OPTIMIZING A TRANSFORMATION SYSTEM USING SPHEROPLASTS OF *ESCHERICHIA COLI* 15T⁻

by

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B. S., University of Wisconsin--Eau Claire, 1967

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Microbiology

Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1972

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Major Professor

THIS BOOK CONTAINS NUMEROUS PAGES WITH THE ORIGINAL PRINTING BEING SKEWED DIFFERENTLY FROM THE TOP OF THE PAGE TO THE BOTTOM.

THIS IS AS RECEIVED FROM THE CUSTOMER.

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INTRODUCTION

In strains of Escherichia coli two of the three types of genetic exchange known to occur in bacteria, transduction and conjugation, occur as natural biologic phenomena. Transduction is genetic transfer mediated by a bacteriophage vector. In E. coli, occurrences of transduction have been reported with a number of bacteriophages including λ (Morse, 1954), P1 (Lennox, 1955), and 363 (Jacob, 1955). Transduction has been utilized in research mainly as a tool for the analysis of genetic fine structure and function. Conjugation in E. coli involves actual cell to cell contact between different cells and the subsequent one-way transfer of genetic material from the donor (male) cell to the recipient (female) cell. This male character is demonstrated only in those cells that harbor a sex factor such as F factors, which may exist in the cells either in an autonomous state (F or F') or incorporated into the host genome (Hfr), colicin factors or R factors. Most research involving conjugation has been done utilizing strains of E. coli harboring the F factor in the Hfr state, and has been carried out to discover the exact mechanism of conjugation and the role each partner plays in the transfer of the genetic material. Work has also been done using Hfr strains to map the relative order of genes on the E. coli chromosome and to estimate their relative distances from one another.

The third type of genetic transfer, transformation, which is the process whereby a cell takes up naked DNA from its environment, integrates it into its own genome, and expresses it as "self information", does not occur in E. coli; however, if such a system were developed it could become a useful research tool. Although not a true system of transformation, spheroplasts of E. coli have been infected with naked DNA of several bacteriophages.

Guthrie and Sinsheimer (1960) have developed a phage DNA infection system using E. coli K12W6 treated with lysozyme and EDTA to prepare spheroplasts. Benzinger et al., (1967) have used osmotic shock procedures to prepare spheroplasts of E. coli C capable of taking up bacteriophage DNA and producing mature phage. If spheroplasts of E. coli can take up and replicate bacteriophage DNA and produce bacteriophage, perhaps it is possible that these spheroplasts could take up and replicate a somewhat larger piece of non-bacteriophage DNA such as an R factor or an F factor.

With a transformation system some of the problems of studying DNA replication of such sex factors in the recipient after conjugation could be eliminated. For instance, the replicative act could be separated from the conjugal act since transformation would not involve cell to cell contact. The necessity of compatible donors and recipients would no longer be a factor, and therefore DNA from other species of bacteria that will not conjugate with E. coli or that will yield only a small number of recipients could be studied. Isotope labeling of the R factor or F factor could be simplified since the necessity of labeling only the plasmid would no longer be present, as cells could be grown in media containing label and then the plasmid could be harvested and separated from any host chromosomal DNA. R factor or F factor DNA could be isolated in great quantities and frozen and thus be ready at all times for experimentation. Also, this means of storing sex factor DNA could serve to add genetic and maintenance stability because in many organisms spontaneous loss of an R factor can occur, especially in stock cultures which are not often being challenged with antibiotic.

Many additional research questions can be asked, and perhaps answered by the use of a transformation system. Will spheroplasts take up the

plasmid as double stranded DNA, or as a single stranded molecule as occurs in conjugation, or not at all? If the DNA is taken up will it be expressed and replicated and thus be passed on to daughter cells or will it simply be degraded? If double stranded DNA enters and is replicated, will both strands serve as templates, or will one be degraded and the other strand preferentially copied? If the spheroplast contains a plasmid closely related to that attempting to enter, will the DNA be taken up? Also, studies of the effects of different chemical or physical agents on the plasmid DNA could be carried out in this system that could not be carried out in any other as readily. As previously stated, this system could be an exciting tool for use in future experimentation and research.

STATEMENT OF THE PROBLEM

The research described in this thesis is one contribution to an overall research effort aimed at developing an Escherichia coli transformation system which will employ purified sex factor DNA and a strain of E. coli which has been well characterized in terms of its chromosomal DNA synthesis. The purpose of this specific study was to compare various procedures for preparing revertable spheroplasts of a DNA restriction deficient strain of E. coli 15 T^- (555-7) which was maximally competent for infection with purified DNA of the bacteriophage ØX174.

The choice of E. coli 15 T^- (555-7) was made because of the extensive accumulation of information on the mechanism of its chromosomal DNA replication [Lark, C. (1966); Lark, K. G. (1966); Lark, Repko, and Hoffmann (1963); Bird and Lark (1968)]. A restriction deficient strain was chosen to avoid host DNA restriction (Arber and Linn, 1969) of sex factor DNA which is ultimately to be isolated from another species, Proteus mirabilis. The particular restriction deficient strain chosen has been well characterized (Lark and Arber, 1970).

An assay for competence based on infection by bacteriophage ØX174 DNA was chosen because the assay depends upon entry of large, biologically active DNA molecules and because the assay is simple, relatively inexpensive, fast and sensitive.

REVIEW OF LITERATURE

Spheroplasts

A primary characteristic of bacteria and other procaryotic cells is the presence of a cell wall. This cell wall confers rigidity to cells, acts as a girdle and osmotic protector, and has as an integral component a single large molecule, murein, which serves as the backbone of the cell wall structure. Except for certain unusual strains of cell wall deficient bacteria, L-forms (Salton, 1964), cell wall integrity is necessary for cell function and growth, unless precautions are taken to maintain cells in an osmotically protected environment. Cell wall can be removed by a number of methods, but in Gram negative organisms total wall removal is not usually attained. Such incompletely denuded cells are termed spheroplasts. Since spheroplasts contain some residual cell wall, it is possible under certain conditions for cell wall regeneration to occur (Sinsheimer, 1968) with apparently normal cells resulting.

Spheroplasts have permeability properties which differ from normal cells. Heppel (1968) reports that spheroplasts prepared by osmotic shock or lysozyme-EDTA release a number of degradative enzymes: (a) an alkaline phosphatase; (b) a 5' -nucleotidase; (c) an acid phosphatase; (d) a cyclic phosphodiesterase; and (e) an RNA inhibited DNase. Only about 4% of the cellular protein is released. Spheroplasts also demonstrate increased uptake of certain molecules; (a) actinomycin D; (b) carbamyl phosphate; (c) nucleoside triphosphates; and (d) o-nitrophenyl-galactoside.

The most conventional methods employed for spheroplast formation are osmotic shock and lysozyme-EDTA treatment. Osmotic shock procedures consist of rapid dispersion of bacterial cells from a medium with a high osmotic strength such as medium with a high concentration of sucrose into a medium

of low osmotic strength such as water. The procedures were first attempted during the study of enzymes that may be located at or near the cell surface of Escherichia coli (Neu and Heppel, 1965; Nossal and Heppel, 1966; Anraku and Heppel, 1967). One of the first attempts to use osmotic shock spheroplasts in a transformation system was reported by Nossal and Heppel (1966) using λ bacteriophage DNA. Benzinger et al. (1967) carried out some extensive studies on a transformation system utilizing ØX174 DNA and osmotic shock spheroplasts of E. coli. When spheroplast concentrations were kept constant, infectivity was directly proportional to the DNA concentration. A plateau was reached at a DNA concentration of 1×10^9 molecules/ml. When the DNA concentration was kept constant the infectivity was directly proportional to the spheroplast concentration from 5×10^6 to 1×10^9 cells/ml. No reported studies of DNA replication have been carried out using osmotic shock spheroplasts.

Lysozyme-EDTA spheroplasts have been studied and utilized in research to a much greater extent. The first attempts to infect spheroplasts were carried out to add proof to the fact that DNA is the only necessary viral fraction to obtain replication in the host. Cells treated with lysozyme only were infected with a DNA preparation from bacteriophage T_2 (Spizizen, 1957; Fraser et al., 1957). With more advanced methods of DNA purification and the addition of EDTA in preparing spheroplasts, better infectivity and clearer results were obtained. The first infection systems with these improved techniques involved spheroplasts of E. coli and the DNA of bacteriophage ØX174 (Guthrie and Sinsheimer, 1960; Sekiguchi et al., 1960). ØX174 DNA has been characterized by Sinsheimer (1959b) and by Fiers and Sinsheimer (1962a,b,c). The DNA is a single stranded circular molecule with a molecular weight of 1.7×10^6 daltons, and there is one DNA molecule per virus.

Purification procedures are also described (Sinsheimer, 1959a; Sinsheimer, 1966).

Further work with spheroplasts included a study of the environmental factors that influenced the infection process and to optimize the system in relation to these factors. Separate projects were carried out by Huppert et al., (1962) and Guthrie and Sinsheimer (1963). Huppert studied the effects of albumin concentration, Mg^{++} concentration, and the presence of extraneous DNA. Guthrie and Sinsheimer determined the effects of and the optimum levels of sucrose, bovine serum albumin, $MgSO_4$, temperature, other salts such as CsCl, NaCl, and KCl, and Tris buffer at various stages of the spheroplast preparation and of the infection process. The level of interference by foreign nucleic acid was determined. Almost total inhibition of infection occurs when 40 ug/ml of deproteinized uninfected E. coli DNA and RNA are present. Levels of 4 ug/ml or less do not inhibit infection.

The mode of entry of the DNA into spheroplasts is not understood, but it is not simply a passive penetration of the DNA into the spheroplast. Uptake of the DNA continues for about 10 min after the DNA and spheroplasts are mixed, and in studies with P^{32} labeled ØX174 DNA, about 10% of the DNA is taken up (Guthrie and Sinsheimer, 1963). Only approximately 1 in 10 of the DNA molecules taken up establish an infection, however. The assay is proportional up to a point of saturation and can therefore be used in quantitative studies. Sinsheimer (1968) reports that with variation in the amounts of lysozyme and EDTA and in the time of treatment with these agents, competent spheroplasts of any strain of E. coli can be obtained.

Spheroplasts have been infected not only with the single stranded ØX174 DNA but also with the replicative form (RF) DNA of ØX174 (Sinsheimer et al., 1962), with the DNA of the bacteriophage λ (Meyer et al., 1961;

Young and Sinsheimer, 1967), and with the RNA of RNA viruses (Davis et al., 1964; Strauss, 1964). In general the efficiency of infection is greater for single stranded DNA than for double, for smaller DNA than larger, and for DNA than for RNA.

Infection of E. coli spheroplasts with viral DNA has been used rather extensively to study the replication of the viral DNA. The first bacteriophage DNA studied in this manner was ØX174. It was determined that when E. coli C spheroplasts were infected with ØX174 DNA in the presence of chloramphenicol, the single stranded DNA is converted to a double stranded replicative form (Sinsheimer et al., 1962; Hayashi et al., 1963). The infectious ØX174 RF DNA is a ring shaped molecule as shown by its resistance to exonuclease action (Burton and Sinsheimer, 1963) and by electron microscopy (Kleinschmidt et al., 1963; Burton and Sinsheimer, 1963; Chandler et al., 1964). Ultracentrifugal analyses show that preparations of ØX174 RF contain a mixture of at least two components (Burton and Sinsheimer, 1965; Sinsheimer et al., 1965). Component I (21s) is an intact ring shaped double stranded DNA and component II (16s) is a double strand with at least one single-strand break (Burton and Sinsheimer, 1965; Ray et al., 1966). Infection of spheroplasts has also afforded similar studies using the RNA of RNA bacteriophage MS-2 and the DNA of λ bacteriophage.

Bacterial Plasmids

Bacterial plasmids are a non-chromosomal genetic element which are transmissible from one cell to another by infection, and may be propagated either independently in the cytoplasm or as a part of the chromosome after insertion. Plasmids include temperate bacteriophages, F factors, R factors, and Colicin factors. The two discussed briefly here are F factors and

R factors.

Genetic material can be transferred from one bacterium to another through a process termed conjugation (Lederberg and Tatum, 1946; Tatum and Lederberg, 1947). It was later discovered that this transfer was mediated by a genetic element named F factor (Cavalli, Lederberg and Lederberg, 1953; Hayes, 1952; Hayes 1953). The presence of the F factor in a cell allows that cell to become a genetic donor and also causes the cell to produce new appendages known as sex pili (Brinton, 1959; Brinton et al., 1964). It is not certain that these pili are the actual route of gene transfer (Brinton, 1965) or if they simply aid in cell to cell contact.

The F factor can exist in three states: free within the cytoplasm of the cell (F^+), integrated within the host chromosome (Hfr) (Wollman, Jacobs and Hayes, 1956), or free within the cytoplasm, but with a small portion of chromosomal DNA attached (F') (Hirota and Sneath, 1961). In all of these states the sex factor mediates the unidirectional transfer of DNA from the donor cell to the recipient cell. The state of the DNA during conjugation and transfer in the donor and the recipient has been the object of much study. Jacob, Brenner and Cuzin (1963) demonstrated that DNA replication was involved immediately before, during, or immediately after transfer. Autoradiographic techniques later showed that only one strand of DNA synthesized before mating was transferred (Gross and Caro, 1966). In work with minicells of E. coli, Cohen, Fischer, Curtiss and Adler (1968) found that the single stranded DNA transferred was later recoverable in a double stranded molecule. These results were confirmed by Ohki and Tomizawa (1968) who further showed that the second strand was synthesized in the recipient. The strand transferred is always the one which has the 5' end toward the transfer origin (Ohki and Tomizawa, 1968;

Rupp and Ihler, 1968). The DNA of the sex factor in the F^+ state in the donor is a covalently closed double stranded circular DNA molecule (Freifelder, 1968a,b). Vapnek and Ruff (1970) have obtained covalently closed double stranded circular DNA from the recipient as well as the donor. They state that one strand is transferred to the recipient where a complement to this strand is synthesized and a covalently closed double stranded DNA molecule is formed. The other strand of the sex factor, which remained in the donor, forms a new covalently closed double stranded DNA molecule with a new strand synthesized during the transfer process.

The R factor is also a covalently closed double stranded circular DNA molecule. It is most like the F' factor, since it is comprised of genes determining drug resistance and others (RTF) conferring the ability to conjugate and to transfer the factor to a new host (Watanabe, 1963a). Falkow, Haapala and Silver (1969) have estimated the molecular weight of the R factor R1 at 60×10^6 daltons. The approximate molecular weight of an F factor is 45×10^6 daltons (Freifelder, 1968a). It has been reported that in E. coli only a single copy of the R factor is present (Rownd, Nakaya and Nakamura, 1966), however in Proteus there may be as many as 40-60 copies of the R factor components (Falkow, Haapala and Silver, 1969). From Proteus mirabilis, Falkow, Haapala and Silver have isolated three molecular species which they suggest are the RTF, the linkage group conferring resistance, and a composite of these two. In E. coli only the composite molecule has been found. Cohen and Miller (1970) also found three independently replicating closed circular species of DNA in Proteus. Haapala and Falkow (1971) obtained an E. coli mutant thought to harbor only the RTF fraction and transferred this RTF to Proteus. There was no resistance to drugs developed but the Proteus was now sensitive

to male specific phage MS-2.

The frequency of transfer of R factors by conjugation are low in comparison to F (Watanabe, 1963b). This is due to the presence of a repressor in cells harboring R factors (Meynell and Datta, 1966, 1967). R factors are classified as fi^+ or fi^- depending upon their ability to inhibit the function of F (fi=fertility inhibition). Mutants have been isolated which have lost the ability to repress the frequency of conjugation (drd) (Meynell and Datta, 1967). In the Rl-drd studied conjugation occurred at 300 times the frequency of the wild type.

Transmissible drug resistance was first discovered in Japan (Watanabe, 1963) after isolation of a Shigella flexneri resistant to four drugs. Because the multiply resistant strains were found in increasing numbers, it was suggested that the resistance was being transferred to Shigella from E. coli. Watanabe and Fukasawa (1961) confirmed that this multiple resistance was indeed being transferred from E. coli by cell to cell contact. Datta (1962) found R factor in strains of Salmonella typhimurium causing gastroenteritis in London. It has now been shown that R factors are quite prevalent in strains of Salmonella, Shigella and E. coli around the world. The R factor can be transferred among the Enterobacteriaceae (Harada et al., 1960), Vibrio sp. (Kuwabara et al., 1963; Baron and Falkow, 1961), Pasteurella sp. (Ginoza and Matney, 1963), and Serratia marcescens (Rownd et al., 1966).

MATERIAL AND METHODS

STRAINS:

Escherichia coli K12W6	Obtained from R. Sinsheimer, California Institute of Technology
E. coli C	Obtained from R. Sinsheimer, California Institute of Technology
E. coli 15T$^-$ (555-7) arg$^-$ met$^-$ trp$^-$ thy$^-$	Obtained from K. G. Lark, Kansas State University
E. coli 15T$^-$, 65r$^-$m$^-$ arg$^-$ met$^-$ trp$^-$ thy$^-$ (referred to as 65r$^-$m$^-$)	Obtained from C. Lark, Kansas State University and designated 2365 in Lark and Arber, 1970.
E. coli 15T$^-$, 65r$^-$m$^-$/ØX174 (smooth)	Mutant isolated according to the procedure described below.
E. coli J5-3 met$^-$ pro$^-$ R1-19drd (This strain harbors the derepressed R factor R1-19 which confers resistance to ampicillin, kanamycin, streptomycin, chloramphenicol, and sulfonamide.)	Obtained from E. Meynell, The Lister Institute of Preventive Medicine, University of London, and described in Meynell and Datta, 1967.
Proteus mirabilis PM-1 lac$^-$ nic$^-$	Obtained from S. Falkow, Washington University School of Medicine and Dentistry.
P. mirabilis PM-1 R1-19drd	Obtained as described below.

MEDIA:

The growth media for osmotic shock spheroplasts consisted of 0.08 M NaCl and 1% (W/V) proteose peptone. The storage media contained 0.035 M Tris buffer, pH 8.5, and 0.38 M sucrose. The DNA adsorption media consisted of 0.1 M NaCl, 0.1 M sucrose, 5 mM $MgSO_4$, 1 mM Tris buffer, pH 8.5, 1 mM $CaCl_2$, and 0.5% bovine serum albumin. The bottom layer agar consisted of 15 g of bacto-agar, 10 g peptone, 5 g NaCl per liter of distilled and deionized water. The pH was adjusted to 7.5-8.0 with 1 N NaOH after autoclaving.

The growth media for lysozyme-EDTA spheroplasts was M9 media, plus any specific requirements of the organism. M9 consisted of 7 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 1 X 10^{-4} M $CaCl_2$, 1 X 10^{-3} M $MgSO_4$, and 0.4% glucose diluted to 1 liter with distilled and deionized water. The $CaCl_2$, $MgSO_4$, and glucose were prepared at 100X concentration and autoclaved separately. Required amino acids for E. coli $15T^-$ were added after autoclaving at concentrations of 100 ug/ml arginine, and 50 ug/ml methionine and tryptophan. Thymine was added at 4 ug/ml after autoclaving.

The base media in preparation of lysozyme-EDTA spheroplasts was PA medium as described by Guthrie and Sinsheimer (1960). It consisted of 10 g casamino acids, 10 g nutrient broth, 1 g glucose, and 100 g sucrose diluted to 1 liter with distilled and deionized water. The dilution media for the "growth tube" was PAM, which is PA media plus 0.2% $MgSO_4$ added after autoclaving.

Bacto-agar, proteose peptone, Mac Conkey's agar, casamino acids, nutrient broth and bacto-peptone were obtained from Difco, Detroit, Michigan. Amino acids, 30% bovine serum albumin, Tris(hydroxymethyl) amino methane (TRIS), lysozyme (eggwhite, 3X crystallized), sulfonamide, kanamycin SO_4 and ethylene diamine tetraacetic acid (EDTA) were obtained from Sigma, St. Louis, Missouri. Tetracycline HCl U.S.P. was purchased from American Cyanamid, Pearl River, New York. Sodium ampicillin was a gift from Bristol Laboratories, East Syracuse, New York. Chloramphenicol was a gift from Parke-Davis, Detroit, Michigan. All other chemicals were commercially available, reagent grade.

The media used in the titration of bacteriophage was a nutrient agar base plate (8 g nutrient broth, 15 g bacto-agar, distilled and deionized water to 1 liter), and nutrient soft agar as the top layer (8 g nutrient

broth, 5 g bacto-agar, distilled and deionized water to 1 liter). In those experiments exploying E. coli 15T$^-$, 4 ug/ml of thymine was added to the base plate agar and the top layer agar.

Other reagents necessary for preparing lysozyme-EDTA spheroplasts are as follows: various concentrations of lysozyme dissolved in 0.25 M Tris, pH 8.1, and made fresh for each experiment, 1.5 M sucrose, 30% bovine serum albumin, 4% EDTA, 10% $MgSO_4$, and 0.05 M Tris, pH 8.1.

ISOLATION OF 65r$^-$m$^-$/ØX174:

Two-tenths ml of 65r$^-$m$^-$ and 0.1 ml of ØX174 bacteriophage (1.3×10^{11} pfu/ml) were mixed in 2.0 ml of nutrient soft agar which contained 4 ug/ml thymine and plated to nutrient agar plates. The plates were incubated overnight at 37 C. Colonies of resistant organisms (65r$^-$m$^-$/ØX174) were picked and restreaked to nutrient agar plates for several successive days to free the bacteria from adsorbed bacteriophage. The organisms were then retested with ØX174 as described above to reaffirm their resistance to the virus. Two colony types were obtained. The smooth variety proved to be more resistant than the rough variety, and thus was used in subsequent experimentation.

E. coli 15T$^-$, 65r$^-$m$^-$, and 65r$^-$m$^-$/ØX174 were tested periodically to determine if the strains were still arg$^-$ meth$^-$ trp$^-$ by culturing the organisms in M9 media plus the required amino acids and thymine eliminating one of the required components in each successive tube and checking for growth after overnight incubation and after 48 hrs incubation at 37 C. The strains show no growth in the tubes without arginine, tryptophan and thymine, but show a +2 growth (based on a 0 - +4 scale) in 48 hrs in the media without methionine.

TESTING RESTRICTION AND MODIFICATION PROPERTIES:

The 65r$^-$m$^-$ and 65r$^-$m$^-$/ØX174 organisms were tested for the lack of restriction characteristics by performing a mating using these cells as females and Proteus mirabilis PM-1 R1-19drd as the male transferring the R factor R1-19drd. (PM-1 R1-19drd was obtained by transferring the R factor R1-19drd from E. coli J5-3 R1-19drd to P. mirabilis PM-1 followed by selection of lac$^-$ swarming colonies on Mac Conkey's agar containing antibiotics at 22-25 ug/ml).

CULTURE AND PURIFICATION OF BACTERIOPHAGE ØX174:

ØX174 phage was prepared according to R. Sinsheimer (1969) with a few modifications as follows: two liters of M9 + amino acids + 4 ug/ml thymine + 0.5% casamino acids were innoculated with 30 ml of a 16 hr overnight culture of 15T$^-$. The culture was incubated at 37 C with vigorous aeration until the cell concentration reached approximately 3 X 10^8 cells/ml. At that time 1.0 ml of a 2 X 10^{12} virus/ml stock culture was added (a multiplicity of 3 phage/cell). Thirty minutes later, 10 ml of 0.1 M EDTA, pH 7.0 was added and about 10 min later lysis began. After 3.75 hrs of incubation, the lysate was transferred to a separatory funnel and 400 ml of 40% polyethylene glycol (Carbowax 6000-obtained from Union Carbide Corporation) and 126 ml of 20% sodium dextran sulfate 500 (obtained from Pharmacia, Uppsala, Sweden) were added (one ml of the lysate was saved and titered for phage yield). The separatory funnel contents were mixed thoroughly and refrigerated at 4 C for 44 hrs. The interface was separated from the top and bottom phases and centrifuged. The sediment was then resuspended in 15 ml of saturated sodium borate at 4 C and mixed for 16 hrs at 4 C. After centrifugation, the supernatant was saved and the sediment

was washed with 5.0 ml of saturated sodium borate and pooled with the supernatant. To the pooled borate supernatant 1/10 volume of 5X tryptone (5 g/100 ml) was added. CsCl was then added (0.625 g of CsCl/g of solution) to raise the density to 1.41. The virus suspension was centrifuged in a Spinco Model L centrifuge in the 40 rotor at 30,000 rev/min for 24 hrs at 6 C. A visible band was noted and collected. The purified virus was dialyzed against saturated sodium borate and stored in saturated borate solution.

EXTRACTION OF BIOLOGICALLY ACTIVE ØX174 DNA:

Five ml of redistilled phenol was equilibrated with an equal volume of saturated borate solution at room temperature. ØX174 virus (in saturated borate) (1.5 ml) containing 0.01 ml of 30% bovine serum albumin was added to 1.5 ml of borate saturated phenol solution and vortex mixed approximately 30 seconds, after which the sample was centrifuged at room temperature in a swinging bucket rotor to separate phases. The aqueous phase was re-extracted with an equal volume of borate saturated phenol solution, centrifuged and the aqueous phase was placed on ice. The phenol phases were pooled and washed with $\frac{1}{2}$ volume of saturated borate, centrifuged, and the aqueous phase was added to the previous aqueous phases on ice. The pooled aqueous phases were washed 5X with equal volumes of fresh ice-cold ethyl ether. This ether washed aqueous phase was bubbled (at ice water temperature) with O_2 free nitrogen to remove the ether. The aqueous phase was dialyzed against three 500 ml changes of 0.1 M Tris, pH 8.1, in the cold. The DNA content of the final preparation was determined from ultraviolet absorption. A solution for which A_{260} is 1.0 contains 1.3×10^{13} molecules/ml or 36 ug/ml (Sinsheimer, 1968).

PREPARATION OF OSMOTIC SHOCK SPHEROPLASTS:

This procedure is basically that of Benzinger et al. (1968) with some modification. Forty ml of peptone growth media were innoculated in a shaker flask with a fresh overnight culture of the organism being tested. The flask was shaken at 37 C and the culture monitored until the concentration of organisms was approximately 5 X 10^8/ml. The organisms were sedimented at 12,100 X g for 10 min and the pellet was resuspended in 20 ml of sterile distilled water at 20 C. The organisms were again sedimented and the pellet resuspended in 3 ml of cold storage medium. The changes of media from growth media to water to storage media constitute osmotic shocks. The spheroplasts were stored at 37 C for approximately 4 hours, and then tested to determine their ability to take up ØX174 DNA. The spheroplasts were then stored at 5 C for 20 hrs and retested.

TESTING FOR COMPETENCE OF OSMOTIC SHOCK SPHEROPLASTS BY INFECTIOUS CENTER PLATING:

Five-hundreth ml of ØX174 DNA at various concentrations was added to 0.45 ml of DNA adsorption media prewarmed to 37 C. One-tenth ml of osmotic shock spheroplasts were added to each tube. After 15 min incubation at 37 C, 3.0 ml of soft agar and 0.1 ml of $15T^-$ indicator were added to each infection tube. This was poured over a bottom layer agar plate and incubated until plaques could be scored.

PREPARATION OF LYSOZYME-EDTA SPHEROPLASTS:

This procedure is basically that of Guthrie and Sinsheimer (1963) with some modification. Twenty ml of growth media in a shaker flask was innoculated with a fresh overnight culture of the organism to be tested in a 1:50 dilution.

The culture was shaken at 37 C and monitored until the organisms were at a concentration of approximately 5 X 10^8 cells/ml. The organisms were sedimented at 12,100 X g for 10 min after which the pellet was resuspended in 0.35 ml of 1.5 M sucrose. The following were added in order with gentle mixing after each addition: 0.1 ml 30% bovine serum albumin, 0.02 ml lysozyme in 0.25 M Tris, pH 8.1, (concentration varied with the experimentation), 0.04 ml 4% EDTA, and 10.0 ml PA media. This was incubated at room temperature for a specific time period depending on experimentation (7-28 min). Two-tenths ml of 10% $MgSO_4$ was added to complex the EDTA and stop the spheroplasting reaction. This is the spheroplast stock.

PREPARATION OF SHOCKED LYSOZYME-EDTA SPHEROPLASTS:

One ml of spheroplast stock was added to 40 ml of M9 dilution media, mixed gently and centrifuged for 5 min at 12,100 X g. The pellet was resuspended in 1.0 ml of PAM. This is the "shocked" spheroplast stock.

TESTING FOR COMPETENCE OF LYSOZYME-EDTA SPHEROPLASTS BY ASSAY OF MATURE PHAGE YIELD:

Two-tenths ml of the spheroplast preparation being tested was added to 0.2 ml of appropriately diluted ØX174 DNA prewarmed to 37 C. After 20 min of incubation at 37 C, 1.6 ml of PAM media was added to each tube. This is the "growth tube". The growth tube was incubated an additional 100 minutes at 37 C. The spheroplasts were ruptured by freezing and thawing in dry-ice-acetone or in a food-freezer, and the phage was titered.

VIABILITY PLATING:

Viability counts were taken at various times by diluting the treated organisms in M9 media and plating to nutrient agar plus 4 ug/ml thymine.

The plates were incubated at 37 C until the colonies could be scored. All plating was done in duplicate.

RESULTS AND DISCUSSION

Bacteria and bacteriophages possess enzymatic mechanisms for recognizing their own DNA and for destroying foreign DNA. These mechanisms are termed modification and restriction (Arber and Linn, 1969) and presumably serve as a protective mechanism. Since the overall research effort, of which this study is a part, is aimed at developing an Escherichia coli transformation system which will employ sex factor DNA isolated from another strain, Proteus mirabilis, it was considered necessary to use a restriction deficient strain of E. coli. Table I list the results of tests to show the restriction properties of 15T$^-$ and the restriction deficient mutant 65r$^-$m$^-$ in a conjugation system employing P. mirabilis PM-1 R1-19drd as a donor. The results indicate that 65r$^-$m$^-$ more readily incorporates the R factor. To avoid confusion of competence assays by reinfection of incomplete spheroplasts by maturing bacteriophage in the growth tube, it was considered necessary to select a strain for spheroplasting which was resistant to ØX174. Table I compares the receptivity of 65r$^-$m$^-$ and 65r$^-$m$^-$/ØX174 to R1-19drd and shows them to be essentially identical. Therefore 65r$^-$m$^-$/ØX174 was the organism chosen for this study.

Since newly acquired resistance to antibiotics is the most exacting method for determining the incorporation of an R factor, it was necessary to find a system of infection that would allow the organism to take up R factor DNA and still be able to grow and multiply to form drug-resistant colonies. Nossal and Heppel (1966) report that osmotic shock procedures cause release of enzymes in E. coli and an altered permeability to actinomycin D.

TABLE I: TESTING THE RESTRICTION DEFICIENT CHARACTER OF $65r^-m^-$ and $65r^-m^-/\phi X174$

The restriction deficient character of E. coli $65r^-m^-$ and $65r^-m^-/\phi X174$ was confirmed by comparing the ability of each to accept an R factor from Proteus mirabilis. P. mirabilis PM-1 lac^- nic^- R1-19drd was used as the donor strain transferring the R factor R1-19drd to E. coli $15T^-$, $65r^-m^-$, and $65r^-m^-/\phi X174$. R1-19drd confers on the cells resistance to ampicillin, kanamycin, streptomycin sulfate, chloramphenicol and sulfonamide. Fresh cultures of each strain were prepared. The E. coli strains were cultured in M9 + 100 ug/ml arginine + 50 ug/ml methionine and tryptophan + 4 ug/ml thymine + 1% casamino acids and PM-1 R1-19drd was cultured in M9 + 1% casamino acids + 50 ug/ml nicotinamide. After overnight incubation the PM-1 R1-19drd was diluted 2:11 in fresh media and incubated two hours, after which 0.5 ml of this PM-1 R1-19drd culture was added to 4.5 ml of $15T^-$, $65r^-m^-$, and $65r^-m^-/\phi X174$ overnight cultures. The mixtures were incubated at 37 C with frequent gentle mixing for 1 hr. After this incubation period the mixtures were diluted in M9 and plated to determine the concentration of donor cells (PM-1 R1-19drd-plated to M9 + 50 ug/ml nic + 50 ug/ml leu + 22-25 ug/ml each of amp, kana, strep, chlor, sul + 1.5% bacto agar), recipient cells (females-plated to M9 + amino acids + thymine), and converted recipient cells (females R^+-plated to M9 + amino acids + thymine + 22-25 ug/ml each of amp, kana, strep, chlor, sul).

	$15T^-$	$65r^-m^-$	$65r^-m^-/\phi X174$
Females (org/ml)	1.2×10^9	8.7×10^8	9.7×10^8
PM-1 R1-19drd (org/ml)	1.6×10^7	1.7×10^7	1.8×10^7
Females $R^\pm$ (org/ml)	3.5×10^3	1.0×10^6	9.5×10^5
Females R^+/PM-1 R1-19drd	2.2×10^{-4}	5.9×10^{-2}	5.3×10^{-2}
Females R^+/Females	2.9×10^{-6}	1.2×10^{-3}	1.0×10^{-3}

[In a comparable mating experiment using $15T^-$ leu^- R1-19drd (1.2×10^7 org/ml) as male donor cells and $15T^-$ (6.0×10^8 org/ml) as female recipient cells, 1.0×10^6 org/ml female R^+ cells were obtained giving a Female $R^+/15T^-$ leu^- R1 -19drd ratio of 8.3×10^{-2}]

Yet cells treated in this fashion do not need to be osmotically protected and therefore can multiply to form colonies on plating media. Benzinger et al. (1968) used osmotic shock to infect E. coli C with ØX174. We, therefore, investigated the potential of the Benzinger technique in developing a transformation system. Table II lists the results of assays of competence for ØX174 DNA using osmotic shock procedures on $65r^-m^-$ and $65r^-m^-$/ØX174. The infectivity using $65r^-m^-$ was too poor to be useful, and no infection at all could be obtained with ØX174 DNA using $65r^-m^-$/ØX174.

Since the osmotic shock procedure could not provide adequate levels of infectivity, another system had to be found. It was apparent that a more drastic treatment to the cell wall was necessary to render the cells capable of taking up the viral DNA. The next step was to test Sinsheimer's lysozyme-EDTA spheroplasting procedures. Figure 1 illustrates an assay testing a proven system: E. coli K12W6 spheroplasts and ØX174 DNA. The cells were treated for 15 min with 2 mg/ml lysozyme. The curve shows a high degree of competence and further shows that there is a definite proportional relationship between the concentration of ØX174 DNA molecules added to the growth tube and the concentration of mature phage particles produced. Saturation does occur as is evidenced by the deviation from proportionality at a DNA concentration of greater than 4.4×10^9 ØX174 molecules/ml. Sinsheimer (1968) states that at concentrations less than 5×10^4 ØX174 molecules/ml reproducibility of results is poor. The ratio of mature phage output to DNA input in this assay is 1.3×10^{-2}.

After it was evident that good levels of infectivity could be attained in this laboratory using E. coli K12W6 spheroplasts, it was necessary to optimize a system for $65r^-m^-$/ØX174 spheroplasts by determining the optimal concentration of lysozyme and the optimal length of time for treatment of

TABLE II: COMPETENCE OF OSMOTIC SHOCK SPHEROPLASTS

Fresh osmotic shock spheroplasts of $65r^-m^-$ and $65r^-m^-$/ØX174 were prepared and stored at 37 C for 4 hrs followed by 20 hrs storage at 4 C. After each of these storage periods the ability of these cells to take up ØX174 DNA was tested as follows: ØX174 DNA at the concentrations indicated in the table was added to 0.45 ml of DNA adsorption media prewarmed to 37 C. After which 0.1 ml of the osmotic shock spheroplasts being tested were added. After 15 min at 37 C, 3.0 ml of soft agar and 0.1 ml of $15T^-$ indicator was added. This was poured over a bottom layer agar plate and incubated at 37 C until plaques could be scored. This system scores for infectious centers and does not reflect the amplification of numbers shown in later figures (eg. Figs. 1 and 2).

ØX174 DNA molecules/ml	4.4×10^{11}	4.4×10^{10}	4.4×10^{9}	4.4×10^{8}
	$65r^-m^-$			
Infectious centers 4 hrs at 37 C	97	18	2	0
Infectious centers 20 hrs at 4 C	29	3	0	0
	$65r^-m^-$/ØX174			
Infectious centers 4 hrs at 37 C	0	0	0	0
Infectious centers 20 hrs at 4 C	0	0	0	0

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Figure 1. Yield of mature ØX174 phage from K12W6 spheroplasts as a function of the concentration of ØX174 DNA added. An overnight culture of K12W6 was diluted 1:50 in 20 ml of fresh M9 growth medium. The cell concentration was allowed to reach 5 X 10^8 cells/ml. Spheroplasts were prepared by resuspending the centrifuged cells in 0.35 ml of 1.5 M sucrose and then adding in order: 0.1 ml bovine serum albumin, 0.02 ml 2 mg/ml lysozyme, 0.04 ml 4% EDTA, and 10 ml PA media. After 15 min at room temperature, 0.2 ml 10% $MgSO_4$ were added. Two-tenths ml of spheroplasts were added to 0.2 ml aliquots of ØX174 DNA at concentrations ranging from 4.4 X 10^{11} to 4.4 X 10^4 molecules/ml prewarmed to 37 C. After 20 min incubation at 37 C, 1.6 ml of PAM were added to each infection tube. The tubes were incubated an additional 100 min, frozen, thawed, and plated to determine the number of bacteriophage produced. E. coli C was used as indicator.

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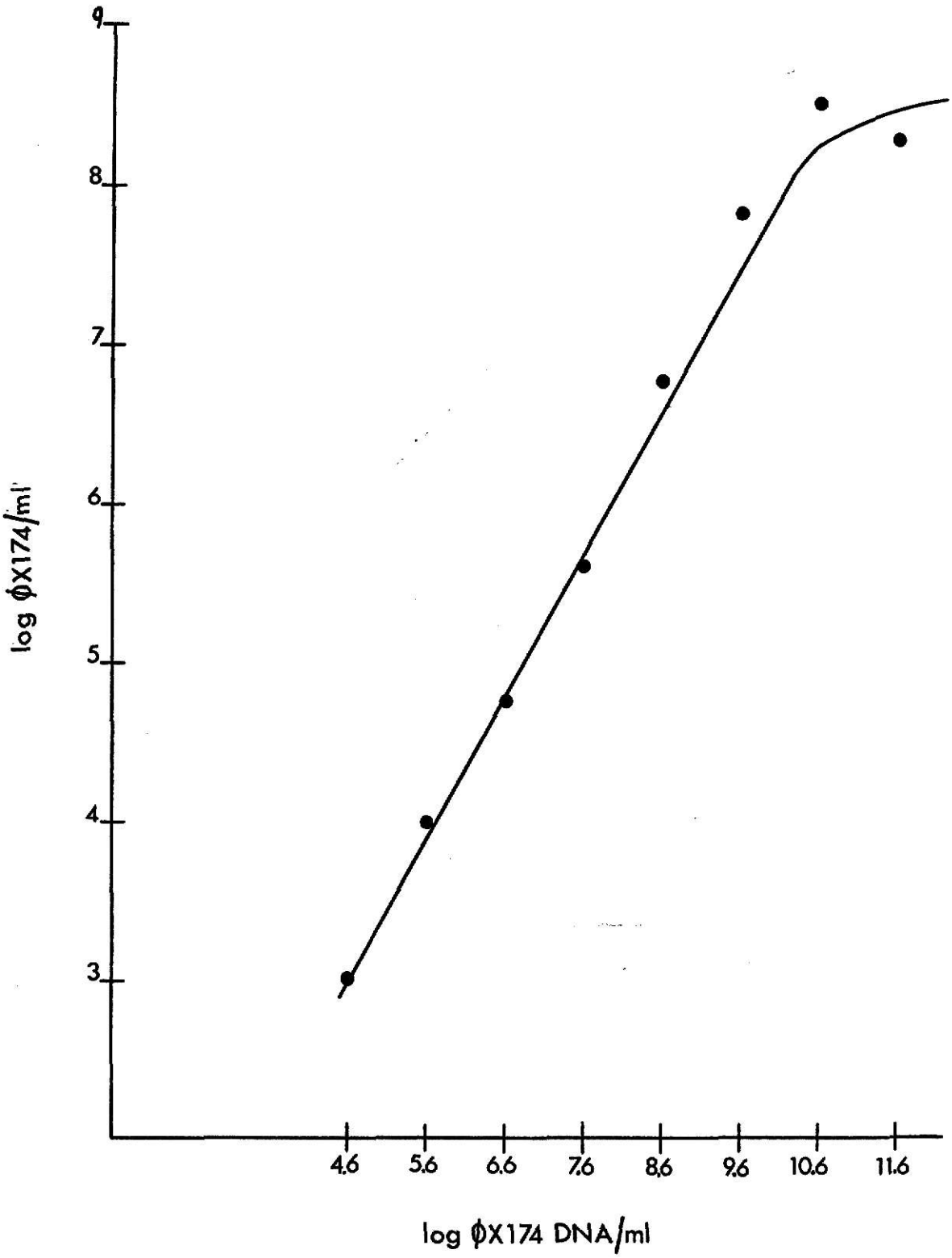
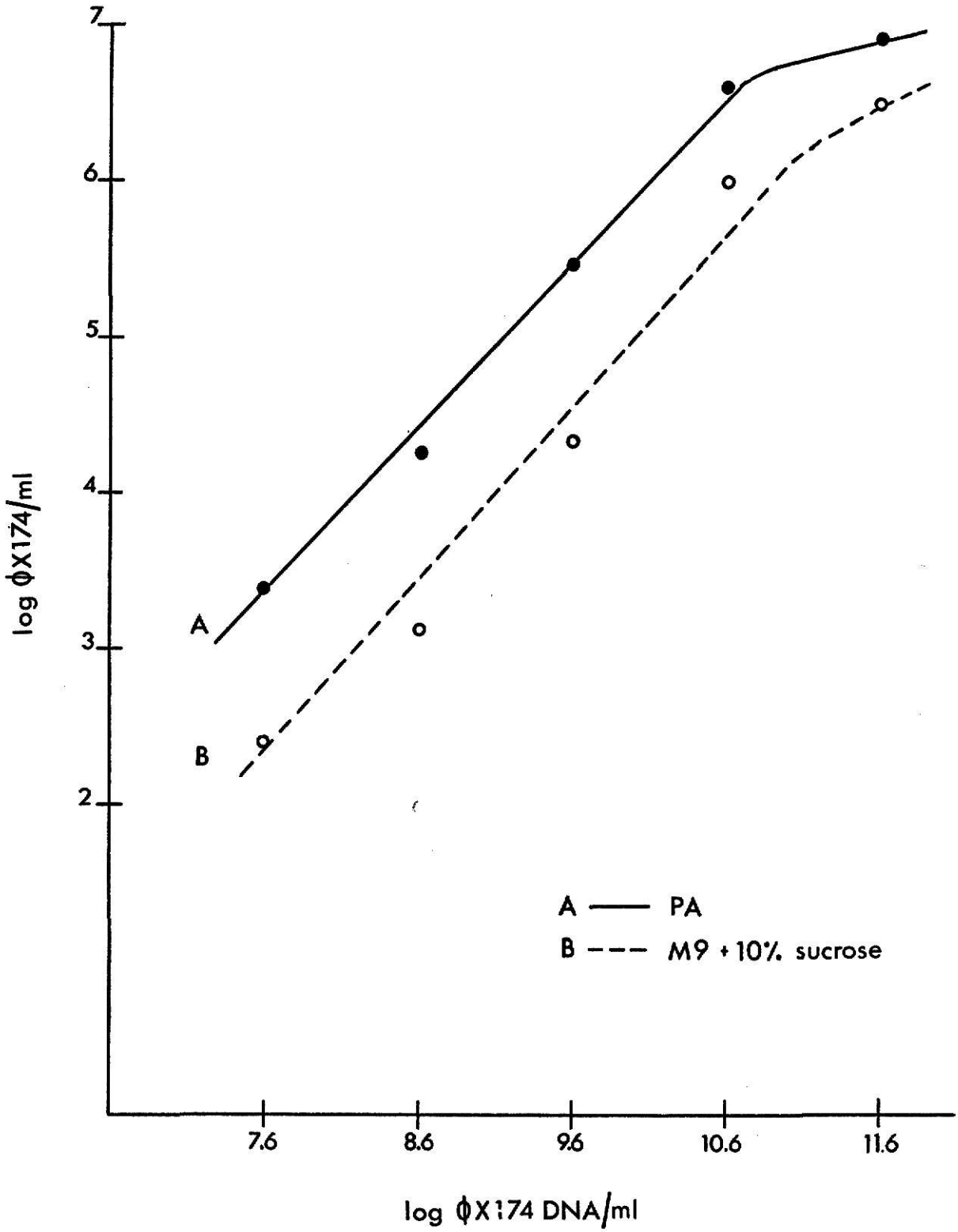

9
8
7
6
5
4
3
log ϕX174/ml
4.6
5.6
6.6
7.6
8.6
9.6
10.6
11.6
log ϕX174 DNA/ml

these cells. Before carrying out experimentation to determine these optimums, the range of ØX174 DNA concentration at which assays would be most reliable using $65r^-m^-$/ØX174 spheroplasts was determined. This step was necessitated by the fact that a sensitive and valid assay for permeability to ØX174 DNA depends upon addition of a less than saturating amount of DNA. Curve A in Figure 2 illustrates an assay using spheroplasts of $65r^-m^-$/ØX174 prepared in PA medium using 20 mg/ml lysozyme treatment for fifteen minutes. There is a proportional relationship between DNA input and mature phage production and also the same saturation phenomenon occurs at a concentration of DNA greater than 4.4×10^9 molecules/ml (compare to Figure 1). Therefore, for further experimentation, a DNA concentration of 4.4×10^9 molecules/ml was considered optimal. In this assay the ratio of phage output to DNA input was 7×10^{-5}. The same assay was carried out in M9 medium plus 10% sucrose as is illustrated in Curve B in Figure 2. Even though in this case there was a decrease in the level of infectivity (the ratio of phage output to DNA input is 5×10^{-6}), the results show it is possible to use a completely defined media in experimentation of this sort.

After determining a concentration of ØX174 that would allow reliable assays (4.4×10^9 molecules/ml), experiments were conducted to determine the optimum concentration of lysozyme for spheroplast preparation. Figure 3 illustrates such an assay in which various concentrations of lysozyme were used to treat $65r^-m^-$/ØX174 for 15 minutes. This study demonstrates that a greater yield of mature phage can be obtained when spheroplasts are treated with increasingly higher concentration of lysozyme. Adequate infectivity was obtained when 2 mg/ml of lysozyme was used, but a slightly higher level was obtained with 20 mg/ml. Therefore 20 mg/ml of lysozyme produced spheroplasts with the greatest levels of competence.

Figure 2. Yield of mature phage from $65r^-m^-$/ØX174 spheroplasts as a function of the concentration of ØX174 DNA added. An overnight culture of $65r^-m^-$/ØX174 was diluted 1:50 in fresh M9 growth medium. The cell concentration was allowed to reach 5 X 10^8 cells/ml. Spheroplasts were prepared by resuspending the centrifuged cells in 0.35 ml of 1.5 M sucrose and then adding in order: 0.1 ml 30% bovine serum albumin, 0.02 ml 20 mg/ml lysozyme, 0.04 ml 4% EDTA, and 10 ml PA media. After 15 min at room temperature, 0.2 ml of 10% $MgSO_4$ was added. Two-tenths ml of spheroplasts were added to 0.2 ml aliquots of ØX174 DNA at concentrations ranging from 4.4 X 10^{11} to 4.4 X 10^7 molecules/ml prewarmed to 37 C. After 20 min incubation at 37 C, 1.6 ml of PAM were added to each infection tube. The tubes were incubated an additional 100 min, frozen, thawed, and plated to determine the number of bacteriophage produced. The same procedure was followed to determine the infectivity when using defined media. The PA was replaced with M9 + 100 ug/ml arginine + 50 ug/ml methionine and tryptophan + 4 ug/ml thymine + 10% sucrose. E. coli $15T^-$ was used as indicator.


7
6
5
4
3
2
log ϕX174/ml
A
B
A —— PA
B --- M9 + 10% sucrose
7.6
8.6
9.6
10.6
11.6
log ϕX174 DNA/ml

It was then necessary to find the optimum length of time for the treatment. Figure 4 outlines the effect of the variation in time of lysozyme treatment using 20 mg/ml lysozyme. This assay indicates that maximum infectivity can be attained when cells are treated for 14 minutes. Therefore, it was determined that for maximum competence for ØX174 DNA using $65r^{-}m^{-}$/ØX174, the cells should be treated with 20 mg/ml of lysozyme for approximately 14 minutes.

However, maximum infectivity was not the only consideration. In future work with R factors, those cells that have incorporated the R factor must be able to grow and multiply on antibiotic plating media in order to be enumerated. Complete spheroplasts are those spheroplasts that must be osmotically protected and cannot grow into colonies due to the large amount of cell wall destroyed by the lysozyme (Salton, 1964). Treatment with 20 mg/ml of lysozyme for 14 minutes resulted in the greatest level of infectivity presumably because at that concentration and time the greatest number of cells were made into complete spheroplasts, but the spheroplasts were not yet traumatized to such an extent that their levels of competence was lowered as in those cells which had been treated for 21 and 28 minutes. With a lower lysozyme concentration and less treatment time partial spheroplasts, that is cells that are still competent but do not need to be osmotically protected should be obtained.

The assay illustrated in Figure 3 shows that treatment with 2 mg/ml of lysozyme gave adequate levels of infectivity. Figure 5 shows that a treatment time of 14 minutes gives optimum infectivity, but that treatment for 7 minutes causes only a slight decrease in infectivity. It was assumed then that $65r^{-}m^{-}$/ØX174 can be treated with 2 mg/ml of lysozyme for 7 minutes and still show adequate levels of infectivity.

Figure 3. Yield of mature phage as a function of concentration of lysozyme used to prepare spheroplasts. An overnight culture of $65r^{-}m^{-}$/ØX174 was diluted 1:50 in 40 ml of fresh M9 growth medium. The cell concentration was allowed to reach 5 X 10^{8} cells/ml. Two 20 ml samples were centrifuged and each pellet was resuspended in 0.35 ml of 1.5 M sucrose. To each, 0.1 ml 30% bovine serum albumin was added. The samples were halved (4 preparations) and to each was added 0.01 ml of lysozyme at various concentrations (20 mg/ml, 2 mg/ml, 0.2 mg/ml, 0.02 mg/ml), 0.02 ml 4% EDTA, and 5 ml PA. After incubation at room temperature for 15 min, 0.1 ml of 10% $MgSO_4$ was added. Then 0.2 ml of each spheroplast preparation was added to 0.2 ml of ØX174 DNA at a non-saturating concentration of 4.4 X 10^{9} molecules/ml. After 20 min incubation at 37 C, 1.6 ml of PAM was added to each infection tube. The tubes were incubated an additional 100 min, frozen, thawed, and plated to determine the number of bacteriophage produced.

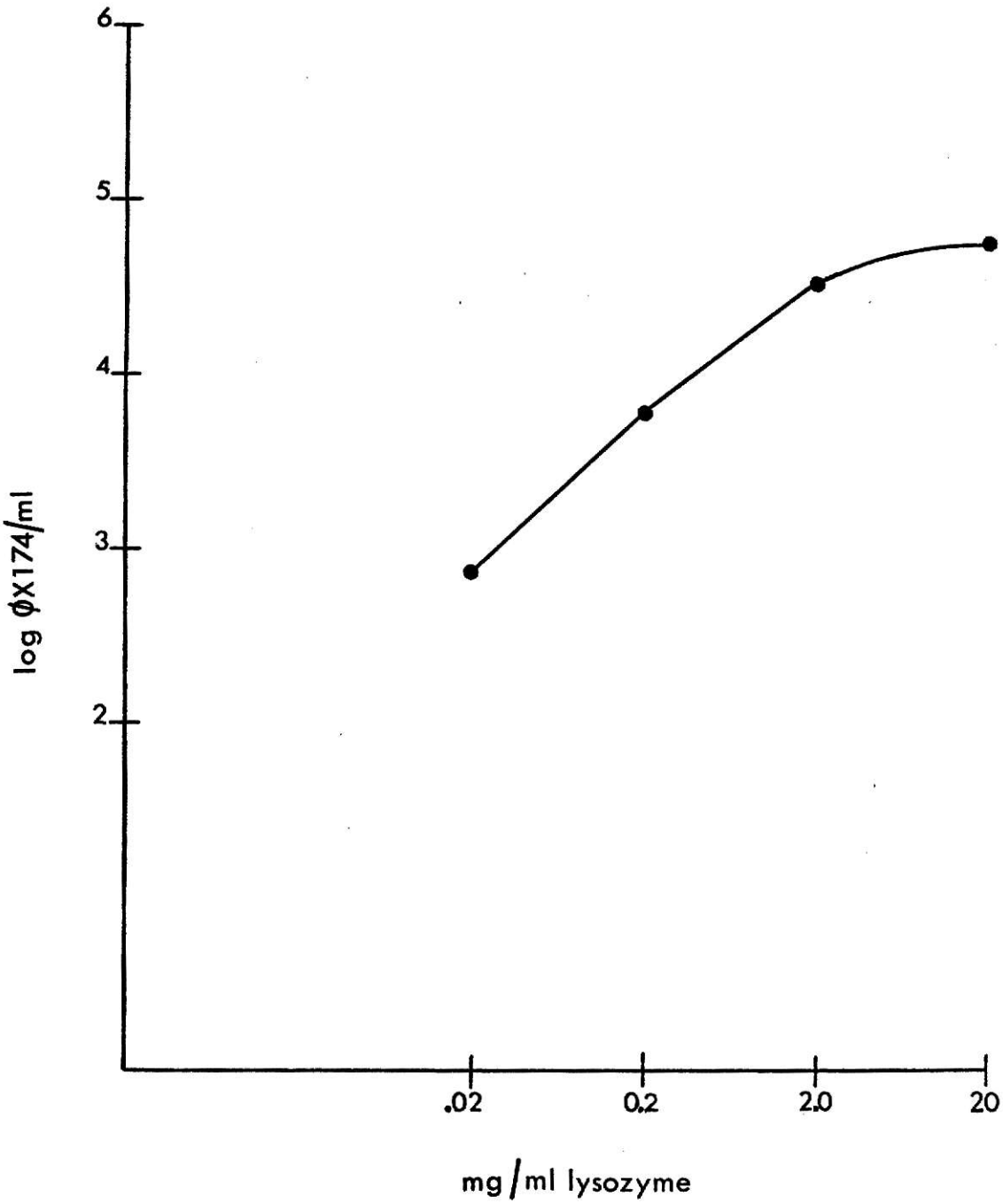
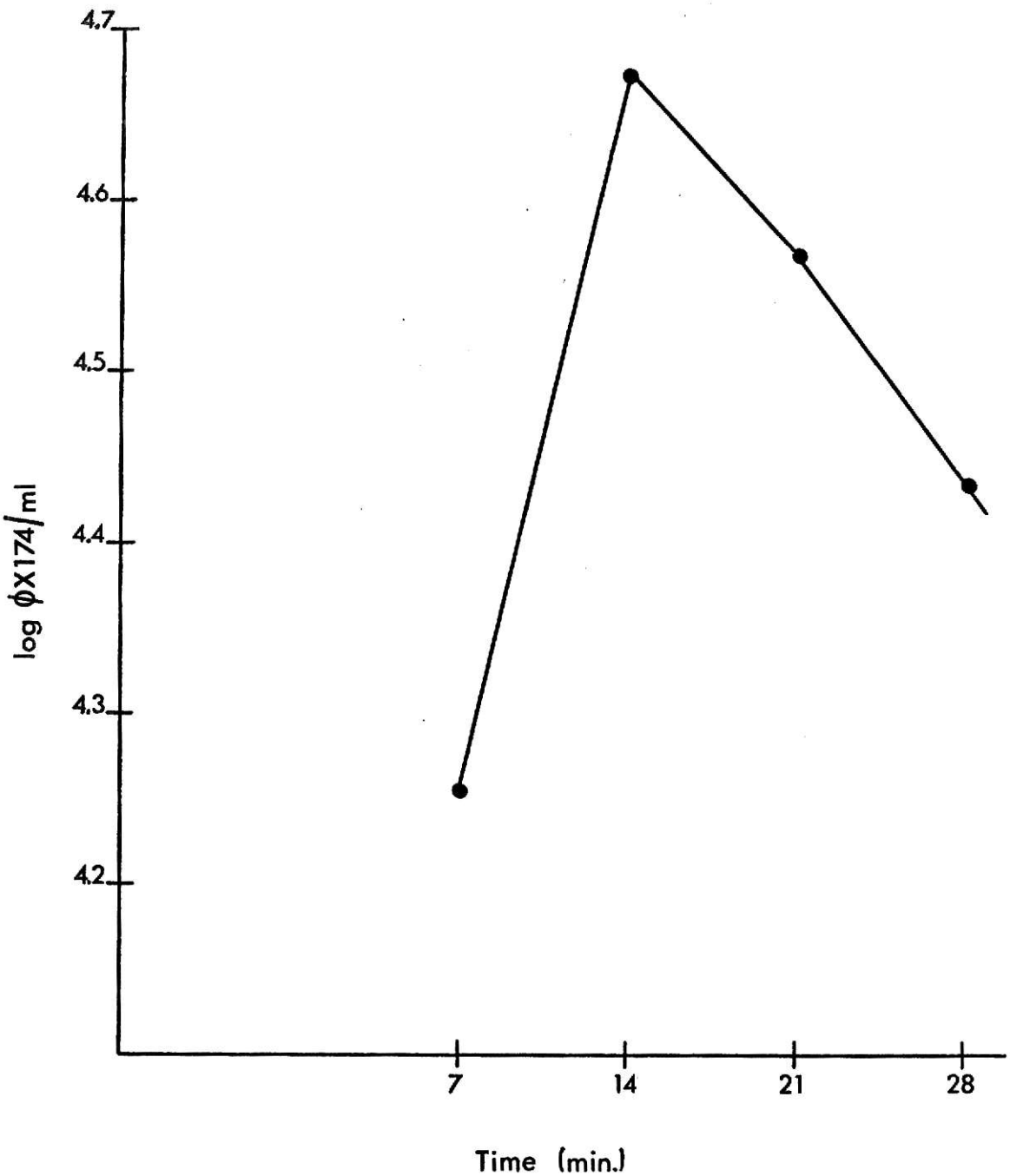

6
5
4
3
2
log ϕX174/ml
.02
0.2
2.0
20
mg/ml lysozyme

Figure 4. Yield of mature phage as a function of time of lysozyme treatment of spheroplasts prepared using 20 mg/ml lysozyme. An overnight culture of $65r^{-}m^{-}$/ØX174 was diluted 1:50 in fresh M9 growth medium. The cell concentration was allowed to reach 5×10^{8} cells/ml. Spheroplasts were prepared by resuspending the centrifuged cells in 0.35 ml of 1.5 M sucrose and then adding in order: 0.1 ml 30% bovine serum albumin, 0.02 ml 20 mg/ml lysozyme, 0.04 ml 4% EDTA, and 10 ml PA media. After 7, 14, 21, and 28 min at room temperature, 2.5 ml was removed from the spheroplast preparation tube and 0.05 ml 10% $MgSO_4$ was added. Two-tenths ml of spheroplasts were added to 0.2 ml of ØX174 DNA at a concentration of 4.4×10^{9} molecules/ml prewarmed to 37 C. After 20 min at 37 C, 1.6 ml of PAM were added to each infection tube. The tubes were incubated an additional 100 min, frozen, thawed, and plated to determine the number of bacteriophage produced.


4.7
4.6
4.5
4.4
4.3
4.2
log ϕX174/ml
7
14
21
28
Time (min.)

As well as adequate levels of infectivity, better viability of cells can be attained using 2 mg/ml and 7 minutes as is shown in Table III. When using 2 mg/ml of lysozyme as compared to 20 mg/ml of lysozyme, there is a 10 fold increase in the number of cells capable of producing colonies on solid media.

In order to rid the spheroplast preparations of those cells that are not capable of multiplying to form colonies, the preparation was diluted 1:40 in M9 media. This constituted an osmotic shock. Those cells that could survive this shock treatment were assumed to be those capable of producing colonies on solid media.

The results of an infectivity assay using shocked spheroplasts and not shocked spheroplasts treated with 2 mg/ml of lysozyme for 7 minutes are shown in Figure 6. The assay indicates that the same proportionality and saturation phenomenon occur with the shocked spheroplasts, and that the level of infectivity is lowered only slightly. The ratio of phage output to DNA input for the spheroplast stock is 7×10^{-5}. The ratio for the shocked spheroplast preparation was 3×10^{-5}.

It must be noted here that individual spheroplast preparations varied greatly in their competence. All precautions were taken to prepare spheroplasts in exactly the same manner using the same reagents, times and temperatures, and still infectivity levels varied greatly from day to day. Due to this variation, comparison of assays (levels of infectivity) carried out on different spheroplast preparations was not possible.

Experimentation, therefore, has established that DNA permeable, colony forming cells of the modification and restriction deficient strain $15T^-$, $65r^-m^-$ can be prepared. These DNA permeable cells should be suitable for use in developing an R factor transformation system.

Figure 5. Yield of mature phage as a function of time of lysozyme treatment of spheroplasts prepared using 2 mg/ml lysozyme. An overnight culture of $65r^{-}m^{-}$/ØX174 was diluted 1:50 in fresh M9 growth medium. The cell concentration was allowed to reach 5 X 10^8 cells/ml. Spheroplasts were prepared by resuspending the centrifuged cells in 0.35 ml of 1.5 M sucrose and then adding in order: 0.1 ml 30% bovine serum albumin, 0.02 ml 2 mg/ml lysozyme, 0.04 ml 4% EDTA, and 10 ml of PA media. After 7, 14, 21, and 28 min at room temperature, 2.5 ml was removed from the spheroplast preparation tube and 0.05 ml of 10% $MgSO_4$ was added. Two-tenths ml of spheroplasts were added to 0.2 ml of ØX174 DNA at a concentration of 4.4 X 10^9 molecules/ml prewarmed to 37 C. After 20 min at 37 C, 1.6 ml of PAM was added to each infection tube. The tubes were incubated an additional 100 min, frozen, thawed, and plated to determine the number of bacteriophage produced.

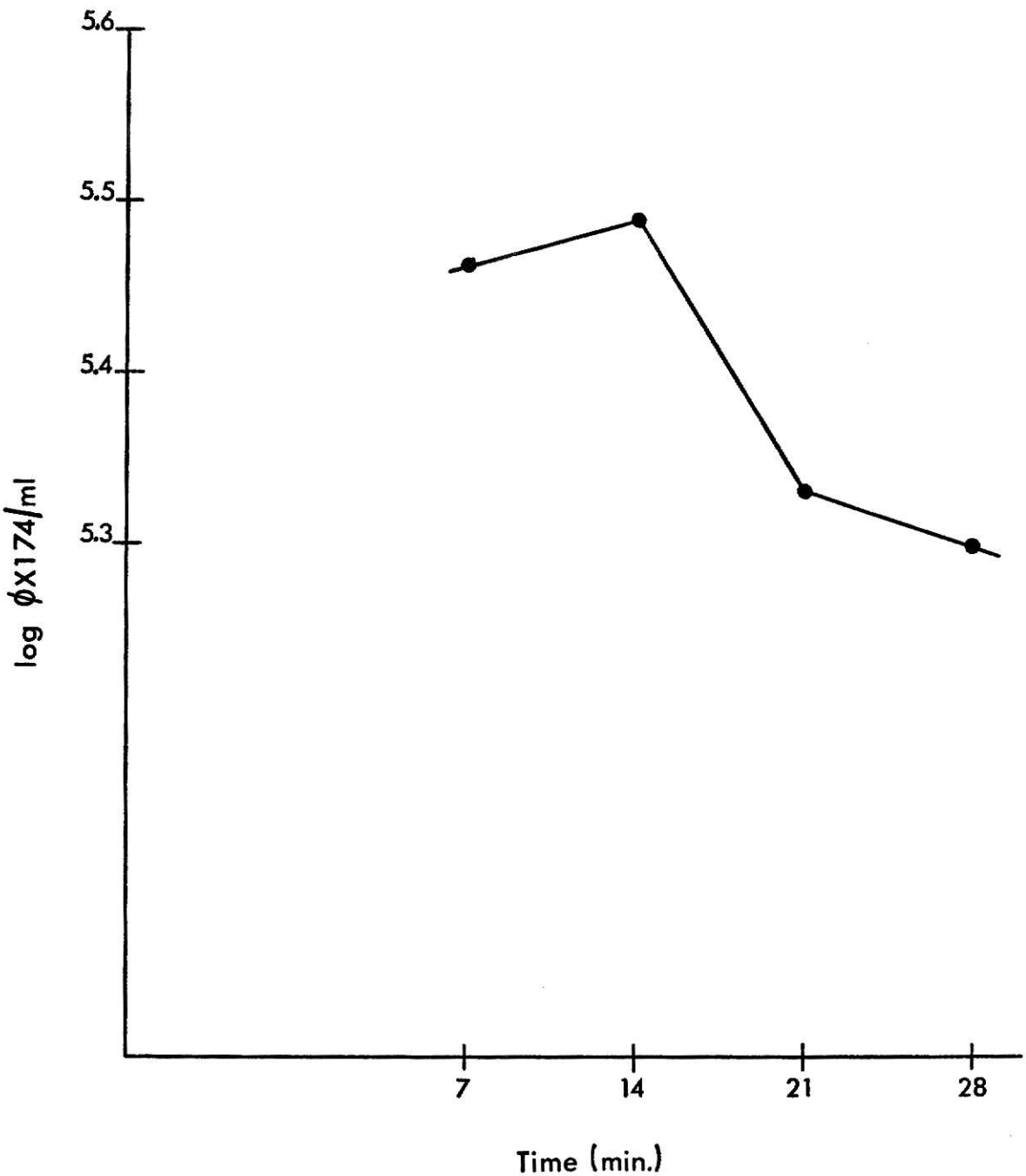
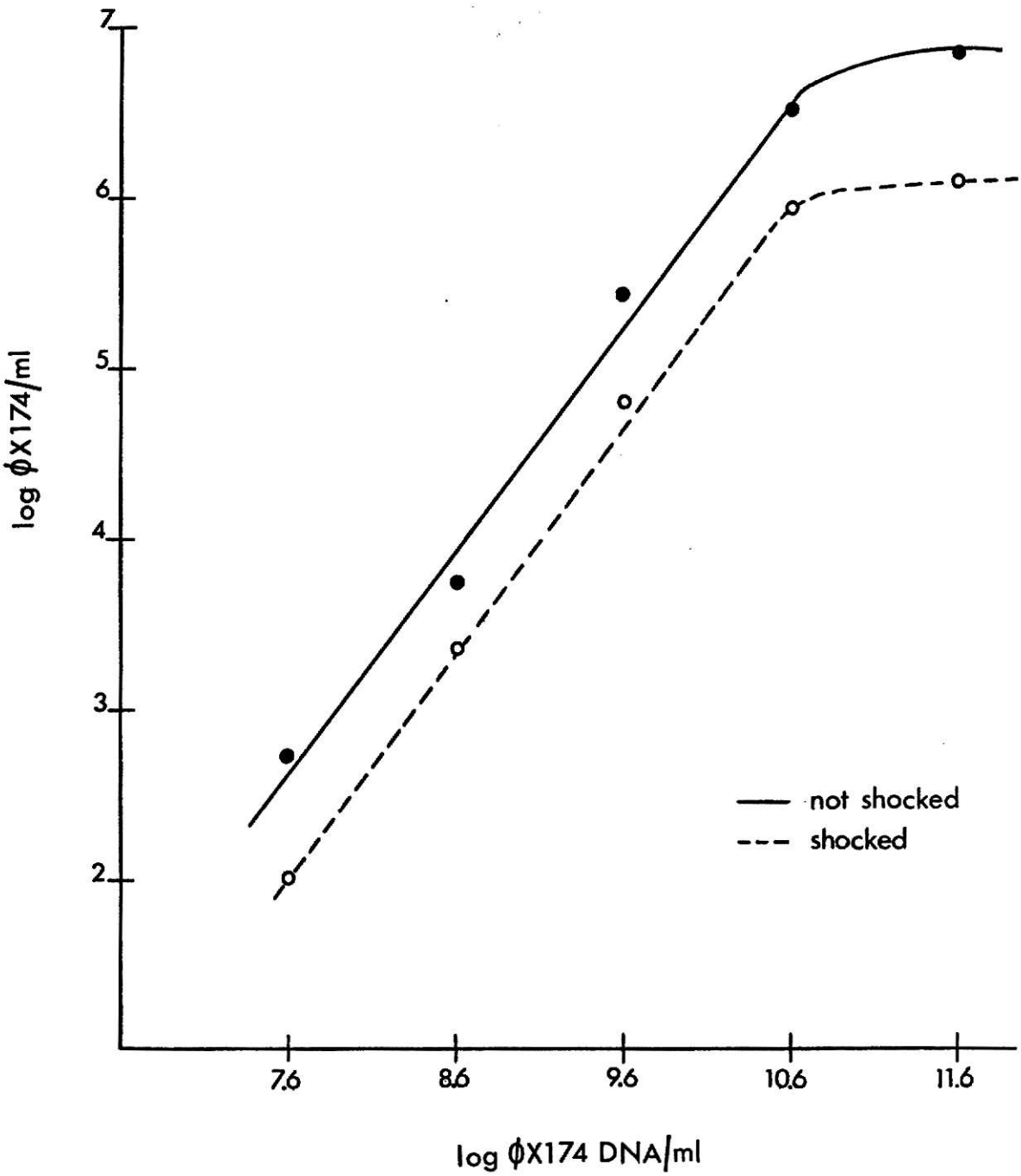

5.6
5.5
5.4
5.3
log ϕX174/ml
7
14
21
28
Time (min.)

TABLE III: VIABILITY TESTING ON SPHEROPLAST PREPARATIONS BEFORE AND AFTER SHOCK

Viability platings were carried out during spheroplast preparation at the times indicated in the table. Cell preparations treated with 20 mg/ml of lysozyme for 7 min and 14 min and cell preparation treated with 2 mg/ml of lysozyme for 7 min and 14 min were tested. Viability platings were also done after each of these preparations was osmotically shocked (diluted 1:40 in M9 medium). For viability plating, the cells were diluted in M9 media, after which 0.1 ml of the appropriate dilutions were mixed in 2 ml of soft nutrient agar, and layered onto a nutrient agar + 4 ug/ml thymine base plate. Plates were incubated at 37 C approximately 24 hrs. Plating was done in duplicate.

	20 mg/ml		2 mg/ml	
	7 min	14 min	7 min	14 min
Initial Culture (org/ml)	5.2×10^8	5.2×10^8	5.2×10^8	5.2×10^8
Spheroplast stock (org/ml)	5.6×10^6	7.2×10^6	5.6×10^7	4.9×10^7
Shocked spheroplasts (org/ml)	5.9×10^6	5.0×10^6	3.0×10^7	4.1×10^7

Figure 6. Yield of mature phage as a function of the concentration of ØX174 DNA added. An overnight culture of $65r^{-}m^{-}$/ØX174 was diluted 1:50 in fresh M9 growth media. The cell concentration was allowed to reach 5 X 10^{8} cells/ml. Spheroplasts were prepared by resuspending the centrifuged cells in 0.35 ml of 1.5 M sucrose and then adding in order: 0.1 ml 30% bovine serum albumin, 0.02 ml 2 mg/ml lysozyme, 0.04 ml 4% EDTA, and 10 ml PA media. After 7 min at room temperature, 0.2 ml 10% $MgSO_4$ was added. One ml of spheroplasts stock was diluted 1:40 in M9 medium, centrifuged, and resuspended in 1.0 ml of PAM. These spheroplasts were considered shocked spheroplasts. Two-tenths ml of shocked and not shocked spheroplasts were added to 0.2 ml of ØX174 DNA at concentrations ranging from 4.4 X 10^{11} to 4.4 X 10^{7} molecules/ml prewarmed to 37 C. After 20 min incubation at 37 C, 1.6 ml of PAM was added to each infection tube. The tubes were incubated an additional 100 min, frozen, thawed, and plated to determine the number of bacteriophages produced.


7
6
5
4
3
2
log ϕX174/ml
7.6
8.6
9.6
10.6
11.6
log ϕX174 DNA/ml
not shocked
shocked

SUMMARY

Studies were carried out to optimize a system of preparing revertable spheroplasts of a restriction deficient strain of Escherichia coli 15T$^-$, 65r$^-$m$^-$ which are permeable to naked ØX174 DNA. Experimentation involved the testing of procedures known to cause permeability changes in E. coli.

Osmotic shock procedures were tested and yielded inadequate levels of infectivity for the proposed research, and these methods were abandoned.

Lysozyme-EDTA induced changes in permeability yielded adequate levels of infectivity and lysozyme-EDTA preparation of spheroplasts was studied to determine optimum concentrations of lysozyme and times of lysozyme treatment to produce spheroplasts of maximum infectivity and recoverability. Treatment of 65r$^-$m$^-$/ØX174 with 20 mg/ml of lysozyme for 14 min yields spheroplasts of maximum competence and poor recovery for colony formation. These spheroplasts could be used in transformation research when incorporation and integration of the DNA could be tested for by chemical or biological assay rather than by growth on solid media. Treatment of 65r$^-$m$^-$/ØX174 with 2 mg/ml lysozyme for 7 min with subsequent osmotic shock produces partial spheroplasts with good levels of infectivity and revertability.

ACKNOWLEDGMENTS

I wish to express my appreciation to Dr. James Urban for his help and encouragement during the course of this study.

I also want to acknowledge the support of the other members of my graduate committee, Dr. Charles Hedgcoth and Dr. Richard Consigli.

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OPTIMIZING A TRANSFORMATION SYSTEM USING SPHEROPLASTS OF *ESCHERICHIA COLI* 15T⁻

by

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B.S., University of Wisconsin--Eau Claire, 1967

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Microbiology

Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1972

This study was one contribution to an overall project aimed at developing an Escherichia coli transformation system which will employ purified sex factor DNA. Various procedures for preparing revertable spheroplasts of a DNA restriction deficient strain of E. coli 15T⁻ which was maximally competent for infection with purified DNA were tested. The assay for competence was based on infection by ØX174 DNA.

Osmotic shock procedures known to cause permeability changes in E. coli were tested. The levels of infectivity with ØX174 DNA were inadequate for the proposed research, and these methods were abandoned.

Lysozyme-EDTA induced changes in permeability yielded adequate levels of infectivity with ØX174 DNA. Studies were carried out to determine the optimum concentration of lysozyme and time of lysozyme treatment to produce spheroplasts of maximum infectivity and recoverability. Treatment with 20 mg/ml of lysozyme for 14 min yielded spheroplasts of maximum competence but poor recovery for colony formation. These spheroplasts could be used in transformation research when incorporation and integration of the DNA could be tested for by chemical or biological assay rather than by growth on solid media. Treatment with 2 mg/ml lysozyme for 7 min with subsequent osmotic shock produced partial spheroplasts with good levels of infectivity and recoverability.