Joan Mae Nippert. PHAGE-ASSOCIATED LYSOZYME OF <u>STAPHYLOCOCCUS</u> <u>AUREUS</u>:
ITS CHARACTERIZATION AND USE IN GENETIC TRANSFORMATION (Under the direction
of Dr. Wendall E. Allen) Department of Biology, October, 1974.

Attempts were made to use phage-associated lysozyme (PAL) obtained
from cells of <u>Staphylococcus</u> <u>aureus</u> strain PS53 (International Phage-
Typing Series) lysed by staphylococcal phage 53 to obtain biologically
active DNA to be used in the transformation of tetracycline resistance
and DNase activity. Characterization of PAL included determination of:
the effect of reducing agents on activity, the optimal conditions of
protein precipitation, the effect of age and storage conditions on
activity, and its lytic activity on various strains of <u>S</u>. <u>aureus</u> alone
and in combination with other physical and chemical treatments. Using
1-methyl-3-nitro-1-nitrosoguanidine, mutants having either increased or
decreased nuclease activity were isolated. A mutant resistant to tetra-
cycline and having increased nuclease activity was selected as a donor
strain and a mutant sensitive to tetracycline and having decreased
nuclease activity was selected as a recipient strain. The transformation
of neither of these characteristics was observed in any of the experiments
attempted.

PHAGE-ASSOCIATED LYSOZYME OF

STAPHYLOCOCCUS AUREUS: ITS CHARACTERIZATION

AND USE IN GENETIC TRANSFORMATION

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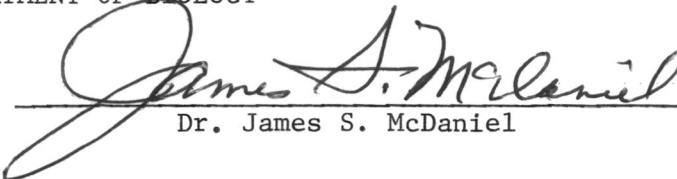
by

Joan Mae Nippert

APPROVED BY:

SUPERVISOR OF THESIS *Wendall E. Allen*
Dr. Wendall E. Allen

CHAIRMAN OF THE DEPARTMENT OF BIOLOGY

James S. McDaniel
Dr. James S. McDaniel

DEAN OF THE GRADUATE SCHOOL

Joseph G. Boyette
Dr. Joseph G. Boyette

520259

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Dedicated

to

My Father, Robert

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INTRODUCTION

Transformation, the transfer of genes in the form of isolated nucleic acid from a donor to a recipient cell, has been extensively studied for a number of bacterial genera such as <u>Streptococcus</u>, <u>Diplococcus</u>, <u>Bacillus</u> and <u>Escherichia</u>. Only recently was transformation reported for <u>Staphylococcus</u> (Nomura, et al 1971). Two problems exist that delayed transformation among the staphylococci: (1) many strains produce extracellular nuclease destroying the transforming ability of the DNA and (2) cells of staphylococci are contained by a thick cell wall that must be ruptured to recover the donor DNA. Treatments affording cell lysis usually were so severe as to destroy transformational activity of the DNA recovered. Sonstein, Hammel and Bondi (1971) reported the isolation of an enzyme associated with phage infection, phage-associated lysozyme (PAL), active in lysing metabolizing, unaltered staphylococcal cells. The purpose of this study is to isolate and characterize PAL and use it to obtain donor DNA for use in transformation. Mutants of staphylococci deficient for extracellular nuclease will be isolated and employed as recipients. The combination of PAL treatment for isolation of donor DNA and use of DNase-deficient mutants as recipients may permit extensive transformation of staphylococci.

LITERATURE REVIEW

There are various methods available for use in genetic analysis of
bacteria. All involve the active or passive transfer of only parts of the
total bacterial genome from one cell (donor) to another (recipient). The
process of sexual conjugation (mating characterized by the temporary fusion
of mating partners) cannot be used for genomic analysis of Staphylococcus
aureus since staphylococcal cells are asexual. Transduction, the transfer
of genetic material from one bacterium to another mediated by bacteriophage,
can be performed for S. aureus as was shown by Morse (1959). Transfection,
the infection of competent cells with donor DNA extracted from viruses pre-
viously grown in donor cells, has been performed with S. aureus (Sjöström
et al, 1972).

The chemical nature of the cell wall of the gram-positive bacterium
S. aureus has been characterized. The staphylococcal cell wall is a thick
tough covering (Mandelstam and McQuillan, 1968), consisting of mucopeptides,
polysaccharides, and teichoic acids. In S. aureus, a large part of the
cell wall is the mucopeptide backbone of alternating N-acetylglucosamine
and N-acetylmuramic acid residues (Davis et al, 1967). According to
Mandelstam and McQuillan (1968), every muramic acid residue is substituted
and the interpeptide chain bridges are exclusively made by glycine units
from the terminal amino group of lysine on one mucopeptide chain to the
carboxyl group of the terminal D-alanine on an adjacent mucopeptide chain.
The teichoic acids are linked alpha and beta to the N-acetylmuramic acid
sugars (Mandelstam and McQuillan, 1968).

The thickness, rigidity and composition of the staphylococcal cell

wall presents a formidable barrier to breach in order to obtain materials inside the cells. Physical methods required to rupture bacterial cells are so destructive that DNA obtained after their use has lost all genetic function (Hayes, 1968). Davie and Brock (1966) reported the ability of teichoic acid to confer resistance to activity by lytic enzymes on bacterial cell walls. Lysostaphin, a protein with a molecular weight of 30,000 produced by an unusual staphylococcal strain (Schlinder and Schuhardt, 1964, 1965), is highly active against S. aureus, resulting in the hydrolysis of the N-acetylglucosamyl linkages (Browder et al, 1965) and rapid lysis of staphylococci regardless of their metabolic state (Schlinder and Schuhardt, 1964). Riggs and Rosenblum (1969) used lysostaphin-induced spheroplasts as recipients for phage DNA and showed successful transfection. They later (Riggs and Rosenblum, 1969) reported transfection of two strains of S. aureus with DNA in a hypertonic medium only when the cell walls of the recipients had been partially disrupted with lysostaphin. The discovery and characterization of the staphylolytic enzyme, lysostaphin, by Schlinder and Schuhardt (1965) therefore provided a mild method for releasing the nucleic acids from staphylococcal cells.

Some lysins are only effective after the cells have been subjected to sensitizing treatment. Virolysins act on actively metabolizing staphylococcal cells only after the cells have been altered by heat, acetone treatment, ultraviolet irradiation or bacteriophage "sensitization" (Ralston et al, 1955). Bacteriophage-induced lytic enzyme (BILE) active on staphylococcal cells has been reported by Ralston et al (1964) and Doughty and Mann (1967). This lysin was released upon lysis of staphylococcal cells after infection with specific staphylococcal phages (Strominger and Ghuysen,

1967). Purified phages themselves did not cause lysis but in combination with BILE produced rapid lysis (Ralston et al, 1964).

A staphylococcal phage-associated lysin (PAL) active on metabolizing unaltered staphylococcal cells was reported by Sonstein, Hammel and Bondi (1971). This enzyme was released upon lysis of staphylococcal strain PS53 infected with its homologous typing phage 53 and was optimally active at pH 6.5 and 30C. Its lytic activity was greatly enhanced by the addition of reducing agents. It had peptidase activity and resulted in the production of spheroplasts. According to Sonstein, Hammel and Bondi (1971), PAL, BILE and virolysin were all active in solubilizing isolated cell walls, however, only PAL was active in solubilizing viable, actively metabolizing, whole staphylococcal cells without prior alteration or sensitization.

Phage-associated lysozyme is not the first example of a bacteriophage-induced enzyme capable of solubilizing the cell walls of intact bacteria. A similar enzyme has been reported which is active against Streptococcus faecalis var. liquefaciens (Bleiweiss and Zimmerman, 1961) and another which is active against Streptococcus pyogenes Group A, Type 1 (Kühnemund, 1972). A "virolysin" and CWSE-80, bacteriophage-induced cell wall - solubilizing enzymes, were described by Doughty and Mann (1967), active in solubilizing isolated cell walls of S. aureus without prior alteration or "sensitization".

In 1928, the process of transformation in pneumococcal cells was discovered by Griffith. It was not until 1944, however, that Avery, MacLeod and McCarty showed that DNA conveyed genetic information from one bacterium to another in this process. Transformation involves the penetration of a fraction of the donor's total DNA, obtained by chemical extraction or nat-

ural cell lysis, into a related bacterial cell; and replacement, through
a process of recombination, of a specific nucleotide sequence in the
recipient's genome. Transformation was shown to be quantitatively related
to the integrity of the DNA molecule since denaturation of the DNA molecule
resulted in the loss of transforming activity (Avery, MacLeod and McCarty,
1944). According to Braun (1965), five events occur during transformation:
(1) binding of donor DNA to the recipient cell, (2) uptake of donor DNA,
(3) association or pairing of the transforming DNA with the corresponding
nucleotide sequence of the recipient chromosome, (4) integration by recom-
bination, and (5) replication of the integrated new information. It pro-
vides the only method of relating the effects of physical and chemical changes
in the structure of DNA with its biological activity (Hayes, 1968).

DNA must be a certain minimum size (10^6 to 10^7 molecular weight) and
double stranded for uptake by a bacterial cell (Davis et al, 1967). As
the DNA is absorbed by the cell, the strands of the DNA separate and only
one strand penetrates the cytoplasm (Davis et al, 1967). As reported by
Hayes (1968), at concentrations of DNA below 100 ng/ml, the number of trans-
formants in a culture of 4×10^8 cells/ml is directly proportional to the
concentration of DNA. Above 100 ng/ml, increases of DNA concentrations
fail to result in any proportional increase in the number of transformants.
Once DNA saturation occurs, transforming molecules compete for available
receptor sites on the cell wall, so that no further transformants are
obtained with increasing DNA concentrations (Hayes, 1968).

Not all recipient cells can be transformed. According to Stent (1971),
"competency" is the capacity of a bacterium to take up DNA from its surround-
ings. Competency of several bacterial species, such as Diplococcus

pneumoniae, Haemophilus influenzae and Bacillus subtilis, is greatest at
the end of the exponential phase of the growth cycle (Hayes, 1968). Accord-
ing to Ravin (1965), the DNA of one species will only transform bacteria
of the same or a very closely related species. The inability of DNA to
transform appeared to be due not to failure to penetrate the recipient
bacteria but failure to effect transformation once inside (Ravin, 1965).
The recent reports of functionalization of eucaryotic genes in microbial
cultures, however, indicates the potential for transformation is not yet
realized (Chang and Cohen, 1974).

As reported by Davis et al (1967), a number of species of bacteria
have been found to be transformable, however, not until recently has trans-
formation been observed in Staphylococcus aureus (Nomura et al, 1971).
The main obstacle in effecting transformation was in obtaining high molecular
weight, biologically active DNA which was resistant to the action of staphy-
lococcal deoxyribonuclease (Lindberg, Sjöström and Johansson, 1972). Omenn
and Friedman (1970) described a method for the isolation of mutants of S.
aureus lacking extracellular nuclease activity. According to Omenn and
Friedman (1970), the possibility of successful transformation in S. aureus
has been enhanced with the availability of a nuclease-deficient mutant of
S. aureus for use as a recipient strain.

Information on transformation in staphylococci has been limited and
results have been inconsistent. Blobel (1961) indicated some success in
the isolation and characterization of DNA from S. aureus. Klesius and
Schuhardt (1968) reported their inability to effect transformation with
numerous DNA preparations. Dobrzanski, Oswiecki and Jagielski (1968)
reported successful intergeneric transformation with Staphylococcus as a

donor and Streptococcus as a recipient. They succeeded in transforming
the streptomycin-resistance marker, but failed to transfer the penicillin-,
novobiocin-, erythromycin- and oxytetracycline-resistance markers.

Initial reports of transformation in S. aureus were limited to trans-
mission of plasmidal resistance markers such as streptomycin and metal ions
(Imshenetiskii et al, 1960; Lambina, 1961; Komarov, 1962; Smirnova, Gubenko
and Ribakowa,1969). Nomura et al (1971) reported the induction of hemo-
lysin synthesis, a chromosomal marker, by transformation in S. aureus.
Lindberg et al (1972) reported a method for genetic transformation of
S. aureus with phenol-extracted DNA. For selective genetic markers, they
attempted to transform the plasmidal characters erythromycin, penicillin,
and cadmium resistance, and the chromosomal markers of thymine and cytidine
independence.

A recurring problem in staphylococcal genetics is to determine whether
a marker is coded by a chromosomal or plasmidal gene. Recently, Lindberg
and Novick (1973) reported the application of transformation to differentiate
between plasmidal and chromosomal markers.

Sjöström, Lindberg and Philipson (1973) reported that the ability of
a culture to develop competency during growth differed between the wild-
type strain and a nuclease-deficient mutant. The wild-type strain showed
maximum uptake of DNA in the early exponential-growth phase where no extra-
cellular nuclease activity was observed. The nuclease-deficient strain
showed competency at this time but also showed competency later in the
exponential-growth phase. Recently, the process of transfection has been
used to determine the requirements for competence in Staphylococcus aureus
8325 (Rudin et al, 1974). In S. aureus 8325, an absolute requirement for

lysogeny for phage Ø11 existed for competence induction for both trans-
fection and transformation.

MATERIALS AND METHODS

Cultures

The parent culture of <u>Staphylococcus</u> <u>aureus</u> (PS53), a propagating
strain of the International Phage Typing Series, was obtained from the
Sylvana Co., Milburn, N.J. 07041. The culture of <u>S</u>. <u>aureus</u> strain 7-8
used in this study was isolated from the infected teat of a cow with
mastitis. Both cultures were maintained at 4C on Trypticase Soy Agar
(TSA), Baltimore Biological Laboratories (BBL), Baltimore, Md.

Cultures having lost the ability to produce DNase were obtained by
the procedure of Omenn and Friedman (1970). <u>S</u>. <u>aureus</u> strain 7-8 was
inoculated into 10 ml of BBL Trypticase Soy Broth (TSB) and incubated
overnight at 37C. After incubation, the culture was centrifuged (Sorvall
SS-3) at 5,000 x g for 10 minutes. The cells were washed once with 0.07M
sodium phosphate buffer, pH 7.0, then suspended in 9 ml of the same buffer.
A solution containing 200 µg of 1-methyl-3-nitro-1-nitroso-guanidine (NTG,
Aldrich Chemical Co., Milwaukee, Wisconsin) per ml water, was sterilized
by filtration through a 0.45 µ filter (Millipore Filter Corp., Bedford, Mass.)
and added to the cell suspension to a final concentration of 100 µg/ml.
The suspension was then incubated at 37C for 30 minutes. One milliliter
samples were diluted in 50 ml of TSB and incubated overnight at 37C. After
incubation, 5 ml of dimethyl sulfoxide (Fisher Scientific Co., Fairlawn, N.J.)
were added to enhance the viability of cells upon freezing and thawing.
Some samples were stored at -70C for use later. Mutants not producing DNase
were defined by qualitative and quantitative assays.

Strains of <u>S</u>. <u>aureus</u> selected for transformation experiments lacked

deoxyribonuclease (DNase) production and were sensitive to tetracycline.

DNase production was determined by methods described below. The tolerance

of the cultures to tetracycline was determined by placing one drop of an

overnight TSB culture of the strain into each of a series of tubes contain-

ing 5 ml of TSB and concentrations of tetracycline (Roerig Division, Chas.

Pfizer and Co., Inc., New York) ranging from 50-100 mg/ml, and incubating

overnight at 37C. The concentration having increased turbidity after incu-

bation indicated the greatest amount of tetracycline tolerated by that

culture. In addition, all cultures were streaked on TSA plates containing

3% defibrinated sheep blood (Robbin Laboratories, Chapel Hill, N.C.),

incubated overnight at 37C followed by 1 hour at 4C, and then observed for

beta-hemolytic activity (evidenced by a clear zone upon cooling).

<div align="center">Assays of DNase Activity</div>

Qualitative Assays

Three qualitative assays were performed for DNase activity. One

assay involved the use of DNA-AO test medium. Dilutions of mutagen-

treated cultures were prepared in saline solution to give approximately

100 colonies per plate when streaked on DNA-acridine orange (AO) agar.

This medium was prepared by adding 40 µg of AO/ml (Fisher Scientific Co.)

to DNase Test Agar (BBL) before autoclaving. This medium had a brilliant

yellow-green fluorescence. Release of nuclease from a colony was indicat-

ed by a dark halo of defluorescence (nuc+). Fluorescence persisted

around nuclease-deficient colonies (nuc-). Presumptive nuc- colonies were

selected from DNA-AO agar according to the method of Morave et al (1969),

inoculated into a rich liquid medium, incubated for 24 hours at 37C, and

kept at 4C for use as stock cultures. The rich liquid medium had the
following ingredients per liter of distilled water; 30 gm Casamino acids
(Nutritional Biochemical Corporation, Cleveland, Ohio), 10 mg lactic acid
(Fisher), 2.4 gm Na_2HPO_4 (Mallinckrodt Chemical Works, St. Louis, Mo.),
0.4 gm KH_2PO_4 (Fisher), 0.006 gm $FeSO_4 \cdot 7H_2O$ (Mallinckrodt), 0.006 gm citric
acid (Mallinckrodt), and 5 gm yeast hydrolysate (Nutritional Biochemical
Corporation). The pH was adjusted to 7.6-7.8 by adding 1N NaOH (Fisher).
A second assay was the toluidine blue test. In this test dilutions of
growth from the NTG-treated cultures were streaked onto DNase Test Agar
plates and incubated overnight. Plates containing isolated colonies were
flooded with 0.1% (w/v) Toluidine Blue O in water (Fisher Scientific Co.).
A pink halo developed immediately around colonies which produced DNase while
no such halo occurred with nuc- colonies. A third assay was the hydrochloric
acid test. As in the toluidine blue test, colonies on DNase Test Agar plates
were flooded with normal hydrochloric acid. DNase-positive colonies showed
a distinct clear zone around the growth, while nuc- colonies showed no
clearing.

Quantitative Assay

Quantitation of nuclease activity of each culture was determined spectro-
photometrically (Cuatrecasas et al, 1967). Salmon DNA (Sigma Chemical Co.,
St. Louis, Mo.) was used as the substrate. Fifty micrograms of DNA were
dissolved in 1.0 ml of 0.025 M Tris-HCL buffer, pH 8.8, containing 0.01 M
$CaCl_2$. The cells of an overnight culture grown in the rich liquid media
previously described were centrifuged (Sorvall SS-3) at 5,000 x g for 10
minutes. Thirty microliters of supernatant were added to the DNA-Tris buffer

mixture in a quartz cuvette and the absorbance at 280 nm determined at 13-second intervals for 2 minutes with a Gilford spectrophotometer (Model 2000). Increase DNase activity of the mixture was indicated by an increase in absorbance (280 nm).

Phage Methods

Propagation and Titration of Bacteriophage

Growth from an overnight TSA slope culture of <u>Staphylococcus aureus</u> PS53 was suspended in 2 ml of TSB to a concentration of approximately 10^9 cells/ml. To 5 ml of melted and cooled (50C) TSB containing 0.3% Agar (BBL), 0.2 ml of cell suspension, 0.1 ml $CaCl_2$ (20,000 µg/ml stock solution), and 0.1 ml of Staphylococcal Phage 53 stock suspension (International Phage Typing Series, purchased from Sylvana Co.) were added. The suspension was mixed and poured over the surface of a solid agar layer (TSA + 400 µg/ml $CaCl_2$) in a petri dish. The cultures were incubated in an upright position for 5 hours at 37C. The semisolid layer was emulsified in 5 ml of TSB, transferred to a 15-ml centrifuge tube and centrifuged (Model CL, International Equipment Co., Needham Heights, Mass.) for 10 minutes (setting of "7"). The liquid portion was then filtered through a 0.45 µ filter (#7102, BBL). The filtrate containing the phage harvest was aseptically transferred to a sterile container and stored at 4C until used. Titration of the numbers of phages per ml was done as follows: One drop of an overnight TSB culture of <u>S</u>. <u>aureus</u> propagating strain 53 (PS53) was smeared over the surface of a TSA plate with a sterile glass spreader. The numbers 1-8 were written clockwise around the bottom of the dish. Dilutions of the phage harvest from 10^{-1} to 10^{-8} were prepared with sterile water, 0.01

ml of each spotted on the cell lawn in the numbered positions, and the plate incubated overnight at 37C. The titer, expressed as plaque-forming units/ml (pfu/ml), was determined by counting the individual plaques which appeared in a position and multiplying this number by the dilution factor appropriate to that position.

Sensitivity of S. aureus Strains to Staphylococcal Phage 53

Twenty-eight strains of S. aureus were individually tested for resistance to staphylococcal bacteriophage 53 by the following method. One drop of phage 53 preparation (9.25×10^8 pfu/ml) was placed on a lawn of each S. aureus strain on a TSA plate containing 400 µg/ml of $CaCl_2$ and incubated overnight at 37C. Strains found to be resistant showed no evidence of lysis.

Preparation of Phage-Associated Lysozyme (PAL)

A modified method of Sonstein, Hammel and Bondi (1971) was used as a basic procedure for the preparation of PAL. Two flasks, each containing 50 ml of TSB plus $CaCl_2$ (400 µg/ml), were inoculated from overnight TSA cultures of S. aureus PS53 and incubated overnight at 37C. The content of each flask was emptied into 1000 ml of TSB containing $CaCl_2$ (400 µg/ml). This large culture was incubated on a rotary shaker for 2 hours at 37C followed by the addition of 10 ml of staphylococcal bacteriophage 53 harvest (containing 9.25×10^8 pfu/ml) to the culture. Incubation was continued until visual clearing occurred (approximately 90 minutes). During the incubation period the contents were gently stirred with a magnetic stirrer.

The 1100 ml of lysate was concentrated to approximately 100 ml using ultrafiltration (Amicon Model 200, Lexington, Mass.) with a 150 mm UM 10 membrane (Amicon). Bottled nitrogen gas was used to maintain 40 psi

pressure during the ultrafiltration process. Cellular debris was removed from the unfiltered lysate concentrate by centrifugation at 25,000 x g for 15 minutes. This and all subsequent centrifugations were performed at 5C using a Sorvall SS-3 centrifuge unless otherwise indicated. Ammonium sulfate, recrystallized from EDTA solution to remove metal ions but not EDTA free(Sigma), was added to the supernatant to 60% saturation (36.1 g/100 ml) and the preparation stored at 4C for 24 hours. In subsequent preparations, mixtures were stored for 5 hours, and retained greater enzyme activity. The resulting precipitate was collected by centrifugation at 13,000 x g for 15 minutes, resuspended in one-twentieth the volume of 0.9% NaCl and recentrifuged at 13,000 x g for 15 minutes to remove insoluble material. Ammonium sulfate was then added to the liquid to 50% saturation (29.1 g/100 ml) and the preparation stored at 4C for 24 hours. The resulting precipitate was removed by centrifugation at 13,000 x g for 15 minutes and dissolved in 10 ml of 0.07 M NaH_2PO_4 buffer, pH 7.0. After a final centrifugation at 13,000 x g for 15 minutes, the liquid was dialyzed for 48 hours against 25 volumes of 0.9% NaCl in 0.07 M NaH_2PO_4 buffer, pH 7.0. The resulting dialysate was refrigerated at 5C until used.

Modifications of the above procedure included applying the enzyme preparation (after 48-hour dialysis) to a 4 x 33 cm Sephadex G-200 column (Pharamacia Fine Chemicals, Piscataway, N.J.) equilibrated with 0.07 M NaH_2PO_4 buffer, pH 7.0. Protein-containing fractions were identified spectrophotometrically (Coleman, Model 124, absorbance of 280 nm). Fractions from each elution peak containing protein were pooled, lyophilized and refrigerated until used (5C). Additionally, ammonium sulfate-grade III (Sigma) treated to reduce metal ions, and ammonium sulfate-enzyme grade

(Nutritional Biochemical Co.) were each used to effect protein precipitation in separate PAL preparations. Another method of protein precipitation involved adding an equal volume of cold acetone (-20C) to the flask of lysate previously cooled in an ice bath. The mixture was gently stirred for 5 minutes and the precipitate collected on Whatman #1 paper (Arthur Thomas Co., Philadelphia, Penn.) in a Buchner funnel with vacuum. The precipitate was dissolved in 10 ml of lysis medium (LM) containing 0.05M NaH_2PO_4 buffer, pH 6.5, and 0.2% $MgSO_4 \cdot 7H_2O$ after which the solution was dialyzed overnight against 10 volumes of LM and refrigerated at 5C until used.

Bacteriolytic assay of PAL activity

The activity of each PAL preparation obtained above was determined by the method of Sonstein, Hammel and Bondi (1971). Cellular substrate was prepared by inoculating fifty milliliters of TSB, in a 250-ml flask, with an overnight TSB culture of S. aureus PS53 and incubating at 37C for 4 hours. The cells were harvested by centrifugation at 5,000 x g for 10 minutes; washed three times with 0.9% NaCl; suspended in 5 ml of LM and used on the day of preparation.

The assay of PAL activity on whole, washed cells was performed as follows. The following "Klett" tubes were prepared: two tubes contained 2 ml of a PAL preparation and 2.8 ml of LM and two contained 4.8 ml of LM. All tubes were placed in a water bath and the temperature equilibrated to 30C for 15 minutes. Then 0.2 ml and 2.0 ml of the thrice-washed cells was added to one tube of each mixture and the contents mixed. Turbidity readings were made immediately upon the addition of the substrate (zero time) and at 20-minute intervals for 2 hours with a Klett-Summerson photoelectric colorimeter

(Klett Mfg. Co., Inc., N.J.) equipped with a 540 nm filter. In one trial two additional controls were included. One tube contained 2 ml of phage 53 (9.25 x 10^8 pfu/ml) and 2.8 ml of LM, and a second tube contained 2.8 ml of LM and 2 ml of cell lysate. This lysate was obtained by disruption of cells of an overnight TSB culture of S. aureus PS53 in a French press (American Instrument Co., Silver Springs, Maryland) at 20,000 lbs. pressure at ice-bath temperature. Substrate was added and turbidity readings were determined as above.

In all graphs, the percent reduction in optical density was plotted from 0 to 120 minutes after the cell suspension was added to the test mixture. Rates of activity (R) were determined for each curve by computing the reduction in turbidity in a 20-minute interval during the period of greatest decrease (steepest slope).

In separate assays, 0.05M mercaptoethanol and 0.05M cysteine-HCL were added to the LM to observe any effect of the reducing agent on PAL activity. The stability of the enzyme preparations was determined by comparing the lytic activity of the enzyme preparations both frozen (stored at -20C) and lyophilized (stored at room temperature) after storage intervals of 1, 4, 8, and 12 weeks.

The lytic assay described above was used to test the activity of a PAL preparation on: (1) strains found to be resistant to phage 53, (2) S. aureus propagating strain 53 and (3) S. aureus strain 146p, sensitive to phage 53, kindly donated by Riaz-ul Haque, University of Illinois, Medical Center, Chicago, Illinois.

PAL-Induced Release of DNA from Staphylococcal Cells

To quantitatively assay for DNA released from the cells upon treatment with PAL, Burton's diphenylamine test (1956) was used. Steam-distilled di-phenylamine (1.5 g) was dissolved in 100 ml of redistilled acetic acid and 1.5 ml concentrated H_2SO_4. The solution was stored in the dark. Immediately before use 0.10 ml of aqueous acetaldehyde (16 mg/ml) was added for each 20 ml of reagent needed. The complete reagent was used on the day of preparation.

A standard curve was prepared for DNA analysis. A stock solution of calf thymus DNA (Sigma) at a final concentration of 200 μg DNA/ml was pre-pared. Dilutions containing 50, 100, and 150 μg/ml were made in sterile water from the stock solution. Two milliliters of each of the four concentrations of DNA were pipetted into test tubes containing 2 ml of 0.5 NH_4ClO_4 and 4 ml of Burton's complete reagent. The tubes were then incubated at 30C. After exactly 18 hours of incubation, the absorbance at 600 nm was determined using a double-beam spectrophotometer (Coleman 124).

Transformation

Isolation of Donor DNA

Deoxyribonucleic acid to be used in the transformation experiments was prepared from an overnight culture of S. aureus strain HB-2' cells grown in 150 ml of TSB. The donor cells were washed three times with 0.9% NaCl and suspended in 5 ml of LM. PAL (2 ml) was added and the suspension incubated with shaking for 60 minutes at 37C. The cell suspension was treated with protease (2 mg/ml, repurified from Streptomyces griseus, (type VI-Sigma)

at 37C for 60 minutes followed by the addition of 0.4 ml sodium dodecyl
sulfate (5% w/v in 45% ethanol) and shaking for 30 minutes at room temp-
erature.   Treatment of donor cells with various agents and conditions,
after and before PAL exposure, was effected to enhance the lytic activity:
2) adding trypsin (6 mg/ml, Fisher Scientific Co.) after the 30-minute
shaking with sodium dodecyl sulfate and further incubating for 60 minutes;
3) treating with trypsin as above followed by freezing (-20C) and thawing;
4) two periods of freezing at -20C and thawing before treating with PAL;
5) treating with trypsin prior to treating with PAL; 6) suspending the cells
in distilled water for 10 minutes at room temperature prior to PAL treat-
ment (osmotic shock); and 7) treating with egg white lysozyme (6 mg/ml,
Grade 1, Sigma) before PAL treatment.

Preparations obtained from any of the above treatments were mixed with
an equal volume of redistilled phenol (Fisher) saturated with 0.01M Tris
HCl buffer, pH 8.1.   The mixtures were shaken by hand for 15 minutes at room
temperature.   The resulting emulsions were broken by centrifugation (5,000
x g for 10 minutes).   The aqueous phase was collected and the phenol extrac-
tions repeated three times.   Residual phenol was removed from the aqueous
phase by the addition of 10 ml of ethyl ether.   The volatile phenol-ether
mixture was removed by active aeration.   Nucleic acids were then precipitated
by gently mixing the solution with two volumes of cold 95% ethanol.   The
resulting precipitate was collected by a 5-minute centrifugation (Interna-
tional Model Cl, setting of "7") and dissolved in 5 ml of 0.01 dilution of
SSC (0.15M sodium chloride and 0.015M sodium citrate, pH 7.2).   To remove
RNA, ribonuclease-A (Type I-A, Sigma) was added to a final concentration of
50 µg/ml and the mixture incubated for 30 minutes at 37C.   DNA was repreci-

pitated by the addition of 2 volumes of cold 95% ethanol, collected as before, and dissolved in 2.5 ml of a 0.01 dilution of SSC. Finally, an equal volume of a 0.1 dilution of SSC was added. The concentration of DNA was determined by the diphenylamine method, and the solution stored at 5C until used (not exceeding 7 days).

Procedure

For the transformation experiments, the recipient, S. aureus strain MB^{-2}(1), was grown overnight on a TSA plate at 37C. Cells from the culture were suspended in TSB to produce a reading of 0.100 using a Spectronic 20 (Bausch and Lomb) spectrophotometer (absorbance, 525 nm). One milliliter of cell suspension was added to each of two test tubes containing 10 ml of TSB. The suspensions were incubated at 37C with shaking for three and seven hours, respectively. At the end of the incubation periods, the cells were washed in 0.1M CaCl$_2$ and resuspended in 0.1M CaCl$_2$ at 10^9 cfu/ml. In the transformation experiments, 0.1 ml of the donor-DNA solution was mixed with 0.9 ml of cell suspension and the mixture incubated with shaking at 37C for 20 minutes. The cells were then centrifuged (International Model C1, setting of "7") and resuspended in 2 ml of TSB after which 1.0 ml was plated on TSA containing tetracycline (5 µg/ml) and 10^6 and 10^7 dilutions were streaked on DNA-AO plates. In later transformation experiments the 2-ml cell suspension was incubated 4 hours at 37C before diluting and plating. All the plates were incubated for 48 hours at 37C, and the colonies counted. Controls in the experiments included; (1) plating the recipient culture without DNA treatment, (2) adding DNase (Sigma, 50 µg/ml in 5mM MgCl$_2$) to the tube containing donor DNA and incubating for 10 minutes before adding the cells,

(3) plating 0.1 ml of the donor DNA solution on a lawn of S. aureus strain MB^{-2}(1) on a TSA plate containing 400 µg CaCl$_2$/ml medium (viral activity control), (4) plating 0.1 ml of the transformation mixture on BHI agar (BBL) containing tetracycline (5 µg/ml) and 0.2M sodium citrate (transduction control), and (5) plating 0.1 ml of the transformation mixture on TSA containing 400 µg/ml CaCl$_2$ and tetracycline at a concentration of 5 µg/ml (control for lytic activity).

RESULTS

Selection and Characterization of DNase Mutants

The results of assays for the qualitative deoxyribonuclease (DNase) prod-
uction by derivatives of S. aureus strain 7-8 are presented in Table 1. Since
the results for the three qualitative assays for DNase activity were iden-
tical, the toluidine blue test was employed for initial screening of isolates.

The amount of increase in absorbance (280 nm) reflected the quantita-
tive increase in DNase activity of each strain tested (Table 2). Mutants were
obtained from each parent showing less DNase production than their parent.
Mutants of a parent varied significantly from each other in DNase production.
As an example, quantitative nuclease production by mutants of strain $7-8AT^+P^+B^+$
showed up to a sixty-two fold reduction compared to the parent and varied over
fifty-fold among the mutants. Mutants of strain IB^- showed as much as six-
fold less nuclease production than the parent strain. The mutant $1B^-_1$ showed
three-fold less nuclease production than its parent. The parent strain MB^-
showed moderate nuclease production. Mutants of this strain exhibited up to
an eight-fold reduction in nuclease activity. A seven-fold difference in
DNase activity was detected among its mutants. The parent $2B^-tet^s$ displayed
the highest nuclease production of all cultures. Mutants of this culture
showed little difference in nuclease production among themselves but represented
up to a four-fold reduction in activity as compared to the parent. Mutants of
the parent strain HB^- were the only mutants which showed an increase in nuclease
production compared to the parent. Strain HB^-_2, HB^-_1, and HB^-_2, showed a
two to a four-fold increase in nuclease production. One of these mutants,

Table 1. Qualitative DNase Production by <u>S</u>. <u>aureus</u> Strain 7-8 and Mutants.

	Qualitative DNase Production		
Strain Number	Acridine Orange	Toluidine Blue	HCL
7-8AT$^+$P$^+$B$^+$	+	+	+
IB$^-$	+	+	+
1B$^-$	+	+	+
2B$^-$tet^s	+	+	+
MB$^-$	+	+	+
HB$^-$	−	−	−

Table 2. Quantitative DNase Production by Derivatives of $\underline{S.}$ $\underline{aureus}$ Strain 7-8.

$\underline{S.}$ $\underline{aureus}$ Strain Number		Quantitative DNase Production[1]
Parent	Mutant	
$7\text{-}8AT^+P^+B^+$		0.186
	$7\text{-}8(1)$	0.137
	$7\text{-}8(2)$	0.163
	$7\text{-}8(3)$	0.103
	$7\text{-}8(4)$	0.003
	$7\text{-}8(6)$	0.034
IB^-		0.256
	IB^-_1	0.090
	IB^-_2	0.046
$1B^-$		0.085
	$1B^-_1$	0.027
$2B^-tet^s$		0.307
	$2B^-tet^s(1)$	0.100
	$2B^-tet^s(2)$	0.178
	$2B^-tet^s(3)$	0.074
MB^-		0.218
	$MB^-(1)$	0.027
	$MB^-(2)$	0.064
	$MB^-(3)$	0.187
HB^-		0.091
	HB^-_1	0.068
	HB^-_2	0.142
	HB^-_3	0.039
	HB^-_4	0.075
	HB^-_5	0.052
	$HB^-_{1'}$	0.166
	$HB^-_{2'}$	0.392
	$HB^-_{3'}$	0.087

[1]Increase in absorbance (280 nm) for 2 min.

$HB^-_{2'}$, showed the highest nuclease production of all cultures assayed.

Five of the mutants of strain HB^-, HB^-_1, HB^-_3, HB^-_4, HB^-_5, and $HB^-_{3'}$, showed

a reduction in nuclease production.

The results of tests of the parents and mutant cultures for beta-hemo-

lytic (hot-cold lysin) activity on sheep blood agar and sensitivity to

Staphylococcal Typing Phage 53, presented in Table 3, show no correlation

existed between beta-hemolysin production and resistance to Staphylococcal

Phage 53. Cultures $7-8AT^+P^+B^+$, $7-8T^+P^-B^+$, $7-8(4)$ and $HB^-_{2'}$ were beta-hemo-

lytic and strains $7-8T^+P^-B^+$, IB^-_2, $2B^-tet^s(3)$, HB^-_1, HB^-_4, HB^-_5 and $HB^-_{2'}$

were sensitive to Staphylococcal Phage 53. Hemolytic activity (other than

beta-hemolytic) did not correlate with resistance to Typing Phage 53. It

was interesting to note that mutant strain $HB^-_{2'}$, which showed four-fold

more nuclease production than its parent HB^-, acquired, in addition, beta-

hemolytic activity.

Characterization of Strains for Transformation Experiments

Initially, we intended to select strains for transformation experiments

on the basis of both DNase and beta-hemolytic activity. The strains chosen

were 7-8, 7-8(4), 7-8(6), $MB^-(1)$, $HB^-_{2'}$ and HB^-_5. Transformants resistant

to antibiotics would be readily detected as contrasted to beta-hemolytic or

DNase activity. Therefore, the above strains were tested for tetracycline

resistance (Table 4). The parent strain 7-8 and the DNase mutants 7-8(4),

7-8(6) and $HB^-_{2'}$ grew in media containing tetracycline concentrations of

40 μg/ml while strains $MB^-(1)$ and HB^-_5 were sensitive to concentrations of

5 μg/ml. Strain $HB^-_{2'}$ was chosen as the donor for transformation experiments

and $MB^-(1)$ was chosen as the recipient strain. A summary of all the charact-

Table 3. Beta-Hemolysin Production and Sensitivity to Staphylococcal
Typing Phage 53 by S. aureus strains.

Culture	Zone of Hemolysis (mm)	Beta-Hemolysin Production	Resistance to Phage 53
$7\text{-}8AT^+P^+B^+$	12	+	R'
$7\text{-}8AT^+P^-B^+$	10	+	S
7-8(1)	0	−	R
7-8(2)	0	−	R
7-8(3)	2	−	R
7-8(4)	6	+	R
7-8(6)	3	−	R
IB_-	0.25	−	R
IB_{-1}	1	−	R
IB_{-2}	0.25	−	S
$1B_-$	1.25	−	Not Tested
$1B_{-1}$	0.25	−	R
$2B_-tet^s$	0.25	−	R
$2B_-tet^s(1)$	1.25	−	R
$2B_-tet^s(2)$	0.25	−	R
$2B_-tet^s(3)$	1	−	S
MB_-	0.25	−	R
$MB_-(1)$	0.25	−	R
$MB_-(2)$	0.25	−	R
$MB_-(4)$	0.25	−	R
HB_-	0.25	−	R
HB_{-1}	0.25	−	S
HB_{-2}	0	−	R
HB_{-3}	0.25	−	S
HB_{-4}	0.25	−	S
HB_{-5}	0.50	−	R
$HB_{-1'}$	8.00	+	S
$HB_{-2'}$	0.50	−	R
$HB_{3'}$			

' R = resistant to Staphylococcal Typing Phage 53
  S = sensitive to Staphylococcal Typing Phage 53

" 0 = no hemolysis of any type

Table 4. Tolerance to Tetracycline of Strains of <u>S</u>. <u>aureus</u> Selected for
Use in Transformation Experiments.

Strain	Maximum Concentration of Tetracycline Tolerated in Growth Medium
7-8	40 µg/ml
7-8(4)	40 µg/ml
7-8(6)	40 µg/ml
MB$^-$(1)	< 5 µg/ml
HB$^-_2$,	40 µg/ml
HB$^-_5$	< 5 µg/ml

Table 5. Summary of Characteristics of Strains Chosen for Transformation.

Strain	DNase Production	Beta-Hemolysin Production	Tetracycline Tolerance	Staphylococcal Phage 53
HB$^-_2$, (donor)	+	+	40 µg/ml	Sensitive
MB$^-$(1) (recipient)	–	–	< 5 µg/ml	Resistant

eristics investigated of those strains used in the actual transformation experiments is given in Table 5. Strain HB^-_2, is: (1) a producer of both DNase and beta-hemolysin, (2) sensitive to tetracycline concentrations above 40 µg/ml and (3) sensitive to lysis by Staphylococcal Phage 53. Strain $MB^-(1)$ is: (1) not a producer of DNase or beta-hemolysin, (2) sensitive to tetracycline concentrations below 5 µg/ml and (3) resistant to Staphylococcal Phage 53.

Lytic Activity of PAL Preparations

Figure 1 shows the effect of a ten-fold difference in the concentration of PAL on whole cells of S. aureus strain 53. No lag in PAL activity was observed in the control or experimental systems. The rate of activity (R) for 0.2 ml of PAL (0.25/min) was one-half the rate of activity for 2.0 ml of PAL (0.50/min). Thus, a ten-fold increase in concentration of PAL resulted in a doubling of the rate of lytic activity.

The effects of the addition of the reducing agents; 0.05M cysteine-HCl or 0.05M mercaptoethanol (ME) on the lytic activity of PAL are shown in Figure 2. No lag in lytic activity was observed. The activity of the PAL preparation with mercaptoethanol (R=0.84/min) was 1.5 times that of the control (PAL alone) while cysteine-HCl enhanced activity nearly three-fold (R=1.50/min).

A comparison of the curves in Figure 3 shows PAL preparation filtered through a Sephadex G-200 column had less than half the activity of the unfiltered preparation (R=0.50/min and R=1.20/min, respectively). The unfiltered PAL preparation showed a lag in enzyme activity that was not observed for the filtered preparation.

FIGURE 1. THE EFFECT OF CONCENTRATION OF PAL ON LYTIC ACTIVITY FOR
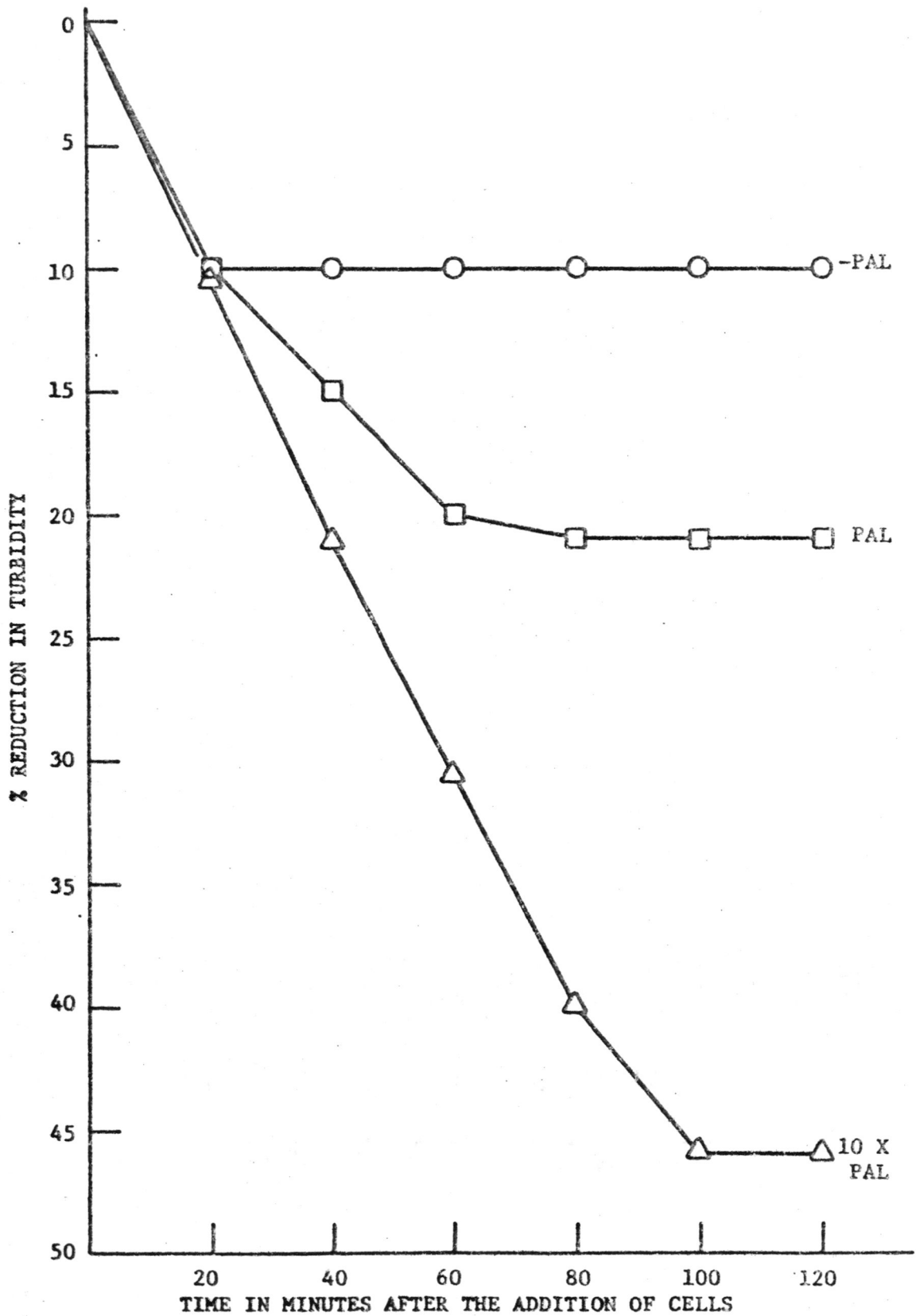WHOLE CELLS OF STAPHYLOCOCCUS AUREUS STRAIN 53

FIGURE 1

FIGURE 2. THE EFFECT OF REDUCING AGENTS ON THE LYTIC ACTIVITY OF PAL

FOR WHOLE STAPHYLOCOCCUS AUREUS STRAIN 53

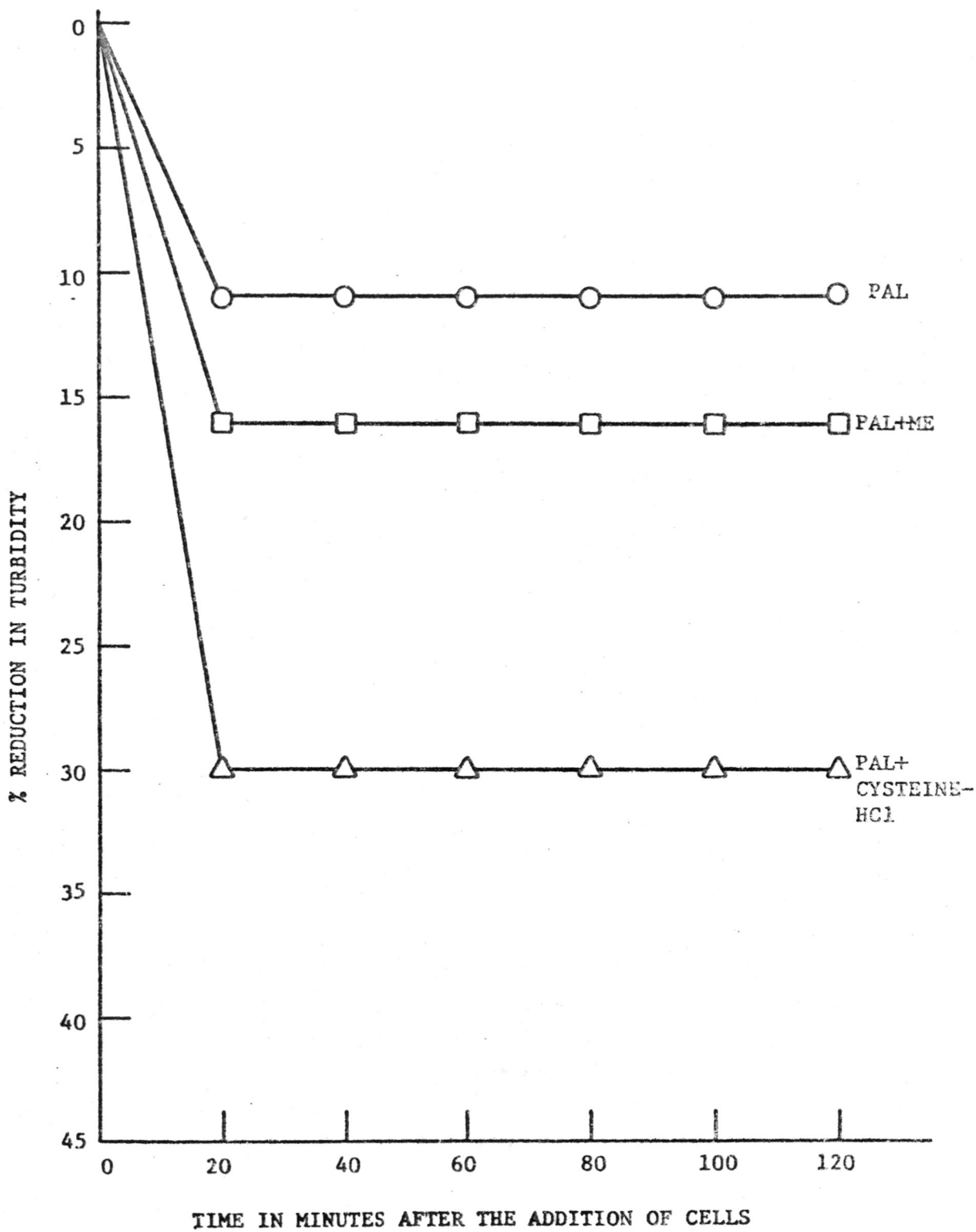TIME IN MINUTES AFTER THE ADDITION OF CELLS

FIGURE 2

FIGURE 3.   EFFECT OF GEL FILTRATION WITH SEPHADEX G-200 ON LYTIC ACT-

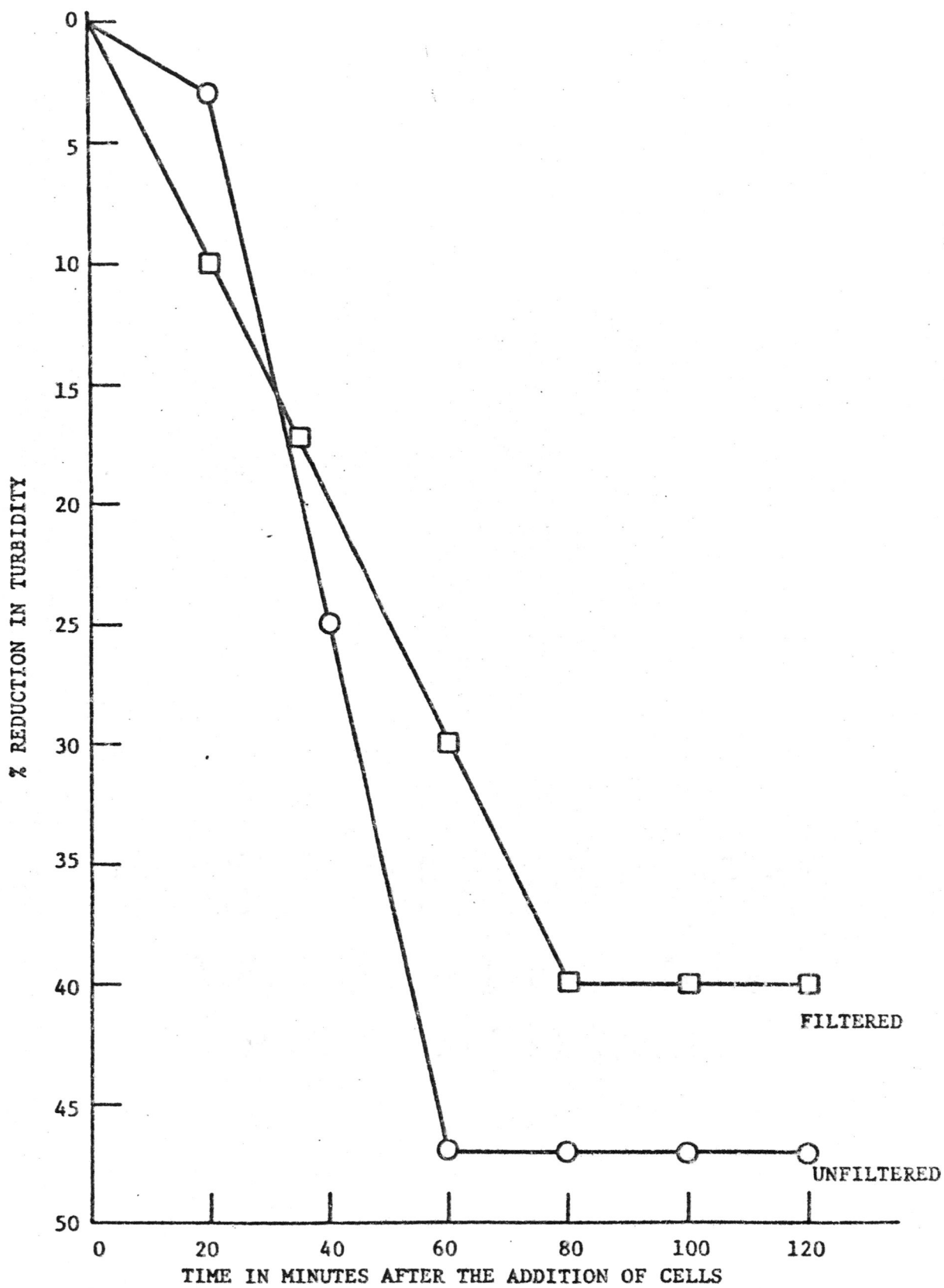IVITY OF PAL FOR WHOLE CELLS OF S. AUREUS STRAIN 53

FIGURE 3

FIGURE 4. THE EFFECT OF AGE AND STORAGE CONDITION ON LYTIC ACTIVITY

OF PAL ON WHOLE CELLS OF <u>STAPHYLOCOCCUS</u> <u>AUREUS</u> STRAIN 53
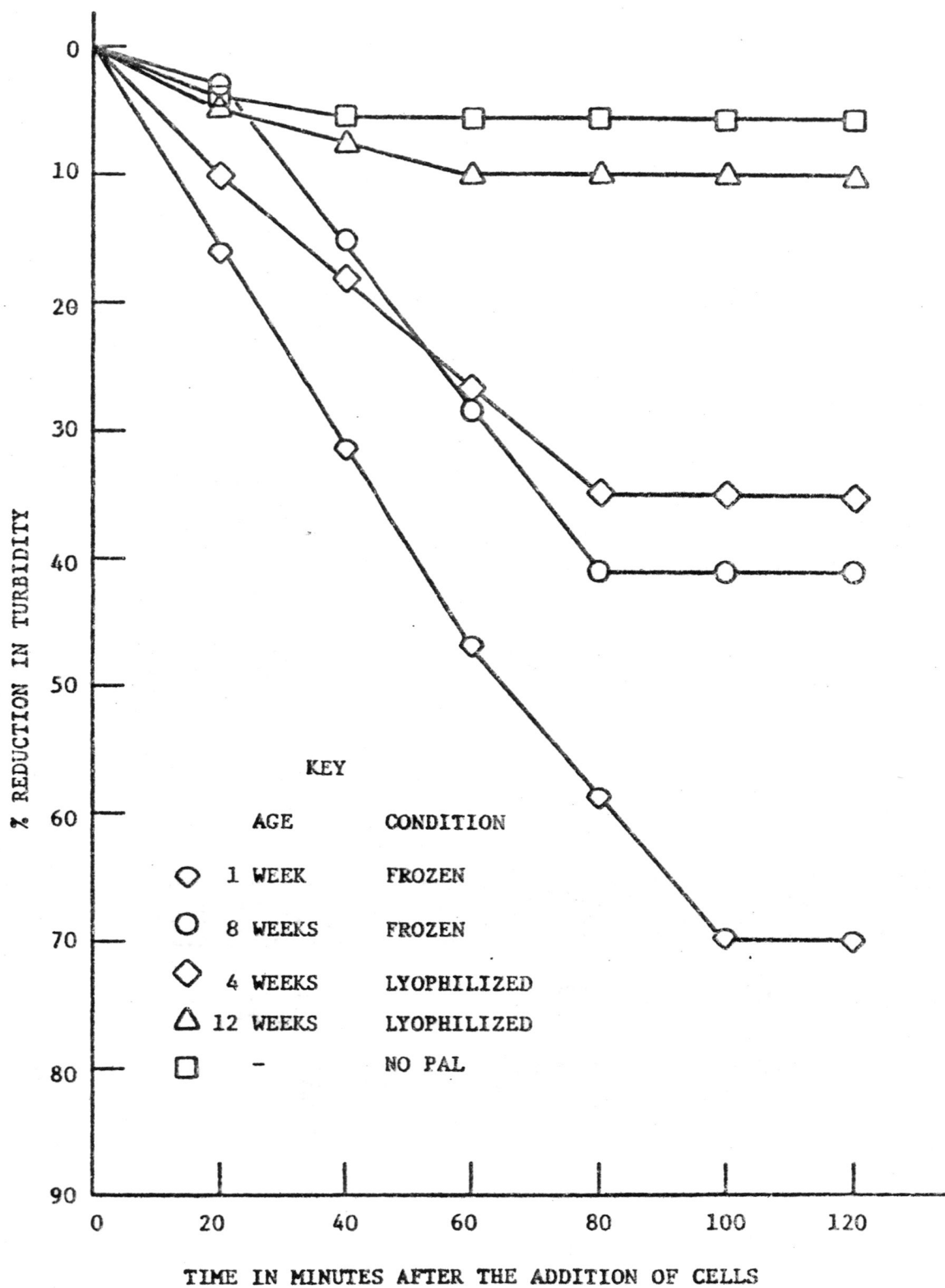
FIGURE 4

Figure 4 shows age and storage conditions directly affected the lytic activity of PAL on whole cells of S. aureus strain 53. A three-fold loss in PAL activity occurred upon storage of the lyophilized preparation from 4 to 12 weeks (R=0.45/min and R=0.15/min, respectively). A slight reduction in activity occurred when storage of the frozen preparation was increased from 1 week to 8 weeks (R=0.85/min and R=0.75/min, respectively). The oldest frozen preparation was the only preparation that showed a lag in enzyme activity. The frozen preparations had at least twice the lytic activity as the lyophilized PAL preparations. The preparation frozen for only one week showed at least three-fold greater activity than the other preparations.

The effect of methods of precipitation on the lytic activity of PAL on whole cells of S. aureus strain 53 is shown in Figure 5. The preparation of PAL obtained by cold acetone precipitation showed the least lytic activity (R=0.30/min). The PAL preparation obtained by precipitation with ammonium sulfate grade III from Sigma, showed only slight lytic activity (R=0.50/min). The PAL preparation obtained by precipitation with ammonium sulfate from Nutritional Biochemical Co., showed approximately the same lytic activity as the preparation obtained with ammonium sulfate from Sigma (recrystallized from EDTA solution to remove metal ions but not EDTA free) and stored for 24 hours at 4C (R=0.85/min and R=1.00/min, respectively) but produced about 21% less lysis. Reducing the time of storing the PAL preparations from 24 to 5 hours increased the rate of lytic activity from 1.0/min to 2.5/min respectively. Also the amount of lysis was increased by 20%.

The effect of PAL on strains of S. aureus resistant to Staphylococcal Typing Phage 53 varied from strain to strain (Figure 6). The parent strain

FIGURE 5.   THE EFFECT OF METHODS OF PRECIPITATION ON THE LYTIC ACTI-

VITY OF PAL ON WHOLE CELLS OF <u>STAPHYLOCOCCUS</u> <u>AUREUS</u> STRAIN 53

METHOD OF PRECIPITATION

◇ ACETONE

○ AMMONIUM SULFATE (NBC) 5 HOURS

☐ AMMONIUM SULFATE (SIGMA) 24 HOURS

△ AMMONIUM SULFATE (SIGMA) 5 HOURS

◇ AMMONIUM SULFATE (SIGMA) GRADE III

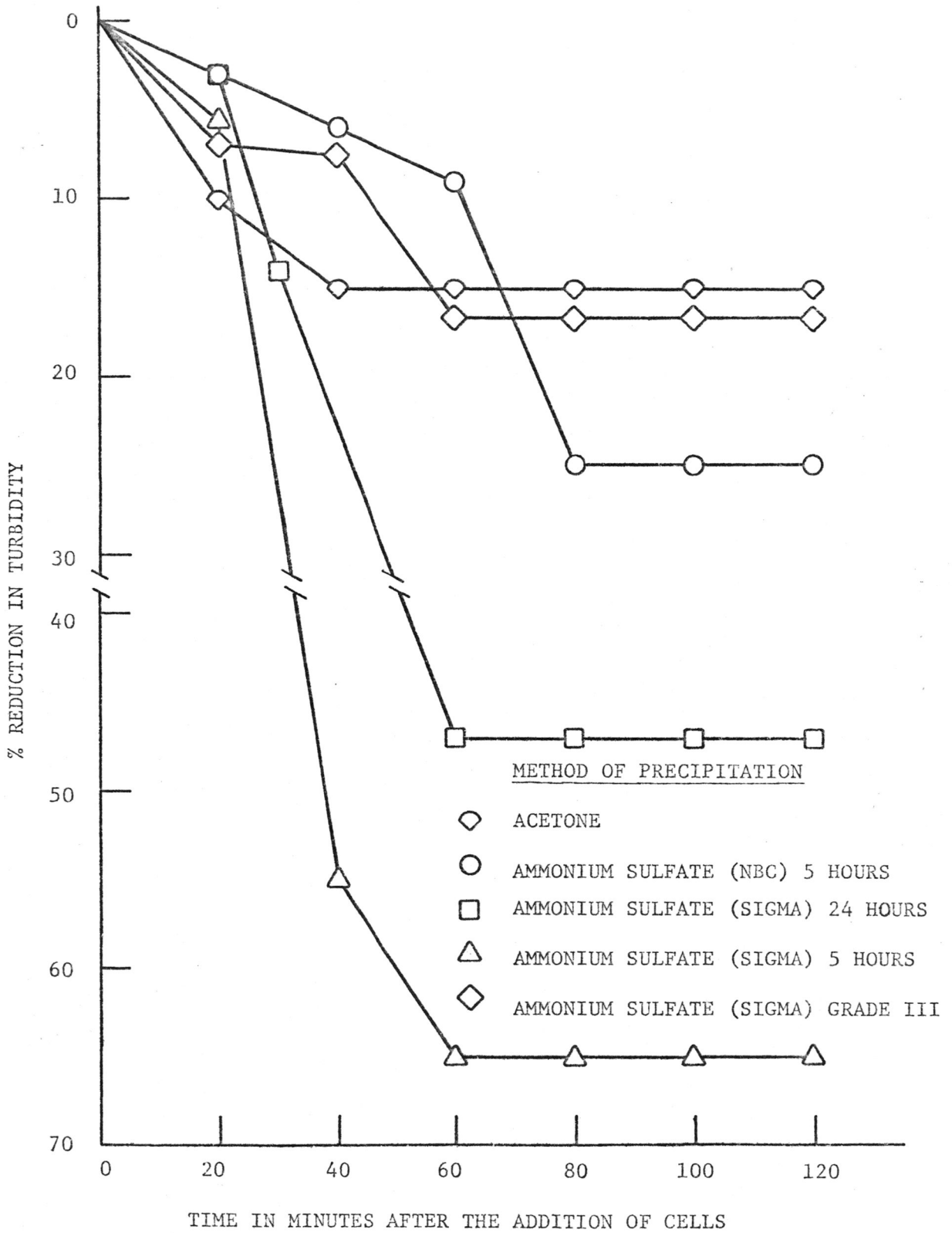TIME IN MINUTES AFTER THE ADDITION OF CELLS

FIGURE 5

FIGURE 6. PAL ACTIVITY ON STRAINS OF S. AUREUS RESISTANT TO STAPHY-

LOCOCCAL TYPING PHAGE 53
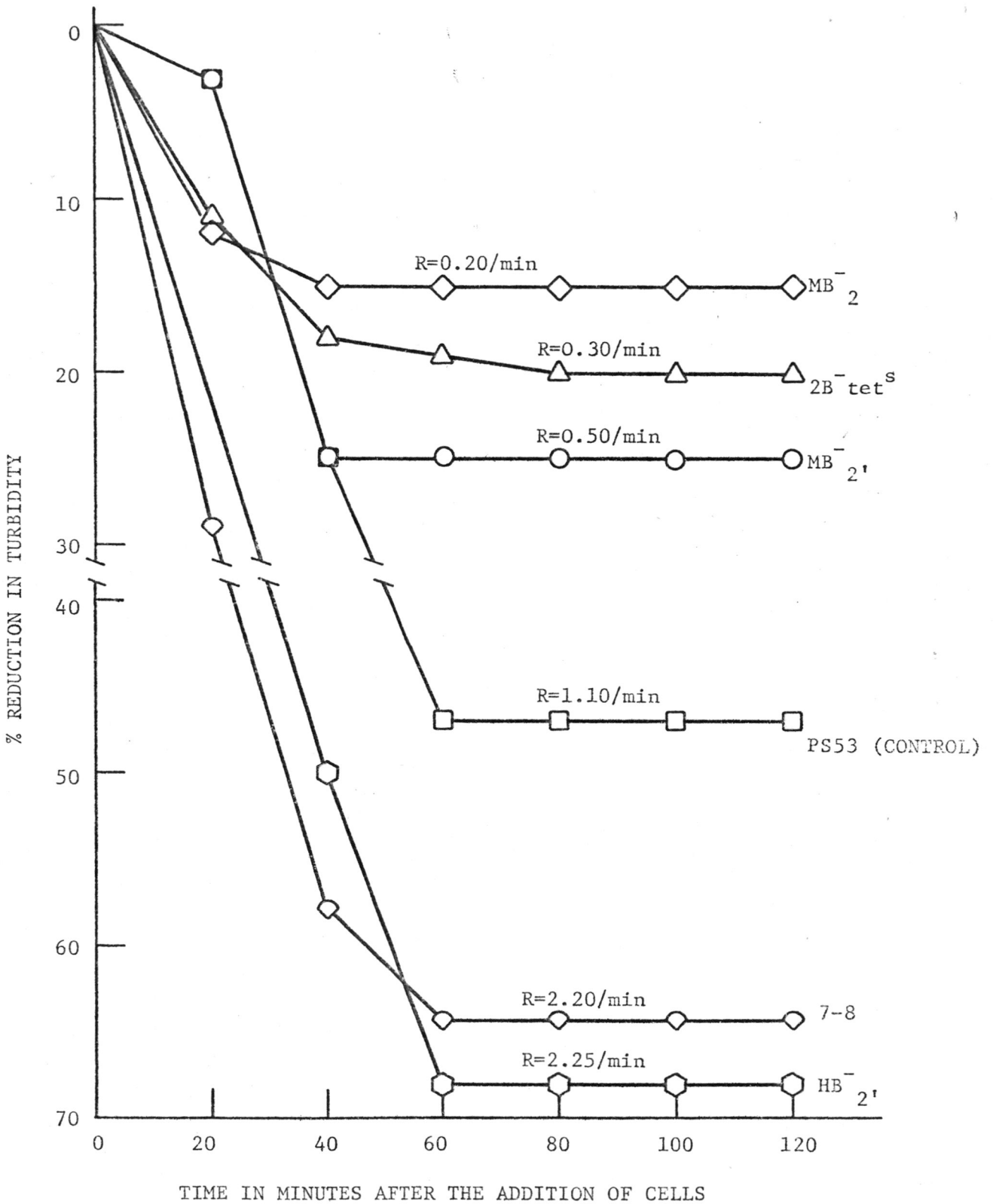
FIGURE 6

FIGURE 7. THE EFFECT OF CYTOPLASMIC CONTENTS OF MECHANICALLY LYSED

STAPHYLOCOCCAL CELLS ON WHOLE CELLS OF $\underline{S}$. $\underline{AUREUS}$ STRAIN HB$^{-}_{2}$,

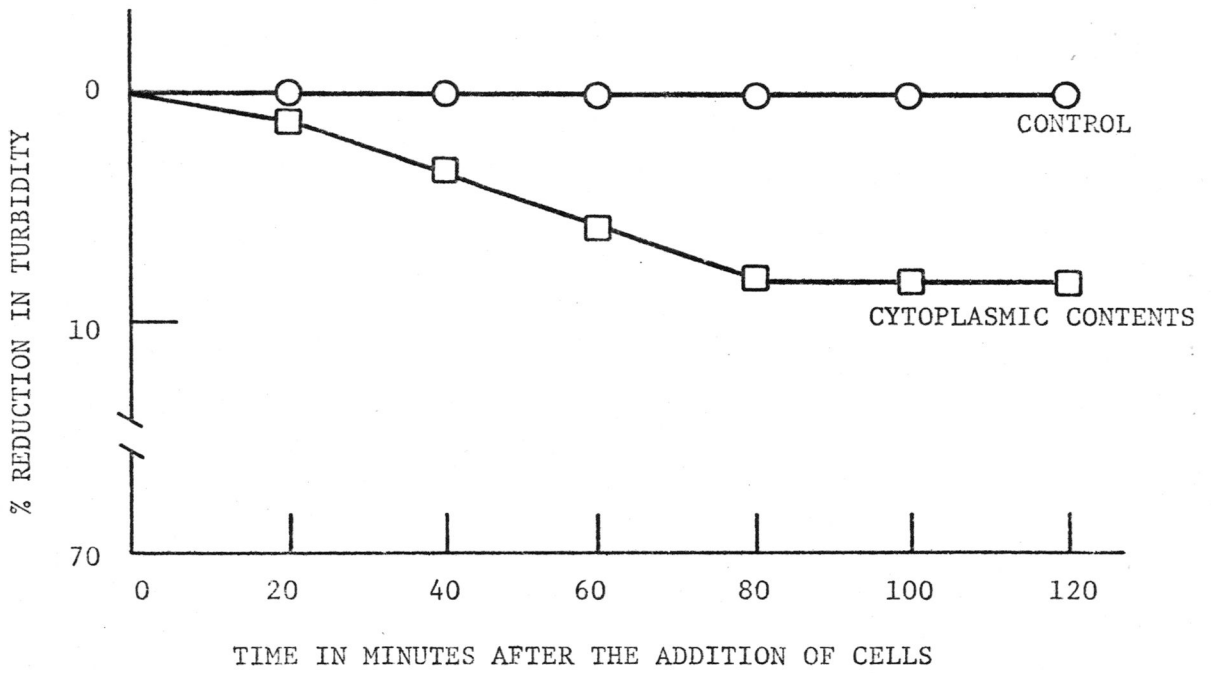FIGURE 7

7-8 was four times as sensitive to PAL as mutant strain $MB^-_{2'}$, seven times as sensitive as mutant strain $2B^-tet^s$, and seven and one-half times as sensitive as mutant strain MB^-_2. The mutant strain $HB^-_{2'}$ and the parent 7-8 were both highly sensitive to PAL action. A lag in activity occurred for $MB^-_{2'}$ and PS53.

Figure 7 shows the effect of contents of mechanically lysed staphylococcal cells on whole cells. The cytoplasmic contents had negligble lytic activity for whole cells (R=0.10/min).

DNA Obtained from Cells Lysed with PAL

The results of pre- and post-treatments with other physical or chemical conditions in combination with PAL on the amount of DNA freed is shown in Table 6. Treatment with: (1) trypsin, followed by PAL; (2) PAL, followed by trypsin; (3) trypsin, freezing and thawing, and then PAL; neither increased nor reduced the DNA released. Pretreatment of cells with osmotic shock or lysozyme in combination with PAL yielded some increase in the amount of DNA released.

Transformation by DNA Obtained from PAL-Treated Cells

Three experiments to transform tetracycline resistance and DNase activity from _S. aureus_ strain $HB^-_{2'}$ to the recipient strain $MB^-(1)$ were conducted. From the 3600 colonies screened for tetracycline resistance and DNase activity, no transformants were recovered in either the transformation mixtures incubated for twenty minutes or four hours. These colonies were not screened for beta-hemolytic activity. Controls conducted simultaneously showed no evidence

of lethal effect or transduction.

Table 6. Effect of Pre- and Post-Treatment Conditions on the Amount
of DNA Released from Cells of $\underline{S}$. $\underline{aureus}$ Strain HB-2, Exposed
to PAL.

Treatment	Absorbance (600 nm)*
PAL	.110
Trypsin, PAL	.110
PAL, Trypsin	.110
Trypsin, Freeze and Thaw, PAL	.110
Lysozyme, PAL	.120
Osmotic Shock, PAL	.135

*Any increase in absorbance above the value for PAL alone (0.110)

indicated increased amount of DNA present.

DISCUSSION

Since the method of DNA preparation is critical to its transforming capacity (Lindberg et al, 1972), we felt PAL treatment may allow the cell wall of staphylococci to be removed, freeing the DNA, without destroying its transforming capacity. Lysostaphin is highly active against S. aureus but it is expensive and not readily available whereas PAL can be produced readily in the laboratory.

In 1971, Sonstein, Hammel and Bondi reported the isolation of staphylococcal PAL and determined the dose-response relationship. In their experiments, a doubling in the concentration of PAL resulted in a doubling of the rate of turbidity reduction of a suspension of cells. In the experiments reported here, a ten-fold increase in concentration of PAL resulted in a 23% increase in lytic activity (percent reduction in turbidity) as is seen in Figure 1, indicating the rate of PAL activity in our preparations was substrate limited using washed cell concentrations prepared according to Sonstein, Hammel and Bondi (1971). Since our preparations appeared sufficiently active, the experiments were not repeated using less PAL or greater substrate concentration (cell density).

Sonstein, Hammel and Bondi (1971) reported that lytic activity of PAL was greatly enhanced by the addition of reducing agents. They reported addition of 0.05M cysteine-HCL resulted in a 20% increase in PAL activity. By comparison, the addition of 0.05M mercaptoethanol to the lysis medium in the work reported here enhanced PAL activity by 53% and the addition of 0.05M cysteine-HCl enhanced PAL activity by 172% (Figure 2). Our results verified the ability of the reducing agents to enhance the PAL activity previously reported by Sonstein et al (1971). The same authors reported

the partial purification of PAL by gel filtration through a Sephadex G-200 column. Our PAL preparations purified by gel filtration through a Sephadex G-200 column showed 3 times less activity than did crude preparations (Figure 3) possibly due to retention within the column or loss of activity. The loss of activity by PAL upon gel filtration contraindicated the use of this method of purification in our experiments requiring highly active lytic preparations without regard to purity.

The effect of age and storage condition on the lytic activity of PAL was determined because it is well known that there is a slow inactivation of enzyme on standing even under the most favorable conditions (Dixon and Webb, 1964). The results showed that frozen preparations had over twice the lytic activity as lyophilized PAL preparations. Frozen enzyme preparations showed little decrease in lytic activity upon storage for two months. Therefore, freezing is the better of the two methods of preservation of PAL investigated.

The activity of preparations of PAL varied. One source of variation was the ammonium sulfate used for protein precipitation in the preparation of PAL (Figure 5). Sonstein, Hammel and Bondi (1971) did not specify the type of ammonium sulfate used for their protein precipitations. In the work reported here, ammonium sulfate (Sigma), recrystallized from EDTA solution to remove metal ions but not EDTA free, was more effective in precipitation of active PAL than either PAL obtained from protein precipitation using ammonium sulfate grade III treated to reduce metal ions (Sigma) or with enzyme grade ammonium sulfate from Nutritional Biochemical Corporation. The decreased lytic activity of PAL obtained with the latter two ammonium sulfate types could be due to its containing a greater concentration

of inhibitory metal ions. Many investigators have reported reagents,
especially ammonium sulfate, are apt to produce an appreciable inhibition
of enzymes (Dixon and Webb, 1964). Acetone was shown to be ineffective
in precipitating active PAL (Figure 5). Since a significant decrease was
observed in PAL activity when both protein precipitations were done at
24 hour intervals as compared to the lytic activity of PAL when the protein
precipitations were done at 5 hour intervals, the use of the shorter pre-
cipitation period is recommended. As reported by Dixon and Webb (1964),
enzyme purifications should be carried out as rapidly as possible. We
found the additional 38 hours between protein precipitations did result in
inactivation of the enzyme (Figure 5).

It was reported that PAL caused lysis of cells of all strains of staphy-
lococci tested (Sonstein, Hammel and Bondi, 1971). This was also true for
all strains we exposed; however, the activity of PAL was shown to vary
(Figure 6). Possibly, the differences in lytic activity of PAL on various
strains are a result of minor differences in cell-wall structure of the
strains. These strains were obtained following exposure to a chemical
mutagen and thus could possibly by cell-wall mutants. In this respect,
further characterization of PAL is needed, especially with regard to the
determination of its mode of action.

In order to demonstrate that the"PAL" we were using was phage-associated,
a cell-free extract of uninfected cells of S. aureus strain 53 was tested
on intact S. aureus cells. No lytic activity was associated with the cyto-
plasmic contents (Figure 7). This seems to indicate no PAL is present in
cells of S. aureus strain 53 unless they are infected with Typing Phage 53.

The use of S. aureus in transformation analysis of genetic fine structure

has been difficult due in part to the resistance of the staphylococcal cell wall to previously employed means of disruption. Treatment of the staphylococcal cell wall with trypsin alone, freezing and thawing, osmotic shock or lysozyme (which hydrolyzes B 1,4-glycosidic linkages in the mucopolysaccharide wall) do not produce lysis of staphylococcal cells. Treatment of whole cells with PAL in combination with these methods which would be expected to affect the cell wall, indicated that pretreatment of cells by osmotic shock and lysozyme in combination with PAL resulted in the release of slightly more DNA from the cell than PAL alone or PAL used with trypsin (Table 5).

According to Lindberg et al (1972) high nuclease activity had been considered the main obstruction in effecting transformation in S. aureus. Mutants lacking extracellular nuclease activity may make it possible to extensively utilize transformation in genetic studies of S. aureus. In the present study, nuclease-deficient mutants of S. aureus strain 7-8 were isolated after treatment with 1-methyl-3-nitro-1-nitrosoguanidine and used as recipients in subsequent transformation experiments. Mutants acquiring beta-hemolytic activity as well as having four-fold increase DNase production, as compared to the parent strain 7-8, were also obtained by the same treatment. These findings seem to correlate with those of Omenn and Friedman (1970) who noted a loss of beta-hemolytic activity with a loss of DNase production. We felt it necessary for the recipient strain of S. aureus also to be resistant to infection by Typing Phage 53. Since PAL was produced from cells lytically infected with Typing Phage 53 and subsequently used to lyse donor cells to obtain the DNA for use in transformation, there could have been viruses in the DNA preparation capable of lysing the transformants.

Three separate transformation experiments were attempted using donor
DNA obtained from cells lysed by PAL. None of the experiments yielded any
transformants of tetracycline resistance or DNase activity. One factor
that could explain the failure to effect transformation was the variability
in the concentrations of PAL in the preparations. The concentration of
PAL used to lyse donor cells was approximately one-half that used in the
determination of the effects of pre- and post-treatment conditions on the
amount of DNA freed (Table 6). Another explanation could be that the DNA
obtained using PAL for lysis was biologically inactive. The method of
Marmur (1961) has been routinely used to extract DNA for use in transforma-
tion. However, Lindberg et al (1972) reported DNA obtained by the method
of Marmur (1961) did not always have biological activity. In view of recent
findings by Rudin et al (1974) discussed below, it is also possible that
the recipient cells were not competent.

The transformation experiments were conducted for S. aureus strain 53
according to the methods described by Lindberg et al (1972) for S. aureus
strain 8325 at 37C. Rudin et al (1974) reported temperature and pH affected
competence for transformation in S. aureus strain 8325. These same authors
reported the optimal pH and temperature for transformation were 6.75 to 7.0
and 30C, respectively, and that only staphylococcal cells lysogenic for
the phage Ø11 could be transformed. Our experiments were performed prior
to the publication of Rudin et al (1974), therefore, the relationship of
lysogeny to transformation for our strains was not investigated. No evidence
of lysogeny has been shown for S. aureus strain 7-8 used in these experiments,
although, it has been repeatedly tested in our lab for evidence of prophage
by exposure of cells to UV irradiation, ethidium bromide and mitomycin C

(unpublished data). No efforts have been made to specifically identify phage Ø11 in S. aureus strain 7-8.

In view of this new information of conditions necessary for optimum competency of S. aureus strain 8325 for transformation, it would be worthwhile to repeat the transformation attempts at different temperatures and pH. It would also be interesting to investigate whether a requirement of lysogeny for a particular phage exists for competence induction in S. aureus strain 53.

SUMMARY

Crude phage-associated lysozyme (PAL) was obtained by ultrafiltration and precipitation with ammonium sulfate from a culture of <u>Staphylococcus aureus</u> PS53 (International Phage Typing Series) at the time of lysis by staphylococcal typing phage 53. Ammonium sulfate (Sigma) recrystallized from EDTA solution to remove metal ions but not EDTA free was most effective in the precipitation of active PAL compared to other preparations of this salt or acetone. Addition of the reducing agents 0.05M mercapto-ethanol and 0.05M cysteine-HCL to the lysis medium enhanced the lytic activity of PAL for this strain by 53% and 172%, respectively. Preparations of PAL obtained after gel filtration on a Sephadex G-200 column showed a three-fold reduction in lytic activity compared to the crude preparations.

Results of the effect of age and storage conditions showed from frozen preparations of PAL retained over twice the lytic activity of lyophilized samples. Little decrease in lytic activity was observed during storage in the frozen condition for two months. PAL caused lysis of all strains of <u>S. aureus</u> exposed; however, the activity was shown to vary from strain to strain. No lytic activity was associated with cell-free extracts of uninfected cells of <u>S. aureus</u> PS53 obtained by mechanical disruption and subsequently tested on intact <u>S. aureus</u> cells.

Preparations of PAL were used to produce lysis of donor cells to obtain DNA for use in transformation. Treatment of donor cells with PAL in combination with pretreatment by osmotic shock and lysozyme resulted in the release of slightly more DNA than treatment with PAL alone; PAL and trypsin; or PAL, trypsin, freezing and thawing. Nuclease-deficient, tetracycline-sensitive mutants of <u>S. aureus</u> were isolated after treatment with 1-methyl-3-nitro-1-nitrosoguanidine for use as recipients. None of the three separate attempts at transformation of tetracycline resistance and DNase activity into these mutants were successful.

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