A MECHANISM FOR THE SUCROSE STIMULATION OF THE GENETIC TRANSFORMATION OF *BACILLUS SUBTILIS*

A Dissertation

Presented to

the Faculty of the Department of Biology

---

in Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Richard Haines Peetz

January 1976

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ABSTRACT

A study of the sucrose stimulation of genetic transformation in Bacillus subtilis has presented experimental evidence related to the part played by the cytoplasmic membrane and mesosomes in the binding of transforming deoxyribonucleic acid to competent cells.

The transformation stimulation by 0.3M sucrose was found to be limited to cells already competent, to affect equally all genetic markers tested, to be limited to the reversible binding phase of the uptake process and inhibited by very low and very high temperatures. The pH optimum remained the same as the control, as did the ionic strength optimum, but the methylene blue dye adsorption, i.e. the negative charges on the cell, decreased in the presence of sucrose. The stimulating effect was found to be associated with the cells, not the medium or the DNA. It was decreased by lipolytic enzyme treatment and decreased the amount of residual DNase in supernatants of competent cells. Cells with and without sucrose responded similiarly to dinitrophenol inhibition of active transport.

Single-strand transformation responded to sucrose the same as the native DNA system.

Lactose, mannose and galactose, also stimulating sugars, demonstrated the same reversible binding phase specificity and low temperature inhibition of the stimulatory effect, thus suggesting that most, if not all, sugars that stimulate genetic transformation have a common mode of action.

The data was discussed in relation to existing ideas of mesosomal involvement in DNA uptake and a mechanism dedeloped for the interaction of stimulating sugars and mesosomes resulting in an increase in genetic transformation.

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"Discertingly few laymen - even few college graduates - really understand what the scholar means by 'truth'. It is not a citadel of certainty to be defended against error; it is a shady spot where one eats lunch before tramping on. The professional thinker enjoys where he is, but he also looks forward to new vistas around the next bend, over the next crest."

- Lynn White, Machina ex Deo

Part I

INTRODUCTION

Review of the Literature

Information transfer in biological systems by means of relatively pure chemical preparations has developed into an area of great importance since the discovery of the deoxyribonucleic acid (DNA) mediated genetic transformation of bacteria by Griffith (1928) and its further clarification by Avery and associates (1944; see also Downie, 1972, for an historical review). The process has been studied in great detail in a large number of organisms since then, as witnessed by the many review articles concerning all or specific parts of the transformation process (Ravin, 1961; Taylor, 1965; Spizizen et al, 1966; Epstein, 1968; Lacks, 1968a; Tomasz, 1969a,b; Erickson, 1970; Hotchkiss and Gabor, 1970; Archer, 1973; Notani and Setlow, 1974). The complexity of the phenomenon reveals itself if one considers that one Hemophilus influenzae cell, 1-2 microns in length by 0.8 microns in diameter, takes into its interior about 5 DNA molecules, 10-20 microns in length, within a few minutes time. To increase the complexity, the cell already contains 2 DNA genomes, each 400 microns in length.

The transformation process may be roughly divided into 2 phases: first the adsorption of the donor DNA molecules onto the recipient cell surface and its transport into the cell interior; second is the integration and subsequent genetic expression. Many steps and physical/chemical interaction are involved in these two phases, as will be seen in the following survey of the literature. Due to the large amount of literature available, an attempt will be made to limit the survey to bacterial DNA mediated genetic transformation.

There are certain restraints on the donor DNA molecule, not as a mechanism for information transfer, but as related to its "read-out" device, the recipient cell. The donor DNA may be obtained from chemical extraction in the laboratory or may arise by natural causes, such as cell lysis (Ottolenghi and Hotchkiss, 1962; Takahashi, 1962; Ottolenghi-Nightingale, 1969), or from the release of DNA from actively growing cells (Ephrati-Elizur, 1968; Ranhand and Cole, 1971). Data concerning the length of a DNA molecule versus transformability arranges itself into two hypotheses:

1) Transformation is only possible when the DNA molecule is longer than a given length (Litt et al, 1958).

2) There is a continuous increase in transformation as a function of length of the DNA molecule (Cato and

Guild, 1968).

Some data supports both hypotheses (Nicolaieff and Chevallier, 1970), but in general, for the DNA molecule to be biologically active, the molecular weight must be greater than 7.7×10^4 to 3×10^5 daltons, although the steps following the uptake portion of the transformation process are much more sensitive to decreases in length (Lerman and Tolmach, 1957; Szybalski and Opara-Kubinska, 1966; Barnhart and Herriott, 1963; Lacks, 1968; Randolph and Setlow, 1972; Morrison and Guild, 1972). The configuration may be the normal double stranded helix or, under certain cultural manipulations, single stranded DNA may be taken up and expressed at reduced frequency by Hemophilus influenzae (Postel and Goodgal, 1966, 1967), Diplococcus pneumoniae (Miao and Guild, 1970) and Bacillus subtilis (Chilton, 1967; Chilton and Hall, 1968; Tevethia and Mandel, 1970, 1971; Tevethia and Caudill, 1971).

In these three transformation systems either strand of donor DNA can transform (Guild and Robinson, 1963; Roger et al, 1966a; Chilton, 1967; Rudner et al, 1968; Goodgal and Notani, 1968; Gurney and Fox, 1968; Peterson and Guild, 1968). B. subtilus DNA (Rudner et al, 1968, 1969; Rudner and Remeza, 1973a,b) and D. pneumoniae DNA (Roger et al, 1966a,b; Roger, 1968) can be fractionated into light and heavy complementary

strands via methylated albumin-kieselguhr chromatography and the individual strands utilized as donor DNA. Peterson and Guild (1968) report more transmission of drug resistance by heavy strands of fractionated pneumococcal DNA. The complimentary single strands may be renatured to form a biologically active double stranded molecule (Strauss, 1970; Rudner and Remeza, 1973b).

Double stranded DNA with overlapping single strand ends transforms poorly or not at all (Williams and Green, 1972). Some transformable strains take up synthetic polydeoxyribonucleotides but not polyribonucleotides or hybrid polymers (Setlow et al, 1965; Ciferri et al, 1970; Williams and Green, 1972).

Transforming DNA is usually homologous or "self" DNA, but D. pneumoniae (Lerman and Tolmach, 1957; Rebeyrotte and Latarjet, 1967) H. influenzae (Shaeffer et al, 1960; Steinhart and Herriott, 1968; Setlow and Boling, 1972; Scocca, 1974) H. parainfluenzae (Notani and Setlow, 1972) and B. subtilis (Bodmer and Ganesan, 1964) all bind DNA from different species and that DNA will compete to a varying extent with "self" transforming DNA for uptake sites on the cell. The foreign DNA is usually not genetically expressed. There are some exceptions, usually marker specific, in that antibiotic resistance is readily transferred while biochemical markers

are not (see Notani and Setlow, 1974, for a review on heterospecific transformation; Sanchez et al, 1975). Restriction enzymes are responsible for the degradation of foreign DNA in bacterial cells (see Archer, 1973, and Notani and Setlow, 1974 for reviews; Seto et al, 1975). B. subtilis shows some specificity in uptake and extent of degradation of heterologous DNA (Piechowski et al, 1975), while in Neisseria meningidicus the binding is apparently specific for homologous DNA only (Jyssum et al, 1971).

Heating streptococcus cultures decreases discrimination against heterospecific DNA for a short period. Inhibition of nucleic acid synthesis, but not protein synthesis, prevents recovery from the heat induced nondiscrimination (Ravin and Ma, 1972; Dedish and Ravin, 1974). Strauss and Marone (1967) show that extracts of transformable B. subtilis show little degrading activity on homologous transforming DNA, but heating the extracts briefly allowed homologous DNA to be degraded. This might indicate the presence of a heat labile nuclease inhibitor.

A bacterial cell must be in a physiological state called "competence" to be able to take up and express exogenously added DNA. The term is rather vague in that the transformation system for each species has unique requirements for approaching the competent state. Whether competence differs

in each system, or whether different pertubations are required in each system to achieve the same state is not known. The point of maximum competence in relation to the growth curve varies with each system. D. pneumoniae shows maximum competence during exponential growth (Hotchkiss, 1954; Tomasz, 1966) and remains competent only for a short time (Tomasz, 1966); streptococci, at the beginning of exponential growth (Perry and Slade, 1963); B. subtilis and H. influenzae, near stationary phase (Anagnostopoulos and Spizizen, 1961; Goodgal and Herriott, 1961) with B. subtilis remaining competent for several hours (Kammen et al, 1966b) while Neisseria (Catlin, 1960; Lie, 1965) and Micrococcus (Moseley and Setlow, 1968) show competence throughout the growth curve.

One method of studying the competent state is to determine the effects of various chemical and physical agents on the transformation process. This method is widely used, and to summarize the vast amount of data in this particular area, tables are utilized. Table I lists the physical/chemical agents tested against B. subtilis, Table II for H. influenzae, Table III for D. pneumoniae, Table IV for Streptococcus and Table V for miscellaneous transformation systems. All the Tables list the overall effect on transformation frequency of each agent and, if given in the data, where specifically

the agent acts. Effects are listed as S=stimulation, I= inhibition, N=no or very little effect.

Very few physical and chemical agents give the same responses in all the transformation systems. The Tables indicate that protein synthesis, but not DNA synthesis is required for competence development or DNA uptake or integration in all systems except N. meningiticus, where protein synthesis is apparently not necessary (Jyssum, 1969a). B. subtilis shows peaks of protein synthesis correlating with peaks of transformation frequency (Tichy, 1972). RNA synthesis is usually required in all the systems in that blockage will indirectly suppress protein synthesis. Part of the protein synthesis requirement is due to the production of a "competence factor" by competent cells. This factor is discussed later.

One property of competent cells that is common to several transformation systems is autolysis. Autolysis is found in competent, but not noncompetent cells of D. pneumoniae (Mosser and Tomasz, 1970), Streptococci (Ranhand et al 1971a; Ranhand, 1973) and B. subtilis (Young et al, 1963; Young and Spizizen, 1963; Young et al, 1964; Young, 1966; Horvath, 1968). Poorly transformable strains of B. subtilis show less autolytic activity than highly transformable strains (Young and Spizizen, 1963) and Young (1966) notes that the lytic

TABLE I

FACTORS AFFECTING BACILLUS SUBTILIS TRANSFORMATION

Compound	Site of Action: Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Refererence
acriflavin	I		I	I		Ayad, 1969
chloramphenicol	I					D'Aquino, 1974; Felkner
tetracyclines	I					and Wyss, 1970
streptomycin	S (kills non-competent cells)					Mergeay, 1972
pronase	S					Goldsmith, 1970
nonoptimal generation time	I				I	Horvath, 1968b
aeration decrease	I				I	Ibid.
Actinomycin D	I					Kammen et al, 1966b
puromycin	I	N		N	I	Ibid.
amino acid mixture	S (stabilizes newly transformed cells)					Kammen et al, 1966a
sodium poly-phosphate	S					Kohiyama and Saito, 1960
caffeine	I	N	I		N	Mazza, 1972
EDTA	I	I				Morrison, 1971; Chilton and Hall, 1968
penicillin pre-treatment (cells)	S			N		Nester, 1964

I = inhibition S = stimulation N = no or very little reaction

TABLE I - BACILLUS SUBTILIS - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
phenethyl alcohol	I	I		N		Richardson and Leach, 1967, 1969a; Urban and Wyss, 1969
toluene	N					Richardson and Leach, 1969a
DNA bound protein	S			S		Lostia et al, 1972
membrane associated DNA	S					Snyder
Mg^{+2}	S	S			S	Bott and Wilson, 1967; Stewart, 1968; Bott and Wilson, 1968
histidine	S				S	Bott and Wilson, 1968
tryptophan	S				S	Ibid.
arginine	S				S	Ibid.
valine	S				S	Ibid.
lysine	S				S	Ibid.
threonine	S				S	Ibid.
glycine	S				S	Ibid.; Kohoutova, 1973
aspartic acid	S				S	Ibid.
methionine	S				S	Ibid.
phenylalanine	N					Ibid.
cysteine	N					Ibid.
serine	N					Ibid.
tyrosine	N					Ibid.
glutamic acid	I					Ibid.
alanine	I					Ibid.
leucine	I					Ibid.

TABLE I - BACILLUS SUBTILIS - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
isoleucine	I					Bott and Wilson, 1968
proline	I					Ibid.
D-lysine	I					Ibid.
diaminopimelic acid	I					Ibid.
6-(p-hydroxyphenylazo)-uracil	N					Dubnau and Cirigliano, 1973b
monofluoroacetate	I				I	Felkner and Wyss, 1970
7-azatryptophan	I				I	Ibid,
B-mercaptoethanol	S				S	Anagnostopoulos and Spizizen, 1961; Stewart, 1968
acridine orange	I		I	I		Stewart, 1968
phleomycin	I			I		Ibid.
glucose phosphate	S					Young, 1963
glycerol phosphate	S					Ibid.
potassium phosphate	I					Young, 1963; Stewart, 1968
variation with brand of K_2HPO_4	IS					Horvath, 1972
glycerol	I				I	Bott and Wilson, 1968
succinate as carbon source	I				I	Ibid.
acetate as carbon source	I				I	Ibid.

TABLE I - BACILLUS SUBTILIS - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
Fe^{3+}	I				I	Bott and Wilson, 1968
Mn^{2+}	I				I	Ibid.
potassium sulfate	S				S	Ibid.
sodium periodate	I	I		N		Polsinelli and Barlati, 1967
thymine starvation in Thy$^-$ cells	N					Archer and Landman, 1969
thymine starved DNA	I			I		Sicard and Venema, 1969
a-picolinic acid	N					Bott and Wilson, 1968
mercuric ions	I	I (reversible phase)				Groves et al, 1974
methyl mercuric ions	I	I "				Ibid.
phenyl mercuric ions	I	I "				Ibid.
cadmium	I					Ibid.
dinitrophenol	I	I				Young and Spizizen, 1963
nalidixic acid	N					Pedroni et al, 1972
pyruvate as carbon source	N					Young and Spizizen, 1963
lactate as carbon source	N					Ibid.
glucose as carbon source	N					Ibid.
spermine	S	S				Tabor, 1961

TABLE I - BACILLUS SUBTILIS - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
Ca^{2+}	S				S	Young and Spizizen, 1963
Ba^{2+}	S				S	Ibid.
Sr^{2+}	S				S	Ibid.
Cu^{2+}	I				I	Ibid.
Zn^{2+}	I				I	Ibid.
Ni^{2+}	I				I	Ibid.
nonoptimal pH	I				I	Ibid.
nonoptimal temp.	I				I	McCarthy and Nester, 1969
sucrose	S (concentration dependent)					Peetz, 1966
maltose	S "		"			Ibid.
lactose	S "		"			Ibid.
ribose	S (slight)					Ibid.
glucose	N					Ibid.
electrical stimulation	S					Ibid.
2-deoxy-D-ribose	I					McLemore, 1974
D-ribulose	I					Ibid.
L(-)sorbose	I					Ibid.
glucose-6-phosphate	I					Ibid.
D-glyceraldehyde	S,I (concentration dependent)					Ibid.
D(+)xylose	S,I "		"			Ibid.
D(+)galactose	S,I "		"			Ibid.
L-glucose	S,I "		"			Ibid.
B-D-glucose	S,I "		"			Ibid.

TABLE I - BACILLUS SUBTILIS - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
L-mannose	S,I	(concentration dependent)				McLemore, 1974
D-fucose	S,I	"	"			Ibid.
D(+)glucoseamine HCl	S,I	"	"			Ibid.
isomaltose	S,I	"	"			Ibid.
DLglyceraldehyde	S					Ibid.
D-ribose	S					Ibid.
D(+)mannose	S					Ibid.
L-fucose	S					Ibid.
a-lactose	S					Ibid.
saline	I					Iijima and Ikeda, 1969
oxidized spermine	I			I		Persky et al, 1967
u.v. light	I			I		Ibid.
nitrogen mustards	I			I		Ibid.
mitomycin C	I			I		Ibid.
carzinophillin	I			I		Ibid.
3,4-benzpyrene	I	I				Maher et al, 1971
3,4-bp/9,10-di-methyl-1,2-benzanthracene	I	I				Ibid.
nitric acid	I	I		I		Bresler et al, 1970
dimethylsulfate	I	I		I		Ibid.
hydroxylamine	I	I		I		Ibid.

TABLE I - BACILLUS SUBTILIS - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
growth in $^{15}N^{3}H$ medium	I				I	Laird et al, 1968
cold shock	I	I (irreversible binding)				Strauss, 1970
KCN	I	I "	"			Young, 1963; Strauss, 1970
excess cell wall precursors	I					Spizizen, 1959; Bott and Wilson, 1968
excess competence substance	I					Kohoutova, 1973

TABLE II

FACTORS AFFECTING HEMOPHILUS INFLUENZAE TRANSFORMATION

		Site of Action				
Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
reduced aerobic maintenance	S				S	Goodgal and Herriott, 1961; Miller and Huang, 1972
lactate	S	S (binding)				Miller and Huang, 1970, 1972; Ranhand, 1969, 1970
inosine (added early)	I				I	Ibid.
Inosine (added late)	S	S (binding)				Ibid.
NAD	I				I	Miller and Huang, 1972
glycerol as carbon source	I				I	Ibid.
periodate	I	I (binding)		N		Ranhand and Lichstein, 1966
L-valine	I				I	Spencer and Herriott, 1965
deoxyinosine	S				S	Ranhand and Herriott, 1966
pyruvate	S				S	Ibid.
silver ion	N			N		Wilhelm and Daune, 1969

I = inhibition S = stimulation N = no or very little reaction

TABLE II - HEMOPHILUS INFLUENZAE - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
chloramphenicol	I				I	Goodgal, 1958; Stuy, 1962; Leidy et al, 1962; Spencer and Herriott, 1965; Ranhand and Lichstein, 1969
8-azaguanine	I				I	Ibid.
novobiocin	I				I	Ibid.
erythromycin	I				I	Ibid.
streptomycin	I				I	Ibid.
penicillin	N	reduces viability and				Ibid.
polymyxin	N	competence proportionally				Ibid.
D-cycloserine	N					Ibid.
6-azauracil	N					Ibid.
L-aspartic acid	S				S	Leidy et al, 1962; Spencer and Herriott, 1965
L-cysteine	S				S	Ibid.
L-argenine	S				S	Ibid.
L-glutamic acid	S				S	Ibid.
L-citrulline	S				S	Ibid.
Mg^{2+}	S	S			S	Leidy et al, 1962; Goodgal and Herriott, 1961
Ca^{2+}	S	S			S	Ibid.
EDTA	I	I				Goodgal and Herriott, 1961
bovine albumin	N					Leidy et al, 1962

TABLE II - HEMOPHILUS INFLUENZAE - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
thiamine	N					Miller and Huang, 1972
pantothenate	N					Ibid.
uracil	I				I	Ibid.
dinitrophenol	I	I				Stuy, 1962; Barnhart and Herriott, 1963
arsenate	I	I				Stuy, 1962
high cell conc.	S				S	Goodgal and Herriott, 1961
phosphate	I	I				Ibid.
citrate	I	I				Ibid.
iodoacetate	I	I				Barnhart and Herriott, 1963
sodium azide	I	I				Ibid.
high ionic strength	I					Barnhart and Herriott, 1961, 1963
nonoptimal pH	I					Barnhart and Herriott, 1963
nonoptimal temp.	I					Barnhart and Herriott, 1961
cAMP	S				S(?)	Wise et al, 1973
sodium citrate	I	I				Stuy, 1972
low ionic strength	-	I				Stuy, 1972
inosine + pyruvate	S				S	Ranhand, 1970
inosine + fructose	S				S	Ibid.
inosine + glucose	S				S	Ibid.
inosine + mannose	N					Ibid.
inosine + mannitol	N					Ibid.
inosine + galactose	N					Ibid.

TABLE II - HEMOPHILUS INFLUENZAE - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
inosine + sorbose	N					Ranhand, 1970
inosine + sorbitol	N					Ibid.
inosine + glucose-amine	N					Ibid.
inosine + rhamnose	N					Ibid.
inosine + D(-) arabinose	N					Ibid.
inosine + D(+) arabinose	N					Ibid.
inosine + xylose	N					Ibid.
inosine + fucose	N					Ibid.
putrescine	I	N(cells)		I		Tiesler, 1972
cadaverine	I	I "		I		Ibid.
sperimidine	I	I "		I		Ibid.
protamine sulfate	I	S "		I		Ibid.
histone sulfate	I	I "		I		Ibid.
Rivanol	I	I "		I		Ibid.
proflavin sulfate	I	I "		I		Ibid.
Atebrine	I	I "		I		Ibid.
pacatalhydrochloride	I	I "		I		Ibid.
methylene blue	I	I "		I		Ibid.
Verophenphosphate	I	I "		I		Ibid.
Nobrium	I	I "		I		Ibid.

TABLE II - HEMOPHILUS INFLUENZAE - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
adamantanhydro-chloride	I	I	"	I		Tiesler, 1972
Berberinchloride	I	I	"	N		Ibid.
ethidiumbromide	I	I	"	I		Ibid.

TABLE III

FACTORS AFFECTING DIPLOCOCCUS PNEUMONIAE TRANSFORMATION

	Site of Action					
Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
casein hydrolysate	S	N			S	Fox and Hotchkiss, 1957; Tomasz, 1966, 1970; Tomasz and Mosser, 1966; Lacks and Greenberg, 1973
sucrose (as withheld carbonsource)	I	I			I	Lacks and Greengerg, 1973
Ca^{2+}	S	S			S	Fox and Hotchkiss, 1957; Lacks and Greenberg, 1973
Mg^{2+}	S	S			S	Tomasz and Mosser, 1966; Lacks and Greenberg, 1973
Chloramphenicol	I	N			I	Fox and Hotchkiss, 1957; Tomasz and Mosser, 1966; Tomasz, 1966, 1970
actinomycin D	I		I		I	Tomasz and Mosser, 1966
puromycin	I				I	Tomasz, 1970
rifamycin	I				I	Ibid.
tryptazan	I				I	Ibid.
fluorodeoxyuridine	N					Ibid.

N = no or little reaction S = stimulation I = inhibition

TABLE III - DIPLOCOCCUS PNEUMONIAE - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
iodoacetate	I	I				Tomasz, 1970
sodium azide	I	I				Ibid.
no energy source	I	I				Ibid.
thymidine starvation in Thy⁻ cells	N					Ibid.
uridine	S				S	Tomasz and Mosser, 1966
valine	S				S	Ibid.
leucine	S				S	Ibid.
isoleucine	S				S	Ibid.
glutamine	S				S	Ibid.
choline	S	S				Ibid.
glucose as carbon source	S				S	Bott and Wilson, 1968
sucrose as carbon source	S				S	Ibid.
sodium malonate	N					Ibid.
sodium fluoroacetate	N					Ibid.
ethanolamine	I				I	Tomasz, 1968; Tomasz et al, 1971
N-methyl ethanolamine	I				I	Tomasz, 1968
acridine orange	I			I		Lerman, 1964
serum albumin	S					Fox and Hotchkiss, 1957

TABLE III - DIPLOCOCCUS PNEUMONIAE - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
nonoptimal temp.	I	I				Fox and Hotchkiss, 1957
KCl (0.2M)	S	S				Kohoutova, 1965
NaCl (0.2M)	S	S				Ibid.
D-glucosamine	I (binds to competence substance)					Kohoutova and Kocourek, 1973, 1974
D-galactosamine	I "	"	"			Ibid.
D-glucose	S (only one concentration tested)				S	Ibid.
D-galactose	S "					Ibid.
N-acetyl glucos-amine	S				S	Ibid.
N-acetyl galactos-amine	I					Ibid.
D-fructose	I					Ibid.
D-mannose	S				S	Ibid.
D-arabinose	S				S	Ibid.
L-fucose	S				S	Ibid.
peptides(casein)	S				S	Sirotnak, 1971
albumin	S					Thomas, 1955, 1957
pneumococcal teichoic acid	I				I	Rejholcová et al, 1974
gamma rays	I			I(s.s. breaks)		Thorsett and Hutchinson, 1971
inhibition of high DNase activity	S	S				Kohoutova, 1967a, b

TABLE IV

FACTORS AFFECTING STREPTOCOCCUS SPECIES TRANSFORMATION

Compound	Total Effect	Site of Action: Uptake/ Binding	Integration	DNA	Competence Development	Reference
adenine	I				I	Osowieki and Dobrzanski, 1968
guanine	I				I	Ibid.
cytosine	I				I	Ibid.
uracil	I				I	Ibid.
thymine	I				I	Ibid.
bovine serum albumin (V)	S				S	Pakula, 1965; Ibid.
human serum albumin	S				S	Osowieki and Dobrzanski, 1968
fibrinogen	S				S	Ibid.
human albumin ("Behring")	N					Ibid.
egg whitw albumin	N					Ibid.
protamine	N					Ibid.

S = stimulation I = inhibition N = no or very little reaction

TABLE IV - STREPTOCOCCUS - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
gelaitn	N					Osowieki and Dobrzanski, 1968
DL-serine	S				S	Ibid.
L-glutamine	S				S	Ibid.
DL-cysteine	S				S	Ibid.
swine serum	S				S	Lawson and Gooder, 1970
D-cycloserine	I				I	Perry, 1972
peptides	S				S	Pakula and Iler, 1969
sulfhydryl reagents	I				I	Ranhand, 1974a
sodium metaperiodate	I	I				Ranhand, 1974b
cationic detergents	N	I				Deddish and Slade, 1971
SDS	N	N				Ibid.
excess DNA	I (cells)					Piechowska and Shugar, 1967

TABLE V

FACTORS AFFECTING MISCELLANEOUS TRANSFORMATION SYSTEMS

Compound	Total Effect	Site of Action: Uptake/ Binding	Integration	DNA	Competence Development	Reference
			NEISSERIA MENINGIDITIS			
ethidium bromide	I	I				Jyssum and Gunderson, 1971
acriflavin	I	I				Ibid.
hydroxyurea	N					Jyssum
nalidixic acid	N					Ibid.
chloramphenicol	N					Jyssum and Gunderson, 1971
			MICROCOCCUS LYSODEIKICUS			
$MgSO_4$ $\leq 10^{-2}$M	S	($>10^{-2}$, I)			S	Kloos, 1969
$CaCl_2$ "	S	"			S	Ibid.
$BaCl_2$ "	S	"			S	Ibid.
$SrCl_2$ "	S	"			S	Ibid.
			BACILLUS LICHENIFORMIS			
phosphate	S				S	Throne and Stull, 1966
glycerol	S				S	Ibid.
glucose	I				I	Ibid.

S = stimulation I = inhibition N = no or very little reaction

TABLE V - MISCELLANEOUS - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
			PASTEURELLA NOVICIDA			
Ca^{2+}	S				S	Tyeryar and Lawton, 1970
Mg^{2+}	N	(with no Ca^{2+} present)				Ibid.
Sr^{2+}	N	"	"	"		Ibid.
Ba^{2+}	N	"	"	"		Ibid.
			STAPHLOCOCCUS AUREUS			
nonoptimal pH	I				I	Rudin et al, 1974
nonoptimal temp.	I				I	Ibid.
Ca^{2+}	S				S	Ibid.
Ba^{2+}	S	($Ca^{2+} \gg Ba^{2+} = Mg^{2+}$)			S	Ibid.
Mg^{2+}	S				S	Ibid.
Fe^{2+}, Fe^{3+}	I				I	Ibid.
trypsin	S					Markov et al, 1973
pepsin	I					Ibid.
E-30	I					Ibid.
EDTA	N					Ibid.
SDS	S (slight)					Ibid.
gentian violet	I (slight)					Ibid.
basic fuchsin	I					Ibid.
			PSEUDOMONAS AERUGINOSA			
agitation	I					Khan and Sen, 1967
starvation	I					Ibid.

TABLE V - MISCELLANEOUS - Contd.

Compound	Total Effect	Uptake/ Binding	Integration	DNA	Competence Development	Reference
			RHIZOBIUM TRIFOLII			
alanine	S				S	Drozänska and Lorkiew, 1973
glycine	S				S	Ibid.
serine	S				S	Ibid.
			ESCHERICHIA COLI			
$CaCl_2$	S					Cosloy and Oishi, 1973

activity increases during the development of competence. Stewart (1970) feels that the increase in lytic activity is DNA induced since DNase halts lytic activity. The site of autolytic degradation is limited to the equatorial region in Streptococcus (Shockman et al, 1967) and in pneumococcus (Tomasz et al, 1971; Seto and Tomasz, 1975).

Some of the general characteristics of competent cells in the B. subtilis transformation system are:

a) The competent state is not associated with a particular phase of the cell division cycle (Vermeulen and Venema, 1974).

b) Cultures inoculated with different numbers of bacteria give different rates of appearance of competence (Horvath, 1967).

c) Competence is associated with a resistance to the bactericidal action of penicillin (Nester and Stocker, 1963; Nester, 1964; McCarthy and Nester, 1967; Singh and Pitale, 1974).

d) The cells have an increased heat sensitivity (McDonald, 1970; Archer, 1973).

e) The cell surface develops an increased negative charge as measured by methylene blue adsorption and membrane filter fractionation (Jensen and Haas, 1963a, b).

f) There is maximum binding of proteins such as anti-DNA antibody, DNase, cytochrome C and serum albumin to the cell surface (Erickson et al, 1969).

g) The cell wall composition changes (Young et al, 1963) as does the membrane morphology (Wolstenholme et al, 1966).

h) The cells contain more galactosamine (Young et al, 1964) which reaches peak concentration at the time of maximum competence (Young, 1965).

i) In general, competent cell walls are weaker than noncompetent cell walls (Miller et al, 1967).

j) DNA synthesis decreases before the point of maximum competence (Dooley et al, 1971) and appears to stop at the competence peak (Barlati, 1972).

k) Newly transformed cells have reduced DNA, RNA, but not protein synthesis (McCarthy and Nester, 1967; Laird et al, 1968; Erickson, 1970; Dooley et al, 1971).

l) Competent cells release DNA, RNA and protein into the culture medium (Sinha and Iyer, 1971) and can be transformed by excreted DNA (Streips and Young, 1974).

m) Abortive transformation may occur if a lesion in the histidine pathway exists (Jensen, 1968).

n) The competence peak varies with time in relation to the type of DNA involved in uptake. The more homo-

logous the DNA, the earlier the competence peak (Epstein, 1968).

o) The competent cells lose their photoreactive capacity (Kelner, 1964).

p) Competence may be maintained at a high level in continous culture (Eichhorn et al, 1968; Lopez et al, 1970).

Some characteristics of competent D. pneumoniae are:

a) Less DNA is synthesized and less lysine (a cell wall constituent) is incorporated than other amino acids (Ephrussi-Taylor and Freed, 1964).

b) The cells leak DNA (Ottolenghi and Hotchkiss, 1962) and other intramembrane cell components (Seto and Tomasz, 1975).

c) Acid conditions cause the competent cells to agglutinate (Tomasz and Zanati, 1971) and form clumps (Tomasz, 1966).

d) The cells maintain normal K^+ pump operation (Tomasz, 1971).

e) Competence involves an increased heat sensitivity (Lack and Greenberg, 1973).

f) A DNase activity is present which is optimally low in activity in highly transformable strains and high in strains that show unstable transformation

(Kohoutova, 1967a).

g) Competence seems to result from cyclical processes other than cell division (Hotchkiss, 1954).

h) The replacement of choline by ethanolamine in the cell wall removes autolysis and transformability (Tomasz, 1968).

Competent Streptcocci demonstrate the following characteristics:

a) There is a one protein difference between competent and noncompetent cells that is not due to the competence substance (Ranhand et al, 1970).

b) Less DNA and more protein is synthesized, and a different rate of growth is apparent, as is penicillin resistance (Pakula and Spencer, 1971).

c) Competent cells have a reduced capacity to absorb methylene blue (Pakula et al, 1970).

d) Spontaneous release of DNA occurs (Ceglowski and Dobrzanski, 1974).

e) The cells are no longer receptive to competence factor after the competence peak has passed (Pakula, 1965a).

f) Acid conditions cause a high precipitability (Pakula et al, 1970) although Ceglowski and Dobrzanski (1974) show data indicating a strain in which both

competent and noncompetent cells will agglutinate at low pH.

Nontransformable B. licheniformis is rendered transformable by transducing into its genome a glutamyl polypeptide minus marker from a transformable strain (McCuen et al, 1971).

The change from competency to noncompetency in N. meningitidus involves a change of replication origin as well as direction of replication (Jyssum, 1969b) and some strains demonstrate an easily lost competence, perhaps indicating cytoplasmic inheritance (Jyssum and Lies, 1965; Jyssum, 1966). This is confirmed in that competence is genetically lost upon treatment with acriflavin or ethidium-bromide (Jyssum, 1971; Jyssum and Gundersen, 1971).

Bøure and Frøholm (1971) find much higher transformation frequencies in strains of Moraxella nonliquifaciens, Mycobacterium bovix and Neisseria gonorrhoreae which have fimbriae.

Lindberg and Novick (1973) note an apparently plasmic specific transformation on Staphlococcus aureus.

The number of competent cells in a population is difficult to determine. Bouyant density studies in Renografin gradients (Cahn and Fox, 1968; Hadden and Nester, 1968),

electron microscopy (Javor and Tomasz, 1968) and autoradiography (Somma and Polsinelli, 1970) all indicate that only about 20% of B. subtilis cells are actually transformable or transfectable. H. influenzae appears to be about 95% competent by ultra-violet sensitivity (Beattie and Setlow, 1969). Competent B. subtilis cells may be separated from noncompetent cells because they are less dense. Singh and Pitale (1967, 1968) indicate that this is due to the competent cells being uninucleate. This is confirmed to a certain extent by the single target killing of competent B. subtilis as opposed to the multitarget killing of noncompetent cells (Epstein, 1971). Hadden and Nester (1968) indicate that water permeability or production of mesosomes might cause the density difference. Competent B. subtilis sediments more slowly on sucrose density gradients than do noncompetent cells (Richardson and Leach, 1969b).

Substances termed "competence factors" or "competence substances" have been found in the cultural filtrates of competent cells of various species. These substances enable noncompetent cells to undergo genetic transformation. The D. pneumoniae factor is sensitive to proteolytic enzymes, insensitive to lysozyme, RNAase, DNAase; is relatively heat resistant and has a molecular weight of about 10,000 (Tomasz

and Hotchkiss, 1964; Tomasz, 1965a,b, 1966; Kohoutova et al, 1968). The substance is produced in proportion ot the number of cells present, has its activity increased by mercaptoethanol and causes DNA to bind irreversibly (Tomasz and Mosser, 1966). It appears to induce leakage of intramembrane components (Seto and Tomasz, 1975). The substance appears free in the medium (Tomasz and M-sser, 1966) and contains an inactivating activity toward transforming DNA (Kohoutova et al, 1968, 1970; Lipavska et al, 1972). Lacks and Greenberg (1973) feel that the DNA inactivating activity is located on the surface of the cell. The proteolytic inhibition of the substance does not affect cells already competent. Pneumococcal competence substance does not act on Hemophilus or Bacillus, but will act on Streptococcus viridans (Tomasz, 1965).

The B. subtilis competence substance is heat labile, protease sensitive, polypeptide in nature, and will cause noncompetent cells to become competent (Charpak and Dedonder, 1965; Akrigg et al, 1967a,b; Pariiskaya and Pukhova, 1967; McCarthy and Nester, 1969). Ayad and Shimmin (1974) show that the factor exists in two forms, one active and one inactive. The inactive form may be activated by freeze-thawing. Goldsmith et al (1970) feels that the substance is surface bound since the cells must be washed, i.e. osmotically

shocked, to remove it. Cell walls are degraded by the substance. Analysis shows that the lytic factor is identical with the autolytic enzyme N-acetylmuramyl-L-alanine amidase located in the cell wall (Akrigg and Ayad, 1969, 1970; Ayad and Shimmin, 1973). A nuclease activity is also present (Ayad and Shimmin, 1973), which digests one strand of a DNA duplex, and is associated with mesosomal vesicles (Ayad and Shimmin, 1974). Joenje et al (1974, 1975) and Joenje and Venema (1975) also show exonuclease activity associated with membrane vesicles. Joenje et al (1972) isolated a competence substance apparently different from the lytic competence enhancing factor since it is relatively resistant to heat, repeated freeze-thawing and to protease activity.

Competent Bacillus cereus cells produce a substance that causes the cells to bind DNA irreversibly. Cells with the substance bind more methylene blue than washed cells (Felkner and Wyss, 1964).

The competence factor of Bacillus stearothermophilus alters the sedimentation properties of the cells when bound and appears to interact with membranes as well as whole cells. This interaction with membrane appears to be the first, and rate limiting, step leading to irreversible DNA binding (Streips and Young, 1971). There is no lytic activity

associated with this substance and there are noncompetent mutants which do not release the substance (Streips and Welker, 1971).

The Hemophilus influenzae competence substance is dialyzable, insensative to RNase, DNase, proteolytic enzymes and only slightly affected by heat. Puromycin inhibits production of the substance (Barnhart, 1967).

Competent Streptococci produce a substance which provokes competence in noncompetent cells (Pakula and Walczak, 1963) and is protease labile, temperature, RNase and lysozyme resistant (Dobrzanski and Osowiecki, 1967; Ranhand et al, 1971b; Leonard et al, 1970; Leonard and Cole, 1972). Studies of the purified substance show it to be a basic protein of about 5,000 molecular weight with no lipids, phosphorus, carbohydrates or sulfur containing amino acids (Osowieki et al, 1969; Leonard and Cole, 1972). Glutamate is required for competence development but not competence factor production. Its activity is destroyed by antiserum to competent cells (Leonard et al, 1970) and the factor is transmitted as a genetic package (Nalecz et al, 1970; Pakula et al, 1972). A DNA inactivating factor is also present which is not affected by proteolytic enzymes, RNase or lysozyme, but is inhibited by citrate and EDTA (Nalecz and

Dobrzanski, 1972). The addition of purified competence substance to noncompetent streptococci does not inhibit overall growth or rates of DNA and protein synthesis, but does reduce the rates of synthesis of RNA and peptidoglycan (Horne and Perry, 1974).

In pneumococcus (Ziegler and Tomasz, 1970) and in Streptococcus (Perry, 1974) the respective competence factors have been shown to bind specifically to sites on the cell membrane. From the data of Landman and Knott (1968), the same result may be inferred for B. subtilis. The binding sites on the Streptococcal membrane are very sensitive to SDS, chloroform-methanol, sodium metaperiodate and phospholipase D, suggesting that they consist of lipid, carbohydrate and protein (Perry, 1974). Kohoutova and Kocourek (1973, 1974) show that D-glucosamine and D-galactosamine will compete with the cell binding sites for competence substance in pneumococcus. Phytohemagglutinins will also inhibit competence development. This might indicate the presence of aminated sugars at the receptor sites.

The binding of competence substance to the intact cell membrane is a reversible one and is not sufficient in itself to make noncompetent cells competent, since at low pH, competence substance will bind but competence expression is

sharply reduced (Ziegler and Tomasz, 1970).

Leonard (1973) suggests that a competence substance-membrane complex may function to bind DNA. In B. subtilis, the competence factor does not alter the binding of DNA to membrane vesicles (Joenje et al, 1974), while in B. stearothermophilus the competence factor seems to induce affinity for DNA binding to membranes (Streips and Young, 1971).

Rabbits injected with competent pneumococci (Nava et al, 1963; Tomasz and Beisen, 1965), Streptococci (Pakula, 1965), N. catarrhalis (Snyder and Otero, 1970), H. influenzae (Bingham and Barnhart, 1973) or B. subtilis (Erickson et al, 1969) produce serum containing a low titer of antibodies specific for antigens on the surface of the competent cells. This antibody will inhibit the transformation process, whereas antibody to noncompetent cells will not. Antibody against Streptococcal competence factor will also inhibit DNA uptake (Pakula et al, 1970). Competent cell antibody will block competence substance binding to noncompetent cells made competent by the addition of competence substance (Pakula, 1967). Tomasz and Beiser (1965) conclude that the competence substance in pneumococcus induces the appearance of the "competence" antigen.

The addition of transforming DNA to a culture of competent cells results in the initial binding of a quantity of

DNA proportional to the competence of the culture (Fox and Hotchkiss, 1957; Lerman and Tolmach, 1957; Green, 1964; Levine and Strauss, 1965). This initial binding is rapid and reversible in that bound DNA may be removed by washing or by DNAase action (Fox and Hotchkiss, 1957; Lerman and Tolmach, 1957; Levine and Strauss, 1965). Since the bound DNA can be removed from the cell surface by shear forces, it may be concluded that the DNA is extended spatially with a terminal attachment rather than bound as a coiled mass (Dubnau and Cirigliano, 1972b). Kinetic studies in B. subtilis indicate that by terminating transformation by washing, transformants appear linearly, while if termination is by DNAase, transformants appear nonlinearly. This lag period before the appearance of transformants in the DNAase studies is independent of the marker studied, independent of the DNA concentration, increases with decreasing temperature (Levine and Strauss, 1965) and increases with assays for linked markers and for markers of increasing map units apart (Strauss, 1965, 1966). This phenomenon in B. subtilis, pneumococcus (Gabor and Hotchkiss, 1966) and N. meningitidis (Jyssum, 1969c) is interpreted to mean that the DNA molecule enters the cell in a lengthwise manner. Electron microscopy of pneumococcus undergoing genetic transformation visualized

this longitudinal entry of the DNA molecule (Tomasz and Stoeckenisus, 1963).

The DNA is next bound irreversibly, as defined by DNAase and washing insensitivity. The rate at which the DNAase insensitive setp is attained is temperature dependent in B. subtilis (Leviňe and Strauss, 1965) and in Streptococcus (Ravin and Ma, 1972), thus indicating possible enzyme involvement. Arrhenius plot calculations result in an activation energy of 13.9 kcal. for the reversible to irreversible binding reaction (Levine and Strauss, 1965).

The salt concentration optimum for reversible uptake is lower than that of irreversible uptake, perhaps indicating an ionic interaction in the reversible step (Barnhart and Herriott, 1963).

Studies in B. subtilis show that if competent cells have their walls removed through lysozyme action, no transformation will occur, but if the protoplasts thereby formed are allowed to start reverting to bacillary form, i.e., form some cell wall material, transformants will appear (Peetz, 1966; Tichy and Kohoutova, 1968; Tichy, 1968; Tichy and Landman, 1969; Wilson and Bott, 1970; Miller, 1972). D. pneumoniae also shows this phenomenon (Tomasz, 1972). Treatment with limited amounts of lysozyme, which only partially removes the cell wall, results in the stimulation of com-

petence (Prozorov, 1965; Hirokawa and Ikeda, 1966; Tichy and Landman, 1969; Wilson and Bott, 1970).

Microscopic autoradiography indicates that DNA uptake appears centered around the growing points of the cell in B. subtilis and pneumococcus (Javor and Thomas, 1968; Tomasz et al, 1971). Substitution of ethanolamine for choline into competence medium rapidly inhibits competence. Thus, on a biochemical basis, Wagner (1964) and Briles-Bavak and Tomasz (1970) postulate that DNA enters a region of the cell where growth is occurring.

Hotchkiss (1954) and Miller and Landman (1965) first suggested that mesosomes might be involved in the transformation process. Electron microscopic autoradiography of B. subtilis shows that a majority of the disintegration tracts from labeled DNA appears associated with mesosomal material during uptake (Wolstenholme et al, 1966; Vermuelen and Venema, 1974a,b) and after uptake (Wolstenholme et al, 1966; Piechowsak and Fox, 1971; Harris and Barr, 1971; Dooley and Nester, 1973; Awert and Venema, 1973; Vermeulen and Venema, 1974a,b). The same technique also shows that mesosomes in B. subtilis increase in number and/pr type near or in the phase of maximum competence (Wolstenholme et al, 1966; Vermeulen and Venema, 1974a,b). Nuclear material has long been known to be attached to membrane material (Ryter, 1968;

Ivarie and Pene, 1973). Fractionation of competent B. subtilis cells immediately after uptake indicates that the donor DNA is bound to the membrane (Young, 1967; Harris and Barr, 1971). Ayad et al, (1973) and Joenje et al (1974, 1975) show data suggesting that DNA binds preferentially to mesosomal vesicles from competent B. subtilis rather than noncompetent vesicles. Tichy and Landman (1969) show evidence indicating that B. subtilis cells without mesosomes cannot undergo genetic transformation. The DNA apparently does not bind to cell walls of B. subtilis (Joenje et al, 1974), while it does bind to cell walls of Streptococcus (Deddish and Slade, 1971). Pneumococcus shows binding of DNA to the membranes of competent cells only. However, if the cells are physically damaged mechanically or enzymatically, noncompetent cells will bind DNA and competent cells will bind even greater amounts of DNA (Seto et al, 1975). This indicates that hidden DNA binding sites might exist. Irreversible binding is characterized by stabilization, due to the presence of glucose (Seto et al, 1975). Isolated walls and protoplasts of competent pneumococci do not bind DNA appreciably)Seto et al, 1975). Seto et al (1975) postulates that the DNA binding sites in pneumococcus are membrane components, perhaps protein, stabilized by polysaccharide, perhaps cell

wall material. In Streptococcus, Ranhand (1974b) indicates that the DNA binding sites contain amino acids.

Estimates of the number of DNA uptake sites are vague at best. D. pneumoniae appears to have 30-80 sites (Fox and Hotchkiss, 1957), H. influenzae 2-8 (Stuy and Stern, 1964; Stachura et al, 1968; Scocca et al, 1974) and B. subtilis 20-50 (Singh, 1972). Somma (1970) indicates that a competent B. subtilis cell at saturating DNA conditions can fix 3-6 molecules of molecular weight 1.6×10^7, which equals the irreversible binding of 0.9×10^{-10} to 1.4×10^{-10} ug of DNA, while Bresler et al (1963) suggests the incorporation of 500-600 molecules of molecular weight $6-8 \times 10^6$, equaling approximately 6×10^{-15} g DNA. Each competent H. influenzae cell can take up 5-7 molecules of DNA of molecular weight approximately $1,5 \times 10^7$ (Barnhart and Herriott, 1963) and D. pneumoniae may take up more than one bacterial DNA equivalent (Fox, 1957).

Transformation in Streptococci can be "helped" by saturating concentrations of nontransforming DNA in the presence of limiting concentrations of transforming DNA (Chen and Ravin, 1966; Chen, 1971). This phenomenon is thought to be due to the presence of some component of the neopeptone transformation medium which aggregates the transforming DNA.

The addition of excess nontransforming DNA frees the transforming DNA from the aggregate (Metzer and Ravin, 1972).

Penetration of the cell wall and penetration of the cell membrane appear to be two separate steps. EDTA can inhibit selectively the intracellular transport of DNA without altering the normal binding to receptors (Seto and Tomasz, 1974). Cyanide inhibits transformation in B. subtilis after irreversible DNA binding, suggesting that only the membrane transport step is energy-dependent (Strauss, 1970).

Following binding, random double strand clavage occurs in B. subtilis (Dubnau and Cirigliano, 1972a; Awert and Venema, 1973) and pneumococcus (Morrison and Guild, 1973), resulting in fragments which are external to the membrane and accessible to pancreatic DNAase (Dubnau and Cirigliano, 1972a). These fragments are precursors of the fragments which will be integrated into the host genome (Dubnau and Cirigliano, 1972b; Davidoff-Abelson and Dubnau, 1973b). One or two minutes after adding DNA to competent cells, single stranded DNA is recoverable from the cell interior (inaccessible to external DNAase) in B. subtilis (Harris and Barr, 1969, 1971; Piechowska and Fox, 1971; Davidoff-Abelson and Dubnau, 1973a,b) and in pneumococcus (Lacks,

1962; Lacks and Carlson, 1967; Morrison and Guild, 1973). This conversion to single strand configuration is termed the "eclipse phase" in pneumococcus (Fox, 1960) and in B. subtilis (Venema et al, 1965) in that extraction of this DNA results in very little biological activity and if labeled donor DNA is utilized, one half of the label appears in acid soluable form in pneumococcus (Lacks, 1962) and B. subtilis (Dubnau and Cirigliano, 1972a; Lacks and Greenberg, 1973; Morrison and Guild, 1973). A protein binding activity that seems to protect single strand DNA from nuclease attack is present in competent B. subtilis cultures (Eisenstadt et al, 1975). Isolated vesicles of B. subtilis have a double stranded endonuclease on the surface which causes the appearance of similar "eclipse DNA" (Joenje et al, 1974). No eclipse phase is noted in H. influenzae (Stuy, 1965; Voll and Goodgal, 1965)or H. parainfluenzae (Notani and Setlow, 1972). No significant amount os single stranded donor DNA is found and no label in acid soluble form (Notani and Goodgal, 1966), but LeClerc and Goodgal (1974) and LeClerc and Setlow (1975) find that the DNA is partially single stranded following uptake. No single stranded DNA or acid soluble products are found in N. meningitidis (Jyssum et al, 1971).

A size dependent degradation occurs immediately after entry and before integration. In D. pneumoniae, clevage of larger donor molecules is greater than cleavage of smaller donor molecules. The cleavage approaches zero at 7.7×10^4 daltons (Morrison and Guild, 1972b). It is not clear if this size is limiting in integration or some preceeding step. Above this size, the probability of integration increases with DNA length in D. pneumoniae (Cato and Guild, 1968), H. influenzae (Nicolaieff and Chevallier, 1970) and B. subtilis (Morrison and Guild, 1972a). Guild et al (1968) demonstrates than random independent switching events occur with a definite frequency per unit length of DNA. The probability of integration of a marker rises from 0 to 50% as length increases.

The donor DNA that is integrated into the recipient DNA is single stranded (Fox and Allen, 1964; Bodmer and Ganeson, 1964; Notani and Goodgal, 1966), but the mechanism of the integration process is not known. The donor DNA is physically inserted into the recipient chromosome by pairing with a homologous recipient strand (Bodmer and Ganeson, 1964; Pene and Romig, 1964; Harris and Barr, 1969; Ayad and Barker, 1969; Dubnau and Davidoff-Abelson, 1971) and either strand of the recipient DNA is capable of pairing with an

appropriate donor DNA strand (Louarn and Sicard, 1969). Once the single strand stage is reached after uptake, about 75% of the DNA becomes associated with recipient DNA in B. subtilis (Davidoff-Abelson and Dubnau, 1973b). Early in the integration process the donor recipient complex possesses single strans breaks which are later repaired (Dubnau and Davidoff-Abelson, 1971). The complex appears to be non-covalently bonded in B. subtilis (Dubnau and Davidoff-Abelson, 1971; Dubnau and Cirigliano, 1973a) and in Hemophilus (LeClerc and Setlow, 1974). Erickson and Braun (1968) and Erickson and Copeland (1972) feel that integration occurs at the DNA replication point, but much evidence exists against this hypothesis in that transformation readily occurs with DNA replication inhibited chemically or mutationally (Laird et al, 1968; Archer and Landman, 1969; Hadden, 1973 for example). Membrane fractions from B. subtilis are enriched with recombinant DNA, suggesting that pairing or recombination might occur on or close to the membrane (Dooley and Nester, 1973).

Transformation is inhibited by the presence of prophage in Streptococcus (Parsons et al, 1973; Pakula et al, 1973) and B. subtilis (Peterson and Rutberg, 1969; Yasbin and Young, 1972; Yasbin et al, 1973; Garro, 1974; Garro and Law, 1974; Yasbin et al, 1975). There appears

to be less irreversibly bound DNA. The data indicates either an alteration of the cell surface or a differential inactivation of bacterial genes after lysogeny begins or after prophage derepression. Transformation in H. influenzae is not altered by the presence of prophage (Setlow et al, 1973), while Staphlococcus aureus can be transformed only in the lysogenic state (Rudin et al, 1974).

Mutant strains of transformable species exist with lesions at various points along the transformation process. These have been utilized to study the various portions of the binding-uptake-integration scheme. The types of mutations in the various transformation systems are as follows:

H. influenzae -

a) no transfer of donor DNA to recipient DNA (Notani et al, 1972; Postel and Goodgal, 1972a)

b) donor DNA remains degraded after uptake (Notani et al, 1972)

c) cells transform well with denatured DNA but will not bind native DNA (Caster et al, 1970)

d) cells bind neither denatured nor native DNA (Caster et al, 1970)

e) cells bind both denatured and native DNA, but donor DNA is integrated poorly (Caster et al, 1970; Postel and Goodgal, 1972b)

f) cells bind denatured but not native DNA, but transformation frequency is very low (Caster et al, 1970)

g) cells bind native DNA but no transformation occurs (Postel and Goodgal, 1972a)

h) cells form only slightly fewer recombinant molecules, but transformation is severly reduced (Kooistra and Venema, 1970)

i) cells transform better for simple markers than for complex or linked markers (Caster and Goodgal, 1972)

B. subtilis -

a) cells cannot be transformed with bacterial DNA nor transfected with phage DNA (Polsinelli and Fiettam, 1970)

b) no transformation with bacterial DNA, but could be transfected with phage DNA (Polsinelli and Fietta, 1970)

c) cells have normal donor-recipient complex, but decrease in donor marker replication (Davidoff-Abelson and Dubnau, 1971)

d) normal levels of transformation in cells with APTase dependent nuclease activity removed (Dubnau et al, 1973)

e) normal levels of transformation in DNA polymerase I deficient mutant (Laipis and Ganesan, 1972; Hadden, 1973)

f) cells with rec 1 lesion transformed 5% of control and with rec 2 lesion transformed 25% of control (Hoch et al, 1967)

g) competent cells do not excrete DNA, RNA or protein, also do not transform (Sinha and Kyer, 1971)

D. pneumonia -

a) mutant lacking ATP dependent DNAase transforms with 1/6 efficiency of control, but forms the normal amount of donor-recipient complexes (Vovis and Buttin, 1970; Vovis, 1973)

b) no transformability change in cells lacking endonuclease and native DNA exonuclease activity (Lacks, 1970)

c) mutant does not require competence substance when grown in dilute culture or in presence of trypsin (Lacks and Greenberg, 1973)

d) cell binds DNA reversibly, but has entry blocked (Lacks et al, 1974)

e) mutant with binding blocked (Lacks et al, 1974)

f) removal of major DNA endonuclease activity blocks entry (Lacks et al, 1975)

Streptococcus -

a) three different nontransformable strains that still produce active competence substance (Pakula et al, 1971)

b) normally nontransformable strains harbored transformable mutants (Starosciak et al, 1972)

Neisseriae -

a) mutants with low transformability which will give wild type transformation frequencies if competence substance is added externally (Rytir and Tichy, 1972)

This research problem will deal with sugar stimulation of transformation, therefore a survey of this particular field is in order.

The interaction of D-glucosamine and D-galactosamine with the competence substance is reported by Kohoutova (1973) and Kohoutova and Kocourek (1974). These same authors also briefly mention the stimulation of transformation by various hexoses, but neglect giving any experimental particulars or data. Lopez et al (1972), working with competent B. subtilis in a chemostat, mentions briefly that increases in transformation frequency are higher if the cells are grown in a medium isotonic with respect to pseudo-spheroplasts osmotic requirements. Taketo and Kuno (1969) find

that an E. coli strain becomes susceptible to DNA transfection if plasmolyzed in 23% sucrose and 0.6% polyvinylpyrolidone. Finally, Kingsvik and Santilli (1970) find that the infectivity of tobacco mosaic virus RNA, not the virion, is increased in the presence of sucrose. The sucrose is sprayed on the leaves with the RNA. They feel that the sucrose aids in the cell penetration phase of the infectuous process.

Statement of Problem

This problem involves a study of the concentration dependent stimulation of genetic transformation in bacteria by certain sugars. Sucrose will be utilized as a model sugar, mainly because the stimulation effect was discovered when using sucrose, and sucrose is used quite freely in a microbiology laboratory as a seemingly inert osmotic buffer and for gradients in separation techniques and for concentrating various preparations.

Descriptive parameters will be ascertained, such as required exposure times, kinetic studies, preincubation studies, physical/chemical characteristics, etc., in the hope that a tentative site of action will appear. The sites of action are limited to the cell and/or medium and/or DNA. Once a tentative site of action is determined, experiments will be designed with the idea of pinpointing the mechanism of action.

It is hoped that the study of this stimulation phenomenon, or perhaps the phenomenon itself, will aid in solidifying the tenuous knowledge concerning the transport of informational molecules into living cells rather than adding to it.

Part II

MATERIALS AND METHODS

Organisms

The strains of *Bacillus subtilis* utilized were:

1) strain W23, prototrophic, as the DNA donor
2) strain 168, auxotrophic for adenine (ade^-), leucine (leu^-) and mathionine (met^-), as the DNA recipient

Chemicals

All chemicals utilized were reagent quality or better. Sugar reaction concentrations were made by dilution of sterile, concentrated stock solutions directly into the transformation medium. Where solubility limitations precluded the preparation of concentrated stock solutions, the sugars were dry sterilized by heat or by ultra-violet light.

Media

Cultures were maintained on tryptose blood agar base slants. Strains were streaked for isolation on tryptose blood agar base plates 24 hours prior to utilization in an experiment.

All chemically defined media were based on the minimal salts medium of Anagnostopoulos and Spizizen (1961)

TABLE VI

MEDIA SUPPLEMENTS FOR BACILLUS SUBTILIS 168 $A^{-}L^{-}M^{-}$

Supplement	S-2H	S-3H
Adenine	50 ug/ml	5 ug/ml
Leucine	25 ug/ml	5 ug/ml
Methionine	25 ug/ml	5 ug/ml
Casamino acids	.02g/100ml	.01g/100ml
a, a' - di - pyridal	--	20 ug/ml

with modifications as per Levine and Strauss (1965) and bacterial strain requirements (see Table VI). Solid media consisted of chemically defined media plus 1.5g purified agar per 100 ml.

Controls

Purity of bacterial strains was tested routinely utilizing typical biochemical and morphological tests.

Controls were made to determine any spontaneous reversion of the auxotrophic markers to prototrophy, sterility of the media, glassware and dilution blanks and any adverse effects of the various supplements on cell viability.

DNA Extraction

The phenol extraction procedure of Saito and Miura (1963) was modified as follows: cell lysis was accomplished by suspending the washed cell pellet in 30 ml. of NaCl-EDTA containing sucrose (20g/100ml) and lysozyme (100ug/ml). After incubation of 45 minutes at 37°C, lysis was accomplished by the addition of tris-SDS buffer as called for in the method. For control purposes, some cell sampels were lysed by freezing and thawing as called for in the method since sucrose effects were to be studied. The final DNA solution was stored over chloroform at 4°C and dialysed against 100 volumes of minimal medium for 24 hours at 4°C before use.

Chemical Determinations

DNA concentration was determined by the technique of Burton (1956), RNA by the orcinol reaction (Brown, 1946) and protein by the procedure of Lowry (1957).

Plating Procedures

In the interest of plating speed, a drop plate method (Miles and Misra, 1938) was utilized with the following modification: disposable plastic tuberculin syringes, with the finger tabs removed, were fitted with 22 gauge, 1 inch hypodermic needles and rubber squeeze bulbs. Each syringe-needle unit was then calibrated for drop volume utilizing supplemented minimal medium. The density of this medium was determined by using a pygnometer. Drop volume ranged from 0.0117ml to 0.0133ml. Repeated cleaning and autoclaving at 10 psi, 110°C, did not alter drop volume significantly.

Agar preparation was critical for adequare results using the drop plate method. Plates were dried in a 37° incubator for 24 hours with the lids closed or 4 hours with the lids open. On a properly dried plate, a drop would spread to a circle roughly 8mm in diameter. Up to 36 drops could be placed in a standard petri dish, and up to 15 large colonies and 50 micro-colonies could be counter per drop. A minimum of 6 drops per sample were plated.

A statistical analysis of the drop plate method as compared to other plating methods may be found in the literature (Badger, 1960) and Joys and King (1967) state that no difference in transformation frequency was noted in using a drop method versus smear plate method.

All experiments were repeated at least one time on separate days.

Transformation and Assay Procedures

The transformation procedure was that of Levine and Strauss (1965). DNA was added to a final concentration of 50ug/ml to competent cells obtained by the above procedure. This mixture was allowed to incubate at 37°C in a shaking water bath for 30 minutes at which time DNase (prepared in 5uMolar $MgSO_4$ and filter sterilized) was added to a final concentration of 50ug/ml. This mixture was then incubated for 10 minutes at 25°C, then serially diluted in minimal salts medium and the appropriate dilutions plated on solid medium to indicate the genetic transformation of the cells from auxotrophy to prototrophy with respect to each of three genetic markers, combinations of two of the three markers, or all three markers. Viable counts were made by plating the appropriate dilution on minimal medium containing the three auxotrophic requirements. Plates were incubated at 37°C for 24 hours to 36 hours before counting.

Transformants were scored as a frequency in cells/ml, as percent transformation $\left(\frac{\#\text{transformants}}{\#\text{viable cells}} \times 100\right)$, or as a ratio equal to $\frac{\text{modified transformation frequency}}{\text{unmodified transformation frequency}}$.

If the effects of various substances on the transformation process were to be determined, these substances were added to the competent cells at the same time as the DNA or as per experimental requirement.

Kinetic studies were made by using DNA at less than saturating conditions and withdrawing samples from a large volume of reaction mixture at the required time intervals and placing the samples into tubes containing DNase.

Studies involving pH changes were accomplished by dialysing the DNA against dialysate of the appropriate pH and altering the pH of the competent cell culture by means of sterile H_2SO_4 or KOH, at 1.0M each, as required. Sulfate and potassium ions are normal components of the transformation medium.

Changes in ionic strength were made by the addition of sterile K_2SO_4. Calculation of ionic strength (I) was made by use of the formula

$$I = \frac{1}{2} \sum_i C_i Z_i^2$$

where C_i is the concentration of each ion i and Z_i is the charge on each ion i.

Methylene blue adsorption studies involved the suspension of a cell pellet in a solution of methylene blue known absorbance (at 660nm), usually 0.120. The cells were incubated as required, then centrifuged. The absorbance of the supernatants were determined and an approximation of the amount of methylene blue adsorbed by the cells obtained by subtracting the new absorbance from the original absorbance (Jensen and Haas, 1963).

Estimation of Competent Cell Number

The number of competent cells in a culture was estimated by the unlinked marker technique of Nester and Stocker (1963).

Single Strand Transformation

Single strands of transforming DNA were obtained by heat denaturation of purified native DNA (Roger et al, 1966) and separated by methylated albumin kieselguhr, prepared as per Mandell and Hershey (1960). The stepwise gradient technique of Sueoka and Cheg (1962) was utilized for elution. Single strandness was verified by utilizing hydroxyapatite (prepared as per Miyazawa and Thomas, 1965) column chromatography with single strands eluting at 0.10-0.15M phosphate and native DNA eluting at 0.2-0.22M phosphate (Bernardi, 1969). Light and heavy chains were identified by correlation of elution profiles obtained here with those of Rudner

et al (1968, 1973a,b) and Terraso (1971). Transformation utilizing single strands was accomplished by utilizing the technique of Chilton (1967) and Chilton and Hall (1968).

Physical Properties of Liquid Medium

A capillary tube vapor pressure manometer was constructed (see diagram I, Appendix A for relevant construction details and calibration details) in order to determine if the addition of various substances to the transformation mixture would alter the physical properties of the media in a way that correlated with alterations in the transformation frequency. With this instrument, the vapor pressure depression due to each added substance could be determined. From this value, approximations for osmotic coefficients and activity coefficients could be calculated if desired.

Molalities and osmolalities were calculated as described in standard physical chemistry texts.

Physical Properties of DNA

The effects of substances added to the transformation mixture on the conformation of the transforming DNA were studied in two ways:

1) Hyper-/hypochromic spectral shifts were determined by a point scan of the 270nm absorption peak

of the DNA, DNA plus supplement and supplement alone.

2) A thermoelectric vapor pressure osmometer was designed to determine if the supplements and the DNA were undergoing any molecular association. Briefly, the circuitry consisted of a D.C. Wheatstone bridge with two of the bridge arms being thermisters with high negative termperature coefficients. The output of this bridge is fed into a two stage, direct coupled D.C. amplifier wi h a current null indicator read out (see Appendix B for circuitry and calibration details). In use, one drop of solvent is placed on one thermister bead, a drop of solution on the other thermister bead. Both beads are then placed in a solvent saturated atmosphere whose ambient temperature is $\pm$ .02°C. Since the vapor pressure (and the chemical potential) of the solvent in the drop of solution is lower than that of the pure solvent, solvent vapor will condense on the solution drop at a greater rate than on the solvent drop. As soon as a steady differential rate of condensation is established, a differential heating is also established since condensation releases heat to the

surroundings. This temperature (as resistance) differential, on the order of 10^{-10}A, moves the indicator off null. The resistance change required to null the indicator, when taken through the required mathematics, can be utilized to calculate a number of coefficients if desired. The resistance changes obtained for solvent, solvent plus DNA, solvent plus supplement and solvent plus DNA plus supplement, could then be related as to the presence or absence of intermolecular associations.

Part III

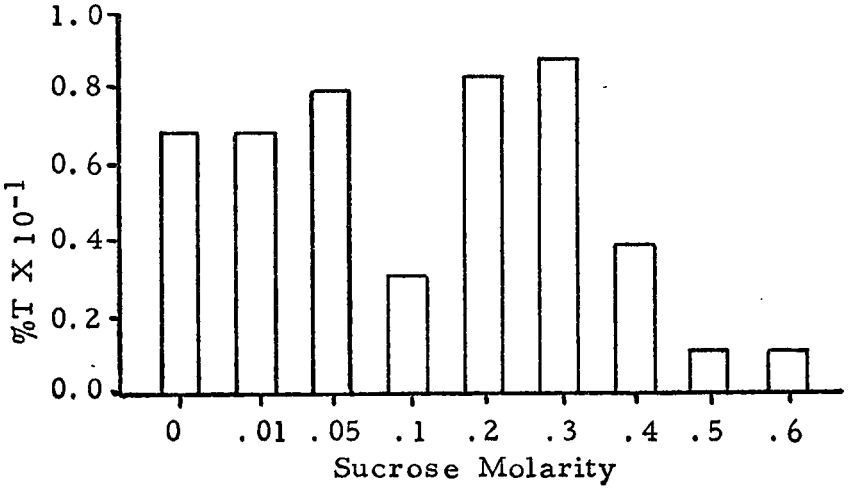
RESULTS

DNA Chemical Analysis

A typical DNA extraction resulted in approximately 200 ug/ml DNA, 20 ug/ml RNA and 30 ug/ml protein. The DNA extraction procedure utilizing sucrose resulted in transformation frequencies identical to the literature extraction procedure.

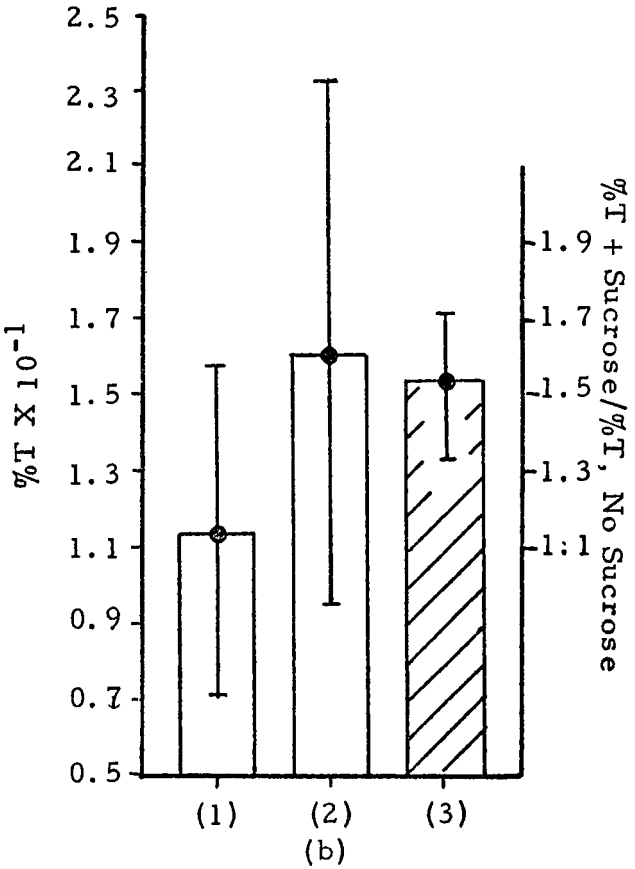
Sucrose Concentration versus Percent Transformation

The effect of increasing concentrations of sucrose on the percent transformation of the Ade marker is depicted in FIGURE I (a). The stimulation peak at 0.3M was chosen for analysis. A statistical analysis of 14 experiments concerning the percent transformation in the presence and absence of 0.3M sucrose, utilizing the Students *t* test (Lacey, 1953), suggested that the results of the stimulation effect are significant at the 0.1% level. FIGURE I (b) shows the standard deviation of the percent transformation of the 14 experiments with respect to (1) transformation without sucrose and (2) transformation with 0.3M sucrose. The filled column (3) represents the standard deviation of the ratio of the %T with 0.3M sucrose to the %T without sucrose per experiment for a 14 experiment sample. This date indi-



(a)

FIGURE I



(b)

FIGURE I (a) INDICATES THE EFFECT OF INCREASING SUCROSE CONCENTRATION ON PERCENT TRANSFORMATION. FIGURE I(b) INDICATES THE STANDARD DEVIATION OF 14 EXPERIMENTS WITH (1) BEING TRANSFORMATION WITHOUT SUCROSE, (2) BEING TRANSFORMATION WITH 0.3M SUCROSE AND (3), THE FILLED COLUMN, INDICATING THE STANDARD DEVIATION OF THE RATIO %T + SUCROSE/%T, NO SUCROSE, CALCULATED PER EXPERIMENT.

cates that while the normal and stimulated transformation undergoes typical fluctuations in %T, the ratio of the stimulated %T to the control stays relatively constant.

Marker Specificity of Sucrose Effect

FIGURE II demonstrates typical transformation of the Ade, Leu and Met genetic markers in the presence and absence of 0.3M sucrose. The marker frequency ratios can be seen to remain relatively constant, suggesting that no marker specificity is apparent.

Number of Competent Cells

The calculation of the percent competent cells by the unlinked marker technique, although of questionalbe value, indicates a 1.5 to 2.5 fold increase in the number of competent cells in the presence of 0.3M sucrose. It should be noted that this technique does not discriminate between an increased number of competent cells and an increased competency of cells already competent.

The Effect of DNA Concentration on Sucrose Stimulation

Typical DNA saturation curves in the presence and absence of 0.3M sucrose, for the three genetic markers utilized, are shown in FIGURES III (a), (b) and FIGURE IV. The stimulation can be seen at both limiting and saturating

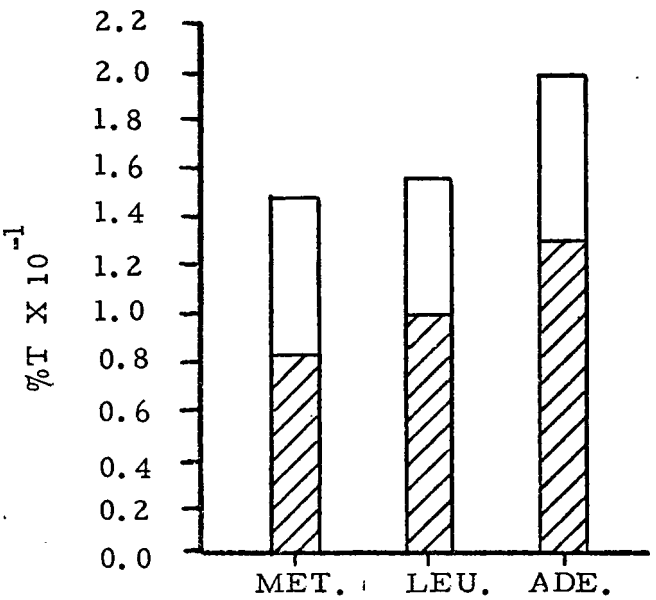


FIGURE II

THE PERCENT TRANSFORMATION OF THREE GENETIC MARKERS IN THE ABSENCE OF SUCROSE (FILLED COLUMNS) AND IN THE PRESENCE OF 0.3M SUCROSE (OPEN COLUMN EXTENSIONS).

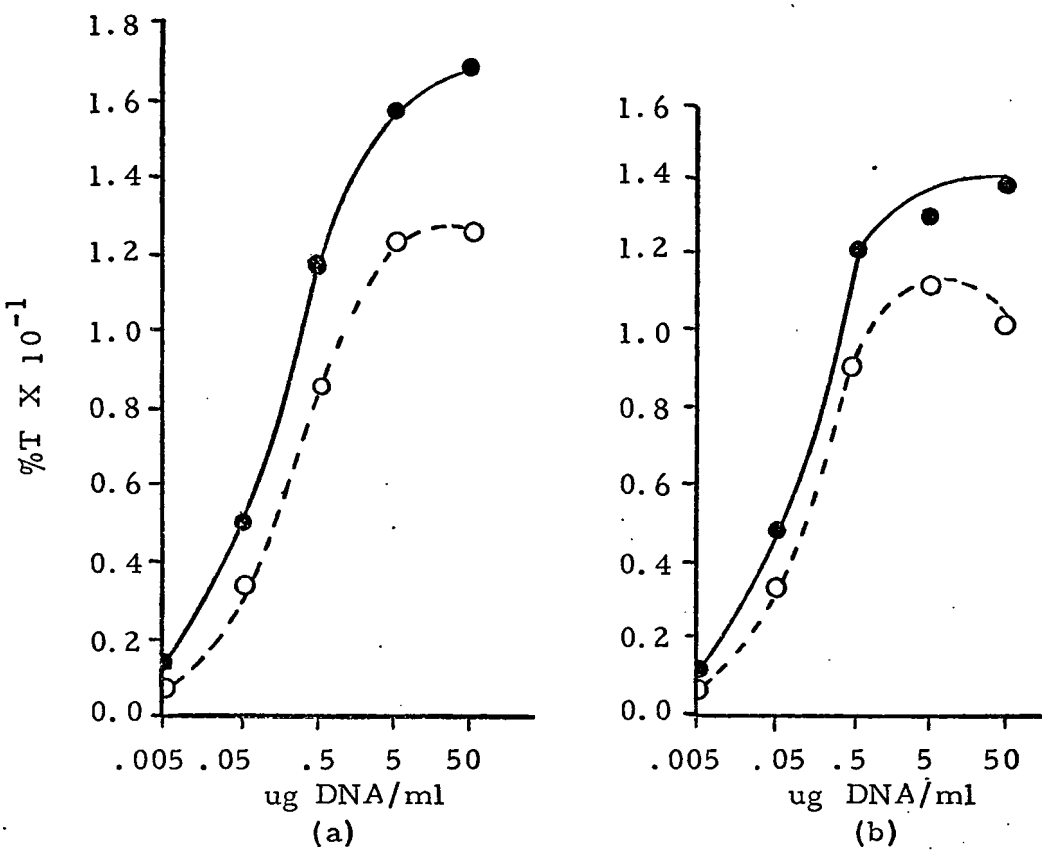


FIGURE III

SATURATION CURVES FOR ADENINE (a) AND LEUCINE (b) IN THE PRESENCE (●——●) AND ABSENCE (○- - -○) OF 0.3M SUCROSE WITH RESPECT TO PERCENT TRANSFORMATION.

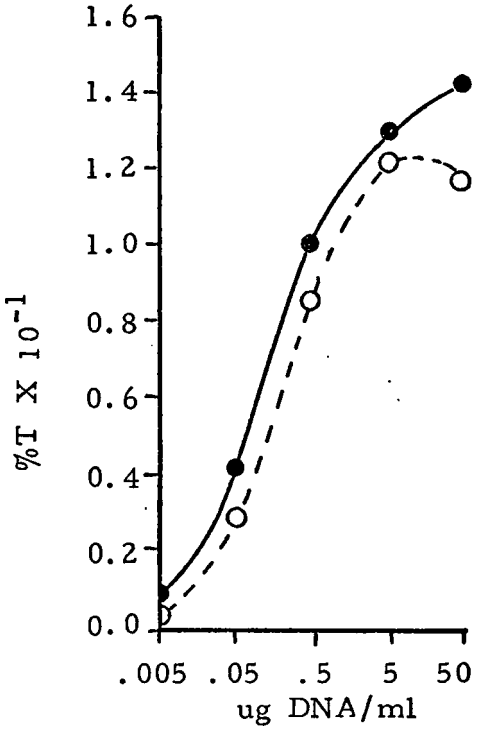


FIGURE IV

SATURATION CURVE FOR METHIONINE IN THE PRESENCE (●——●) AND ABSENCE (O- - -O) OF 0.3M SUCROSE WITH RESPECT TO PERCENT TRANSFORMATION.

levels of DNA. The break to saturating levels of DNA appears to occur at approximately the same concentration. The data indicates that sucrose causes the appearance of more transformants per unit of DNA.

Sucrose Effect on the Kinetics of Transformation

The appearance of Ade transformants with time in the presence and absence of 0.3M sucrose is indicated in FIGURE V. Limiting concentrations of DNA are utilized and samples assayed for transformants at the indicated intervals. Approximately 40% more transformants appear per unit time in the presence of sucrose. If the cells are preincubated in 0.3M sucrose for ten minutes and the same experiment performed, FIGURE VI typically results. A further increase in the number of transformants appearing with time can be seen. If the DNA is preincubated ten minutes with 0.3M sucrose, FIGURE VII typically results. No real significant difference appears with DNA preincubation while a significant difference occurs if the cells are preincubated with sucrose. This might suggest that DNA is not the site of action of the stimulation effect.

Long Term Incubation

The effect of long term incubation of the cell-DNA reaction mixture on Ade transformation in the presence and absence of 0.3M sucrose is shown in FIGURE VIII. Saturating

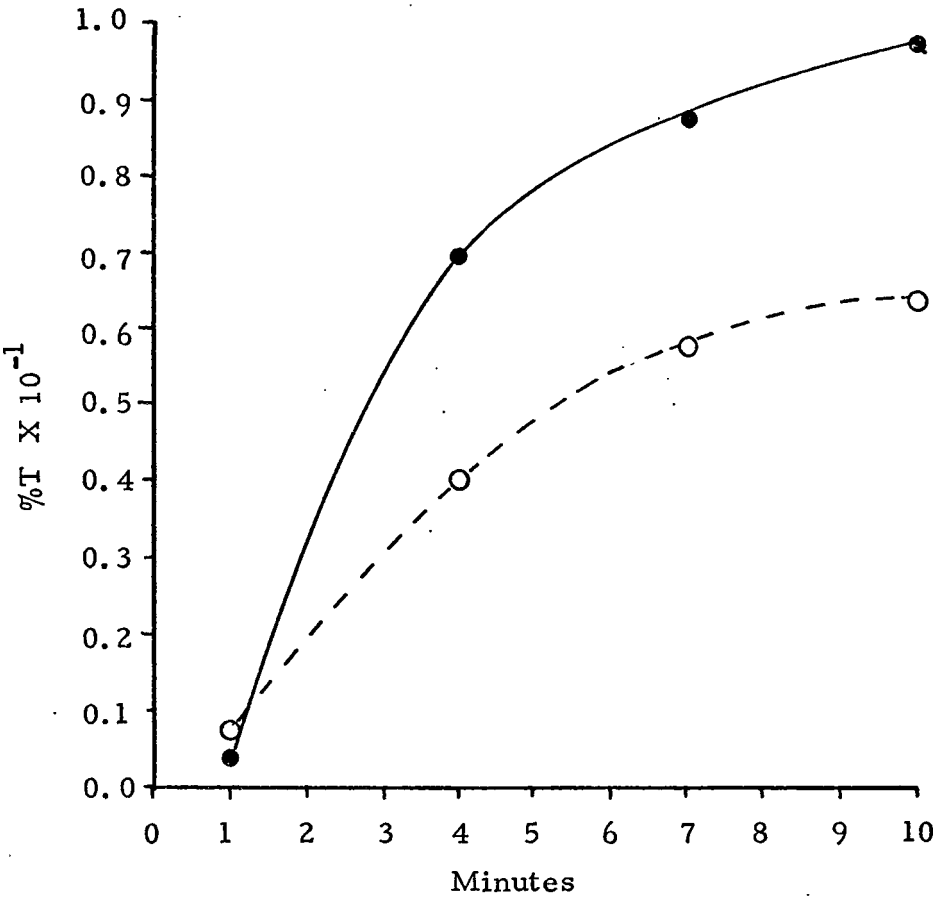


FIGURE V

THE APPEARANCE OF TRANSFORMANTS WITH TIME IN THE PRESENCE (●—●) AND ABSENCE (○- -○) OF 0.3M SUCROSE.

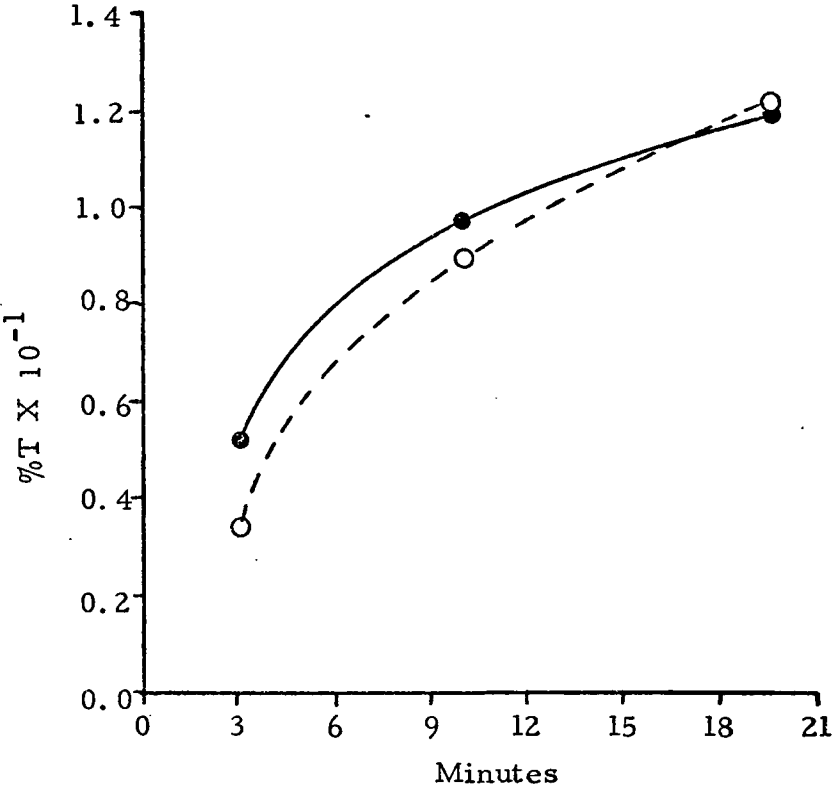


FIGURE VI

THE APPEARANCE OF TRANSFORMANTS WITH TIME IN CELLS PREINCUBATED WITH 0.3M SUCROSE FOR 10 MINUTES (●——●) AND IN CELLS WITH NO PREINCUBATION (○- -○).

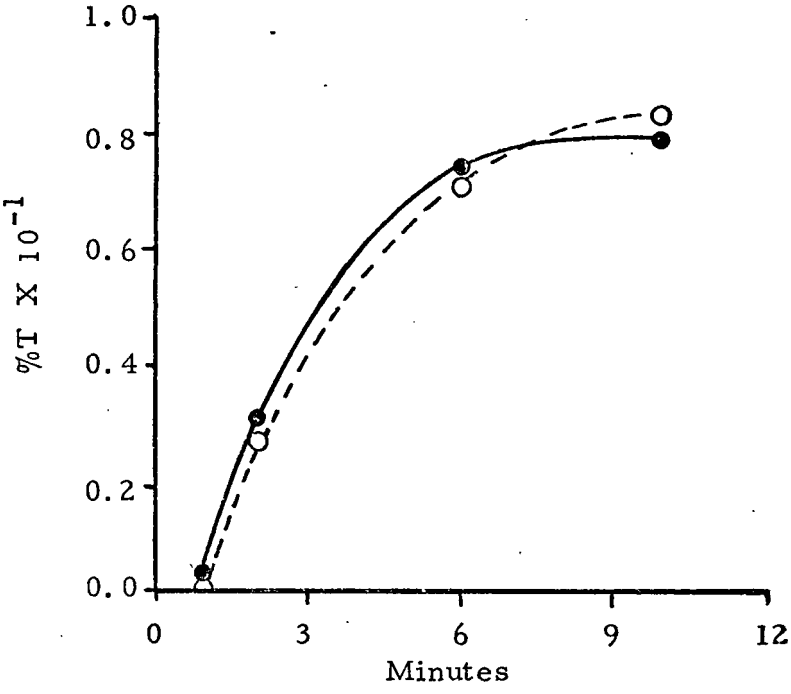


FIGURE VII

THE APPEARANCE OF TRANSFORMANTS WITH TIME WITH THE DNA PREINCUBATED IN 0.3M SUCROSE FOR 10 MINUTES (●——●) AND WITHOUT PREINCUBATION (O---O).

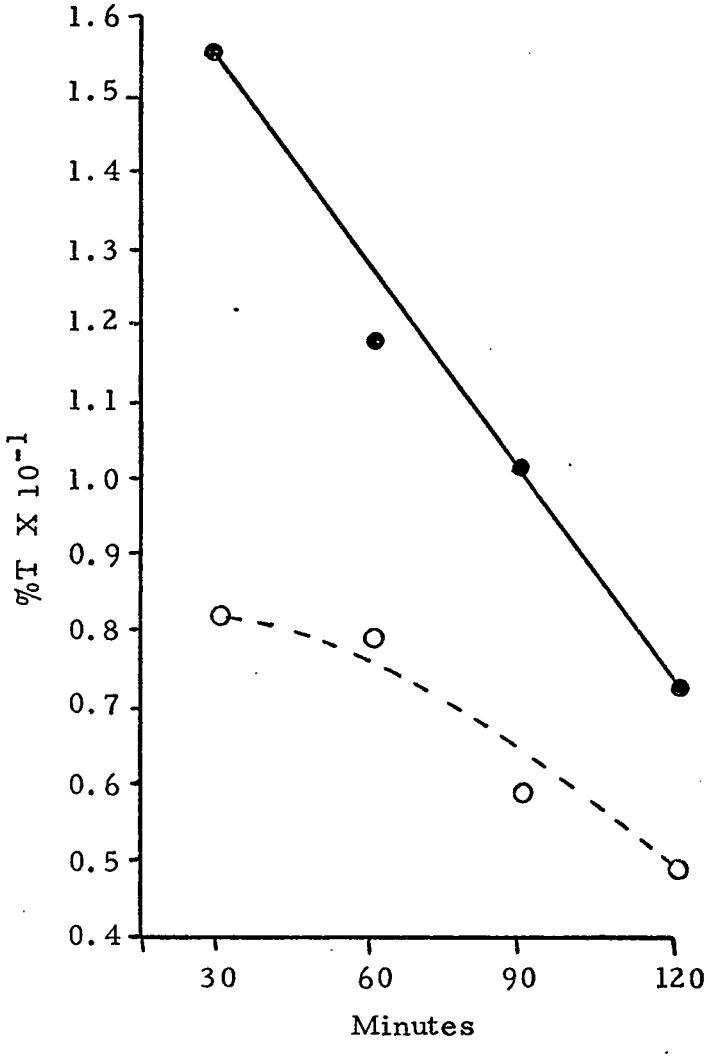


FIGURE VIII

THE EFFECT OF LONG TERM INCUBATION ON THE PERCENT TRANSFORMATION IN THE PRESENCE (●——●) AND ABSENCE (O- - -O) OF 0.3M SUCROSE.

levels of DNA were utilized and the reactions terminated in the usual manner at the indicated time intervals. Both samples show a progressive decrease in number of transformants with increasing time, but the samples with sucrose present show a greater rate of decline than the control samples.

The Effect of RNA on the Stimulatory Effect

The presence of RNA, obtained by utilizing a DNA sample untreated with RNase, does not interfere with the stimulation of transformation by sucrose.

Competent Cell Concentration Effect on Sucrose Stimulation

The competence regime utilized here involves a dilution or "stepdown" at stationary phase (170-190 Klett Units) for maximum competence to develop. If the dilution occurs earlier in the growth curve, fewer competent cells develop. FIGURE IX (b) shows typical results for the Ade marker of samples diluted at 100, 130 and 180 Klett Units and transformed in the presence and absence of 0.3M sucrose. Transformants in each case increase throughout the growth curve in both control and treated samples. FIGURE IX (a) indicates that the ratio of the %T, + sucrose, to the %T, - sucrose, remains relatively constant no matter how many competent cells are present. This suggests that only competent cells are affected by the sugar. To further test

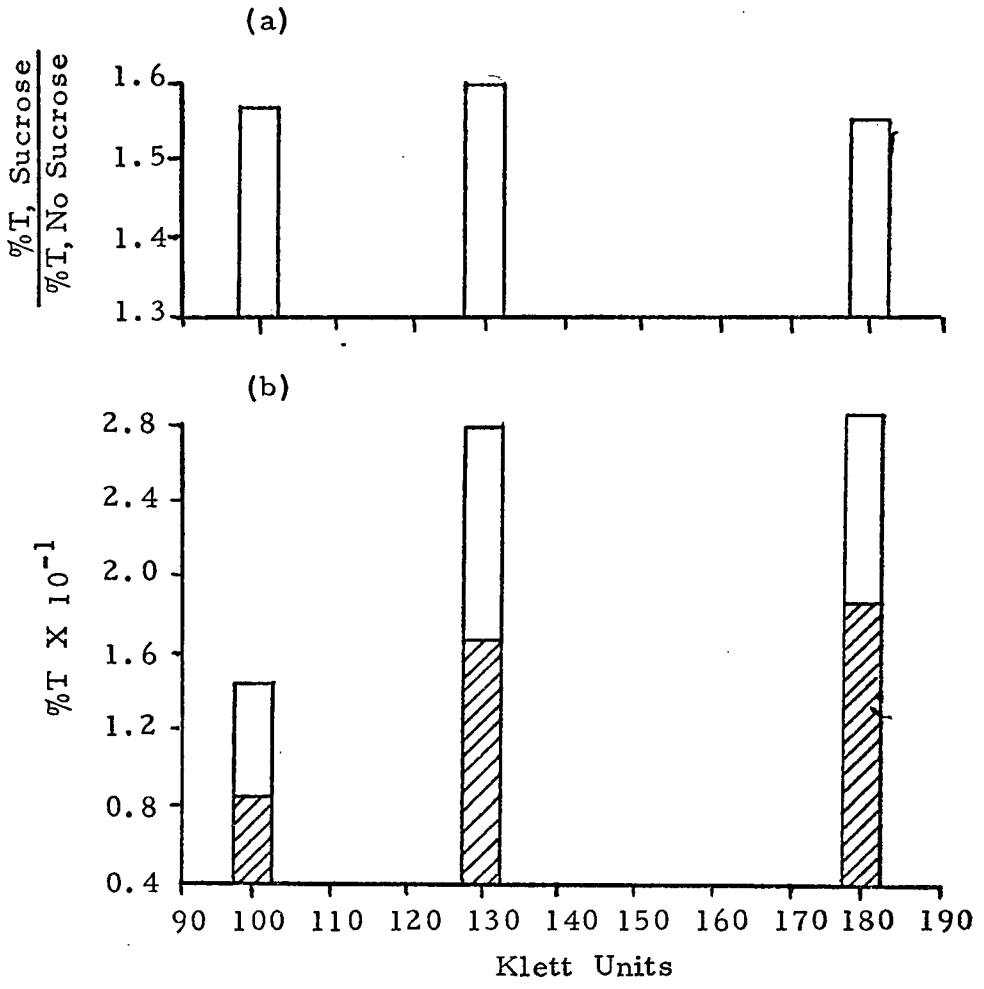


FIGURE IX

(a) THE RATIO OF THE PERCENT TRANSFORMATION WITH 0.3M SUCROSE TO THE PERCENT TRANSFORMATION WITHOUT SUCROSE. (b) THE PERCENT TRANSFORMATION WITH (OPEN COLUMN EXTENSIONS) AND WITHOUT (FILLED COLUMNS) 0.3M SUCROSE AT VARIOUS TIMES THROUGHOUT THE GROWTH CURVE.

this idea, an attempt was made to transform the normally incompetent W-23 strain of B. subtilis in the presence of sucrose. No transformation was apparent.

Sucrose Effect on pH Optimum

Typical results of percent transformation of the Ade marker in the presence and absence of 0.3M sucrose with increasing pH is shown in FIGURE X. This data indicates that the pH 6.8 optimum for transformation in this organism is maintained in the presence of sucrose. The sucrose effect appears to be removed or inhibited at high pH.

Sucrose Effects on Ionic Strength Optimum

The ionic strength was increased from the I=0.35 of the normal transformation medium in intervals to I=1.25. Typical results for the Ade marker are shown in FIGURE XI. The control samples show a small peak at I=.38 and a larger one at I=.95, while the samples treated with sucrose show a large peak at I=.38 and a decrease in the stimulatory effect at higher ionic strengths.

Time Required For Sucrose Stimulation

Sucrose was added to reaction mixtures at various times throughout the 30 minute incubation period to determine the approximate time required for the stimulation effect to occur. FIGURE XII indicates typical results for this

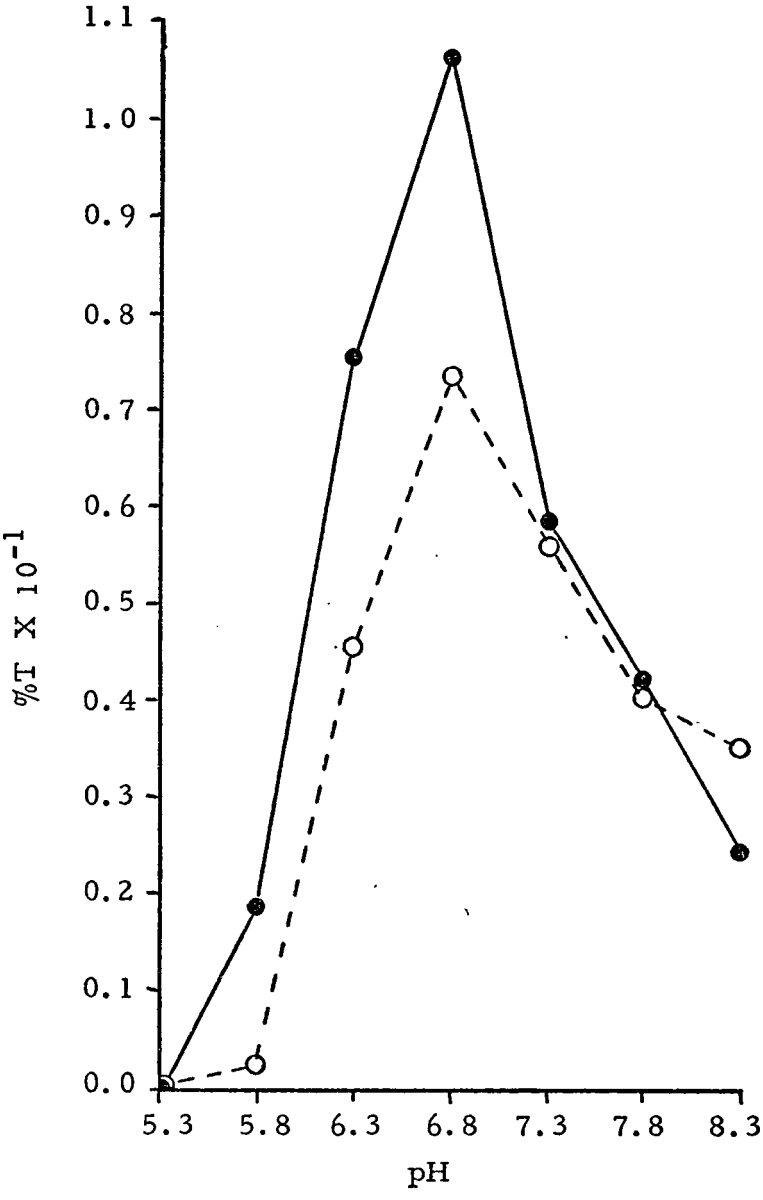


FIGURE X

THE pH OPTIMA FOR GENETIC TRANSFORMATION IN THE PRESENCE (●——●) AND ABSENCE (O- - -O) OF 0.3M SUCROSE.

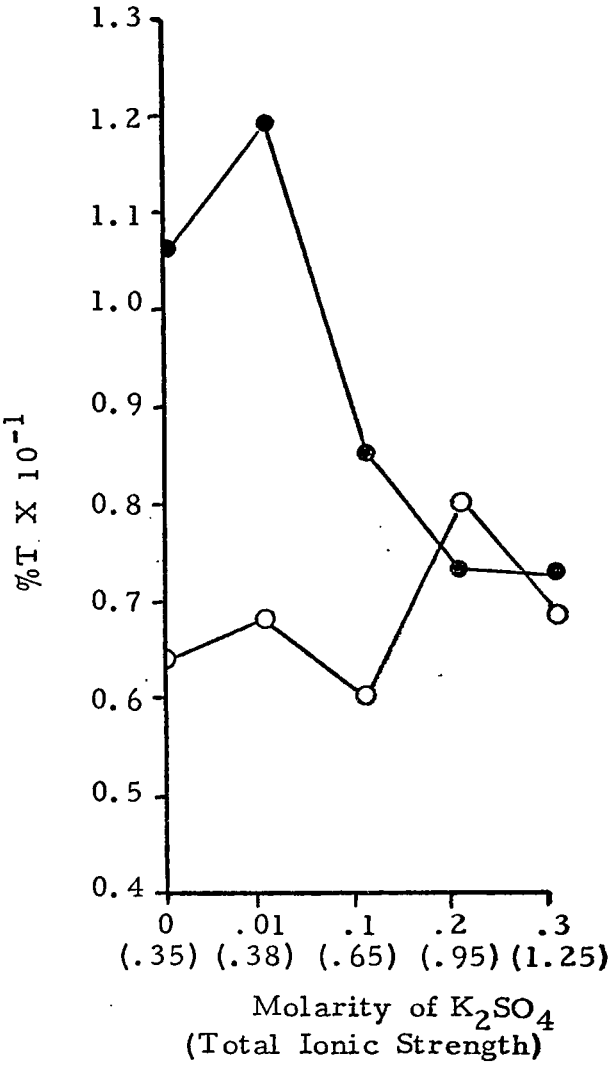


FIGURE XI

THE IONIC STRENGTH OPTIMA IN THE PRESENCE (●——●) AND ABSENCE (○——○) OF 0.3M SUCROSE.

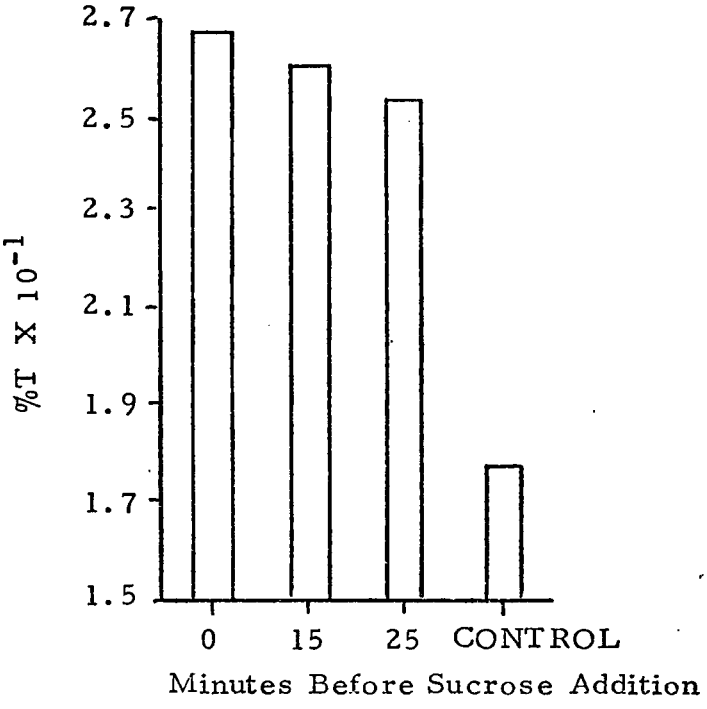


FIGURE XII

THE EFFECT OF SUCROSE ADDITION AT VARIOUS TIMES DURING THE 30 MINUTE INCUBATION PERIOD ON THE PERCENT TRANSFORMATION.

experiment with the Ade marker. The full stimulation effect occurs with a full thirty minute incubation period. Shorter incubation periods with the sucrose demonstrate slightly decreasing stimulation, although the major stimulation occurs within the first 5 minutes of contact.

Time of Sucrose Action Within the Transformation Process

In order to determine when the sucrose acts in relation to the various phases of the transformation process, the following experimental arrangement was utilized:

Competent cells were divided into three samples. At time "0", cells + DNA were added to tube #1, cells + DNA + sucrose to tube #2 and cells + DNA added to tube #3. After 15 minutes incubation, DNase was added to all tubes to terminate transformation. Ten minutes later sucrose was added to tube #3 and after 10 more minutes of incubation, all samples were plated. Typical results of this type of experiment for three genetic markers is shown in FIGURE XIII. The addition of sucrose after DNase treatment not only removes the stimulation effect, but also reduces the transformation frequencies to slightly below those of the control. This data suggests that the sucrose acts before irreversible binding occurs.

The Effect of Low Temperature on Sucrose Stimulation

It was found that incubating a cell-DNA-sucrose

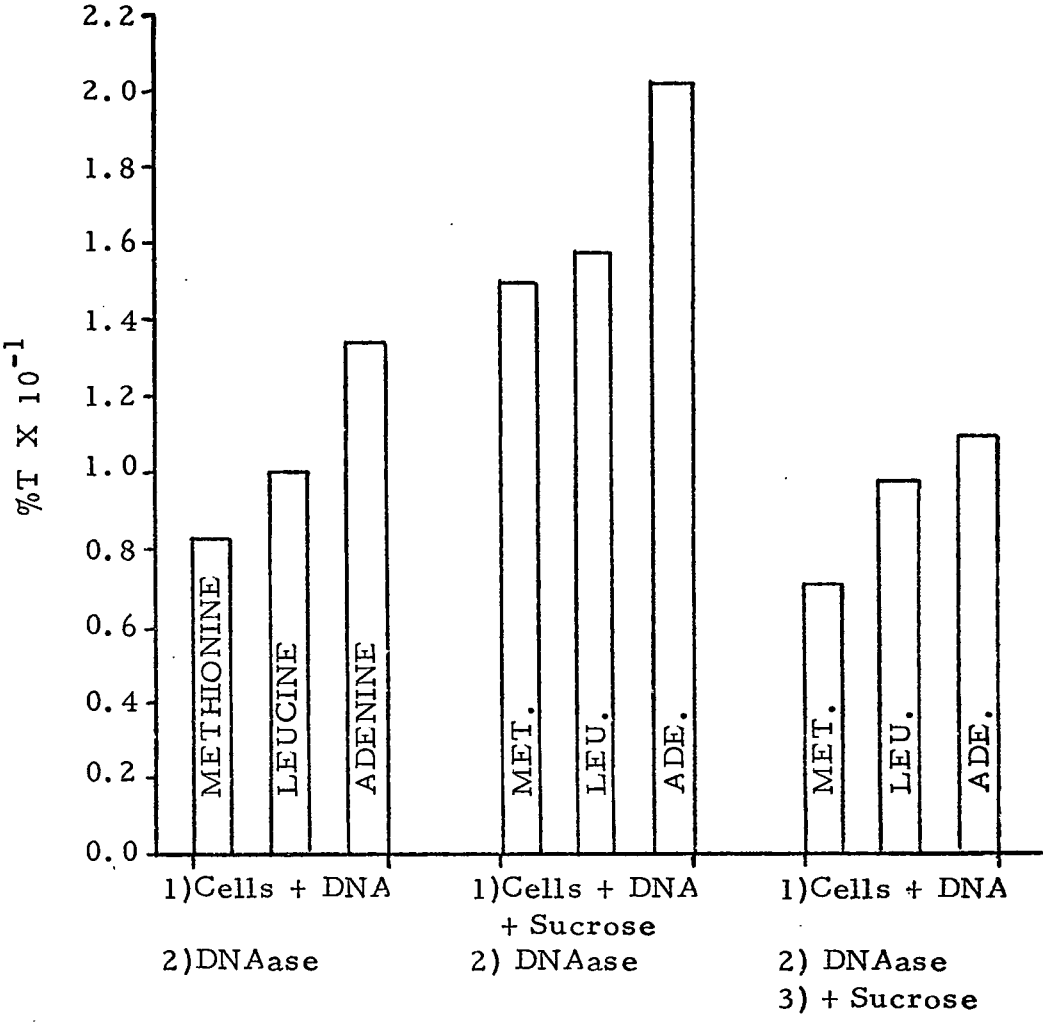


FIGURE XIII

THE DETERMINATION OF THE TIME OF ACTION OF 0.3M SUCROSE IN THE TRANSFORMATION PROCESS.

1) STIMULATION PRIOR TO DNAase ACTION= REVERSIBLE BINDING PHASE STIMULATION.
2) STIMULATION AFTER DNAase ACTION = IRREVERSIBLE BINDING PHASE STIMULATION.

reaction mixture at 5°C for 30 minutes would eliminate the stimulating effect without altering control transformation frequencies. This allowed a series of experiments to be performed which indicated upon which reactant(s) of the transformation reaction mixture the sucrose acted. Three samples were utilized in the experiments. For the first experiment, cells + DNA were added to tube #1, cells + DNA + sucrose to tube #2 and cells + DNA to tube #3 at time "0". All samples were rapidly mixed and then incubated at 5°C for 30 minutes. Then, after incubating 10 minutes at 37°C, sucrose was added to tube #3, all tubes incubated 20 minutes at 37°C, and the reaction terminated as usual. Typical results for Ade are shown in FIGURE XIV, indicating that a) the cold eliminates the stimulation only if sucrose is present in the 5° incubating mixture, b) sucrose added to reaction mixture after cold incubation still causes stimulation and c) the low temperature reduced the transformation frequency slightly below that of the control. The same experiment, but with DNA, not cold incubated, added after the 37° incubation period, gave identical results, suggesting that DNA is not the site of action of the sucrose.

The previous experiment demonstrated that if a complete reaction mixture, i.e. cells, DNA and sucrose in

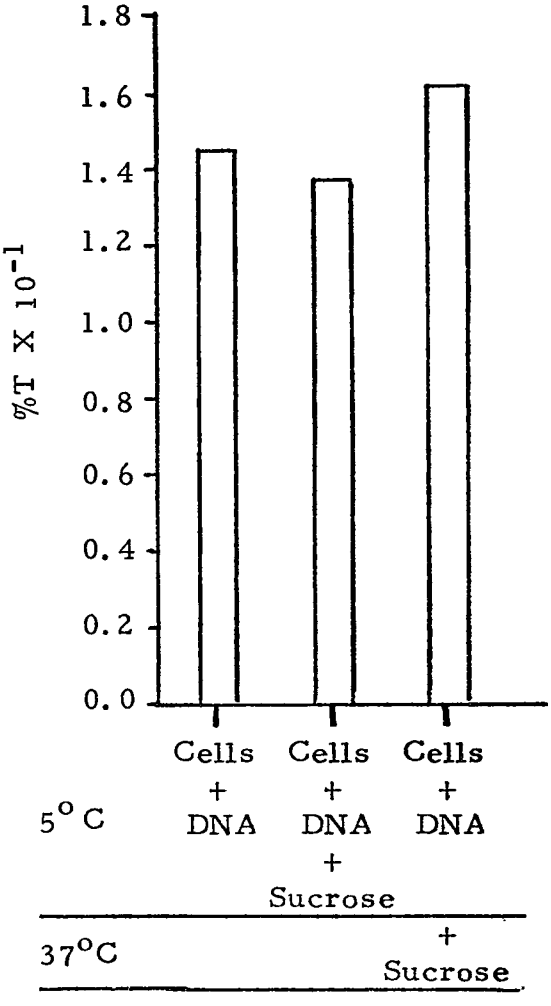


FIGURE XIV

THE EFFECT OF COLD ON THE SUCROSE STIMULATION OF GENETIC TRANSFORMATION.

transformation medium, or just cells + sucrose in transformation medium, was incubated at low temperature, the sugar stimulation of transformation is removed. The next experiment was arranged such that 2 tubes were utilized, with cells in tube #1 and cells + sucrose in tube #2 at time "0". Both tubes were mixed and incubated at 37° for 5 minutes to allow the "sucrose effect" to take place, then centrifuged at 8000 RPM at 25° and the supernatants pipetted into sterile tubes. The cell pellets were stored at room temperature while the supernatants were incubated at 5° for 30 minutes. After warming at 37° for 10 minutes, the supernatants were added to their original pellets, DNA added to both tubes, and after a 15 minute incubation at 37°, the reactions were terminated as usual. Typical results for Ade are given in FIGURE XV. This data suggests that a) the stimulatory effect is removed by cold treatment of supernatants of competent cells treated with sucrose and b) confirms that the presence or absence of DNA does not alter the removal of sugar stimulation by low temperature.

The next experiment was arranged identically to the previous one except that the cells were centrifuged and supernatants collected before sucrose addition to one supernatant. Both samples were then incubated at 5° for 30

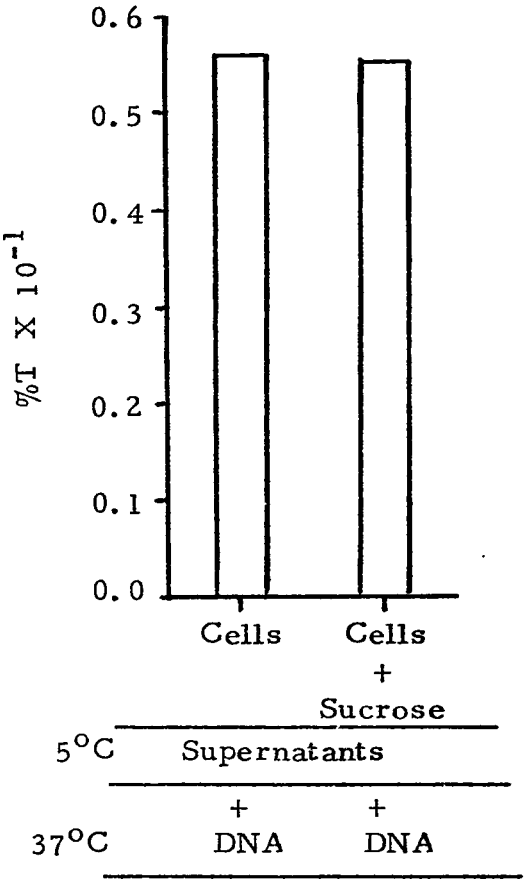


FIGURE XV

THE EFFECT OF LOW TEMPERATURE ON THE SUPERNATANTS OF UNTREATED CELLS AND OF CELLS TREATED WITH 0.3M SUCROSE WITH RESPECT TO THE SUCROSE STIMULATION OF GENETIC TRANSFORMATION.

minutes, warmed at 37° for 10 minutes, supernatants and original cell pellets combined, DNA added and reaction mixtures incubated for 10 minutes. The reactions were terminated as usual. Typical results for the Ade. marker are shown in FIGURE XVI. The data indicates that sucrose stimulation still occurs, thus suggesting that the competent cell medium plays no role in the stimulatory effect unless cells are present during sucrose treatment.

Effects of High Temperature on Sucrose Stimulation

It was found that treatment of reaction mixtures at 95°C for 10 minutes would also eliminate the stimulatory effect, but transformation frequencies were reduced far below that of unheated or cold treated samples. Therefore, this technique was not utilized to study the site of action of the sugar.

Sucrose Effects on the Physical-Chemical Characteristics of the Medium

Various physical and chemical characteristics of the medium were studied in order to confirm the idea of the medium playing no role in the sucrose stimulation effect. Competent cell medium was examined by vapor pressure manometry in the presence of increasing concentrations of sucrose. No correlation was found between fluctuations of transfor-

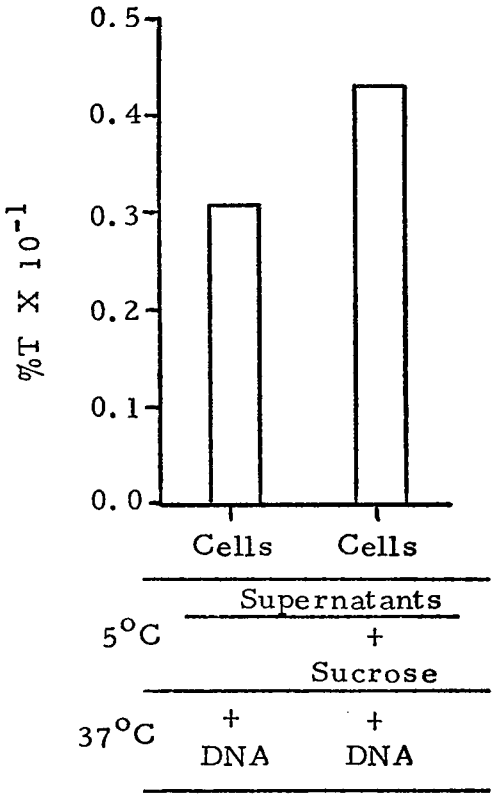


FIGURE XVI

THE EFFECT OF LOW TEMPERATURE ON UNTREATED SUPERNATANTS AND ON SUPERNATANTS TREATED WITH 0.3M SUCROSE WITH RESPECT TO THE SUCROSE STIMULATION OF GENETIC TRANSFORMATION.

mation frequency at the various sucrose concentrations and increase in vapor pressure depression with increasing sucrose concentration. This indicates that properties which can be calculated utilizing the vapor pressure depression data, such as activity coefficients and osmotic pressure, also do not correlate with transformation fluctuations. Properties based on solute and solvent concentrations, such as chemical potentials and osmolarities, and physical properties such as viscosity were also found not to correlate with transformation fluctuations.

Sucrose Effects on DNA

DNA was examined spectroscopically in the 246nm to 270nm range in the presence and absence of 0.3M sucrose. A hypochromic shift was noted that was not accounted for by the subtraction of the sucrose absorption spectrum from the DNA + sucrose absorption spectrum. This phenomenon is depicted in FIGURE XVII (a). FIGURE XVII (b) demonstrates the same experiment utilizing D-glucose, a non-stimulating sugar, in place of sucrose. The hypochromic shift is not noted. A similar experiment utilizing D-galactose, a transformation stimulating sugar, also revealed the hypochromic shift (FIGURE XVII (c)). The galactose concentration of 0.3M is not optimal for stimulation. Sucrose, a non-stimulating

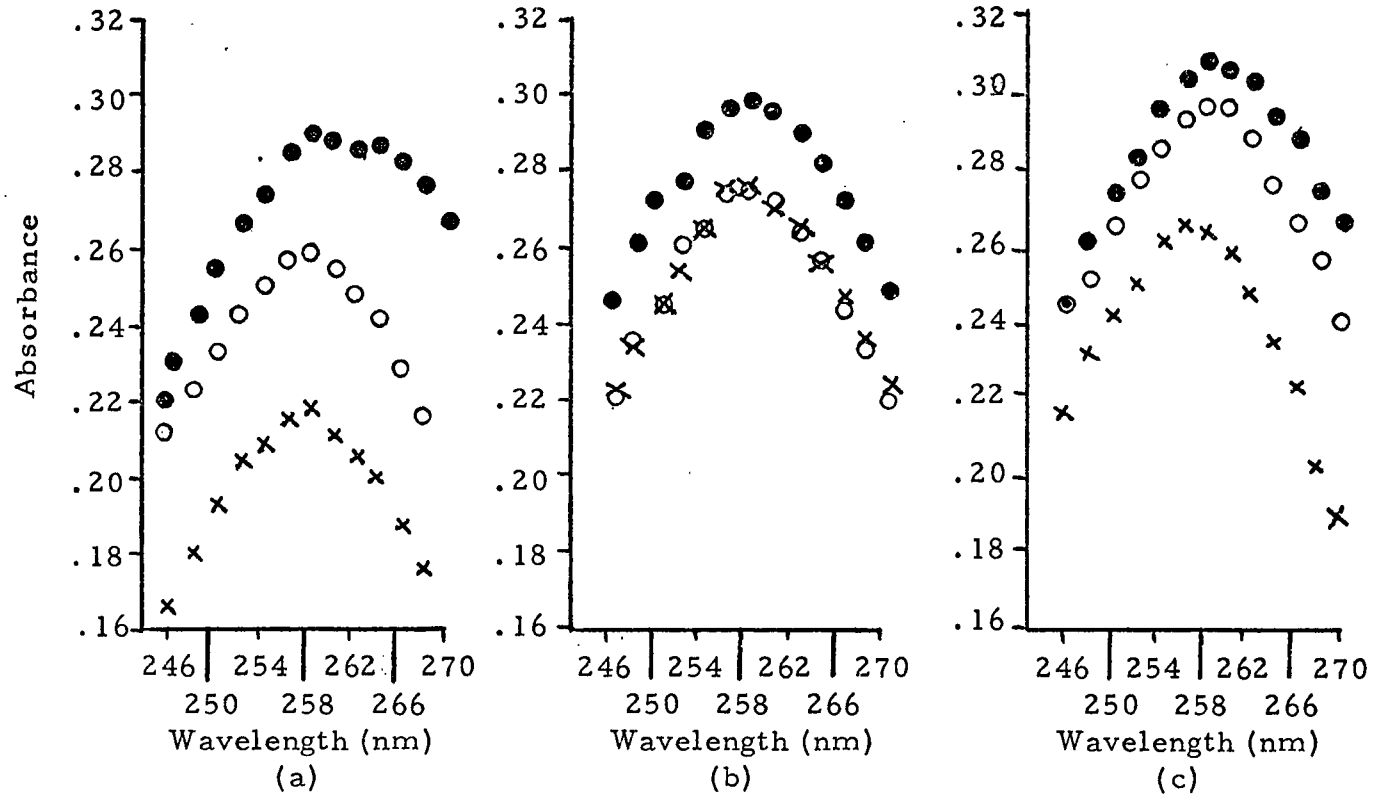


FIGURE XVII

THE EFFECT OF THE SUGARS SUCROSE (a), GLUCOSE (b) AND GALACTOSE (c), AT 0.3 MOLARITY, ON THE ABSORPTION SPECTRUM OF DNA. DNA ABSORPTION = o o o, DNA + SUGAR ABSORPTION = ● ● ●, DNA + SUGAR ABSORPTION MINUS SUGAR ABSORPTION = × × × .

concentration of 0.6M, also showed the hypochromic shift. Thermoelectric vapor pressure osmometry of sucrose and DNA separately and in combination demonstrated no molecular interactions to be occurring at the sensitivity level of this particular instrument (see Table VII).

Sucrose Effects on the Cell

The cell response to sucrose in the medium was studied in various ways. Spectroscopic scans, from 900nm to 350nm, made of cell suspensions in the presence of increasing concentrations of sucrose demonstrated regular decreases in absorbance at all wavelengths with no significant deviations.

The adsorption of methylene blue by cells was utilized to determine changes in the number of negative charges on the competent cell in the presence and absence of sucrose. FIGURE XVIII (a) depicts the difference in absorption between sucrose treated cells and control cells. Also included are treated and untreated cells which have been incubated at 5^{o} for 30 minutes. Initial methylene blue adsorbance at 660nm was 0.12. Therefore, the lower the absorbance of the cell supernatants, the more methylene blue was adsorbed, and the greater the negative charge on the cell. Sucrose, in warm and cold cells, decreases the amount of methylene blue adsorbed. FIGURE XVIII (b) shows the effect of in-

TABLE VII

THERMOELECTRIC VAPOR PRESSURE OSMOMETRY

OF SUCROSE AND DNA SOLUTIONS

Solution on bead (water on other bead)	ΔR (ohms)
1) Water	00
2) DNA (50ug/ml)	710
$\Delta R_2 - \Delta R_1 = 710$	
3) 0.3M sucrose	4990
4) 0.3M sucrose + DNA (50ug/ml)	5730
$\Delta R_4 - \Delta R_3 = 740$	

$\Delta R_4 - \Delta R_3 < \Delta R_2 - \Delta R_1$ = intermolecular association

$\Delta R_4 - \Delta R_3 \geq \Delta R_2 - \Delta R_1$ = no association

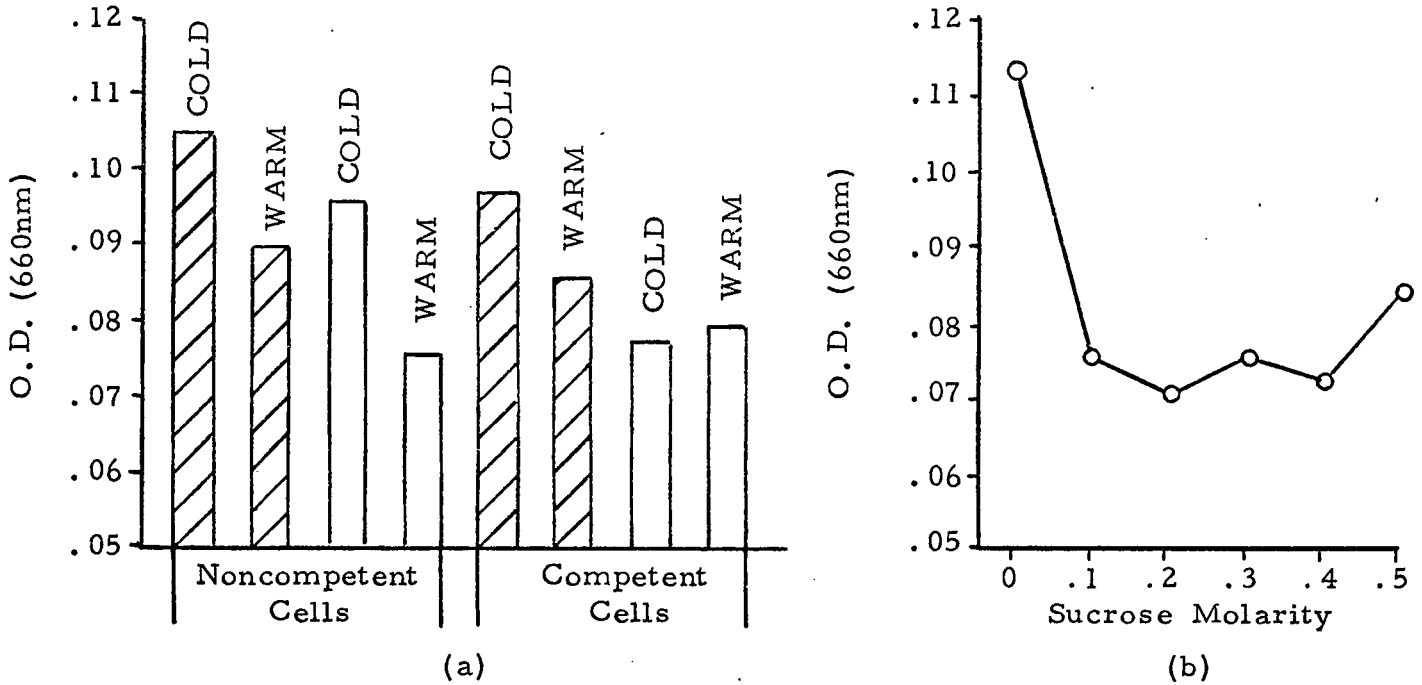


FIGURE XVIII

THE ADSORPTION OF METHYLENE BLUE DYE ONTO CELLS: (a) A COMPARISON OF CELLS TREATED WITH 0.3M SUCROSE (FILLED COLUMNS) WITH UNTREATED CELLS (OPEN COLUMNS) AT 5°C AND 37°C. UNDER COMPETENT AND NONCOMPETENT CONDITIONS. (b) METHYLENE BLUE ADSORPTION WITH INCREASING SUCROSE CONCENTRATION.

creasing sucrose concentration on methylene blue adsorption. Initial absorbance was 0.113. The absorbance can be seem to remain relatively constant until 0.5M is reached, where it increases slightly.

The active transport of DNA into the cell in the presence of sucrose was studied by utilizing 2, 4-dinitrophenol, and inhibitor of the energies of active transport. A concentration of 1.5mM was utilized since its effect of viability was minimal. FIGURE XIX shows typical results of the effect of the inhibitor in the presence and absence of 0.3M sucrose. The results indicate that transformation in the presence and absence of sucrose is reduced 10-15% in both cases.

The cells were next treated with lipolytic enzymes in the presence and absence of 0.3M sucrose. It was felt that if the sucrose caused additional exposure of membrane material, this material would be suseptible to the action of lipolytic enzymes. Cell samples were treated with 0.001, 0.0001 and 0.00001 gm/ml of wheat germ lipase, pancreatic lipase and phospholipase C. FIGURE XX shows typical results of cell samples treated with 0.001 gm/ml of each enzyme. All three enzymes reduced the ratio of transformation without sucrose. Wheat germ lipase reduced it to 1.2-1.3, pan-

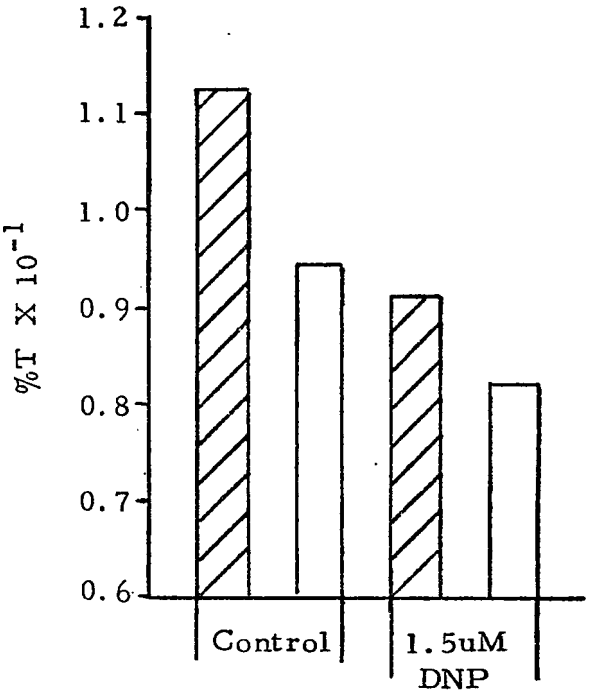


FIGURE XIX

THE EFFECT OF 2,4-DINITROPHENOL, AN INHIBITOR OF ACTIVE TRANSPORT, ON THE SUCROSE STIMULATION OF TRANSFORMATION. FILLED COLUMNS = CELLS TREATED WITH 0.3M SUCROSE, OPEN COLUMNS = UNTREATED CELLS.

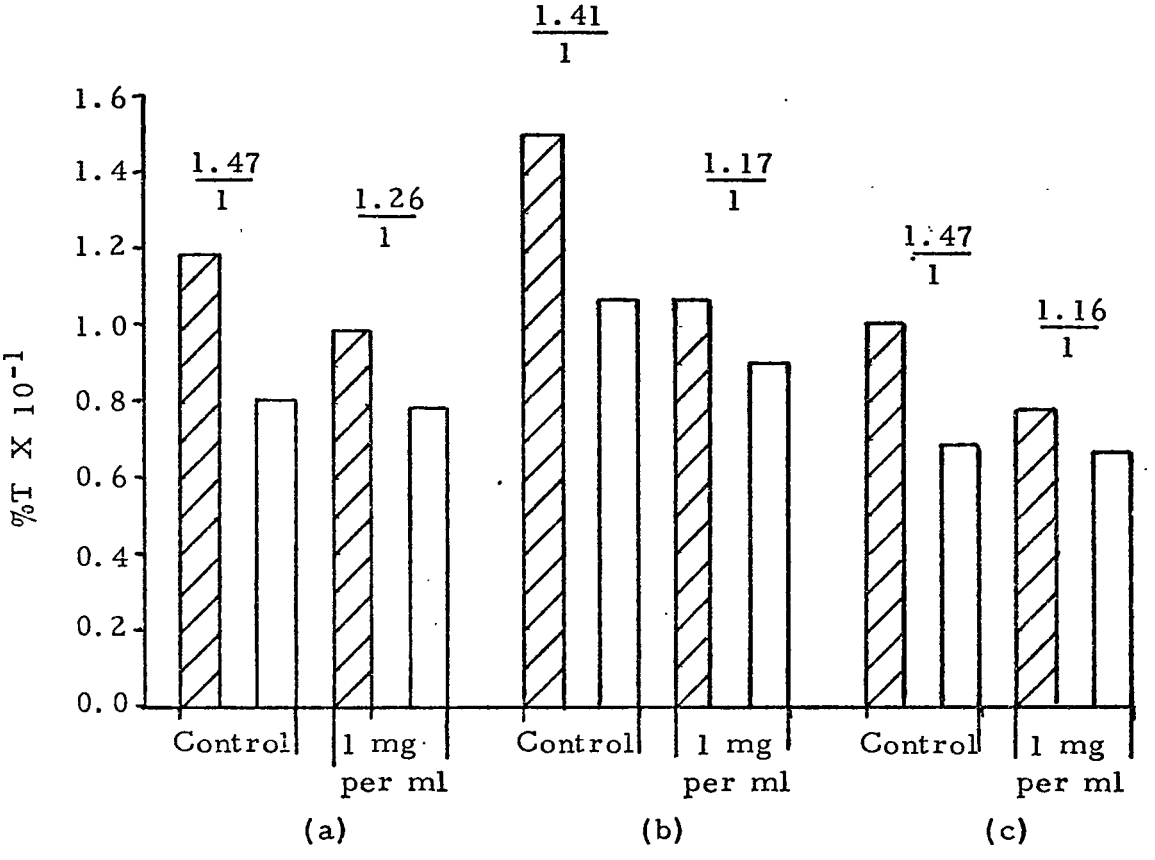


FIGURE XX

THE EFFECT OF VARIOUS LIPOLYTIC ENZYMES ON THE STIMULATION OF TRANSFORMATION BY 0.3M SUCROSE. UNTREATED CELLS (OPEN COLUMNS) AND SUCROSE TREATED CELLS (FILLED COLUMNS) WERE EXPOSED TO WHEAT GERM LIPASE (a), PANCREATIC LIPASE (b) AND PHOSPHOLIPASE C (c). THE RATIO ABOVE EACH PAIR OF BAR GRAPHS REPRESENTS THE PERCENT TRANSFORMATION IN THE PRESENCE OF SUCROSE TO THE PERCENT TRANSFORMATION WITHOUT SUCROSE.

creatic lipase to 1.05-1.17 and phospholipase C to 1.0.-1.16 from the control value of 1.4-1.5. All three enzymes reduced the percent transformation with and without sucrose present, phospholipase and pancreatic lipase causing the greatest reduction. Wheat germ lipase and phospholipase C caused some reduction in viability.

The effect of sucrose on the residual DNase activity found in competent cell cultures was examined by utilizing three aliquots of competent cells, two of which were left as is and sucrose added to the third to 0.3M concentration. All aliquots were then incubated at 37^{o} for 30 minutes. Three 0.9ml samples from each aliquot were centrifuged at room temperature. The supernatants were placed in sterile tubes and 0.1ml DNA of 0.5 ug and 0.005 ug per milliliter concentration added to respective tubes of each aliquot. All samples were incubated for 10 minutes at 37^{o}, after which the supernatants were added back to their original cell pellets. Sucrose was added to one set of one of the untreated aliquots and all tubes were incubated at 37^{o} for 30 minutes. The usual termination procedures were followed. FIGURE XXI indicates the effect of the preincubation of dilute transforming DNA in competent cell filtrates in the presence (b) and absence (a) of 0.3M sucrose. The percent

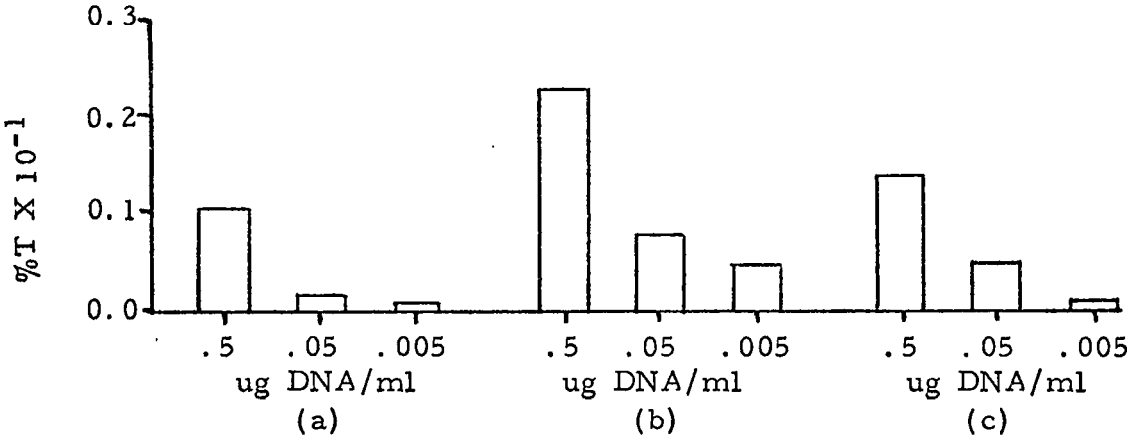


FIGURE XXI

THE EFFECT OF SUCROSE ON THE RESIDUAL DNASE ACTIVITY IN COMPETENT CELL FILTRATES. (a)- THE PREINCUBATION OF DILUTE TRANSFORMING DNA IN FILTRATE. (b)- THE PREINCUBATION OF DILUTE DNA + 0.3M SUCROSE IN FILTRATE. (c) - THE ADDITION OF SUCROSE AFTER PREINCUBATING DILUTE DNA IN FILTRATE.

transformation can be seen to be increased in the presence of sucrose. FIGURE XXI (c) indicates the normal stimulation by sucrose when it is added to preincubated dilute DNA in the transformation reaction mixutre.

In order to determine, albeit indirectly, if the sucrose is altering the activity of the DNase or is perhaps decreasing the amount of DNase present in the medium, the activity of DNase I was tested in the presence of 0.3M sucrose. DNase I, 50ug/ml, was added to DNA of O.D. 260 = .584 and the increase in absorbance determined spectrophotometrically at one minute intervals. FIGURE XXII shows typical results of the enzymatic cleavage of DNA by DNase I, at 25°C, in the presence of 0.3M sucrose (X—X) and absence of sucrose (□—□). To control for the increased viscosity decreasing the rate of reaction, the hydrolysis was also tested in the presence of a non-stimulating sugar, glucose (o-o). As would be expected, the increased viscosity does decrease the reaction somewhat, but the sucrose does not exhibit any more inhibition of DNase I activity that does glucose.

The Effect of a Non-stimulatory Sugar on Sucrose Stimulation

Increasing concentrations of glucose were placed in a series of cell samples containing 0.3M sucrose and the usual transformation procedure followed. FIGURE XXIII shows

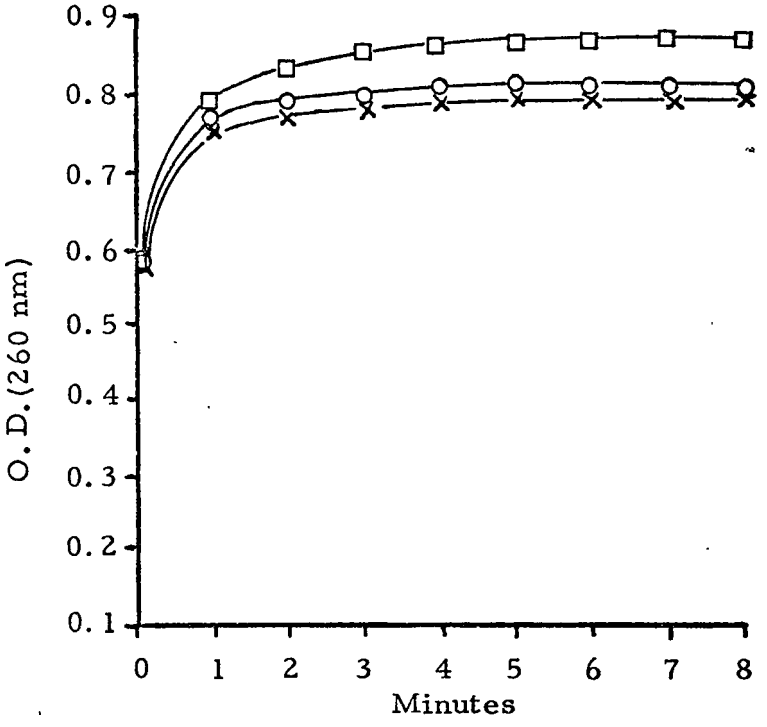


FIGURE XXII

THE ENZYMATIC HYDROLYSIS OF DNA BY DNASE I WITH TIME IN THE PRESENCE OF 0.3M SUCROSE (×——×), 0.3M GLUCOSE (o—o) AND THE ABSENCE OF ANY AGENT (□—□).

typical results of this type of experiment, where the percent transformation decreases with increasing concentration of glucose. The viability remains unchanged.

"Structural" Changes in Sucrose Effects of Stimulation

If sucrose is added in the form of its component parts, a-D-glucose and fructose at 0.3M each, a synergistic stimulation occurs. A further stimulation occurs if the concentrations are dropped to 0.15M each, thereby making the sucrose solution and the component solution have the same osmolarity. This is depicted in FIGURE XXIV (d). Substitution of β-Dglucose (c) for α-D-glucose resulted in a slight increase in %T, as did substitution of galactose or mannose (FIGURE XXIV (a) (b). It should be noted that these three sugars are stimulatory sugars (McLemore, 1974), and this could account for the increase in stimulation. Fructose, galactose and mannose also reduce total viability while maintaining or sometimes increasing the number of transformants.

The Effect of Sucrose on Single-strand Transformation

FIGURE XXV indicates the transformation, for the Ade marker, of single strands of donor DNA in the presence and absence of 0.3M sucrose. All DNA solutions were diluted to O.D. $_{260}$=.05 (2ug/ml) before use. The light strands (a) and

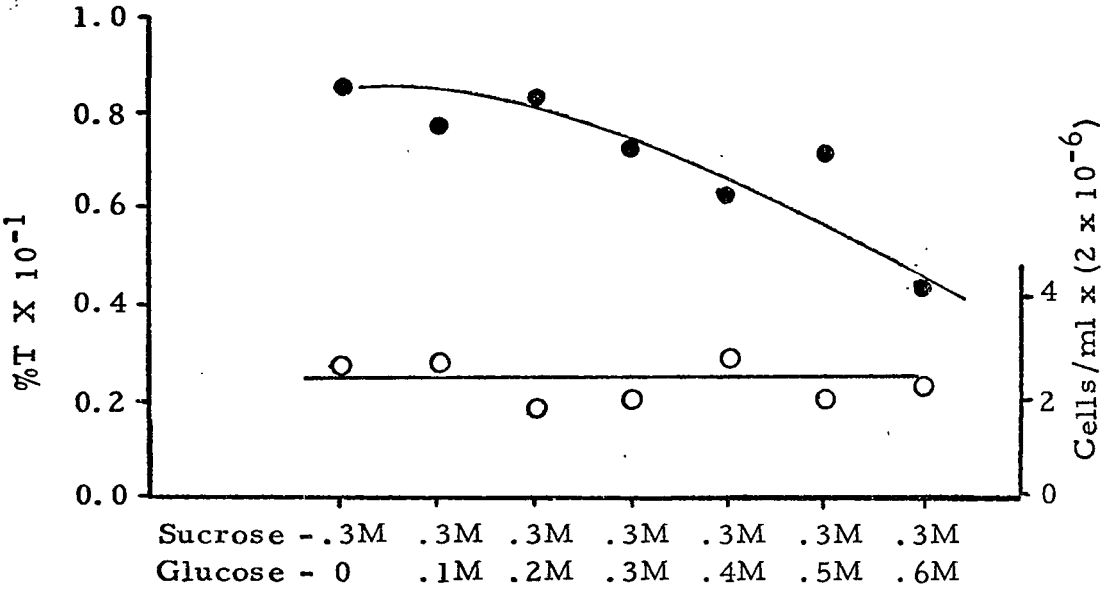


FIGURE XXIII

THE EFFECT OF GLUCOSE, A NONSTIMULATING SUGAR, ON THE SUCROSE STIMULATION OF GENETIC TRANSFORMATION. CLOSED CIRCLES INDICATE THE PERCENT TRANSFORMATION WITH 0.3M SUCROSE AND INCREASING GLUCOSE CONCENTRATION. OPEN CIRCLES INDICATE THE VIABLE COUNT AT THE SAME SUGAR CONCENTRATIONS.

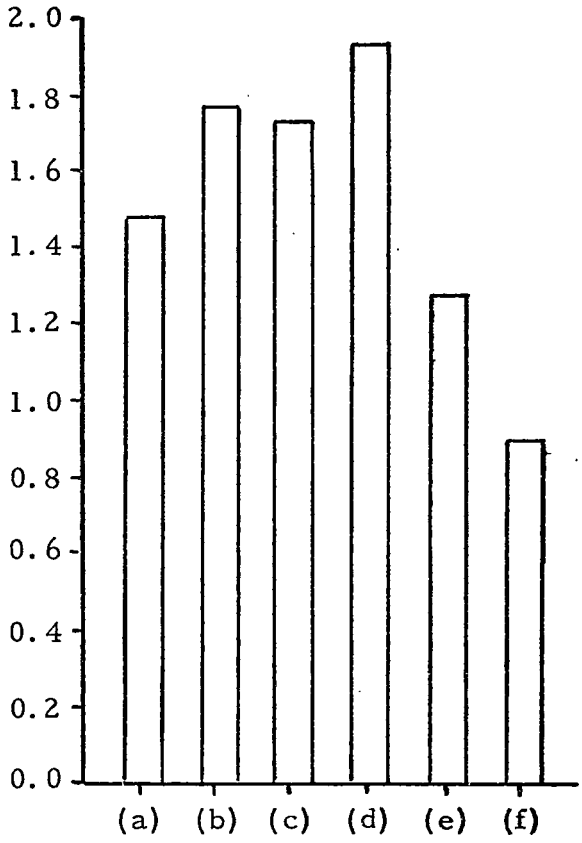


FIGURE XIV

THE EFFECT OF VARIOUS COMBINATIONS OF MONOSACCHARIDES RESEMBLING THE STRUCTURE OF SUCROSE ON TRANSFORMATION.

(a) 0.15M FRUCTOSE + 0.15M GALACTOSE
(b) 0.15M FRUCTOSE + 0.15M MANNOSE
(c) 0.15M FRUCTOSE + 0.15M B-D-GLUCOSE
(d) 0.15M FRUCTOSE + 0.15M a-D-GLUCOSE
(e) 0.3M SUCROSE
(f) NONE

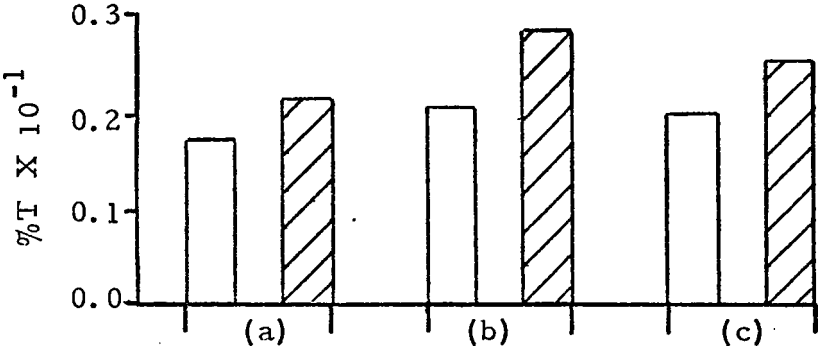


FIGURE XXV

SINGLE-STRAND TRANSFORMATION IN THE PRESENCE (FILLED COLUMNS) AND ABSENCE (OPEN COLUMNS) OF 0.3M SUCROSE WITH (a) BEING THE LIGHT CHAIN, (b) BEING THE HEAVY CHAIN IN THE PRESENCE OF 0.001M EDTA AND (c) BEING THE HEAVY CHAIN TRANSFORMED UNDER DOUBLE-STRAND CONDITIONS.

heavy strands (b) were transformed in the presence of 0.001MEDTA and in (c) heavy strands were transformed under conditions for native double strans transformation.

The fact that the heavy strand gives identical transformation frequencies in the presence and absence of EDTA indicates that double strand contamination is minimal in the single strand preparations. The sucrose increases the percent transformation for the Ade. marker in both light and heavy strand.

Do Other Sugars Stimulate Similiarly?

Sucrose is a disaccharide fermented by B. subtilis. A nonfermented disaccharide, lactose, a fermented hexose, mannose, and a nonfermented hexose, galactose, are all stimulatory sugars (Peetz, 1966; McLemore, 1974). These sugars were tested to determine if they acted similiarly to sucrose with respect to a) action at the reversible phase of the transformation process and b) the sensitivity of the stimulation to cold temperature. FIGURE XXVI shows typical results of the testing of 0.5M lactuse, 0.7M mannose and 1.4M galactose for these requirements. The concentrations utilized are those showing peak stimulation (Peetz, 1966; McLemore, 1974). All three sugars show no activity if added after DNase treatment, and all three show cold sensitivity.

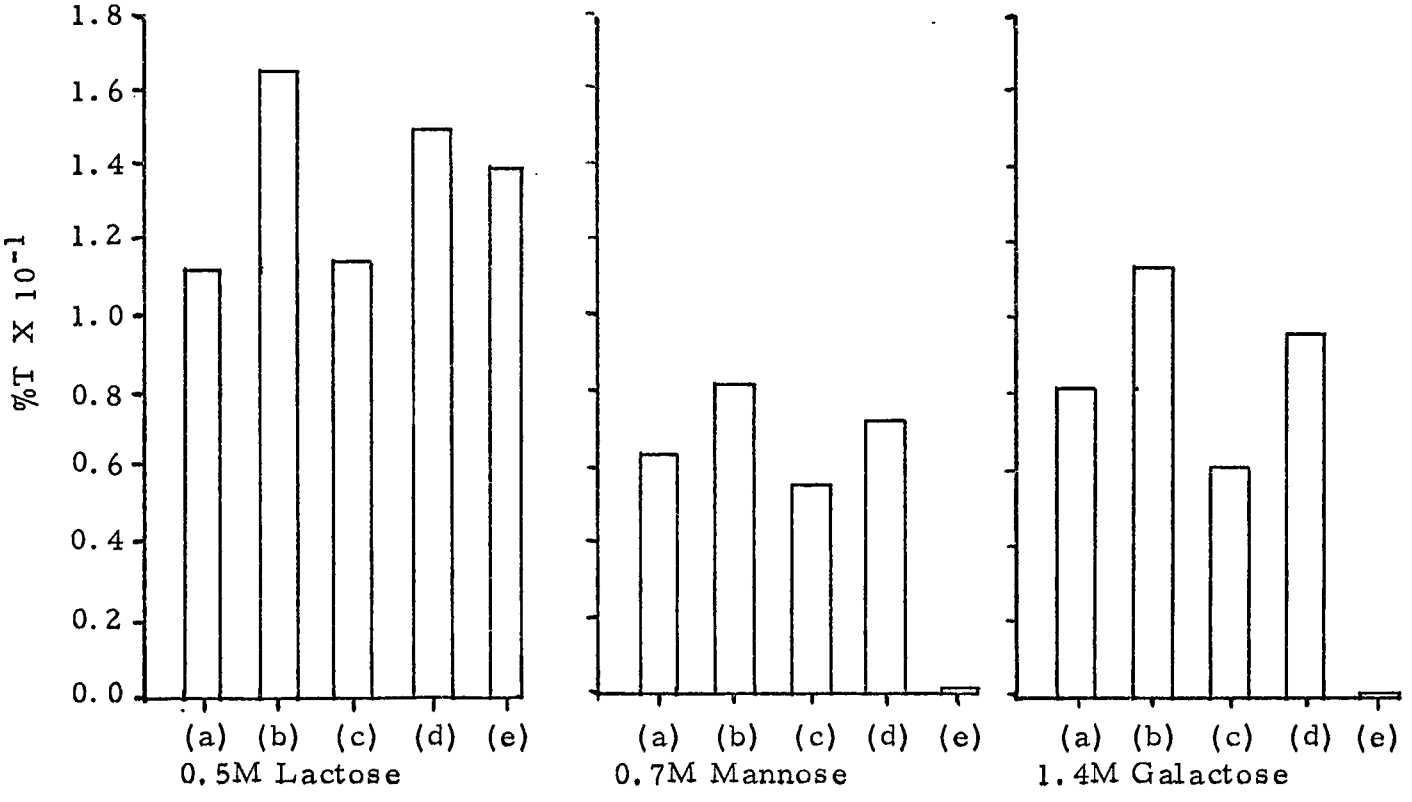


FIGURE XXVI

THE EFFECT OF THREE STIMULATING SUGARS ON TRANSFORMATION WITH RESPECT TO TIME OF ACTION AND COLD SENSITIVITY OF THE STIMULATION. (a) = CELLS + DNA, (b) = CELLS + DNA + SUGAR, (c) = SUGAR ADDITION AFTER DNASE TREATMENT, (d) = CELLS + DNA AT 5°C, (e) = CELLS + DNA + SUGAR AT 5°C

The hexoses show an extreme cold sensitivity in the presence of the sugars. Transformation is reduced far below control.

Part IV

DISCUSSION

The data given here confirms the validity of the sugar stimulation of genetic transformation in bacteria as put forth by Peetz (1966) and McLemore (1974) in B. subtilis, and mentioned by Kohoutova (1973) and Kohoutova and Kocourek (1974) in D. pneumoniae.

Saturation curves (FIGURES III, IV), attempts to stimulate transformation in a normally incompetent strain of Bacillus and competent cell number studies (FIGURE IX), all indicate that only competent cells are affected by the sugars with respect to stimulation of transformation. The effect is relatively rapid with most of the effect occurring within the first 5 minutes of contact with the sugar (FIGURE XII).

Newly transformed cells are quite delicate (Kammen et al, 1966a; Joys and King, 1967; Joenje et al, 1972) and a reasonable explanation or the sugar effect would be the stabilization of potential transformants. Some evidence opposing this hypothesis is available in the literature in that diluting and plating transformants in hypertonic protoplast/L-form media does not increase the transformation frequency, nor does very gentle handling with respect to

plating and pipetting methods (Joys and King, 1967). No protection is shown against the bactericidal action of the DNA itself (Piechowska and Shugar, 1967; Stewart and Marmur, 1970) as evidenced in FIGURE VIII, where not only do cells incubated long term with sucrose show a decrease in number of transformants, but also show a greater rate of decline than the control cells.

The DNA itself is not affected by the sugars, at least with regard to interactions in the medium leading to transformation. All three markers are affected equally by the sucrose (FIGURE II) while alterations of DNA structure by heat (Roger and Hotchkiss, 1961), for instance, allows significant increases in certain markers before decrease due to collapse of the helix. More direct evidence for DNA not being the site of action of the sugars is found in:

1) FIGURE VII, which demonstrates that preincubation of the DNA with sucrose does not result in a significant increase in the number of transformants appearing with time.

2) FIGURES XIV, XV, which show that the cold sensitivity of the sugar stimulatory effect occurs whether or not DNA is present.

3) FIGURE XX, which indicates that lipolytic enzymes decrease the stimulation.

Certain sugars do have an effect on DNA conformation as evidenced by the hypochromic spectral shifts in the presence of these sugars (FIGURE XVII). This is undoubtedly due to a dehydration phenomenon which increases the pitch of the helix. This is a relatively common phenomenon, evidenced in the literature for sucrose and glycerin (Blout and Asadourian, 1954). Lewin (1974) gives an excellent review and theoretical discussion of water intercalation and DNA conformation. The X-ray crystallographic diffraction patterns of DNA depend almost solely on the relative hydration of the molecule. Rudner et al (1967) show the expected opposite result to the sucrose dehydration hypochromic shift by showing a hyperchromic shift in the absorption spectrum by dialysing DNA against pure water, thus increasing hydration and decreasing the pitch. Continued dialysis results in denaturation of the helix.

The sugar effect is apparently limited to areas outside the cell, i.e. from the membrane outwards. This is evidenced by FIGURE XIII, which demonstrates that only the reversible phase of DNA uptake is stimulated. FIGURE XXVI

implies this indirectly in that both fermentable and non-fermentable sugars will stimulate transformation, thereby hinting that metabolic parameters are not directly involved. A conceivable explanation would be that the relatively high osmotic pressure exerted by the sugar is "overriding" the normal DNA transport mechanism and forcing the DNA into the cell another way. FIGURE XIX suggests that this is not the case in that the normal and stimulated DNA transport are both reduced approximately the same amount by 2, 4-dinitrophenol, an energy uncoupler. It is known from the literature (Richardson, 1969) that the drilling of random holes in the membrane with .05% toluene alters permeability but not transformation. Physical-chemical characteristics of the medium are not related to transformation fluctuations with increasing sugar concentrations, so the cell itself is left as the site of action of the sugars. This is confirmed by the low temperature studies (FIGURES XV, XVI), which suggest that cells must be treated with sucrose for inhibition of stimulation by low temperature to occur. Cold treatment of cell supernatants alone with sucrose elicits no inhibition.

A survey of the literature yields one fundamental effect of hypertonic sugar solutions on bacterial cells: the mesosomes of the cell are induced to extrude from the

cytoplasm and into the space between the cell wall and the cell membrane (Fitz-James, 1965; Weibull, 1965; Ryter, 1968 - a review; Remsen, 1968). Mesosomes, as discussed in the Introduction, have long been postulated to be involved with transformation. Electron microscopy of bacteria in hypertonic solutions, when attacked by the enzyme lysozyme, indicates breaks occurring in cell wall material at various areas of the cell surface, including some areas overlying extruded mesosomes (Ryter, 1968; Shockman, 1968b; Ellwood and Tempest, 1972). Shockman (1968a) gives evidence that autolytic enzymes, especially those in B. subtilis, hydrolyze the same linkage in the peptidoglycan as lysozyme. The autolytic enzymes have their action limited to the growing points of the cell, while lysozyme acts more universally. Again, as discussed in the Introduction, DNA uptake seems limited to the growing points of the cell. Akrigg et al (1968) postulated that an autolytic event occurring in the vicinity of a mesosome would allow a surface bound (reversibly bound?) DNA molecule more exposure to mesosome and would cause uptake. If this hypothesis is taken as a base, a more efficient DNA/membrane association might be obtained in several ways:

1) increase the number of autolytic cell wall breaks, thereby increasing the probability of a

break occurring in the vicinity of a mesosome.

2) increase the number of mesosomes, thereby increasing the probability that a mesosome will occur under an autolytic break.

There is evidence in the literature for item 1) in that low concentrations of lysozyme will enhance transformation (Prozorov, 1965; Tichy and Landman, 1969), and treatment of cells with autolysin increases DNA binding (Seto et al, 1975). Evidence for item 2) is available in that electron microscopy of cells approaching competence indicates that the types number of mesosomes increases as competence increases (Wolstenholme et al, 1966; Vermeulen and Venema, 1974a,b). It is here that the sugar effect might be placed. The number of mesosomes is not being changed, but the area encompassed by the extrued mesosome is effectively larger than that encompassed in the invaginated position. This increase in mesosome area, if indeed the cause of the stimulation of transformation, should result in an increased sensitivity to lypolytic enzymes. This is the case as is indicated in FIGURE XX. Inhibition of transformation by the lipases is not as great as expected, but steric or accessibility hindrances might explain this. The pH optimum remains unchanged (FIGURE X) in the presence of sucrose, but the amount of

methylene blue adsorbed decreases as compared to the control (FIGURE XVIII), thereby indicating a decrease in the negative charge of the competent cells. Since the pH optimum is the same, but fewer negative charges are apparent, a rearrangement or change in accessability of charge groups might be suggested. More indirect evidence for this idea is given in the ionic strength studies (FIGURE XI), where the control cells show a small peak of transformation activity at 0.01M K_2SO_4 and a larger peak at 0.2M, while sucrose stimulated cells show a single large peak at 0.01M and an inhibition of activity at higher concentrations. This suggests an alteration in charge availability induced by the sucrose. The 0.2M peak in the control cells confirms in B. subtilis the 0.2M Na^+/K^+ stimulation of transformation reported by Kohoutova (1965) in pneumococcus.

The low temperature removal of the sugar stimulation has literature validity if one accepts the "mesosome hypothesis". Fitz-James (1965), Neale and Chapman (1969, 1970) give evidence that mesosomal deterioration and cell wall changes occur if cells are chilled.

As indicated in the literature survey, Seto et al (1975) shows evidence in pneumococcus that competent cells have a large number of sequestered DNA binding sites that

are uncovered by damage to the outer wall by mechanical means or autolysis treatment. The hypertonic sucrose treatment falls in with this concept quite nicely. In this same paper, Seto et al show that hypertonic sucrose treatment of cells allows substantial loss in DNA binding capacity. This confirms the inhibitory portion of FIGURE I in that the concentration of sucrose utilized by Seto et al falls roughly in the 0.6M portion of the concentration spectrum.

Up to this point, a hypothesis has developed which suggests that the sucrose has increased, or possibly just changed, the membrane/mesosomal exposure to autolytic breaks in the cell wall. This alteration is accompained by a change in the charge number/distribution. A question that may now be asked is "what sites or activities are being uncovered or masked by the osmotically induced change?" Joenje and Venema (1975) in B. subtilis and Kohoutova (1967), Lacks and Greenberg (1973) and Lacks et al (1975) in pneumococcus report a surface bound exonuclease activity that is necessary for transformation. They also suggest that the enzyme is loosely bound to mesosomes and that endonuclease activity is also present. DNase activity is also found in cultural filtrates of B. subtilis (Kohoutova, 1967), pneumococcus (Ottolenghi and Hotchkiss, 1962; Kohoutova, 1967) and

Streptococcus (Nalecz and Dobrzanski, 1972). Kohoutova (1967) gives evidence that hypertonic treatment of competent pneumococcus by NaCl or KCl inhibited DNase activity in filtrates. Inhibition of low DNase activity in highly transformable strains resulted in a decrease in transformation, while inhibition of high DNase activity in low transformation strains resulted in an increase in transformation. The relationship of the DNase activity associated with competence substances (see Introduction) with the DNases just mentioned is not known. FIGURE XXI indicates that transforming DNA incubated with competent cell filtrates has much less biological activity than DNA incubated in filtrates of sucrose treated competent cells. Treatment of DNA with DNase I in the presence of sucrose or glucose (a non-stimulating sugar) shows almost identical reactions of slightly less activity than the control. This is not significant for this study since a stimulating and non-stimulating sugar gave the same results. Thus, it may be tentatively suggested that the sucrose does not directly affect the activity of the DNase. This must be qualified by the assumptions that other solubilized DNases react similarly to DNase I and "immobilized" DNases (on cell surfaces) will also react to sucrose in the same way. Since

the activity apparently is not affected, perhaps the quantity of DNase released into the medium is altered. It may be postulated from the data of Seto et al (1975) that loosely bound DNases exist at sites on membrane material, but only specific DNase sites, or perhaps sites with a particular type of DNase, allow, in combination with competence factor, irreversible binding of DNA. The other sites are purely nucleolytic. The early work of Barnhart and Herriott (1963) gives some indirect evidence supporting this in that reversible binding has a different (lower) ionic strength optimum than irreversible binding. The addition of sucrose to transforming cells after DNase treatment, thus leaving only irreversible bound DNA, consistently results in transformation frequencies lower than those of the control (FIGURE XIII), as does cold treatment in the presence of sucrose (FIGURES XIV, XV). This removal of DNA from DNase protection is probably mechanical and might indicate movement of binding sites in that alteration of the binding sites by sucrose would result in an overall decrease in transformation, which is not the case. Thus, a movement of cell membrane material may be envisioned, induced by the osmotic properties of the sucrose and resulting from extrusion of mesosomal material between the wall and membrane. This movement would bring

an area of membrane into the autolytic break containing not more DNA binding sites, but fewer plain nucleolytic sites (no nucleases have been reported in mesosomal preparations - Reusch and Burger, 1973 - a review). This could account for the reduced hydrolysis of DNA preincubated in supernatants of sucrose treated cells and the increased transformation, since the DNA is less likely to be hydrolysed before irreversible binding. Less hydrolysis of the DNA should show up as an increase in the double transformation of unlinked markers due to more, or longer, pieces of DNA becoming irreversibly bound. This indeed occurs and is reflected in the 1.5 to 2.5 fold increase in the number of "competent cells". This number is calculated using the ratio of single transformants of unlinked markers to the double transformants of the same markers.

The difference in the binding sites could be the ability to bind competence factor, since Tomasz (1971) shows evidence that the first step in conversion to competence is the penetration of competence factor molecules through the cell wall and association with receptors on the membrane.

The sucrose also stimulates single-strand transformation. Tevethia and Cautill (1971) give evidence that competence for single-strand and double strand DNA uptake arises

simultaneously. They also indicate that single and native DNA competes for the same uptake sites. This is also supported by Seto et al (1975). Tevethia and Mandel (1971) demonstrate the presence of a DNase for single strand DNA on competent B. subtilis. Thus, the single-strand system gives similar results to the native DNA system, thereby supporting the developing mechanism.

The postulated mechanism is summarized below and in FIGURE XXVIIa,b.

1) A normal competent cell contains autolytic breaks in the cell wall exposing membrane material (FIGURE XXVIIa). This membrane material has nucleases loosely bound to it. Competence factor may bind to nuclease sites adjacent to, or part of, a mesosome. Here, the DNA is ionically bound reversibly, then irreversibly, as one strand is hydrolysed and the other strand becomes transported into the mesosome. The other sites also bind DNA reversibly, but it is merely hydrolyzed.

2) The same competent cell in the presence of an appropriate concentration of a stimulating sugar shows the same autolytic break in the wall (FIGURE XXVIIb), but the exposed membrane material is changed due to

evagination of the mesosome between the wall and membrane. A mesosomal DNA binding site is still present, but fewer, or masked, solely nucleoytic sites are present, thus allowing more DNA to be transported.

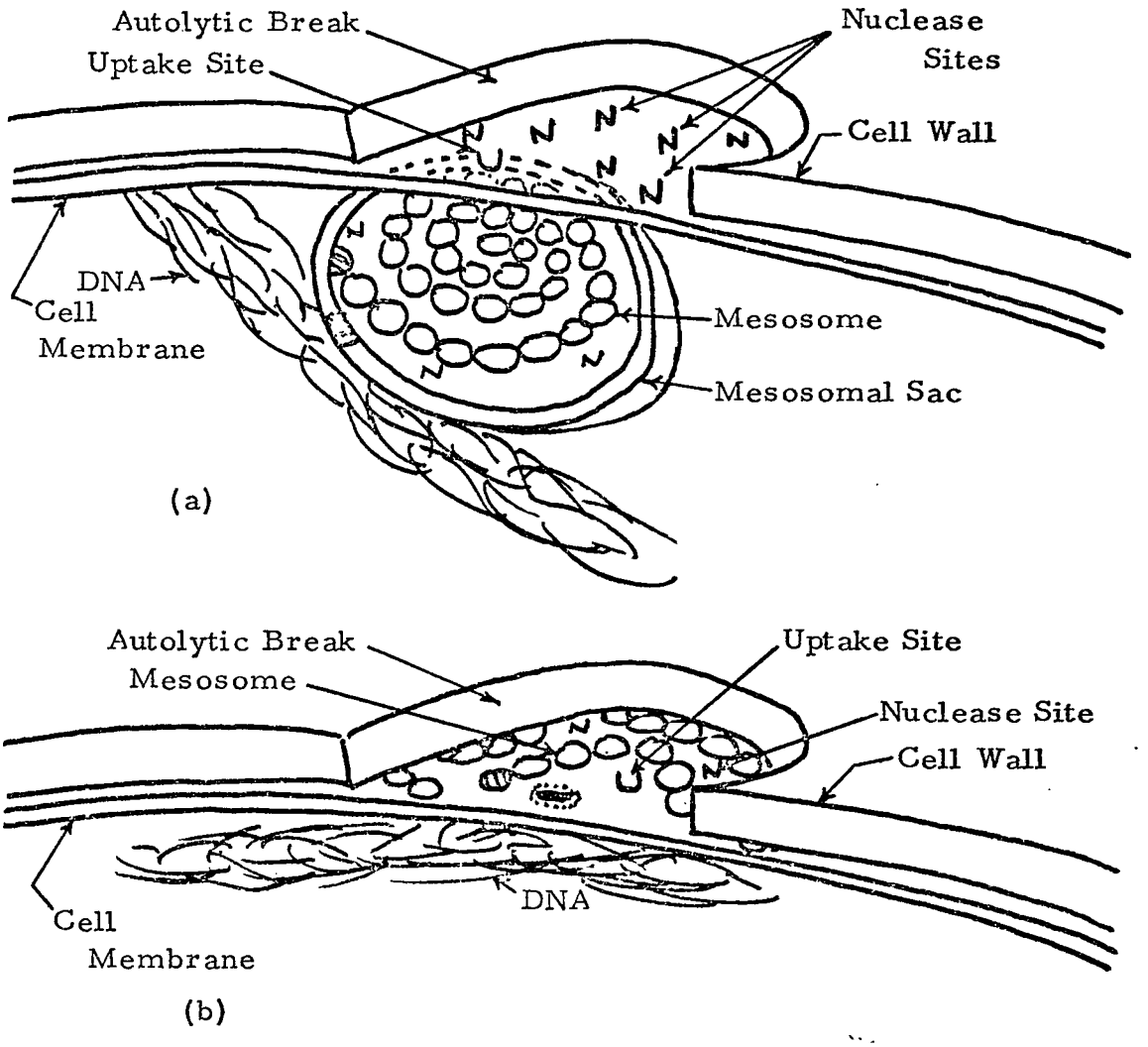


FIGURE XXVII

IDEALIZED REPRESENTATION OF AN AUTOLYTIC BREAK IN A COMPETENT CELL WALL IN THE ABSENCE (a), AND PRESENCE (b) OF A STIMULATING SUGAR. IN (b) THE MESOSOME HAS EXTRUDED INTO THE PERIPLASMIC SPACE MASKING OR "MOVING" THE NUCLEASE SITES.

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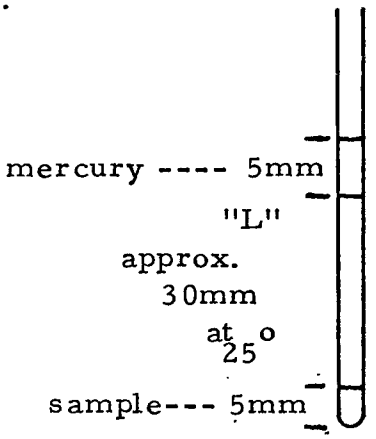
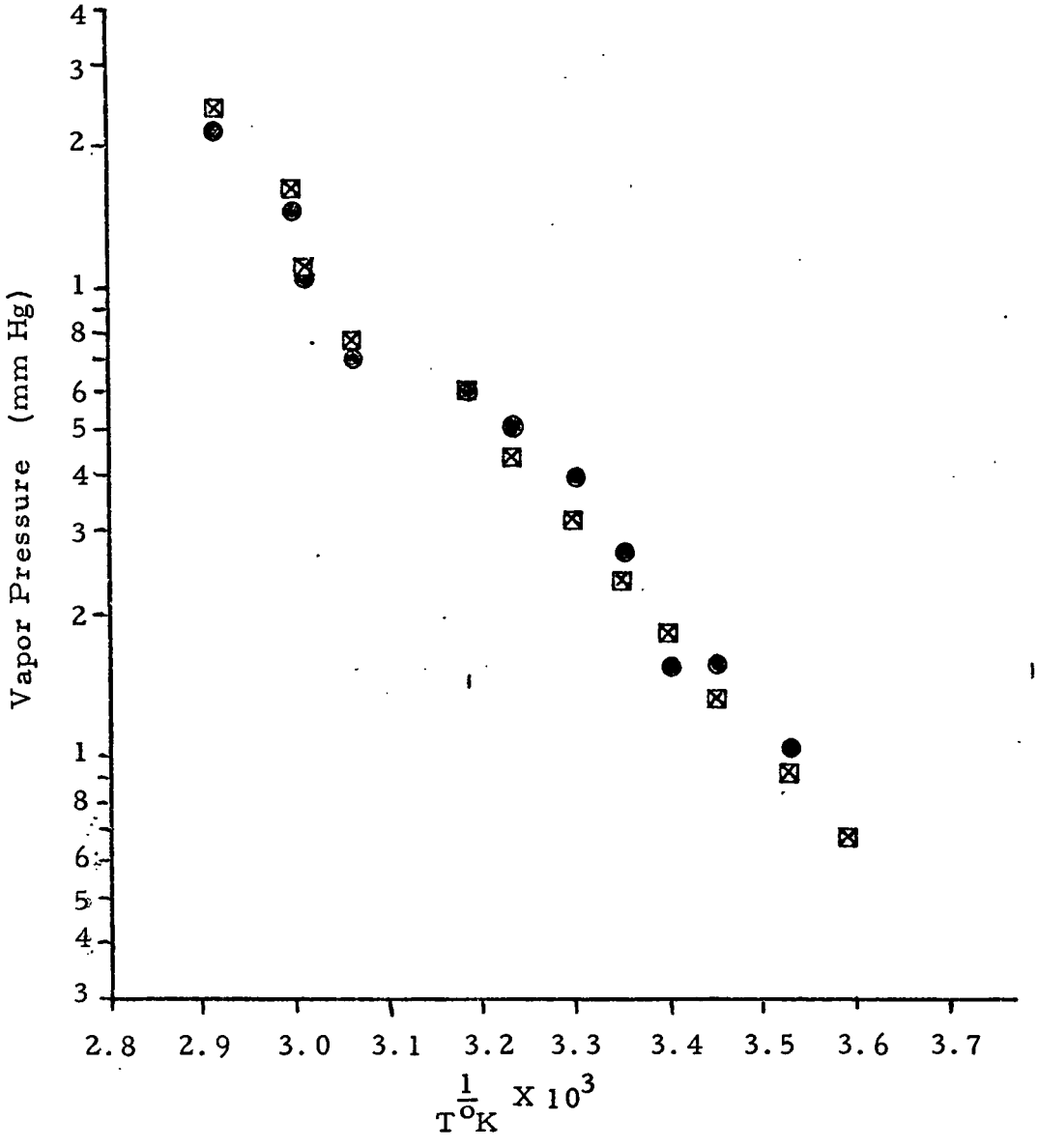
APPENDIX A

Capillary Tube Vapor Pressure Manometer

Glass capillary tubes, about 1.5mm i.d., were sealed at one end with a micro-burner. The tube contents were arranged as per the diagram.

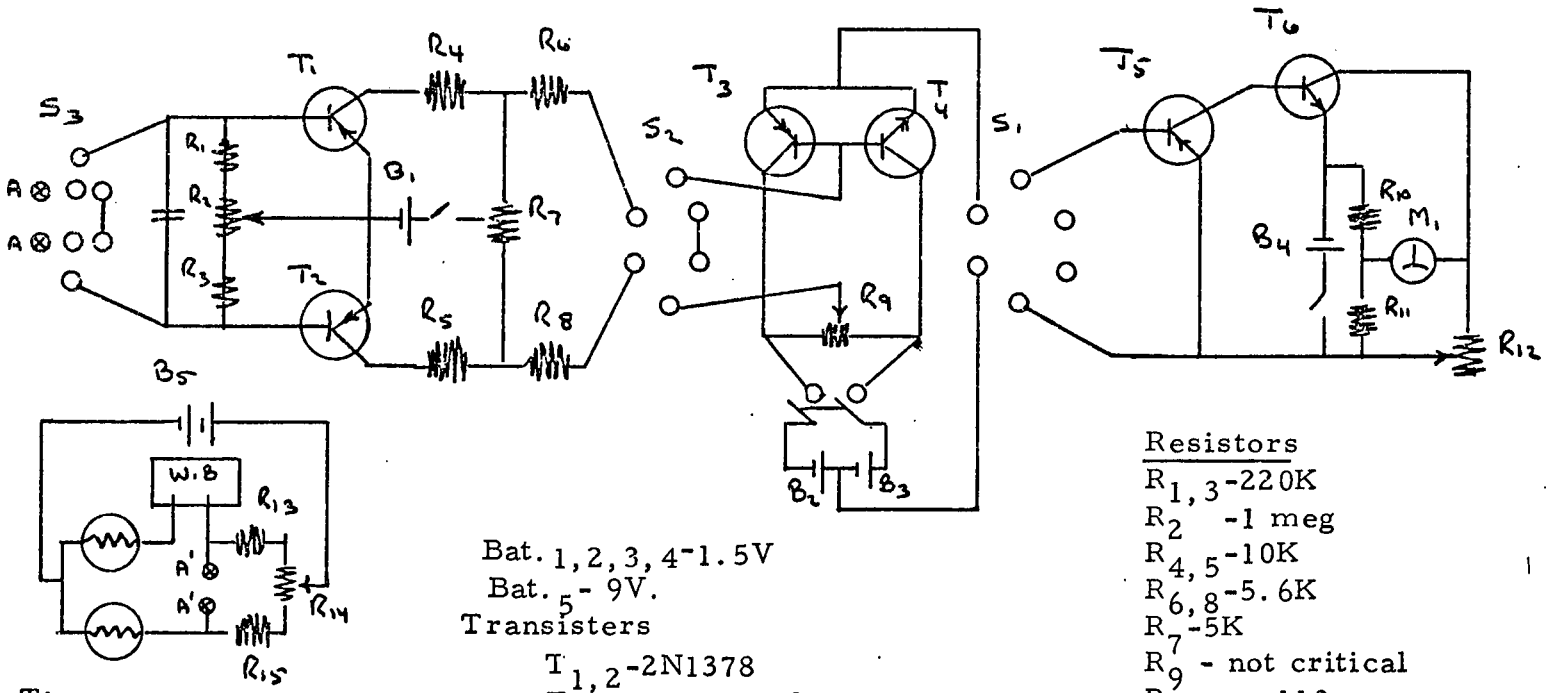


A series of 6 tubes were used per sample. All tubes were placed in an ice water bath in a temperature controlled container. The temperature was then raised in 5° increments and the change in L measured and recorded.



CALIBRATION CURVE FOR A CAPILLARY TUBE VAPOR PRESSURE MANOMETER UTILIZING WATER AS THE CALIBRATING FLUID. EXPERIMENTAL VALUES ARE REPRESENTED BY ● ● , LITERATURE VALUES BY ⊠ ⊠ .

APPENDIX B



Thermistors -
any 100K bead

W. B. = Wheatstone Bridge

Bat. 1, 2, 3, 4 - 1.5V
Bat. 5 - 9V.

Transisters

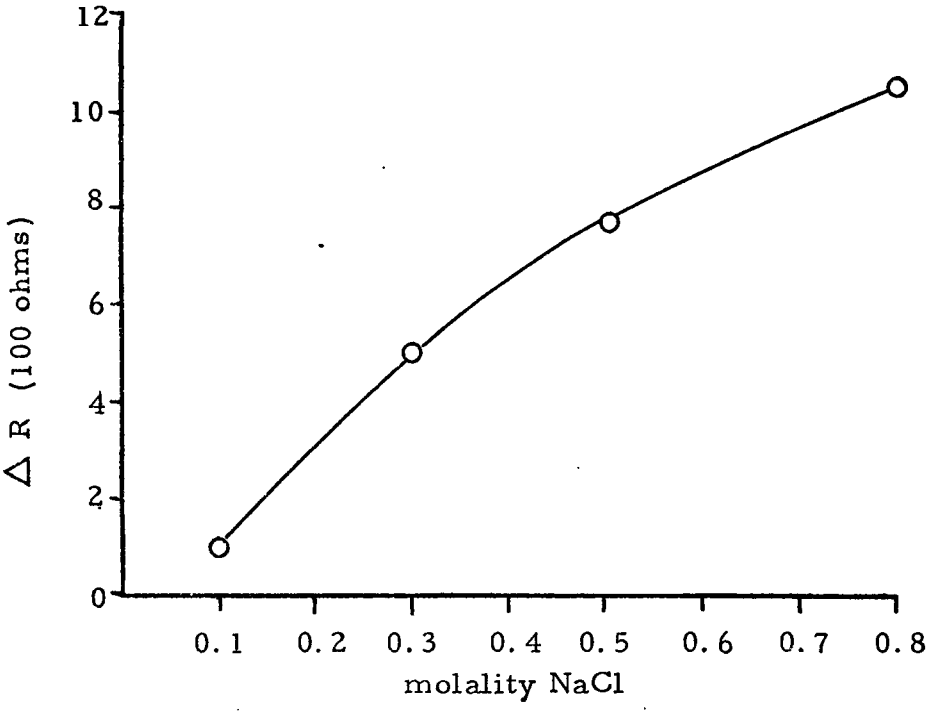
$T_{1,2}$ - 2N1378
$T_{3,4}$ - any complementary
T_5 - 2N34
T_6 - 2N35

Resistors

$R_{1,3}$ - 220K
R_2 - 1 meg
$R_{4,5}$ - 10K
$R_{6,8}$ - 5.6K
R_7 - 5K
R_9 - not critical
$R_{10,11}$ - 110
R_{12} - 10K

Meter - 50-0-50 uA

SCHEMATIC OF THE THERMO-ELECTRIC VAPOR PRESSURE OSMOMETER.



CALIBRATION CURVE FOR A THERMO-ELECTRIC VAPOR PRESSURE OSMOMETER. IF MOLALITY IS CINVERTED TO OSMOTIC CONCENTRATION (NUMBER OF IONS X OSMOTIC COEF.) THE PLOT ASSUMES A STRAIGHT LINE.