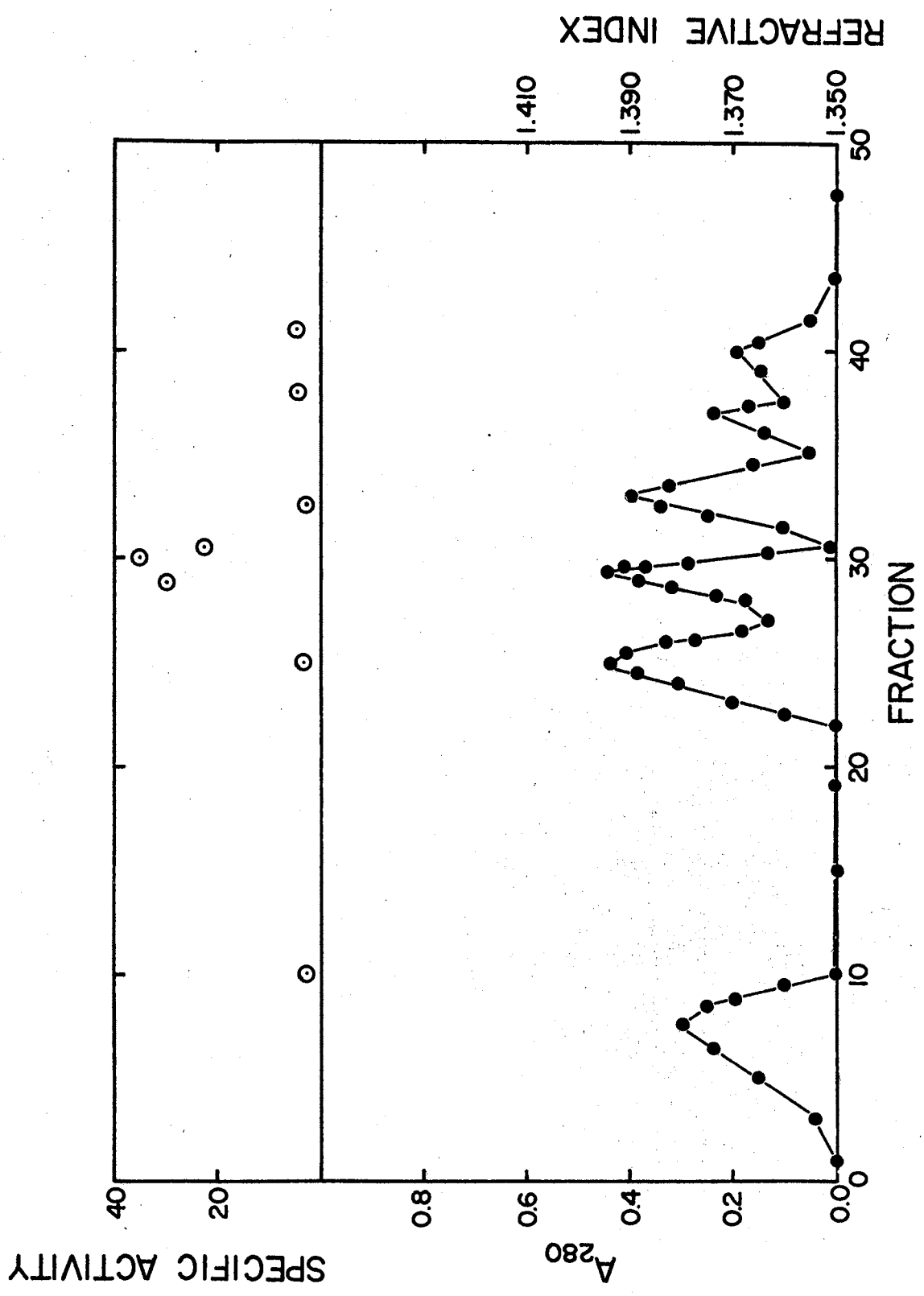
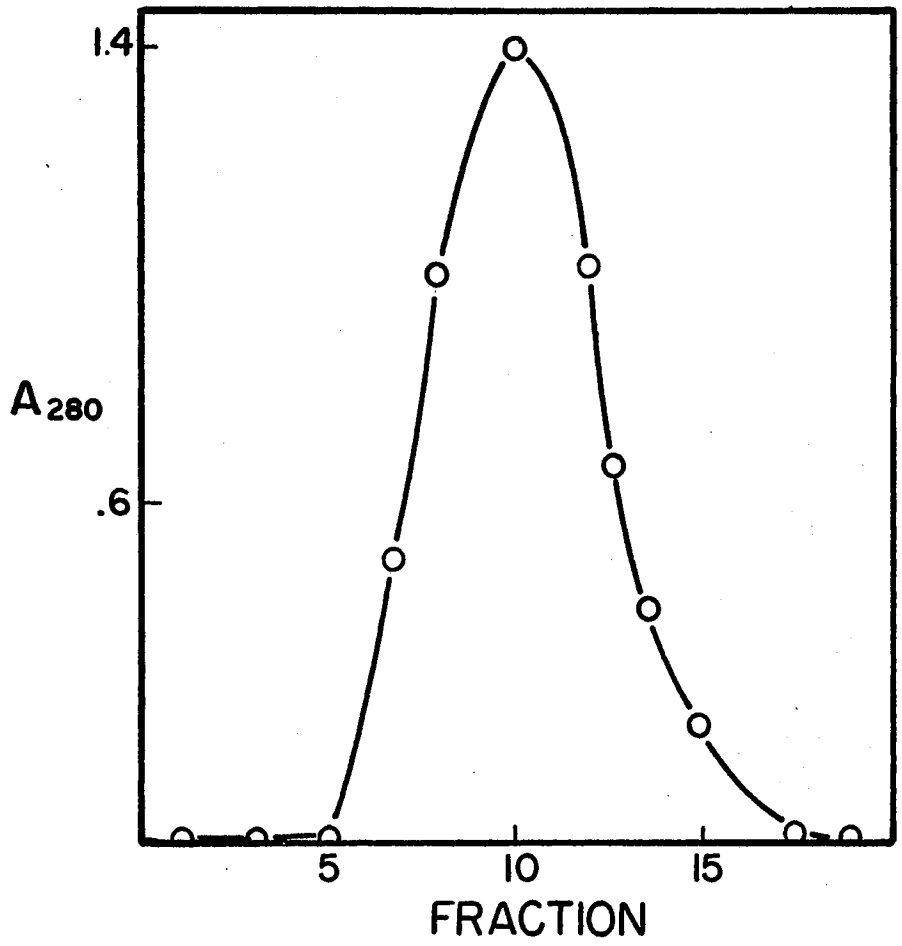

REFRACTIVE INDEX
1.410
1.390
1.370
1.350
FRACTION
0
10
20
30
40
50
A_{280}
0.0
0.2
0.4
0.6
0.8
SPECIFIC ACTIVITY
40
20

Figure 18. DNA-Cellulose Chromatography of Shock Fluid

Shock fluid (1.0 ml) was layered on the DNA-cellulose column, which was prepared according to Alberts, et al. (118). Fractions of 1.0 ml were collected and the A_{280} was determined. The specific activity of the peak was 3.5.


1.4
A280
.6
5
10
15
FRACTION

activity eluted off with the solvent front, and the specific activity of the peak was 3.5. Hence, no competence factor or protein was bound to the column. This indicates that the competence factor of the shock fluid does not interact with DNA.

CHAPTER V

DISCUSSION

The first step in the process of bacterial transformation involves a modification of the cell surface which augments binding of DNA. This is followed by the active transport of transforming DNA into the recipient cell by an energy dependent process. Cells which bind, transport, and are later transformed by DNA are designated competent. Unfortunately, the state of competence is still undefined in molecular terms. The procedures for obtaining maximum competence are empirical and vary from laboratory to laboratory.

In B. subtilis, maximum competence occurs at the end of exponential growth and is maintained longer than in the Pneumococcus and Streptococcus systems. The requirements for competence development are the glucose minimal medium, the auxotrophic nutritional requirements, magnesium ions, and the chelation of cupric ions. Small amounts of L-amino acids, which are not required for growth, stimulate competence development (histidine, tryptophan, valine, glycine, aspartic acid, threonine, methionine, lysine, and arginine) (45).

Extracellular activating factors function in the Pneumococcus (72), Streptococcus (62,64) and B. subtilis (65,66) transformation systems. These factors, which allow or stimulate transformation, are loosely defined as competence factors (CF). The modes of action of the competence factors are unclear, but an interaction between the competence factor

and the cell surface seems certain.

Table II shows that subjection of competent B. subtilis cells to a cold osmotic shock treatment results in a 70-90% reduction in transformability and also a decrease in DNA transport. For maximum reduction of transformability the competent cells must be suspended in a sucrose-Tris, pH 7.1, EDTA solution for 10 minutes and then instantaneously suspended in cold 10^{-5} M $MgCl_2$ (Tables II, III). Neu (119) found no substitute for Tris buffer in the shocking of E. coli; Table IV shows that phosphate buffer cannot substitute. Magnesium ions were required for retention of viability. Washing of competent cells with medium also reduces transformability (Table VII); however, the osmotic shock treatment is more effective. Both of these treatments might either remove a surface-located substance or denature a surface protein involved in DNA transport. The osmotic shock treatment has little effect on the rates of uptake, accumulation, and incorporation of leucine, uridine, and thymidine (Figures 3, 4, 5). The slight retardation of thymidine uptake should make no difference in transformability since Bodmer (53) has shown that DNA synthesis is not required for transformation.

Since competent B. subtilis cells are physiologically different from the great majority of the population (36), they might be more osmotically labile. Joys and King (120) have found a physical state during which potential B. subtilis transformants are more osmotically fragile; however, the conditions of the experiments reported in this paper are not comparable to those found associated with the lability to physical forces. The purpose of the experiment reported in Table VII was to determine whether the potential transformants were more sensitive

to lethal effects of the osmotic shock procedure. When potential transformants are treated with the osmotic shocking procedure after exposure to DNA, there was only a slight reduction in the transformants recovered.

Competent cells may be made deficient in DNA transport by phenethylalcohol treatment (81,121,122), by periodate treatment (123), and by genetic mutation (39). The current study shows similar results with osmotic shocking. Do all of these approaches produce the same deficiency? Can the transformability be restored? Can competence factors restore transformability? What are the molecular details of the transport reaction?

The irreversible binding of DNA and transformability can be restored by suspending the shocked cells in the supernatant solution of a competent culture. Addition of shock fluid to shocked cells resulted in little or no increase in either DNA binding or transformation frequency.

Phenethylalcohol (PEA) decreases the transformability of competent cells by 60-70% with the cell membrane being the probable site of action. After removal of PEA, suspension of the treated cells in supernatant solution results in a substantial reversal of PEA inhibition (Table XII). Shock fluid also restores the transformability lost by PEA treatment.

To explore further the biological properties of the supernatant factor and the shock factor, a low transforming strain (C^-) was treated with the shock fluid factor (Table X). The shock fluid factor enhanced irreversible binding of DNA, and there was an increased transformation frequency of 2-20 fold depending upon experimental conditions (Table XXII). Similar results were observed when supernatant solution was

used.

An incubation period of 30-40 minutes at 37° in supernatant solution was required for maximum recovery of transformability by shocked cells. Protein synthesis and messenger RNA synthesis were required for any recovery from shocking (Figure 6).

Maximum enhancement of transformation frequency in C^- cells was achieved by the addition of an optimum amount of shock fluid in 30 minutes at 37° (Table XXIII). There was still significant stimulation of transformation when the incubation was at 4° (Table XXIV). This suggested that a biosynthetic step was not required for activation. The activation process depends on the shock fluid concentration. When shock fluid is added to transformation medium at the beginning of the competence development period, competence does not appear (Table XXV). This suggests that something in the shock fluid may prevent the synthesis or release of a factor necessary for irreversible uptake of DNA.

The supernatant solution contained little extracellular material besides protein (20 µg/ml) and 260 nm absorbing material (Table XIII). Ten protein bands were discernable by polyacrylamide gel electrophoresis (Figure 9). The supernatant solution was examined for DNase, RNase, protease, and autolytic activity; none of these enzymes were detected.

The shock fluid contained about 300 µg protein per milligram of dry shock fluid and large amounts of material with an absorption maximum at 260 nm (Table XXVI). Sixteen protein bands were detected by polyacrylamide gel electrophoresis (Figure 12). As with supernatant solution, no DNase, RNase, protease or autolytic activity could be demonstrated in the shock fluid. To detect autolytic activity, cell walls were incubated with either shock fluid or supernatant solution at 37°.

There was no substance in the shock fluid or supernatant solution which produced autolysis nor did they influence the normal cell wall bound autolytic activity (Figures 8, 11).

The factor of the supernatant solution was stable for months at 4^{o} but lost activity rapidly as the temperature exceeded 80^{o} (Table XV). The supernatant factor was stable from pH 6.5 to 7.5 (Table XVI). The factor was destroyed by proteolytic enzymes but was unaffected by RNase (Table XVII). Total loss of activity occurred upon dialysis.

The shock fluid factor was stable for months when lyophilized and for several weeks at 4^{o} in solution. The factor was stable from pH 6.0 to 7.5 but was progressively inactivated as temperatures exceeded 45^{o} (Tables XXVIII and XXIX). Complete loss of activity occurred when shock fluid was incubated with proteolytic enzymes while RNase had no effect.

The supernatant solution was concentrated about 10-fold by rotary evaporation, but loss of activity occurred upon lyophilization. Transformation frequencies above the unshocked control were achieved with optimal amounts of concentrated supernatant solution (Table XVIII). Concentration of the supernatant solution was essential for further purification.

Fractionation of supernatant solution by Sephadex G-25 chromatography proved successful and the elution volume of the factor indicated a probable molecular weight of less than 10,000 (Figure 10). The factor was stable for a few days. Fractionation by ammonium sulfate precipitation or by carboxymethyl cellulose column chromatography, resulted in little purification and there was drastic reduction in stability. Addition of stabilizing agents such as glycerol, and variations in

experimental conditions did not improve the stability of the factor.

The shock fluid was also chromatographed on a Sephadex G-25 column (Figure 13). The competence factor activity eluted with the void volume and a 3-fold increase in specific activity occurred. Polyacrylamide gel electrophoresis of this peak produced only one major band and three minor protein bands (Figure 14). The factor was stable at 4^{o} for several days at this stage. The shock fluid was chromatographed on a Sephadex G-200 column with little purification (specific activity of 7.5) (Figure 15). The elution volume of the competence factor corresponded to a molecular weight of about 40,000 (Figure 16). The active peak of the G-200 column was not as sharp as would be expected of a pure protein. Since the factor was surface-located, cell wall components such as teichoic acid could be present. However, no increase in resolution of the peak was obtained upon recycling. The factor, as eluted from the G-200 column, was extremely labile as opposed to crude shock fluid. Both ammonium sulfate precipitation and DEAE column chromatography were performed on the shock fluid, but the purification was inferior to that obtained with either G-25 or G-200 column chromatography. The crude shock fluid was fractionated by sucrose gradient (5-20%) centrifugation (Figure 17). The specific activity of the factor from the sucrose gradient was 35 which represented about a ten-fold purification of the factor from shock fluid. In addition, the factor was stable for about a week when prepared by fractionation of shock fluid on the sucrose gradient. This indicates that the sucrose may stabilize the factor.

The competence factors of Pneumococcus (72), Streptococcus (62,64), and B. subtilis (65,66) are all sensitive to proteolytic enzymes, which

is consistent with either a protein or a polypeptide nature. Both the shock fluid factor and supernatant factor are inactivated by proteolytic enzymes. The molecular weight of the pneumococcal competence factor is 10,000 (72) while that of the streptococcal competence factor is 5000-7000 (67). The molecular weights of the competence factors found in the shock fluid and supernatant solution are 40,000 and about 10,000, respectively.

The competence factors of Streptococcus (67) and B. subtilis (66) are lyophilized for storage. The Pneumococcus competence factor (72) is stored frozen. The factor present in the supernatant solution is unstable to lyophilization and is stored at 4^{o}. The shock fluid factor is stored as a lyophilized powder. The streptococcal factor (64) loses only 22% of its activity when heated to 100^{o} for 10 minutes. Ninety minutes at 100^{o} is required for complete loss of activity. Tomasz and Mosser (72) report that the pneumococcal competence factor is destroyed completely in 20 minutes at 100^{o}. The competence factor from the Marburg strain of B. subtilis loses its activity after 5 minutes at 100^{o} according to Charpak and Dedonder (65). The competence factors described in this work are considerably more heat labile. The competence factor of Streptococcus, as described by Dobrzanski and Osowiecki (64) is stable from pH 2 to 11. In fact, stability is prolonged at pH 11. The pneumococcal competence factor has a pH optimum around pH 7.9-8.1, but its stability is increased at pH 10 or 11. The competence factors found in the shock fluid and supernatant solutions in the present study are stable from pH 6.0 to 7.5.

The elucidation of the mechanism of action of the various competence factors is impeded by their lack of stability during extensive

purification. Dobrzanski and Osowiecki (64) describe the streptococcal factor as being stable for 20 days while Tomasz and Mosser (72) describe their factor as extremely labile when dissolved in saline or H_2O with a half-life of 1 and 2 hours at 30° and 0°, respectively. By adjusting the pH to 10 or 11 the factor loses 20% of its activity in 4 hours at 0°. The crude shock fluid and supernatant solution factors are relatively stable until purified and this greatly handicaps further purification and chemical and physical studies.

Kohoutova, Brana and Holubova (124) reported that their Pneumococcus competence factor preparation inactivates transforming DNA with a DNase-like action. There is no good evidence for an interaction between any competence factor and transforming DNA. Our findings are in support of this observation. When the shock fluid was passed over a DNA-cellulose (118) column, no interaction was detected (Figure 18). In addition, neither the presence of supernatant solution nor shock fluid had any effect on the thermal transition curve for B. subtilis DNA.

The correlation of autolytic activity with competence factor activity is of particular interest for two reasons. Young (40) has suggested a role for an autolytic enzyme (an N-acylmuramyl-L-alanine amidase) in competence development. Akrigg and Ayad (75) found autolytic activity in their competence factor preparation which they obtained from a water wash of B. subtilis. They suggested that their competence factor may be the autolytic enzyme. Lytic activity has not be demonstrated in any other competence factor preparation. There is no autolytic activity in either the supernatant solution or shock fluid (Figures 8, 11).

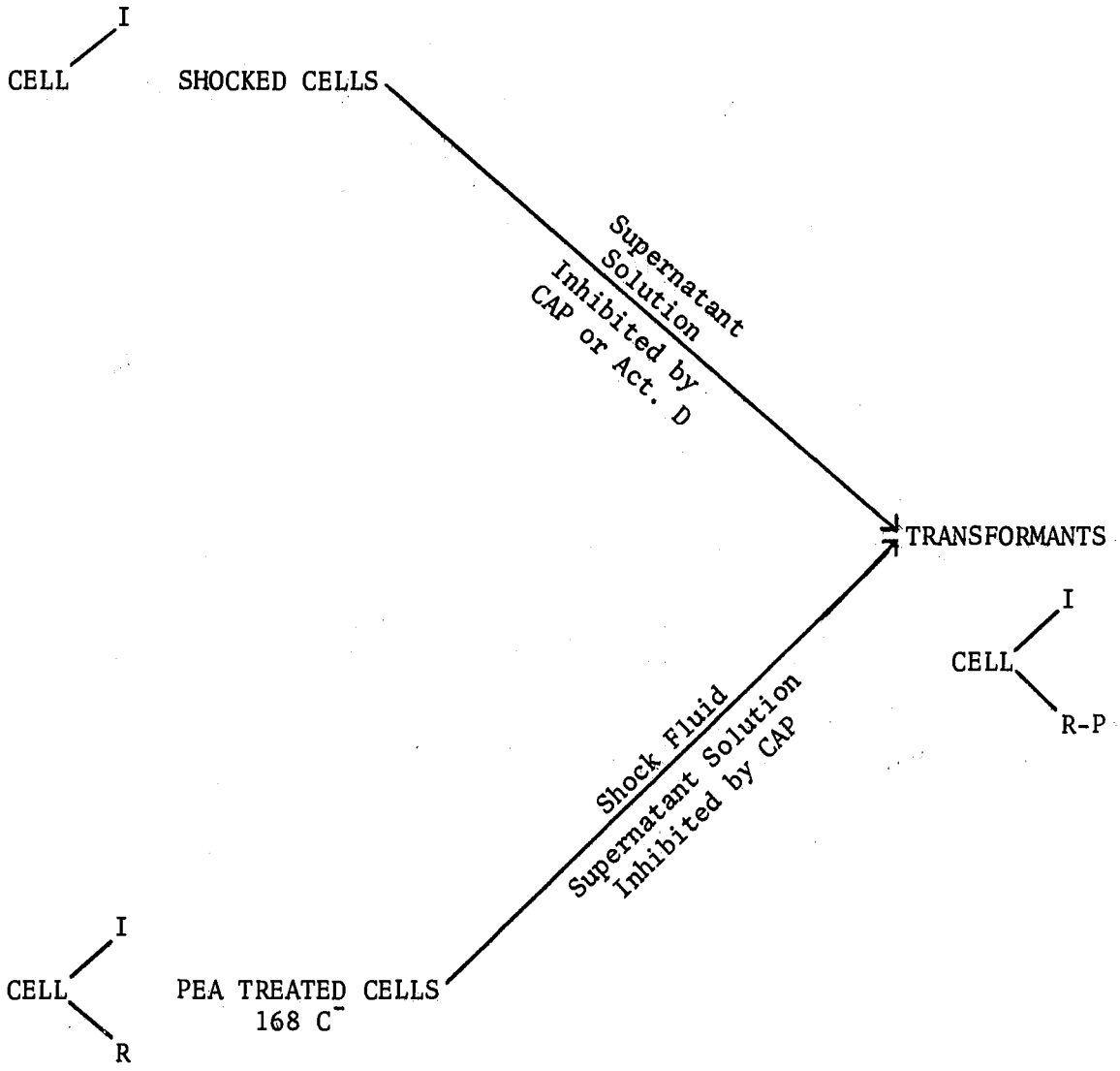
The competence factors of the supernatant solution and the shock fluid differ in three important aspects: (1) the molecular weight of

the supernatant factor is 10,000 or less while the molecular weight of the factor in shock fluid is 40,000; (2) the supernatant solution will restore shocked cells to their normal level of transformability while shock fluid is inactive; (3) the factors differ in stability to lyophilization. The shock fluid enhances the transformability of C^- cells much more than supernatant solution does.

These observations suggest that there may be two competence factors in the B. subtilis system. How they modify the cell to promote DNA uptake is unknown. Since DNA can be bound to cell wall prepartions, this might be the first reaction in the approach of transforming DNA to the cell. None of the treatments studied in this thesis reduced the reversible binding. Therefore, the transport reaction must involve at least two stages since two differentiable factors were demonstrated in this thesis.

Figure 19 shows a scheme which can account for the observations reported in this thesis. Transforming DNA is initially bound reversibly to the cell surface at site I (initial) and is DNase sensitive. This site might be located on the cell wall since the data in Table XII indicates a binding of DNA to the cell wall of competent cells. This I site is not damaged by either the osmotic shock treatment or phenethyl alcohol treatment. Uncertainty exists about whether or not this is an obligating intermediate in the transformation process. A second component which is designated R (DNA receptor) is postulated because of the distinctness of response of shocked cells and low competence strains to supernatant solution and shock fluid. Both the supernatant solution from competent cells and the shock fluid contain a protein factor (P) which restores transformability to low competent strains and to

Figure 19. A Schematic Representation of the Involvement of the Competence Factors of the Supernatant Solution and Shock Fluid With the Cell Surface



Molecular Weight Restores Stable	Shock Fluid 40,000 PEA and C^- Lyophilization	Supernatant Solution 10,000 All Flash Evaporation

phenethyl alcohol treated cells. When cells are osmotically shocked, the R component is denatured and either remains in position or is removed. Thus, shock fluid would not be capable of reactivating shocked cells. Spontaneous release of R into the supernatant solution occurs during growth which produces a solution containing both R and P; hence, transformability can be restored by this preparation to osmotically shocked cells. It is indicated that both restoration processes requires protein synthesis (inhibitable by phenethylalcohol).

SUMMARY

A cold osmotic shock treatment was developed which decreased the irreversible binding of DNA and subsequent genetic transformation of B. subtilis by 70-90% without affecting the viability of the cells. The first stage of the shock treatment consisted of suspension in a solution of sucrose, Tris, and EDTA, all of which were necessary for maximum reduction of transformation. Rapid suspension of the cells in a cold $MgCl_2$ solution constituted the second stage. The shock treatment appeared specific for the irreversible binding step, since it had little effect on the reversible binding of DNA.

The transformability of shocked cells was restored by suspending the cells in the supernatant solution from a competent culture. Maximum restoration resulted when the cells were incubated with supernatant at 37° for 30 minutes prior to the addition of DNA. The factor in the supernatant solution was spontaneously released as competence developed, but the shock fluid did not restore transformability to shocked cells.

Inhibition of transformation by treatment of cells with phenethylalcohol was reversed by either the supernatant solution or shock fluid. Competence was enhanced 2-20-fold in a low competence strain after a short incubation at 37° with either the shock fluid or supernatant solution.

Both the supernatant factor and the shock fluid factor were stable from pH 6.0 to 7.5, but deterioration was rapid outside this range. The supernatant factor was stable up to about 80°, while the factor

of the shock fluid was progressively inactivated as temperatures exceeded 55°. Both factors were destroyed by proteolytic enzymes.

Both factors were stable for several months when stored at 4° in the crude form. However, upon purification the factors became very labile. Though purification was not complete, Sephadex G-25 column chromatography was the most desirable method for fractionation. The partially purified factors remained stable for several days.

The location and site of action of both factors are at the bacterial surface, and both are essential for the irreversible uptake of DNA. The factor in the shock fluid is stable to lyophilization, but the supernatant factor is inactivated by lyophilization and can be concentrated only by flash evaporation. The supernatant factor and the factor of the shock fluid also differ in their molecular weights which are 10,000 and 40,000, respectively. The third outstanding difference is that while both the supernatant solution and the shock fluid will enhance transformability in a low transforming strain and will reverse PEA inhibition of transformation, only the supernatant solution will restore transformability to shocked cells. A scheme accounting for these observations was proposed.

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