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BIOCHEMICAL, GENETIC, AND ENVIRONMENTAL FACTORS AFFECTING GENETIC TRANSFORMATION OF ESCHERICHIA COLI K 12

GREGORY ERNEST STEINKRAUS

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BIOCHEMICAL, GENETIC, AND ENVIRONMENTAL FACTORS AFFECTING GENETIC TRANSFORMATION OF *ESCHERICHIA COLI* K 12

by

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B.S., Upsala College, 1969

A THESIS

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To my Parents.

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TABLE OF CONTENTS

LIST OF TABLES viii

LIST OF FIGURES ix

ABSTRACT xi

I. REVIEW OF LITERATURE 1

II. MATERIALS AND METHODS 14

1. Bacterial and Phage Strains 14

2. Media 17

3. Radioactive Labeling of Transforming DNA 18

4. Isolation of Transforming DNA 19

5. Assay of Radioactivity 22

6. Preparation of Phage and Phage DNA 22

7. Optical Density Readings 25

8. Transformation 25

9. Spheroplast Formation 26

III. RESULTS 27

1. Effect of Calcium on the Transformation of *E. coli* K 12 27

2. Effect of DNA Isolation Procedure on Transformation 34

3. Effect of DNA Concentration on Transformation 45

4. Effects of External Treatments on Transforming Preparation 45

5. Kinetics of DNA-Recipient Cell Interaction .. 49

6. Effect of Inorganic Ions other than Calcium on Transformation 52

7. Effect of Temperature on Transformation 52

8. Effect of pH on Transformation 55

9. Effect of Polyamines on Transformation..... 55

10. Effect of Spheroplasted Recipient Cells on Transformation..... 61

11. Effect of Polyamines on Spheroplasted Recipient Cells in Calcium Deficient and Supplemented Media..... 61

12. Effect of Protamine on Transformation of Spheroplasted and Non-spheroplasted Cells of *E. coli* K 12 treated with Calcium..... 63

13. Effect of O_2 Tension on Transformation..... 63

14. Effect of Phospholipids and Phospholipid Synthesis on Transformation..... 64

15. Transformation as a Function of DNA Source... 64

16. Effect of Preincubation Temperature on the Uptake of ^{32}P Labeled Donor DNA..... 69

17. Effect of Protein Synthesis on Uptake of DNA. 76

18. Transfection of Calcium Treated Recipient Cells..... 76

19. Transfection of *E. coli* K 12 with $^{\lambda}$*plac*5... 76

IV. DISCUSSION..... 85

1. Effect of Calcium, Spermine and Protamine and Spheroplasted Recipient Cells..... 85

2. DNA Isolation..... 90

3. DNA-recipient Cell Interaction..... 95

4. Temperature..... 96

5. pH..... 96

6. DNA Source..... 96

7. Uptake of DNA..... 97

8. O_2 Utilization Characteristics..... 97

9. DNA Concentration..... 98

10. Transfection..... 99

11. Conclusion..... 100

LITERATURE CITED..... 101

LIST OF TABLES

1. Bacterial and Phage Strains..... 15

2. Relationship between Donor DNA Molecular Weight and Transformation..... 40

3. Effects of External Treatments on Transforming Preparation..... 48

4. Effect of pH on the Transformation of *E. coli* K 12..... 60

5. Effect of Spheroplasted Recipient Cells on Transformation..... 62

6. Effect of O_2 Tension on the Transformation of Calcium Treated *E. coli* K 12 Spheroplasts..... 65

7. Effect of the Source of the Donor DNA on Transformation of Various Strains of *E. coli*..... 70

8. Transformation of *E. coli* K 12 Cells with DNA Isolated from Previously Transformed *E. coli* K 12 Cells..... 71

9. Transfection of Calcium Treated Cells of *E. coli* K 12 by λ and Ø80 DNA..... 81

10. Transfection of Calcium Treated *E. coli* K 12 Spheroplasts by λ and Ø80 DNA..... 82

11. Transfection of *E. coli* K 12 with λ*plac*5..... 83

LIST OF FIGURES

1. Diagramatic Representation of Special Features in the Transformation of E. coli K 12..... 13

2. Transformation in E. coli K 12 with Donor DNA Isolated by the Method of Marmur..... 29

3. Transformation in E. coli K 12 with Donor DNA Isolated by the Method of Oishi and Cosloy..... 31

4. Transformation in E. coli K 12 with Donor DNA Isolated by the Method of Worcel and Burgi..... 33

5. Neutral Sucrose Gradient Profiles of Transforming DNA Isolated by the Method of Worcell and Burgi.... 36

6. Sarkosyl Release of Folded E. coli DNA from Membrane-associated Complex..... 39

7a. Arithmetic plot of the number of lac^+ transformants versus the reciprocal of the molecular weight..... 42

7b. Expansion from Figure 7a for molecular weights in excess of 10^8 daltons..... 44

8. Relationship between Donor DNA Concentration and the Number of lac^+ Transformants..... 47

9. Relationship between Recipient Cell Contact with Donor DNA and Yield of lac^+ Transformants.... 50

10. The Effect of Magnesium and Barium on Transformation in E. coli K 12..... 54

11. Effect of Temperature on the Transformation of E. coli GR-1..... 57

12. Effect of Temperature on the Transformation of E. coli JC 7626..... 59

13. Effect of Phospholipids and Phospholipid Synthesis on Transformation..... 68

14. Effect of Preincubation Temperature on the Uptake of 32^P Transforming DNA in JC 7626..... 73

15. Effect of Preincubation Temperature on the Uptake of 32^P Transforming DNA in GR-1..... 75

16. Effect of Inhibition of Protein Synthesis on the Uptake of Transforming DNA in JC 7626..... 78

17. Effect of Inhibition of Protein Synthesis on the Uptake of Transforming DNA in GR-1..... 80

ABSTRACT

BIOCHEMICAL, GENETIC, AND ENVIRONMENTAL FACTORS AFFECTING GENETIC TRANSFORMATION OF *ESCHERICHIA COLI* K 12

by

Gregory E. Steinkraus

The nature of the transformation process in *Escherichia coli* K 12 was investigated by examining various factors affecting the efficiency of transformation. Most important in the demonstration of this genetic phenomenon was treatment of the recipient cell with calcium. In contrast to previous *E. coli* transformation systems where sheared, median molecular weight DNA was employed (Oishi and Cosloy, 1972; Wackernagel, 1973), unsheared, high molecular weight DNA was the most effective. The intracellular status of an ATP-dependent DNase was of critical significance. A mutant deficient in this enzymatic activity (*recB*$^-$ *recC*$^-$), yet capable of genetic recombination due to one or two indirect suppressor mutations (*sbcA*$^-$ or *sbcB*$^-$), enhanced transformation. A second enzymatic activity associated with the transformation process was DNA polymerase I. A mutant (*polA*ex1) deficient in the 5'-3' exonucleolytic activity of the enzyme, yet possessing the 3'-5' exonucleolytic activity and the DNA polymerizing activity, also enhanced transformation. By the use of a glycerol auxotrophic mutant,

phospholipids and phospholipid synthesis neither enhanced nor inhibited the transformation process. For all transformation reactions, the limiting concentration of transforming DNA was 25 ug/ml. Although 40 C was the optimal temperature for transformation, incubation at 3 C for 25 min was required immediately prior to incubation at 40 C. Additionally, only isogenic DNA was effective in the transformation of *E. coli* K 12 cells. The optimal pH was 8.2. With regard to oxygen utilization characterization, transformation was seen to occur under aerobic conditions. The highest number of *lac*$^+$ transformants resulted subsequent to both calcium and spermine (or protamine) treatment of spheroplasted GR-1 recipient cells. With a specialized transformation system using DNA isolated from specialized transducing phages (λ*plac*5 and Ø80*plac*1), the transformation frequency was lower than that observed with bacterial DNA.

REVIEW OF THE LITERATURE

The genetic material of one bacterial cell may be transferred to a second bacterial cell by three general mechanisms. These mechanisms are characterized by the mode of transfer, and are seen to include: (1) conjugation (mating); (2) transduction (virus-mediated transfer), and (3) transformation (uptake of naked bacterial DNA). Within the phenomenon of transformation there exists a closely related mechanism known as transfection. The term transfection is applied to the uptake of naked bacteriophage DNA by bacterial cells.

Transformation is an intercellular process whereby a genetic fragment (exogenote) from a donor cell, obtained by either chemical extraction (Braun, 1965), as is usually the case, or through spontaneous excretion (Braun, 1965; Hayes, 1966; Streips and Young, 1973), penetrates into a competent recipient cell. The competent state is defined as the ability of recipient cells to take up exogenous DNA. Once located intracellularly, the exogenous DNA, if sufficiently related to the DNA of the recipient cell, undergoes integration via recombination with a homologous segment of the recipient cells genome (endogenote). If the newly integrated sequence of nucleotides (exogenote) differs in part from the replaced nucleotide sequence of the recipient, new information will have thus been transformed to the recipient cell and its resulting progeny.

In general, transformable bacterial cells are capable of taking up naked DNA from just about any source (Radding, 1973). Genetic recombinants, however, are formed only if the exogenous DNA is from a closely related organism (Radding, 1973).

Bacterial genetic transformation began in 1928 with the accidental observance of the phenomenon by a British health official concerned with the epidemiology of pneumococcal pneumonia. While experimenting with the etiological agent [Diplococcus (Streptococcus) pneumoniae] of human lobar pneumonia, Griffith (1928) observed that subsequent to his injection of mice with either a live culture of a non-encapsulated (non-virulent) type II pneumonococcus, or with a heat killed suspension of a capsulated (virulent) type I pneumonococcus, none of the mice, although exquisitely sensitive to this bacterial pathogen, died. In contrast, when a mixture of the above two pneumococcal preparations was injected, a small percentage of mice developed the disease and died. Griffith (1928) was able to isolate from the mice which had died, pure cultures of encapsulated (virulent) pneumococci possessing type I characteristics. Griffith (1928) had thereby shown that it was possible to transform a non-encapsulated (non-virulent) type II pneumococcus, into an encapsulated (virulent) type I pneumococcus, by *in vivo* passage, in the presence of heat killed type I cells. Griffith (1928) hypothesized that the chemical nature of the transforming principle was either protein of polysaccha-

ride. A few years later, a similar transformation was achieved in vitro (Dawson and Sia, 1931). In the following years, a deeper analysis of the phenomenon and the chemical nature of the transforming principle became a reality when Alloway demonstrated that a cell-free extract of virulent pneumonococci could transform non-virulent cells (Alloway, 1933).

Still, the chemical nature of the transforming principle eluded researchers. A breakthrough finally came in 1944 when a group of researchers presented an impressive collection of evidence pointing to deoxyribonucleic acid (DNA) as the transforming principle (Avery, MacLeod and McCarty, 1944). To further substantiate their findings, this group reported that the transforming principle was unaffected by either ribonuclease (RNase) or proteolytic enzymes (McCarty and Avery, 1946). The transforming principle was, however, destroyed by the action of deoxyribonuclease (DNase) (McCarty and Avery, 1946). Additionally, that this active fraction owed its activity to DNA was further verified when Hotchkiss purified the transforming DNA to less than 0.02% protein (Hotchkiss, 1949).

Gradually, DNA became recognized as the molecular species involved in transformation. Shortly thereafter, the process of transformation itself was accordingly recognized as a genetic phenomenon (Ephrussi-Taylor, 1951b).

Since its initial demonstration with pneumococcus, transformation has been attained with only a limited number

of bacterial species. The majority of these species are seen to belong to the following bacterial genera: Bacillus, Diplococcus, Hemophilus, Neisseria, Staphylococcus and Streptococcus (Spizizen, Reilly and Evans, 1966; Hotchkiss, 1970). With regard to the Enterobacteriaceae, genetic transformation has proved to be largely unsuccessful. The question naturally arises as to why this is so especially when one considers the enormous amount of information which has been obtained with members of the Enterobacteriaceae, and in which so many well characterized mutants are available. Again, this is especially perplexing with regard to the genus Escherichia. Thus, the remainder of this discussion will focus on the genus Escherichia and its apparent lack of participation in transformation.

Genetic transformation of E. coli, as well as other members of the Enterobacteriaceae, is negligible when compared to other bacterial transformation systems, for example, the systems of Bacillus, Diplococcus, and Hemophilus (Hotchkiss, 1970). This is not to say, however, that transformation in Escherichia coli is non-existent.

It was known since the early 1960's that DNA, extracted by phenol treatment from temperate coliphages such as lambda, 434 and P2, penetrated, in the presence of "helper phage", sensitive cells of E. coli (Kaiser and Hogness, 1960). Specifically, Kaiser and Hogness demonstrated genetic transformation of E. coli by the simultaneous infection of sensitive cells by naked lambda dg DNA and intact lambda phage particles.

Later in the 1960's, this phenomenon was again demonstrated by Mandel (1967). The exact role played by the "helper phage" remains to be elucidated. It was proposed that the entry of the DNA of the "helper phage", and the presence of the intact "helper phage", were requirements for competent cell formation (Takano and Watanabe, 1967). Furthermore, in order for the transfecting coliphage DNA to successfully infect sensitive cells, the nucleic acid must possess at least one free cohesive end (Kaiser and Inman, 1965). In addition, a correlation existed between the specificity of the cohesive ends of the "helper phage" DNA and the transfecting coliphage DNA with the ability of the phage to serve as a helper for the transfecting coliphage DNA (Kaiser and Wu, 1968; Mandel and Berg, 1968). Thus, the infectivity of the transfecting coliphage DNA depended on a homology between its own cohesive ends and the cohesive ends of the "helper phage" DNA.

Permeability changes in the cell wall of E. coli occurred subsequent to their being rendered competent by the simultaneous infection of "helper phage" and naked transfecting DNA (Mandel, 1967). Such an observation prompted researchers to determine the effects of both monovalent and divalent cations on the cell wall permeability of E. coli, and its resulting influence on the uptake of exogenous DNA. It was subsequently demonstrated that DNA, extracted from the temperate phages P2 and lambda, penetrated sensitive cells of E. coli in the absence of "helper phage", but in the presence of calcium ions (Mandel and Higa, 1970). Man-

del and Higa thereby demonstrated that E. coli K 12, when grown in calcium-supplemented P medium (Kaiser, 1962), was capable of taking up exogenous DNA. Additionally, transfection of E. coli spheroplasts by ØX174 (Guthrie and Sinsheimer, 1963) and lambda (Young and Sinsheimer, 1967), has also been successful. An important point with regard to these transfection experiments is that the survival of the transfected cells is not required as the production of viable phage particles is assayed with an appropriate bacterial indicator strain (Cohen, Chang and Hsu, 1972).

Closely related to the phenomenon of transfection is the phenomenon of transformation with R-factor DNA. Following the work of Mandel and Higa (1970) dealing with the uptake of lambda DNA in E. coli cells treated with calcium, similar treatments of E. coli cells also rendered them capable of expressing purified R-factor DNA (Cohen, Chang and Hsu, 1972).

Before discussing the most recently verified aspects of genetic transformation in E. coli, let me briefly consider a reportedly highly successful, but presently defunct, approach to this problem.

In 1957, Chargaff, Schulmann and Shapiro stated that "the complete isolation of DNA from E. coli is not easy". This led researchers to believe that the reason their attempts at showing genetic transformation in E. coli were largely unsuccessful was because the methods available for the isolation of the transforming DNA yielded products of

unsatisfactory quality.

Directing their investigations along the line of an unsatisfactory extraction procedure, Mehta, Rege and Sreenivasan (1962) reportedly demonstrated that a DNA preparation could be obtained from cells of E. coli MacLeod which was capable of transforming, to nutritional independence, the vitamin B_{12} or methionine-requiring strain of E. coli 113-3 of Davis and Mingioli (1950).

This work was later extended and the frequency of transformation attained was in the vicinity of 4.8 to 5.0 percent (Avadhani, Mehta and Rege, 1969). The transformation observed was a strain-specific phenomenon being dependent on both the donor and recipient cells. Additionally, it was emphasized that the procedure for the preparation of the transforming DNA was critical to its biological activity. Finally a special emphasis was placed on the pH value during the lysis of the donor cells (Avadhani, Mehta and Rege, 1969).

With regard to the competency of the recipient cells, this is another property of the transformation system, the implications of which are seen to vary with different strains. In E. coli, the competent state coincides with the middle of the logarithmic phase of growth (Avadhani, Mehta and Rege, 1969). At this stage, the phosphodiesterase activity of the population was at a minimum (Avadhani, Mehta and Rege, 1969).

In recapitulation then, the frequency of transformation for single genetic markers was approximately 5%.

When this percentage is compared to the frequency of transformants for single markers in the transformation systems of Diplococcus, Hemophilus, and Bacillus, the results appear as follows. With regard to the Diplococcal (Fox and Hotchkiss, 1957) and Hemophilus (Goodgal and Herriot, 1961) systems, the frequency of single genetic marker transformants, at saturating levels of transforming DNA, may be as high as 5% or more. In the case of the Bacillus system, the frequency of transformants for single markers is only between 0.1 to 0.5%, while that of competent cells is less than 10% (Nester and Stocker, 1963). Duplication of the 5% E. coli transformation system as reported by Avadhani, Mehta and Rege, has not occurred.

During studies on the mechanism of action of an ATP-dependent DNase, numerous researchers (Wright and Buttin, 1969; Oishi, 1969; Barbour and Clark, 1970; Goldmark and Linn, 1970), observed that the enzyme did not attack double-stranded, nicked or unnicked, circular DNA. In contrast, however, this enzyme led to extensive degradation of linear DNA molecules. This discovery proved quite illuminating as the source of the genetic transforming material in transformation systems other than E. coli were fragments of the donor bacterial genome. Therefore, the repeated failures to transform E. coli could be a function of the extensive degradation of the exogenous transforming DNA by this ATP-dependent DNase (Oishi, 1969).

In the studies that followed, Cosloy and Oishi

demonstrated that transformation for various chromosomal markers could be effected at a frequency of 10^{-6} subsequent to calcium treatment of recipient E. coli cells deficient in both the ATP-dependent DNase (recB$^-$ recC$^-$) and also exonuclease I (sbcB$^-$) (Oishi and Cosloy, 1972; Cosloy and Oishi, 1973). From their investigations, Cosloy and Oishi (1973) suggested that the previous failures to demonstrate genetic transformation in E. coli were attributable to two major factors. First of all, there was the limited penetration of the exogenous transforming DNA into the recipient cells. Secondly, there was the presence of the ATP-dependent DNase which degrades all incoming, linear, double-stranded, transforming DNA before it is able to reach the recombination machinery of the recipient cell. Oishi and Cosloy felt, thereby, that they had solved these two major problems associated with the previous repeated failures at showing genetic transformation in E. coli by, first of all, treating the surface of the recipient cells with calcium, and secondly, by employing a strain of E. coli (recB$^-$ recC$^-$ sbcB$^-$) which lacked the ATP-dependent DNase (recB$^-$ recC$^-$) but which possessed a functional recombination system due to the presence of the sbcB indirect suppressor mutation (Cosloy and Oishi, 1973).

Further studies into the genetic basis of this transformation system showed that a molecule of the ATP-dependent DNase was composed of two different proteins. Each protein was coded for by two different genes, and the genes

were designated as recB and recC (Lieberman and Oishi, 1973). A mutation in either gene was seen to result in the complete loss of degradative enzymatic activity (Oishi, 1969; Barbour and Clark, 1970). In addition, there was an extensive reduction in the ability of the cell to carry out genetic recombination (Clark and Margulies, 1965; Howard-Flanders and Theriot, 1966; Clark, 1967). Although this enzyme possesses degradative activity for linear double-stranded DNA molecules and is a necessary component of the recombination mechanism of the cell, the absence of this ATP-dependent DNase led to an increased efficiency of transformation (Clark, 1973). If, therefore, this ATP-dependent DNase, also known as exonuclease V, is a recombination enzyme, then an explanation of genetic recombination in its absence is necessary.

In the early 1970's, two groups of researchers discovered two classes of indirect suppressor mutations of recB and recC mutations (Barbour, Nagaishi, Templin and Clark, 1970; Kushner, Nagaishi, Templin and Clark, 1971). These suppressor mutations, designated sbcA$^-$ and sbcB$^-$, restored genetic recombination to normal levels, while at the same time, failed to restore the ATP-dependent DNase activity (Clark, 1971). The product of the sbcB gene was identified as exonuclease I (Kushner et. al., 1971). In the situation where both the ATP-dependent DNase and exonuclease I are absent (recB$^-$ recC$^-$ sbcB$^-$), what occurs is the "opening up" of a new pathway for genetic recombination (Clark, 1971). Such a new pathway for genetic recombination has been termed

RecF (Clark, 1971).

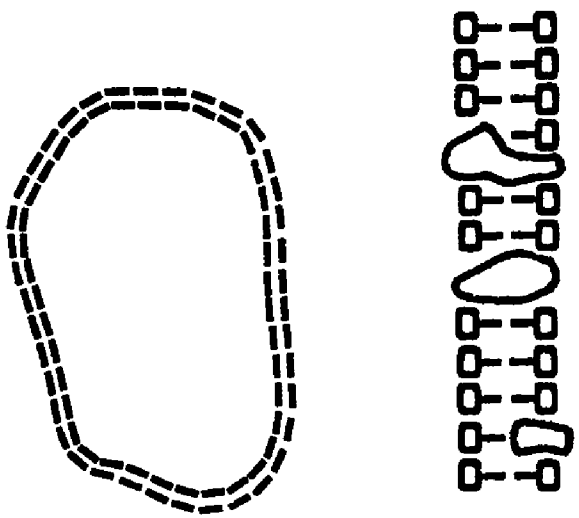
sbcA mutations were first described by Barbour et. al. (1970) by virtue of their ability to suppress, indirectly, the recombination and repair deficiencies associated with recB and recC mutations. As such, triple mutant (recB$^-$ recC$^-$ sbcA$^-$) strains were as proficient in recombination and repair as wild type (recB$^+$ recC$^+$ sbcA$^+$) strains. In the case of sbcA$^-$ mutants, an ATP-independent DNase (exonuclease VIII) was present in higher concentrations as compared to sbcA$^+$ cells (Barbour et. al., 1970). The sbcA$^+$ state is dominant to the sbcA$^-$ state. Dominance of sbcA$^+$ is consistent with the view of Clark (1971) that sbcA regulates the structural gene (recE) for exonuclease VIII, and that mutations derepress recE. Exonuclease VIII reverses the multifunctional effect associated with recB$^-$ recC$^-$ mutations. Furthermore, exonuclease VIII is a new enzyme with only an exonucleolytic function which in its partially purified form preferentially digests double-stranded DNA over heat denatured double-stranded DNA (Clark, 1974). This enzyme participates in the RecE pathway of bacterial recombination (Clark, 1974).

The results of several investigators suggested that E. coli K 12 could be transformed for various chromosomal markers at a frequency of 10^{-6}, provided that the ATP-dependent DNase is nonfunctional, and providing that the capacity for genetic recombination be maintained by the presence of a second recombination pathway, for example, RecF or RecE.

During this discussion, several unique aspects of the genetic transformation process in *E. coli* have been elucidated. Such special features can be most easily understood by referring to the following diagramatic representation (Figure 1).

Cosloy and Oishi (1973), have suggested that treatment of the recipient cells with calcium, and the intracellular status of the ATP-dependent DNase, are the major factors which precluded the attainment of genetic transformation in *E. coli*. However, it should be emphasized that enzymatic activity, similar to exonuclease V (ATP-dependent DNase), has been found in *Bacillus subtilis* (Clark, 1973), *Diplococcus pneumoniae* (Vovis and Buttin, 1970), and *Hemophilus influenzae* (Smith and Friedman, 1972), all of which are easily transformable. Thus, might there be yet another unique aspect of the problem of genetic transformation in *E. coli*?

Figure 1. Diagramatic representation of special features in the transformation of *E. coli* K 12.



Exogenous transforming DNA	Calcium treated membrane	Rec pathway (phenotype)	Genotype	Transformation frequency
		RecBC	*recB*$^{+}$ *recC*$^{+}$ *sbcB*$^{+}$	None
		RecF	*recB*$^{-}$ *recC*$^{-}$ *sbcB*$^{-}$	High
		RecF RecBC	*recB*$^{+}$ *recC*$^{+}$ *sbcB*$^{-}$	Low
		RecE	*recB*$^{-}$ *recC*$^{-}$ *sbcA*$^{-}$	High
		RecE RecBC	*recB*$^{+}$ *recC*$^{+}$ *sbcA*$^{-}$	Low

MATERIALS AND METHODS

Bacterial and Phage Strains

The bacterial and phage strains employed in this study, along with their genetic characteristics and sources, are presented in Table 1. The unique characteristics of each strain are as follows:

Escherichia coli K 12 polAexl. (RS5052) contains normal amounts of DNA polymerase I, but greatly reduced levels (3% of wild type) of the 5'-3' exonuclease activity. Additionally, it is a temperature-sensitive, conditional lethal mutant which does not form colonies on tryptone-yeast extract agar plates supplemented with methyl methane sulfonate. Furthermore, it exhibits reduced survival on exposure to ultraviolet light irradiation, and also, like other polA mutants, does not support plaque formation by lambda mutants defective in general recombination pathways (red^-). It does, however, support the growth of a wild type lambda. This mutant was chosen for study with regard to the transformation phenomenon because of its increased frequency of recombination, the so called "hyper rec" phenotype (Konrad and Lehman, 1974).

Escherichia coli K 12 recB12 recC22 leu-6 ara-14 his-4. (JC 5519) exhibits greatly reduced levels of recombination. This strain participates in transformation at a frequency of less than 1% that observed in JC 7626.

Table 1. Bacterial and phage strains.

Escherichia coli K 12 Strains	Genetic Characteristics	Source
RS5052	*polA*ex1 *ara*$^-$ *lac*$^-$ *thyA*$^-$	E.B. Konrad I.R. Lehman
GR-1	GR-1 *lac*$^-$ *str*^{S}	C.C. Hsu D.F. Fox
EC-12	*xthA*$^-$ *lac*$^-$ *str*^{S} F-	C. Milcarek B. Weiss
EC-12L	*lac*$^+$ *endo* I$^-$ *str*^{S}	H. Durwald H. Hoffmann-Berling
JC 5519	*recB21 recC22 leu-6 ara-14 his-4*	A.J. Clark
JC 7626	*recB21 recC22 sbcB15 leu-6 ara-14 his-4 lac*$^-$ F-	S.D. Cosloy M. Oishi
JC 5176	*recB21 recC22 sbcA6 gal*$^-$ *end*$^-$ *str*^{S} *lac*$^-$	S.D. Barbour
DG75	*lac*$^+$ *thy*$^-$ *leu*$^-$ F-	A. Worcel E. Burgi
ATCC 25254	wild type	ATCC
ATCC 25256	*met*$^-$ lysogenic for lambda	ATCC
Phage Strains		
Lambda	lambda*plac*5	J.R. Beckwith
Phi 80	Ø*plac*1	J.R. Beckwith

Escherichia coli K 12 recB12 recC22 sbcB15 leu-6 ara-14 his-4 lac$^-$ F-. (JC 7626) is a multiple-auxotrophic strain which lacks both the ATP-dependent DNase (recB$^-$ recC$^-$) and exonuclease I (sbcB$^-$). Because of the sbcB suppressor mutation, this strain retains the capacity for genetic recombination (Cosloy and Oishi, 1973).

Escherichia coli K 12 recB21 recC22 sbcA6 gal$^-$ end$^-$ str^S lac$^-$. (JC 5176) has another type of suppressor of the recB$^-$ recC$^-$ mutation. The introduction of this sbcA$^-$ mutation initiates the appearance of a new type of DNase whose activity is ATP independent. Genetic transformation is also possible with this strain (Barbour and Clark, 1970).

Escherichia coli K 12 GR-1 lac$^-$ str^S. (GR-1) is a glycerol requiring mutant of E. coli K 12 with a specific defect in L-glycerol-3-phosphate dehydrogenease (E.C.1.1.1.8). Glycerol and unsaturated fatty acid auxotrophic mutants behave differently from the temperature-sensitive phospholipid mutants. When the former are deprived of their growth factor, they continue to synthesize proteins and nucleic acid for approximately 80 min. This is not the case with regard to phospholipid mutants. When an unsaturated fatty acid mutant is starved for unsaturated fatty acids, saturated fatty acids are still made, resulting in the continued synthesis of phospholipids containing only saturated fatty acids. In contrast, when glycerol auxotrophic mutants are deprived of glycerol, net phospholipid synthesis is immediately halted; however, in-

hibitation of protein and nucleic acid synthesis is not terminated for approximately 80 min.

Escherichia coli K 12 lac^{+} endo I^{-} str^{S} F-. (EC-12L) is deficient of endonuclease I of Escherichia coli. Such cells are viable, demonstrating that Escherichia coli can survive the loss of endonuclease I (Durwald and Hoffman-Berling, 1968).

Escherichia coli K 12 xthA^{-} lac^{-} str^{S} F-. (EC-12) is defective in exonuclease III. Exonuclease III acts on duplex DNA from either the 3' end or the 5' end. In addition, it exhibits a phosphomonoesterase action on a 3'-phosphate terminus (Brutlag and Kornberg, 1972).

Escherichia coli K 12 F- leu^{-} thy^{-}. (DG75) reportedly gives excellent yields of folded chromosomes (Worcel and Burgi, 1972).

plac5 and Ø80placl. These two virus strains are specialized transducing phages which carry the lac operon (Beckwith and Signer, 1966). The genomes of these phages contain approximately 5 to 10% lac operon DNA along with viral genetic material, and almost always, other bacterial genes (Shapiro et. al., 1969). Thus, DNA extracted from these lac transducing particles is enriched approximately 100-fold for lac operon DNA as compared with the DNA extracted from whole bacteria.

Media

The medium employed for the growth of all strains

of E. coli was Trypticase Soy Broth without Dextrose (TSB w/o D) (Bioquest/BBL. Cockeysville, Maryland) .

H medium was employed for the production of phage lysates and consisted of the following/liter: Bacto tryptone (Difco, Detroit, Michigan), 10 g; NaCl 8 g; and Bacto agar (Difco) 12 g. H top agar for titering the lysate consisted of the same ingredients as H medium except that 8 g of agar were used.

Plates employed for the identification of lac^+ transformants were made according to the formula of Davis minimal salt agar (Davis and Mingioli, 1950) supplemented with 1% lactose.

DNA buffer consisted of the following: 0.10M tris-HCL (pH 7.5), 0.001M EDTA.

Radioactive Labeling of Transforming DNA

^3H labeling of donor cells was achieved by growing the cells for three generations in the above mentioned medium with the addition of 100 uCi (^3H) thymidine/ml (New England Nuclear, Boston, Massachusetts).

^{32}P labeling of the donor cells was achieved by growing the cells for three generations in the above mentioned medium with the addition of 50 uCi/ml of $H_3\ ^{32}PO_4$ (New England Nuclear, Boston, Massachusetts).

Where the T4 phage DNA was labeled with ^{14}C, the bacteria were grown for three generations in Bacto-minimal

Broth Davis w/o Dextrose (Difco) with the addition of 10 uCi/ml of [^{14}C] thymine (New England Nuclear, Boston, Massachusetts).

Isolation of Transforming DNA

Fifteen milliliters of trypticase soy broth without dextrose in 175 mm by 22 mm test tubes were inoculated with donor bacteria and incubated at 25 C for 10 h. A 250 ml Erlenmyer flask containing 40 ml of broth medium was inoculated with 10 ml of culture. The donor cells were grown to midlog phase with constant aeration on a rotatory shaker at 37 C. When strain RS5052 was used as the source of the transforming DNA growth to midlog phase was achieved with constant aeration on a rotatory shaker at 32 C. Subsequently, the cells were chilled to 2 C in a dry ice-acetone bath, and harvested quickly by centrifugation (8,000 x g, 10 min).

Lysis of the donor cells was achieved by the procedure of Stonington and Pettijohn (1971) as modified by Worcel and Burgi (1972). The pellet resulting from the centrifugation was resuspended in 4.0 ml of solution A, the composition of which was as follows:

0.01M Tris-HCl (pH 8.2)

0.01M Sodium Azide

20% (w/v) Sucrose (Schwarz/Mann, Orangeburg, NY)

0.1M Sodium Chloride.

Following solution A, 1.0 ml of solution B was added. The composition of solution B was as follows:

0.12M Tris-HCl (pH 8.2)

0.05M Trisodium-EDTA

4mg/ml Egg-white Lysozyme (Sigma, St. Louis, Mo.).

Fresh preparations of solution B were employed in all extractions. After careful mixing, the suspension was removed from ice, and the cells lysed by the addition of 5 ml of solution C was as follows:

1% Brij 58 (Pierce, Rockford, Illinois)

0.4% Sodium Desoxycholate (Sigma, St. Louis, Mo.)

1.0M Sodium Chloride

0.01M EDTA

In most of the experimental runs, a 0.1% diethyl pyrocarbonate solution was added just prior to the addition of solution C. The lysis mixture was then held at room temperature until clearing resulted, usually within 10 to 20 min. Precautions were taken to preclude shearing of DNA. Additionally, the mixing of the components of the lysis mixture was achieved by the slow rotatory motion. The lysate was centrifuged in the cold at 4000 x g for a period of 5 min. The pellet of this low centrifugation contained approximately 10% of the DNA of the lysate (Worcel and Burgi, 1972). If the lysis was carried out on ice a larger portion of the cellular DNA was precipitated out with the insoluble debris (Worcel and Burgi, 1972).

To obtain consistent lysates, the following additional modifications were made. Treatment with lysozyme was carried out on ice for a period of exactly 30 sec. Sub-

sequent to this incubation period, the Brij-desoxycholate-NaCl-EDTA solution was added, and the temperature of the lysate kept constant at 25 C. This was achieved by immersing the tubes containing the lysate in a constant temperature water bath.

The folded membrane-free chromosomal DNA was then purified by sedimentation through a neutral sucrose gradient. The supernatant from the lysate was layered on a 5 ml, 10 to 30% (w/v) sucrose gradient containing 0.01M Tris-HCl (pH 8.2), 1.0M NaCl, 0.001M EDTA, and a 0.001M beta-mercaptoethanol. Centrifugation was carried out in an SW50 rotor of a Beckman ultracentrifuge for 30 min at 100,000 x g and 4 C. In most experiments, ^{14}C labeled T4 phage DNA was employed as an internal marker. The phage DNA was extracted by heating T4 phage for 15 min at 65C in a 1% sarkosyl (Sigma, St. Louis, Missouri) -0.05M EDTA solution.

Fractions to be employed in the transformation procedure were collected at 0 C after puncturing the bottom of the tubes with a 1-mm internal diameter needle. Fractions were then diluted with 0.2M Tris (pH 8.2). A 0.001M NaCl solution was required to preserve the folded conformation of the DNA in the lysate. However after purification, the DNA was relatively stable in 0.15M NaCl-0.015M trisodium citrate (Stonington and Pettijohn, 1971).

Concentration of the DNA solution was determined by the method of Lacks, Greenberg and Carlson (1967). At an absorbance of 1.0, measured at 260 nm, the concentration

of the DNA solution was 50 ug/ml (Lacks et. al., 1967).

Assay of Radioactivity

The ^{32}P-labeled DNA fractions were routinely collected into vials and counted in the following scintillation fluid: Triton X-100 (Packard), 166 ml; 2,5 diphenyloxazole (Packard PPO), 1g; 1, 4 bis-2-(4, ethyl-5-phenyloxazolyl)-benzene (Packard dimethyl POPOP), 100 mg; and deionized water, 1000 ml (Zsigray, Miss, and Landman, 1973). This mixture was stirred for 30 min and then filtered through Whatman #1 filter paper. While under storage at room temperature, the solution was protected from light. Samples were counted in a Packard Tri-Carb liquid scintillation counter, model number 3330 and with a gain setting of 7%. The ^{14}C fractions were routinely collected into vials and counted in PCS liquid scintillation fluid (Amersham/Searle, Arlington Heights, Illinois).

Preparation of Phage and Phage DNA

High titer stocks of non-labeled phage were prepared according to the plate method of Hershey, Kalmanson and Bronfenbrenner (1943a). Phage (1-2x10^5 PFU/ml) were preadsorbed to 10^8 bacterial cells growing in TSB without Dextrose. The preadsorption was carried out by adding 0.1 ml of the appropriate dilution of *plac*5 or Ø80*placI* lysate to 0.2 ml of an exponentially growing culture containing 3-5x10^8 cells/ml. Here, the procedures were the same for both *plac*5 and Ø80*placI*

with the exception that the preadsorption for phage plac5 was carried out in 0.01M $MgSO_4$. Both phage types adsorb poorly to cells of E. coli (Miller, 1972); however, when preadsorption was carried out with concentrated cells, greater amounts of virus were adsorbed, and the infections more synchronous (Miller, 1972). Preadsorption was performed in 20 small test tubes at 37 C for 10 min. Subsequently, 2.5 ml of molten H-top agar (at 45 C) was added to each tube, and immediately poured over the surface of an H plate. As an alternative, preadsorption was carried out in batch. The H agar was diluted before use by adding 15-20 ml broth to 100 ml of H top agar. The agar was then allowed to harden, and the plates incubated in an upright position at 37 C for 8-10 h. The top agar layer of each plate was scraped off with a bent glass rod and placed into a large plastic centrifuge tube. Five drops of chloroform were then added to each tube and the tubes were shaken vigorously for a period of 30 sec. The tubes were then allowed to stand for several min. After standing, the cell debris was pelleted (10,000 x g, 10 min) in a Beckman desk-top centrifuge and the supernatant decanted and saved. To the resulting supernatant, 2 drops of chloroform were added and the supernatant stored in the cold.

The titer of the lysate was determined by making serial dilutions of the lysate. From the following dilutions: 10^{-4}, 10^{-6}, 10^{-7}, and 10^{-8}, 0.1 ml of each dilution was preadsorbed with 0.1 ml of a fresh overnight culture of sen-

sitive E. coli. Each preadsorption mixture was incubated for 10 min at 37 C. Each mixture was plated onto the surface of H plates with undiluted H-top agar. The plates were allowed to dry, incubated overnight at 37 C in an inverted position, and the number of PFU/ml in the original lysate computed.

Preparation of phage DNA was carried out in screw-cap tubes, employing 1 ml of phage suspension. To each tube was added 1 ml of neutralized phenol, after which the tubes were placed on a roller for a period of 30 min at 25 C. The tubes were then cooled in an ice bath and centrifuged at 6000 x g for 5 min at 5 C. The lower phenol phase was removed with a Pasteur pipette. Phenol treatment of the H_2O phase was performed two additional times, the second time the tubes were on the roller for 15 min, whereas in the third phenol treatment, the tubes were on the roller for 5 min. The water phase, which contained the DNA, was transferred to dialysis sacs and dialyzed 4 times against 100 ml DNA buffer. Each dialysis was carried out in the cold for a period of 2 h.

The concentration of the DNA was determined by the method of Lacks, Greenberg and Carlson (1967).

Molecular weight was determined by sedimentation through neutral sucrose gradients as previously mentioned.

Various molecular weight preparations were achieved by shearing with a 0.1 ml pipette.

Optical Density Readings

Optical density readings of both donor and recipient cultures were recorded using a Spectronic 20 spectrophotometer set at a wavelength of 550 nm. Ten min after culture inoculation, 0.25 ml was withdrawn and placed into a small, thick walled sterile test tube. This sample was mixed with an equal volume of cold TSB without Dextrose. In addition, a viable cell count was determined by dilution and plating on TSA plates followed by incubation at 37 C for 24 h. Viable cell count determination was initiated from time zero and continued at 20 min intervals. The 0.1 ml aliquots required for plating were withdrawn from the sample tube before the optical density readings were made.

Transformation

Fifteen milliliters of TSB w/o D were inoculated with recipient *E. coli* and incubated at 25 C for 10 h. Ten milliliters of culture were inoculated into a 250 ml Erlenmyer flask containing 40 ml of broth medium. The recipient cells were grown to the middle of the exponential phase (O.D. 2.180) with constant aeration on a rotatory shaker at 35 C, after which the cells were chilled to 0 C, harvested by centrifugation (6,000 x g, 10 min), washed once in optimal concentration (0.02M, 0.03M, 0.04M) $CaCl_2$, and resuspended in 50 ml of optimal concentration (0.02M, 0.03M, 0.04M) $CaCl_2$. The cells were then placed at 3 C for 25 min. One tenth milliliter of recipient cell suspension was then transferred

to tubes containing 0.8 ml of TSB w/o D. At this time, 0.25 ml of a given DNA preparation was added and the mixture incubated at 40 C for either 35 or 50 min. The reaction was terminated by the addition of 0.5 ug DNase/ml. Duplicate 0.1 ml samples were then plated onto the surface of Davis Minimal Salt agar plates supplemented with 1% lactose. Trypticase soy agar plates were employed for determining the number of recipient cells in the transformation reaction.

When transformation was performed with RS5052, the procedure was as above except that the incubation temperature did not exceed 32 C. Incubation time was 35 min.

When transformation was performed with GR-1, the procedure was as above except that the growth medium was supplemented with glycerol (Sigma, St. Louis, Missouri) (1ml/50 ml). When such cells were ready for transformation, they were washed once with fresh TSB without Dextrose, and resuspended in this medium.

Recipient cell concentrations in all transformations were 1-2 x 10^9 colony forming units/ml.

Spheroplast Formation

The procedure for obtaining spheroplasted recipient cells was the same as that used for the isolation of transforming DNA with the exception that the spheroplasting procedure was terminated prior to the addition of solution C.

RESULTS

Initial investigations into the transformation of *E. coli* were based on the previous observation by Mandel and Higa (1970) and Oishi and Cosloy (1972) that cells of *E. coli* K 12 could be transformed for various chromosomal markers at a frequency of 10^{-6} following treatment with calcium. Although this frequency is not competitive with those obtained with other bacterial systems, it nonetheless represents an improvement with respect to previous studies which could not be duplicated. Calcium treatment of recipient cells is therefore, the starting point for the present investigations. From this base, many additional parameters were considered with regard to their possible enhancement or inhibition of the transformation process in *E. coli* K 12.

Effect of Calcium on the Transformation of *E. coli* K 12

There exists an absolute requirement for calcium in the demonstration of genetic transformation in *E. coli* K 12 (Mandel and Higa, 1970). This point cannot be over emphasized, as cells of *E. coli* K 12 not treated with calcium, fail to participate in the transformation process. Based upon this requirement, all transformation reactions, unless otherwise stated, were performed in the presence of calcium. In this study, the optimal calcium concentration (Figures 2, 3 and 4) was 0.02M for *E. coli* strains GR-1 and RS5052. *E. coli* strain JC 7626 and *E. coli* strain ATCC 25254

Figure 2. Effect of calcium on the transformation of *E. coli* strains GR-1, RS5052, JC 7626, and ATCC 25254. Donor DNA, at a concentration of 25 µg/ml, was EC-12L, and was isolated by the method of Marmur (1961). ●——●, GR-1; △——△, RS5052; ○——○, JC 7626; □——□, ATCC 25254.

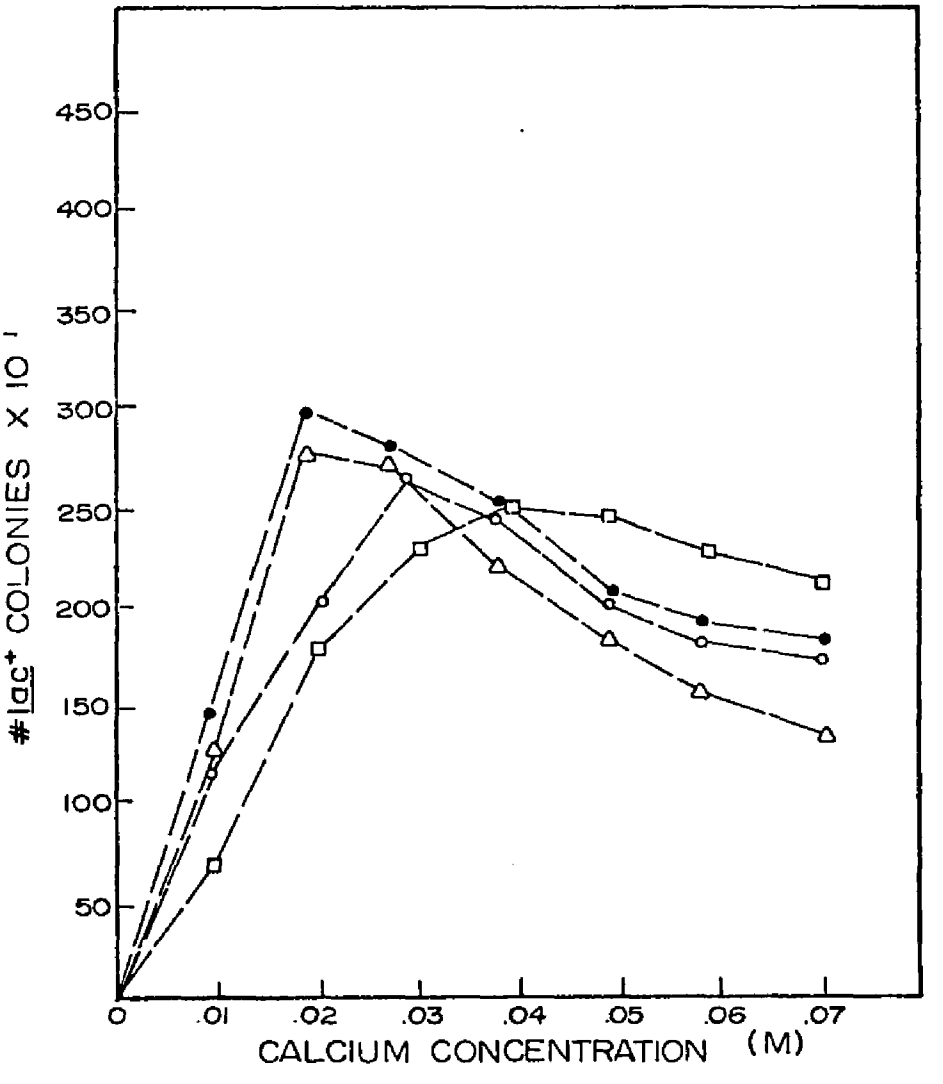

#lac+ COLONIES X 10 1
450
400
350
300
250
200
150
100
50
0
.01
.02
.03
.04
.05
.06
.07
CALCIUM CONCENTRATION (M)

Figure 3. Effect of calcium on the transformation of E. coli strains GR-1, RS5052, JC 7626, and ATCC 25254. Donor DNA, at a concentration of 25 µg/ml, was EC-12L, and was isolated by the method of Oishi and Cosloy (1972). •——• , GR-1; Δ——Δ, RS5052; o——o, JC 7626; □——□, ATCC 25254.

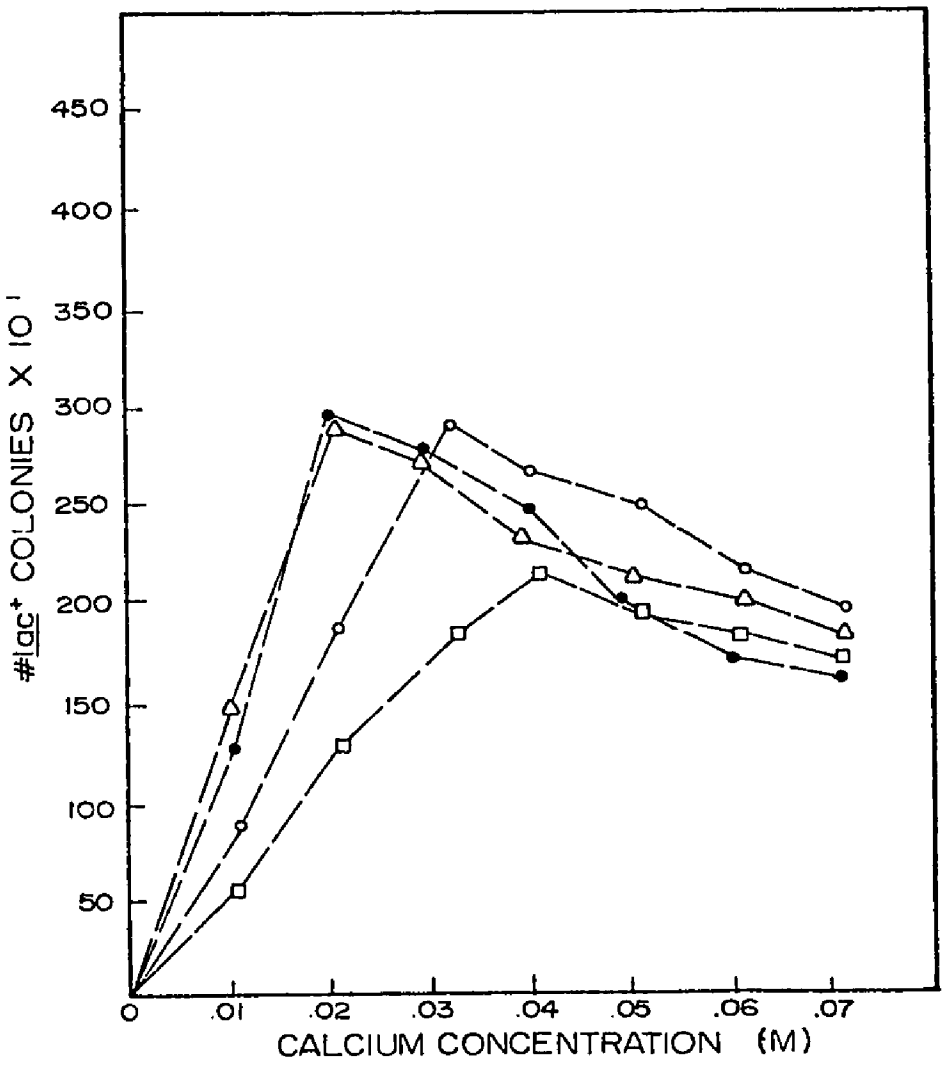
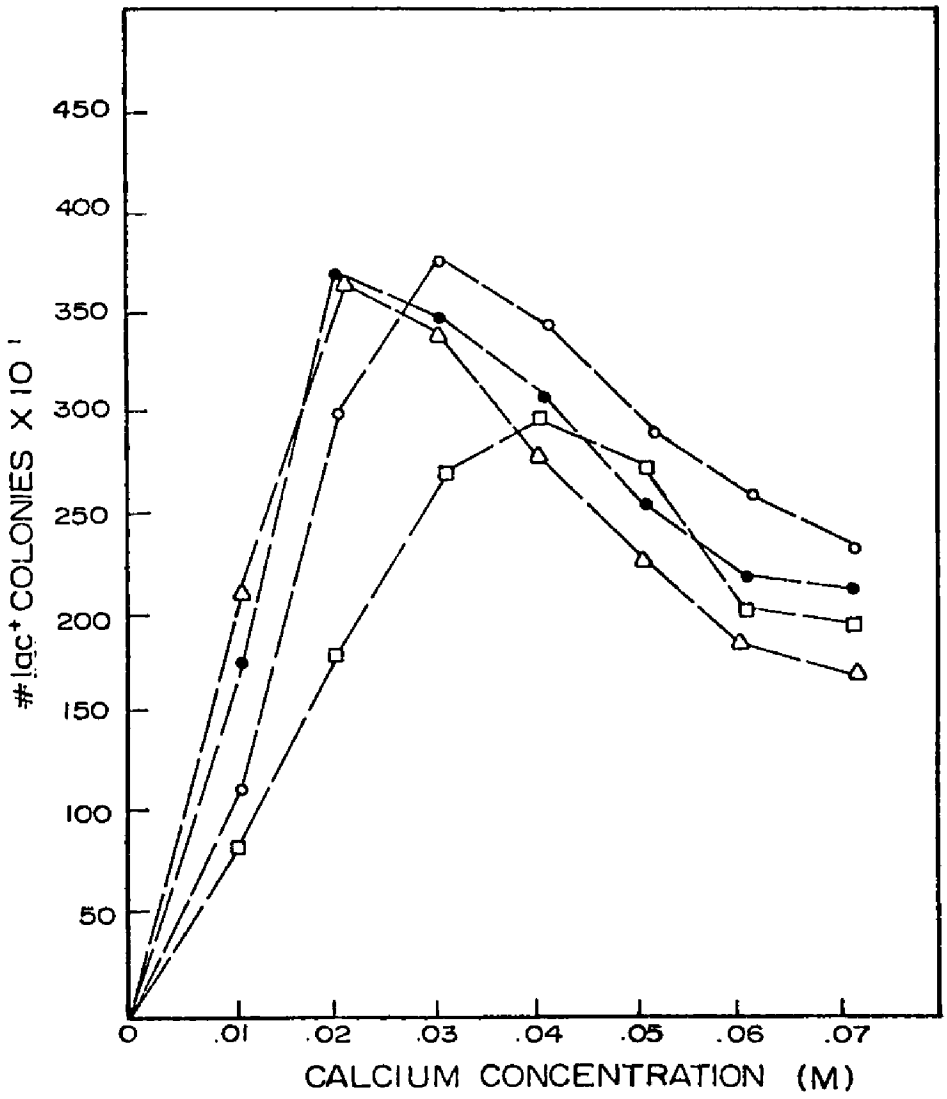

#lac+ COLONIES X 10 1
450
400
350
300
250
200
150
100
50
0
.01
.02
.03
.04
.05
.06
.07
CALCIUM CONCENTRATION (M)

Figure 4. Effect of calcium on the transformation of *E. coli* strains GR-1, RS5052, JC 7626, and ATCC 25254. Donor DNA, at a concentration of 25 µg/ml, was EC-12L, and was isolated by the method of Worcel and Burgi (1972). ●———●, GR-1; △———△, RS5052; o——o, JC 7626; □——□, ATCC 25254.


450
400
350
300
250
200
150
100
50
#lac+ COLONIES X 10 1
0
.01
.02
.03
.04
.05
.06
.07
CALCIUM CONCENTRATION (M)

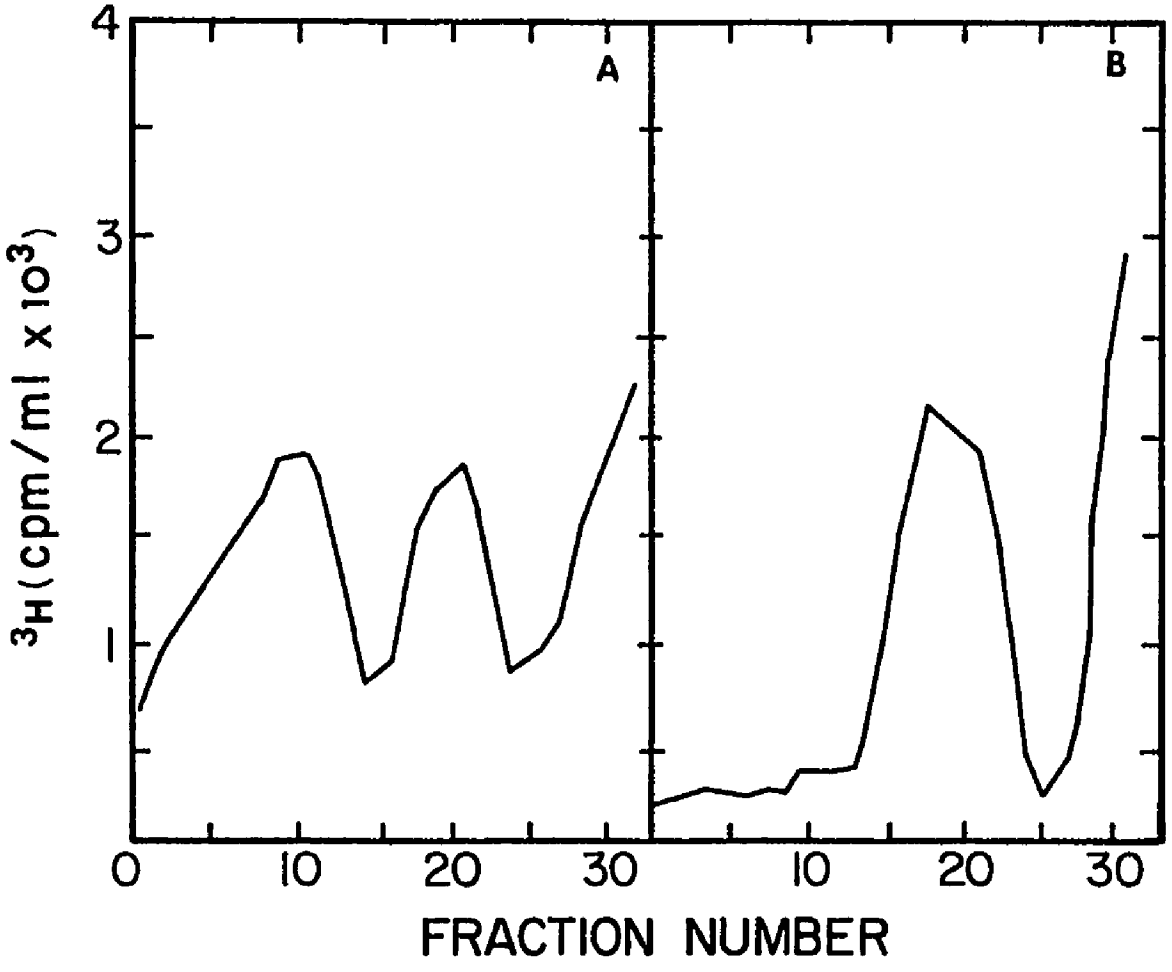
required 0.03M and 0.04M calcium concentrations respectively.

Effect of DNA Isolation Procedure on Transformation

The genetic material of E. coli is a double stranded, closed, circular molecule of DNA (Cairns, 1963). Intracellularly, this DNA is packaged into small nuclear bodies which are seen to occupy only a fraction of the cell volume (Ryter, 1968). Additionally, these nuclear bodies are not bound by nuclear membranes (Ryter, 1968).

The method by which DNA is extracted from the intact host was considered an important aspect in E. coli K 12 transformation. To obtain transforming molecules of maximal size, a modification of the method of Worcel and Burgi (1972) was employed. This method, which represented a modification of the method of Stonington and Pettijohn (1971), consisted of gentle lysis of lysozyme induced E. coli spheroplasts with non-ionic detergents in 1M NaCl. The temperature at which the E. coli spheroplasts were lysed had a marked effect on whether the DNA was membrane-associated or membrane-free. Lysis at 15 C produced membrane-associated folded chromosomes with sedimentation coefficients ranging between 3000s and 4000s (Figure 5a). Lysis at 25 C produced membrane-free folded chromosomes with sedimentation coefficients ranging between 1300s and 2200s (Figure 5b). The faster sedimenting complexes have been referred to as the membrane-associated folded chromosomes (Worcel and Burgi, 1972),

Figure 5. Neutral sucrose gradient profiles of [^{3}H] thymidine labeled DNA released from E. coli DG 75 following lysis at 15 C (a) and 25 C (b). Centrifugation was performed at 100,000 x g for 20 min.


4
3
2
1
0
A
B
10
20
30
10
20
30
^3H(cpm/ml x 10^3)
FRACTION NUMBER

while the slower sedimenting complexes represented the membrane-free folded chromosomes (Worcel and Burgi, 1972). Incubation of the membrane-associated folded chromosomes with 1% sarkosyl released the folded chromosomes from the membrane (Figure 6).

Following the isolation of the transforming DNA, investigation into the transformation process was directed at the molecular weight of the donor DNA. Oishi and Cosloy (1972) indicated that the DNA which was most effective in the demonstration of genetic transformation in E. coli K 12 possessed a molecular weight of 6.0×10^6. The method employed by Oishi and Cosloy for the isolation of their transforming DNA was a limited modification of the method originally formulated by Marmur (1961). Both methods yielded approximately the same number of lac^+ transformants (Figures 2 and 3). In contrast, the method of Worcel and Burgi (1972) yielded a greater number of lac^+ transformants (Figure 4).

From the results shown in Table 2, as the molecular weight of the transforming DNA was reduced, there occurred a decrease in the number of lac^+ colonies.

A plot of the average number of lac^+ transformants for all three strains produced by different molecular weight DNA's against the reciprocal of the molecular weigths, gives a linear relationship for molecular weight values up to 10^8 daltons (Figure 7a).

Figure 6. Sarkosyl release of folded chromosome from membrane-associated complex. Rerun of the chromosome from figure 1(a) after treatment with 1% sarkosyl. Treatment with sarkosyl was carried out in 1.0M NaCl, on ice, for 15 min.

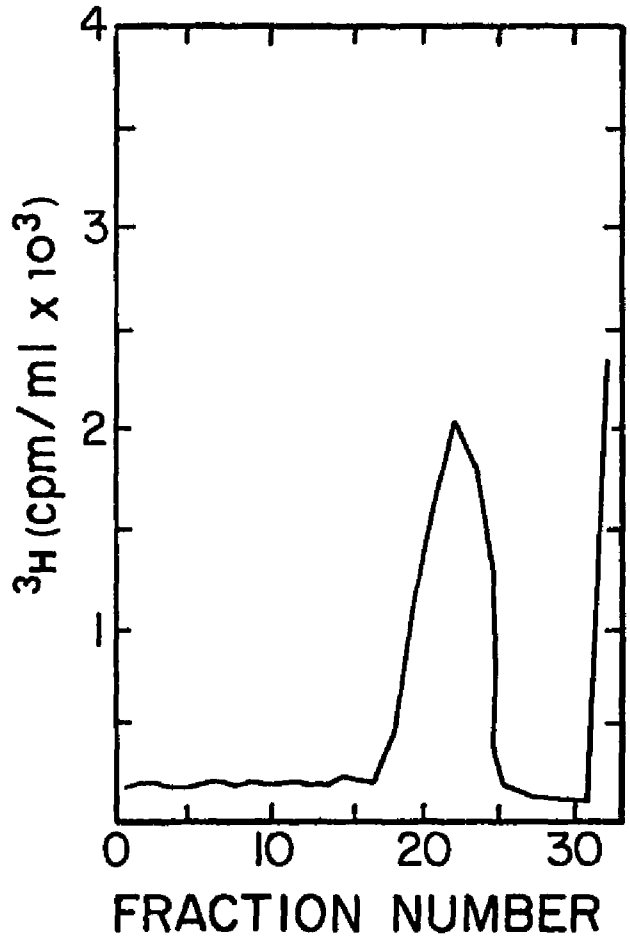

4
3
2
1
0
10
20
30
^{3}H (cpm/ml x 10^3)
FRACTION NUMBER

Table 2. Relationship between donor DNA molecular weight and transformation

Mol. Wt. of DNA (Daltons)[a]	Number of *lac*$^+$ colonies formed with recipients[b]		
	GR-1	RS5052	JC 7626
2.9×10^9	3.6×10^3	3.4×10^3	3.4×10^3
2.1×10^9	3.4×10^3	3.3×10^3	3.1×10^3
3.1×10^8	3.1×10^3	3.0×10^3	3.0×10^3
1.5×10^8	3.1×10^3	2.8×10^3	2.7×10^3
2.5×10^7	2.7×10^3	2.7×10^3	2.4×10^3
1.1×10^7	2.0×10^3	2.2×10^3	2.1×10^3
3.9×10^6	1.7×10^3	1.6×10^3	1.8×10^3
2.7×10^6	1.5×10^3	1.3×10^3	1.4×10^3

[a]Molecular weight of donor DNA was determined by neutral sucrose gradient centrifugation. A 10% to 30% (w/v) gradient containing 0.01 M Tris-HCl (pH 8.2), 1M NaCl, 0.001M EDTA, and a 0.001M beta-mercaptoethanol, was employed. Centrifugation was carried out in a SW50 rotor of a Beckman ultracentrifuge at 100,000 x g for 30 min at 4 C. In most experiments, ^{14}C-labeled T4 phage DNA was added to the lysate as an internal marker (Erikson and Szybalski, 1964). Molecular weights were estimated with the Burgi and Hershey relationship (1963).

[b]Donor DNA was obtained from EC-12L.

Figure 7a. Arithmetic plot of the number of *lac*$^+$ transformants versus the reciprocal of the molecular weight. The number of *lac*$^+$ transformants represents the average number of transformants for all three strains produced at different molecular weights. The average number of transformants was obtained from Table 2.

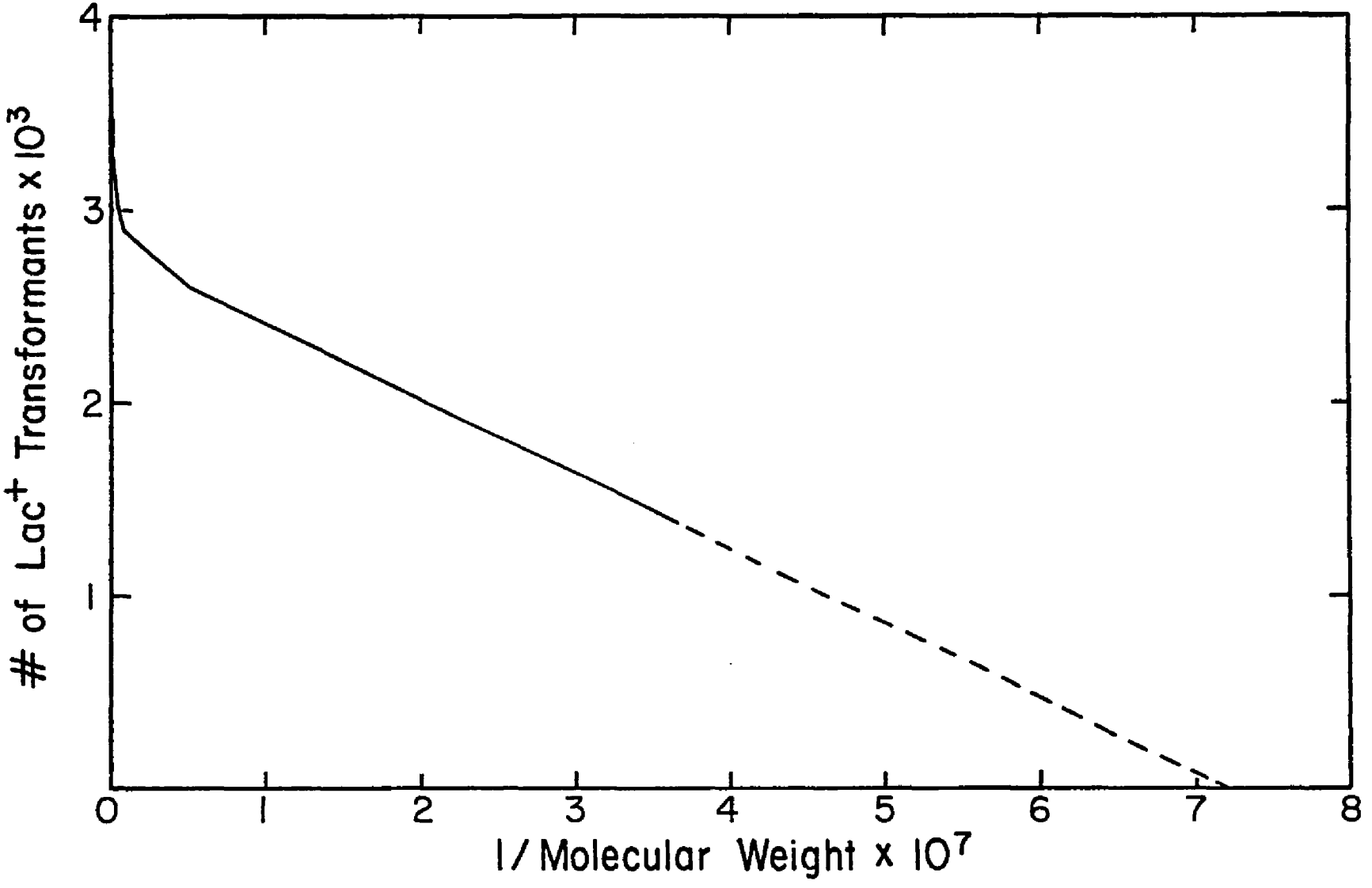
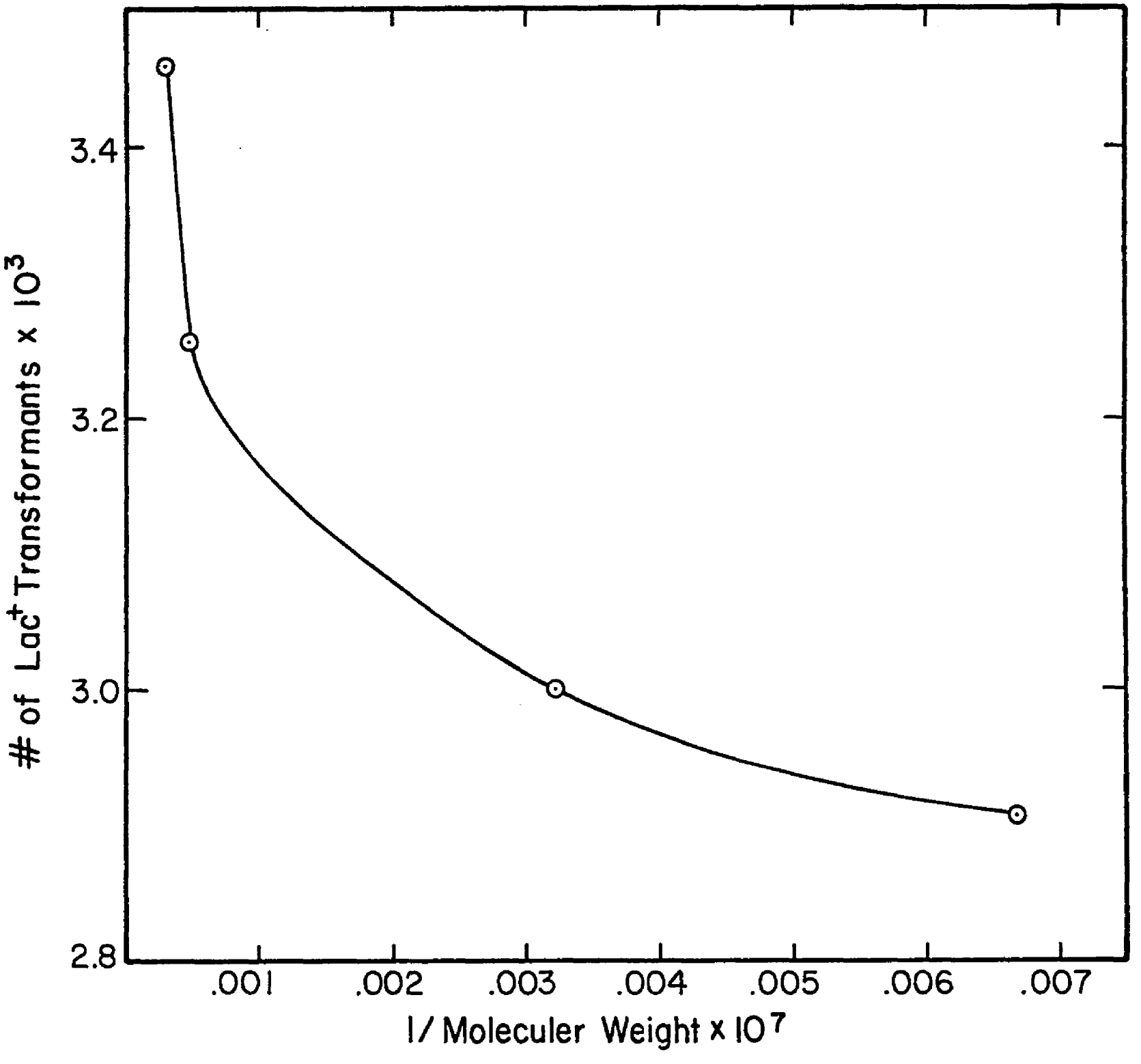

# of Lac+ Transformants x 10^3
4
3
2
1
0
1
2
3
4
5
6
7
8
1/ Molecular Weight x 10^7

Figure 7b. Expansion from Figure 7a for molecular weights in excess of 10^8 daltons.


3.4
3.2
3.0
2.8
# of Lac+ Transformants x 10^3
.001
.002
.003
.004
.005
.006
.007
1/ Moleculer Weight x 10^7

Effect of DNA Concentration on Transformation

Information had been obtained from other bacterial transformation systems which indicated that over a range of low concentrations of transforming DNA, the number of transformants is linearly related to the concentration (Hayes, 1966a). For example, in the transforming system of Yersinia novicida, the yield of transformants obtained from increasing concentrations of donor DNA was linear up to 5 ug/ml (Tyeryar and Lawton, 1970). A saturation plateau was reached upon the addition of higher concentrations of DNA. With regard to E. coli, Oishi and Cosloy (1972) determined the limiting concentration of donor DNA to be between 10 and 20 ug/ml.

In this study, the number of lac^+ transformants (dose-response curve) was proportional to the 1.4 power of the DNA concentration for all three strains examined (Figure 8). Additionally, all three bacterial strains exhibited the same limiting concentration of transforming DNA (Figure 8).

Effects of External Treatments on Transforming Preparation

By studying the effects of external treatments on the transforming preparation, insight into the nature of active components can be obtained. As shown in Table 3, treatment of the transforming preparation with DNase all but eliminated transforming activity, whereas treatment with

Figure 8. Relationship between donor DNA concentration and the number of $\underline{lac}^+$ transformants. Donor DNA was EC-12L. •——•, JC 7626; o——o, RS5052; Δ——Δ, GR-1.

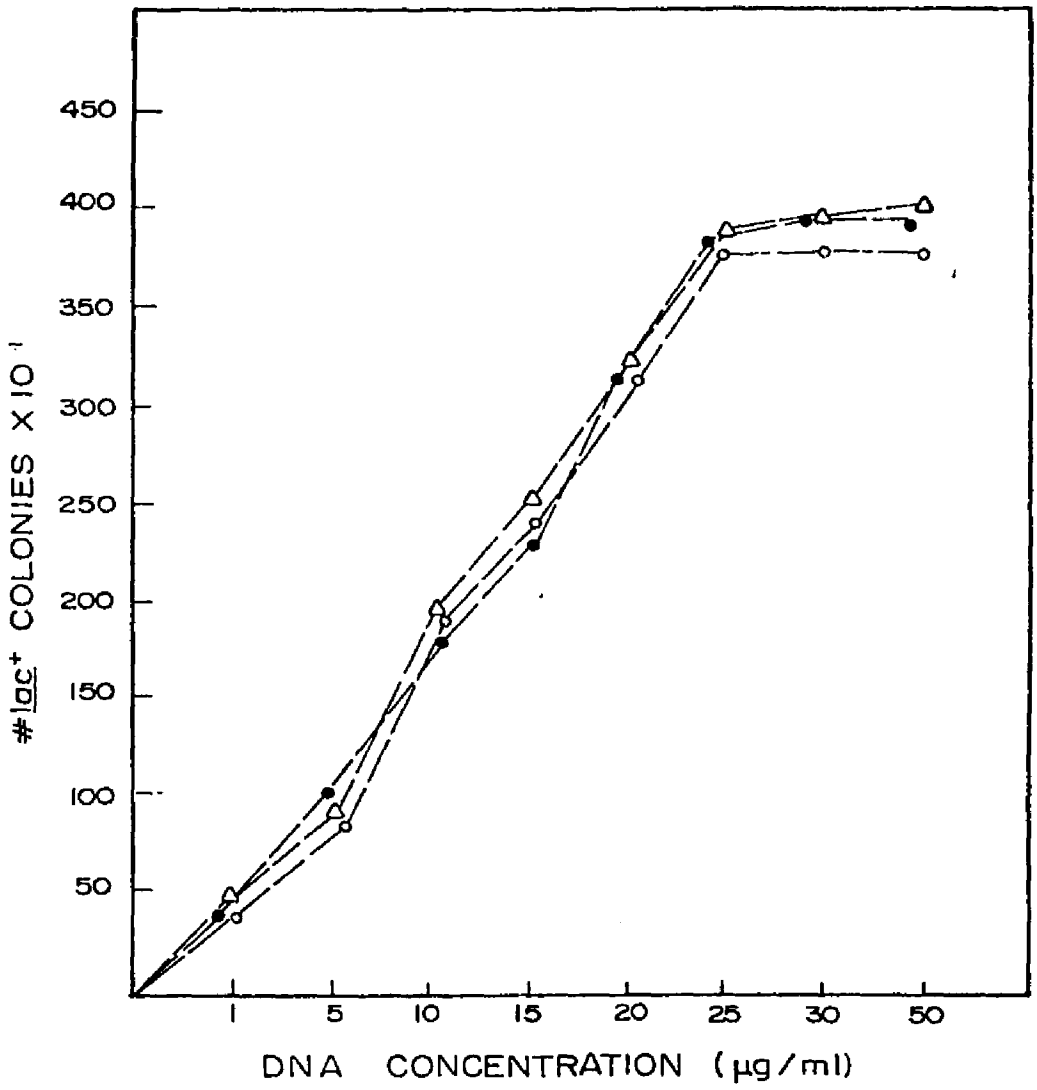

450
400
350
300
250
200
150
100
50
#lac+ COLONIES X 10⁻¹
1
5
10
15
20
25
30
50
DNA CONCENTRATION (µg/ml)

Table 3. Effects of external treatments on transforming preparation[a]

Treatment	Number of lac^+ colonies formed with GR-1
DNase (1.0 ug/ml)	1.0×10^0
RNase (25 ug/ml)	3.5×10^3
Pronase (250 ug/ml)	3.4×10^3
Sonication	1.0×10^0
Denaturation	2.0×10^0
Denaturation-Renaturation	2.0×10^1
No treatment	3.4×10^3
No DNA	1.0×10^0

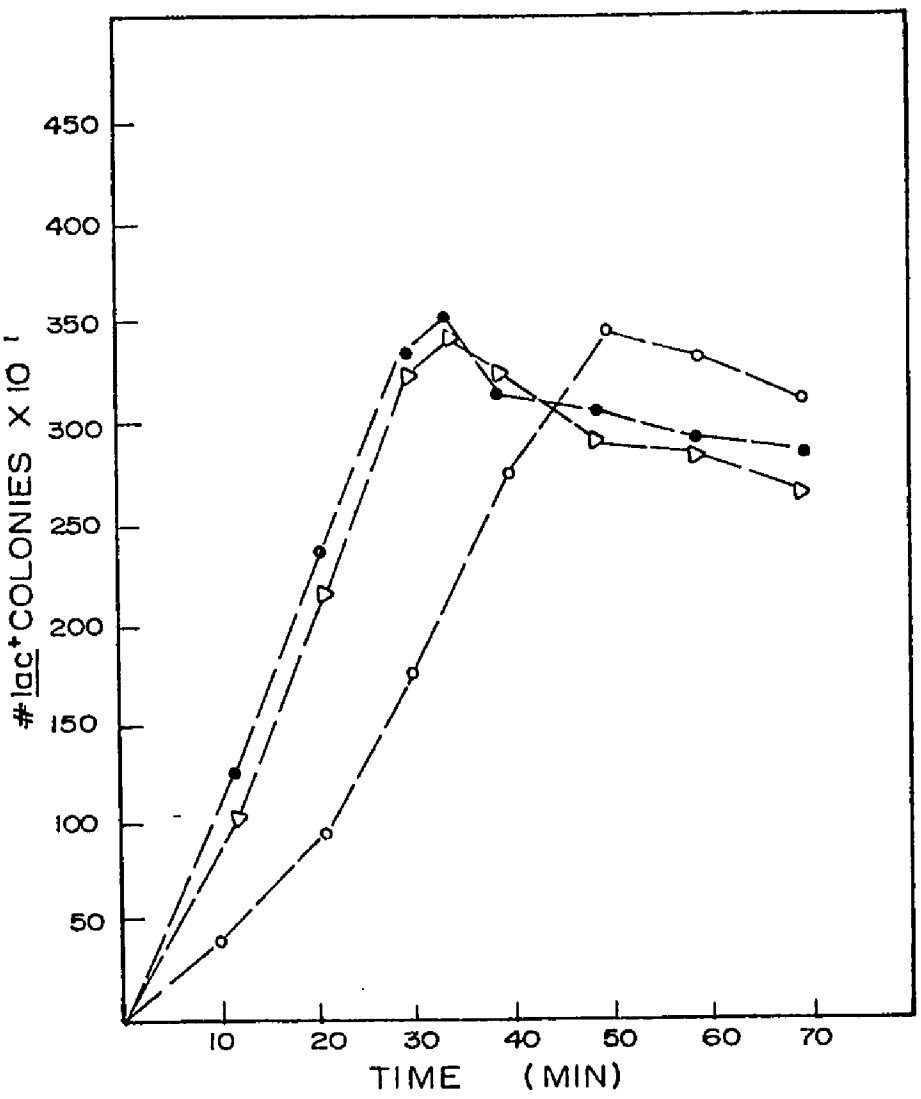
[a] Donor DNA was isolated from [illegible] and was employed at a concentration of 25 ug/ml. Transformation was performed as per materials and methods. Growth of lac^+ colonies was detected on Davis minimal salt agar plates supplemented with 1% lactose. All treatments were performed in 0.1M standard saline citrate (SSC). Treatment with crystaline pancreatic DNase (Worthington Biochem, Freehold, N.J.) was at 37 C for 20 min in SSC with the addition of 0.02M calcium. Treatment with crystaline pancreatic RNase A (Worthington Biochem, Freehold, N.J.) was performed at 37 C for 20 min. For sonicated DNA, a Raytheon model DF 101 sonic oscillator was used for 5 min. The oscillator was run at maximum capacity. Denatured DNA was obtained by heating a solution for 5 min at 100 C, followed by rapid cooling in an ice water bath. Denatured-renatured DNA was obtained by heating as above and subsequently subjecting the solution to a slow cooling process in a water bath finally bringing the solution to room temperature over a 10 h period. Pronase, grade B, (Calbiochem, La Jolla, California) treatment was performed at 37 C for 15 min.

RNase or pronase had virtually no effect. Transforming activity can also be lost subsequent to sonication or denaturation of the donor preparation. These results indicate, therefore, that the biologically active principle in the transforming preparation was DNA.

Kinetics of DNA-Recipient Cell Interaction

It has been reported by Tyeryar and Lawton (1970) that the maximal yield of *Yersinia novicida* transformants required a 30 min contact period between the recipient cells and the transforming DNA. Additionally, further incubation of the recipient cells with donor DNA did not increase the yield of transformants. In the present study, following the cold incubation period (25 min at 3 C), the optimal number of *lac*$^+$ transformants resulted from a 35 min contact period with donor DNA for strains JC 7626 and RS5052 (Figure 9). In contrast, the maximal yield of *lac*$^+$ transformants with the GR-1 strain, occurred after approximately 50 min of contact between the cells and the transforming DNA (Figure 9). In both cases, the number of *lac*$^+$ transformants did not increase following further incubation with transforming DNA.

Figure 9. Relationship between recipient cell contact with donor DNA and yield of *lac*$^+$ colonies. One tenth ml of recipient cells were placed in a series of test tubes containing 0.8 ml of broth medium. Two and one half tenth ml of a 25 µg/ml donor DNA preparation was then placed into each tube and allowed to react with the recipient cells for varying periods of time after which the reaction was terminated by the addition of 0.5 µg/ml DNase. The DNase was allowed to react with the incubation mixture for 15 min at 32 C, after which 1 ml samples were plated onto the surface of Davis minimal salt agar plates supplemented with 1% lactose. The plates were read after 24 h at 35 C.●———●, GR-1;o———o, RS5052;Δ———Δ, JC 7626.


450
400
350
300
250
200
150
100
50
#lac+COLONIES X 10 ⁻¹
10
20
30
40
50
60
70
TIME (MIN)

Effect of Inorganic Ions other than Calcium on Transformation

In view of the fact that calcium ions enhance the transformation process of E. coli K 12, subsequent investigation was directed at the role of other divalent and monovalent cations in the transformation process. The results indicate, that the monovalent cations Li^+, K^+, or Na^+, do not replace calcium. No lac^+ transformants were detected when these were present and calcium was not (data not shown). The same observation was noted for Co^{++}, Cu^{++}, Fe^{++}, or Zn^{++}. The above monovalent and divalent cations, even in combination with calcium, did not enhance the transformation process. In the presence of either Mg^{++} or Ba^{++} ions, strains GR-1 and RS5052 showed a small number of lac^+ transformants (Figure 10). It should be emphasized, however, that neither of these two divalent cations were capable of either substituting for calcium or enhancing transformation. The third recipient strain employed, JC 7626, failed to yield any lac^+ transformants in the absence of calcium (data not shown).

Effect of Temperature on Transformation

In the only other reproducible E. coli transformation system, Oishi and Cosloy (1972) demonstrated that the optimal temperature for the transformation of their

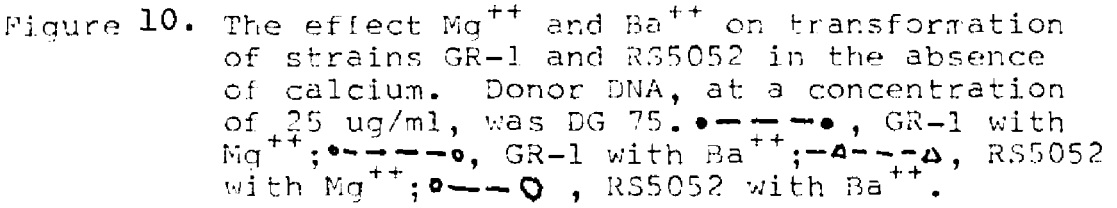
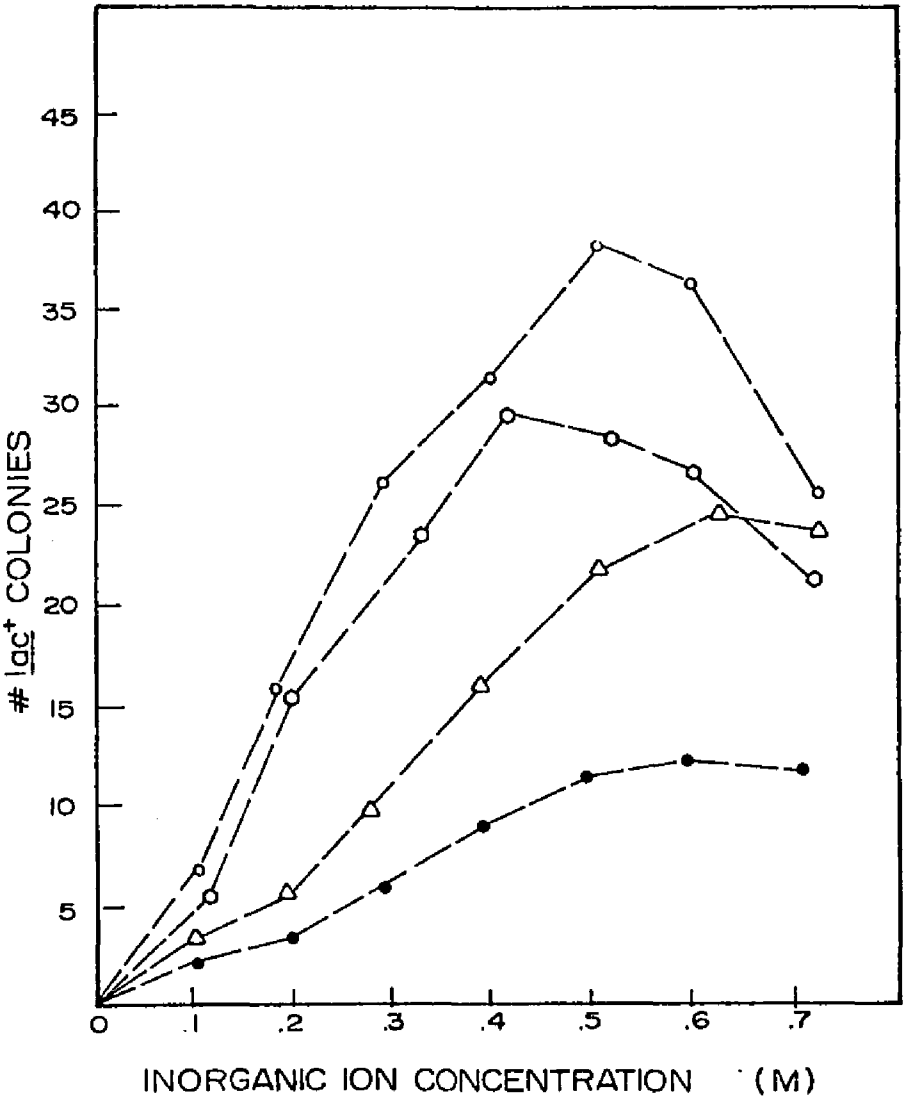


Figure 10. The effect Mg^{++} and Ba^{++} on transformation of strains GR-1 and RS5052 in the absence of calcium. Donor DNA, at a concentration of 25 ug/ml, was DG 75. •—•, GR-1 with Mg^{++}; •---•, GR-1 with Ba^{++}; —Δ—Δ, RS5052 with Mg^{++}; o—o, RS5052 with Ba^{++}.


45
40
35
30
25
20
15
10
5
0
.1
.2
.3
.4
.5
.6
.7
lac+ COLONIES
INORGANIC ION CONCENTRATION (M)

strains of E. coli was 42 C. The maximum period of time at this temperature was 15 min. Temperatures above 42 C did not yield any additional transformants. In my study, however, the number of lac$^+$ GR-1 transformants increased up to a temperature of 40 C (Figure 11). Temperature above 40 C did not yield any additional lac$^+$ transformants. In addition, following the cold incubation period (25 min at 3 C), the period of time required to obtain maximal yields of lac$^+$ transformants at 40 C was 50 min (Figure 11). With regard to the JC 7626 strain, the maximum temperature was also 40 C (Figure 12). In contrast to the GR-1 strain, however, the maximal yield of transformants occurred within 35 min.

Effect of pH on Transformation

One E. coli transformation study indicated that the optimal pH for successful transformation of the organism was 9.5 (Avadhani, Mehta and Rege, 1969). In contrast to these findings, my studies indicated that the optimal pH for enhancing the number of lac$^+$ transformants was 8.2 (Table 4).

Effect of Polyamines on Transformation

DNA is a strong polybasic acid due to its highly polar phosphate groups. These phosphate groups are located on the outer edges of the double helix and are capable of forming ionic bonds, not only with divalent cations such

Figure 11. Effect of temperature on transformation of strain GR-1. Donor DNA, at a concentration of 25 ug/ml, was EC-12L.

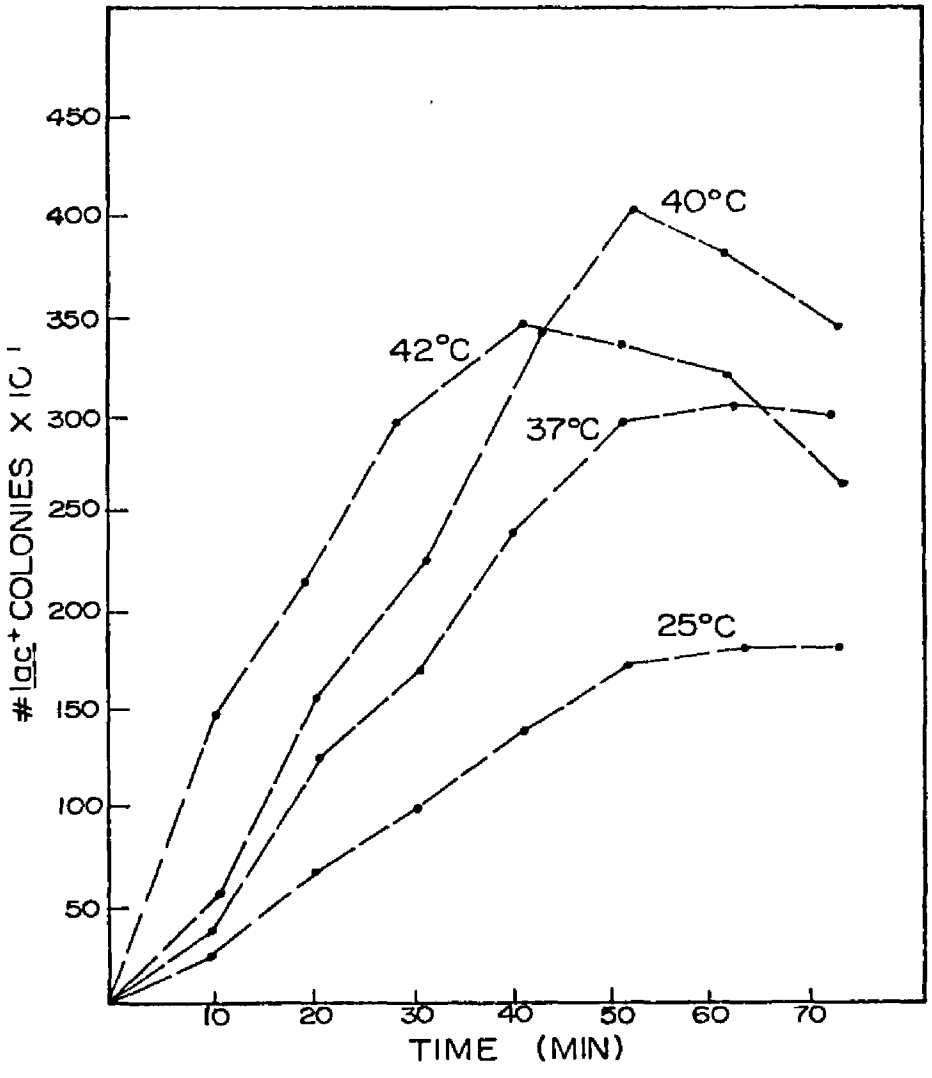

450
400
350
300
250
200
150
100
50
lac+ COLONIES X 10⁻¹
40°C
42°C
37°C
25°C
10
20
30
40
50
60
70
TIME (MIN)

Figure 12. Effect of temperature on transformation of strain JC 7626. Donor DNA, at a concentration of 25 ug/ml, was EC-12L.

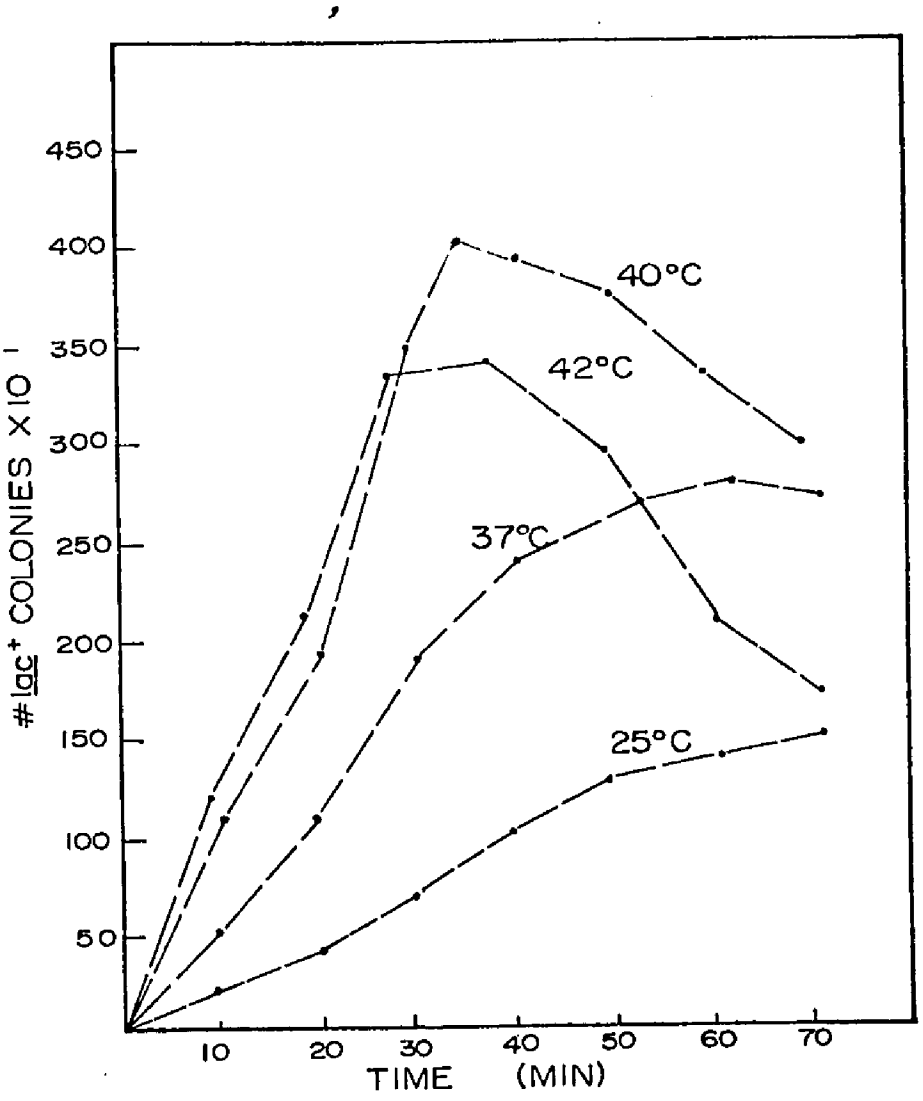

450
400
350
300
250
200
150
100
50
#lac+ COLONIES X10 -1
40°C
42°C
37°C
25°C
10
20
30
40
50
60
70
TIME (MIN)

Table 4. Effect of pH on the transformation of *E. coli* K 12[a]

pH	Number of *lac*$^+$ colonies formed with		
	GR-1	RS5052	JC 7626
6.2	2.6×10^3	2.5×10^3	2.1×10^3
6.4	2.6×10^3	2.4×10^3	2.3×10^3
6.6	2.8×10^3	2.6×10^3	2.3×10^3
6.8	2.8×10^3	2.8×10^3	2.5×10^3
7.0	3.1×10^3	3.2×10^3	2.9×10^3
7.2	3.5×10^3	3.4×10^3	3.0×10^3
7.4	3.9×10^3	3.7×10^3	3.2×10^3
7.6	4.1×10^3	4.0×10^3	3.5×10^3
7.8	4.2×10^3	4.0×10^3	3.6×10^3
8.0	4.4×10^3	4.1×10^3	4.1×10^3
8.2	4.5×10^3	4.3×10^3	4.3×10^3
8.4	4.3×10^3	4.0×10^3	4.2×10^3
8.6	4.0×10^3	3.7×10^3	3.9×10^3
8.8	3.6×10^3	3.4×10^3	3.6×10^3

[a] Donor DNA, at a concentration of 25 ug/ml, was EC-12L. An appropriate volume of either 2N HCl or 2N NaOH was added to achieve the desired pH. Following either a 35 min or 50 min incubation period with the donor DNA at 40 C or 32 C, the reaction was terminated by the addition of 0.5 ug/ml DNase.

as calcium and magnesium, but also with the polycationic amines putrescine, spermidine, and spermine (Lehninger, 1970). Of the three polyamines tested, only spermine seemed capable of enhancing the transformation of E. coli K 12. In addition, when calcium was deleted from the system, spermine was incapable of substituting for the divalent cation (data not shown).

Effect of Spheroplasted Recipient Cells on Transformation

Following the work on intact recipient cells, the effect of spheroplasting was investigated. Table 5 shows that spheroplast formation enhances the transformation process in E. coli K 12. Although the number of lac^+ transformants did not increase by any substantial margin, there was an increase in the total number of lac^+ transformants as a function of spheroplasting the recipient cells (Table 5). Table 5 also reveals that calcium plays just as important a role in spheroplasted recipient cells as it does in non-spheroplasted recipient cells. The control volume for calcium treated, non-spheroplasted cells were 4.5×10^3 for strain GR-1, 4.3×10^3 for strain RS5052 and 4.2×10^3 for strain JC 7626.

Effect of Polyamines on Spheroplasted Recipient Cells in Calcium Deficient and Supplemented Media

Of the three polyamines, only spermine seemed capable

Table 5. Effect of spheroplasted recipient cells on transformation[a]

Number of *lac*$^+$ colonies formed with calcium treated			Number of *lac*$^+$ colonies formed with non-calcium treated		
GR-1	RS5052	JC 7627	GR-1	RS5052	JC 7626
5.4×10^3	5.2×10^3	5.3×10^3	1.9×10^1	2.0×10^1	2.2×10^1

[a] Donor DNA, at a concentration of 25 ug/ml, was DG 75. Treatment with lysozyme (4 mg/ml Egg-white lysozyme) was carried out on ice for a period of 3 min.

of enhancing the transformation process of E. coli K 12 spheroplasts treated with calcium. The number of lac$^+$ transformants formed as a result of the interaction of spermine treated spheroplasted recipient cells in the presence of calcium is greater than that observed for the interaction of spermine treated non-spheroplasted recipient cells in the presence of calcium. In the transformation of spheroplasted recipient cells not treated with calcium, the number of lac$^+$ transformants is less than that of the spheroplasted cells treated with calcium. Here, however, the number of lac$^+$ transformants is greater than the number observed in non-spheroplasted cells in the absence of calcium (data not shown).

Effect of Protamine on Transformation of Spheroplasted and Non-spheroplasted Cells of E. coli K 12 Treated with Calcium

Protamine seems to participate in a manner like spermine in the transformation process. A similar number of lac$^+$ transformants were formed as a result of the addition of either spermine or protamine to either spheroplasted or non-spheroplasted recipient cells (data not shown).

Effect of O_2 Tension on Transformation

In the Bacillus subtilis transformation system, the optimal number of transformants appear only when the reaction is carried out under aerobic conditions (Young and Spizizen, 1963). In the present study, the effect of O_2

tension on the transformation reaction was investigated by incubating spheroplasted recipient cells in varying concentrations of sodium thioglycollate (Difco, Detroit, Michigan). The optimal number of *lac*$^+$ transformants appeared in response to concentrations of the reducing agent ranging between 0 to 400 mg/l (Table 6). Such concentrations of reducing agent are equatable to aerobic through microaerophilic conditions. Additionally, a number of *lac*$^+$ transformants were obtained in the range of 500 to 700 mg/l of reducing agent.

Effect of Phospholipids and Phospholipid Synthesis on Transformation

The effect of phospholipids and phospholipid synthesis on transformation was investigated by employing a glycerol auxotrophic mutant. Depriving this mutant of glycerol leads to an immediate termination of net phospholipid synthesis. By effectively inhibiting net phospholipid synthesis, neither enhancement nor inhibition of the transformation process in *E. coli* K 12 was achieved (Figure 13).

Transformation as a Function of DNA Source

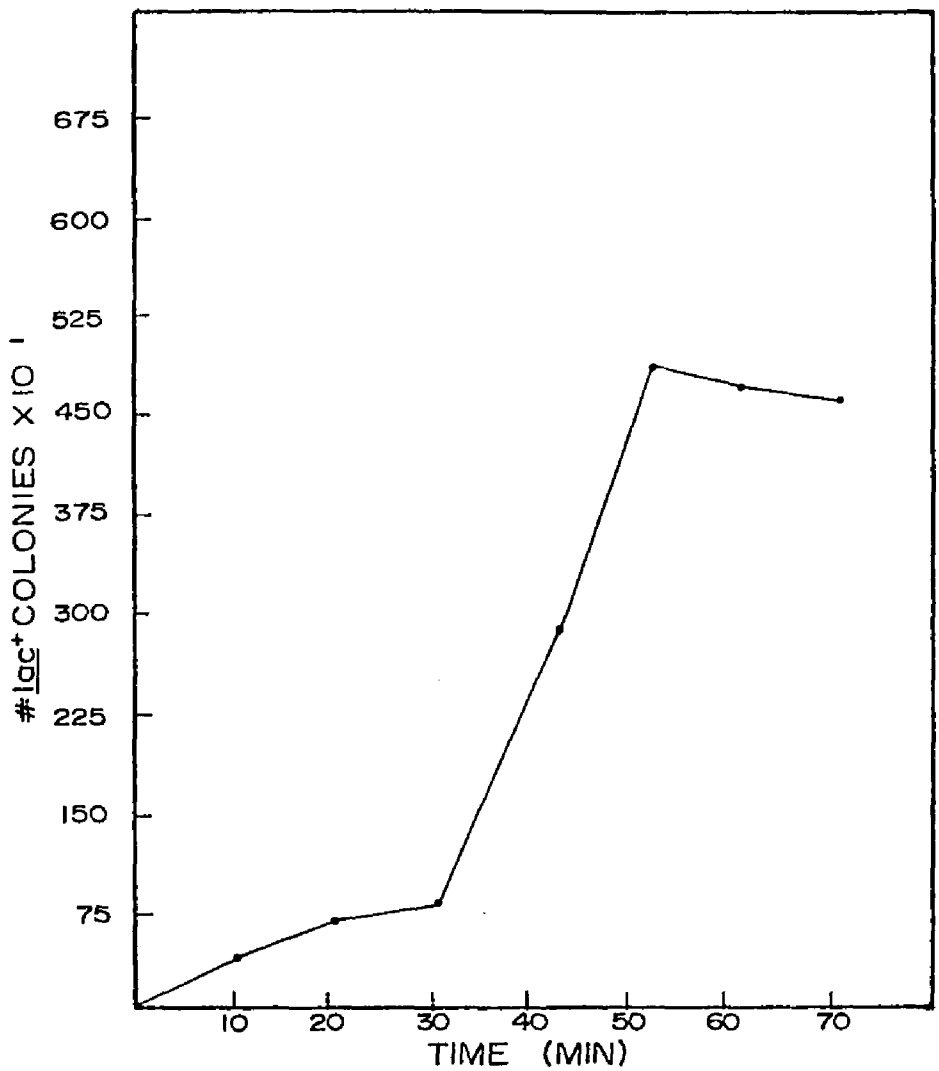
Another aspect of the transformation process investigated was the effect of the source of donor DNA. The only DNA preparations effective in transforming *E. coli*

Table 6. Effect of O_2 tension on the transformation of calcium-treated *E. coli* K 12 spheroplasts[a]

Sodium Thioglycollate (mg/liter)	Number of *lac*$^+$ colonies formed with		
	GR-1	RS5052	JC 7626
1000	1.8×10^3	1.9×10^3	1.5×10^3
900	2.1×10^3	1.9×10^3	1.9×10^3
800	2.2×10^3	2.1×10^3	2.0×10^3
700	4.8×10^3	4.4×10^3	4.2×10^3
600	4.5×10^3	4.1×10^3	3.8×10^3
500	4.9×10^3	4.8×10^3	4.6×10^3
400	5.4×10^3	5.2×10^3	4.9×10^3
300	5.4×10^3	5.3×10^3	5.1×10^3
200	5.2×10^3	5.3×10^3	5.1×10^3
100	5.3×10^3	5.4×10^3	5.0×10^3
0	5.4×10^3	5.3×10^3	5.1×10^3

[a] Donor DNA, at a concentration of 25 ug/ml, was DG 75. Subsequent to visible growth, the recipient cells were washed once and then resuspended in fresh broth medium containing varying concentrations of the reducing agent. Incubation in this second medium was carried out for 20 min prior to the addition of the transforming DNA.

Figure 13. The effect of phospholipids and phospholipid synthesis on transformation. Recipient cells were grown in TSB without dextrose supplemented with 10 ml/liter glycerol. Spermine was added in a concentration of 0.005M. Upon reaching the desired cell density, the cells were washed twice in TSB without dextrose not supplemented with glycerol. Following 25 min at 3 C, EC-12L transforming DNA was added at a concentration of 25 ug/ml, and the reaction incubated at 40 C.


675
600
525
450
375
300
225
150
75
#lac+ COLONIES X 10 1
10
20
30
40
50
60
70
TIME (MIN)

K 12 cells were DNA preparations obtained from E. coli K 12 (Table 7). Additionally, DNA preparations from closely related species, including E. coli B, possessed essentially no transforming activity. This finding was extended into the two fungal and other bacterial species examined.

Transformation of E. coli K 12 cells with DNA isolated from previously transformed E. coli K 12 cells verified that the DNA remained biologically active and was capable of transforming the original genetic marker (Table 8). JC 5176 DNA, not possessing the lac region, was not capable of initiating transformation.

Effect of Preincubation Temperature on the Uptake of 32^P Labeled Donor DNA

When recipient cultures are preincubated in tsb w/o d at 25 C and 40 C, and then maintained at these temperatures subsequent to the addition of 32^P transforming DNA, uptake of 32^P DNA is linear; however, the rate is greater at the higher temperature (Figures 13 and 14). Incubation of a recipient culture at 40 C, followed by a shift to 25 C with the addition of transforming DNA, the rate gradually decreases so that subsequent to a 10 min to 20 min lag period the maximal rate as observed at 25 C is reached. Conversely, when a recipient culture at 25 C is shifted at 40 C, the rate of uptake is initially low, and only after a 12 min period does it increase to the degree observed in a 40 C culture.

Table 7. Effect of the source of the donor DNA on transformation of various strains of *Escherichia coli*[a]

Source of DNA	Number of *lac*$^+$ colonies formed with		
	GR-1	RS5052	JC 7626
Escherichia coli K 12 DG 75	5.0×10^3	5.2×10^3	5.0×10^3
Escherichia coli K 12 EC-12L	5.1×10^3	5.1×10^3	4.9×10^3
Escherichia coli B *lac*	6.0×10^0	4.0×10^0	2.0×10^0
Bacillus subtilis 168 *lac*	2.0×10^0	1.0×10^0	2.0×10^0
Hemophilus influenzae Rd *lac*	1.0×10^0	3.0×10^0	3.0×10^0
Pseudomonas aeruginosa ATCC 17423 *lac*	6.0×10^0	8.0×10^0	5.0×10^0
Azotobacter vinelandii ATCC 12837 *lac*	3.0×10^0	3.0×10^0	1.0×10^0
Penicillium roqueforti ATCC 10110 *lac*	2.0×10^0	3.0×10^0	0.0×10^0
Aspergillus niger ATCC 16888 *lac*	4.0×10^0	1.0×10^0	0.0×10^0

[a] Donor DNA was employed at a concentration of 25 ug/ml. The molecular weight of the transforming *E. coli* DNA preparations was 2.9×10^9, whereas the molecular weight of the remaining bacterial and fungal species was not determined although the DNA was extracted by the procedure described in materials and methods. Spermine was added in a concentration of 0.005M.

Table 8. Transformation of *Escherichia coli* K 12 cells with DNA isolated from previously transformed *Escherichia coli* K 12 cells[a]

Source of DNA	Number of *lac*$^+$ colonies formed with	
	GR-1	RS5052
RS5052 *lac*	4.8×10^3	4.7×10^3
GR-1 *lac*	5.1×10^3	4.8×10^3
JC 7626 *lac*	4.6×10^3	4.5×10^3
JC 5176 *lac*$^-$	0.0×10^0	1.0×10^0
GR-1 *lac*	4.8×10^3	4.7×10^3
JC 7626 *lac*	4.6×10^3	4.9×10^3
JC 5176 *lac*$^-$	0.0×10^0	1.0×10^0
RS5052 *lac*	4.9×10^3	5.0×10^3
JC 7626 *lac*	5.0×10^3	4.8×10^3
GR-1 *lac*	4.9×10^3	4.9×10^3
RS5052 *lac*	4.7×10^3	4.5×10^3

[a]Concentration of the donor DNA preparations was 25 ug/ml, while transformation was performed as per materials and methods. Spermine was added in a concentration of 0.005M.

Figure 14. The effect of preincubation temperature on the uptake of transforming DNA. Recipient cells were JC 7626. Cultures grown at 40 C were preincubated for 25 min at 25 C and 40 C. On addition of ^{32}P-DNA at time zero, samples at each temperature were instantaneously shifted to 25 and 40 C. ●——●, 40 C throughout; □——□, 40 C then 25 C; ○——○, 25 C then 40 C; △——△, 25 C throughout.

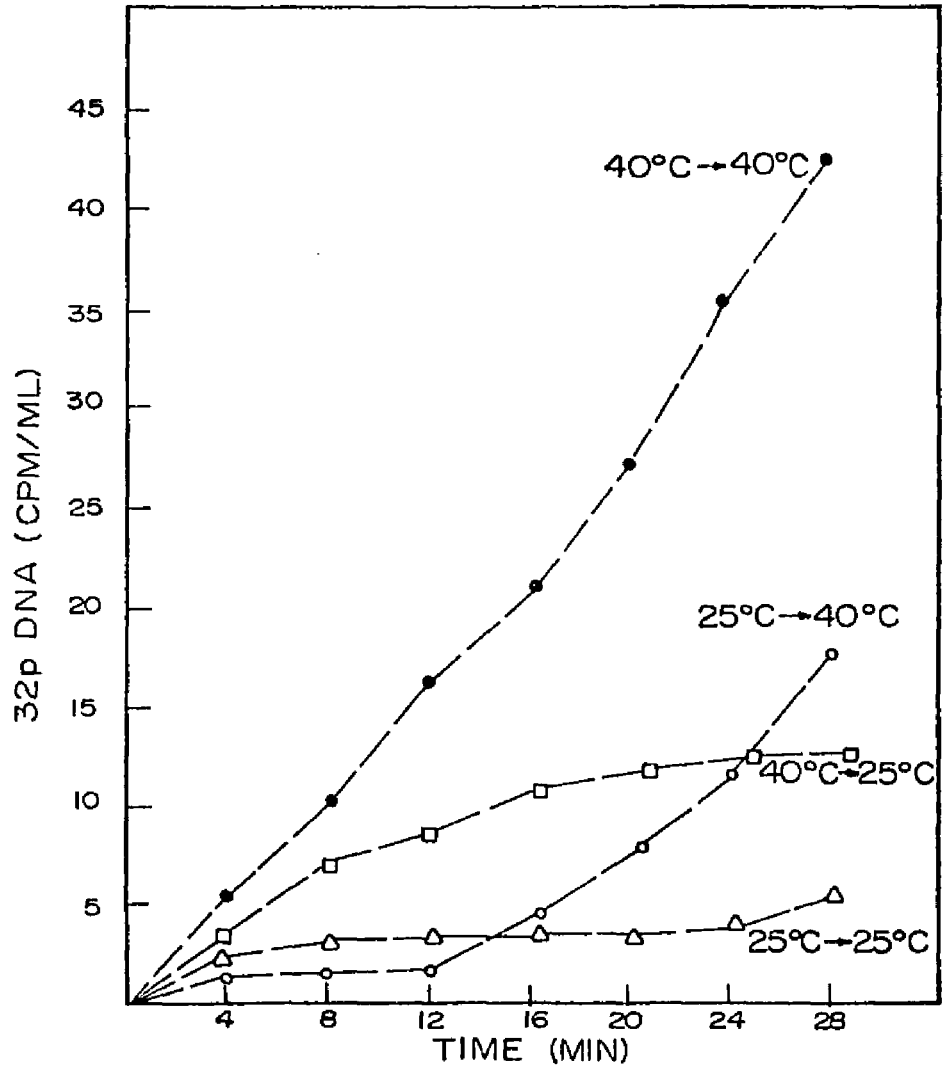
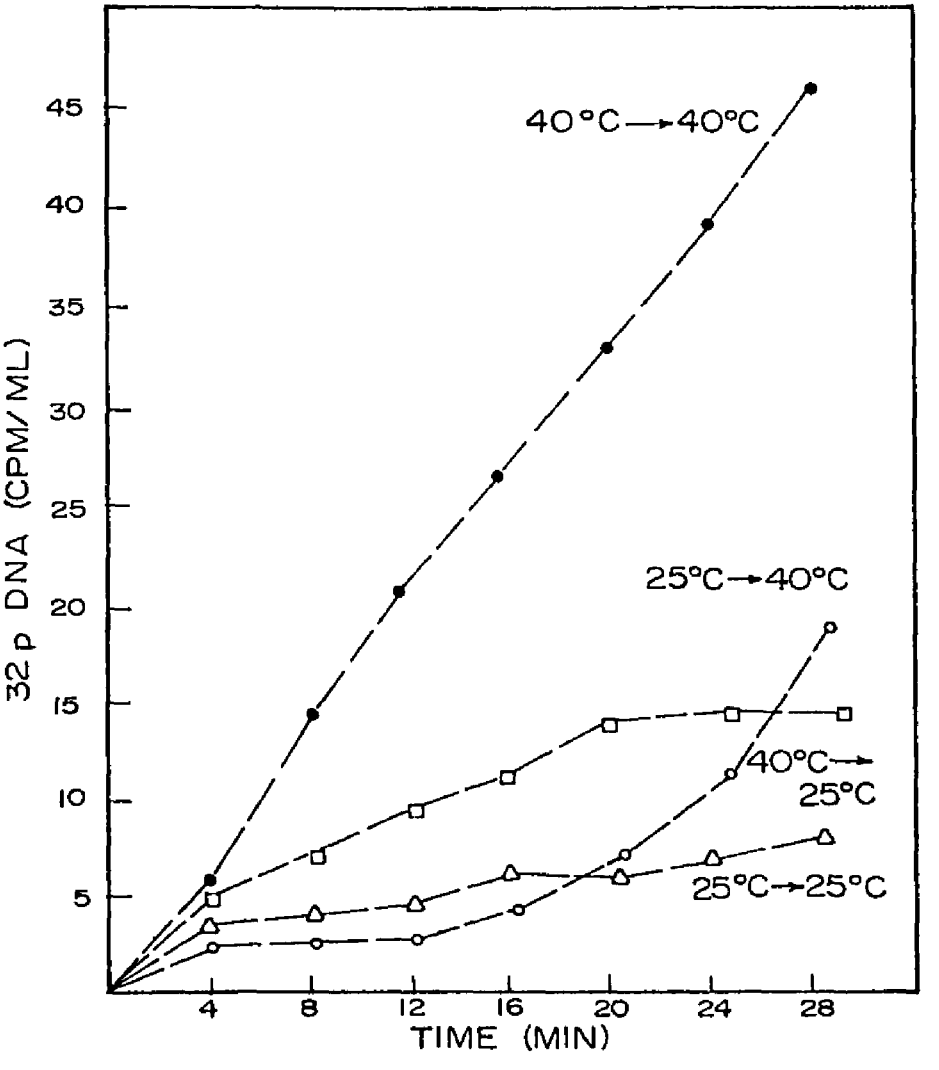

45
40
35
30
25
20
15
10
5
32P DNA (CPM/ML)
40°C→40°C
25°C→40°C
40°C→25°C
25°C→25°C
4
8
12
16
20
24
28
TIME (MIN)

Figure 15. The effect of preincubation temperature on the uptake of transforming DNA. Recipient cells GR-1. Cultures grown at 40 C were preincubated for 25 min at 25 C and 40 C. On addition of ^{32}P-DNA at time zero, samples at each temperature were instantaneously shifted to 25 C and 40 C. ●— —●, 40 C throughout; □— —□, 40 C then 25 C; ○— — —○, 25 C then 40 C; △— —△, 25 C throughout.


45
40
35
30
25
20
15
10
5
32 p DNA (CPM/ML)
40°C→40°C
25°C→40°C
40°C→
25°C
25°C→25°C
4
8
12
16
20
24
28
TIME (MIN)

Effect of Protein Synthesis on Uptake of DNA

Protein synthesis is not required for the uptake of transforming DNA in either the JC 7626 strain (Figure 16) or the GR-1 strain (Figure 17). Both strains were sensitive to chloramphenicol at 1 mg/ml.

Transfection of Calcium Treated Recipient Cells

A final question with regard to genetic transformation in E. coli concerns whether or not enrichment for the genetic marker under investigation increases the frequency of transformation. To answer this question, the transfection of spheroplasted and non-spheroplasted cells of E. coli by λplac5 and Ø80placl DNA was studied. The number of lac$^+$ transformants, formed in response to the enriched (low molecular weight) transfecting DNA, were not as high in comparison to the number of lac$^+$ transformants formed in response to the unenriched (high molecular weight) DNA (Table 9). This same observation can be seen with regard to the transfection of spheroplasted recipient cells (Table 10). Contrasting volumes for unenriched high molecular weight DNA can be found in Table 2.

Transfection of E. coli K 12 with lambdaplac5

Because λplac5 and Ø80placl are capable of plaque formation (Shapiro, et al., 1969), potential transformants might be destroyed (lysed) following the uptake of viral DNA. When PFUs were determined rather than trans-

Figure 16. The effect of protein synthesis on the uptake of transforming DNA in JC 7626. Recipient cells were grown in TSB without dextrose at 40 C. The cells were washed once in the above broth medium and then resuspended in the same medium with or without calcium. Chloramphenicol, at a concentration of 1 mg/ml, was added to one sample 5 min prior to the addition of the transforming DNA. ^{32}P-DNA was added at time zero. o——o, complete medium with calcium; □——□, complete medium with calcium and chloramphenicol; •——•, medium without calcium.

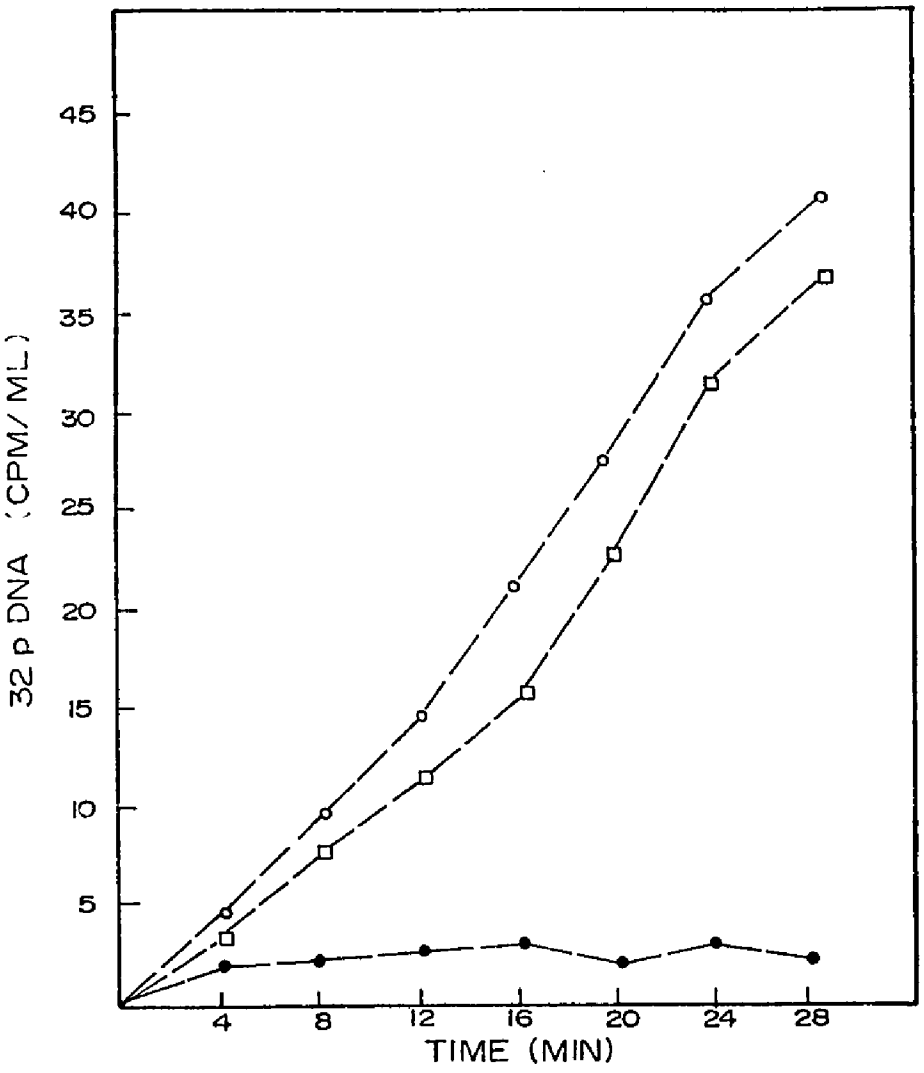

45
40
35
30
25
20
15
10
5
32 P DNA (CPM/ML)
4
8
12
16
20
24
28
TIME (MIN)

Figure 17. The effect of protein synthesis on the uptake of transforming DNA in GR-1. Recipient cells were grown in TSB without dextrose at 40 C. The cells were washed once in the above broth medium and then resuspended in the same medium with or without calcium. Chloramphenicol, at a concentration of 1 mg/ml, was added to one sample 5 min prior to the addition of the transforming DNA. ^{32}P-DNA was added at time zero. o——o, complete medium with calcium; □——□, complete medium with calcium and chloramphenicol; ●——●, medium without calcium.

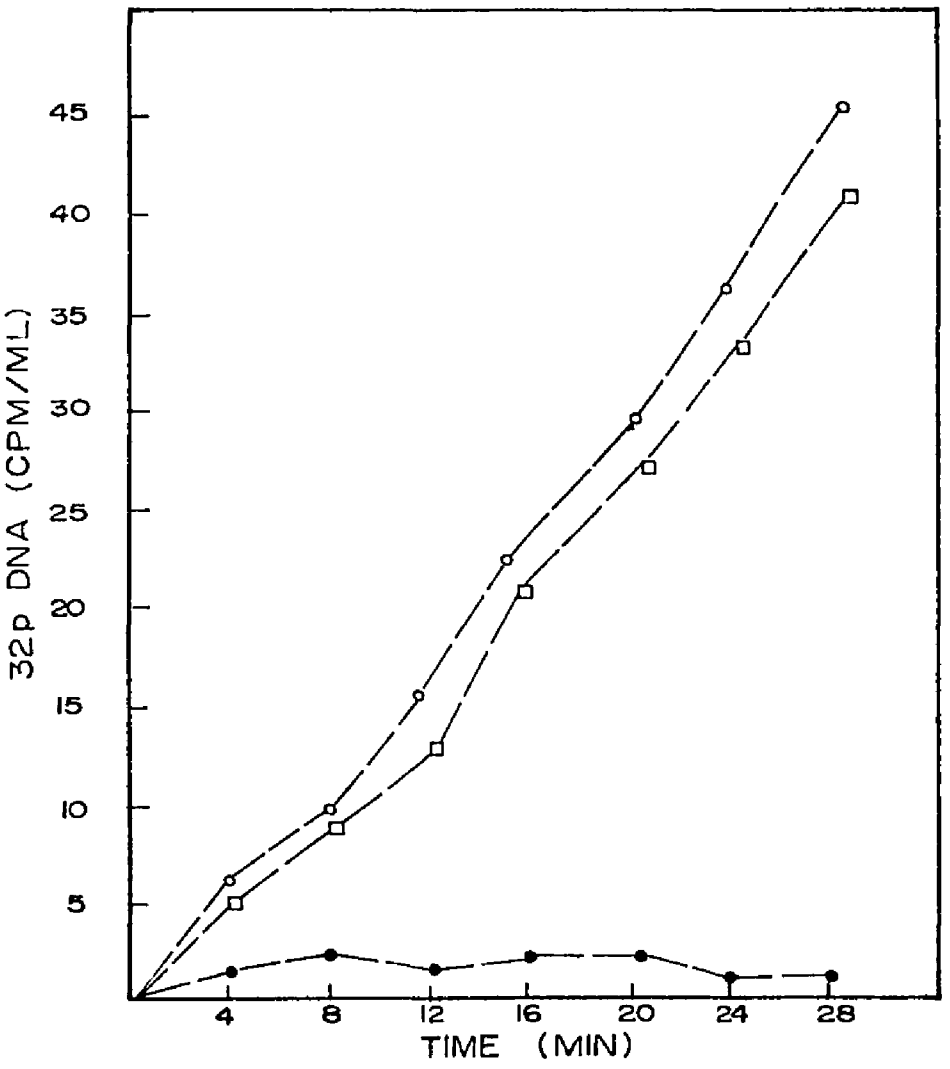

45
40
35
30
25
20
15
10
5
32P DNA (CPM/ML)
4
8
12
16
20
24
28
TIME (MIN)

Table 9. Transfection of calcium treated cells of *E. coli* K 12 by λ and ø80 DNA

Bacteriophage[a]	Molecular weight[b]	Number of *lac*$^+$ transfectants formed with[c] GR-1 (λ)	ATCC 25256
λ *plac*5	25×10^6	9.0×10^1	1.1×10^1
	10×10^6	5.0×10^1	1.3×10^1
	15×10^5	2.3×10^0	1.0×10^0
	10×10^4	1.0×10^0	0.0×10^0
ø80*plac*1	20×10^6	6.1×10^1	1.3×10^1
	10×10^6	5.8×10^1	1.2×10^1
	10×10^5	4.5×10^0	2.0×10^0
	10×10^4	0.0×10^0	0.0×10^0

[a] Donor DNA, at a concentration of 25 ug/ml, was λ*plac*5 and ø80*plac*1.

[b] Molecular weight was determined by the distance weight equation of Burgi and Hershey

[c] Recipient cells, at a concentration of 1.2×10^9, were GR-1 (λ) and ATCC 25256

Table 10. Transfection of calcium treated E. coli K 12 spheroplasts by λ and Ø80 DNA.

Bacteriophage[a]	Molecular weight[b]	Number of lac$^+$ transfectants formed with[c] GR-1 (λ)	ATCC 25256
λplac5	25×10^6	5.4×10^1	1.6×10^1
	10×10^6	3.5×10^1	1.3×10^1
	15×10^5	2.9×10^0	1.4×10^0
	10×10^4	1.1×10^0	0.0×10^0
Ø80placl	20×10^6	5.1×10^1	3.9×10^1
	10×10^6	3.9×10^1	1.6×10^1
	10×10^5	1.3×10^0	0.0×10^0
	10×10^4	0.0×10^0	0.0×10^0

[a]Donor DNA, at a concentration of 25 ug/ml, was λplac5 and Ø80placl.

[b]Molecular weight was determined by the distance weight equation of Burgi and Hershey.

[c]Recipient cells, at a concentration of 1.2×10^9, were GR-1 (λ) and ATCC 25256.

Table 11. Transfection of *E. coli* K 12 with λ plac5[a]

Recipients	Indicator Strain	Number of transfectants/ml
GR-1 (λ)	GR-1 (λ)	4.0×10^2
	RS5052	1.2×10^3
RS5052	GR-1 (λ)	1.1×10^3
	RS5052	1.7×10^3

[a]Transfection was performed using H agar and an overlay of H top agar. One tenth ml of phage was incubated with two tenths ml of recipient cells. The concentration of indicator strain cells was 0.2 ml.

formants results of Table 16 were obtained. Thus, the potential transformants were indeed being destroyed (lysed) by the specialized transducing phage DNA.

DISCUSSION

Effect of Calcium, Spermine and Protamine and Spheroplasted Recipient Cells

One of the most essential parameters for transformation in Escherichia coli K 12 is the requirement of the recipient cells for calcium (Oishi and Cosloy, 1972). The absolute requirement for calcium could easily be demonstrated since E. coli K 12 is capable of growth in chemically defined media. That calcium treatment of recipient cells is essential for transformation was first reported, not for a transformation system, but for a transfection system (Mandel and Higa, 1970). In 1972, Oishi and Cosloy demonstrated calcium-mediated transformation in E. coli. Additionally, stimulation of the transformation process by calcium, by calcium and magnesium, and by calcium, magnesium and barium has been observed with D. pneumoniae, (Fox and Hotchkiss, 1957), H. influenzae (Goodgal and Herriott, 1961), and B. subtilis (Young and Spizizen, 1963), respectively.

At present it is not possible to determine whether the requirement for calcium is related to a neutralization of the electronegativity of the cell wall, a partial dissociation of the membrane structure, a cofactor requirement in an enzymatic reaction, a factor required for initiation of penetration by exogenous DNA, a neutralization of the charge on the transforming DNA, or a stabilization of the transforming DNA.

Cosloy and Oishi (1973) have postulated that calcium ions increase the permeability of the cell surface through either a neutralization of the electronegativity of the cell wall, or through a partial dissociation of the membrane structure. If either of these were the case, then other methods known to increase the permeability of the cell surface should also enhance transformation frequencies. However, both treatment with EDTA (Leive, 1965) and osmotic shock (Neu and Heppel, 1966) were examined and neither of these two methods enhanced transformation.

Calcium ions could possibly be required for initiating the penetration of exogenous DNA molecules into recipient cells. Magnesium ions are known to play such a role in the B. subtilis system (Morrison, 1971).

There exists several lines of evidence, although indirect, which favor the hypothesis of DNA stabilization. First of all, ions which stimulate and inhibit the incorporation of DNA are, in general, similar to those which stabilize and destabilize DNA (Eichhorn, 1962). Secondly, in the transformation systems of both D. pneumoniae and B. subtilis, heated DNA, although biologically active, is not as readily incorporated into competent cells (Lerman and Tolmach, 1959; Young and Spizizen, 1961). Thirdly, a critical molecular weight is necessary for the incorporation of DNA (Litt, Marmur, Ephrussi-Taylor, and Doty, 1958).

In addition to calcium ions, a molecular species capable of DNA stabilization is spermine. Spermine is also capable of a neutralization of the charge on the DNA mole-

cule. Furthermore, spermine is capable of enhancing the transformation process in E. coli. Such enhancement, however, is not possible in the absence of calcium. That spermine is the only polyamine seen to participate in the transformation of E. coli K 12 is interesting because, of the three polyamines, only spermine has not been found in bacteria (Davis et. al., 1973). It is known, however, that exogenous spermine, which is present in both yeast and meat extracts, can be taken up by E. coli subsequent to which it replaces endogenous spermidine (Davis et. al., 1973). Although the function of polyamines within a cell are not clearly defined, all three polyamines readily bind to nucleic acids in vitro (Lehninger, 1970). As a result of such binding, the polyamines are seen not only to effect a neutralization of charge on these highly anionic polymers, but are also capable of stabilizing the conformation of nucleic acids through the formation of reversible, ionic cross-links (Lehninger, 1970). Additionally, transforming DNA in the presence of spermine possesses an increased stability to heat inactivation and retains a high degree of activity for genetic transformation (Tabor, 1961). This increased stability to heat inactivation is a function of the ionic cross-links. Likewise, nitrogen mustard cross-linked, B. subtilis DNA retains a high degree of activity for genetic transformation (Kohn and Green, 1966). At present, however, there is no information to definitively support the contention that enhancement of transformation by spermine results from

either its neutralization of charge or its ability to form stabilizing ionic cross-links with DNA. Furthermore, enhancement of transformation by spermine is not possible in the absence of calcium.

Spheroplasted as well as protamine treated recipient cells enhance the transformation process in E. coli K 12. Additionally, the need for calcium in either spheroplasted or protamine treated recipient cells is the same as that required in non-spheroplasted, non-protamine treated recipient cells.

The addition of protamine sulfate to spheroplasts of E. coli K 12 is seen to stimulate transfection. This is true not only for T1 phage DNA (Hotz and Mauser, 1969), but also for DNA from lambda, ØX174 (replicative form), fd (replicative form), and T7 (Benzinger, Kleber and Huskey, 1971). The effect of protamine is, in all cases, specific for double-stranded DNA.

Several possible hypotheses for the mechanism of action of protamine could be advanced. First of all, protamine, like spermine, could lead to a stabilization of DNA by virtue of its ability to bind to the polymer. This is unlikely because addition of protamine to the transfecting DNA is sufficient to inhibit transfection (Benzinger et. al., 1971). In the present study, similar findings were obtained following the addition of spermine to the transforming DNA. Thus, nucleic acid stabilization does not appear to be the mechanism involved in facilitating the uptake of the trans-

forming DNA. Secondly, protamine could inhibit a nuclease or nuclease complex capable of degrading the exogenous transfecting or transforming DNA before such DNA could reach the recombination machinery of the recipient cell. This is a possibility because some nucleases are precipitated by protamine sulfate in the presence of cellular nucleic acids (Sadowski and Hurwitz, 1969). Such nuclease activity, especially deoxyribonuclease activity, can also be inhibited in E. coli by streptomycin (Adams, 1959). Bacterial nucleases can greatly limit the efficiency of both transfection and transformation, and no such activity seems to be more destructive than the nuclease complex encoded by the recB and recC genes found in E. coli. This nuclease complex appears to possess at least 4 different enzymatic activities: an ATP-dependent double-stranded exonuclease; an ATP-dependent single-stranded exonuclease; an ATP stimulated single-stranded endonuclease; and an ATPase (Goldmark and Linn, 1972). The double-stranded exonuclease activity seems to predominate (Benzinger, Enquist and Shalka, 1975). Additionally, both circular single-stranded and circular double-stranded molecules seem to escape degradation (Benzinger et. al., 1975). Thus, the possibility that protamine enhances transfection or transformation by nuclease inhibition would appear to be a viable explanation with regard to the E. coli transformation system.

With regard to spheroplasting the recipient cells, such a procedure could facilitate entry to exogenous DNA by

releasing not only various binding proteins, but also hydrolytic enzymes, for example, proteases, nucleases, etc., associated with the periplasmic space.

Thus, the exact role or mechanism involved in the enhancement of transformation by calcium, spermine, protamine and spheroplasting remains unclear. The only conclusion which can be drawn with confidence is that, without calcium, transformation in E. coli K 12 cannot be demonstrated. Additionally, whatever enhancement spermine, protamine or spheroplasting possess, without calcium, the effect is minimal.

DNA Isolation

In all other bacterial transformation systems, the transforming DNA has been obtained through chemical extraction procedures. Additionally, the chemical extraction procedures so employed were not known for their gentle treatment of the DNA. Accordingly, might such crude extraction procedures which yield DNA fragments, the average molecular weight of which is approximately 4.0×10^6 (Radding, 1973), be partly responsible for the inability to demonstrate transformation in E. coli K 12? To answer this question, a method for the isolation of transforming DNA was found which was not only easily performed and gentle, but which also yielded intact, circular, double-stranded E. coli K 12 DNA. As such, the DNA most effective in the demonstration of transformation in E. coli K 12 possessed a molecular weight of approx-

imately 2.9 x 10^9. Such DNA represents the intact E. coli chromosome. Additionally, as the molecular weight of the transforming DNA decreases, so do the number of lac$^+$ transformants. Thus, indirectly speaking, the method employed for the isolation of the transforming DNA, and directly speaking, the molecular weight of the transforming DNA, exert an influence on the transformation process in E. coli K 12. These results are in disagreement with the findings of Cosloy and Oishi (1973). The method Cosloy and Oishi employ for the isolation of their transforming DNA is basically that of Marmur (1961). This method exposes the DNA to a greater degree of both chemical and mechanical destruction as compared to the modified method of Worcel and Burgi (1972). Nevertheless, Cosloy and Oishi state that the DNA most effective in the transformation of E. coli possesses a molecular weight of approximately 6.0 x 10^6. In other bacterial transformation systems, unsheared, high molecular weight DNA is most efficient for transformation. This is substantiated by the findings Bettinger and Young (1975) where the efficiency of transformation in B. subtilis was increased between 3 to 10 fold by the use of high molecular weight DNA. Cosloy and Oishi support their findings that lower molecular weight DNA is more effective in the transformation of E. coli by suggesting that such DNA more effectively penetrates the cell envelope barrier. They suggest that the lower the molecular weight of the transforming DNA, the greater the degree of penetration, and there-

fore, the greater the efficieny of transformation. In contrast, my study showed that the efficiency of transformation decreases as the molecular weight of the transforming DNA decreases.

A central question concerning transforming DNA is; does transforming activity depend on the size of the molecule? The least active molecular weight DNA may reflect either the minimum size required for effective attachment to a site on the bacterial cell or a combination of this and the minimum size required for effective integration following penetration into the bacterial cell. If there does exist a critical or minimum size, we must understand its significance. This is necessary if quantitative interpretations of transformation experiments are to be made. Previous data on the mean transforming activity versus the reciprocal molecular weight of sheared DNA have been interpreted on the assumption that there existed a specific DNA region which, if intact, had 100 per cent activity, and if not, had zero activity (Litt, et al., 1958; Fox, 1962; Gurney, 1965). The data found in these studies, all of which were for the pneumococcal system, were consistent with the existence of a qualitative "all-or-nothing" critical size of active transforming DNA. The critical values so obtained were 1.1×10^6 (Litt, et al., 1958), 0.6×10^6 (Fox, 1962), and 0.7 to 0.9×10^6 (Gurney, 1965). Because of such reports, the question was raised as to whether or not activity existed at smaller molecular weights. Using a

pneumococcal system, Cato and Guild (1968) demonstrated transforming activity for several single markers in DNA fragments of molecular weights 290,000 ± 30,000. Although the probability of transformation was very low, the activity was not due to contamination from larger sizes of DNA (Cato and Guild, 1968). This figure sets an effective lower limit on the size of DNA active for transformation in this system. It is not an "all-or-nothing" critical size, however, since above this minimum the activity remains size-dependent. Looking at this question as it applies to my E. coli system, it must first be emphasized that the plot of figure 7a has two slopes. Thus, in the E. coli system, there exists two critical molecular weights. Extrapolation to zero biological activity in the range below 10^8 daltons yielded a critical molecular weight of 1.4×10^6 daltons, whereas extrapolation to zero biological activity in the range 10^8 to 2×10^9 daltons yielded a critical molecular weight of 5×10^7 daltons. The data presented here is, therefore, consistent with the idea that the probability of transformation by an individual DNA fragment is a size-dependent variable. Both my E. coli system and the D. pneumoniae system of Cato and Guild (1968) are similar in showing transformation by an individual DNA frgament to be size-dependent. However, the two systems differ in that

the E. coli system is diphasic, whereas the D. pneumoniae system is monophasic. This may reflect the fact that high molecular weight DNA's have not as yet been examined in the pneumococcal system. With regard to the E. coli system, the sharp increase in activity at the highest molecular weights could possibly be explained on the basis of molecular conformation. High molecular weight DNA would possess a more looped conformation as opposed to linear low molecular weight DNA. Worcel and Burgi (1972) determined that for unbroken DNA's in the 10^9 molecular weight range, the number of loops per DNA molecule was approximately 80. By dividing the critical molecular weight (5×10^7) into the molecular weight which yielded the highest number of lac$^+$ transformants, the approximate number of loops per genome for my E. coli system was determined to be approximately 58. This value correlates fairly well with the value (80) obtained by Worcel and Burgi (1972). Therefore, it is possible that high molecular weight DNA more effectively participates in the transformation of E. coli as a function of its looped conformation. Furthermore, it may be that the looped segments of the transforming DNA are involved in attaching to sites on the bacterial cell.

DNA-recipient Cell Interaction

With regard to the kinetics of the DNA-recipient cell interaction, the time periods involved are fairly much characteristic of bacterial transformation systems in general (Spizizen, et al., 1966). Noteworthy in this study is the fact that the periods of contact between cells and DNA are slightly longer in comparison to previous reports for other bacterial systems.

Prior to the addition of the transforming DNA, recipient cells required a 25 min incubation period at 3 C. Without this cold incubation period, transformation frequencies were reduced. Why this is so remains to be elucidated. One plausible explanation could be that there occurs a selective decrease in the activity of a specific nuclease or nuclease complex. Such a slowed nuclease activity might possibly allow for initiation of recipient cell-DNA complexes which are resistant to endogenous nuclease destruction. Another possible explanation is that the cold incubation period liberates the nuclease activity present in the periplasmic space of E. coli. What can be said, however, is that his preliminary cold incubation appears to be unique for the E. coli K 12 transformation system.

Temperature

Temperature is another parameter which is characteristic for bacterial transformation systems in general. The optimal temperature for all recipient strains employed in this study was either 40 C or 32 C. Overall, the mechanism by which temperature exerts its influence might possibly be on the penetration of the transforming DNA into the recipient cells. This explanation might not be that strong as Strauss (1970) has shown that an early step in the entry of exogenous DNA into B. subtilis is energy dependent. Alternatively, temperature could possibly affect the recombination process. Whatever, the exact mechanism remains to be elucidated.

pH

Another influential parameter in the transformation process is pH. All biological phenomena require an optimal pH, and genetic transformation in E. coli K 12 is no exception. The optimal pH for all strains employed in this study was 8.2. This is an interesting point because if transformation in E. coli is a species specific phenomenon, how could one account for the identical pH and temperature values obtained in my experiments.

DNA Source

When looking at the transformation process as a

function of the source of the donor genetic material, only DNA from E. coli K 12 was capable of transforming E. coli K 12 cells. It is of interest to note that even DNA from E. coli B cells was incapable of transforming E. coli K 12 cells. The most reasonable explanation for this result may by the different restriction-modification systems possessed by the two different strains of E. coli (Meselson, Yuan and Heywood, 1972).

Transformation employing DNA isolated from previously transformed cells indicated that such DNA remained biologically active through the transfer process. It would be of interest to determine if there existed any limit to the number of transfers which could performed.

Uptake of DNA

With regard to the rate of uptake of the exogenous DNA, we see that such a rate is greater at 40 C than 25 C. Overall, however, these results might indicate that it is not the rate of uptake per se which is greater at 40 C, but simply that there is greater uptake by recipient cells of exogenous DNA at 40 C as opposed to 25 C.

O_2 Utilization Characteristics

In B. subtilis (Young and Spizizen, 1963), D. pneumoniae (Fox and Hotchkiss, 1957), and H. influenzae (Goodgal and Herriott, 1961), the incorporation of DNA is influenced by the requirement for strict aerobic conditions. In

comparison, incorporation of DNA in E. coli K 12 was seen to take place under aerobic conditions. In contrast, however, incorporation was also seen to take place under more facultative conditions.

DNA Concentration

Over a range of low DNA concentrations, the number of transformants is linearly related to the concentration of the DNA. As the concentration of DNA is increased, a plateau is reached whereby further increases in DNA concentration do not result in an increased number of transformants. Thus, there is a limit to the amount of DNA which can be adsorbed by a single receptive bacterium. While transformation in Y. novicida exhibits a saturation plateau of 5 ug/ml (Tyeryar and Lawton, 197), transformation in the E. coli strains of Oishi and Cosloy (1972) exhibit saturation plateaus ranging between 10 to 20 ug/ml. In my study, the saturation plateau for all strains was 25 ug/ml. Based on saturating DNA concentrations, Oishi and Cosloy (1972) employ a 2 ug/ml DNA concentration. In my study, a 6.25 ug/ml DNA concentration was employed. All concentrations are well within their respective linear ranges.

DNA concentration, increases as the 1.4^{th} power of the number of lac^{+} transformants. Such a result can

be interpreted in two different ways; First, that the activity of the transforming DNA is a function of the degree of dilution. At high dilution (low concentration) of the polymer the stability of the molecule would be reduced. Reduced stability would result from the fact that low concentration DNA is more easily degraded. Such degradation would reduce the molecular weight (size) of the molecule to the point of minimal transforming activity. In contrast, low dilution (high concentration) of the polymer would increase the stability of the molecule. Increased stability, conferring a greater resistance to degradation, would result in greater transforming activity. A second explanation could be that the average recipient cell is receiving more than one molecule of transforming DNA. The more DNA which penetrates a recipient cell the less likely all of that DNA will be degraded by nucleases and therefore, the greater the probability that the genetic marker under investigation will integrate into the recipient cells genome.

In conclusion, both interpretations explain the observed relationship between the number of *lac*$^+$ transformants and DNA concentration on the basis of minimizing the damage to transforming DNA and thereby maintaining the highest degree of activity.

Transfection

Transformation was also performed with DNA isolated

from specialized transducing phages (lambdaplac5 and ø80placl). These phage carry all or part of the lactose (lac) operon from the E. coli chromosome in place of a phage chromosomal segment that includes the immunity operon (Shapiro, et al., 1969). Transformation with such phage DNA is expected to be more efficient than that observed with bacterial chromosomal DNA. This higher efficiency is due to the higher concentration (100-fold increase) of lac genetic material in the phage DNA. Ths results indicate, however, that such phage DNA is not more efficient in transformation of E. coli K 12. Here, a higher number of transformants were not expressed because the cells so transfected were being lysed by the virulent phage DNA. Additionally, such phage could not be repressed for lytic functions due to the deletion of the immunity operon.

Conclusion

Overall, the results of this investigation indicate that strains of E. coli K 12 can be successfully transformed for the beta-galactosidase marker at a frequency of 10^{-4} to 10^{-5}. Such a frequency of transformation represents a small but definite improvement over previous E. coli transformation systems. Still there is the need for continued investigation into this genetic phenomenon as it pertains to this gram-negative bacterial species. Such continued investigations can lead not only to a better understanding of the transformation phenomenon as a means of genetic transfer, but also to a more precise explanation of the mechanism involved in genetic recombination.

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