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LARRY K. McKANE

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GROUP A STREPTOCOCCAL BACTERIOPHAGES AND LYSOGENIC CONVERSION

APPROVED BY

Joseph J. Ferretti

William Gilbert

R. E. McCallen

M. Lerner

Leon Unger

DISSERTATION COMMITTEE

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CHAPTER I

INTRODUCTION

Although participation of bacteriophages has been indicated in the pathogenesis of several bacterial diseases, the mechanisms by which this influence is initiated in the lysogenic complex has been largely unexamined. Toxinogenic Corynebacterium diphtheriae attracted extensive study following the discovery of prophage beta control on the production of exotoxin in 1951 (1), and is now considered the classic model of lysogeny involvement in human disease. The only other thoroughly investigated example of phage conversion concerns the change of antigenic determinants of Salmonella Group E following lysogeny by certain unrelated temperate phages. Most of the remaining examples were described only recently, with no fewer than 24 reports of phage conversion in the previous 15 years.

The discovery that scarlatinal strains of group A streptococci were also phage producers appeared in 1964 but the original observations on the transfer of toxinogenicity by a filterable

streptococcal agent was reported 47 years ago (2), and still awaits widespread scientific efforts to elucidate the mechanism by which phage affects the production of erythrogenic toxin. Technical difficulties in working with streptococcal phages have proven a deterent to investigators otherwise interested in the pathogenesis of the many streptococcal disease states which may be attributable to the lysogenic complex. Recent advances in streptococcal phage technology, however, facilitate the study of this phage-host interaction as a potential system for understanding the genetics and metabolism of the streptococci.

The biological properties of three temperate streptococcal bacteriophages and the effects of lysogeny on their group A streptococcal hosts are examined in this study. The principle objective is to detect possible phage conversion systems affecting antigenic determinants or extracellular products, and to further investigate the role of temperate phage in the production of erythrogenic toxin. Since the virulence is due in part to the antigenic structure and numerous extracellular products of the streptococci, an understanding of the effects of bacteriophage on the nature of these constituents will perhaps expand our appreciation of bacteriophages as "virulence factors" in streptococcal diseases.

CHAPTER II

REVIEW OF THE LITERATURE

Perhaps the earliest indication of bacteriophage influence on the production of bacterial disease was in 1893 when San felice reported that nontoxic tetanus bacilli became toxinogenic when grown in cell-free culture media containing wastes of toxigenic tetnun (3). Although the report predated the discovery of bacteriophage, it was perhaps the first example of bacteria acquiring different properties following interaction with what was subsequently shown to be phage. Possible mechanisms for phage mediated conversions include selection of a mutant genotype in a population of cells susceptible to virulent bacteriophage (4), and transduction of bacterial genes via bacteriophage capsids or virions from a previously lysed host into a recipient cell (5). Especially interesting, however, is a process by which a high frequency of bacteria infected by a specific bacterial virus, usually temperate or semitemperate, acquire characteristics not obviously pertinent to lysogeny or the productive phage cycle. This phenomenon is termed "phage conversion."

Originally designated "lysogenic conversion" by Lederberg, this phenomenon was defined as a "permanent change in one or more properties of a cell brought about by lysogenization with a temperate virus" (6). The definition was later modified to limit the event to changes unrelated to normal phage functions involving lysogeny or lysis, but still exclusively to lysogenic cells (7). Both definitions, however, failed to account for "pseudolysogenic conversion".

The distinction between pseudolysogeny and lysogeny was recognized in 1925 by McKinley (8). Three unique characteristics have since been described for the pseudolysogenic relationship: 1) the phage deoxyribonucleic acid (DNA) fails to integrate into the bacterial chromosome (9); 2) only a portion of the cells in any culture contain phage DNA (9); and 3) the phages cannot be induced (9). This phage-host relationship is found in several possible forms. The first is commonly known as the carrier state (10), resulting from a latent period of phage infection that spans several generations in the host (11, 12, 13). Phages that establish the carrier state in their hosts are labeled semi-temperate to describe the hypothetical mechanism by which partial immunity in the infected cell retards vegetative phage replication. A second form of pseudolysogeny is associated with a temporary inability to adsorb certain virulent bacterial viruses. An example of this type of relationship is found when Shigella dysenteriae is infected with virulent phage T7 (13). During lysis, an enzyme is

released that removes phage receptors from many of the uninfected cells, rendering them resistant to phage infection. The result is an equilibrium between phage and bacteria, resembling a "phage producing", or lysogenic culture. The non-lytic secretion of the filamentous phages fd and fr by Escherichiae coli accounts for a third form of pseudolysogeny (14).

The incidence of pseudolysogeny is not uncommon in nature. Twort and de Herelle's original phages proved to be pseudolysogenic. Furthermore, conversion of bacterial properties following pseudolysogeny is similar to that of lysogenic conversion. This was recognized in 1959 by Barksdale, who proposed the term "phage conversion" to include both lysogenic and pseudolysogenic changes, as well as those alterations which occur following infection with virulent mutants of lysogenic converting phages (7).

The first clear indication that phage played a role in determining phenotype of bacteria was published in 1951. The discovery that only strains of Corynebacterium diphtheriae lysogenic for, or infected with bacteriophages carrying the tox^+ gene were capable of toxin biosynthesis led to the prototype system for the study of viral induced bacterial toxins (1, 15, 16, 17). Although these lysogens acquired the herditary ability to produce diphtheria exotoxin, the implication that the tox gene itself was introduced with the infecting temperate virus (phage beta) was not unchallenged. Other

possibilities included the derepression of a gene on the bacterial genome by phage infection; the splitting of a bacterial gene coding for a nontoxic molecule by integration of prophage beta, resulting in a smaller toxic molecule; or the interaction of precursor products of the bacterial and phage genes, forming the active toxin. Several ingenuous approaches that provided evidence supporting the existence of the tox gene on the phage genome still failed to unequivocally prove this theory of genetic control (18, 19, 20, 21). Conclusive proof was provided by Murphy and coworkers who demonstrated that the genome of phage beta served as a template for the biosynthesis of active toxin in an in vitro protein synthesis system, using extracts of Escherichia coli (22).

Much of the knowledge concerning phage conversion is derived from extensive scientific investigation of the Corynebacterium prophage relationship. Characteristics of this system include the following:

1) viral DNA synthesis is accompanied by intracellular appearance of toxin (23);
2) extracellular toxin is released before lysis (23);
3) toxin synthesis stops before lysis (23);
4) conditions preventing viral maturation increase toxin yield (24).

5) ultraviolet induction of prophage beta enhances toxin production (24);

6) toxin is produced only when the iron in the medium is reduced to a critically low level (25);

7) iron deficiency retards viral maturation, thus extending the latent period of phage infection (23);

8) only one antigenic type of toxin is produced by several species of Corynebacterium lysogenic for prophage beta (23);

9) toxin is synthesized during the lytic cycle of a virulent mutant of prophage beta (20);

10) toxin is produced whether phage is integrated or free in the cytoplasm, regardless of the state of the cytoplasmic repressor of phage replication (26);

11) replication of phage DNA is not necessary for toxin production (27);

12) kinetics of toxin production of a temperature sensitive mutant of phage beta at restrictive temperature indicates that only parental phage DNA functions in toxin production (27);

13) no supression of toxin synthesis occurs when iron is added to the E. coli in vitro toxin synthesis system (22); and

14) a specific inhibitor of the expression of the tox gene is present in both lysogenic and nonlysogenic strains of C. diphtheriae (22).

A second intensively studied phage conversion system was discovered in 1953, and involved the somatic antigens of Salmonella anatum and S. typhimurium (28, 29). At least nine temperate phages were capable of converting Salmonella O antigens to new specificities. The transition was detected serologically within five minutes following phage infection and persisted as long as the cells contained the prophage (30). Once lysogeny was established, the lysogens were no longer able to absorb homologous phages, since the lipopolysaccharide (LPS) O antigen was the specific phage receptor. These new antigens have been subjected to thorough chemical analysis, revealing various changes in the terminal trisaccharides of the LPS molecules. The conversion of S. anatum E_1 strain to E_2 by phage epsilon 15 (E^{15}) involved the loss of an acetyl group from galactose of the O antigen, and concomitant conversion of the α-galactose linkage to a β-linkage. A second conversion system, working in concert with the E^{15} example, was discovered by Uchida who noticed S. anatum E_1 could be infected by phage E^{34} only when first converted to S. anatum E_2 by phage E^{15} (32). Phage E^{34} was capable of further conversion of E_2 cells to E_{34} cells. The O antigen resulting from E^{15} infection was shown to be the substrate for chemical modification to

the antigen characteristic of group E_{34} Salmonella, a double lysogen which cannot adsorb either phage (30). This step involved the glucosylation of the E_2 trisaccharide moiety (33), requiring previous infection by phage E^{15} (34).

An accumulation of information has partially elucidated the genetic mechanisms responsible for these antigenic conversions. The ability of the cells cured of prophage to revert to their preinfection antigenic specificities indicates that the original bacterial gene(s) governing antigenic specificities were not replaced by phage-associated genetic elements (35). Neither integration of phage into the bacterial chromosome, phage induction, or phage replication were necessary prerequisites to conversion by phage E^{15}, since virulent phage mutants and lysogen mutants incapable of producing infective phages still produced antigen 15 and failed to adsorb phage E^{15} (29, 36, 37).

Following phage infection with E^{15}, three genetic functions appear to affect the resulting conversion:

1) the transacetylase responsible for the attachment of the acetyl group to the galactose of the original O antigen is inhibited by a cytoplasmic component which can be isolated from the lysogens (38);
2) the formation of α-linkages is somehow inhibited (38);
3) new β-linkages are generated (38).

The three functions have been found to be independent of one another, since they can be separated by mutations which affect only one of the genetic functions (39). This evidence also suggests that the structural genes for these functions reside on the phage genome.

As more phage conversion systems are reported, the potential medical significance of this phenomenon becomes increasingly apparent. This is well demonstrated by the genus Staphylococcus, which has provided investigators with the greatest number of phage conversion illustrations. The earliest report of possible antigen conversion was made in 1936 when a staphylococcal strain was described that lost the ability to adsorb the phage for which it was lysogenic, and was interpreted as representing a change in the surface structures of this organism (40). More recently, evidence was presented that group A phages caused an increased production of Panton-Valetine leucocidin by Staphylococcus aureus (41). The level of this cytotoxic product was again reduced by curing the cells of the phage, the growth rate remaining unaffected. Similarly, it has been established that coagulase producing strains of staphlococci are lysogenic, and a relationship exists between the presence of prophage and production of penicillinase (42) and alpha toxin (42, 43). In another study, lysogenization of all 31 nontoxinogenic strains of staphylococci resulted in the acquired capacity for production of enterotoxin A, the toxic agent in staphylococcal food poisoning (44). Gamma hemolysin of

staphylococci has also been reported to be induced by lysogenic conversion (45).

Phage conversion does not necessarily imply the appearance of new characteristics, as illustrated by the lysogenic conversion of staphylococci to the loss of beta hemolysin by serological group F phages (46). This loss, however, was always accompanied by the concomitent acquisition of the ability to synthesize staphylokinase when wild type phages were involved, and both genetic loci were active in the prophage state. Another phage conversion system has been reported that results in the loss of beta hemolysis by staphylococci, with no apparent effect on staphylokinase (47). Recently, Duval-Iflah demonstrated that three L54 bacteriophages can suppress lipase production in Staphylococcus pyogenes group III strains by lysogenic conversion (48).

Three reports have described phages capable of altering production of a "tween-splitting" enzyme in staphylococcal cultures selected for virulence from hospital material (49, 50, 51). Another possible form of phage conversion was demonstrated by eight investigators, who independently showed that lysogeny of straphylococcal strains resulted in increased susceptibility to phage infection (48). Finally, there appears to be a correlation between phage 71 and the production of type 71 bacteriocin, which has been found only in phage lysates of staphylococci (52).

In addition to the previous examples, at least nine additional genera of bacteria are possibly affected by phage conversion. Clostridium botulinum type D, has been shown conclusively to require phage DEβ infection prior to the appearance of the corresponding toxin (54). Lytic release of the toxin was ruled out as an explanation for this dependence, since cell breakage of phage-free cultures failed to increase toxin levels. Toxic cultures cured of phage by acridine orange became nontoxic, but regained this property on reinfection with phage DEβ. Initial indications are that phage CEβ exerts a similar influence on the production of toxin by type C Clostridium botulinum (53, 55).

Antigenic conversion of lysogenic strains of Pseudomonas aeruginosa (56) and Rhizobium trifolii (57) appears to parallel the situation of the Salmonella O antigens. The former was initially detected by differences in opsonizing capacity of mouse serum for lysogenic and phage-free strains of Pseudomonas. Serological analysis of both P. aeruginosa and R. trifolii reveal at least one antigenic difference attributable to lysogeny. Such alterations of cell surface properties may also be responsible for the phage induced change in colony morphology of Bacillus megaterium from smooth to rough, due to a variation in which chains of bacilli pile up to form the colony (58). Bacillus subtilis may be influenced by phage conversion to the extent that pseudolysogens no longer possess

the capacity for transformation (59). Colonies of Brucella abortus change from bluish gray in the phage-free state, to white following the establishment of the phage carrier state (60). Some biological properties of Mycobacterium have been found to be altered by phage conversion (61).

Two further examples have been provided by circumventing genetic barriers. Coliphage P1CM, which carries a marker for resistance to chloramphenicol, can successfully lysogenize Pasturella pestis or P. pseudotuberculosis, conferring antibiotic resistance to virtually all the resultant lysogens. Although no viable phage could be induced from the converted strains, superinfection immunity provided an index of phage presence in the cells (60). Certain strains of Shigella dysenteriae are receptive to coliphage T7, which establishes a pseudolysogenic relationship in this host. The normally lactose-negative Shigella are converted to lactose positive as long as the cell harbors the phage, and persists even when the T7 stock has been grown for several generations on the Shigella strain itself, i.e., in the absence of a bacterial marker (62).

Coliphages are also mediators of phage conversion in their native host species. Lysogenic conversion by phage P2 results in an increased sensitivity of E. coli to 5-fluorodeoxyuridine (63). Cell division in E. coli has also been shown to be influenced by phage, in that replication of the bacterial chromosome is under the control of

phage P2 (64). Lysogens of prophage lambda are capable of blocking superinfection or multiplication of phage T4rII, whereas nonlysogens are sensitive to T4rII infection (65). If this can be shown to be unrelated to phage P2 replication or maintenance of lysogeny, then it is a true phage conversion event (the gene is separable from the CI repressor region by cis-trans analysis) (65). Perhaps viral interference, or superinfection exclusion, may be considered a form of phage conversion common to nearly every phage sensitive species of bacteria.

Inhibition of transformation to streptomycin and rifampicin resistance in group H streptococci by lysogeny is similar to the system in Bacillus subtilis, and may prove to be another example of true phage conversion (66). Another potential phage conversion system of the genus Streptococcus concerns Streptococcus mutans, one study establishing a 100 percent correlation between cariogenicity and phage production following induction with ultraviolet light, mitomycin C, or N-methyl-N'-nitro-N-nitrosoguanidine (NG) (67). In addition, all phages isolated in the investigation were morphologically similar. However, attempts to repeat these experiments have failed in several laboratories (J. J. Ferretti, personal communication).

Various streptococcal bacteriophages have also been found to affect changes in group A streptococcal characteristics. One such example is the formation of phage-hyaluronidase following infection by any one of several temperate phages capable of penetrating the

bacterial hyaluronic acid capsule. This hyaluronidase is immunologically distinct from the bacterial enzyme produced by phage-free strains, and appears to be a product of phage multiplication, in a manner similar to the synthesis of phage-associated muralysin by lysogenic group C streptococci following induction (68). A second example of phage influence on streptococcal extracellular products is the reported relationship between infection with temperate phage and the occurrence of fibrinolysin in the medium (69).

Cellular structures of the streptococci have also been found in an altered state following viral infection. Virulent phage influence led to the development of group A streptococcal populations having two of the characteristics of the most virulent strains, i.e., possession of M antigen and ability to produce hyaluronic acid capsules (71). Since virulent group A phages lack the capacity for capsule penetration, this effect would seem partially the result of selection by the phage for the resistant encapsulated cells. A high rate of change to mucoid "survivors" (characteristic of M antigen presence and encapsulation) suggests factors other than straight mutation and selection, perhaps an induction phenomenon due to a phage-host interation (70).

Another cellular structure, namely, the group specific carbohydrate, may be modified by phage influence. Zabriskie reported a nonlysogenic group A strain ($T25_3$) was changed from phage

A25 sensitive to A25 resistant (characteristic of group A-varient mutants) following infection by a temperate phage isolated from an A-variant strain (B940) (72). Although Zabriskie could not repeat this observation, Fischetti's report of an 83 percent lysogeny rate among A-variant strains supports Zabriskie's findings (72).

In 1927 Frobisher and Brown reported that a filterable agent isolated from scarlatinal strains of hemolytic streptococci could induce the formation of erythrogenic toxin in nonscarlatinal strains (2). Two decades later, these observations were confirmed by Bingel (73). Finally, in 1964, Zabriskie reported his findings on the role of phage in the production of erythrogenic toxin (74, 75). His data indicated a similar phage-host relationship as that for the C.diphtheria studies. Using skin tests on shaved rabbit backs and subsequent erythematous responses as an index of toxin production, he demonstrated possible phage-directed toxin synthesis. A "cured" strain of scarlatinal streptococci which he found incapable of both phage and toxin production was restored to toxinogenicity on reinfection with the original phage. A marked enhancement in free phage and a 20 fold increase in erythrogenic toxin concentration were detected following irradiation with ultraviolet light.

Lytic release of preformed intracellular toxin fails to explain the mechanism of conversion since the peak toxin activity was detected 6 to 7 hours after the appearance of maximum phage titer.

In addition, lytic infection of non-scarlatinal strains with virulent bacteriophage, and cellular disruption studies revealed no change in the amount of toxin released following these procedures. The possibility that ultraviolet irradiation might elevate the level of all extracellular products in general was eliminated by measurement of streptococcal deoxyribonuclease (DNase) levels released from both lysogenic and phage-free strains following exposure. The culture supernatant fluid of the phage free strain was reported to produce twice the DNase of the lysogen. Zabriskie speculates that this decrease of DNase production following lysogeny may reflect the redirection of cellular processes toward the production of toxin at the expense of other extracellular products (72).

The nature and properties of erythrogenic toxin has attracted considerable study. The toxin has three immunological forms, types A, B, and C (77); type A being the best understood. This toxin is a heat labile glycoprotein with a molecular weight of 29,000 daltons and containing about 20 percent hyaluronic acid. One of the major difficulties in toxin related studies is development of a convenient assay technique. Whereas the purification of diphtheria toxin was aided greatly by in vitro toxin-antitoxin flocculation tests, one of the only dependable assays for erythrogenic toxin is still the skin test devised by its discoverers, the Dicks, in 1924 (78). A problem in the reliability of skin testing arises from evidence

indicating that the skin reaction is a hypersensitive response rather than an expression of intrinsic toxicity (76). Kim and Watson's data indicate that a positive skin reaction is dependent on a combination of primary (intrinsic) toxicity plus a delayed hypersensitive response from an immunologically appropriate host (76). Thus, skin test results perhaps depend as much on host factors as on the presence of toxin itself. This rather crude technique has been supplemented with other in vivo assays, such as Watson and Kim's pyrogenic assay (76). None of these methods provide a convenient and indisputable technique for toxin detection.

The biological and clinical significance of erythrogenic toxin is perhaps concealed by its principle name, which reflects its role in the production of the skin rash of scarlet fever, but fails to describe additional biological pathological properties of this agent. Kim and Watson emphasize that the decline in scarlet fever incidence due to immunization is not accompanied by a concomitant decline in the clinical impact of the toxin because of its even greater potential to modify host responses to other infectious agents, as well as to group A streptococci (76). In addition to finding enhanced susceptibility to fatal endotoxic shock, Watson proposed that the toxin may account for tissue damage leading to rheumatic fever (79, 80). No fewer than 11 biological properties associated with the erythematous activity of the toxin. These include cytotoxicity, i.e., inhibition of adult rabbit

macrophages (81) and depression of reticuloendothelial function (82); myocardial and liver necrosis (83); altered membrane permeability (84); and immunosuppression (85).

As already indicated, the possibility for enhanced virulence of the streptococci due to viral influence is not restricted to conversion to the toxic state. This was perhaps first indicated in 1933, when Evans attempted to treat mice infected with streptococcal phage. He discovered the phage-treated mice expired more rapidly than the untreated controls (86). The incidence of lysogeny did, in fact, correspond directly with occurrence of many group A streptococcal diseases, although not generally true for all such outbreaks. Maxted established that the average detection rate of lysogenic strains of group A streptococci was 25 to 30 percent (70), in studies using one or two indicator strains. During epidemics, however, this number increased to a 70 percent frequency of lysogeny of the strains isolated (87), and even 100 percent in other studies (72).

Frequency studies such as these have attempted to establish a relationship between phage influence and the development of non-suppurative post streptococcal sequellae; i. e., acute glomerulonephritis (AGN) and rheumatic fever. The pathogenesis of these diseases appear to be related since both maladies occur following streptococcal infection and both seem to be related to immunological responses of the host. The epidemiology of post streptococcal

glomerulonephritis indicates predominant involvement of type 12 streptococci, whereas, rheumatic fever can follow streptococcal infections of nearly every M type. Post streptococcal glomerulonephritis is described as being an immune complex disease. Among the four proposed mechanisms for AGN pathogenesis, all but one involve a specific streptococcal antigen that either cross reacts with, or is deposited in some complexed form on the mesangium or basement membrane of the glomerulus (88, 89, 90, 91, 92, 93, 94). The alternative explanation suggests an extracellular streptococcal product that reacts with glomerular antigen and causes it to be recognized as foreign (88, 95). Similarly, the pathogenesis of rheumatic fever may be related to an immunological host response, but the evidence is fragmentary. Results of decades of experimental pathology have yet to contribute significantly to an understanding of this disease mechanism.

Antigens with common immunological specificities do in fact reside on the streptococcal cell and the organs involved in these diseases. The group A carbohydrate of streptococci, for example, cross reacts immunologically with mucoprotein of the heart valve (96).

This antibody is elevated in patients with rheumatic heart disease, and declines following surgical excision of the valve. In addition, antibody to M-associated protein (MAP) of group A

streptococci is found in elevated titer in serum from patients with rheumatic fever (97).

It is not difficult to imagine mechanisms of phage influence on the pathogenesis of these sequellae. Lysogeny could, as in the salmonella antigen conversion example, result in the production of antigens characteristic of nephritogenic or rheumatogenic strains. Such strains could also be the result of induction of an extracellular nephritogenic or rheumatogenic substance via phage conversion. Streptolysin O, for example, is known to be cardiotoxic (98). Lysis of susceptible bacteria by certain phages may also contribute to the disease process by exposing new streptococcal antigens. In addition, certain cytolytic substances, i.e., bacteriocins, may be involved in the pathogenesis of rheumatic fever (88), and AGN (100, 101). Bradley's report that bacteriocins are in reality fragments or portions of phage tails lends further support for phage participation in these diseases (102).

Only a few studies have revealed a phage associated pattern consistent with the incidence of these sequellae. Rammelkamp reported those types of group A streptococci most commonly associated with acute AGN were more susceptible to the lytic action of a type 12 virulent bacteriophage (103). These results were supported by similar findings of Potter (104), and later Leonova (105), both using type 12 temperate phages in independent investigations.

Zabriskie proposes that the nephritogenic strains have possibly been cured of lysogeny and become sensitive to phages of the type-specific strain (76). Loss of lysogeny could in turn result in derepression of the genetic information coding for the nephritogenic character(s). Alternatively, according to Maxted, this relationship may reflect type specificity of certain phages rather than an association of specific viral action with nephritogenic strains (106). Another report compared the incidence of lysogeny in strains isolated from areas where there was little or no glomerulonephritis or rheumatic fever and areas where the occurrence of these sequelae was high (72). The incidence of lysogeny was 10 percent and 88 to 100 percent, respectively, suggesting a strong association of lysogeny in areas of streptococcal sequelae. Wannamaker and coworkers, however, could find no consistent differences in several biological parameters between phages isolated from nephritogenic and non-nephritogenic strains (106). In fact, recent investigations have failed to find either common antigenic characteristics of nephritogenic strains (107), or to discriminate between streptococci isolated from patients with and without nephritis (108).

Nonetheless, the antigenic constitution of the group A streptococci has great clinical significance, and a discussion of these surface determinants is important. Several components of the streptococcal cell wall have been extensively studied, i. e.,

1) M protein and related T and R antigens, 2) group specific polysaccharride, 3) mucopeptide, and, to a lesser extent 4) glycerol teichoic acid. The M protein is the major virulence factor of group A streptococci, by virtue of its antiphagocytic properties (109), and strains lacking the M protein are unable to survive in normal human serum (97). Anti-M antibodies enhance phagocytosis while providing long lasting immunity (110). In addition, group A streptococci fall into more than 50 types on the basis of M protein precipitation reactions (111), a finding that has proven a powerful diagnostic tool. Antigens T and R have no apparent biological activity, but are epidemiologically useful.

The group specific carbohydrate forms the basis of grouping the streptococci into groups A through O by agglutination and precipitation with specific antiserum (111). Although most human pathogens are group A or A-variant, group B streptococci have been identified as opportunistic disease producers in urinary tract infections and meningitis (112-115). Other groups associated with A-variant rare human pathogenesis are C, G, and O (115). The group carbohydrate is immunologically distinct from group A. Evidence indicates that the immunodominant sugar residues of the group A carbohydrate are N-acetyl glucosamines linked to a backbone of rhamnose moieties (116). The A-variant molecule on the other hand,

contains no terminal amino sugar residues, the antigenic specificity being determined by the rhamnose moiety itself (116, 117, 118).

The group A antigen is localized as a discrete band exposed to the outer surface of the cell wall (183). It immunologically cross reacts with the hexosamine polymer of the mucopeptide cell wall (119), which has been found to have many biological and toxic properties similar to endotoxin of gram negative bacteria, including pyrogenic, necrotic, and hemolytic activities (120, 121). Group A streptococcal mucopeptide also provides a local Shwartzman reaction (121) and causes extensive carditis in microgram amounts in rabbits (121). Injection of streptococcal mucopeptide into mice enhances resistance to subsequent challenge with virulent group A streptococci (122), but may also intiate shock and death (121). Although immunologically similar, chemical similarities between mucopeptide and group A carbohydrate are restricted to common N-acetylglucosamine residues (110). The previously discussed indication of bacteriophage influence on the construction of group specific carbohydrate, however, acquires increasing significance in light of these similarities.

Phage influence on the severity of streptococcal disease may also depend on transfer of genetic material from one cell to another via a bacteriophage vector, i. e., the process of transduction. Although no conclusive evidence is available to prove that

transduction contributes to the morbidity of bacterial infections in humans, many genetic markers have been transduced that may well be related to the development and treatment of disease. The receipt of genetic information for resistance to such antibiotics as streptomycin, erythromycin, and bacitracin has obvious implications in both the treatment and the identification of group A streptococci. Since antibiotic resistant cells tend to be less able to survive following natural mutation (123), transduction could yield an antibiotic resistant cell which possesses the survival potential of the parental wild type.

Transduction is also proving to be a valuable tool for the elucidation of streptococcal genetics. Phage-mediated genetic transfer was discovered in group A streptococci in 1968 by Leonard and coworkers who reported both temperate and virulent phage mediated transfer of streptomycin resistance (124). Later the number of transductants by virulent phage was increased 10-fold by irradiating phage lysates with ultraviolet light prior to adsorbtion (125, 126). No enhancement occurred, however, by irradiating temperate phage lysates. Malke confirmed these findings in 1969 (127) and increased transduction frequencies by 1) lowering the multiplicity of infection (moi) (127, 125, 126), and by 2)preventing superinfection lysis of the transductants. He accomplished this second task by 1) employing a doubly temperature sensitive phage mutant and transducing at the

restrictive temperature; and 2) taking advantage of the protective role of the hyaluronic acid capsule in preventing phage infection. With this technology, the list of markers transduced increased to include resistance to ultraviolet light irradiation and at least 10 additional antibiotics (128, 129). (J. G. Stuart and J. J. Ferretti, personal communication). Furthermore, linkage relationships have been established by Malke between $\underline{kan}^R$ and $\underline{str}^R$, $\underline{ery}^R$ $\underline{lin}^R$ and $\underline{spec}^R$ (129),and by Stuart and Ferretti between $\underline{rif}^R$ and $\underline{stl}^R$ (personal communication). The only additional marker to be transduced has been the genome of a prophage in a virulent phage mediated transfer (130), i. e., "transfer induction" (125). The same study provided evidence suggesting that lysogenic strains of streptococci were more efficient donors but less efficient recipients in transduction. A partial explanation may be supplied by the finding that interference with virulent phage propagation was a widespread property of streptococcal prophages.

Intergroup transduction has been described between group A and group C streptococci (131) as well as group A and group G strains (132). At least two studies indicated that recipient efficiency in transductional exchanges is related to M type (133, 134). In both reports only 50 percent of the M types tested served as recipients, although the virulent transducing phages rapidly adsorbed to all the strains employed.

Progress in such genetic studies of group A streptococci and the corresponding phages have been hampered by a number of technical barriers. The chaining property of the organisms complicates selection of pure cell lines and the fastidious nature of the streptococci restricts the use of nutritional genetic markers as well as interfering with the formation of confluent bacterial lawns essential for detection of phage plaques. In addition, this phage-host system is often remarkably unpredictable.

Recently, however, some of these difficulties have been circumvented. In studies in the 1950's Kjems incorporated ascitic fluid in his medium (135), and Krause later introduced a dialysate broth which eliminated the need for ascitic fluid (69). Both offered a good nutritional source favoring plaque formation. Todd-Hewitt agar supplemented with normal horse serum produced excellent plaque morphology in the group A phage studies of McKane and Ferretti (150). Mickelson found success in growing group A streptococci in chemically defined medium (136), which was subsequently modified by Friend and Slade to support the propagation of virulent bacteriophages (137). Temperate phage plaques are best detected on a medium that stimulates the production of hyaluronic acid capsules, which yields a mucoid lawn. The dissolved capsular material surrounding the central plaque appears as two distinct halos that aid in plaque detection. These zones were first described by

Kjems (138) for whom the effect is named. Media advantageous for this purpose includes the ascites-supplemented solid medium of Kjems (135) and serum Todd-Hewitt (STH) agar described by McKane and Ferretti (150). Plaques developing on STH agar at 30C possess an additional halo than described by Kjems, and lacked all zones following incubation at 37C. The problem of chain formation was partially resolved by the use of mild sonification (150) or forced pipe-pipetting through the fine orifice of a pipette (127). Chaining still represents a major handicap to investigators of streptococcal genetics and phages.

Another difficulty involved the finding of a suitable phage-free indicator strain of streptococci that would accept heterologous phages. Kjems solved this problem with the selection of a purely mucoid type 12 bacterial strain called K56 which was apparently phage-free since it showed distinct plaques when infected with a broad spectrum of group A bacteriophages (139). Even this strain, however, has been reported to contain phage (R. M. Cole, personal communication). Nevertheless, strain K56 is now accepted as the "universal" indicator for group A phage studies.

Since the pioneer studies of Evans (140-145) and, later, Kjems (87, 135, 138, 139), two categories of group A streptococcal bacteriophages have been described. The virulent phages, originally isolated from sewage (135), are heat stable particles that

require both enzymatic removal of the hyaluronic acid capsule prior to infection, and a living cell for adsorbtion (146). The temperate phages, obtained from cultures of streptococci in 1955 (135), are thermolabile viruses that readily penetrate the bacterial capsule and adsorb to both viable and heat killed cells, as well as to isolated cell walls (146). One step growth curves indicate that both virulent and temperate phages of group A streptococci possess abnormally long latent periods (50-60 min) and small burst sizes of 18-32 plaque forming units (PFU) per infected cell (139, 147, 148). In one study, temperate phages proved more fragile than the virulent phage to moderate physical forces of ultracentrifugation and sonification. Chloroform also exerted a deleterious effect on preparations of the temperate bacteriophages (150). Electron photomicrographs demonstrate similar morphology among all group A phages (72, 138, 102, 148).

Both groups of phages are responsible for the appearance of new extracellular products in the medium (70, 138, 149). In the case of the virulent phages, these "extracellular" products may be due to lytic release of preformed material within the host cell, whereas temperate phages may influence the actual synthesis of at least one of these products in a yet undetermined manner (149).

Although it now appears that phage plays an important role in at least one streptococcal disease, i.e., scarlet fever, the panorama of phage conversion in this organism remains to unfold.

Our understanding of this phage-host relationship is primarily limited to certain aspects of viral replication, as very little information on the bacterial response to phage infection has accumulated. Even the simplest phage-associated bacterial changes await definitive explanation. Many of these viral induced differences elude detection because of inadequate methodology, and yet the host properties may be profoundly influenced by these seemingly subtle alterations.

CHAPTER III

MATERIALS AND METHODS

Organisms

The strains of Streptococcus pyogenes and bacteriophages employed in this study are listed in tables 1 and 2. Bacterial indicator strains for phage assays included K56, a small colony type 12 variant from the Copenhagen phage studies (139), and $T25_3$, a derivitive of strain T25-41 lacking at least one prophage, as well as the ability to produce erythrogenic toxin. The corresponding lysogens of K56; i.e.,K56(2172) and K56(H4489A) contained one of two temperate bacteriophages obtained from streptococci involved in glomerulonephritis epidemics in Cleveland, Ohio and Red Lake, Minnesota. Another lysogen, $T25_3$(T12gl), contained a temperate converting phage that transfers the capacity to produce erythrogenic toxin (75). This phage is referred to as phage T12, denoting its original toxinogenic bacterial donor, strain T12gl. The T12 phage used in this study, however, was isolated in this lab from the $T25_3$(T12gl) strain constructed by J. B. Zabriskie and obtained from Lewis Wannamaker. Another organism, NY5-type 10, elaborated two immunologically distinct toxins, A and B. This strain is a common

TABLE 1

STRAINS OF GROUP A STREPTOCOCCI
USED IN THIS STUDY

Strain	Type	Source	Description and Reference
K56	12	Kjems	broad spectrum phage indicator (139)
K56(2172)	12	McKane & Ferretti	K56 lysogen of phage 2172 (150)
K56(H4489A)	12	McKane & Ferretti	K56 lysogen of phage H4489A (150)
$T25_3$	25	Zabriskie	$\underline{tox}^-$ derivitive of T25-41 "cured" of phage (139)
$T25_3$(T12g1)	25	Zabriskie	$\underline{tox}^+$ $T25_3$ lysogenized by phage T12 (139)
$T25_3$(T12)B	25	McKane	$T25_3$ lysogenized by phage T12 (this study)
NY5type10	10	Dochez & Stevens	$\underline{tox}^+$, produces both type A and B toxins (185)
$T25_3$(H4489A)	25	McKane	$T25_3$ lysogen of phage H4489A (this study)

TABLE 1--Continued

Strain	Type	Source	Description and Reference
9440*str-10*	6	Wannamaker	streptomycin resistant derivitive of 9440 (131)
K56*str-10*	12	Wannamaker	streptomycin resistant derivitive of K56 (131)
9440*AB*^{R}*	6	Stuart & Ferretti	derivatives resistant to either streptomycin (AB=*str*^{R}), rifampicin (AB=*rif*^{R}), erythromycin (AB=*ery*^{R}), or spectinomycin (AB=*spec*^{R}) (this study)
K56*AB*^{R}*	12	McKane	
K56(2172)-*AB*^{R}*	12	McKane	
K56(H4489A)-*AB*^{R}*	12	McKane	

tox^{-} produces little or no erythrogenic toxin

tox^{+} produces erythrogenic toxin

**AB*^{R} antibiotic resistance to either streptomycin (*str*^{R}), rifampicin (*rif*^{R}), erythromycin (*ery*^{R}), or spectinomycin (*spec*^{R}).

TABLE 2

STRAINS OF BACTERIOPHAGE USED IN THIS STUDY

Strain	Type	Source	Description and Reference
2172	12	Wannamaker	isolated from "nephritogenic" streptococci (131)
H4489A	49	Wannamaker	isolated from "nephritogenic" streptococci (131)
T12	12	Griffith	isolated from *tox*$^{+}$ T12gl (151)
T12*cp*^{1}	12	McKane	clear plaque mutant of phage T12 (this study)
H4489A *vir*^{1}	49	McKane	virulent mutant of phage H4489A (this study)
A25	25	Boulgakov	virulent phage isolated from sewage (70)
A25*ts*$^{1-2}$	25	Malke	propagates at 30 C but not at 37 C (127)

source of many standard preparations of erythrogenic toxin. $T25_3$ was relysogenized in this laboratory by phage T12 to construct the strain $T25_3$(T12)B, and by phage H4489A to produce $T25_3$(H4489A). A mutant of phage T12 was selected for its ability to form clear plaques on a bacterial lawn of strain $T25_3$. This phage, called T12<u>cp</u>^{1} is capable of lytic infection only. A similar virulent mutant of H4489A was selected for its capacity to lyse K56(H4489A), a lysogen immune to superinfection by wild type H4489A. Phage A25<u>ts</u>$^{1-2}$, a double temperature sensitive mutant of virulent phage A25, completes the list of bacteriophages.

Group A streptococcal strains resistant to various antibiotics were also utilized. These mutant strains included K56<u>str</u>-10 and 9440<u>str-10</u>, both streptomycin resistant strains isolated as subsurface colonies in agar containing 2.0 mg/ml streptomycin sulfate (L. Wannamaker and S. Skjold; and J. Stuart and J. Ferretti). Strains of K56 and 9440 resistant to the antibiotics rifampicin (0.15mg/ml), erythromycin (0.20 mg/ml), and spectinomycin (0.06 mg/ml) were obtained by Stuart and Ferretti (123). Antibiotic resistant strains of K56, K56(2172),and K56(H4489A) were constructed by transduction of the desired genetic markers from an appropriate 9440<u>AB</u>^{R} donor.

Media

The standard liquid medium (PP broth) chosen for bacterial growth and phage propagation contained 6.0 percent proteose peptone #3

(Difco) and 0.3 percent NaCl (these and all subsequent quantities are final concentrations). Prior to autoclaving (15 lb. pressure for 20 minutes) Na_2HPO4 (0.38%) was added to the medium, after which sterile solutions of each of the following were added: 0.02% $CaCl_2$, 0.05 percent glucose, and 5.0 percent normal horse serum (Gibco). Hyaluronidase (0.1 mg/ml) was added to PP broth for the propagation of virulent phages A25 and A25ts[1-2] on the bacterial strain K56. An alternate liquid medium for bacterial growth was unsupplemented Todd-Hewitt Broth (Difco); this medium, however, was unsatisfactory for phage propagation.

A variety of liquid media were employed for growing tox$^+$ strains when detection of scarlatinal exotoxin was desired. The primary broth for this purpose was a dialysate of Todd-Hewitt medium (TH dialysate) prepared by dialyzing 100 ml of 10 fold concentrated Todd-Hewitt broth (30 g/100 ml distilled H_2O) in 500 ml distilled water for 16 hours at 4.0 C. The dialysate was then autoclaved and completed with the addition of sterile glucose (0.05%). A second medium for this purpose was a modified infusion broth of Stock (153, 154). Brain heart infusion (45.0 g/ 200 ml distilled H_2O) was infused for one hour at 50 C, or until dissolved, and subsequently dialyzed against one liter distilled H_2O for 48 hours at 4 C. Following autoclaving, 50 ml of sterile Stocks buffer was added. The Stock's buffer consisted of glucose (3.0 g), NaHCO3 (2.0 g), Na_2HPO_4 (1.2g), and

TABLE 3

COMPOSITION OF MICKELSON MEDIUM (136)

Component	mg/ml	Component	mg/ml
Amino acids		B vitamins	
Alanine	0.1	Folic acid	0.005
Arginine	0.1	Biotin	0.0025
Asparagine	0.1	p-Aminobenzoic acid	0.1
Aspartic acid	0.1	Thiamine	0.5
Cysteine HCl	0.05	Riboflavine	0.5
Cystine	0.05	Pyridoxal HCl	1.0
Glutamic acid	0.5	Pyridoxamine	0.5
Glycine	0.2	Ca-pantothenate	0.5
Histidine HCl	0.2	Niacin	1.0
Hydroxyproline	0.1		

TABLE 3--Continued

Component	mg/ml	Component	mg/ml
Isoleucine	0.1	Salts	
Leucine	0.1	K_2HPO_4	0.5
Lysine HCl	0.1	KH_2PO_4	0.5
Methionine	0.1	$MgSO_4$	0.1
Norleucine	0.1	$FeSO_4 7H_2 0$	0.004
Phenylalanine	0.1	$MnSO_4 4H_2)$	0.005
Proline	0.1	$ZnSO_4 7H_2O$	--
Serine	0.1	NaCl	0.01
Threonine	0.1	$Na(C_2H_3O_2)_2 3H_2O$	10.0
Tryptophan	0.1	Glucose	10.0
Tyrosine	0.1		

TABLE 3--Continued

Component	mg/ml	Component	mg/ml
Valine	0.1	pH	7.0
Glutamine	--		
Adenine	0.01		
Guanine	0.01		
Uracil	0.01		

Amounts are expressed as final concentration.

TABLE 4

COMPOSITION OF CHEMICALLY DEFINED MEDIUM
OF LEONARD, ET AL (157)

Component	Amount g/liter	Component	Amount g/liter
L-amino acids		Purines and pyrimidines	MS6
Arginine	0.6	Adenine	0.01
Methionine	0.2	Guanine	0.01
Valine	0.2	Uracil	0.01
Leucine	0.2		
Isoleucine	0.2	Salts	
Lysine	0.2	NaCl	0
Serine	0.2	K_2HPO_4	12.0
Tyrosine	0.4	KH_2PO_4	5.2

TABLE 4--CONTINUED

Cysteine	0.2	$(NH_4)_2SO_4$	2.0
Histidine	0.2	$MgSO_4\ 7H_2O$	0.2
Cystine	0.05	$MnSO_4\ H_2O$	0.01
Phenylalanine	0.2	$FeSO_4\ 7H_2O$	0.01
Glycine	0.2	$NaHCO_3$	5.0
Tryptophan	0.2	Glucose	10.0
Glutamic acid	0.5	Water (double distilled) to make 1 liter.	
Alanine	0.2		
Proline	0.2		
Threonine	0.2	pH 7.6	
Aspartic acid	0		
Vitamins			
Niacinamide	0.01		

TABLE 4--Continued

Vitamins		
Thiamine	0.01	
Riboflavin	0.01	
Ca-Pantothenate	0.01	
Biotin	0.003	
p-aminobenzoic acid	0.002	
Pyridoxal .HCl	0.002	
Pyridoxamine	0.002	
Folic acid	0.002	pH 7.6

TABLE 5

SYNTHETIC MEDIUM FOR THE GROWTH OF THE DEXTRAN-PRODUCING STREPTOCOCCI (158)

Amino acids (g/l)		Purines and pyrimidines (mg/l)	
Arginine	0.15	Adenine	5.0
Cysteine HCL	0.05	Guanine	5.0
Cystine	0.05	Uracil	5.0
Glutamic acid	2.00		
Glycine	0.60	Vitamins (mg/l)	
Histidine	0.20		
Isoleucine	0.20	Folic acid	25.0
Leucine	0.40	Biotin	62.5
Lysine	0.60	PABA	100.5

TABLE 5--Continued

Amino acids (g/l)		Purines and pyrimidines (mg/l)	
Methionine	0.20	Thiamine	500.0
Phenylalanine	0.20	Riboflavin	1000.0
Serine	0.10	Pyridoxal HCL	1000.0
Tryptophan	0.10	Calcium Pantothenate	500.0
Tyrosine	0.05	Niacin	1000.0
Valine	0.30	Pyridoxamine	1000.0
Salts (g/l)		g/l	
K_2HPO_4	0.5	Glucose	10.0
KH_2PO_4	0.5	Sodium Acetate	10.0
$MgSO_4$	0.4		

TABLE 5--Continued

Amino acida (g/l)		Purines and Pyrimidines (mg/l)
NaCl	0.02	
$FeSO_4$ $7H_2O$	0.02	
$MnSO_4$ $4H_2O$	0.02	

TABLE 6

COMPOSITION OF HENDERSON AND SNELL'S MEDIUM (156)

Component	Quantity per 100 ml.	Component	Quantity per 100 ml.
Glucose	2 gm.	Ca pantothenate	100
Sodium citrate (U.S.P. XII)	2 gm.	Niacin	100
Sodium acetate (anhydrous)	0.1 gm.	p-Amininobenzoic acid	20
NH_4Cl	0.3 gm.	Biotin	1
K_2HPO_4	0.5	Folic acid	1
Salts C	2 ml.	DL-Alanine	100 mg.
Adenine sulfate	1 mg.	DL-Aspartic acid	100 mg.
Guanine hydrochloride	1 mg.	L-Glutamic acid	100 mg.
Uracil	1 mg.	L-Arginine-HCl	20 mg.
Xanthine	1 mg.	L-Lysine-HCl-H_2O	20 mg.

TABLE 6--Continued

Component	Quantity per 100 ml.	Component	Quantity per 100 ml.
Thiamine	100	Other amino acids L forms	10 each
Riboflavin	100	Or DL forms	20 each
Pyridoxal	20		

l-glutamine (200 mg) dissolved in 50 ml distilled H_2O. A dialysate of proteose peptone #3 (PPD), prepared as was THD but using 60 g/ 100 ml H_2O, was the final medium used for toxin studies.

Chemically defined (synthetic) liquid media were prepared using the four protocols in tables 3-6. With one exception, solutions of glucose and phosphate buffer (pH, 7) were autoclaved separately to supplement the cooled sterile media. The procedure of Leonard, et al., however, called for solutions of glucose, $FeSO_4$, $MnSO_4$, and $NaHCO_3$ to be sterilized by passage through Millipore filters (0.45μ) and added to the medium immediately prior to its use. The pH of all media was adjusted to 7.4 with 1.0 N HCl or NaOH. Further enrichment to enhance growth included addition of the amino acids alanine, proline, threonine and aspartic acid in final concentrations of 1.0 mg/l to those media lacking these nutrients.

The solid medium most commonly employed was Serum Todd-Hewitt (STH) agar. This medium was composed of Todd-Hewitt broth (Difco) adjusted to a pH of 6.9 prior to addition of 1.0 percent bacteriological agar (BBL), 0.038 percent Na_2HPO_4 and subsequent autoclaving. The sterile mixture was supplemented with 5.0 percent normal horse serum (Gibco), and 0.02 percent $CaCl_2$. Detection of phages A25 and A25$\underline{ts}^{1-2}$ plaques on the bacterial indicator strain K56 required the further addition of hyaluronidase (0.04 mg/ml) to STH media used for that purpose. Todd-Hewitt (TH) agar contained

the same concentrations of Todd-Hewitt broth and agar as STH agar, but lacked the other supplements. All solid media were poured into 100 mm plastic petri dishes (about 25 ml/plate), flamed to eliminate bubbles, and dried upside down with lids removed for one hour at 41 C. Ingredients of serum soft agar (SSA) corresponded to those of STH medium, with the agar reduced from 1.0 to 0.7 percent.

Cultural Conditions

Routine bacterial growth in liquid media entailed inoculating 5.0 ml PP broth from stock cultures of bacteria (these stock cultures were sustained on STH agar at 4 C, and transfered to fresh agar each 14 to 21 days). The inoculated PP broth was maintained for 16 hours at 30 C, producing a bacterial culture uniformly in the late log phase of growth. These overnight cultures were employed as daily stocks.

Several attempts were made to stimulate bacterial growth in synthetic media:

1) inoculation of 0.1 ml from a daily stock bacterial culture into 10 ml synthetic medium, and subculturing 1.0 ml inocula each 24 hours at 37 C;
2) adding decreasing amounts of PP broth to fresh synthetic medium with each daily transfer, until growth occurred in unsupplemented synthetic medium; and
3) incubating inoculated cultures in the reduced oxygen tension of a candle jar.

Phage propagation was accomplished by simultaneous inoculation of one drop from a phage preparation containing 10^8 plaque forming units (PFU)/ml, and one drop of an overnight culture of the appropriate bacteria to 5.0 ml of sterile PP broth. With the exception of temperature sensitive phage strain A25ts$^{1-2}$, all phages were propagated at 37 C, centrifuged, and the cells removed by filtration (all filtering procedures employed Millipore membranes with a pore size of 0.45μ). Phages H4489A, 2172, and A25 were propagated on bacterial strain K56 for 4 hours, whereas phage T12 required an additional 2 hour incubation with bacterial strain $T25_3$ for proliferation. Phage A25ts$^{1-2}$ was harvested following 16 hours incubation with K56 at 30 C. Both A25 and A25ts$^{1-2}$ failed to propagate on K56 unless the medium contained 0.1 mg/ml hyaluronidase to remove bacterial capsules. In attempts to obtain higher phage titers, several variations of this protocol were tested. These included 1) propagation in Todd-Hewitt broth, either containing or lacking the supplements used in PP broth, 2) propagating at temperatures of 24, 30, and 37 C, and 3) harvesting the phages after 3, 4, 5, 6, 7, 8, and 16 hour incubation.

Detection of PFU was accomplished by a modification of the phage assay technique of Wannamaker, et al. (159). Confluent bacterial lawns were produced by diluting an appropriate overnight bacterial culture 1:10 in PP broth, and flooding the surface of an STH

plate with 2.0 ml of this cell suspension. Once the excess fluid was removed, the plates were inverted and incubated at 37 C for 30 minutes without covers for drying. The plate was then divided into four sections and a drop of the appropriate phage dilution (0.033 ml) was applied with a Pasteur pipette to each quadrant. In order to determine the undiluted phage titers, the number of infectious centers within a quadrant was multiplied by 30 and adjusted for the dilution. The accuracy of this method was confirmed by comparing titers determined by utilizing calibrated pipettes in a standard agar overlay procedure (160). Assays for A25 and A25$\underline{ts}^{1-2}$ plaques required the addition of hyaluronidase (0.04 mg/ml) to the medium.

Isolation of Bacteriophage Strains

The bacterial strains listed in Table 1, with the exception of the K56 lysogens and the antibiotic resistant strains, were examined for the production of bacteriophage. Preliminary analysis consisted of filtering centrifuged supernatant fluids from PP broth cultures grown overnight at 30 C and 4 hours at 37 C. The filtrates were dropped undiluted onto separate STH agar plates containing standard lawns of strains K56, $T25_3$, $T25_3$(T12gl), 9440 $\underline{str}$-10, or NY5 type 10. Duplicate STH agar plates containing 0.04 mg/ml hyaluronidase were similarly prepared. Following incubation for 16 hours at 30 C, the surface lawns were inspected for infectious

centers. These plaques were aseptically picked, resuspended in 0.3 ml PP broth, and used as an inoculum for propagation in the same bacterial strain supporting the plaque. Standard procedures for propagation were employed, but variations included use of both TH and PP broth, and incubating at 24, 30 and 37 C. Similar preparations of resuspended phage from infectious centers were dropped on surface lawns of each bacterial strain to determine the host range of each bacteriophage, as well as the phage sensitivities of the streptococci.

In an attempt to enhance phage production by the lysogens, actively growing bacterial cultures were exposed to inducing agents. One ml of each overnight bacterial culture was inoculated into 9.0 ml PP broth, and incubated for two hours at 37 C. Five ml of these mid-logarithmic growth cultures were then irradiated under a 15-watt bar type bactericidal ultraviolet lamp for 30 seconds in open glass petri dishes (distance of 24 cm). The nonirradiated portion of the actively growing cultures received 0.1 mg/ml of mitomycin C (Calbiochem). All cultures were then reincubated at 37 C for 3 hours, and the filtrates obtained assayed for phage activity on each bacterial strain. Strain K56(H4489A), which has previously been shown to be inducible by both methods (150), was used as an indicator of induction efficiency.

Concentrating and Purifying Bacteriophage Preparations

The use of ultracentrifugation was investigated to determine its efficiency to concentrate and purify temperate streptococcal bacteriophages. The protocol was similar to that used previously in this laboratory for 2172 and H4489A (150). These two phages were also employed to determine the conditions resulting in maximum phage recovery. Four 13 ml tubes containing high titer PP broth phage preparations were centrifuged at 101,962 x g. One tube was removed each 30 minutes, the supernatant fluid extracted, and the pellet resuspended in 1.3 ml T2 buffer (162). Plaque forming activity was determined for both the supernatant fluid and the resuspended pellet. The purity of the preparations containing maximum phage concentrations was examined by disc gel electrophoresis in 7.0 percent polyacrylamide (Canalco). Protein bands were fixed in 5.0 percent trichloroacetic acid (TCA) and stained with amidoschwartz. Acetic acid (7,0%) served as an effective destaining solvent. Concentration of phages was also attempted by pervaporative dialysis in a Schleicher and Schuell colloidon bag aparatus. Twenty ml of PP broth phage preparations were subjected to constant negative pressure until a 10 fold reduction in volume occurred. The PFU/ml of both the concentrate and the dialysate were ascertained.

In order to obtain highly purified, concentrated phage preparations, two methods were employed. First, ultracentrifugation

(101,962 x g, 72 hour) in CsCl (refractive index, adjusted to 1.5) was attempted using 1.0 ml of a phage sample (7.4×10^8) concentrated and partially purified by centrifugal sedimentation (101,962 x g, 90 minutes). Fractions (0.5 ml) were collected by puncturing the bottom of the cellulose nitrate tube with a 26 gauge needle, and assayed for absorbancy at 280 nm on a Gilford spectrophotometer, model 2400, as well as for phage activity. The second endeavor involved chromatographic separation by Sephadex G 200. Bacteriophage H4489A was first concentrated 10-fold with polyethylene glycol, MW 4000 (161), to a titer of 8.4×10^{10}PFU/ml, resulting in a turbid flocculant precipitate in the lower 10 percent of the final 10 ml volume. Ninety percent of the phage activity in the preparation was associated with this non-cellular precipitate. Following application of this precipitate (containing 1.5×10^{10}PFU/ml) to the G 200 column, 1.0 ml elution fractions were assayed spectrophotometrically at 280 nm, and phage concentrations determined.

Properties of Bacteriophages

Several practical characteristics of the temperate bacteriophages H4489A, 2172 and T12 were determined, although a working knowledge of H4489A and 2172 had been previously established (150). To determine the ideal medium for storage and dilution, stock phage preparations were diluted 10^{-3} in either TH broth, PP broth, THD,

physiological sterile saline (PSS), T2 buffer (162) and tris buffer (163), and maintained at 4C. Plaque forming activity was tested each hour for the initial 4 hours. Subsequent assays were performed every 24 hours.

Loss of viability in the presence of 10 percent chloroform was previously reported for phages H4489A and 2172, although the nature of this sensitivity remained unexamined (150). The effects of chloroform on phage T12 viability was similarly determined in this study. Ten ml of a filtered, cell-free T12 phage preparation was divided into five ml aliquots. One tube served as the control, whereas, 0.5 ml of chloroform was added to the others and shaken vigorously. All preparations were maintained at 4 C. and assayed for infectious centers at 0 time, after 3 hours, and every 24 hours for 18 days.

To elucidate the nature of this instability, the above protocol was repeated for phages H4489A, 2172, and T12 with several variations.

1) The detergent Tween 80 (10%) was substituted for chloroform.
2) Phage preparations diluted 10^{-2} in PP broth pretreated with 10 percent chloroform for 18 days were examined for stability in the absence of the solvent. The treated PP broth was heated for one hour at 60 C. to facilitate removal of chloroform traces prior to the addition of phage.

3) "Resistant" phages still viable after 18 days of chloroform exposure were used as the inoculum for phage propagation. Chloroform sensitivity of the phage in these fresh preparations was then ascertained.

Immunological similarities in the three temperate phages were examined. Rabbit antisera specific for each bacteriophage strain was added separately to all phage preparations in concentrations of 0.0001 percent to 10 percent. Following one hour incubation of the "neutralization" tubes at 37 C, the phage concentration of each mixure was determined. Negative controls entailed substituting preimmune sera for the phage specific hyperimmune sera.

Phage Development

One step growth experiments were performed by the method of Ellis and Delbruck (164), with certain modifications. Immediately following sonification of bacterial cultures to produce single cocci, a phage preparation of H4489A containing 2.4×10^7 PFU was added to 9.5×10^8 viable K56 cells in 10 ml PP broth, at a multiplicity of infection (moi) of 0.025. Following 10 minutes incubation, 0.5 ml phage specific antiserum was added to this adsorption tube. After an additional 10 minutes, the culture was diluted 1000-fold in prewarmed PP broth to produce the first growth tube (FGT). The second growth tube (SGT) represented a further

1:10 dilution after 30 minutes incubation. Samples from the FGT and SGT were withdrawn at 5 minute intervals from the time of dilution, and assayed for infectious centers. The same procedure was employed using 8.9×10^6 PFU and 9.5×10^8 viable K56 chains for 2172 (moi of 0.01), and 7.1×10^6 PFU and 7.2×10^8 viable $T25_3$ chains for T12 (moi of 0.03).

Concurrent development of two phages within the same cell was examined to ascertain any dominance relationships among the three temperate viral strains. Phage H4489A (3.3×10^8 PFU) and 2172 (3.6×10^8PFU) were allowed to coinfect 3.0×10^7 viable K56 chains in 10 ml PP broth. Similar bacterial cultures were inoculated separately with each phage for the singly infected controls. The high total moi of 23.0 promoted multiple phage infection of each bacterium. Following standard propagation protocol, the phages were harvested after four hours. The samples were divided into two 5.0 ml volumes, each of which received 0.5 ml of neutralizing antiserum specific for either H4489A or 2172. The phage concentrations of the remaining viable particles was determined after 30 minute incubation at 37 C. The procedure was repeated for phages T12 (1.2×10^8PFU) and H4489A (1.8×10^8PFU) simultaneously infecting 1.8×10^7 viable chains of $T25_3$ (combined moi of 16.6).

One aspect of phage directed control of bacterial functions was investigated using rifampicin, an antibiotic which interferes

with deoxyribonucleic acid (DNA) dependent ribonucleic acid (RNA) polymerase (165). Overnight cultures of K56 and K56rif^{R} were used as inocula for propagation of phage H4489A on each strain in normal PP broth and in PP broth containing 0.15 mg/ml rifampicin. This concentration of the antibiotic prevents the growth of wild type K56, but not K56rif^{R}. The four cultures were incubated at 37 C for 4 hours, at which time the phages were harvested and assayed for infectious centers. Growth kinetics of both bacterial strains in the presence and absence of rifampicin were determined, using a turbidimetric technique. The absorbancies of the bacterial cultures were measured in a Klett colorimeter with a No. 660 filter. The experiment was repeated for phage 2172.

Adsorption Kinetics

Experiments to determine the kinetics of phage adsorption to nonlysogenic bacteria and homologous lysogens required standardizing overnight bacterial suspensions to differ in viable chain concentrations by no more than 5.0 percent. Phage T12 (1.6×10^{7} PFU) was then added to separate tubes containing 7.5×10^{8} and 7.3×10^{8} viable chains of $T25_3$ and $T25_3$(T12gl) in PP broth prewarmed to 37 C. Samples were removed at intervals, filtered immediately, and the phage titer of the filtrate determined in the usual manner. The experiment was repeated for phages H4489A and 2172 using

8.3×10^6 and 9.3×10^6 phage particles, respectively. The viable count of the K56 inoculum was 2.4×10^8 CFU/ml, and that of the corresponding lysogens was 2.8×10^8 and 7.9×10^8 CFU/ml.

When using a lysogenic bacterial strain in these experiments, it was necessary to distinguish residual (unadsorbed) phage from the normal background phage count produced spontaneously by the lysogen. Controls were established by filtering a sample of the standardized bacterial suspension prior to phage inoculation. The phage concentration of this filtrate represented the background count due to spontaneous induction.

Serological Methods

Antisera against whole cells were produced by a combination of intraperitoneal and intramuscular injections twice weekly for 86 days. The antigens were living cell preparations of S. pyogenes, strains K56, K56(H4489A), K56(2172), $T25_3$ and $T25_3$(T12gl). Each strain was grown at 37 C for 3 hours in 10 ml PP broth, washed and resuspended in 5.0 ml PSS immediately before injection. Cultures were uniformly in late logarithmic phases of growth. The final sera were standardized to an agglutinating titer of 2052.

The titer of the whole cell antisera was determined by tube agglutination, using fresh bacterial suspensions similar to the immunizing preparations as indicators of agglutination. Several

alternative methods for titration of antisera were examined, as well. A microtiter agglutination technique was attempted, initially using bacterial suspensions as the indicator. It was anticipated that adsorption of whole bacteria to goat erythrocytes would facilitate interpretation of the agglutination patterns. Streptococci and goat erythrocytes were washed and suspended in conjugation buffer containing 3.3 percent 1 ethyl-3 (3-diethylaminopropyl) carbodimide (ECDI). The two cell suspensions were combined and maintained at 4 C for 2 hours for coupling to occur (166). Separation of the goat erythrocytes from the bacteria proposed an additional problem. Attempts to resolve this difficulty included differential centrifugation at 132, 6450, and 15,900 x g for 10, 20, and 30 minutes, and high speed centrifugation (29,500 x g) for 10, 20, and 30 minutes in gradients of sucrose (5-20%) for velocity separation, and CsCl (refraction index 1.5) for density separation.

The capacity of the antisera to kill the corresponding bacteria was examined as an alternate method for determining specific antibody concentration. Overnight bacterial cultures were sedimented and resuspended in an equal volume of antisera, and incubated for one hour at 36 C. The viability of the cultures was then ascertained by viable counts of the appropriate dilution of STH agar. Similar cultures received identical treatment, but were exposed to preimmune serum, rather than the serum to be titrated.

The standard agglutination technique involved two fold dilutions of the test serum in 1.5 M phosphate buffered saline (PBS) at a pH of 7.4 (167). Each 1.0 ml dilution tube received one drop (0.033 ml) from a standard bacterial suspension, prepared by inoculating 10.0 ml PP broth with 1.0 ml of an overnight culture of cells, incubating 3 hours at 37 C, washing and resuspending in 1.0 ml PBS. Positive agglutination was indicated by decreased opalescence of the liquid and formation of a pellet with edges curling away from the glass. Gentle agitation resulted in the appearance of large aggregates in positive reactions, and uniform swirls in both negative and control tubes.

In addition to the test tube method, slide agglutination was also employed. One drop each of antisera, or a dilution of antisera, and whole cell antigen suspended in PBS were combined on a microscope slide and agitated. Negative controls using preimmune serum were adjacent to each test site. Agglutination was determined both macroscopically and under a Bausch and Lomb dissecting microscope (3x mag).

Hyperimmune sera to whole bacterial cells were used in precipitin reactions with solubilized somatic antigens of streptococci. Two methods for antigen extraction were applied; i.e., the hot formamide extraction of Fuller (168), and the hydrochloric acid procedure of Lancefield, et al, (169). Using Fuller's protocol, late logarithmic

growth phase bacterial cultures in 10 ml PP broth were standardized turbidimetrically, washed and suspended in 0.2 ml formamide. The tubes were then placed in a 170 C oil bath and gently agitated for 15 minutes. Once the dissolved cells cooled, 0.5 ml acid-alcohol (95 parts absolute alcohol plus 5 parts 2 M HCl) was added and the resulting precipitate removed by sedimentation. Acetone (1.0 ml) added to this supernatant fluid precipitated the group carbohydrate, as well as several protein and teichoic acid antigens. Following centrifugation, this precipitate was dissolved in PBS, and adjusted to a pH of 7.0.

Hydrochloric acid extracts contained primarily M protein, as well as the group carbohydrate and other antigens, to give a complex antigenic mosaic. The procedure was initiated by concentrating 250 ml standardized bacterial cultures to 5 ml in physiological saline containing 1/20 N HCL (final pH 2.0). The tubes were cooled after 10 minutes of immersion in a boiling water bath, and centrifuged. Once the clear supernatant fluid was neutralized with Na_2CO_3 (1.0 M), it was ready for use in immunological reactions.

Initial assays of all antisera for precipitating antibody employed the single direction diffusion test developed by Oudin (170). Equal volumes of sera and 2.0 percent Noble agar were mixed in Durham tubes. Soluble antigen was then applied to the solidified

mixture and incubated for 48 hours at 37 C. The tubes were examined for precipitate with an indirect diffused light source.

A soluble immunodiffusion assay for serum precipitins followed the protocol of Ouchterlony (171). Noble agar (1.0 % in PBS) was uniformly applied (1.8 ml) to the surface of clean glass microscope slides. Wells were cut in the solidified agar and cores removed by suction. The standard configuration of these holes was 3 mm diameter in the center, surrounded by six openings with 2 mm diameters. Antisera diffused from the center well toward the peripheral, antigen-containing wells.

Concentrations of specific precipitins in antisera were ascertained by a quantitative precipitin technique designed by McCarty and Lancefield (172). Solubilized somatic antigen preparations (0.4 ml) were combined with equal volumes of group A or A-variant (group V) specific antisera, incubated for one hour, and maintained at 4 C for 48 hours. The precipitate was sedimented, washed three times in cold saline, and redissolved in 0.4 ml NaOH (0.1 M). Absorbancy at 287 nm was determined in a Gilford spectrophotometer. This method was restricted to quantitative assays of antibody reactions with carbohydrate, and since the absorption of this polysaccharide at 287 nm was negligible, no correction for antigen absorption was required.

Immunoelectrophoresis was utilized for resolving individual precipitin reactions in complex mixures, and for determining relative electrophoretic mobilities of certain antigens. The procedure was carried out essentially according to the micro method of Scheidegger (173), utilizing the LKB immunoelectrophoresis apparatus. Antigen concentrations were adjusted to a dilution conducive to optimal precipitation. Microscope slides were coated with 1.0 percent Noble agar in Gelman buffer (pH 8.0) and were electrophoresed in the same buffer for 50 minutes at 250 volts for 18 slides.

Hyperimmune rabbit antisera were also prepared against culture filtrates of six streptococcal strains. Strains K56, K56(H 4489A), K56(2172), $T25_3$, $T25_3$(T12gl), and NY5 type 10 were incubated at 37 C in 500 ml THD for 24 hours. Growth kinetics were followed turbidimetrically on a Klett colorimeter (660 filter) to insure that all cultures were in stationary phases of growth. Sedimentation of cells was followed by filtering the supernatant fluids through Millipore membranes. These filtrates were placed in dialysis tubing (1.8 in.) and concentrated to 5.0 ml volumes. This was accomplished by covering the filled dialysis tubes with polyethylene glycol (MW 20,000) in separate catheter trays, where they were maintained at 4C for 30 hours. The concentrates were carefully transfered to smaller dialysis tubes (0.38 in.) by puncturing the membrane with a 20 gauge needle and withdrawing the

liquid in a syringe. These samples were extensively dialyzed in physiological saline to eliminate all dialysable components, and subsequently filtered through Millipore membranes. Protein determinations using the method of Lowry (174) provided an index of the relative efficiency of the concentration procedure for each sample.

Immunization of rabbits with these filtrates was accomplished with biweekly intramuscular and intraperitoneal injections of 1.5 ml of filtrate emulsified in an equal volume of Freund's incomplete adjuvant (Difco) for the first three weeks. The same procedure was employed for the next 21 days of immunization except the samples were first suspended in 7.0 percent polyacrylamide and polymerized with riboflavin (0.02%) for 30 minutes in the presence of direct visible light. Such preparations have been reported to be excellent adjuvants (175). The gel suspensions were then emulsified with equal volumes (3.0 ml) of Freund's incomplete adjuvant. The animals were bled by arterial puncture every 10 days, and precipitating antibody assays performed by single direction immunodiffusion (Ouidan method). Serum titers were subsequently determined by tube dilution of antigen in constant serum concentrations.

Antisera against phages H4489A, 2172, and T12 were prepared by seven consecutive daily injections into the ear vein of rabbits, followed by the same procedure one week later. The phages

were prepared by sedimentation in the ultracentrifuge (101, 962 x g, 90 min) and resuspension in 1/10 the original volume of PBS. Daily injections contained 3.3×10^9, 9.6×10^8, and 5.4×10^9 PFU of H4489A, 2172 and T12 respectively. The animals were bled 28 days from the first injection.

Phage-Host Relationships

Stability of the Phage-Host Complex

To determine whether the phage-host complexes were lysogenic or pseudolysogenic, the stability of the relationships established by temperate phages H4489A, 2172 and T12 with the corresponding streptococci was examined. One ml of an overnight culture of K56(H4489A), K56(2172) or $T25_3$(T12gl) was inoculated into 9.0 ml of PP broth containing 10 percent neutralizing antisera specific for the corresponding bacteriophage, and incubated at 37 C for 10 days. Daily samples were removed and sonified in a Bronson Sonifier model W-140 (15 sec, setting 4) to reduce bacterial chain length. Dilutions of the sonified preparations were spread on TH agar to obtain isolated colonies. The presence of phage was ascertained by individually suspending 200 colonies in 0.3 ml PP broth and applying one drop to the surface of STH agar seeded with a lawn of either K56 or $T25_3$. Phage producers lysed the background bacteria immediately surrounding the colony, whereas phage-free

colonies were undetectable. Ten consecutive daily samples were tested.

Phage Induced Effects on Surface Determinants

Antigenic analysis of bacterial surface determinants involved immunoabsorption of each antisera with intact lysogens and corresponding non-lysogens. Absorbing doses were standardized by equating dry weight with the turbidity of the bacterial culture. Dry weight determinations were accomplished by filtering samples of bacterial cultures of known turbidity through thoroughly dried, prewashed membranes. The membranes were subsequently washed with physiological saline and dryed for three days in a desicator oven at 120 C. A saline control and an untreated membrane were included to ascertain the weight of the sodium chloride and any fluctuations in the weights of the membranes themselves.

Organisms used for immunoabsorption were prepared by inoculating 9.0 ml PP broth with 1.0 ml from an overnight bacterial culture. Growth kinetics of these strains were followed turbidimetrically on a Klett colorimeter (660 filter), and cells were harvested in late logarithmic growth stages, similar to the cell suspensions used for immunization.

Preliminary immunoabsorption involved saturation absorption of each serum with 1000 µg of the lysogen and corresponding

phage-free strain. Absorbing suspensions were washed, resuspended in 3.0 ml PBS, and added to equal volumes of antisera. Following one hour incubation at 37 C with periodic agitation, the tubes were placed at 4 C and maintained for 18 hours. The cells and absorbed antibody were then sedimented, and the residual agglutinating titer of each serum determined against the heterologous and homologous strains; that is, using the lysogens and nonlysogens as indicators.

Antisera against K56 and corresponding lysogens were absorbed three consecutive times using the previously described procedure. To facilitate removal of residual antibody from these sera, hydrochloric acid (HCl) was used to increase the antigenicity of the absorbing suspensions (176). The protocol called for HCl (5.0 N) to be added to washed, packed cells (cell volume: acid volume, 1:3), held 10 minutes in a boiling water bath, and cooled to room temperature (RT). After a final pH adjustment to 7.3 with 1.0 N NaOH, the cells were sedimented, resuspended in 3.0 ml PBS, and used as immunoabsorbants as previously described. Overnight incubation in the proteinases,trypsin (1.0 mg/ml) and pepsin (1.0 mg/ml) in PBS,was another method employed to increase absorbancy of these bacterial strains.

Quantitative immunoabsorption involved the same methods, but 2.0 ml aliquots of each serum were mixed with concentrations of heterologous and homologous absorbing strains varying from 150

to 1200 µg/ml. The residual antibody titers were determined by agglutination of the lysogenic and phage-free strains. Quantitative immunoabsorption was performed with $T25_3$ and $T25_3$(T12gl) only.

Solubilized antigens of each strain extracted by hot formamide or HCl were employed in double immunodiffusion assays using the method of Ouchterlony (171). Each antigen preparation was tested with both lysogen and nonlysogen antisera. Undiluted antisera were placed in the center well, whereas the antigen occupied the smaller peripheral wells.

The effects of various agents on agglutingation of $T25_3$ (T12gl) by residual antibody in heterologously absorbed $T25_3$(T12gl) antiserum were examined. Standard indicator suspensions of this lysogen were exposed for 18 hours in PBS to 1.0 mg/ml of the proteolytic enzymes pepsin and trypsin, at pH's of 7.0 and 2.0 at 37 C. Controls on enzyme activity consisted of incubating 1.0 mg/ml human serum albumin (HSA) with and without these proteinases at 37 C. Samples were removed every hour and precipitated with an equal volume of cold 14 percent trichloroacetic acid (TCA). Following centrifugation (6450 x g, 20 min) absorbancy of the supernatant fluid at 280 nm was determined spectrophotometrically. Exposure of $T25_3$(T12gl) suspensions to 10 percent TCA (pH 1.1) to determine if antigenic alterations could be produced by protein

precipitation was accompanied by exposure to HCl (pH 1.1) under similar conditions. The final two agents utilized were 10 percent sodium dodecyl sulfate (SDS) and 10 percent chloroform. Both were incubated with the lysogen suspension for 18 hours at 37 C. All chemically treated preparations of $T25_3$(T12gl) were used as indicators of agglutination in heteroabsorbed antisera.

Group carbohydrates of lysogens and nonlysogens were compared by first determining the agglutinating titers of various Lancefield group-specific antisera using $T25_3$ and $T35_3$(T12gl) as indicators. The sera utilized were specific for group A (obtained from BBL and W.R. Maxted) and group A-variant (E.M. Ayoub) (group V) carbohydrates (C substance). Each antiserum was also employed in double immunodiffusion precipitin tests with solubilized surface antigens. The quantitative precipitin method previously described was used to ascertain concentration differences of various group carbohydrates in the formamide extracts of strains $T25_3$ and $T25_3$(T12gl). Total carbohydrate in the formamide extracts were determined using a modified method of Dische, et al. (177, 178).

The mechanism of this antigenic conversion system was partially characterized by agglutination of modified strains in absorbed antisera under various conditions.

A. The role of bacteriophage adsorption to surface receptor molecules in altering the antigenic specificity was

examined by treatment of all residual sera with 2.0×10^9 PFU of phage T12 for 18 hours at 37 C. The capacity of this phage concentration to neutralize phage-specific antibody was determined by incubating a similar concentration of T12 with 10 percent antiphage serum for 18 hours at 37 C, and the residual phage neutralizing potential of this serum determined. The procedure was repeated until the neutralizing activity in the control serum was exhausted.

B. Bacterial indicator suspensions were preincubated in hyaluronidase (0.1 mg/ml) to remove bacterial capsules prior to agglutination.

C. Strain $T25_3$(T12)B, a lysogen of $T25_3$ and phage T12 constructed in this laboratory, was substituted for $T25_3$(T12gl) as the agglutinating suspension.

D. Another $T25_3$ lysogen, containing phage H4489A instead of phage T12, was also substituted for $T25_3$(T12gl).

Phage Influence on Extracellular and Intracellular Products

Differential analysis of soluble constituents produced by lysogens and nonlysogens of group A streptococci was focused primarily on the production of erythrogenic toxin, which is reportedly associated with bacteriophage infection (149). Development of a

reliable toxin assay initially exploited the erythematous response of animals to intradermal injections of scarlatinal exotoxin (179). The shaved backs of guinea pigs and rabbits (3 to 6 months of age) were used as indicators of erythema. A skin response was considered positive if zones of erythema had a diameter of 10 mm or greater within 48 hours after a 0.1 ml infection. Rabbits were either previously sensitized to extracellular products of $T25_3$(T12gl) or to partially purified streptococcal exotoxin (scarlatinal). Unsensitized rabbits were used, as well. Sensitization required two injections of the antigen emulsified in complete Freund's adjuvant into the foot pads. The 3.0 ml injections were 7 days apart, and 14 days were allowed for the development of hypersensitivity.

An in vitro assay for erythrogenic toxin using serological precipitin reactions was developed, adopting a standard type A toxin preparation provided by Dr. Dennis Watson, University of Minnesota, to use with antiserum against NY5 type 10 culture filtrates. The assay depended on lines of identity between the toxin preparation and an unknown antigen in double immunodiffusion reactions with the NY5 type 10 antiserum. All wells were standardized to similar volume capacities, and agar surfaces were prepared fresh to insure against fluctuations due to evaporation. Since the original toxin preparation was contaminated by a single protein species, appropriate steps were taken to eliminate this contaminant from the

known standard. This entailed diluting the toxin 1:4 in PBS prior to its use in the assay procedure. Validation that the precipitin line eliminated by dilution represented the contaminant required further determinations:

1) Polyacrylamide gel electrophoreses was used for resolution of the original toxin preparation and broken cell extracts of NY5 type 10. These extracts were prepared by washing 50 ml of a stationary growth phase bacterial culture and suspending in 10 ml PBS. The cells were then broken by agitation for 3 minutes in a Bronwill cell disintegrator using 10 g of glass beads (0.25 mm diameter). Heat denaturation was avoided by circulating gas from a CO_2 tank through the chamber and immersing the bottle containing the bacteria in a dry ice-acetone bath at 60 second intervals. The particulate material was removed by centrifugation for 30 minutes at 6450 x g.

2) Immunoelectrophoresis of undiluted and diluted toxin, as well as the concentrated NY5 type 10 filtrates employed antiserum prepared against the latter mentioned filtrates.

The in vitro toxin assay was also useful as a quantitative technique by using 2-fold dilutions of the sample to be analyzed.

The presence of erythrogenic toxin in concentrated culture filtrates and broken cell extracts of six strains [K56, K56(H4489A), K56(2172), $T25_3$, $T25_3$(T12gl), and NY5 type 10] was determined by skin tests and double immunodiffusion. Neutralization of the erythematous response was attempted by combining either toxin, filtrates, or broken cell extracts with equal volumes of antisera against filtrates of $T25_3$(T12gl) or $T25_3$. The mixures were incubated for one hour at 37C prior to intradermal infections in unsensitized rabbits, as well as animals sensitized to toxin A and $T25_3$(T12gl) culture filtrates. No toxin-specific antisera was available.

Further toxin determinations using the in vitro method examined unconcentrated culture filtrates of $T25_3$ and $T25_3$(T12gl), $T25_3$(T12)B, and $T25_3$(H4489A). In addition, the presence of toxin in various cell-free phage lysates was examined following propagation of phages T12, T12cp[1] and A25 on $T25_3$ using both the standard propagation procedure and a modification calling for inoculation of 1.0 ml high titered phage preparations into $T25_3$ cultures in mid-logarithmic stages of growth, and subsequent incubation at 37C for 16 hours.

Kinetics of toxin production at 37 C were determined by inoculating 15 ml PP broth with 1.0 ml from an overnight culture of either $T25_3$, $T25_3$(T12gl), or $T25_3$(T12)B. Samples were

periodically removed and filtered, and concentrations of toxin determined by the quantitative double immunodiffusion method. A similar procedure was employed for the kinetics of toxin production in actively growing $T25_3$ cultures infected with 1.2×10^7 PFU and 5.1×10^7 PFU of phages T12 and T12cp[1].

Polyacrylamide gel electrophoresis (PAGE), double immunodiffusion, and immunoelectrophoresis were employed for comparative analysis of each nonlysogenic strain and the corresponding lysogenic complexes. The standard Canalco method was used in the PAGE studies. Samples were concentrated culture filtrates, broken cell extracts, and phage preparations of H4489A, 2172 and T12 that had been previously sedimented by ultracentrifugation (101,962 x g, 90 min) and resuspended in distilled water. Samples (300 µl) containing 1.3 percent sucrose were applied directly to the surface of the stacking gel. Running time was 75 minutes at 5 milliamperes/gel. The gels were fixed in 5.0 percent TCA, stained with 0.6 percent amido schwartz, and destained in 7.0 percent acetic acid. All destained gels were optically scanned in a Gilford spectrophotometer at 570 nm, using a scanning attachment. Immunelectrophoresis of concentrated filtrates of each lysogen or nonlysogen were run in duplicate with both homologous and heterologous antisera.

A method for isolating antigens unique to either lysogens or nonlysogens was attempted with an affinity immunoabsorption technique. A complex mixture of soluble antigens (concentrated culture filtrates) was passed through a solid phase coupled to purified gamma globulin, which should theoretically retain only those molecules complementary to the affixed antibody. Antisera (10 ml) was precipitated in 50 percent saturated ammonium sulfate and resuspended in 0.01 M potasium phosphate buffer (pH 8.0). This procedure was repeated and the resuspended precipitate dialyzed for 72 hours in 3000 ml 0.01 M potasium phosphate buffer (pH 8.0). The sample was then applied to a DEAE cellulose column (G52) and eluted with a linear ionic gradient (0.01 M to 0.30 M phosphate buffer, pH 8.0) (180). The fractions were monitored for absorbancy at 280 nm. Pooled fractions were concentrated by a colloidin bag apparatus (Schleicher and Schuell) under negative pressure. Samples containing protein from the first two peaks were examined for antibody activity against the corresponding antigens by double immunodiffusion in agar.

The purified active antibody was then coupled to cellulose filter pads (12 mm, Schleicher and Schuell) previously activated with cyanogen bromide as described by Caska, et al. (181), and thoroughly washed. Homologous and heterologous culture filtrates were absorbed by passing samples of varying volumes through the

antibody containing filter pads in Millipore filtering housings. An alternate method of antigen absorption involved placing small pieces of the immunologically active filter pads in various size volumes of culture filtrates and agitating for 18 hours. The presence of antigens in each sample was then determined serologically by diffusion against hyperimmune serum.

Transduction

Transduction of bacterial markers was accomplished by Stuart and Ferretti's modification of Malke's procedure (128). Transducing lysates (1.5 ml) prepared by A25ts$^{1-2}$ propagation on donor bacteria, were irradiated for 60 seconds with ultraviolet light at a distance of 24 cm. The lysates were then combined in 0.3 ml volumes with equal amounts of recipient bacteria in the stationary phases of growth, and incubated for 20 minutes at 37 C to allow phage adsorption to occur. A sample of the incubated preparation (0.1 ml) was thoroughly mixed with 3.0 ml serum soft agar (SSA) and evenly poured onto the surface of STH agar. The petri dishes were incubated for 2 hours at 37C before receiving an additional 3.0 ml of SSA containing the proper concentration of the appropriate antibiotic. Transductants were scored following 18 hours incubation at 37 C.

The purpose of these transductional studies was to ascertain the effects of lysogeny on both donor and recipient efficiency. Donors

in the first experimental procedure were the four 9440 mutants resistant to the antibiotics listed in Table 1. Wild type K56, K56(H4489A) and K56(2172) served as recipients. Antibiotic resistant transductants generated in this experiment were isolated, the stability of the acquired marker confirmed by three subcultures on agar containing lethal concentrations of the antibiotic, and employed as donors of transduction in lieu of the 9440 strains. This procedure was similar to the previous protocol, differing only in the origins of the A25ts$^{1-2}$ lysates employed as mediators of the genetic exchange.

CHAPTER IV

RESULTS

Cultivation of Bacteria in Synthetic Media

All strains of S. pyogenes employed in this study were grown in PP broth, TH broth, and Stock's dialysate medium. Attempts to cultivate bacteria in various synthetic media, however, were less successful (Table 7). Whereas the initial cultures in synthetic media showed moderate growth of strain K56 after 48 hours incubation, further subcultures were successively reduced in turbidity until complete absence of detectable growth occurred after the second or third transfers. Similar results were obtained using strains 9440str-10, K56(H4489A) and K56(2172). It was believed that the early growth was due to nutritional supplementation from the 10.0 percent inoculum (a PP broth culture), and that perhaps a strain could be adapted to growth in defined media by adding decreasing amounts of PP broth with each daily transfer, until growth occurred in unsupplemented synthetic media. Cultures failed repeatedly, however, once concentrations of PP broth dropped below 0.001 percent. In addition, neither increasing the inoculum size for each transfer nor

TABLE 7

TURBIDITY OF A K56 CULTURE IN SYNTHETIC MEDIA AFTER 48 HOURS AT 37 C USING A 10 PERCENT INOCULUM

Number of Sub-Cultures	A	B	C	D
1	64	52	49	45
2	17	8	11	10
3	2	-	-	-
4	-	-	-	-
5	-	-	-	-

Medium
A = Mickelson (136)
B = Leonard, et al (157)
C = Lawton (158)
D = Henderson, et al (156)

incubating in the reduced oxygen tension of a candle jar would stimulate adequate growth of these group A strains in synthetic media.

Isolation and Propagation of Bacteriophages

All bacterial strains examined were found to produce viruses lytic for at least one of the companion streptococcal strains used in this study, with the single exception of strain K56. The host range of each bacteriophage, as well as the phage sensitivities of the bacteria are illustrated in Table 8. Although lysis by K56 culture filtrates could not be demonstrated, similar preparations of this organism have been shown by electron microscopy to contain phage particles (R. M. Cole, personal communication). Since hyaluronidase is known to be required for infection by certain phages (e.g., A25 lysis of strain K56), this enzyme was included to eliminate potential interference from the bacterial capsules. Its presence, however, affected only the lytic patterns of phages A25, NY5 type 10, T12, and $T25_3$ on strain K56. In addition, lysis of strain $T25_3$ by phages A25 and T12 was unaffected by hyaluronidase, perhaps reflecting an absence of capsular material from this bacterial strain. The sensitivity of the K56 lysogens to A25 infection in the absence of hyaluronidase was probably related to the increased production of hyaluronidase known to occur following infection with certain temperate bacteriophages (139). Lysis resulting from spotting filtrates of strains $T25_3$, $T25_3$(T12gl)

TABLE 8

LYTIC PATTERNS PRODUCED BY APPLICATION OF "PHAGE" PREPARATIONS ON VARIOUS BACTERIAL LAWNS

Bacterial Strain	Cell-Free Phage Preparations													
	K56		H4489A		2172		A25		$T25_3$		$T25_c$(T12gl)***		NY5 type 10	
	Hy*	-Hy**	Hy	-Hy	Hy	-Hy	Hy	-Hy	Hy	-Hy	Hy	-Hy	Hy	-Hy
K56	-	-	+	+	+	+	+	-	+	-	+	-	+	-
K56(H4489A)	-	-	-	-	+	+	+	+	-	-	-	-	-	-
K56(2172)	-	-	+	+	-	-	+	+	-	-	-	-	-	-
$T25_3$	-	-	+	+	-	-	+	+	-	-	+	+	-	-
$T25_3$(T12gl)	-	-	+	+	-	-	+	+	-	-	-	-	-	-
NY5 type 10	-	-	+	+	+	+	-	-	-	-	-	-	-	-

*Hy contained 0.40 mg/ml hyaluronidase
**-Hy no hyaluronidase present
***Culture filtrates of $T25_3$(T12gl) are considered to be preparations of phage T12.

+ = lysis
- = no lysis

and NY5 type 10 on K56 lawns was unique in that only a few isolated plaques developed, whereas the other preparations invariably led to confluent clearing of the entire area involved.

Material obtained with a sterile needle from the center of the isolated $T25_3$, $T25_3$(T12gl), or NY5 type 10 plaques was suspended in 0.1 ml PP broth and inoculated with 0.1 ml of an actively growing K56 culture. No propagation occurred in PP broth following 6 hour incubation at 37 C. As expected, however, the culture filtrate of strain $T25_3$(T12gl) containing the phage T12 proliferated moderately on the indicator, $T25_3$. To enhance phage production by these lysogens, the cultures were exposed to ultraviolet (UV) irradiation or mitomycin C. This attempt to induce phage failed to increase the number of plaques detectable by K56 bacterial lawns. The effects of these inducing agents on $T25_3$(T12gl), however, using strain $T25_3$ as the indicator of infectious centers are shown in Figure 1. Both treatments increased phage titers to more than 99.5 percent of the untreated controls within 90 minutes of exposure. The induced bacteriophage was considered to be phage T12.

To determine the optimal conditions for phage T12 propagation on strain $T25_3$ several variations of the standard phage propagation procedure were employed. These included the use of different propagation media as well as various lengths of propagation times. The results of these experiments (Table 9) suggest that phage T12 propagated most

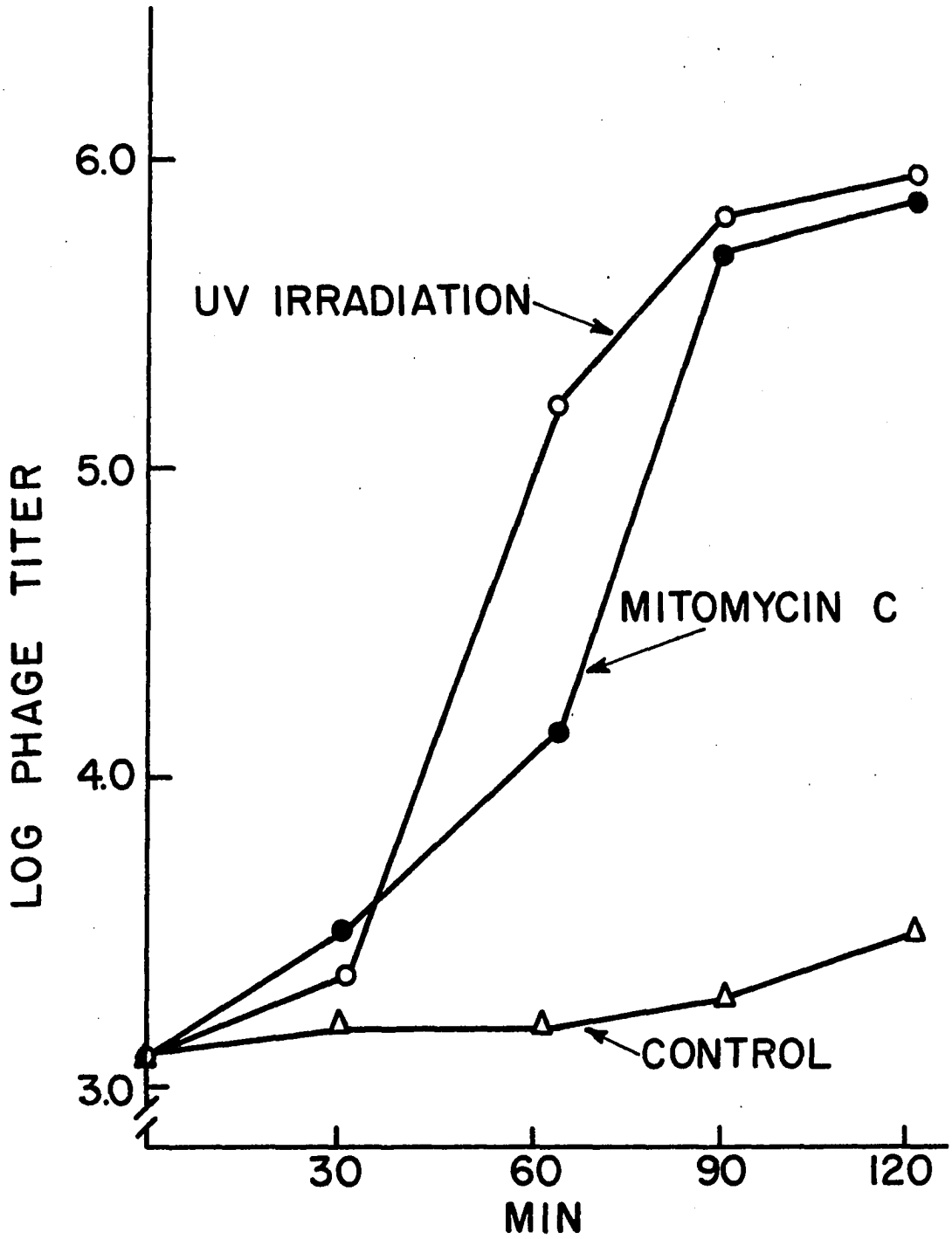


FIGURE I - INDUCTION OF AN ACTIVELY GROWING CULTURE OF $T25_3$ (T12gl)

TABLE 9

TITERS (PFU/ml) OF T12 PHAGE PREPARATIONS PRODUCED UNDER VARIOUS CONDITIONS

Media	°C	PRU/ml following Hours of Propagation Time						
		3	4	5	6	7	8	16
PP Broth	24	-	-	-	-	-	-	1.8×10^7
	30	-	-	-	-	-	2.4×10^6	1.0×10^8
	37	-	1.2×10^7	3.6×10^8	2.5×10^9	1.2×10^9	3.2×10^8	6.0×10^7
TH (+) Broth	24	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	5.8×10^7
	37	-	-	3.0×10^7	1.8×10^8	1.8×10^8	7.6×10^7	3.3×10^7
TH Broth	24	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	9.0×10^6
	37	-	-	-	7.3×10^7	3.6×10^6	-	-

TH (+) = Todd-Hewitt Broth Containing the Supplements in PP Broth

(-) = $< 10^6$ PFU/ml

efficiently in PP broth at 37 C for six hours, to a final concentration of 2.5×10^9 PFU/ml. Incubation time was apparently critical, since 1.0 hour more or less resulted in respective decreases of 50 and 70 percent in phage production. Supplemented TH broth supported less than 7.0 percent of the phage producing capacity of PP broth, a value further reduced to 2.9 percent by the use of unsupplemented TH broth. Similar efforts to apply these modifications to stimulate propagation of phages found in culture filtrates of $T25_3$ and NY 5 type 10 were unsuccessful under all cultural conditions described in Table 9.

The appearance of T12 plaques on an STH agar lawn of $T25_3$ was quite distinct from those previously described for H4489A on similar media with a K56 lawn (150). While both temperate phages showed considerable size variation, only the T12 plaque was characterized by the classical turbid center described for most temperate bacteriophage. In addition, the zones of dissolved hyaluronic acid capsular material around some temperate phage plaques (Kjems effect) was not a feature of T12 lysis on either indicator strain $T25_3$ or K56. This was perhaps attributable to an absence of capsules from the $T25_3$ cells, as indicated by its broad spectrum of phage sensitivity in the absence of hyaluronidase (Table 8). Lysis of K56 by phage T12 was dependant on an exogenous source of hyaluronidase, the presence of this enzyme preventing the formation of zones. Three distinct halos surrounded the H4489A plaque, which was one more than previously reported for a Kjems effect (138).

The notable property of T12cp[1] plaques, the virulent mutant of phage T12, was the absence of the turbid center from the lytic area. Phage 2172 gave rise to plaques of identical morphology as did phage H4489A, but approximately one-half the size.

Properties of Bacteriophages

The stability of these viral preparations under normal storage conditions in various media was previously reported (150). These experiments were repeated on phage 2172, utilizing an increased number of diluents (Figure 2). Maintenance in either PSS or tris buffer resulted in extensive inactivation (99.2 and 99.7 percent respectively) by the sixth day. The phages survived better in TH dialysate and T2 buffer, eventually losing 82.0 and 63.0 percent of the original viability. The ideal media for storage were PP broth and TH broth, both maintaining virtually 100 percent of the initial PFU. Similar inactivation patterns were established for phages H4489A and T12. The storage temperature, however, was -30 C, for H4489A, since a minimum of 80 percent inactivation occurred in PP broth at 4 C.

The deleterious effect of chloroform on phages H4489A and 2172 reported earlier (150) was equally detrimental to phage T12 preparations, as shown in Figure 3. The rapid loss of 99.4 percent of the initial infectivity by the sixth day, and subsequent stabilization

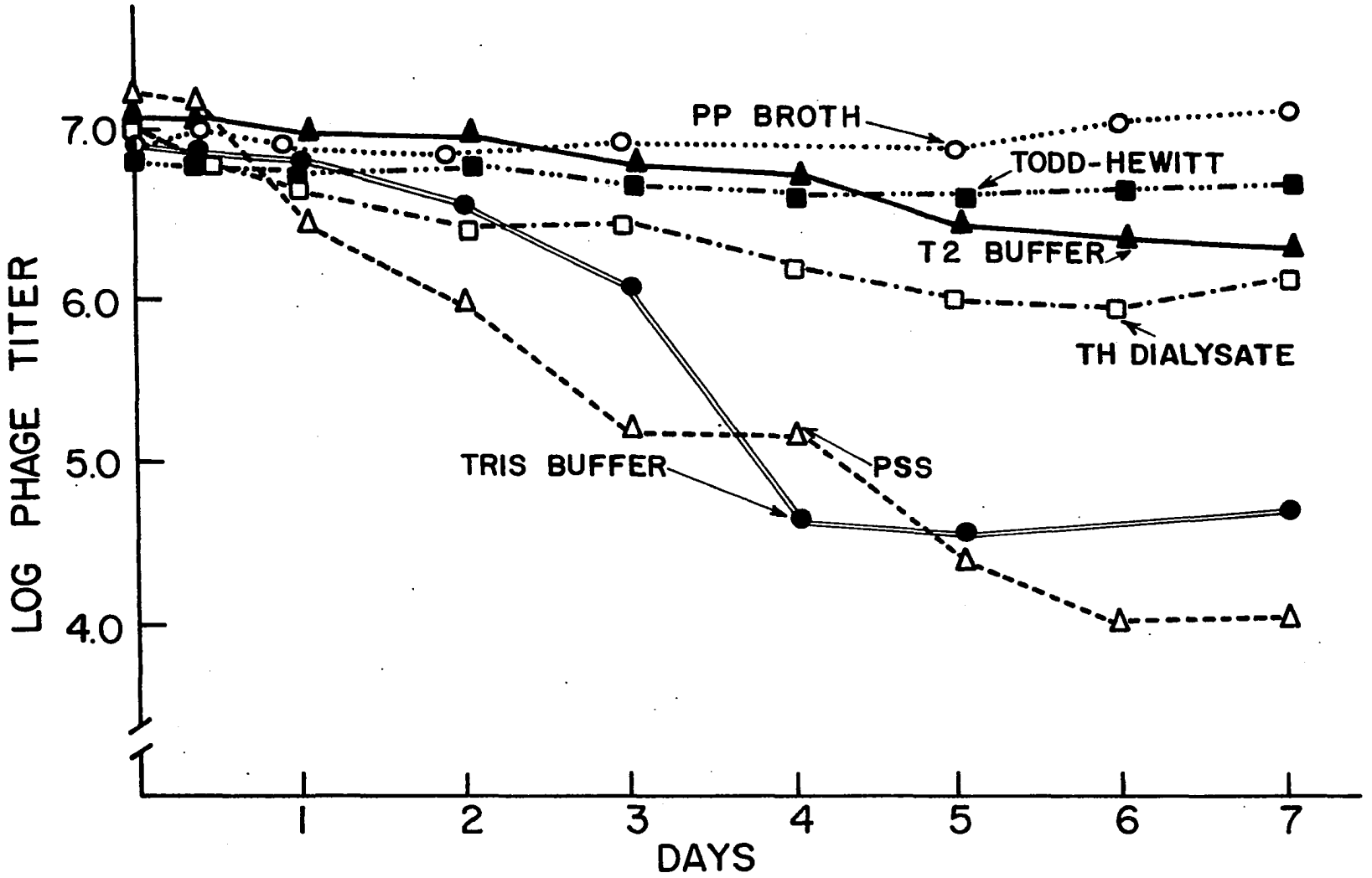


FIGURE 2-STABILITY OF PHAGE 2172 IN SEVERAL DILUENTS AT 4C

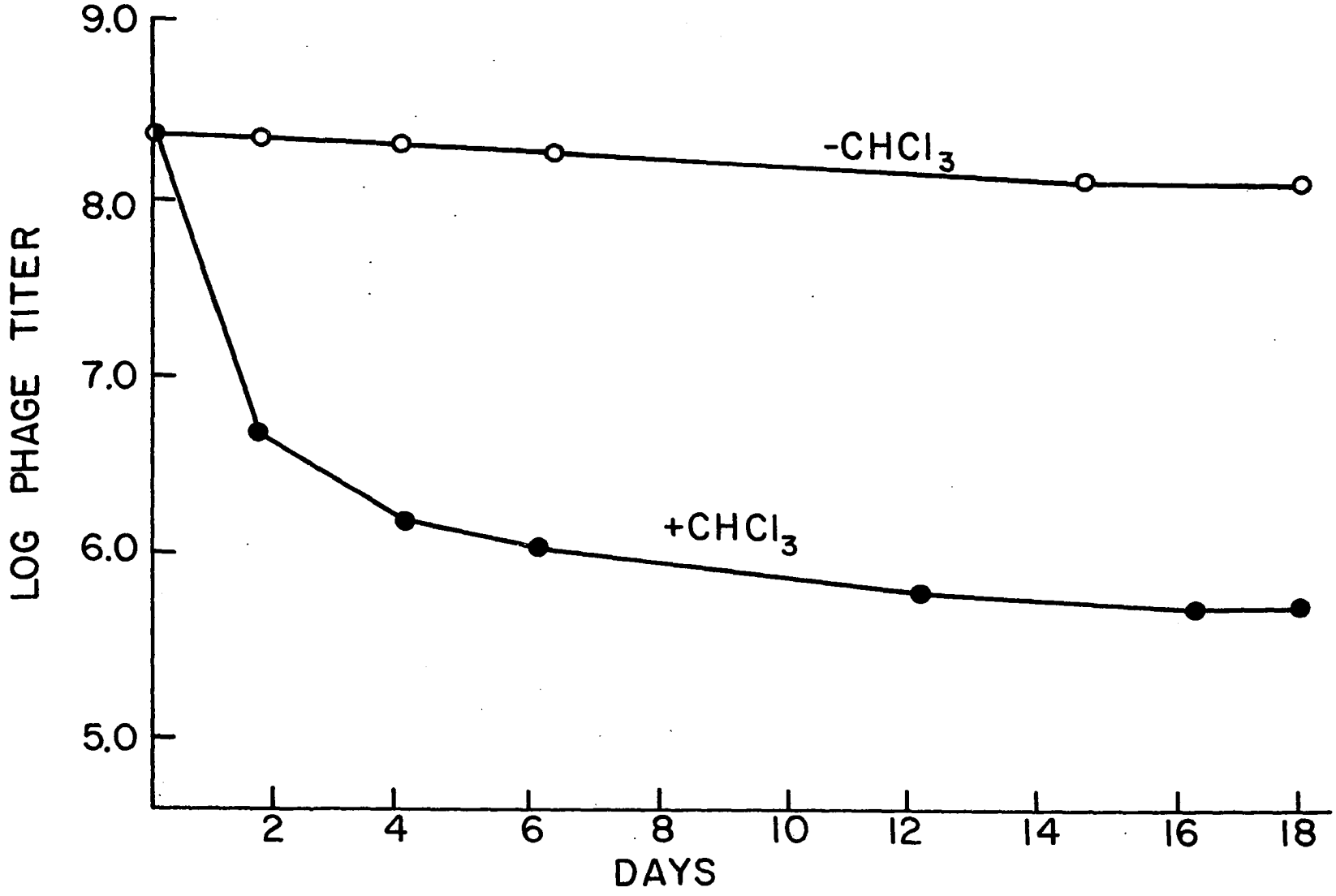


FIGURE 3 - THE EFFECTS OF 10% CHLOROFORM ON PHAGE T12

of the remaining viable phages, mimicked the inactivation kinetics of H4489A and 2172 on exposure to this solvent. Addition of fresh chloroform to the remaining viable phages after 18 days of exposure failed to decrease this persistant titer. Exhaustion of the solvent's activity was therefore an unlikely explanation for the survival of the remaining viruses.

The nature of chloroform inactivation was examined by first determining that the phages were equally stable in PP broth containing the detergent Tween 80 (10%) as in media lacking it. Secondly, phage preparations were maintained in PP broth pretreated with chloroform for 18 days (Figure 4). Although all traces of the solvent were theoretically removed by mild heating, the broth was capable of sustaining the viable phage with an efficiency of only 18 percent of the similar untreated diluent. Whereas this effect was not as extensive as that seen in the presence of chloroform, the same pattern of inactivation was repeated. It is possible that residual chloroform in the PP broth accounted for this phenomenon. An alternate explanation, however, is that the chloroform destroyed a component of the PP broth that was essential for the maintenance of bacteriophage viability. Finally, "resistant" phages still viable after 8 days of chloroform exposure were used as the inoculum for propagation. The resultant viral preparations followed chloroform inactivation kinetics similar to the parental phages. The remaining viable phage, therefore,

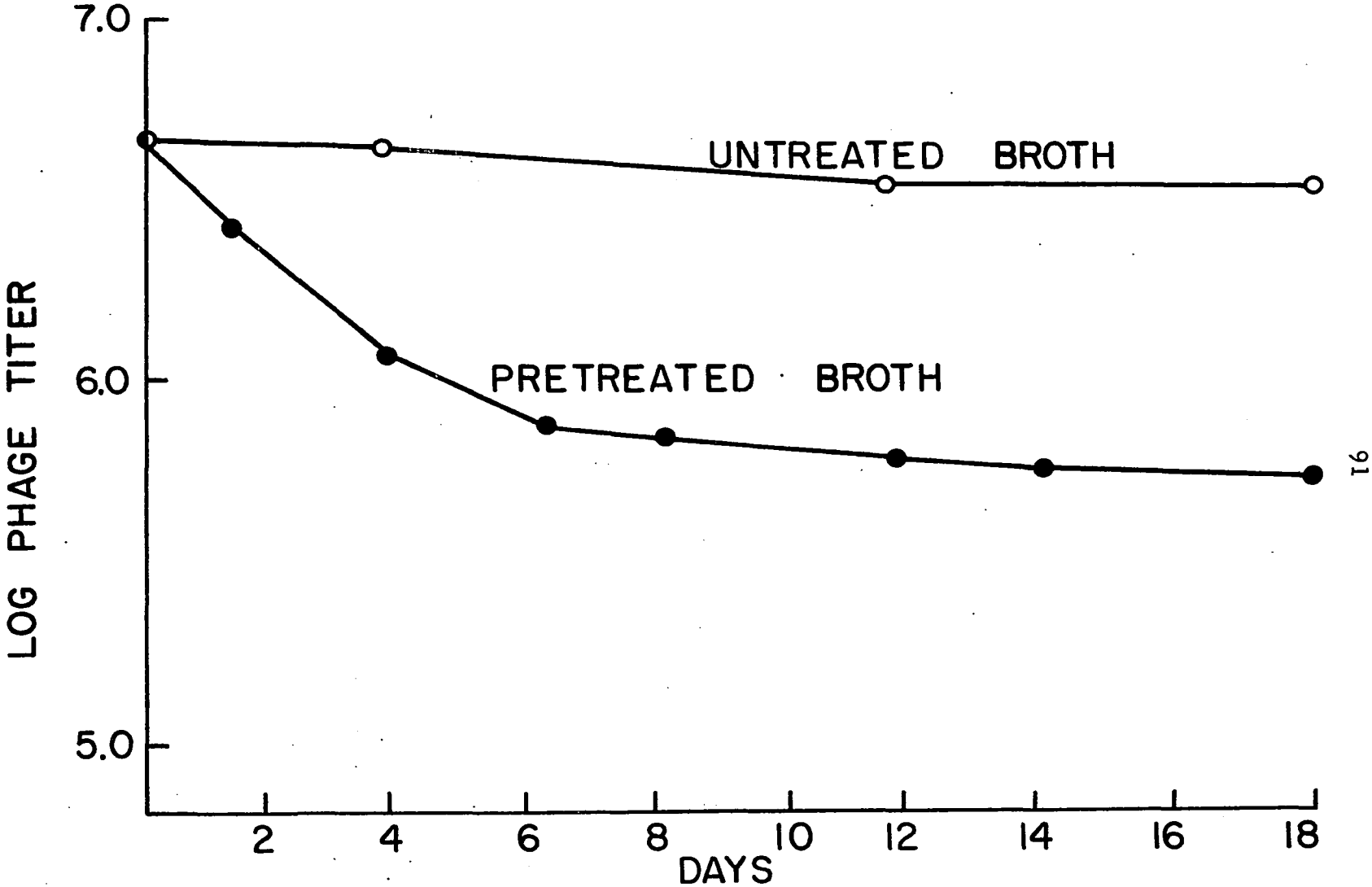


FIGURE 4-PHAGE T12 STABILITY IN PP BROTH PRETREATED WITH 10% CHLOROFORM

probably acquired no inheritable characteristic that rendered them selectively resistant to chloroform.

Immunological similarities in the three temperate phages were examined by determining neutralization of phage viability by homologous and heterologous antisera; that is, by antibody specific for phages H4489A, 2172, or T12. These results, shown in Table 10, indicated the phages to be immunologically distinct from one another, at least in so far as adsorption specificity was concerned. Phages 2172 and T12 showed slight inactivation by antisera specific for phage H4489A and 2172, respectively. Since the reciprocal combinations, i.e., phage H4489A in 2172 antiserum and phage 2172 in T12 antiserum, lead to no loss of infectivity, this marginal neutralization must be viewed skeptically.

Phage Development

In one step growth experiments, low moi's were employed in order to circumvent the problem of multiple infection of bacterial cells. The concentration of phage in the latent period was therefore considered to represent the number of cells singly infected by virus. These results are illustrated in Figure 5, which shows phages H4489A, 2172 and T12 to have respective minimal latent periods of 45, 60, and 55 minutes and burst sizes of 27, 39, and 33 PFU/infected coccus. The use of sonified bacterial cultures prevented immediate

TABLE 10

NEUTRALIZATION OF INFECTIVITY OF PHAGES H4489A, 2172 AND T12 BY HETEROLOGOUS AND HOMOLOGOUS ANTISERA

Serum	Phage	Phage Input (PFU)	Phage Recovered (PFU)	% Neutralization
Anti H4489A	H4489A	2.4×10^7	0	100
	2172	2.1×10^7	1.8×10^7	14
	T12	1.8×10^8	1.8×10^8	0
Pre immune	H4489A	2.4×10^7	2.4×10^7	0
Anti 2172	H4489A	2.4×10^7	2.4×10^7	0
	2172	2.1×10^7	0	100
	T12	1.8×10^8	1.7×10^8	6
Pre immune	2172	2.1×10^7	2.1×10^7	0
Anti T12	H4489A	1.8×10^8	1.8×10^8	0
	2172	2.1×10^7	2.1×10^7	0
	T12	1.8×10^8	0	100
Pre immune	T12	1.8×10^8	1.8×10^8	0

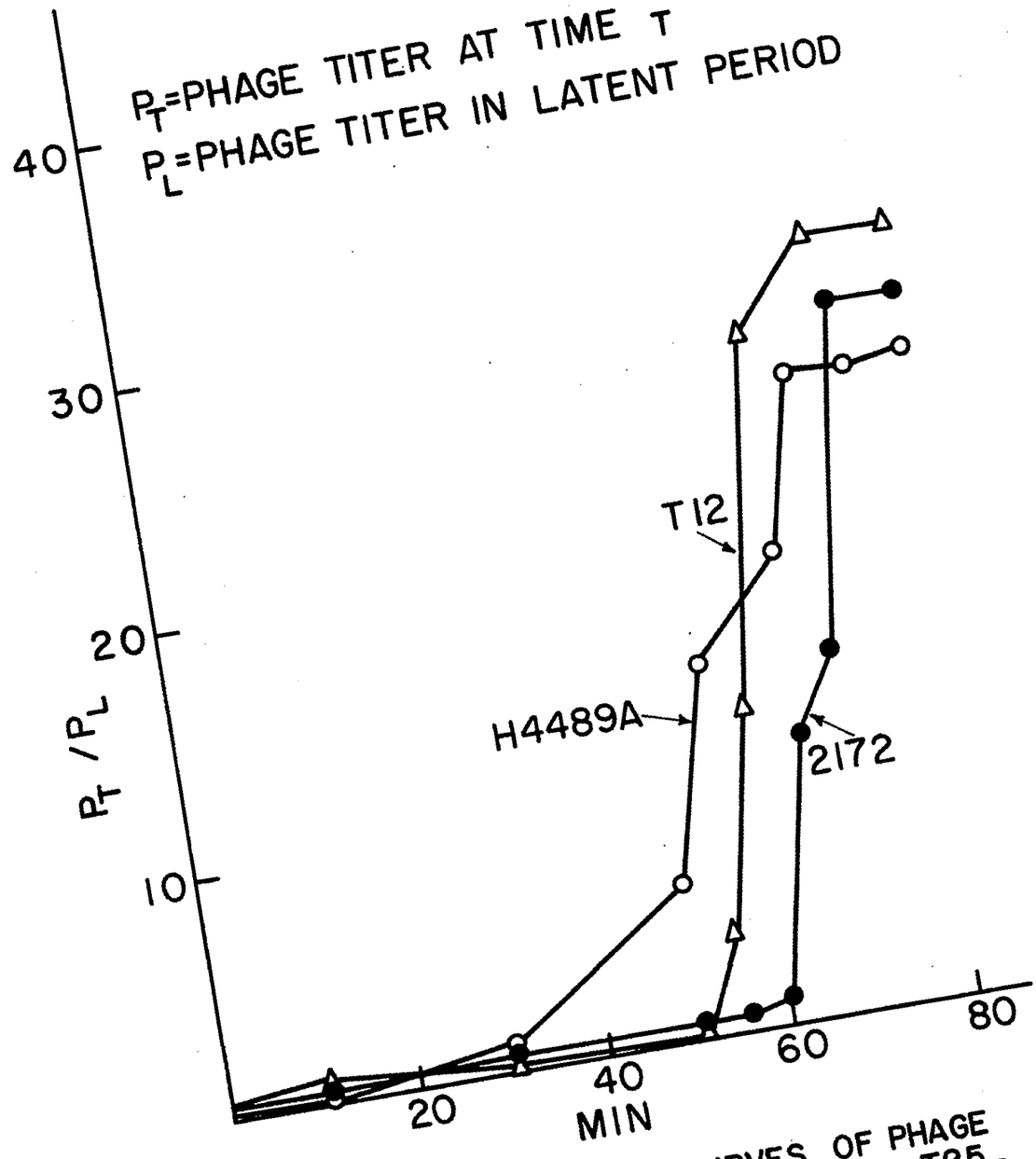


FIGURE 5-ONE STEP GROWTH CURVES OF PHAGE H4489A AND 2172 ON K56, AND T12 ON $T25_3$

reinfection of adjacent cocci, subsequently eliminating secondary bursts.

Viral interference by each of these three phages was examined by concurrent phage development of two phages in the same cell (Table 11). Coinfection of H4489A and T12 in $T25_3$ resulted in normal propagation of phage T12 (1.8×10^8) at the expense of H4489A (7.2×10^6). No dominance relationship was detectable in K56 cultures coinfected with phages H4489A and 2172. Final concentrations of these two phages were 42 and 51 percent of the singly infected control titers, indicating a mutual sacrifice to accommodate propagation of the companion viral strain.

Phages H4489A and 2172 were propagated on K56 and K56$\underline{rif}^R$ hosts in both the presence and absence of rifampicin. Since this antibiotic interferes with DNA-dependant RNA polymerase, a better understanding of this bacterial enzyme's role in phage propagation could perhaps be obtained by this line of investigation. The results are shown in Table 12. Both strains K56 and K56$\underline{rif}^R$ supported phage development efficiently in normal PP broth, producing concentrations above 10^8 in all instances. The presence of rifampicin, however, prevented normal propagation, even on the strain possessing an RNA polymerase insensitive to inhibition by this antibiotic. This is indicated by the production of 3.6×10^5 and 3.3×10^4 viable H4489A and 2172

TABLE 11

CONCURRENT DEVELOPMENT OF PHAGES H4489A AND T12 IN $T25_3$, AND PHAGES H4489A AND 2172 IN K56

Phage	Titer A*	Titer B**	% (A/B x 100)
H4489A	2.4×10^8	7.2×10^6	0.3
T12	1.8×10^8	1.8×10^8	100
H4489A	2.4×10^8	1.1×10^8	42
2172	3.3×10^8	1.7×10^8	51

*Titer A = PFU/ml produced in the culture infected with one phage strain.

**Titer B = PFU/ml produced in the culture coinfected by two phages.

TABLE 12

EFFECT OF RIFAMPICIN ON PROPAGATION OF PHAGES IN STRAIN K56*rif*^{R}

Phage	Bacteria	mg/ml rif	Phage Titer	G(min/gen)
H4489A	K56*rif*^{R}	0.15	3.6×10^5	90
		0.00	3.3×10^8	90
	K56	0.15	9.0×10^3	0
		0.00	1.2×10^8	78
2172	K56*rif*^{R}	0.15	3.3×10^4	90
		0.00	6.9×10^8	90
	K56	0.15	2.1×10^3	0
		0.00	1.2×10^8	78

G(min/gen) = generation time of phage-free K56 and K56*rif*^{R} cultures in mid-logarithmic stages of growth.

per ml by K56$\underline{rif}^R$ in media containing rifampicin. The possibility that this effect was the result of a slower K56$\underline{rif}^R$ growth rate was eliminated by following the growth kinetics of each phage-free culture. Generation times of this strain were unaffected by the addition of rifampicin (1.5 hour). Some propagation, however, did occur on K56$\underline{rif}^R$ in the presence of the antibiotic. This is indicated by the increased titers of 96.7 and 93.1 percent over the H4489A and 2172 concentrations found in the rifampicin inhibited K56 wild type cultures. Nevertheless, inhibition by the antibiotic still retarded phage production in K56$\underline{rif}^R$ cultures by more than 99.9 percent of the same resistant strain in the absence of rifampicin. It would appear therefore that a phage specific RNA polymerase was essential for normal phage propagation.

The stability of the K56 and $T25_3$ phage-host complexes were investigated to determine whether they were lysogenic or pseudolysogenic. Incubation of each lysogenic culture in PP broth containing 10 percent antiserum specific for the corresponding bacteriophage prevented reinfection of any spontaneously occurring phage-free bacteria. To facilitate detection of phage-free cocci in a chain of lysogens, the cultures were sonified prior to spreading for isolated colonies, so that each colony forming unit (CFU) was a single cell. Following daily examinations (for 10 consecutive days) of 200 isolated bacterial colonies for the presence of phage, it was found

that the 2000 colonies selected maintained their association with phage in the presence of viral-specific neutralizing antisera. It was concluded that all three phage-host complexes were truly lysogenic.

Concentrating and Purifying Bacteriophage Preparations

The efficiency of ultracentrifugation to concentrate and purify streptococcal bacteriophages was examined using a sedimentation force of 101,962 x g for durations of 30 minutes to 2 hours (Table 13). Maximum concentrations of viable phages were produced by 90 minutes of centrifugation for all three phages. This was also the time that led to minimum loss of plaque forming activity. Recovery, however, was at best 55 and 72 percent of the bacteriophage input for H4489A and 2172, respectively. Phage T12, on the other hand, appeared insensitive to the excessive sedimentation forces employed, since no decreases in viability occurred.

The concentrated phage preparations produced by 90 minutes sedimentation at 101,962 x g and resuspended in T2 buffer were analyzed for purity by disc gel electrophoreses. Four bands of protein were detected in these samples as indicated in Table 14. The dominant components in the preparations demonstrated the greatest electrophoretic mobilities (R_f,0.30). It seems likely that these proximal bands represented the phage, or at least a phage-related protein. This conclusion was reached after comparing these results with

TABLE 13

EFFICIENCY OF PHAGE CONCENTRATION BY ULTRACENTRIFUGAL SEDIMENTATION (101,962 x g) FOR VARIOUS TIMES

Phage	Min	PFU (S)	PFU (P)	PFU (0)	% Recovery
H4489A	30	1.5×10^9	1.2×10^9	7.5×10^9	37
	60	7.5×10^8	2.1×10^9		39
	90	4.5×10^8	3.7×10^9		55
	120	2.4×10^8	3.0×10^9		42
2172	30	9.9×10^8	1.2×10^{10}	5.1×10^{10}	61
	60	7.5×10^8	2.6×10^{10}		65
	90	1×10^8	3.7×10^{10}		72
	120	7.8×10^7	3.3×10^{10}		70
T12	30	2.1×10^{10}	2.1×10^{10}	4.2×10^{10}	100
	60	1.2×10^{10}	3.0×10^{10}		100
	90	7.9×10^6	4.2×10^{10}		100
	120	1.2×10^6	4.2×10^{10}		100

PFU (S) total PFU in supernatant fluid
PFU (P) total PFU in resuspended pellet
PFU (0) total PFU in original preparation

TABLE 14

QUANTITATIVE REPRESENTATION OF BACTERIOPHAGE PREPARATIONS CONCENTRATED BY ULTRACENTRIFUGATION AND ANALYZED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

R_f	H4489A	2172	T12
0.0	0.14	0.14	0.10
0.15	0.04	0.20	0.02
0.17	0.20	0.44	0.40
0.30	2.9	2.5	3.30

Amounts were determined geometrically from graphic representations obtained by gel scans using a Gilford linear transport. Each value expresses the amount of the individual band as a factor of the average band quantity in the respective gel, i.e.

$$\frac{\text{quantity of band x}}{\text{quantity of average band}}$$

similar electrophoretic determinations of phage-free K56 and $T25_3$ broken cell extracts and culture filtrates. (refer to Tables 24 and 25). These proximal, densely stained bands of the phage preparations were distinct from any components in the cell extracts of filtrates, whereas the three less prominent bands appeared to have counterparts of similar R_f values in the phage-free samples. It is also notable that the three viruses are remarkably similar in electrophoretic mobilities, if these bands did in fact represent the bacteriophages.

Three other methods of concentrating and purifying these bacteriophages were attempted, but proved to be impractical. Concentration by pervaporative dialysis in a Schleicher and Schuell colloidin bag apparatus required 7 days to affect a 10-fold reduction in the original 20 ml volume. In addition, this method produced a viscous liquid containing concentrated nondialyzable impurities as well as viruses. Buoyant density centrifugation in CsCl (refractive index 1.5) was also unsatisfactory for producing highly purified phage concentrates (Figure 6). Although 90 percent of the phage activity was located in 22 percent of the total volume, and optical absorption profiles indicated these fractions to be relatively pure, only 8.2 percent of the phage input was recovered. Sephadex G200 chromatography of a polyethylene glycol (4000) concentrated H4489A phage preparation was attempted to separate the viruses from the viscous turbid precipitate (Figure 7). Although highly concentrated fractions

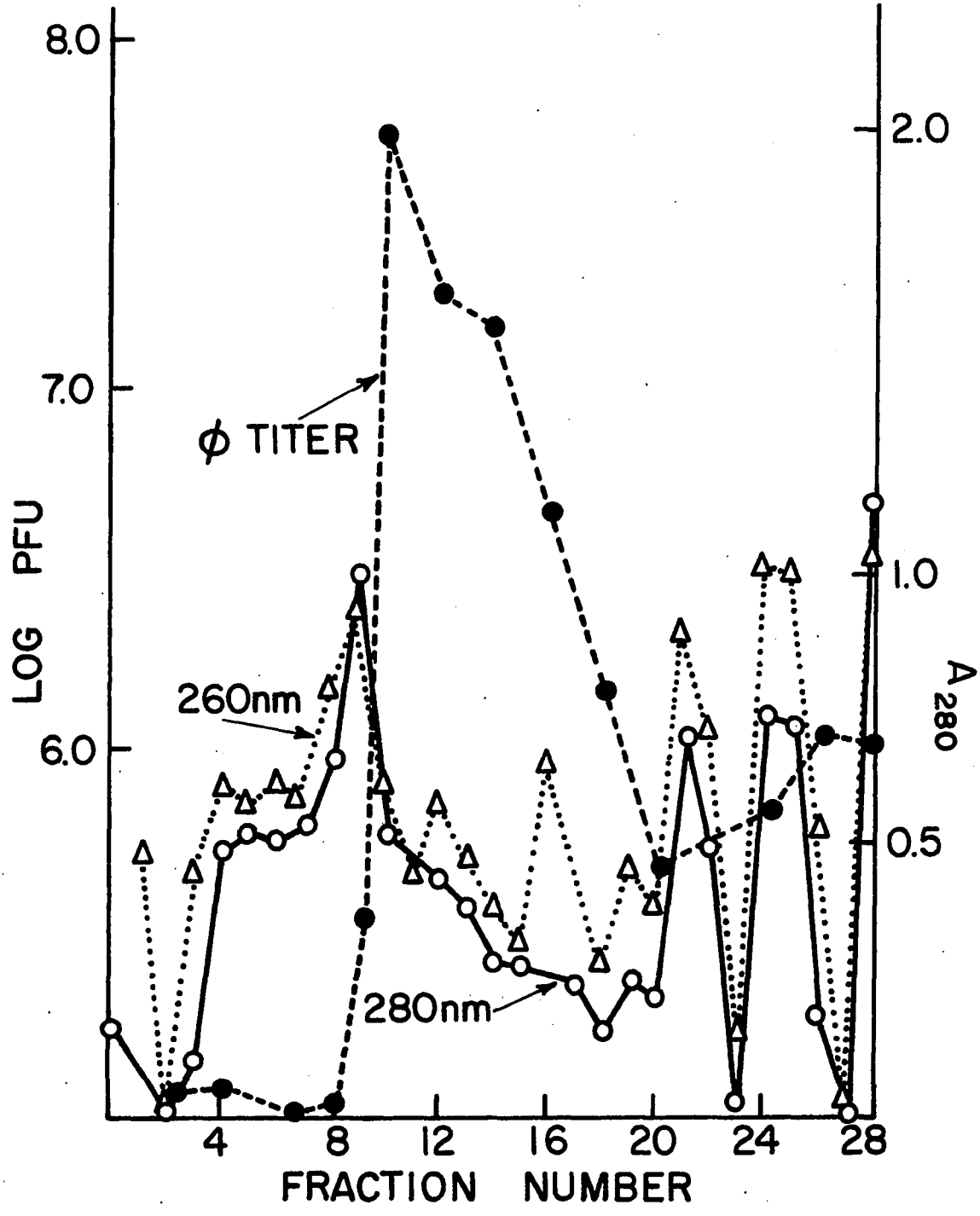


FIGURE 6 - BUOYANT DENSITY CENTRIFUGATION OF PHAGE H4489A IN CsCl

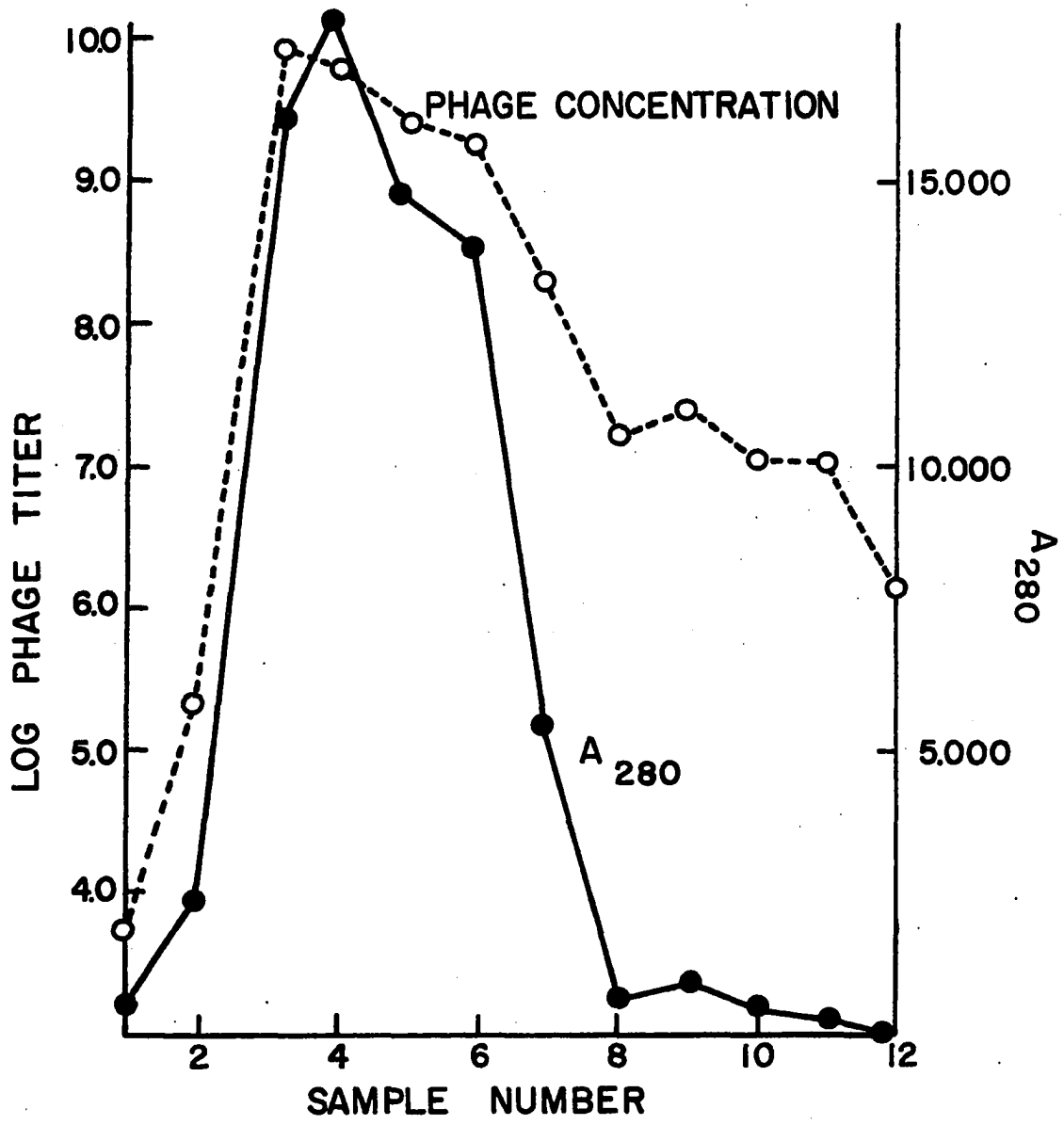


FIGURE 7 -ELUTION PATTERN OF CONCENTRATED PHAGE H4489A ON SEPHADEX G200

were obtained, the abnormally high absorbancy in these fractions reflected the presence of the same impurity as prior to chromatography. The association of phage with the polyethylene glycol precipitate was therefore inseparable by this method. Of the methods attempted, the most efficient for concentrating and purifying bacteriophage preparations was ultracentrifugation for 90 minutes at 101, 962 x g.

Kinetics of Phage Adsorption

Since every phage conversion system described involving altered bacterial surface determinants was accompanied by loss of the corresponding phage sensitive adsorption sites, it was decided to first examine the effects of lysogeny on the rate of phage uptake. Figures 8, 9, and 10 illustrate these adsorption kinetics to lysogenic and nonlysogenic K56 or $T25_3$ cultures. These findings indicate no significant decrease in phage adsorption following infection by any of the three temperate viruses. Calculations of adsorption velocity constants support the graphic data, which shows the greatest variation to be the 14 percent difference in the 2172 system. Therefore, no significant decrease in adsorption of any of the three temperate viruses was associated with lysogeny.

Serological Methods

Establishing the standard agglutination technique for determining concentrations of antibody specific for intact streptococci required

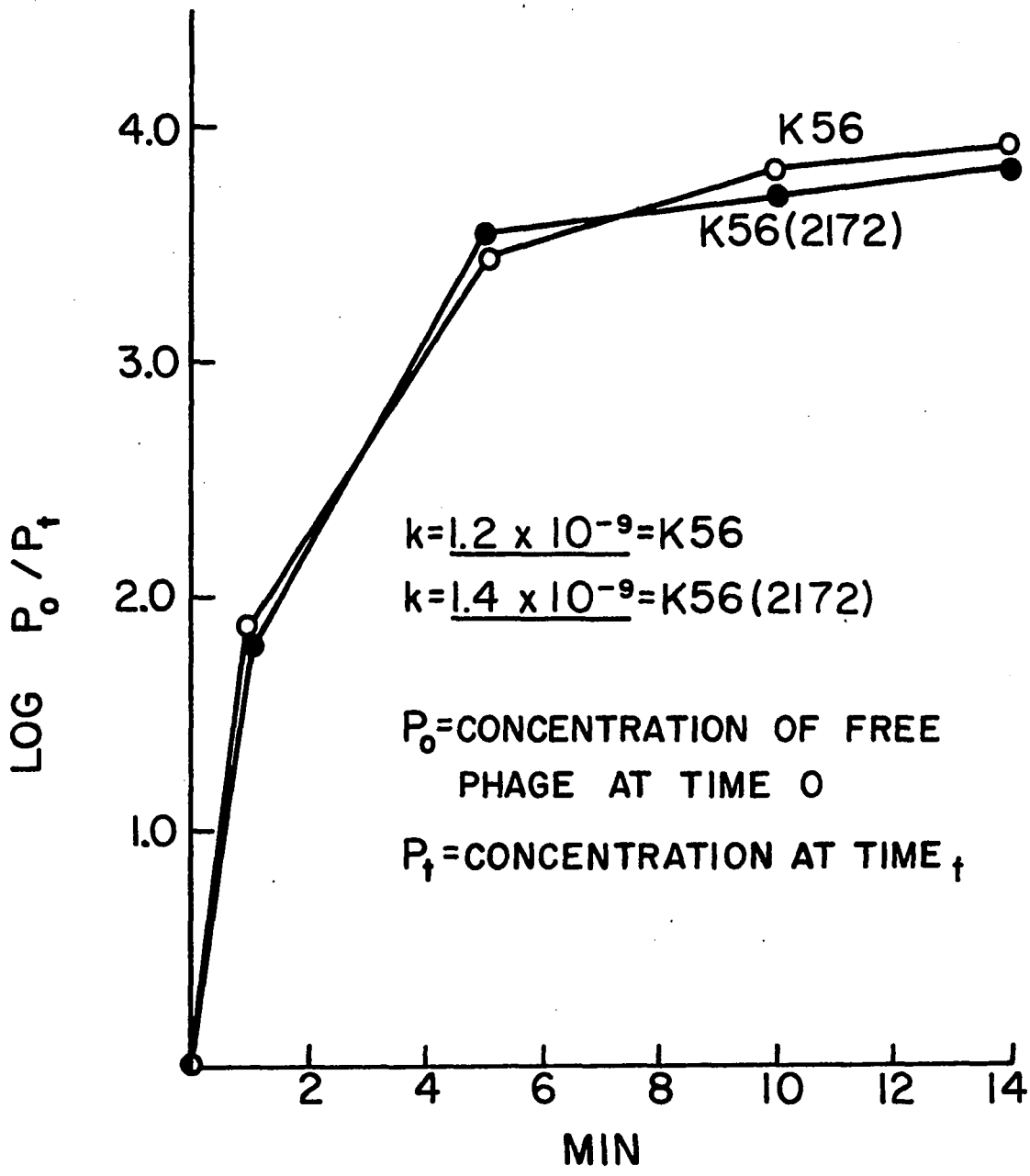


FIGURE 8 - ADSORPTION OF PHAGE 2172 TO K56 AND K56 (2172)

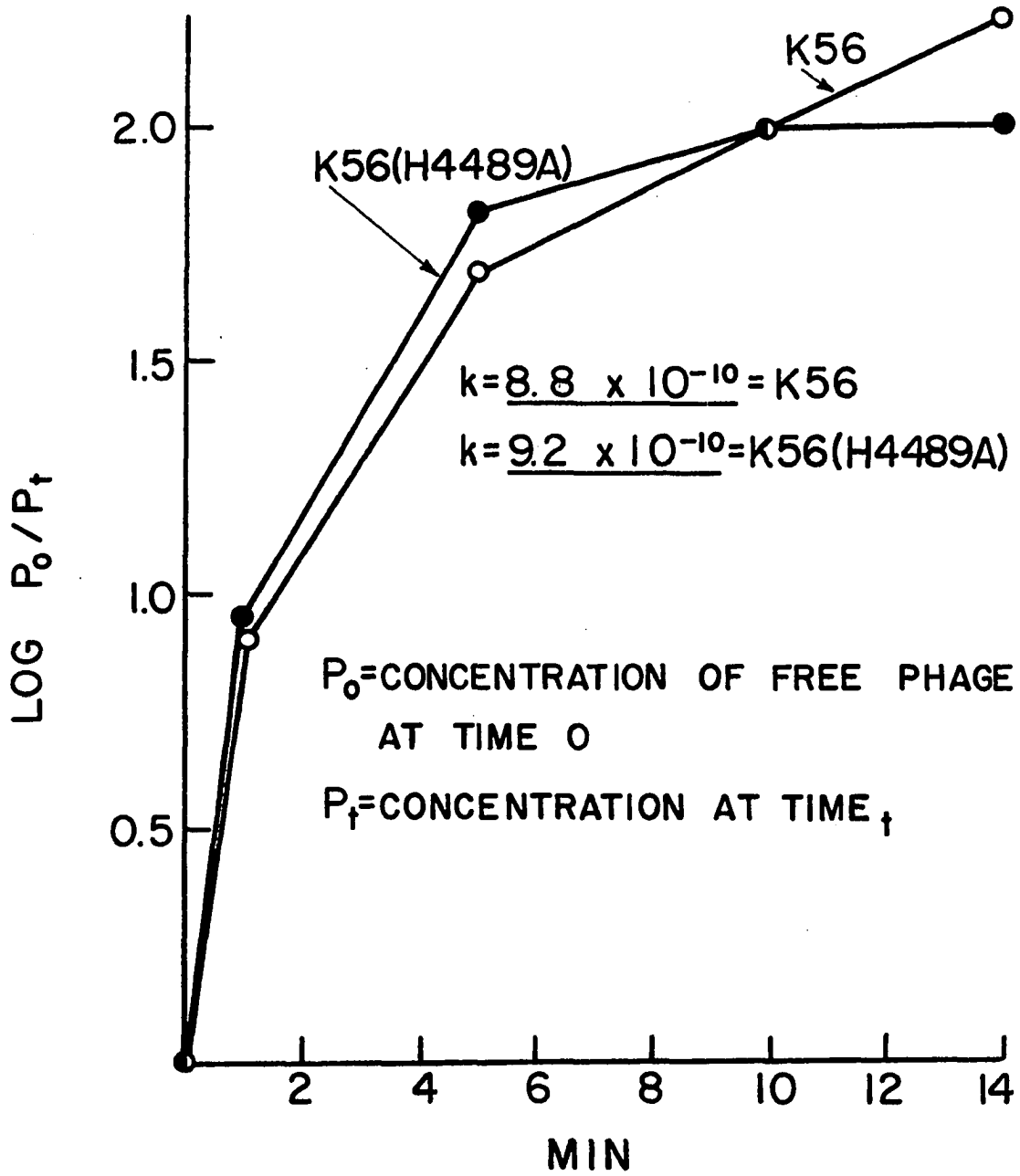


FIGURE 10-ADSORPTION OF PHAGE H4489A TO K56 AND K56 (H4489A)

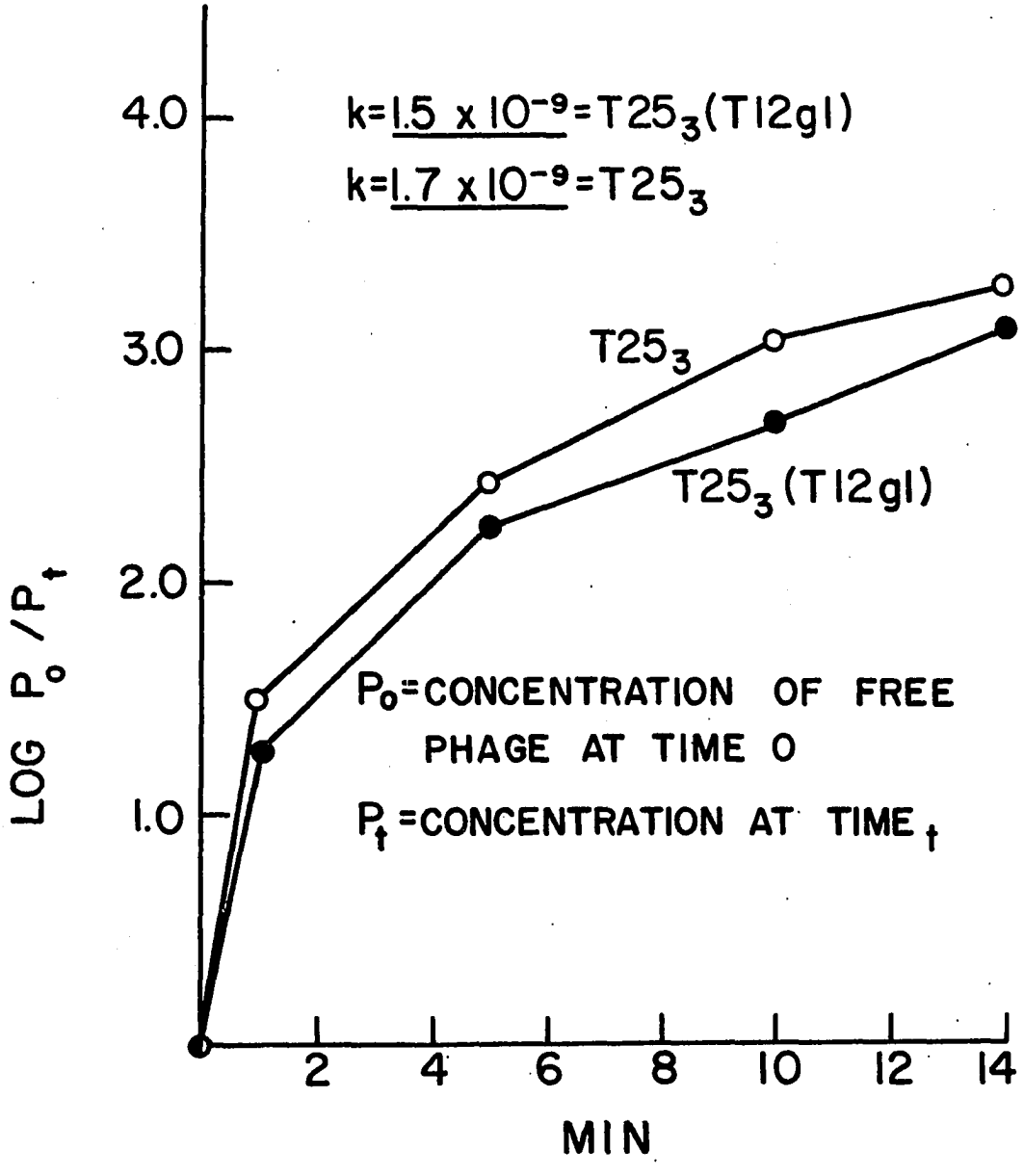


FIGURE 9 -ADSORPTION OF PHAGE T12 TO $T25_3$ (T12gl)

several avenues of approach. Initial agglutination experiments incorporated the indicator bacteria into the diluent prior to diluting the sera. The concentrations of cells in these washed indicator suspensions approximated those of cultures in late logarithmic growth stages. This technique, however, sufficed only for the K56 strains, $T25_3$ and $T25_3$(T12gl) both autoagglutinating in control tubes lacking serum. One attempt to resolve this difficulty involved coupling the bacteria to goat erythrocytes to facilitate interpretation of the agglutination patterns. Subsequent attempts to separate the blood cells from the uncoupled bacteria by differential centrifugation were futile, since no centrifugal force was found to sediment the erythrocytes without concomitant bacterial sedimentation. Microscopic examination revealed the pellets to contain at best 50 percent free bacteria, 25 percent uncoupled goat blood cells, and only 25 percent complexes of erythrocytes and streptococci. Using either velocity or density gradients in the centrifugation steps failed to separate the complexes into discrete bands, but rather distributed the cells throughout the tube. Further efforts to utilize blood cells as indicators were abandoned.

The use of a microtiter agglutination technique using bacterial cells as the indicator was equally unsuccessful. Development of the proper agglutination pattern was apparently prevented by the inability of the nonagglutinated streptococcal chains to form distinct

pellets in the bottom of the wells, as would single cells. The normal chains were perhaps mimicking agglutinated bacteria. Microtiter results using the sedimented duplexes of erythrocytes and streptococci as the indicator were equally ambigous.

The standard agglutination technique was developed by adding varying concentrations of indicator bacteria to the antisera following dilution. It was eventually determined that concentrating bacterial cultures in late logarithmic growth stages, resuspending in PBS at 1/20 the original volume, and adding a single drop from a Pasteur pipette to each ml of diluted serum produced highly reproducible agglutinating patterns. Autoagglutination in preimmune serum or in control tubes lacking serum was eliminated. This method, although apparently reliable, was occasionally evaluated by quantitative slide agglutination, using the same indicator preparation employed in the tube technique. Results rarely differed by more than a single 2-fold dilution factor. Agglutinating titers of all whole-cell antisera were standardized to 2048.

The capacity of the antisera to kill the corresponding bacteria was examined as an alternative method for determining specific antibody concentration. Subsequent findings revealed the antibody to be non-lethal, as no decrease in bacterial viability was noted following incubation in antisera with specific agglutinating titers of 2048 and in preimmune antisera.

Neutralization kinetics of each temperate bacteriophage by specific antiserum are shown in Figures 11, 12, and 13. Each serum diluted 1:10 completely neutralized the infectivity of the corresponding phage preparations containing an excess of 10^7 PFU/ml. These kinetic curves were used to determine the amount of antisera needed in experiments requiring prevention of phage infection.

Effects of Lysogeny on Somatic Antigens

To ascertain whether phage mediated alterations in surface determinants occurred, the standardized antisera prepared against unbroken streptococci were absorbed with standardized doses of bacteria. The concentration of immunoabsorbant was determined using the turbidity vs. dry weight curves illustrated in Figures 14 and 15. Residual agglutinating titers of each serum following immunoabsorption were ascertained using lysogens and nonlysogens as indicators (Table 15). Nonlysogenic $T25_3$ cells failed to remove at least one group of antibodies in the serum prepared against $T25_3$(T12gl). This is indicated by agglutination of the lysogen by this residual antibody (titer=512). Conversely, $T25_3$ antiserum also appeared to contain antibody not removed by $T25_3$(T12gl) absorption (residual titer = 128). The basal level of residual antibody could not be reduced below a titer of 16 by increasing the concentration of the absorbant.

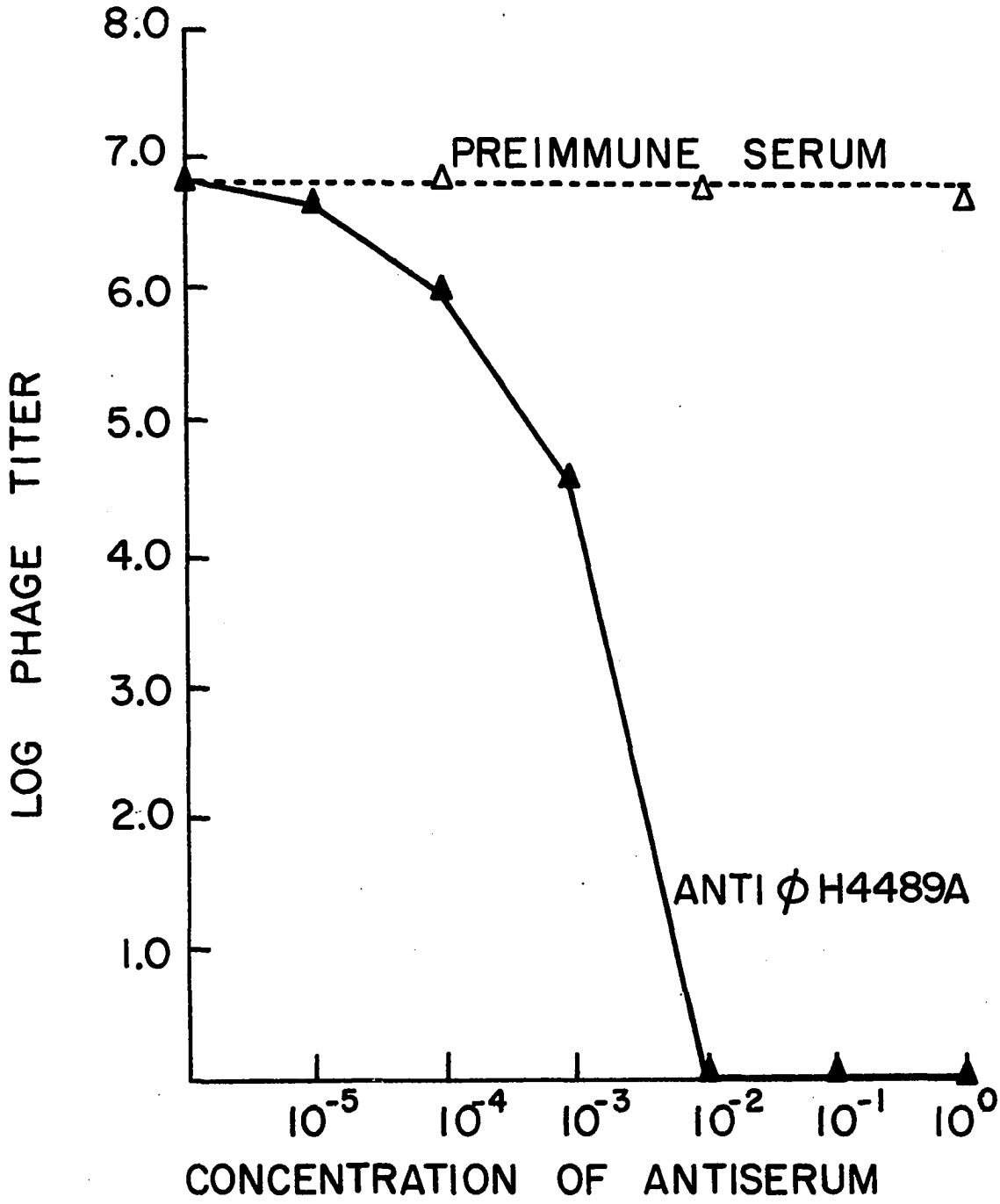


FIGURE 11 -NEUTRALIZATION KINETICS OF PHAGE H4489A BY H4489A SPECIFIC ANTISERUM

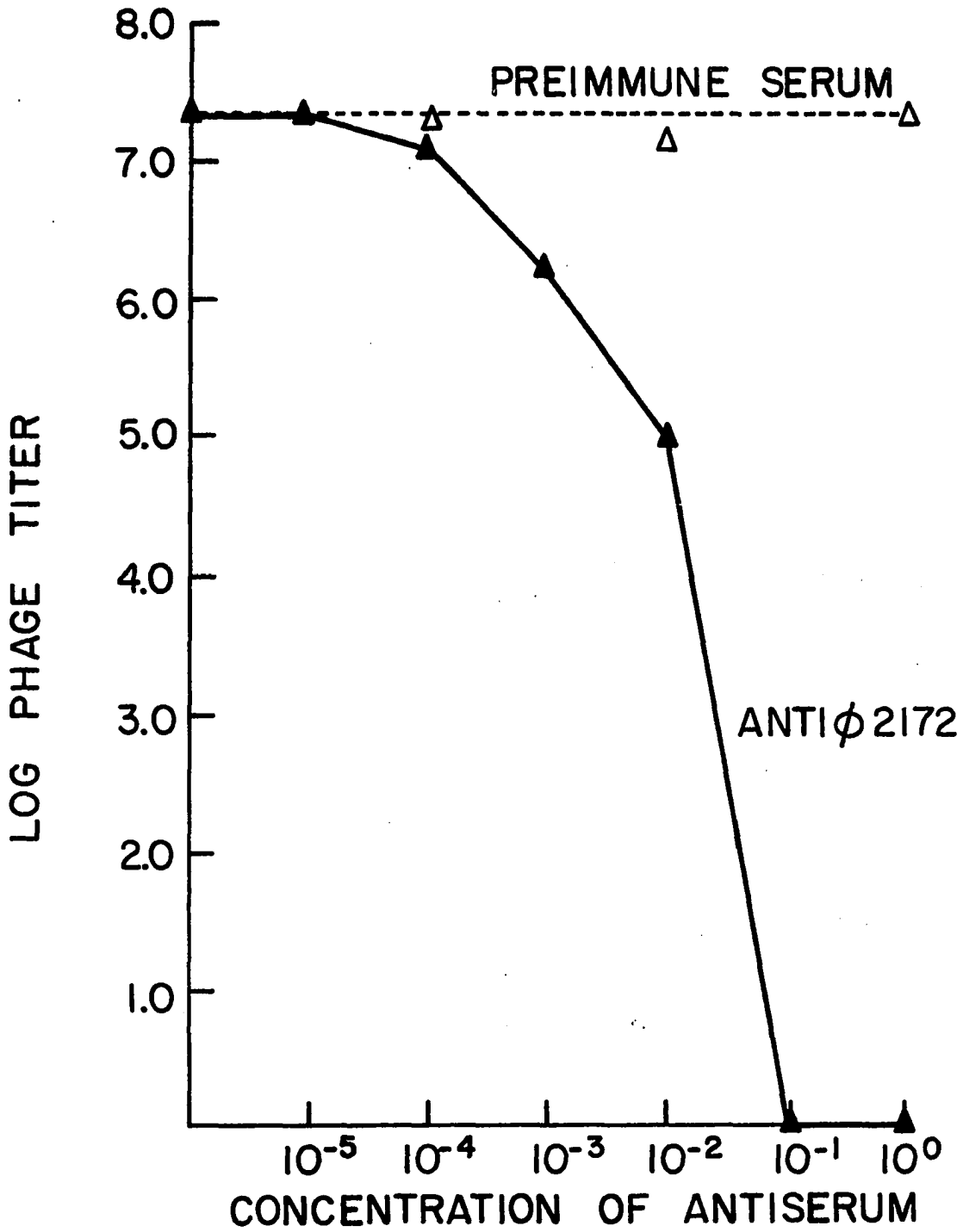


FIGURE 12 -NEUTRALIZATION KINETICS OF PHAGE 2172 BY 2172 SPECIFIC ANTISERUM

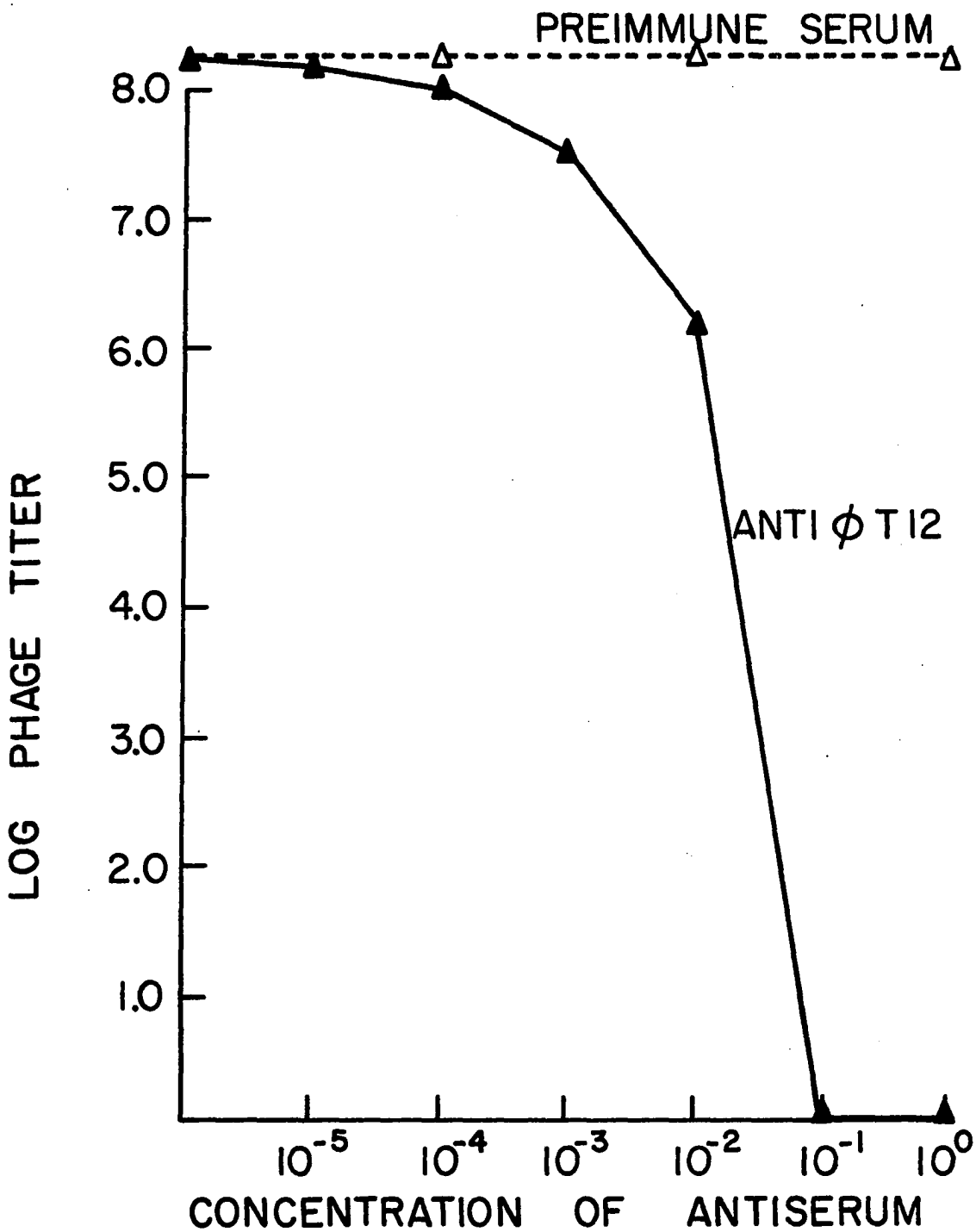


FIGURE 13 -NEUTRALIZATION KINETICS OF PHAGE T12 BY T12 SPECIFIC ANTISERUM

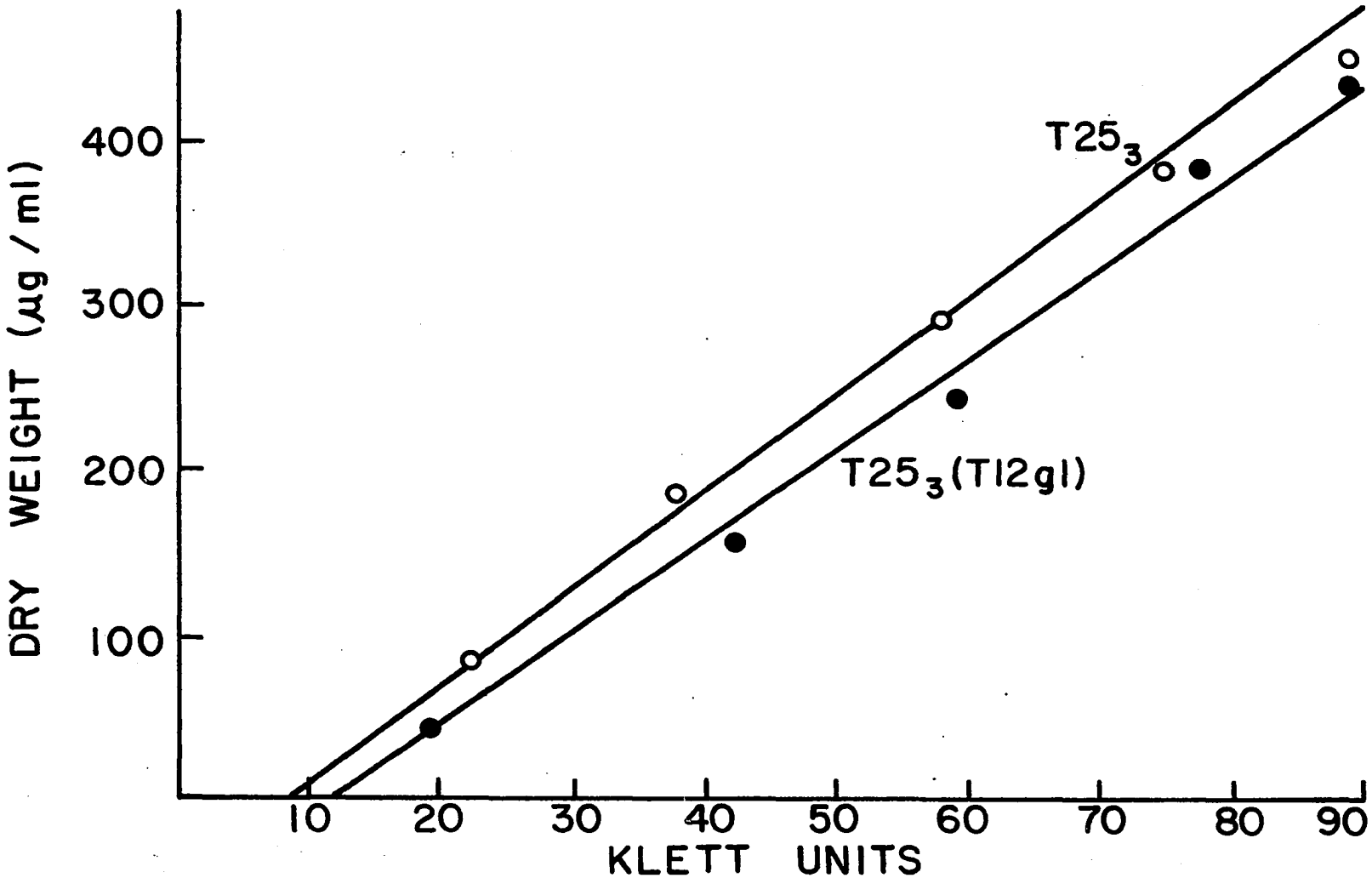


FIGURE 14 -TURBIDITY VS DRY WEIGHT STANDARD CURVE [T25$_3$ AND T25$_3$(T12gl)]

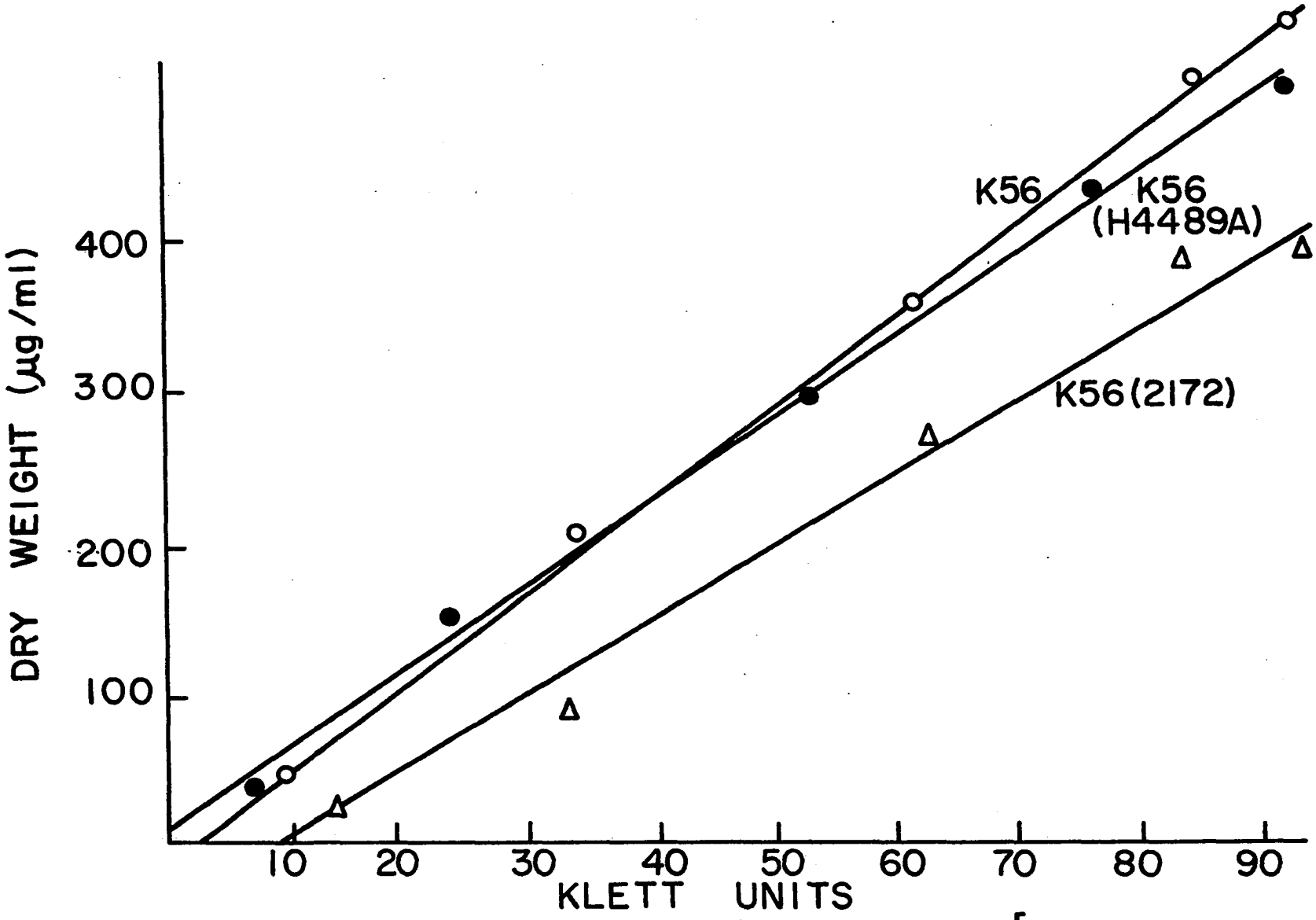


FIGURE 15 -TURBIDITY VS DRY WEIGHT STANDARD CURVE [K56, K56(H4489 A), AND K56 (2172)]

TABLE 15

TITERS OF RESIDUAL AGGLUTININS IN ANTISERA ABSORBED WITH SATURATING DOSES OF HOMOLOGOUS AND HETEROLOGOUS WHOLE CELL ANTIGENS

Antisera	Absorbing Antigen	Agglutinating Antigen	4	8	16	32	64	128	256	512	1024	Residual Titer
Anti $T25_3$	$T25_3$	$T25_3$	-	+	+	-	-	-	-	-	-	16
		$T25_3$(T12gl)	-	+	+	-	-	-	-	-	-	16
	$T25_3$(T12gl)	$T25_3$	-	+	+	+	+	+	-	-	-	128
		$T25_3$(T12gl)	-	+	+	-	-	-	-	-	-	16
Anti $T25_3$ (T12gl)	$T25_3$	$T25_3$	-	+	+	-	-	-	-	-	-	16
		$T25_3$(T12gl)	-	+	+	+	+	+	+	+	-	512
	$T25_3$(T12gl)	$T25_3$	-	+	+	-	-	-	-	-	-	16
		$T25_3$(T12gl)	-	+	+	-	-	-	-	-	-	16
Anti $T25_3$	-	$T25_3$	-	+	+	+	+	+	+	+	+	2048

TABLE 15 --Continued

Antisera	Absorbing Antigen	Agglutinating Antigen	4	8	16	32	64	128	256	512	1024	Residual Titer
Anti $T25_3$ (T12gl)	-	$T25_3$(T12gl)	-	+	+	+	+	+	+	+	+	2048
Preimmune Serum	-	$T25_3$	-	-	-	-	-	-	-	-	-	0
	-	$T25_3$(T12gl)	-	-	-	-	-	-	-	-	-	0

+ = reduction in turbidity of suspension, curling of pellet edges away from bottom of tube, pellet forms clumps when agitated.

Reciprocal quantitative immunoabsorption with the same two bacterial strains and their antisera is shown in Figures 16 and 17. From Figure 16 (antiserum for nonlysogenic $T25_3$) it can be concluded that the lysogenic $T25_3$(T12gl) culture was insufficient in the same, or similar, antigen as the nonlysogenic $T25_3$ to reduce the agglutinating titer to the basal level produced by homologous absorption, even with a sufficiently large absorbing dose (800 µg/ml). The difference in the reciprocal test, using antiserum for lysogenic strain $T25_3$(T12gl), was even more striking (Figure 17). Again, nonhomologous absorption left a large amount of antibody unabsorbed, indicating that lysogenization had resulted in the appearance of at least one new determinant, perhaps with reciprocal loss of the antigenic counterpart from the phage-free strain.

Results of a similar analysis of the K56 system are presented in Table 16. K56(Ø) represents K56(H4489A) in the fourth column and K56(2172) in the final column. The data indicates no significant differences in residual agglutinins in any of the sera. The absorption, however, was inefficient, effecting only a 2-fold decrease in titer, even when the concentration of homologous absorbant was tripled (3600 ug/ml). Furthermore, attempts to increase the antigenicity of the cells by exposure to HCl, pepsin, or trypsin failed to enhance the removal of antibody. Because of this difficulty, quantitative absorption data was inconsequential, and will not be presented.

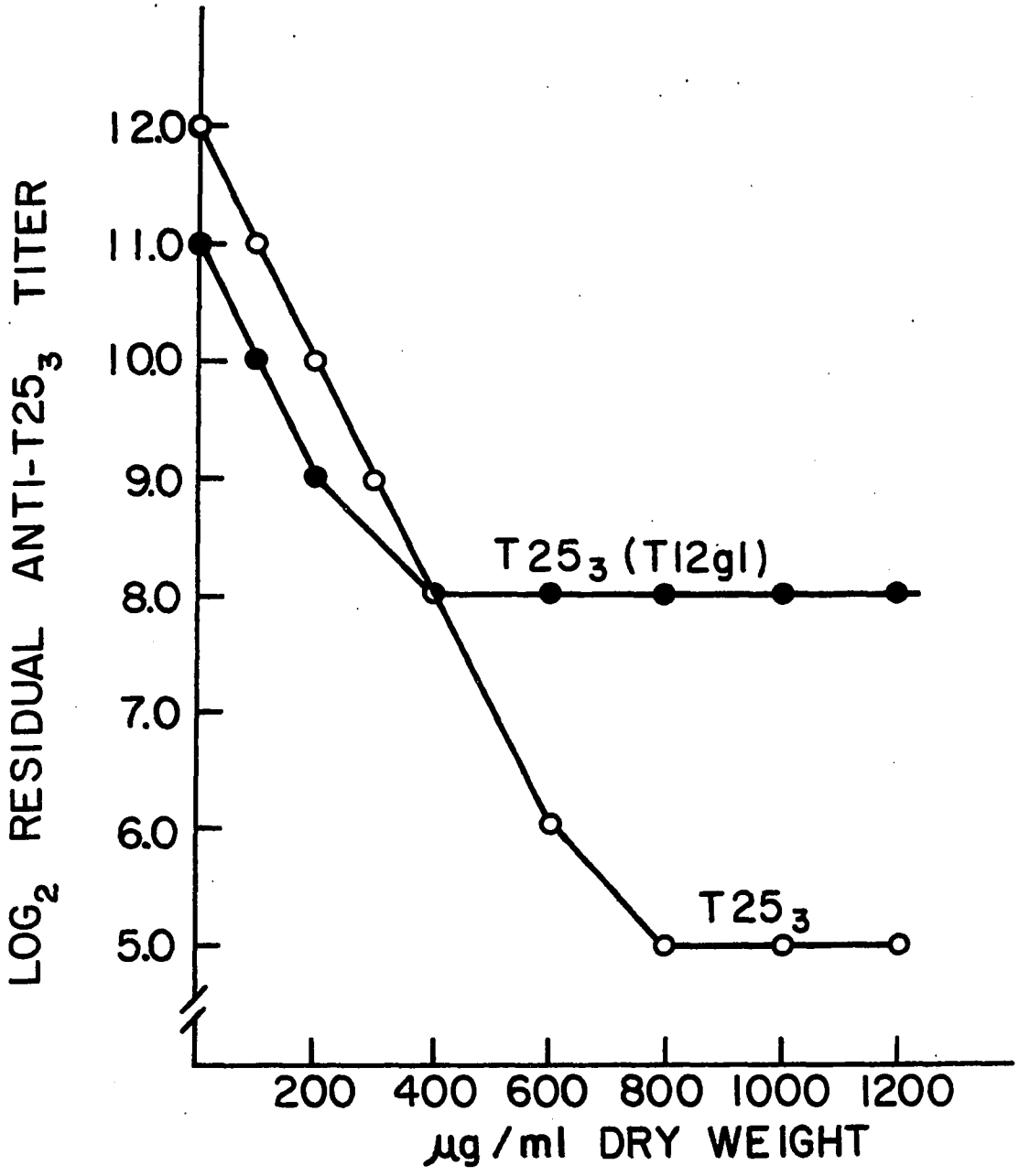


FIGURE 16 - ABSORPTION OF STREPTOCOCCUS PYOGENES $T25_3$ ANTISERUM BY $T25_3$ AND $T25_3$ (T12gl)

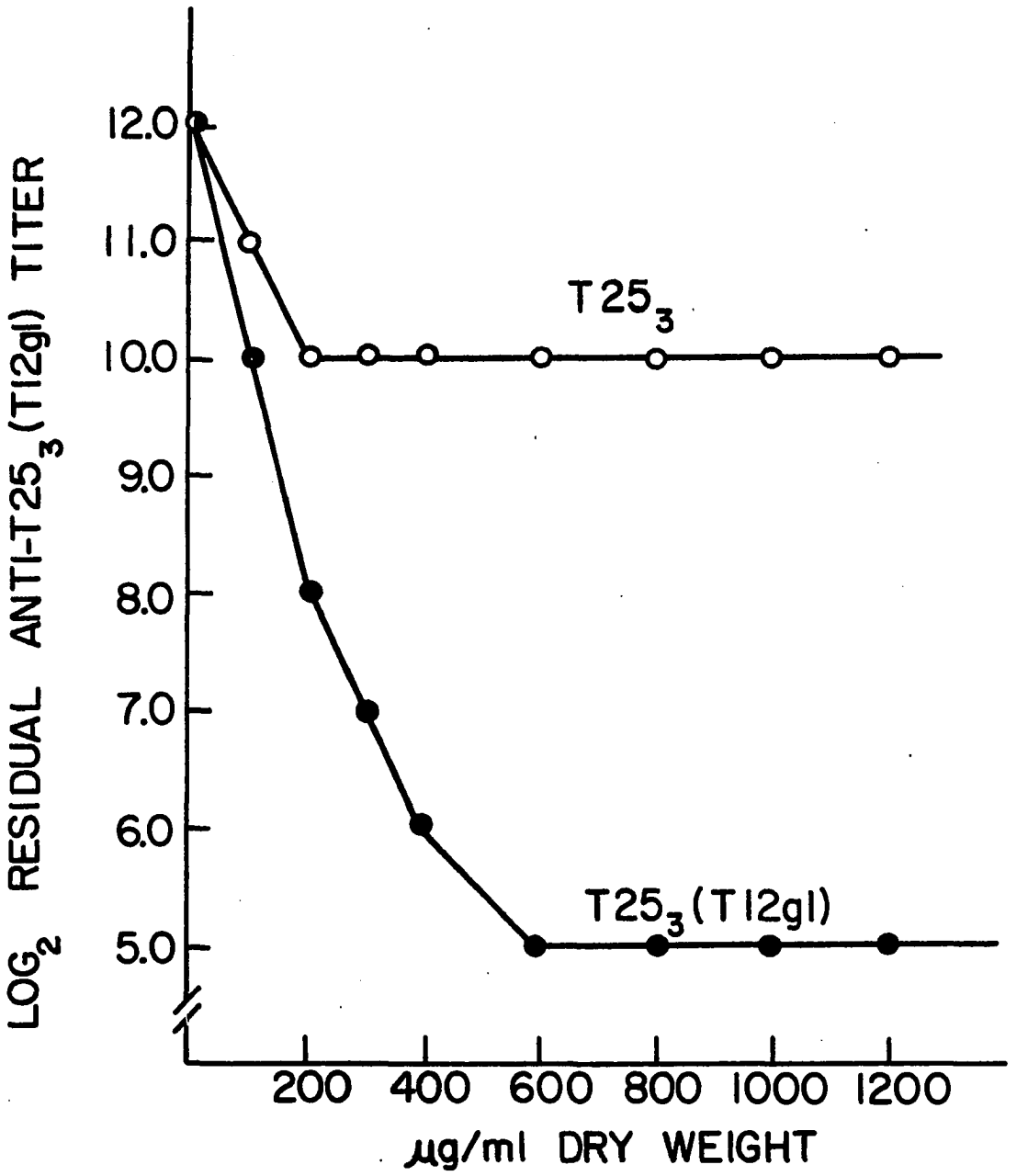


FIGURE 17 -ABSORPTION OF STREPTOCOCCUS PYOGENES T25$_3$(T12gl) ANTISERUM BY T25$_3$ AND T25$_3$ (T12gl)

TABLE 16

TITERS OF RESIDUAL AGGLUTININS IN ANTISERA ABSORBED WITH SATURATING DOSES OF HOMOLOGOUS AND HETEROLOGOUS ANTIGENS

Anti Sera	Absorbing Antigen	Agglutinating Antigen	Residual Titers	
			∅=H4489	∅=2172
Anti K56	K56	K56	512	512
		K56 (∅)	512	512
	K56 (∅)	K56	512	512
		K56 (∅)	512	512
Anti K56 (0)	K56	K56	512	512
		K56 (∅)	512	512
		K56	512	512
		K56 (∅)	512	512
Anti K56	-	K56	2048	2048

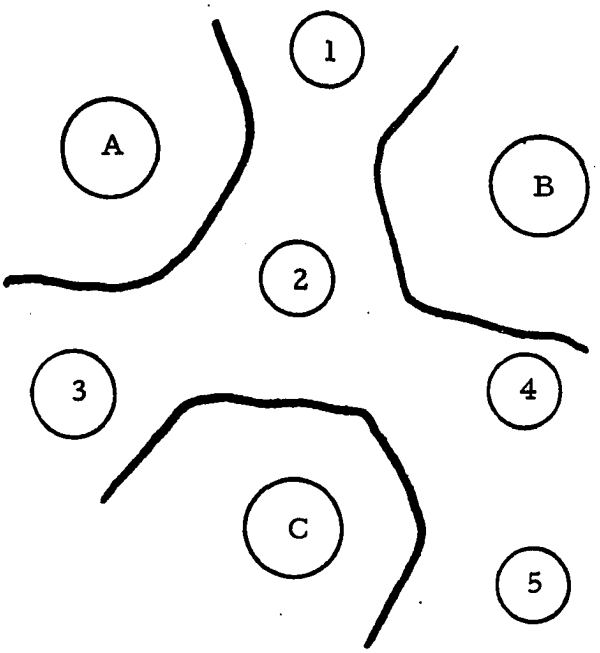
TABLE 16--Continued

Anti Sera	Absorbing Antigen	Agglutinating Antigen	Residual Titers	
			Ø=H4489	Ø=2172
Anti (H4489)	-	K56 (Ø)	2048	2048
Preimmune Serum	-	K56	0	0
	-	K56 (Ø)	0	0

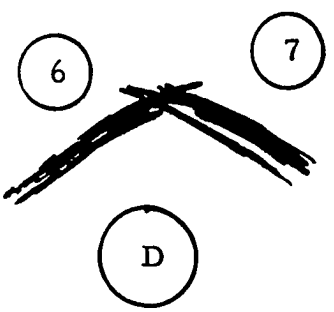
Solubilized antigens of each strain in both the K56 and $T25_3$ systems were employed in double immunodiffusion assays using the method of Ouchterlony. Each antigen preparation was tested with both lysogen and nonlysogen antisera and the results shown in Figure 18. Lines of identity between all antigens present in the preparations of K56 and corresponding lysogens (Figure 20a) support the previous immunoabsorption data indicating no antigenic conversion. In Figure 18-a each antigen well contained a combination of HCl and formamide extracted preparations. However, when $T25_3$ and $T25_3$ (T12gl) antigens were extracted with either formamide (Figure 18-b) or HCl (Figure 18-c), and tested against antisera to respective nonlysogens and lysogens, spur formation revealed the existance of somatic antigens unique to each strain. Furthermore, both extraction procedures are reported to yield primarily a mix of M protein and group carbohydrate.

Attempts to characterize this antigenic conversion chemically involved the use of various agents to destroy the $T25_3$(T12gl) antigen corresponding to the residual agglutinating antibody in the heteroabsorbed serum (Figure 19). The untreated control indicator showed a titer of 512. Enzymatic digestion of surface determinants on the whole cells had no deleterious effects on agglutination when trypsin (1.0 mg/ml) or pepsin (1.0 mg/ml) were employed. The action of pepsin actually enhanced agglutination of the cell suspension perhaps

a.



b.



c.

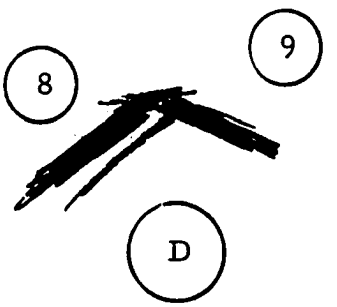


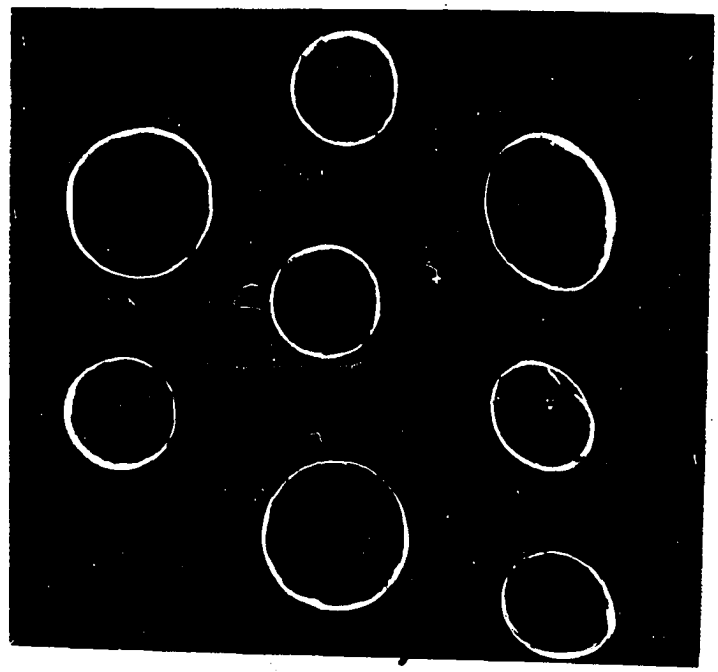
FIGURE 18--GEL DIFFUSION PATTERN USING ANTISERA AGAINST: AND TEST ANTIGENS:

A K56(H4489A)
B K56(2172)
C K56
D $T25_3$ and $T25_3$(T12gl)

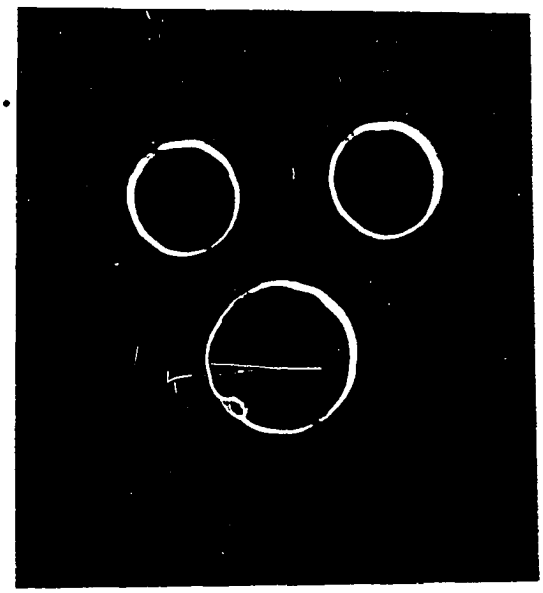
Extracted With

1 & 5 K56(2172) (HCl and Formamide)
2 K56
3 & 4 K56(H4489A) (HCl and Formamide)
6 $T25_3$ (Formamide)
7 $T25_3$(T12gl)
8 $T25_3$ (HCl)
9 $T25_3$(T12gl (HCl)

a.



b.



c.

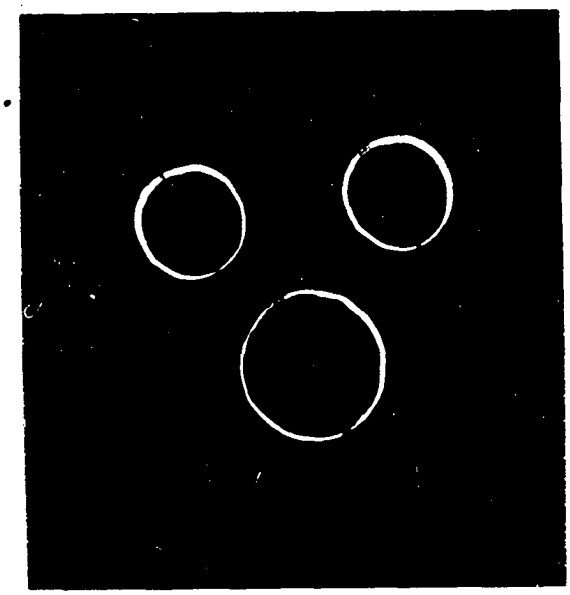


FIGURE 18--GEL DIFFUSION PATTERN USING ANTISERA AGAINST: AND TEST ANTIGENS:

A K56(H4489A)
B K56(2172)
C K56
D $T25_3$ and $T25_3$(T12gl)

Extracted With

1 & 5 K56(2172) (HCl and Formamide)
2 K56
3 & 4 K56(H4489A) (HCl and Formamide)
6 $T25_3$ (Formamide)
7 $T25_3$(T12gl)
8 $T25_3$ (HCl)
9 $T25_3$(T12gl (HCl)

by removal of an overlying protein partially blocking the antigens exposure to antibodies. Activities of the two enzymes were confirmed by examining their capacities to digest human serum albumin, subsequently producing non-TCA precipitable peptide fragments. Figure 20 reveals that both enzymes were actively proteolytic for human serum albumin, indicated by the 2-fold increase in TCA soluble material within 1.75 hours by pepsin and 4.0 hours by trypsin.

Exposure to cold 10 percent TCA resulted in destruction of the distinct agglutinating antigen possibly by precipitation of a protein moiety (Figure 19). However, cold HCl at the same pH had a similar effect as did TCA, suggesting the loss of agglutinating capacity may be due to acid hydrolysis of the antigen rather than specific protein precipitation. The participation of lipid in the specificity of this antigen was contraindicated by the inability of chloroform to destroy its determinant. Sodium dodecyl sulfate, which dissociates protein subunits, also failed to influence agglutination. It was concluded from these experiments that the nature of the somatic antigen in question was neither protein nor lipid.

The possibility that the somatic antigen was either mucopeptide or carbohydrate was further pursued. This was done by determining the agglutination titers of both $T25_3$ and $T25_3$(T12gl) whole cells in Lancefield grouping sera specific for groups A and A-variant (Table 17). The dramatic difference in the titers of the group A serum indicated by the two strains suggests that lysogeny of $T25_3$ by phage T12 was accompanied

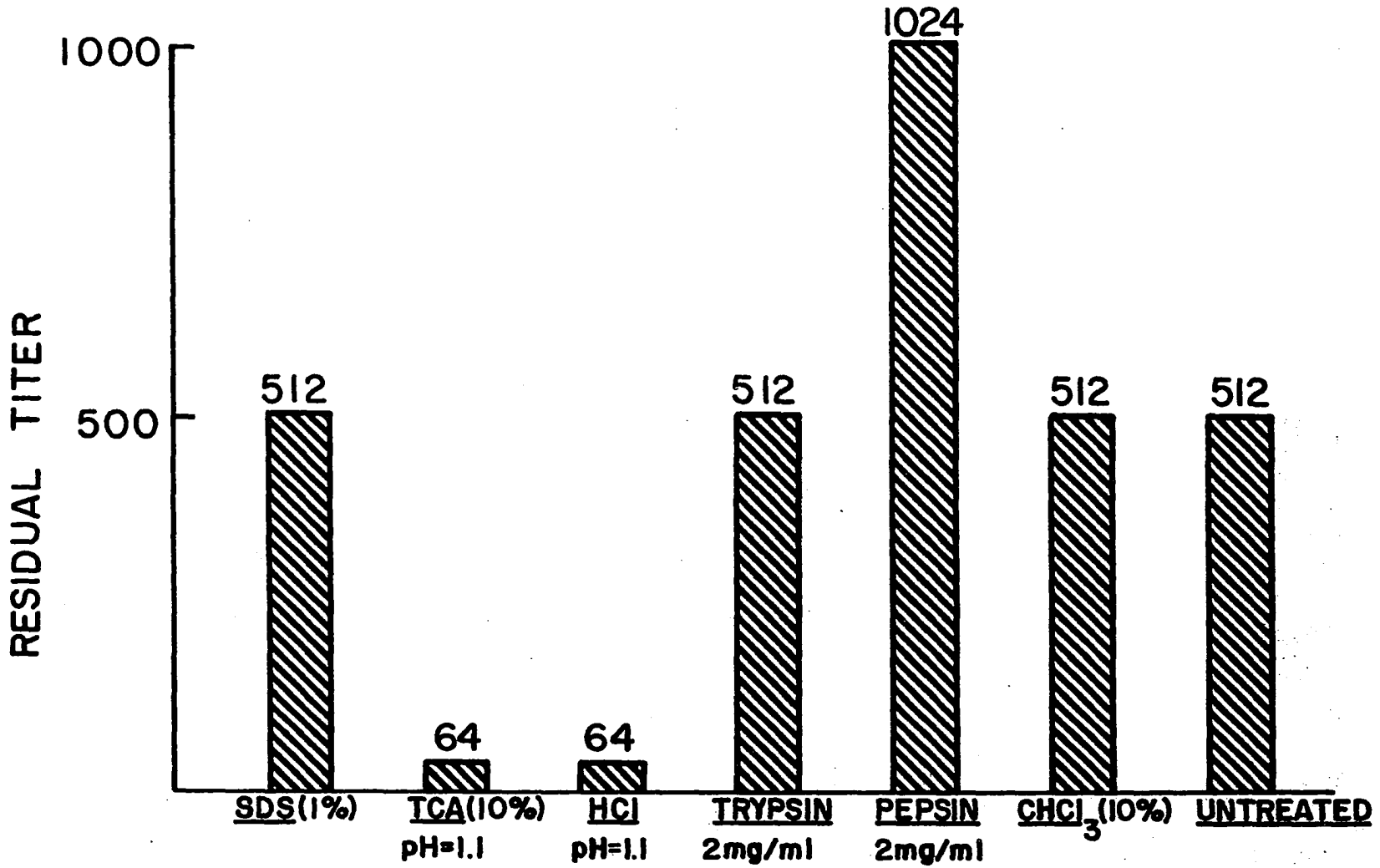


FIGURE 19-EFFECTS OF CHEMICAL AND ENZYMATIC EXPOSURE ON $T25_3$ (T12gl) AGGLUTINATION IN NONHOMOLOGOUSLY ABSORBED ANTISERUM

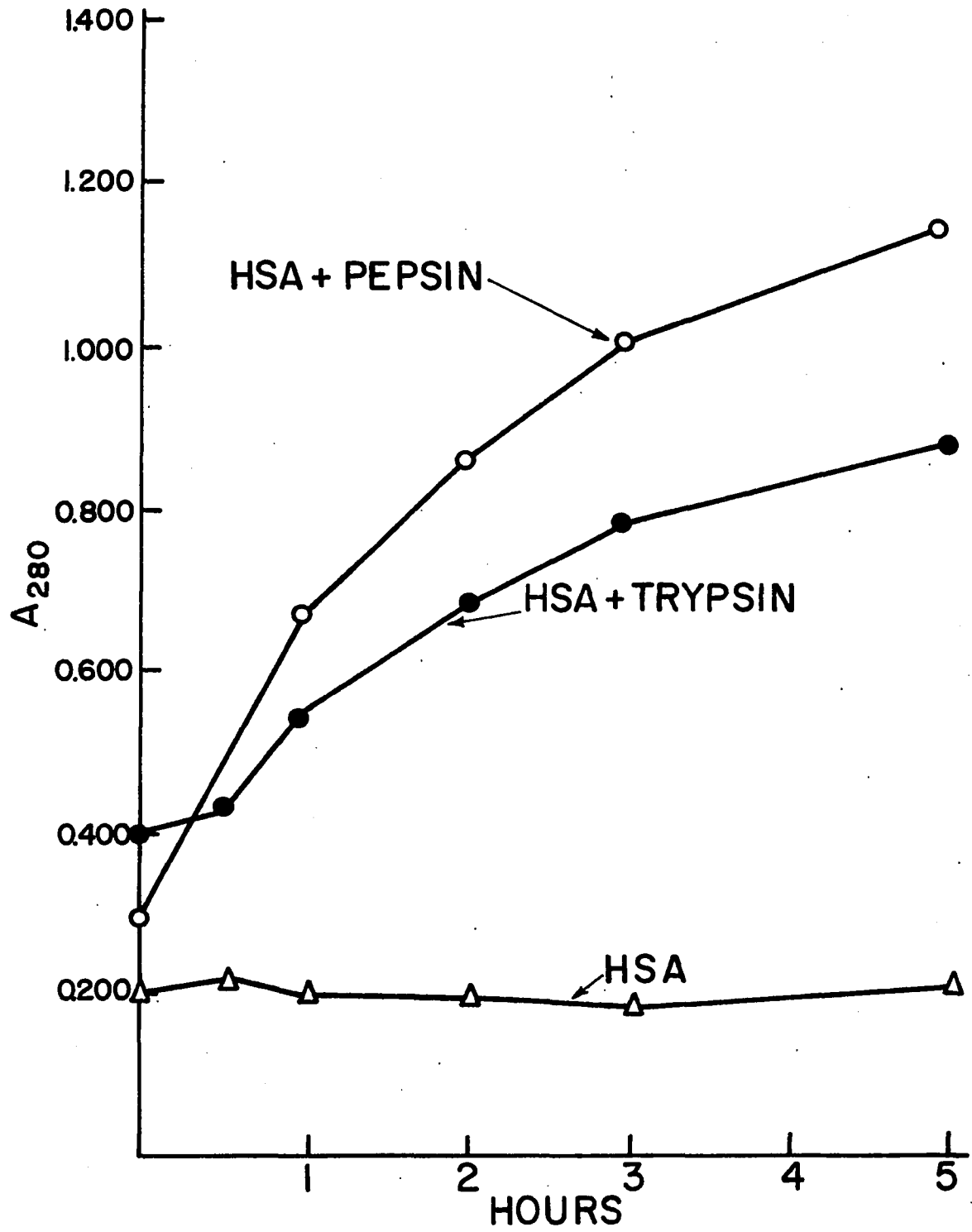


FIGURE20-DETECTION OF TCA SOLUBLE PEPTIDE FRAGMENTS IN SUPERNATANT FLUIDS OF HUMAN SERUM ALBUMIN (HSA) EXPOSED TO PEPSIN AND TRYPSIN

TABLE 17

AGGLUTINATION OF $T25_3$, $T25_3$(T12gl), AND $T25_3$(T12)B IN LANCEFIELD GROUPING SERUM

Serum	Strain	Titer
Anti-Group A	$T25_3$	1024
	$T25_3$(T12gl)	32
	$T25_3$(T12)B	32
Anti-Group V	$T25_3$	512
	$T25_3$(T12gl)	32
	$T25_3$(T12)B	32

by reduction or loss of group A specificity. Support for this conclusion was offered by the finding that artificial lysogenization of $T25_3$ by phage T12 resulted in a strain, $T25_3$(T12)B, with the agglutinating potential of the lysogen rather than the parental phage-free strain. It was also reasoned that group A specificity was possibly replaced by group A-variant since the two polysaccharides differ only in the presence or absence of terminal N-acetyl glucosamine residues. Table 17, however, indicates that $T25_3$ possessed 16 times greater A-variant specificity than did the lysogen. As before, $T25_3$(T12)B appeared similar to $T25_3$(T12gl).

Two quantitative approaches were employed to analyze the differences in group specific carbohydrate possessed by the two strains. Double immunodiffusion using 2-fold dilutions of soluble antigen preparations and undiluted group A specific antiserum suggested that $T25_3$(T12gl) contained no antigen that would form a precipitable complex with group A antibody in agar. Group A specific antigen possessed by $T25_3$, however, was still detectable when diluted 1:4 (Figure 21). The nonlysogen also appeared to contain the greatest amount of A-variant carbohydrate, indicated by the persistance of band formation in dilutions 4 and 8, as well as by the denser precipitin lines that formed in the lower dilutions. The lysogen, however, demonstrated some A-variant specificity, although 4-fold less than $T25_3$.

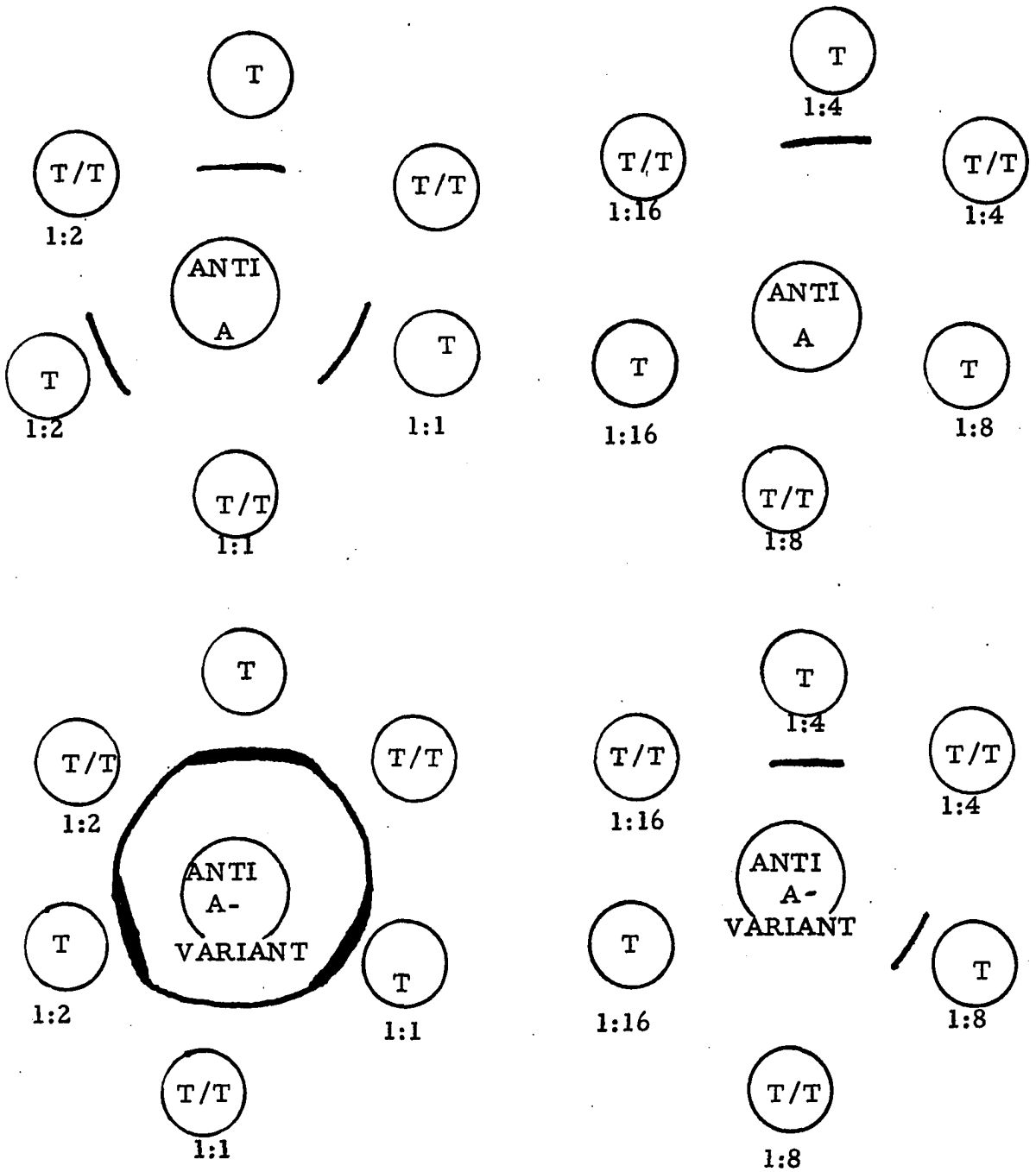


FIGURE 21--A quantitative gel diffusion of solubilized antigens of $T25_3$ and $T25_3$(T12gl) with group A and A-variant antisera.

T = $T25_3$

T/T = $T25_3$(T12gl)

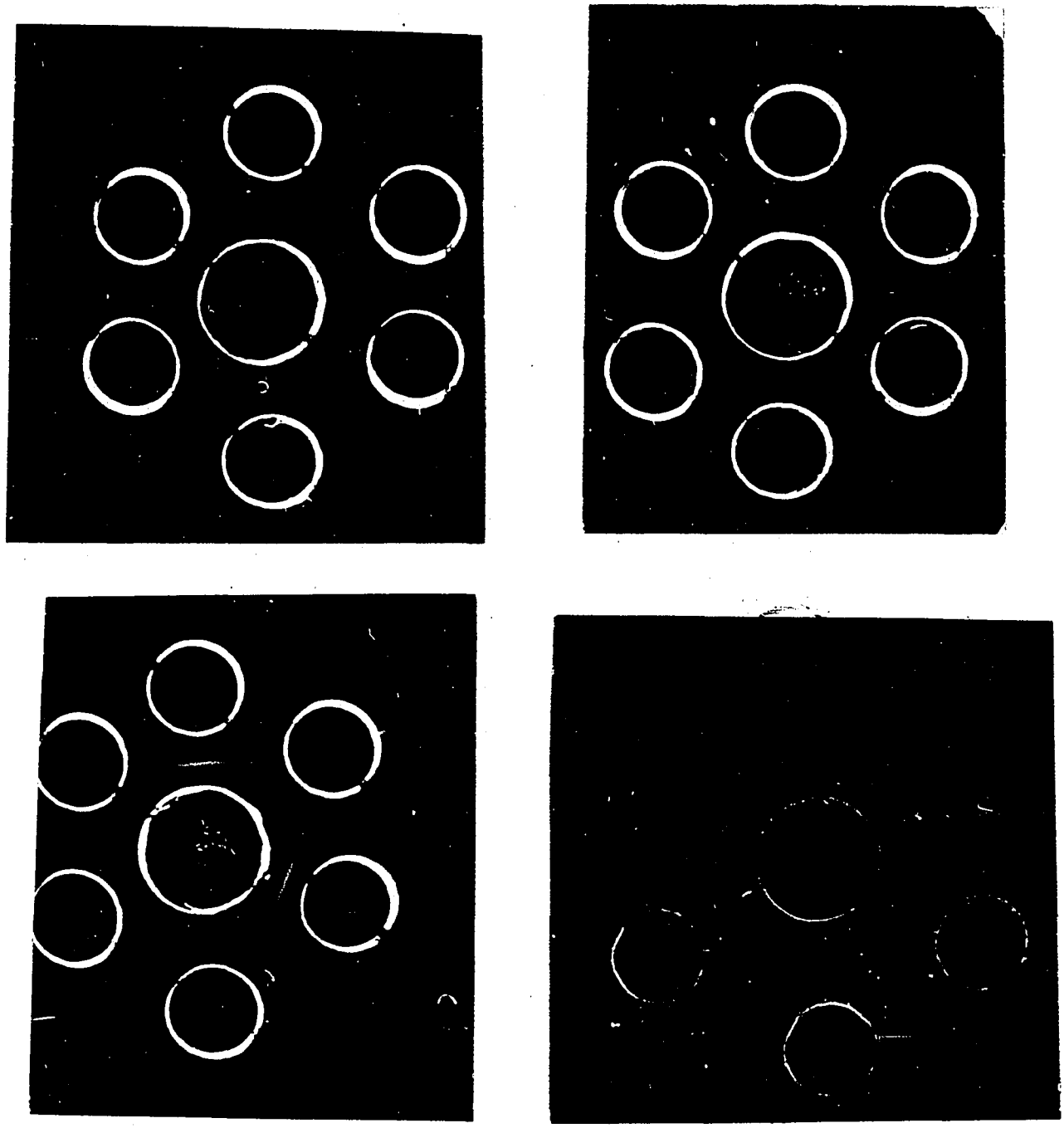


FIGURE 21--A quantitative gel diffusion of solubilized antigens of $T25_3$ and $T25_3$(T12gl) with group A and A-variant antisera.

T = $T25_3$

T/T = $T25_3$(T12gl)

The results of quantitative precipitin analysis in liquid media, however, differed quantitatively from those of the gel diffusion studies (Table 18). These results indicated that $T25_3$ possessed only 31 percent more group A specific carbohydrate than $T25_3$(T12gl). In addition, quantitative differences between the group A-variant polysaccharide of the two strains were only 10 percent compared to the 200 percent determined by gel diffusion. To help resolve this discrepancy, the total carbohydrate in standardized formamide extracts of each strain was determined according to the method of Dische. These values were 1.1 mg/ml in the $T25_3$ preparation and 0.76 mg/ml in the $T25_3$(T12gl) sample (Table 19). The 31 percent difference in these carbohydrate concentrations supported the indications that phage T12 infection reduced group A specificity by approximately one-third. It seems unlikely, however, that this reduction could account for the immunoabsorption data and the 32-fold difference in agglutination of these two strains in group A antisera.

Further characterization of this phage conversion system involved agglutination of modified strains in hetero-absorbed antisera under various conditions (Table 20). Column A represents agglutinin titers in absorbed sera that had been treated with phage T12 prior to agglutination. A comparison of the values in this column with those of the normal unmodified situation (column E) reveals that addition of the phage failed to reduce the residual titer. It was therefore doubtful

TABLE 18

QUANTITATIVE PRECIPITIN ANALYSIS OF STREPTOCOCCAL SOMATIC ANTIGEN COMPLEXES WITH GROUP SPECIFIC ANTISERA

Serum	Antigen	A280
A	$T25_3$	.649
	$T25_3$(T12gl)	.452
A- Variant	$T25_3$	.471
	$T25_3$(T12gl)	.421

TABLE 19

CONCENTRATIONS OF TOTAL CARBOHYDRATE IN FORMAMIDE-EXTRACTED ANTIGEN PREPARATIONS OF $T25_3$ AND $T25_3$(T12g1)

	mg/ml	
Strain	Sample 1	Sample 2
$T25_3$	1.06	1.10
$T25_3$(T12g1)	0.72	0.76

TABLE 20

CAPACITY OF ABSORBED SERA TO AGGLUTINATE $T25_3$ LYSOGENS AND NONLYSOGENS

Antisera	Absorbing Antigen	Agglutinating Antigen	Residual Titers				
			A	B	C	D	E
Anti-$T25_3$	$T25_3$	$T25_3$	16	16	16	16	16
		$T25_3(\emptyset)$	16	16	16	16	16
	$T25_3$ (T12gl)	$T25_3$	128	128	128	128	128
		$T25_3(\emptyset)$	16	16	16	128	16
Anti-$T25_3$(T12gl)	$T25_3$	$T25_3$	16	16	16	16	16
		$T25_3(\emptyset)$	512	512	512	16	512
	$T25_3$ (T12)	$T25_3$	16	16	16	16	16
		$T25_3(\emptyset)$	16	16	16	16	16
Anti-$T25_3$	-	$T25_3$	2048	2048	2048	2048	2048

TABLE 20--Continued

Antisera	Absorbing Antigen	Agglutinating Antigen	Residual Titers				
			A	B	C	D	E
Anti $T25_3$(T12gl)	-	$T25_3(\emptyset)$	2048	2048	2048	2048	2048
Preimmune Serum	-	$T25_3$	0	0	0	0	0
		$T25_3(\emptyset)$	0	0	0	0	0

A = all sera absorbed with $\emptyset$T12 following standard absorption $T25_3(\emptyset) = T25_3$(T12gl)

B = all cells preincubated with 0.1 mg/ml hyaluronidase $T25_3(\emptyset) = T25_3$(T12gl)

C = $T25_3(\emptyset) = T25_3$(T12)B

D = $T25_3(\emptyset) = T25_3$(H4489A)

E = $T25_3(\emptyset) = T25_3$(T12gl)

that phage attachment itself changed the antigenic specificity of the lysogen. Nor did removal of bacterial capsules with hyaluronidase prior to agglutination affect the titers of the residual serum (column B). Substituting the T12 lysogen constructed for this study revealed it to possess similar agglutinating capacities as $T25_3$(T12gl)(column C). The H4489A lysogen on the other hand resembled nonlysogenic $T25_3$ rather than $T25_3$(T12gl) (column D). Lysogeny per se, therefore, did not result in altered antigens.

Effects of Lysogeny on Extracellular and Intracellular Constituents

At least one extracellular product, erythrogenic toxin, has been reported to be influenced by bacteriophage. Further characterization of this conversion system necessitated the development of a reliable assay for the physical presence of the toxin as well as for associated biological activity. Detection of the erythematous property was achieved by intradermal injections into the shaved backs of rabbits. The skin responses were read at 24 and 48 hours post injection. Erythematous activity present in concentrated TH dialysate culture filtrates of several group A streptococcal strains, purified type A scarlatinal exotoxin, and a washed T12 phage preparation is represented in Table 21. Since hypersensitivity plays a possible role in the positive skin response, animals previously sensitized to type A

TABLE 21

ERYTHEMATOUS RESPONSE OF UNSENSITIZED AND SENSITIZED RABBITS TO INTRADERMAL INJECTIONS OF ERYTHROGENIC TOXIN, PHAGE T12, AND VARIOUS CULTURE FILTRATES

Description	Skin Response in Rabbits Sensitized to . . .					
	Type A Toxin		$T25_3$(T12gl) Filtrates		Unsensitized	
	24 hour	48 hour	24 hour	48 hour	24 hour	48 hour
K56	-	-	+1	-	-	-
K56(2172)	-	-	+1	-	-	-
K56(H4489A)	-	-	+1	-	-	-
$T25_3$	-	-	+1	-	-	-
$T25_3$(T12gl)	-	+2	+1	+2	-	+1
$T25_3$(T12)B	-	+2	+1	+2	-	+1
NY5 Type 10	-	+2	+1	+2	-	+1
Sterile THD	-	-	-	-	-	-
Type A Toxin	-	+2	+1	+2	-	+1
ØT12 (Washed)	-	-	-	-	-	-

- = no erythema
+1 = erythema (10 mm diameter) characterized by pink coloration
+2 = erythema (10 mm diameter) characterized by deep red coloration

exotoxin and culture filtrates of $T25_3$(T12gl) supplemented the unsensitized rabbit.

Skin responses after 48 hours showed that the $T25_3$ preparation contained no erythrogenic activity, whereas its lysogenic counterpart produced positive responses regardless of the immunological state of the rabbits, adding support to earlier reports that elaboration of scarlatinal toxin is associated with bacteriophage infection. (A separate experiment showed this skin response to persist through a 1/32,000 dilution of the culture filtrate.) In addition, all rabbits responded positively to filtrates of $T25_3$(T12)B, NY5 type 10, and the purified type A toxin preparation. No erythematous activity was associated with resuspended phage T12 or filtrates of the K56 system. It also appears that at least part of the skin response was attributable to intrinsic (primary) toxicity of the exotoxin, since the unsensitized animal displayed the same pattern of responses as did the sensitized rabbits at 48 hours. However, the reaction was augmented following sensitization, indicating participation of specific hypersensitivity (secondary toxicity) in the development of erythema. Guinea pigs were found to be unsuitable as indicators of erythema, since nonspecific and specific responses were undiscernable.

Serological precipitin reactions formed the basis for the in vitro toxin assay developed for this study. The assay depended on continuous lines of identity between a standard toxin A preparation

and an unknown antigen in double immunodiffision reactions with antiserum against NY5 type 10 culture filtrates. This organism was used for antiserum preparation because it is reported to be a prolific producer of both toxin A and B, and was the source of the toxin in the purified toxin A preparation utilized as the standard in this in vitro technique.

Polyacrylamide gel electrophoresis, however, revealed the purest toxin preparation available to be contaminated by a single molecular species (Figure 22-b). A procedure was subsequently developed to eliminate this contaminant from the known standard, based on the data shown in Figure 22. Three important conclusions could be drawn from these disc gels: 1) toxin A had a greater electrophoretic mobility than did the contaminant, 2) both the toxin and the contaminant had counterparts in the NY5 type 10 filtrates possessing similar R_f's, and 3) the concentration ratio of toxin to contaminant was much higher in the purified preparation than in the culture filtrates, which contained a similar quantity of each. This information was used in coordination with the results of immunoelectrophoresis of the same preparations (Figure 22-c), i.e., 1) two bands of similar intensity were detected in the culture filtrates, whereas the toxin preparation was characterized by one dominant and one faint line of precipitation, probably reflecting the concentrating of toxin by the purification procedure; and 2) the prominant band displayed the greatest electronegative mobility, much

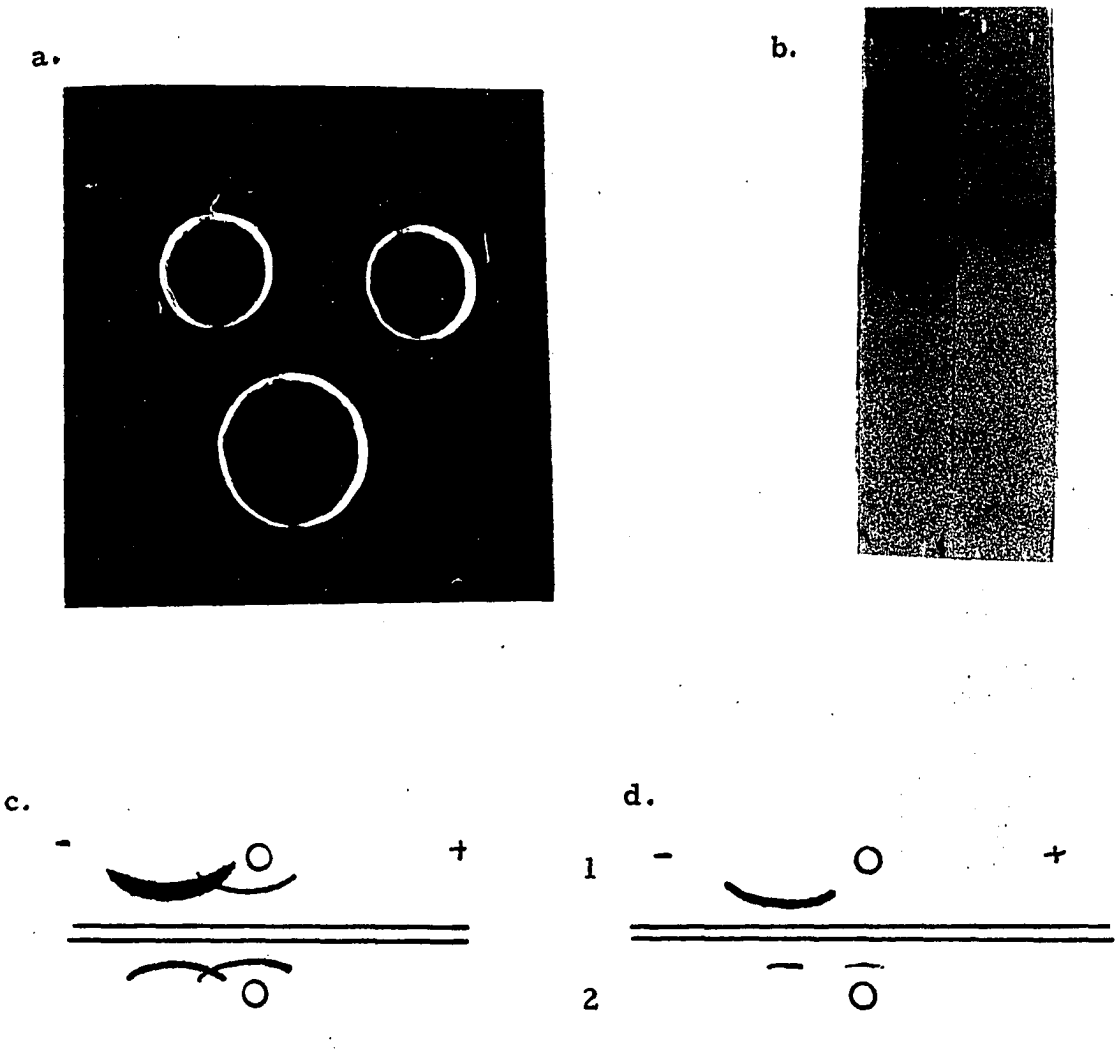


Figure 22--Analysis of purified Type A toxin and culture filtrates of NY5 type 10: (a) Gel Diffusion of 1) Toxin A Preparation 2) NY5 Type 10 Filtrate and 3) Antisera Prepared Against NY5 Type 10 Filtrate; (b) Gel Electrophoresis of 1) Toxin A Preparation and 2) NY5 Type 10 Filtrate; and Immuno-electrophoresis of Toxin A and NY5 Type 10 Filtrates (c) Undiluted (d) and Diluted 1:4.

the same as did the toxin in the disc gel analysis. It was therefore concluded that this electronegative precipitin band represented erythrogenic toxin . Finally, immunoelectrophoresis of successive 2-fold dilutions of the toxin preparation showed the toxin band detectable following a 1:4 dilution whereas the contaminant disappeared after a single doubling of the volume.

The purified known standard for the in vitro toxin assay was subsequently obtained by diluting the original preparation to the limits of detectibility as a band of precipitation by NY 5 type 10 antiserum. Although this technique did not actually remove the contaminant from the standard, it reduced it to one fourth the concentration required for detection by the in vitro toxin assay.

This method was utilized to detect toxin concentrations in culture filtrates of NY5 type 10 grown in THD, Stock's dialysate, or PPD media at 24 and 37 C (Table 22). Following 48 hour incubation, both THD and Stock's media appeared to be equally suited for use in toxin studies, whereas PPD contained a toxin concentration undectable in dilutions of no greater than 1:4. Because of its relative simplicity of preparation, THD was selected as the routine medium for these investigations.

Double immunodiffusion analysis of $T25_3$, $T25_3$(T12gl), and $T25_3$(T12)B filtrates with a mixure of $T25_3$ and $T25_3$(T12gl) antisera reveals spur formation between the nonlysogen and its two lysogenic

TABLE 22

DETECTION OF SCARLATINAL TOXIN A IN CULTURE FILTRATES OF NY5 TYPE 10 GROWN IN VARIOUS MEDIA FOR 48 HOURS AT 24 AND 37 C

Medium	Temp	PTD*
THD	24	16
	37	16
Stocks	24	16
	37	16
PPD	24	4
	37	4

*precipitin test dose (PTD) = highest dilution of sample resulting in a line of identity with the toxin standard (in vitro toxin assay)

counterparts (Figure 23). Both $T25_3$ and $T25_3$(T12gl) filtrates, therefore appear to have contained (an) immunologically distinct counterpart(s). Further analysis using the in vitro toxin assay indicates that whereas the lysogen produced toxin A, the nonlysogen elaborated no immunologically related product detectable by precipitating antibody in NY5 type 10 antiserum (Figure 26).

It is also apparent from Figure 25 that toxin could not be found in broken cell extracts (BCE) of either $T25_3$ or $T25_3$(T12gl) using the in vitro toxin assay. The possibility that inhibition of precipitating antibody was a general property of these extracts was eliminated by demonstrating the ability of each broken cell preparation to precipitate with homologous antisera (Figure 24). Assays for erythematous activity in these broken cell extracts, however, produced results unparalleled to those of the in vitro analysis (Table 23). Both $T25_3$ (broken cell extract) and $T25_3$(T12gl) (broken cell extract) elicited responses similar to the standard type A toxin. The inability of disintegrated K56 preparations to produce a positive response reduces the likelihood that non-specific erythema accounted for the positive response of $T25_3$(BCE). Both the lysogen and nonlysogen contained some specific soluble substance(s) released by cell disintegration.

Neutralization of the erythematous activity in these broken cell extracts was accomplished by incubating them in an equal volume

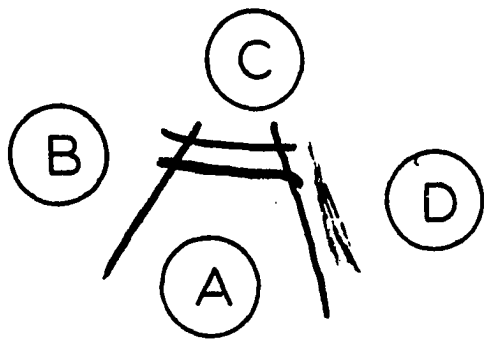


Figure 23--Gel diffusion pattern obtained with a mixure of $T25_3$ and $T25_3$(T12gl) antisera (A) and culture filtrates of B) $T25_3$(T12gl), C)$T25_3$ and D) $T25_3$(T12)B

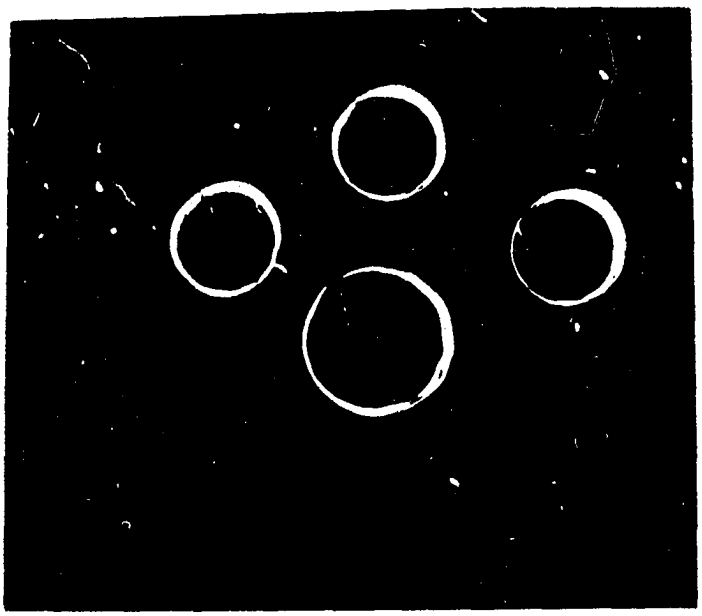
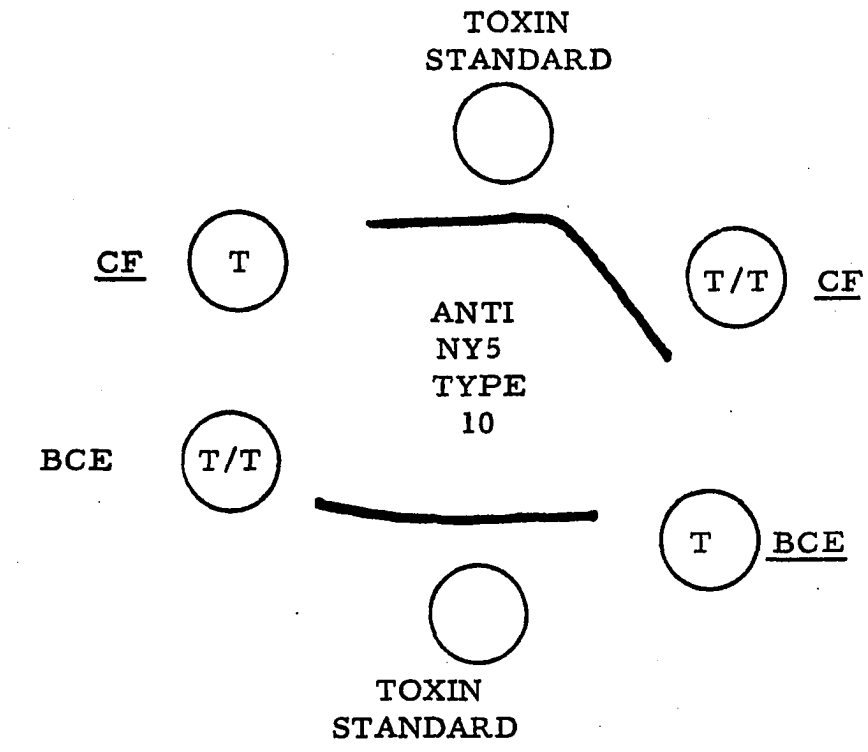


Figure 23--Gel diffusion pattern obtained with a mixure of $T25_3$ and $T25_3$(T12gl) antisera (A) and culture filtrates of B) $T25_3$(T12gl), C)$T25_3$ and D) $T25_3$(T12)B



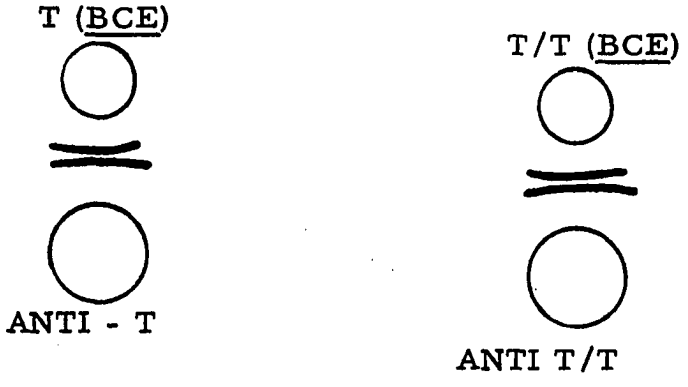
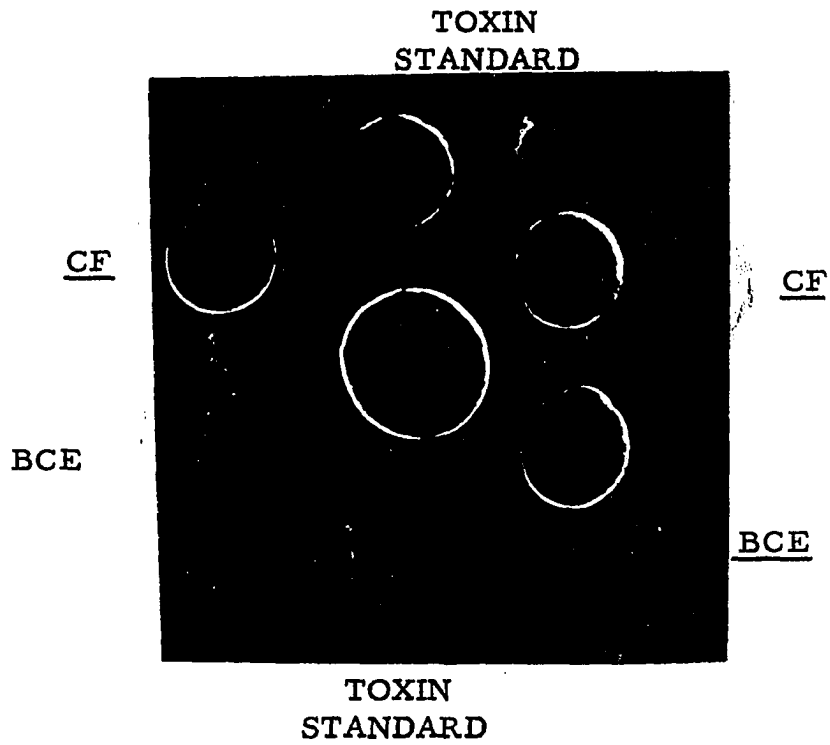


Figure 24--*In vitro* toxin assay for toxin A in culture filtrates (CF) and broken cell extracts (BCE) of $T25_3$(T) and $T25_3$(T12g1)(T/T)



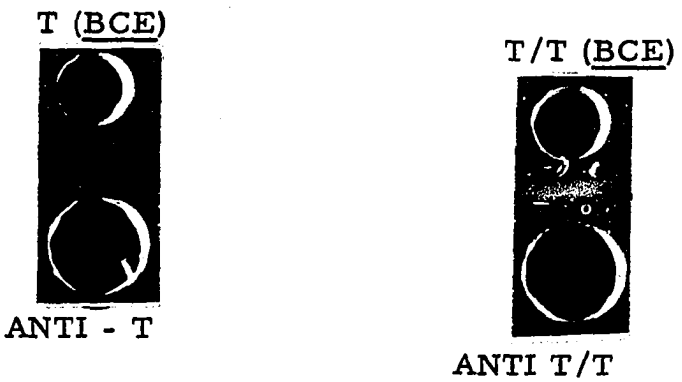


Figure 24--In vitro toxin assay for toxin A in culture filtrates (CF) and broken cell extracts (BCE) of $T25_3$(T) and $T25_3$(T12gl)(T/T)

of antiserum against $T25_3$(T12gl) culture filtrates, which destroyed the scarlatinal properties of toxin A as well (Table 23). On the other hand, analogous $T25_3$ antiserum failed to produce the same effect, suggesting that the two sera differ in the presence of type A specific antitoxin. In addition, the last 6 columns in Table 22 indicate an antigenic similarity between the toxic agents in the disintegrated cell preparations of $T25_3$ and $T25_3$(T12gl), since both are neutralized by antiserum to $T25_3$(T12gl) culture filtrates. Extracellular counterparts of these agents, however, were shown to be immunologically distinct from each other by double immunodiffusion and by differences in antitoxin properties of the corresponding antisera.

Electrophoresis of $T25_3$ and $T25_3$(T12gl) culture filtrates in polyacrylamide gels was employed using the partially purified toxin A preparation as a reference standard (shown in Figure 25, gel 2). These results are presented in Table 24 as R_f values, and the corresponding quantities related to the average band density as a multiplicative factor. Both filtrates had common bands at R_f's of 3.3 and 3.8, corresponding to the contaminant and major component in the toxin A preparation. Whereas toxin was the most abundant extracellular constituent found in the $T25_3$(T12gl) filtrate (6.5), the corresponding product was slightly below the average concentration in the $T25_3$ preparation (0.9). This amount of material possessing the identical electrophoretic mobility as toxin A was significantly more than one would expect from

TABLE 23

ERYTHEMATOUS RESPONSE OF UNSENSITIZED AND SENSITIZED RABBITS TO INTRADERMAL INJECTIONS OF TOXIN AND BROKEN CELL EXTRACTS, AND SUBSEQUENT NEUTRALIZATION BY ANTISERA PREPARED AGAINST CULTURE FILTRATES

	Skin Response in Rabbits Sensitized to . . .					
	Type A Toxin		$T25_3$(T12gl) Filtrates		Unsensitized	
	24 hour	48 hour	24 hour	48 hour	24 hour	48 hour
$T25_3$	-	+2	+	+2	-	+2
$T25_3$(T12gl)	-	+2	+	+2	-	+2
NY5	-	+2	+	+2	-	+2
K56	-	-	+	+2	-	-
Type A Toxin	-	+2	+1	+2	-	+2
Sterile PBS	-	-	-	-	-	-
$T25_3$(T12gl) + anti $T25_3$(T12gl)	-	-	+1	-	-	-
Toxin A + anti $T25_3$	-	+2	+1	+2	-	+2

TABLE 23--Continued

	Skin Response in Rabbits Sensitized to . . .					
	Type A Toxin		$T25_3$(T12gl) Filtrates		Unsensitized	
	24 hour	48 hour	24 hour	48 hour	24 hour	48 hour
Toxin A + anti $T25_3$(T12gl)	-	-	+1	-	-	-
$T25_3$ + anti $T25_3$	-	+2	+1	+2	-	+2
$T25_3$ + anti $T25_3$(T12gl)	-	-	+1	-	-	-

- = no erythema

+1 = erythema (10 mm diameter) characterized by pink coloration

+2 = erythema (10 mm diameter) characterized by deep red coloration

TABLE 24

QUANTITATIVE REPRESENTATION OF STREPTOCOCCAL EXTRACELLULAR PRODUCTS SEPARATED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

R_f	$T25_3$	$T25_3$(T12gl)	Toxin A
0.06	0.2	0.02	-
0.19	0.1	0.10	-
0.23	0.08	0.01	-
0.33	2.7	0.20	0.07
0.38	0.9	6.50	1.9
0.43	0.2	0.01	-
0.50	5.4	1.00	-
0.56	0.1	0.07	-
0.62	0.05	0.07	-
0.66	0.1	0.07	-

Amounts were determined geometrically from graphic representations obtained by gel scans using a Gilford linear transport. Each value expresses the amount of the individual band as a factor of the average band quantity in the respective gel, i.e.

$$\frac{\text{quantity of band x}}{\text{quantity of average band}}$$

a strain incapable of either producing a detectable extracellular erythrogenic substance or reacting with toxin specific precipitating antibody. Support that this compound in these nonlysogen preparations was related to toxin A was provided by the finding that streptococcal pyrogenic exotoxin (SPE) was present in the THD culture filtrates of $T25_3$, according to the standard assay for pyrogenic activity (courtesy of Dennis Watson, University of Minnesota). Erythrogenic toxin and SPE are reportedly synonomous.

Protein concentrations were also determined for each filtrate to allow quantitative comparisons between samples. The total protein in the respective $T25_3$ and $T25_3$(T12gl) preparations was 950 and 810 mg/ml as determined by the Lowry assay procedure. A correction factor of 1.2, therefore, applied to the quantitative data from the gel scans, resulted in a calculation of 7.8 times more extracellular 'toxin' produced by the lysogen than the phage-free strain. It is also noteworthy that of the 10 proteins detected in each sample, toxin was one of only two that was not produced in greater concentrations by $T25_3$ than $T25_3$(T12gl). Apparently, the biosynthetic machinery of the lysogen was altered to favor the production of scarlatinal exotoxin at the expense of the other constituents.

Disc gel electrophoretic patterns of broken cell extracts are presented photographically in Figure 25, and translated into tabular

TABLE 25

QUANTITATIVE REPRESENTATION OF STREPTOCOCCAL BROKEN CELL EXTRACTS ANALYZED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

R_f	NY5-10	Toxin A	$T25_3$(T12gl)	$T25_3$	R_f	ØT12
0.07	0.12	-	1.15	0.19	0.07	0.10
0.15	0.01	-	0.17	0.03	0.15	0.02
0.17	4.08	-	3.70	5.40	0.17	0.40
0.24	0.79	-	1.12	1.21	0.30	3.30
0.33	0.28	0.20	0.84	0.34	-	-
0.38	0.16	6.50	0.09	0.04	-	-
0.46	1.30	-	2.93	2.82	-	-
0.53	0.02	-	0.16	0.06	-	-
0.61	0.01	-	0.01	0.02	-	-
0.82	0.01	-	0.01	0.04	-	-

Amounts were determined geometrically from graphic representations obtained by gel scans using a Gilford linear transport. Each value expresses the amount of the individual bands as a factor of the average band quantity in the respective gel, i. e.,

$$\frac{\text{quantity of band x}}{\text{quantity of average band}}$$

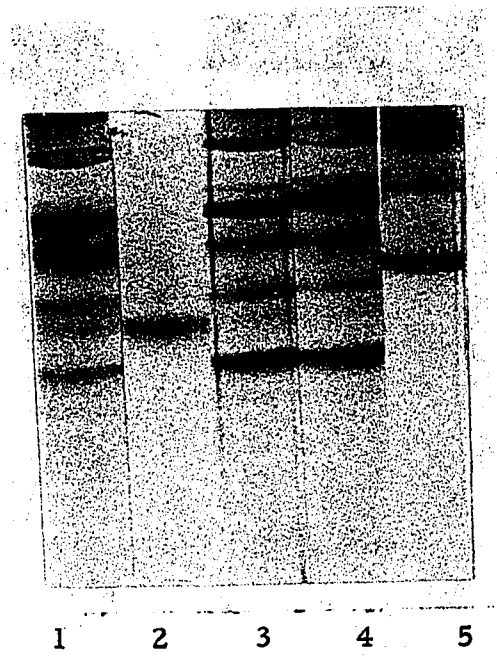
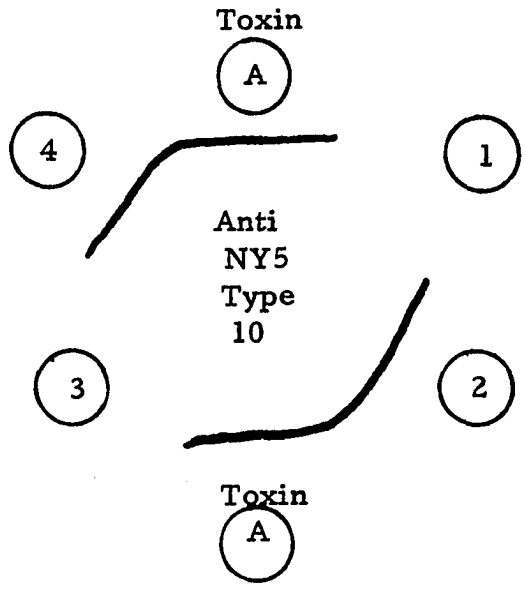


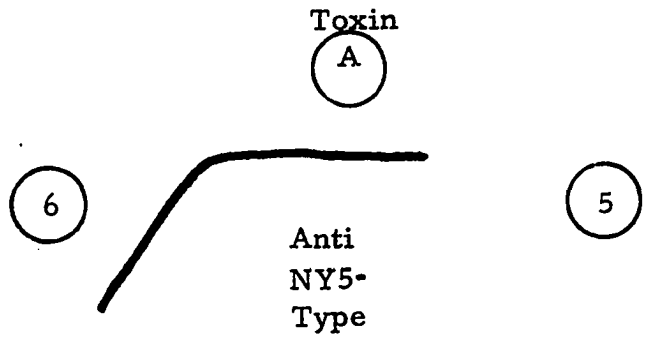
Figure 25--Polyacrylamide gel electrophoretic patterns of broken cell extracts of 1) NY5 type 10, 3, 2) the partially purified toxin A preparation, $T25_3$(T12g1) and 4) $T25_3$. Position 5 represents a washed T12 suspension.

tabular form (Table 25). Toxin A (R_f 0.38) was found to be most concentrated in NY5 type 10 extracts, followed by $T25_3$(T12gl) and $T25_3$ in quantitative ratios of 4.0:2.2:1.0. Whereas the toxin was detected in 7.8 times greater quantities in extracellular products of the lysogen than the nonlysogen, this difference was reduced to 2.2 in the broken cell extracts. The repression pattern of non-toxin biosynthesis by $T25_3$(T12gl) also appears to have been altered, as the concentration of toxin in the disintegrated lysogen preparation was less than 10 percent that of the average protein band in the sample. None of the bands in the washed T12 preparation mimicked the electrophoretic mobility of toxin A, which was therefore probably unrelated to any structural components of the bacteriophage.

The effects of various bacteriophages on the ability of $T25_3$ to produce erythrogenic toxin were studied using the <u>in vitro</u> toxin assay (Figure 26). Lysis by virulent phage A25 produced lysates containing no more detectable toxin than did the phage-free bacterial filtrate. A virulent mutant of phage T12, however, converted $T25_3$ to the toxinogenic state in the process of phage infection and cell lysis. Integration of bacteriophage into the host chromosome, therefore, was not essential for toxin production. Wild type T12 phage infection also resulted in a toxinogenic culture. Propagation of the two T12 phages by standard protocol, however, resulted in the production of no toxin detectable by either method. Only when 1.0 ml of



A-



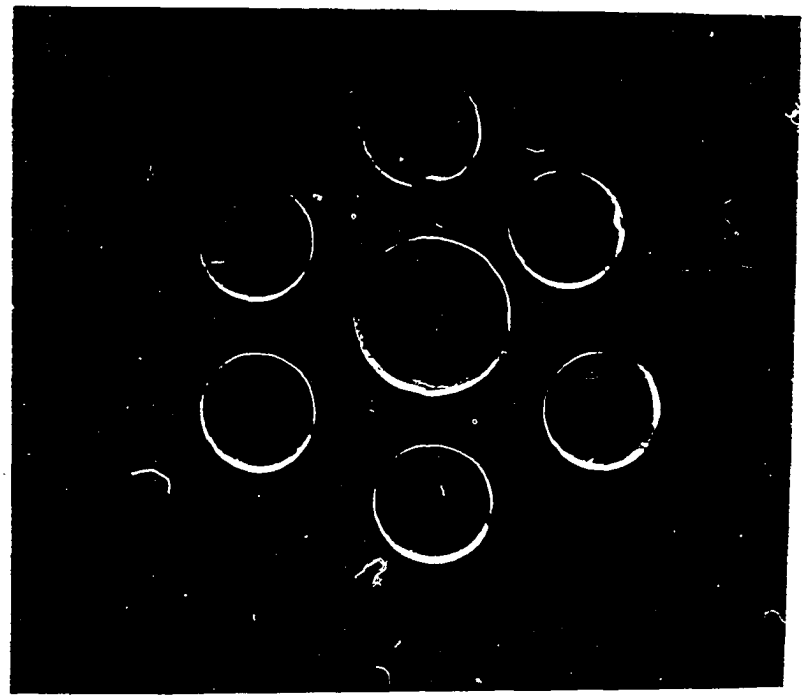
B-

Figure 26--In vitro assay for toxin A in

A. Lysates of $T25_3$ infected with phage
   1. A25
   2. T12
   3. Uninfected
   4. T12 c.p.[1]

B. Culture filtrates of the lysogens

   5. $T25_3$(T12)B
   6. $T25_3$(H4489A)



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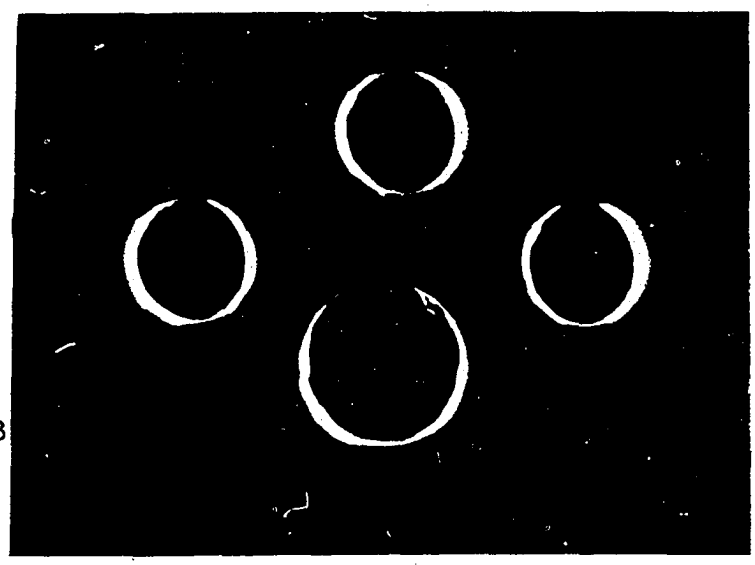

B

Figure 26--<u>In vitro</u> assay for toxin A in

A. Lysates of $T25_3$ infected with phage
   1. A25
   2. T12
   3. Uninfected
   4. T12 <u>c.p.</u> [1]

B. Culture filtrates of the lysogens

   5. $T25_3$(T12)B
   6. $T25_3$(H4489A)

phage was inoculated into $T25_3$ cultures in mid-logarithmic stages of growth and incubated at 37 C for 16 hours could this product be found in the lysates. Figure 26 demonstrates that relysogeny of $T25_3$ with phage T12 resulted in a toxinogenic culture. When repeated with phage H4489A, however, the resulting $T25_3$(H4489A) was incapable of producing toxin A. Lysogeny itself, therefore, was not accountable for conversion to $\underline{tox}^+$.

Kinetics of toxin production by $T25_3$ cultures infected with either phage T12 ot $T12\underline{cp}^1$ are shown in Figures 27 and 28. Maximum toxin levels were detected using the <u>in vitro</u> toxin assay 12 hours following the peak temperate phage titer, suggesting that lysis alone was not responsible for its appearance. Propagation of virulent $T12\underline{cp}^1$ resulted in maximum toxin levels more closely corresponding to the major lytic event, but a 6.0 hour interval still existed.

Attempts to isolate toxin A from culture filtrates of $T25_3$ (T12gl) employed affinity immunoabsorption. The technique was based on the assumption that this product was the only component of the lysogen filtrates not common to the nonlysogen cultures as well. Whereas electrophoretic analysis supports this assumption, the nature of the stain used on the gels (amidoschwartz) was limited to detection of protein moieties. The $T25_3$ antiserum employed as the fixed absorbant was first chromatographed on DEAE cellulose (G52) to purify the gamma globulin (Figure 29). Unlike the usual elution

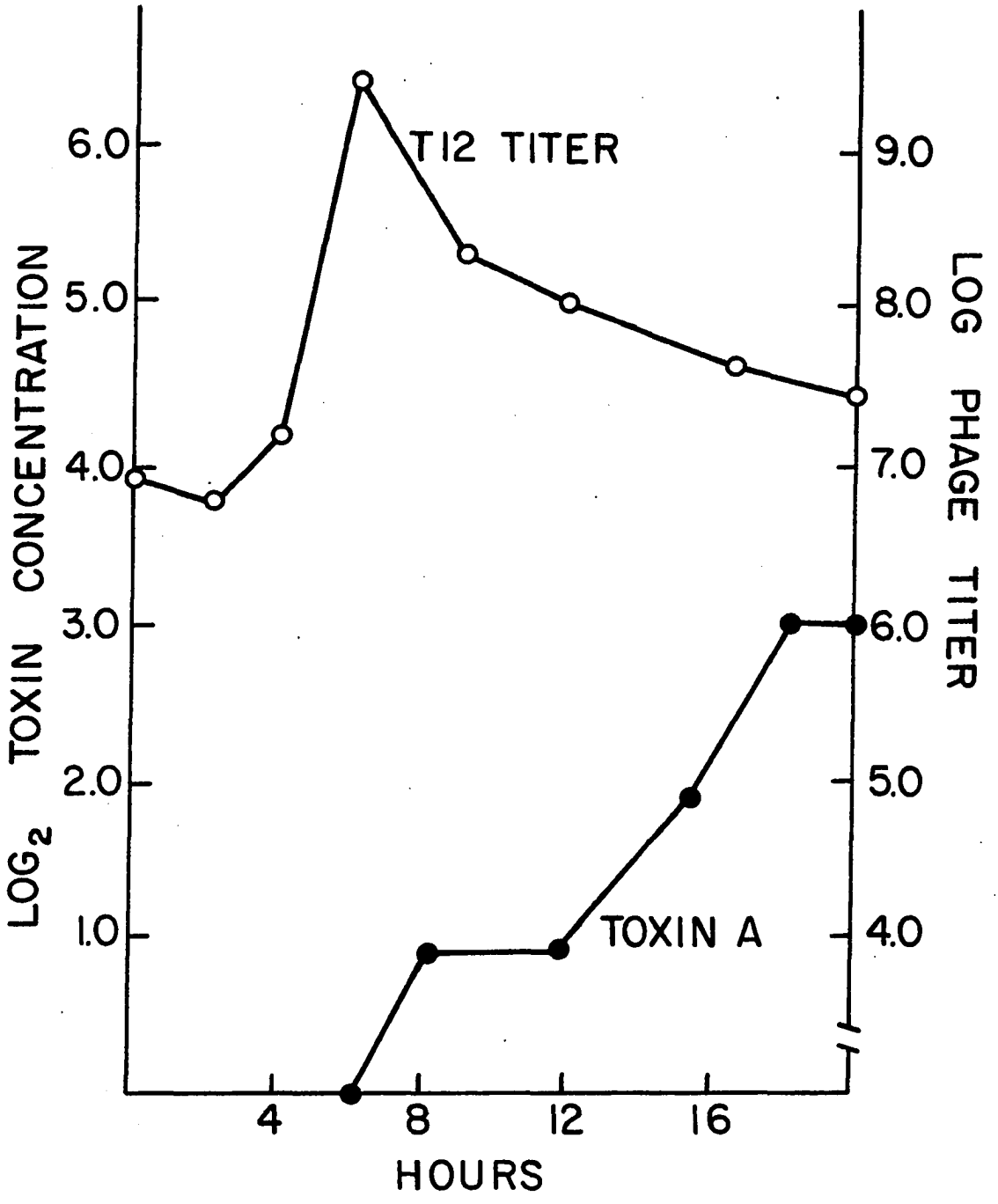

FIGURE 27-KINETICS OF PHAGE T12 AND TOXIN PRODUCTION BY $T25_3$ AT 37C

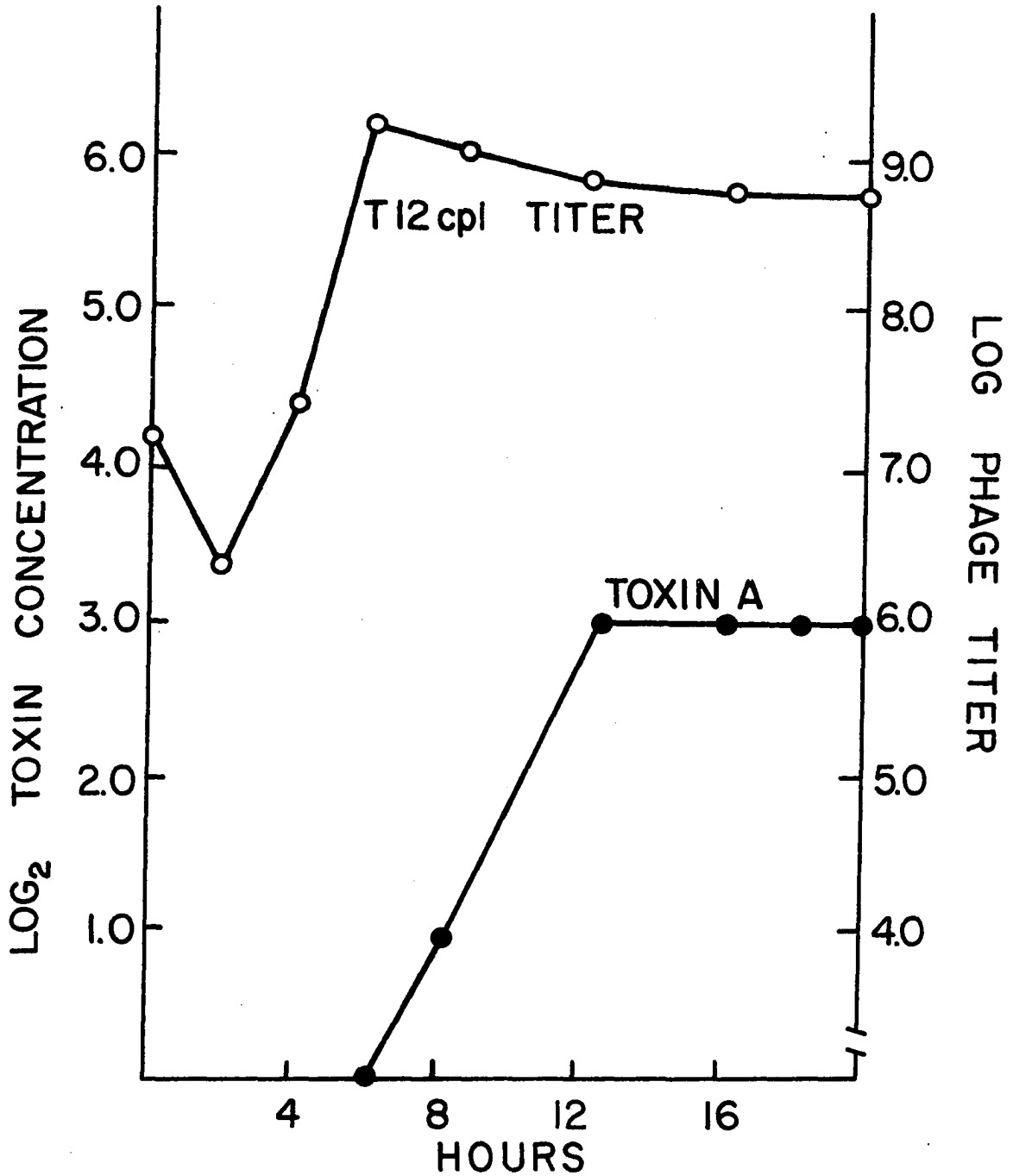

FIGURE 28 - KINETICS OF PHAGE T12cpl AND TOXIN PRODUCTION BY $T25_3$ AT 37C

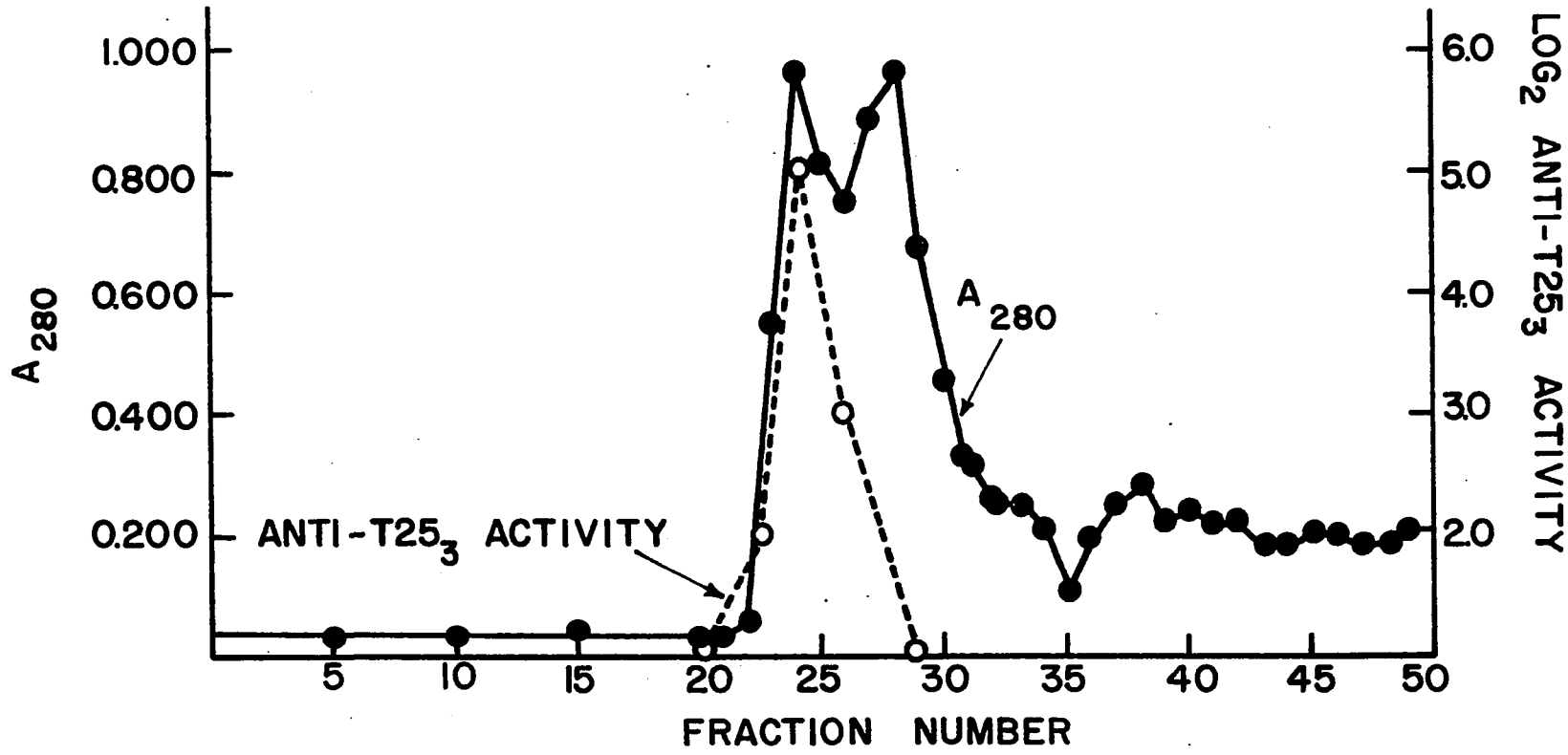

FIGURE 29-ELUTION PATTERN OF $T25_3$ ANTISERUM ON G52 DEAE CELLULOSE

patterns, which show 85 percent of the gamma globulin eluting early as a single large peak, two peaks of similar sizes were detected. The protein concentration of each of the pooled samples following pervaporative reduction to 2.0 ml volumes was determined by 260/280 absorbancy to be 16.5 mg/ml and 18.3 mg/ml, corresponding to fractions nos. 24 and 29. Precipitating antibody against $T25_3$ culture filtrates was exclusive to fraction no. 24. Single direction immunodiffusion in Oudin tubes detected no precipitins in sample no. 29. The active fraction was coupled to cellulose filter pads, which were utilized in Millipore housings to absorb the $T25_3$(T12gl) culture filtrates, as well as $T25_3$ controls. It was anticipated that double immunodiffusion assays using $T25_3$(T12gl) hyperimmune antiserum would indicate a line of identity with toxin A in the absorbed lysogen filtrates, and no reaction with the absorbed nonlysogen preparation. Antigen removal was indeed affected by the procedure, but was equally efficient for all antigens in each sample. No relative increase in toxin concentration could be achieved, even when the immunologically active paper pads were incubated in various volume sizes for 18 hours.

The effects of lysogeny on production of soluble products by K56 was examined using a similar approach as that described for the $T25_3$ system (Table 26). Polyacrylamide gel electrophoresis of K56 K56(H4489A), and K56(2172) culture filtrates, while indicating major decreases in concentrations of the products with R_f's of 0.17 and 0.42

TABLE 26

QUANTITATIVE REPRESENTATION OF STREPTOCOCCAL PRODUCTS SEPARATED BY POLYACRYLAMIDE ELECTROPHORESIS

R_f	Culture Filtrates			Broken Cell Extracts		
	K56	K56 (H4489A)	K56 (2172)	K56	K56 (H4489A)	K56 (2172)
0.07	-	-	-	0.87	0.65	0.93
0.14	0.36	0.01	0.01	0.15	0.10	0.26
0.17	0.73	0.02	0.01	-	-	-
0.20	0.32	0.28	0.02	3.70	2.75	3.34
0.23	0.44	0.59	0.74	0.84	0.82	0.70
0.28	2.63	1.50	2.11	-	-	-
0.31	0.70	0.45	0.53	0.84	0.70	0.65
0.34	-	-	-	0.57	-	-
0.42	1.57	0.30	0.54	1.20	0.96	1.13
0.44	-	-	-	0.33	0.19	0.20
0.58	0.02	0.02	0.02	-	-	-

Amounts were determined geometrically from graphic representations obtained by gel scans using a Gilford linear transport. Each value expresses the amount of the individual band as a factor of the average band quantity in the respective gel, i.e., $\frac{\text{quantity of band x}}{\text{quantity of average band}}$

following lysogeny, revealed no extracellular components unique to either the lysogens of the phage-free strain. These findings were confirmed by the absence of spur formation in double immunodiffusion (not shown). Electrophoretic analysis of the broken cell extracts, however, indicated a protein with an R_f of 0.34 to be unique to the nonlysogenic K56. This product was not excreted, and perhaps represented an intracellular enzyme repressed by infection of H4489A or 2172.

At least three of the extracellular products represented in Table 26 had no counterparts released by cell disintegration (R_f's, 0.17, 0.28 and 0.58). This observation may well be accounted for by their biosynthesis at the cell membrane during the process of excretion. In the broken cell extracts, therefore, they would likely be bound to the membrane and consequently sedimented with the particulate fraction.

Transduction

The effects of lysogeny on K56 transduction efficiency was ascertained by employing four genetic markers for antibiotic resistance. The first experiment examined the capacities of K56, K56(H5589A) and K56(2172) to act as recipients of genetic markers from established 9440 donors (Table 27). While all three strains were excellent recipients in the genetic exchange, K56 invariably displayed the superior ability.

TABLE 27

TRANSDUCTION OF ANTIBIOTIC RESISTANCE MARKERS FROM *S. PYOGENES* 9440 DONORS INTO RECIPIENTS OF *S. PYOGENES* K56, K56(H4489A), AND K56(2172)

Marker	Donor	Recipient	Number of Transductants	Ø Titer (PFU/ml)	T.F. Transduction Frequency	% T.F. of K56	-Ø
Str-10	9440 *str-10*	K56	1.4×10^{2}	5.7×10^{8}	5×10^{-6}	100	2
		K56(H4489A)	8.6×10^{1}		3.1×10^{-6}	62	2
		K56(2172)	8.3×10^{1}		3.0×10^{-6}	60	2
Rif^{R}	9440 *rif*^{R}	K56	2.6×10^{2}	9×10^{8}	5.8×10^{-6}	100	0
		K56(H4489A)	2.3×10^{2}		5.1×10^{-6}	88	0
		K56(2172)	1.5×10^{2}		3.3×10^{-6}	56	0
Spec^{R}	9440 *spec*^{R}	K56	8.0×10^{1}	6.7×10^{8}	2.4×10^{-6}	100	0
		K56(H4489A)	4.0×10^{1}		1.5×10^{-6}	63	0
		K56(2172)	4.2×10^{1}		1.3×10^{-6}	54	0

TABLE 27--Continued

Marker	Donor	Recipient	Number of Transductants	∅ Titer (PFU/ml)	T.F. Transduction Frequency	% T.F. of K56	-∅
Ery^R	9440 ery^R	K56	1.2×10^2	9×10^8	2.7×10^{-6}	100	0
		K56(H4489A)	9.0×10^1		2.0×10^{-6}	74	1
		K56(2172)	6.6×10^1		1.5×10^{-6}	56	0

This is indicated by differences in transduction frequencies (TF) ranging from 12 percent (rif^R) to 46 percent (spec^R) between K56 and its lysogenic counterparts. In addition, K56(H4489A) was 3.0 to 35.0 percent more efficient than K56(2172) as a recipient in this experiment. Lysogeny, therefore, appears to exert a negative influence on the recipient capacity of K56.

Reports of K56 being successfully employed as a donor in transduction are scarce (150), whereas numerous failures have been described. An attempt was made to enhance the efficiency of K56 as a donor of genetic information for transduction by using the K56 lysogens containing appropriate markers (Table 28). Of the four antibiotics utilized, only resistance to rifampicin could be transduced from either K56 or the lysogenic strains, and with less than 90 percent of the frequency using 9440 donors. Transduction frequencies, however, followed a similar pattern as before, i.e., K56rif^R was the most efficient donor, followed by K56(H4489A)rif^R and finally K56(2172)rif^R. Whereas these data confirm reports that nonlysogenic K56 strains serve as poor donors in transduction with A25, one marker (rif^R) was found transducible from K56 at frequencies greater than any K56 donor previously reported.

It was concluded from these experiments that, unlike Malke's findings of increased donor efficiency following lysogenization of K56(130), the presence of temperate bacteriophage interferred with

TABLE 28

TRANSDUCTION OF ANTIBIOTIC RESISTANCE MARKERS FROM K56, K56(H4489A) AND K56(2172) INTO A SENSITIVE K56 RECIPIENT

Marker	Donor	Number of Transductants	Ø Titer	T.F. Transduction Frequency	% T.F. of K56	-Ø
str^R	K56 *str*^{R}	2	4.8×10^8	0	-	1
	K56(H4489A)*str*^{R}	0	5.4×10^8	0	-	0
	K56(2172)*str*^{R}	0	4.5×10^8	-	-	0
rif^R	K56 *rif*^{R}	16	1.0×10^9	3.3×10^{-7}	100	0
	K56(H4489A)*rif*^{R}	15	1.0×10^9	3.3×10^{-7}	91	0
	K56(2172)*rif*^{R}	8	1.3×10^9	1.3×10^{-7}	40	0
spec^R	K56*spec*^{R}	3	6.0×10^8	0	-	0
	K56(H4489A)*spec*^{R}	0	4.5×10^8	0	-	0
	K56(2172)*spec*^{R}	0	6.0×10^8	0	-	0

TABLE 28--Continued

Marker	Donor	Number of Transductants	Ø Titer	T.F. Transduction Frequency	% T.F. of K56	-Ø
ery^R	K56 ery^R	0	3.3×10^8	0	-	0
	K56(H4489A)ery^R	0	4.8×10^8	0	-	0
	K56(2170)ery^R	0	9.5×10^8	0	-	0
str^R	9440 str^R	6.1×10^1	5.4×10^8	1.1×10^{-6}	-	0

the production of transducing particles. Lysogeny also impaired recipient efficiency of all four markers by K56, supporting this portion of Malke's observations. In addition data in Table 28 indicates that propagation of phage A25ts$^{1-2}$ on antibiotic resistant K56, K56(H4489A) or K56(2172) was generally unaffected by the presence of either temperate bacteriophage.

CHAPTER V

DISCUSSION

Many of the biological characteristics of phage T12 were similar to those described for phages 2172 and H4489A in the previous study (150). One striking distinction was the appearance of the plaques; those of T12 contained classical turbid centers while the remaining two temperate phage plaques displayed resistant colonies randomly distributed within the lytic zone. A second distinction, the inability of T12 to produce a Kjems effect, reflected probable differences between the bacterial indicator strains as well as phage. Since the halos are thought to result from degradation of bacterial capsules following lytic release of intracellular hyaluronidase, it is conceivable that the absence of capsular material from $T25_3$ prevented their formation around T12 plaques. Ability to penetrate the capsule should therefore be a property common to all phages capable of generating Kjems effects, since addition of exogenous hyaluronidase to the agar, as required for T12 infection of K56, prevents subsequent zone formation. The absence of hyaluronic acid capsules from $T25_3$ was indicated by its sensitivity to A25 lysis on media lacking

hyaluronidase, whereas K56 was sensitive to both A25 and T12 only in the presence of an exogenous source of the capsular lytic enzyme. The reported increase in extracellular hyaluronidase levels following infection by phages capable of penetrating encapsulated cocci was probably accountable for the efficient A25 lysis of the two lysogenic K56 strains.

The T12 phage strain used in this study was isolated from $T25_3$(T12gl), a lysogen which produced erythrogenic toxin. This lysogen contained another phage, which was present in $T25_3$ as well. While this phage developed plaques on a K56 bacterial lawn, propagation on this or any other strain employed in this study was unsuccessful. Strain NY5 type 10 contained a phage with similar properties of non-proliferation, even following attempts to induce the virus with ultraviolet light or mitomycin C.

The notable stability of phage T12 in the presence of ultracentrifugal forces further distinguished this phage from H4489A and 2172. Virtually all of the T12 infectivity survived the 90 minute concentrating procedure, whereas the other two phages maintained less than 75 percent of the initial viability. The phages were somewhat unstable in all diluents tested except TH and PP broth. The medium subsequently chosen for storage of all three phages was PP broth, since, unlike TH broth, it readily supported viral propagation. This propagation required 6 hours at 37 C for T12, two hours

longer than the duration needed for production of maximum H4489A and 2172 concentrations.

Concurrent phage development of H4489A and T12 within the same cell revealed the T12 to dominate the replicative machinery, inhibiting the development of virtually all the coinfecting virus. Phages H4489A and 2172, on the other hand, were remarkably compatible in the same cell. As a general rule, the degree of similarity among phages is reflected by their capacity for mutual coexistance, indicating the 2172 and H4489A to be more closely related to one another than to phage T12.

All three phages were found to differ immunologically, as determined by the inability of phage specific antisera to neutralize the infectivity of heterologous viruses. Since neutralizing antibody is believed to affect phage adsorption sites, these findings may well imply differences in adsorption specificities. This, in turn, would indicate that competition for bacterial receptor sites was not a factor in coinfection exclusion of H4489A by T12.

Striking similarities among these temperate phages were also discovered. Perhaps the most intriguing was the identical electrophoretic mobilities of all three viruses. This finding, however, may be due to some smaller structural constituent common to all three phages, the intact virion possibly being too large to migrate in the gels. Nontheless, this is the only constituent of the preparations

related to bacteriophage, since it alone was the only component absent from the phage-free bacterial cultures. The apparent absence of phage from the electrophoretic patterns of the lysogens possibly means the rate of spontaneous lysis was too low to produce quantities adequate for detection. This could also be true for a phage-related product, since intracellular phage development following spontaneous induction would occur at very low frequencies compared to that of a culture being propagated. This increase in virion production and number of lytic events (ca. 99.999 percent more) could account for the synthesis and release of phage-related products not found in either the culture filtrates or broken cell extracts of lysogenic streptococci. It seems likely, however, that not just one, but several additional bands would have been detected following a general increase in the level of phage-related products. It is probable, therefore, that this single unique band represented the actual virus.

The three temperate bacteriophages appeared to be uniformly sensitive to chloroform inactivation. Although the nature of this effect wasn't clearly defined, it was perhaps a function of the solvent's effect on the broth rather than the phage itself. Pretreatment of PP broth with chloroform resulted in the loss of 82 percent of its phage maintenance capacity. Either chloroform was still present, or this reflected the destruction of a substance essential to sustaining the long term viability of the virus. Bacteriophage resistance to Tween 80,

which would have probably destroyed most lipid compounds due to its detergent action, indicates that chloroform inactivation likely involved a non-lipid moiety.

It was also apparent that resistance to chloroform was not transmitted genetically, since the progeny of the residual active phages were equally susceptible to the effects of this solvent as were the original sensitive parental viruses. Nor was solvent saturation a plausible explanation for the persistance of residual infectivity, as the addition of fresh chloroform had no further effect on the phage titer.

Similarities in phage development were indicated by one step growth experiments. As with most temperate group A streptococcal bacteriophages, burst sizes were abnormally low (ca. 30 PFU/infected coccus) and minimal latent periods quite long (ca. 60 min). Sonification of bacterial chains eliminated the secondary burst usually described for streptococcal phages by effectuating random distribution of all cocci following dilution, thereby preventing immediate reinfection of adjacent cells in a chain.

During the process of phage development, one unique characteristic was discovered, i. e., transcription from the phage genome was possibly independent of the bacterial RNA polymerase. The inhibition by rifampicin of phages 2172 and H4489A in cells possessing a rifampicin resistant polymerase perhaps indicated that the phage may

utilize a distinct, rifampicin sensitive enzyme, which may be contained within the virion itself. Alternatively, the antibiotic might have been inhibiting some other phage specific function, such as adsorption to the cell surface.

Induction of the T12 lysogens by both ultraviolet light and mitomycin C paralleled the previous methods of inducing H4489A and 2172, in addition to the well studied lysogenic Escherichia coli and Salmonella typhimurium strains. Since semi-temperate bacteriophages are non-inducible, it follows that H4489A, 2172, and T12 were temperate viruses. Verification was provided by the absolute stability of the phage-host complexes in phage specific antisera. Pseudolysogenic cultures would have, on the other hand, contained a significant percentage of phage-free cells, which would have subsequently been isolated as "cured" colonies in conditions preventing reinfection. Sonification of bacterial chains was necessary to locate these phage-free cocci, since one phage-carrier in a chain would have indicated the entire colony forming unit to be a producer of virus. The temperate nature of phages H4489A and 2172 had been previously in doubt, since both produced plaques characterized by random distribution of phage resistant colonies in the zone of lysis, rather than the turbid center which distinguished the plaques of classical temperate phages.

The phage-host complex, $T25_3$(T12gl), was shown to be antigenically altered from the parental nonlysogen. This observation

was unprecedented, since the adsorption of phage to bacteria was not affected by lysogenization of $T25_3$ by the virus. Every known conversion system involving altered bacterial surface determinants has been accompanied by the loss of homologous phage adsorption by the lysogen. Such conversions provide a safeguard, additional to that of cytoplasmic immunity, that prevent lysis by superinfecting virulent mutants of the homologous virus. The selective advantage would obviously favor the survival of both the lysogenic cell and the phage maintained within. Conversion of the immune lysogen to a non-adsorbing form would also increase the efficiency of phage multiplication by preventing wasteful abortive infections.

The advantage of the T12 antigenic converting system, in the absence of superinfection exclusion at the level of phage adsorption, remains an enigma. The phenomenon perhaps provides a limited form of antigenic drift, enabling the lysogen to better escape the immunological defenses of the infected host animal.

The nature of the altered antigen was obscured by two lines of conflicting evidence. First, immunoabsorption of antisera, and titration of the residual agglutinins with heterologous and homologous indicators suggested each strain to possess qualitatively different surface determinants, perhaps reflecting the conversion of a precursor antigen on $T25_3$. Support for these observations was provided by spur formation between $T25_3$ and $T25_3$(T12gl) soluble antigen preparations

used in double immunodiffusion studies. Furthermore, agglutination of $T25_3$ in group A specific antiserum was 32 times greater than that of the lysogen in the same serum. Since this grouping serum presumptively contained only one specificity of antibody, the group A carbohydrate of the nonlysogen was evidently affected by phage infection. Quantitative double diffusion which was much less sensitive, indicated this effect to exceed a factor of four.

Attempts to elucidate the specificity of the reciprocal antigen, however, disclosed that agglutination of the two strains in group A-variant specific antiserum resulted in similar differences in antibody titers as those seen in the group A serum; i.e., a 16-fold decrease following T12 lysogenization. Parallel evidence was again provided by quantitative double diffusion, which detected a four fold decrease in group A-variant specific carbohydrate following lysogeny. These results suggested that a possible generalized reduction in synthesis of the group polysaccharide was associated with T12 infection, but quantitive precipitin assays revealed a total decrease of only 31 percent. This value was subsequently supported by an assay of total carbohydrate in the cell walls of the two strains; i.e., 31 percent more carbohydrate in the $T25_3$ sample than $T25_3$(T12gl).

If the primary effect of this phage conversion system involved altered polysaccharides, the quantitative results indicate the conversion to be relatively insignificant. The group A carbohydrate was not

likely altered to a new antigenic specificity, since the disappearance of the original polysaccharide was accounted for by a similar loss in total carbohydrate. Contrary to the initial indications, therefore, any precursor antigen on $T25_3$ altered by T12 infection was probably not group specific carbohydrate.

The evidence eliminating the role of protein or lipid in determining the antigenic specificity was obtained utilizing a technique that depended on agglutination as the final index. Since nearly all indications of qualitative antigenic differences between the lysogen and nonlysogen were obtained from agglutination experiments, perhaps these results should be re-evaluated, employing an alternative method for determining the chemical nature of the altered antigen. One alternative may be affinity chromatography using nonlysogen antiserum coupled to the solid phase to isolate the unique determinant from soluble $T25_3$(T12gl) antigenic preparations. An isolated compound would be rather easy to chemically qualify. Despite the nature of the antigen involved, however, the spurs of non-identity in double immunodiffusion using antigens extracted from $T25_3$ and $T25_3$(T12gl) provide solid indications of qualitative antigenic modification.

One possible explanation for the apparant conversion of somatic antigens following phage infection was that the viruses provided a selective pressure leading to the proliferation of a cell population resistant to phage adsorption. This possibility was unlikely in

this system, since the population containing the altered determinant was equally susceptible to phage adsorption as was the nonlysogenic culture. Furthermore, addition of phage T12 to the residual agglutinins following heterologous immunoabsorption failed to decrease agglutination of the lysogen in this serum, providing evidence that any antigenic changes were not the result of the attachment of viral capsids to phage receptor sites.

Antigenic conversion of $T25_3$ was mediated by one of the phages also responsible for phage conversion of this cell line to the toxinogenic state. Major differences in toxin production by the lysogen and nonlysogen were detected physiologically (skin test) and serologically (in vitro double immunodiffusion toxin assay). Disc gel electrophoresis revealed this difference to be 7.8 fold in culture filtrates, and only 2.2 fold in the broken cell extracts. Neutralization of erythema using antiserum against $T25_3$(T12gl) culture filtrates indicated the toxic principle in the disintegrated cell proparations of both the lysogen and nonlysogen, as well as purified toxin A, were all antigenically similar to a substance found in the culture filtrates of the toxinogenic lysogen, and missing from similar preparations ot $T25_3$. This "intracellular" toxin, however, while possessing erythrogenic activity, was undetectable by the in vitro assay, suggesting an immunological specificity distinct from its extracellular toxic counterpart. More likely, this d screpancy probably reflected

the superior sensitivity of the skin test assay over the in vitro technique. It is important to realize, however, that the reliability of the physiological parameters in the skin test is still debatable, due to individual variations among animals. The large number of rabbits required for a statistically dependable determination was economically prohibited in this research.

Lytic release of preformed toxin may explain some of these observations. For example, the presence of an erythrogenic substance in both $T25_3$ and $T25_3$(T12gl) that was neutralized by antiserum against $T25_3$(T12gl) culture filtrates suggests that preformed toxin A existed in both cell types but was released only by the lysogenic strain. Additional evidence, however, requires a more complicated explanation for these observations. Lysis of $T25_3$ by virulent phage A25 failed to produce detectable amounts of toxin in the lysates, whereas similar infection with a virulent mutant of phage T12 produced a culture filtrate containing elevated toxin concentrations. Phage T12cp^1, therefore, modified the cell's physiological capacity for extracellular toxin production. In addition, kinetic studies revealed that the appearance of maximum toxin concentrations occurred 6.0 hours after the major lytic event by phage T12cp^1. Electrophoretic analyses of filtrates and broken cell extracts indicate the presence of a molecule with an R_f similar to toxin in both preparations of the nonlysogen. The lysogen was shown to

excrete 7.2 times the amount of this compound than did the phage-free strain, while the differences in intracellular toxin concentrations between the two strains was only 2.2. The additional evidence that the in vitro toxin assay detected toxin in normal, unconcentrated filtrates of $T25_3$(T12gl) but not in broken cell extracts of the same organism, implicated the existence of a phage associated mechanism of concentrating the toxin extracellularly. Finally, double immunodiffusion showed that the nonlysogen produced an extracellular product immunologically distinct from any substance in the $T25_3$(T12gl) filtrate. This may well reflect an extracellular, nontoxic counterpart of toxin A, corresponding to the material with the R_f of 3.8. This substance was perhaps also responsible for the pyrogenic activity found in the $T25_3$ filtrates.

At least one possible mechanism underlying toxin production is suggested by these observations. Toxin A may exist in a preformed state in both the lysogen and the phage-free strains. This toxic substance is the precursor of a nontoxic extracellular product, resulting from modification of the toxin during excretion, a process leading to loss of both erythrogenic toxicity and the original antigenic specificity. The chemical alteration of the intracellular precursor may function to facilitate regulation of its own excretion. The immunologically distinct extracellular product, however, still maintains its original pyrogenic activity. Infection by phage T12, whether resulting in lysis or

lysogeny, may alter the regulatory controls of the excretion process, so that the cell produces excessive extracellular amounts of the material. In the process of deregulation, the substrate is no longer chemically modified when excreted, so that elevated levels of exotoxin A are elaborated.

This proposal also suggests a possible relationship between the two T12 mediated conversion systems of $T25_3$. It is conceivable that a toxin pump, located at the cell surface, may possess characteristics of a somatic antigen. Alteration of this cellular component by T12 infection could affect both its antigenic specificity and its transport functions. The possibility that the somatic antigen and erythrogenic toxin were the same molecule is remote, since toxin A is composed of protein and hyaluronic acid. Not only did experiments contraindicate protein involvement in the antigenic determinant, the ineffectiveness of hyaluronidase to alter the specificity of the somatic antigen reduces the possibility of it possessing a hyaluronic acid moiety.

A schematic representation for toxin production in phage-free and lysogenic bacteria is shown in figure 30. Although this model accounts for the available evidence, it does not preclude the possibility of other explanations.

Several characteristics of the phage conversion systems described in this study parallel those of the classical lysogenic conversion

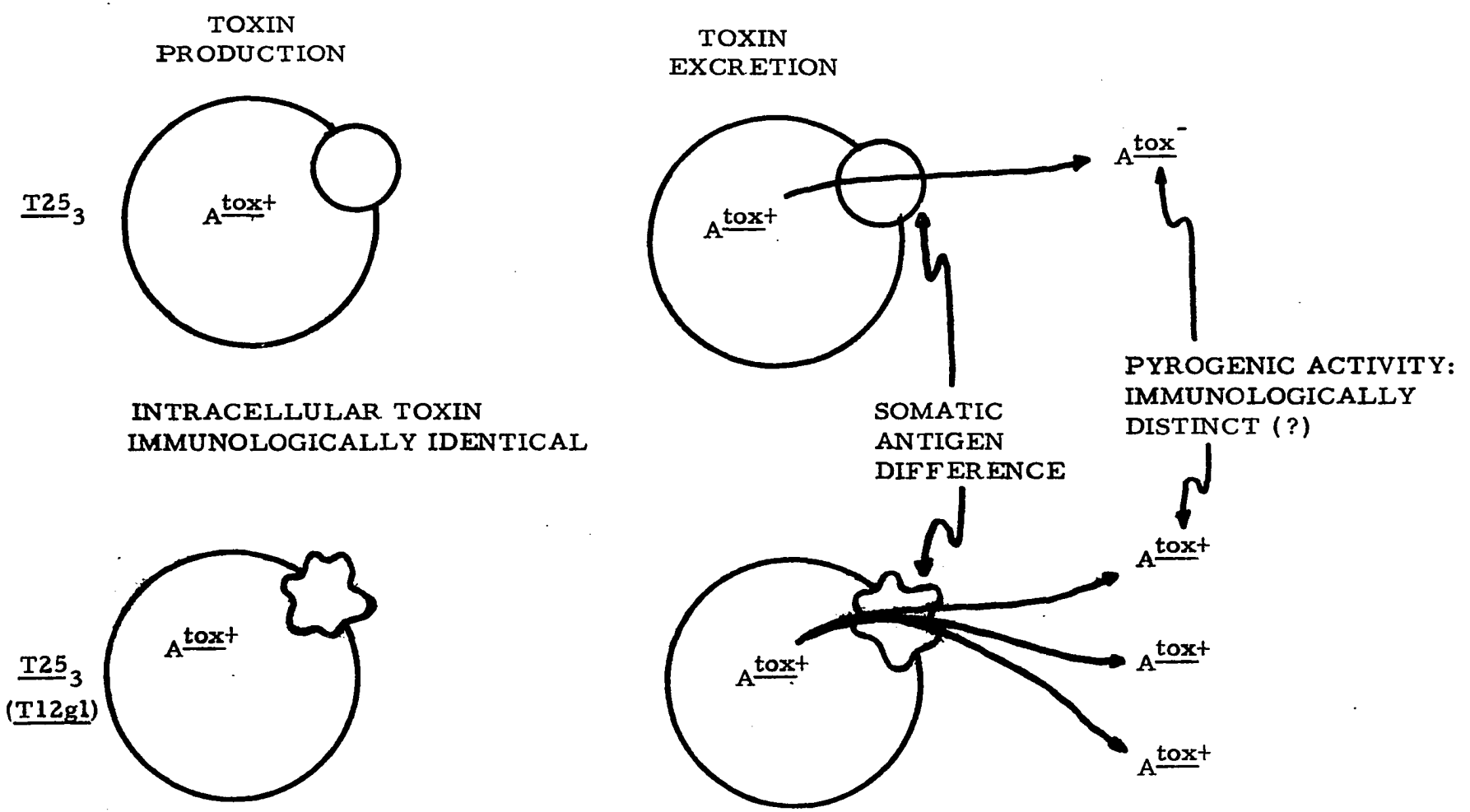

FIGURE 30--Schematic representation of toxin production in phage free and lysogenic bacteria.

models of Salmonella and Corynebacterium. For example, the altered properties occurred only following infection with certain bacteriophages, and lysogeny in general, as with phage H4489A, was not capable of effecting the conversion. The stability of the phage association with the new traits was verified by relysogenization of "cured" lysogens by the same phage. This event was accompanied by the reappearance of the lost characteristic. Whereas infection by nonhomologous, virulent bacteriophages led to no phage conversion, virulent mutants of the converting viruses were all capable of producing phenotypes similar to the lysogen. Integration of the phage genome into the bacterial chromosome was therefore not necessary for expression of the trait.

In addition to these similarities, several differences existed between the streptococcal systems and the classical phage conversion models. For example, extracellular diphtheria toxin was released before lysis by virulent phage beta, whereas erythrogenic toxin was first detected 6.0 hours after the lysis of $T25_3$ by T12cp[1]. Furthermore, while only one antigenic type of diphtheria exotoxin was produced by several species of Corynebacterium lysogenized by prophage beta, three immunological specificities have been described for erythrogenic toxin produced by lysogenic streptococci. Finally, at least one investigator reported a component in purified phage beta preparations with an identical electrophoretic mobility as fragment

B of diphtheria toxin (182), implicating the toxin to have a possible structural function in phage development. No compounds in the purified phage T12 preparations, however, had R_f values similar to toxin A.

The advantage of phage conversion to the bacteriophage is a much discussed theoretical question. In general, those factors which better enable the host bacterium to survive are of subsequent survival value to the virus within. On the basis of current information available concerning erythrogenic toxin, its production seems to impart little or no selective advantage to the bacterium. If this product is unrelated to normal phage functions of lysis or lysogeny, its role becomes even more obscure.

Transduction of antibiotic resistance markers using K56 lysogens and nonlysogens, while producing results similar to previous reports, introduced some original findings, as well. Lysogeny was found to impair recipient efficiency, confirming similar results by Malke. On the other hand, he suggests that for transducing particles to arise, the donor strains must carry prophages which may act by subduing the virulence of A25 in favor of processes leading to the formation of transducing particles. This study, however, disclosed a negative correlation between lysogeny and donor ability. Since the reported incompatibility in propagation of A25 caused by

prophage P5004 was not observed in H4489A and 2172 lysogens, this may account for the differences in these two reports.

The potential phage conversion of strain K56 involving the loss of an intracellular constituent following lysogeny by phages H4489A or 2172 will surely be the subject of future investigations.

Temperate bacteriophages of S. pyogenes are of particular interest since, in addition to any information their study may add to the general field of bacteriophage research, they have a potentially significant role in the pathogenesis of their medically important host.

CHAPTER VI

SUMMARY

The relationship between temperate bacteriophages of group A streptococci and the host bacteria were examined along with certain biological properties of the viruses themselves. Two of the phages (2172 and H4489A) were isolated from nephritogenic strains of Streptococcus pyogenes, whereas the third (T12) was obtained from a toxinogenic lysogen capable of producing the rash of scarlet fever. The corresponding nonlysogen, as well as an additional scarlatinal strain, were found to contain plaque forming units that could not be cultured.

All three phages were found to be similar to other temperate streptococcal viruses described in previous studies. Both phages of nephritogenic streptococci produced plaques characterized by the Kjems effect, but possessing an additional halo than indicated by previous descriptions. The scarlatinal phage plaques, while lacking the characteristic halos, displayed the turbid center common to the well studied temperate phages lambda and P22. Only

the phages capable of penetrating the bacterial capsule developed the Kjems effect.

Concentrated and purified phage preparations were obtained by ultracentrifugation, although subsequent losses of infectivity were common. Phage T12 possessed the greatest stability in the presence of ultracentrifugal forces, but was equally sensitive to the deleterious effects of chloroform as were H4489A and 2172. The mechanism of inactivation by this solvent appeared to be the reduction of the broth's ability to support phage viability.

Intracellular development of these viruses was comparable to most temperate streptococcal bacteriophages, i. e. extended latent periods (45-60 min) and small burst sizes (27-33 PFU/infected coccus). However, transcription of mRNA from viral genomes may have occurred independently of bacterial RNA polymerase, since phage development was inhibited by rifampicin in strains whose polymerase was resistant to this antibiotic. Propagation of phage H4489A was also inhibited by the presence of coinfecting T12, probably the result of viral interference by dissimilar phages. On the other hand, no dominance relationship was observed for coinfecting H4489A and 2172 strains. Although all three phages appeared to possess identical electrophoretic mobilities, they were completely distinguishable by immunological neutralization.

The relationship between each bacteriophage and the respective bacterial strain was determined to be truly lysogenic. The random distribution of phage resistant colonies within the H4489A and 2172 plaques had previously indicated a possible pseudolysogenic bacterial complex followed infection by these two strains.

Three lysogenic conversion systems were examined, two of which were discovered in this investigation. Reciprocal quantitative immunoabsorption revealed that infection of $T25_3$ with phage T12 resulted in a lysogenic complex with (a) somatic antigen(s) not shared by the uninfected strain, which in turn appeared to possess a possible precursor determinant. Spur formation in double immunodiffusion experiments supported these indications. Chemical characterization of the modified antigen eliminated protein or lipid moieties as being essential to immunological specificity. A 32 fold difference in agglutination of the lysogen and nonlysogen in group A specific antiserum indicated the involvement of group carbohydrate in phage conversion. In addition, loss of group A specificity following lysogeny was revealed by quantitative double immunodiffusion assays using the group A specific antiserum and solubilized antigen preparations. Results using group A-variant antiserum in similar experiments indicated a decrease in this antigen, as well. Finally, it was discovered that a 31 percent

reduction in total carbohydrate was associated with lysogeny by phage T12. The specificity of the reciprocal antigen on $T25_3$ (T12gl) remained undetermined. No reduction in the uptake of homologous phage accompanied conversion of the surface determinants.

The phage mediated conversion of nontoxinogenic streptococci to the scarlatinal state was further elucidated in this study, utilizing the same phage-host complex involved in the somatic antigen conversion. The standard skin test method of toxin detection was supplemented by an in vitro toxin assay using double immunodiffusion with purified erythrogenic toxin type A as a known standard. Polyacrylamide gel electrophoresis revealed substances similar to toxin (R_f 0.38) in broken cell extracts and culture filtrates of both the lysogen and nonlysogen. However, the culture filtrates of only the lysogenic $T25_3$(T12gl) possessed erythrogenic activity, which was, in turn, detected in broken cell extracts of both strains. These disintegrated cell preparations, however, were devoid of toxin A detectable by the in vitro toxin assay, probably reflecting the greater sensitivity of the skin test. The erythrogenic activity of both $T25_3$ and $T25_3$(T12gl) was neutralized by antiserum prepared against culture filtrates of the lysogen, but not by similar $T25_3$ antiserum. Streptococcal pyrogenic exotoxin was reportably produced by this nonlysogen. The toxin shared no common electrophoretic mobilities with any components of a washed T12 phage

preparation. A hypothetical model to explain these collective results was presented. Intracellular toxin is perhaps present in both $T25_3$ and $T25_3$(T12gl), but altered to an immunologically distinct, non-erythematous form when excreted by the nonlysogen. Excretion by the lysogen, on the other hand, occurs with no concomitant modification of the toxin.

With both phage conversion systems, the viral mediated changes occurred using the $T25_3$ strain artifically lysogenized with phage T12, but was not apparent when phage H4489A was substituted for T12. Toxin A was elaborated following infection of $T25_3$ by a virulent mutant of phage T12, but not by virulent phage A25 infection.

No detectable conversion of K56 somatic antigens or extracellular products occurred following lysogeny by phages H4489A or 2172. However, the loss of an intracellular constituent following infection by these two phages was discovered using disc gel electrophoresis of broken cell extracts. Transduction of four antibiotic markers of K56 and corresponding lysogens revealed lysogeny to exert a negative effect on both donor and recipient efficiencies. The presence of prophage was not found to interfere with A25 propagation on these strains.

LITERATURE CITED

1. Freeman, V., 1951 Studies on virulence of phage infected strains of Corynebacterium diphtheria. J. Bacteriol. 61:75.

2. Frobisher, M., and Brown, J., 1927 Transmissible toxicogenicity of streptococci. Bull. Johns Hopkins Hospital 41:167.

3. San felice, F., 1893 Z, Hyg. Infectionsk. 14:339.

4. Maxted, W., 1957. The active agent in nascent phage lysis of streptococci, J. Gen. Microbiol. 16:584.

5. Lederberg, J., Lederberg, E., Zinder, N., and Lively, E., 1951. Recombination analysis of bacterial heredity. Cold Spr. Harb. Symp. Quant. Biol., 16:413.

6. Lederberg, E., and Lederberg, J., 1953. Genetic Studies of lysogeny in E. coli. Genetics. 41:51.

7. Barksdale, L., 1958. Lysogenic conversion in bacteria. Bacteriol. Rev. 23:202.

8. McKinley, 1925. Serum antilytique obtenu par immunization contre and bacterie normale, C.R. Soc. Biol. (Paris). 93:1050.

9. Baess, I., 1971. Review of pseudolysogeny. Acta, Pathol. Micro. Scand. 79:428.

10. Fraser, D., 1957, Host range mutants and semitemperate mutants of bacteriophage T3. Virology 3:527.

11. Zinder, N., 1958 Lysogenization and superinfection immunity in Salmonella. Virology, 5:291.

12. Luria, S. Fraser, D., Adams, J. Burrous, J., 1958, Cold Spring Harb. Symp. Quant. Biol. 23:71.

13. Koibong, L., Barksdale, L., Garmise, L., 1961 Phenotypic alterations associated with the bacteriophage carrier state of Shigella dysenteriae. J. Gen. Microbiol. 24:355.

14. Hoffman-Berling, H., Maze, R. 1964 Release of male specific bacteriophages from surviving host bacteria. Virology 22:305.

15. Groman, N. 1953 The change from non-toxigenicity to toxigenicity in Corynebacterium diphtheria. J. Bacteriol. 66:184.

16. Barksdale, W., Pappenheimer, A., 1954 Phage-host relationships in toxigenic diphtheria bacilli, J. Bacteriol. 67:220.

17. Matsuda, M., Barksdale, W., 1967 System for the investigation of the bacteriophage directed synthesis of diphtherial toxin. J. Bacteriol. 93:722.

18. Holmes, R., Barksdale, L., 1969 Genetic analysis of tox^+ and tox^- bacteriophages of Corynebacterium diphtheria. J. Virol., 3:586.

19. Groman, N., 1955 Evidence for the active role of bacteriophage in the conversion of nontoxic Corynebacterium to toxin production. J. Bact. 69:9.

20. Barksdale, L., 1955 Sur quelques bacteriophages de Corynebacterium diphtheriae et leurs hotes. Compt. Rend. 240:1831.

21. Groman, N., Eaton, M., 1955 Genetic factors in Corynebacterium diphtheria conversion. J. Bacteriol. 70:637.

22. Murphy, J. Pappenheimer, A., de Borms, S., 1974 Synthesis of Diphtheria tox gene products in E. coli extracts. Proc. Nat. Aca. Sci. 71:11.

23. Matsuda, M., Barksdale, L., 1966 Phage directed synthesis of diphtherial toxin in nontoxinogenic Corynebacterium Diphtheriae. Nature 210:911.

24. Barksdale, L., Garmise, L., and Horibata, K., 1960 Virulence, toxinogeny, and lysogeny in Corynebacterium diphtheria. Ann. N.Y. Acad. Sci. 88:1093.

25. Pappenheimer, A., 1955 The pathogenesis of diphtheria. Symp. Soc. Gen. Microbiol. 5:40.

26. Uchida, T., Gill, D., Pappenheimer, A., 1971 Mutation in the structural gene for diphtheria toxin by temperate phage beta. Nature, New Biol. 233:8.

27. Matsuda, M., Kanei, C., Yoneda, M. 1972 Expression of tox^+ gene carried by an early mutant (DNA negative mutant) of nonlysogenizing phage beta in nontoxinogenic diphtheria bacilli. Biken Journal 15:111.

28. Izeki, S., Sakai, T., 1953 Artificial transformation of O antigens in Salmonella E. group. I. Transformation by antiserum and bacterial autolysate. Proc. Japan Acad. Sci. 29:121.

29. Zinder, N., 1957 Lysogenic conversion of Salmonella typhimurium. Science 126:1237.

30. Uetake, H., Luria, S., Burrous, J., 1958 Conversion of somatic antigens in Salmonella by phage infection leading to lysis or lysogeny. Virology, 5:68.

31. Uchida, T. Robbins P., Luria, S., 1963 Analysis of the serologic determinant groups of the Salmonella E-group O-antigens. Biochemistry 2:66.

32. Uetake, H., 1957 Lysogenic conversion in bacterial antigens. Proc. Inter. Genet. Symp. Tokyo, Kyota 645.

33. Uetake, H., Higiwara, S., 1960 Somatic antigen 15 as a precursor of antigen 34 in Salmonella. Nature, Lond., 186:261.

34. Uetake, H., Higiwara, S., 1961 Genetic cooperation between unrelated phages. Virology 13:500.

35. Uetake, H., Nakagawa, T. Akiba, T., 1955 The relationship of bacteriophage to antigenic changes in group E Salmonella J. Bacteriol. 69:571.

36. Stocker, B., Lysogenic conversion by the A phages of Salmonella typhimurium. J. Gen. Microbiol. 18:ix.

37. Uetake, H., Luria, S., Burrows, J., 1958 Conversion of somatic antigens in Salmonella by phage infection leading to lysis. Virology 5:68.

38. Robbins, P., Uchida, T., 1965 Chemical and macromolecular structure of 0-antigen from Salmonella anatum strains carrying mutants of bacteriophage epsilon-15. J. Biol. Chem.240:375.

39. Uetake H. Uchida, T 1959 Mutant of Salmonella phage epsilon-15 with abnormal conversion properties. Virology 9:495.

40. Burnet, F., Lush, D., 1936 Induced lysogeny and mutation of bacteriophage within lysogenic bacteria. Australian J. Exp. Biol. Med. Sci. 14:27.

41. van der Vijver, J., van Es-Boon, M., Michel, M., 1972 Lysogenic conversion in Staphylococcus aureus to leucocidin production. J. Virol. 10:318.

42. Blair, J., Carr, M., 1961 Lysogeny in staphylococci, J. Bacteriol. 82:984.

43. Roundtree, p. 1949 The phenomenon of lysogenicity in staphylococci. J. Gen. Microbiol. 3:153.

44. Casman, E., Bergdolf, M. Robinson, J., 1963 Staphylococcal Enterotoxins. J. Bacteriol. 85:715.

45. Clecner, B., Sonea, S., 1966 Acquisition de la prapriete hemolytique de type delta par conversion. Rev. Can. Biol. 25:145.

46. Winkler, K., de Waart, J. and Grootsen, C. 1965 Lysogenic conversion in staphylococci to loss of B-Toxin. J. Gen. Microbiol. 39:321.

47. de Waart, J. Winkler, K. and Grootsen, C., 1962 Lysogenic conversion in staphylococci. Nature,Lond. 195:407.

48. Duval-Iflah, Y., 1972 Lysogenic conversion of the lipase in Staphylococcus pyogenes group III strains. Can. J. Microbiol. 18:1491.

49. Rosendal, K., Burlow P. and Jessen, O. 1964 Lysogenic conversion in Staphylococcus aureus. Nature, Lond. 204:1222.

50. Rosendal, Bulow, 1965 Temperate phages influencing lipase production by staphylococci. J. Gen. Microbiol. 41:349.

51. Bulow, P., 1970 A new epidemic type of Staphylococcus aureus. Microbiol. Scand. B 78:29.

52. Dajani, A., Wannamaker, L., 1973 Kinetic studies on the interaction of phage type 71 staphylococcal bacteriocin with susceptible bacteria. J. Bacteriol. 114:738.

53. Inoue, H., Iida, T., 1970 Japan. J. Microbiol. 14:87.

54. Eklund, M., Poysky, F., Reed, S., 1972 Bacteriophage and the toxigenicity of Clostridium botulinum type D. Nature, New Biol. 235:16.

55. Eklund, M., Poysky, F., Reed, S. Smith, C., 1971 Bacteriophage and the toxigenicity of Clostridium botulinum Type C. Science 172:480.

56. Holloway, B., Cooper, G., 1962 Lysogenic conversion of Pseudomonas aeruginosa. J. Bacteriol. 84:1324.

57. Barnet, Y. Vincent, J., 1970 Lysogenic conversion of Rhizobium trifolii. J. Gen. Microbiol. 61:319.

58. Ionesco, B., 1953. Sur une proprieti de Bacillus megaterium liee a la presence d'un prophage. Compt. Rend. 237:1794.

59. Yasbin, C., Young D., 1972. Transfection in Bacillus subtilis. Biochem, Biophysica. Res. Comm. 47:365.

60. Lawton, W., Molnar, D., 1972. Lysogenic conversion of Pasturella by E. coli phage PICM. J. Virol. 9:708.

61. Bonicke, R., 1970 Phage conversion of biochemical properties of Mycobacterium.Bull. Int. Un Tuberc. 43:217.

62. Barksdale, L., 1959. Lysogenic conversion in bacteria. Bacteriol. Rev. 23:202.

63. Bertani, I., 1964 Lysogenic conversion by bacteriophage P2 resulting in an increased sensitivity of E. coli to 5-fluro-deoxyuridine. Biochim. Biophys. Acta 87:631.

64. Lindahl, G., Hirota, Y., Jacob, F., 1971. Process of Cellular division in E. coli. PNAS 68:2407.

65. Dove, W., 1968 The extent of rII deletions in phage T4. Genet. Res(Camb.) 11:215.

66. Parsons, C., Ranhan, J., Leonard, C., Colon, A., Cole, R., 1973 Inhibition of transformation in group H streptococci by lysogeny. J. Bacteriol. 113:1217.

67. Greer, S., Hsiang, W., Musil, G., Zinner, D., 1973 Viruses of cariogenic streptococci. J. Dent. Res. 6:1594.

68. Maxted, W., 1957 The active agent in nascent phage lysis of streptococci. J. Gen. Microbiol. 16:584.

69. Krause, R., 1958 Studies on bacteriophages of hemolytic streptococci I. Factors influencing the interaction of phage and susceptible host cell. J. Exp. Med. 106:365.

70. Maxted, W., 1964 The Streptococci, Rheumatic Fever, and Glomerulonephritis, ed. Uhr, Williams and Wilkins, Baltimore. 25.

71. Maxted, W., 1955 Influence of phage on Streptococcus pyogenes. J. Gen. Microbiol. 12:484.

72. Zabriskie, J., Reed, S., Fischetti, V., 1972 Streptococci and Streptococcal Diseases: Recognition, Understanding and Management, ed. Wannamaker, L., Matsen, J., New York and London 99.

73. Bingel, K., 1949 Neue Untersuchungen zur Scharlacha tiologie. Deut. Med. Worchschr. 74:703.

74. Zabriskie, J., 1964 The role of temperate bacteriophage in the production of erythrogenic toxin by group A streptococci. J. Exp. Med. 119:761.

75. Zabriskie, J., 1964 The Streptococci, Rheumatic Fever and Glomerulonephritis, ed. Uhr, Williams and Wilkins, Baltimore, Maryland 66.

76. Kim, B., Watson, D., 1972. Streptococci and Streptococcal Diseases: Recognition, Understanding and Management, ed. Wannamaker, L., Matsen, J., New York and London, 33.

77. Hooker, S., Follensby, E., 1934 Studies of scarlet fever. I. Different toxins produced by hemolytic streptococci of scarlatinal origin. J. Immunol. 27:177.

78. Dick, G., Dick G., 1924 A skin test for susceptibility to scarlet fever, J. Amer. Med. Assn. 82:265.

79. Schwab, J., Watson, D., Cromartie, W., 1955. Further studies of group A streptococcal factors with lethal and cardiotoxic properties. J. Infec. Dis. 96:14.

80. Kim, Y., Watson, D., 1970. A purified group A streptococcal pyrogenic exotoxin. J. Exp. Med. 131:611.

81. Heilman, D., Bast, R., 1967. In vitro assay of endotoxin by the inhibition of macrophage migration. J. Bacteriol. 93:15.

82. Hanna, E., and Watson, D., 1968. Host-parasite relationship among group A streptococci. J. Bacteriol. 89:154.

83. Watson, D., 1954. Host factors in experimental streptococcal infections Streptococcal Infections, ed. McCarty M., Columbia Univ. Press, New York, 92.

84. Okada, K., Ayala, G., Sung, J., 1971. Neuropathol. Exp. Neurol. 30:337.

85. Hanna, E., Watson, D., 1968. Host parasite relationships among group A streptococci. J. Bacteriol. 95:14.

86. Evans, A., 1933. Inactiviation of antistreptococcus phage by animal fluids. Publ. Hlth. Rep. 48:411.

87. Kjems, E., 1960. Studies on streptococcal bacteriophages: the occurrence of lysogenic strains among group A hemolytic streptococci. Acta. Path. Microbiol. Scand. 49:199.

88. Seegal, B., Andres, G., Hsu, R., Zabriskie, J., 1965 Pathogenesis of acute glomerulonephritis (abstract) Fed. Proc. 24:100.

89. Michael, A., Drummond, K., Good, R., Vernier, R. 1966 Acute post streptococcal glomerulonephritis, J. Clin. Infect. 45:237.

90. Tresher, G., Semar, M., Ty, A., Sagel, I., Franklin, M., Lange, K., 1970 Antigenic streptococcal plasma membrane components in acute glomerulonephritis. J. Clin. Invest. 49:762.

91. Fish, A., Herdman, R., Michael, A., Pickering, R. Good, R., 1970 Epidemic acute glomerulonephritis, Am. J. Med. 48:28.

92. Feldman, J., Mardiney, M., and Shuler, S. 1966 Immunology and morphology of acute post-streptococcal glomerulonephritis. J. Clin. Infect. 45:237.

93. Tresser, G., Semar, M., McVicar, M., Franklin, M., Ty, A., Sagel, I., and Lange, K. Antigenic streptococcal components in acute glomerulonephritis. Science 163:676.

94. Zabriskie, J., Utermohlem, V., Read, S., Fischetti, V., 1973 Streptococcal related glomerulonephritis. Kidney Int. 3:100.

95. McCluskey, R., Vassali, R., Gallo, G., Baldwin, D., 1966 An immunoflorescent study of pathogenic mechanisms in glomerular disease. J. Clin. Infect. 45:237.

96. Ayoub, E., 1972 Streptococci and Streptococcal Diseases: Recognition, Understanding, and Management, ed. Wannamaker, L., Matsen, J., New York and London 451.

97. Maxted, W., Widdowson, J., Streptococci and Streptococcal Diseases: Recognition, Understanding and Management, ed. Wannamaker, L., Matsen, J., New York and London 251.

98. McCarty, M., 1972 Streptococci and Streptococcal Diseases: Recognition, Understanding, and Management ed. Wannamaker, L., Matsen, J., New York and London 517.

99. Tagg, J., and McGiven, A., 1972. Some possible autoimmune mechanisms in rheumatic carditis. Lancet. 2:686.

100. Kuttner, A., 1966 Production of bacteriocins by group A streptococci with special reference to the nephritigenic types. J. Exp. Med. 124:407.

101. Overturf, G., Mortimer, E., 1970. Studies of the relationship between the production of bacteriocins by group A streptococci and acute glomerulonephritis. J. Exp. Med. 132:694.

102. Bradley, D., 1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol. Rev. 31:230.

103. Rammelkamp, C., 1957. Microbiologic aspects of glomerulonephritis. J. Chronic Dis. 5:28.

104. Potter, E., Moran, A., Poon-King, T., Earle, D., 1968. Characteristics of beta hemolytic streptococci associated with acute glomerulomephritis in Trinidad, West Indies. J. Lab. Clin. Med. 71:126.

105. Leonova, A., Khetenever, M., Chistenkov, N., 1968. Phage sensitivity of hemolytic streptococci isolated from patients with streptococcal infections. J. Microbiol. Epidemiol. Immunobiol. (USSR) 7:104.

106. Wannamaker, L., Skjold, S., Maxted, W., 1970. Characterization of bacteriophages from nephritogenic group A streptococci. J. Inf. Dis. 121:407.

107. Akimova, V., Novosardov, V., Borodiyuk, N., Galachyantz, U., 1973. Study of antigens of "nephritogenic" types of streptococcus, Group A. Zhurnal Mikrobiol. Epidemiologii. I. Immunobiologii. 11:41.

108. Noble, R., Vosti, K., 1973. Biologic and Immunologic comparison of nephritogenic and non-nephritogenic strains of group A, M type 12 Streptococcus. J. Infect. Dis. 128:761.

109. Lancefield, R. 1962 Current knowledge of type-specific M. antigens of group A streptococci. J. Immunol. 89:307.

110. Krause, R., 1972 Streptococci and streptococcal Diseases; Recognition, Understanding and Management, ed. Wannamaker, L., Matsen, J., New York and London 3.

111. Lancefield, R., 1962 Antigenic and biochemical composition of type specific M antigens of group A streptococci. J. Imm. 89:307.

112. Lancefield, R., 1940-41 Specific relationship of cell composition to biological activity of hemolytic streptococci. Harvey Soc. Lect. 36:251.

113. Hood, M., Janney, A., Demeron, G., 1961 Beta hemolytic streptococcus group B associated with problems of the perinatal period. Amer. J. Obstet. Gynecol. 82:809.

114. Eickhoff, T., Klein, A., Daly, A., Ingall, E., Finland, 1964. Neonatal sepsis and other infections due to group B beta-hemolytic streptococci. New Engl. J. Med. 271:1221.

115. Butter, M., de Moor, C., 1967. Streptococcus agalactiae as a cause of meningitis in the newborn, and of bacteraemia in adults. J. Microbiol. Serol. 33:439.

116. McCarty, M., 1969-70 The streptococcal cell wall. Harvey Lect. 65:73.

117. Krause, R., 1963. Symposium on relationship of structure of micro-organisms to their immunological properties. Bacterial Rev. 26:369.

118. Barkulis, S., 1968 Chemical and enzymatic studies on the structure and composition of group A streptococcal cell walls. Current Research on group A Streptococcus, ed. Caravano, R. New York.

119. Karakawa, W., Lackland, H., and Krause, R. 1967. Antigenic properties of the hexosamine polymer of streptococcal mucopeptide. J. Immunol. 99:1178.

120. Abdulla, E., Schwab, J., Cromartie, W., 1966 Biological properties of streptococcal cell wall particles. J. Bacteriol. 91:374.

121. Rotta, J. Bednar, B., 1969 Biological properties of cell wall mucopeptide of hemolytic streptococci. J. Exp. Med. 130:31.

122. Rotta, J., Prendergast, T., Karakawa, W., Harmon, C., Krause, R., 1965 Enhanced resistance to streptococcal infection induced in mice by cell wall mucopeptide. J. Exp. Med. 122:877.

123. Morse, S., 1969 Role of bacteriophages in bacterial resistance to antimicrobials. Ann. Rev. Med. 20:357.

124. Leonard, C., Colon, A., Cole, R., 1968 Transduction in group A streptococci. Biochem. Biophys. Res. Comm. 30:130.

125. Arber, W., 1960 Transduction of chromosomal genes and episomes in E. coli. Virology. 11:273.

126. Benzinger, R., Hartman, P., 1972 Effects of ultraviolet light on transducing phage P22. Virology. 18:614.

127. Malke, H., 1969 Transduction of Streptococcus pyogenes K56 by temperature sensitive mutants of transducing phage A25. Z. Naturforsh. 246:1156.

128. Stuart, J., Ferretti, J., 1973 Transduction of Rifampicin resistance in group A streptococci. J. Bacteriol. 11:709.

129. Malke, H., 1972 Linkage relationships of mutations endowing S. pyogenes with resistance to antibiotics that affect the ribosome. Mol. Gen. Genet. 116:299.

130. Malke, H., 1973 Phage A25-mediated transfer induction of a prophage in Streptococcus pyogenes Molec. Gen. Genet. 125:251.

131. Wannamaker, L., Almquist, S., Skjold, S., 1973 Intergroup phage reactions and transduction between group C and group A streptococci. J. Exp. Med. 137:1338.

132. Colon, A., Cole, R., and Leonard, C., 1972 Intergroup lysis and transduction by Streptococcal bacteriophages, J. Virology. 9:551.

133. Colon, A., Cole, R., Leonard, C., 1970 Transduction in group A streptococci by ultraviolet-irradiated bacteriophages. Can. J. Microbiol. 16:201.

134. Malke, H., Kohler, W., 1973 Transduction among group A streptococci: Transducibility of strains representative of 30 different M types. Zbl. Bakt. I. Abt. Orig. 224:194.

135. Kjems, E., 1955 Studies on streptococcal bacteriophages: techniques of isolating phage-producing strains. Acta. Path. Microbiol. Scand. 36:433.

136. Mickelson, M., 1964 Chemically defined medium for growth of Streptococcus pyogenes. J. Bacteriol. 88:158.

137. Friend, P., Slade, H., 1966 Characteristics of group A bacteriophages. J. Bacteriol. 92:148.

138. Kjems, E., 1958 Studies on Streptococcal bacteriophages: hyaluronidase produced by the streptococcal phage-host system. Acta. Path. Microbiol. 44:429.

139. Kjems, E., 1958 Studies on streptococcal bacteriophages: adsorption,lysogenization, and one step growth experiments. Acta. Path. Microbiol. Scand. 42:56.

140. Evans, A., 1933 Streptococcus bacteriophage, Pub. Hlth, Rep. 48:411.

141. Evans, A., 1934 Streptoccus bacteriophage. Pub. Hlth, Rep. 49:1386.

142. Evans, A., 1934 The prevalence of streptococcus bacteriophage. Science 80:40.

143. Evans, A., 1936 Studies on hemolytic streptocci. J. Bacteriol. 31:423.

144. Evans, A., 1940 Studies on hemolytic streptococci. J. Bacteriol. 40:215.

145. Evans, A., 1942 Studies on hemolytic streptocci. J. Bacteriol. 44:207.

146. Fischetti, V., Barron, B., Zabriskie, J., 1968 Studies on streptococcal bacteriophages II Adsorption studies on group A and group C bacteriophages. J. Exp. Med. 127:489.

147. Friend, R., Slade, H., 1966. Characteristics of group A streptococcal bacteriophages. J. Bacteriol. 92:148.

148. Read, S., 1970. Bacteriophages of group A streptococci. Ph. D. Thesis McGill Univ., Montreal P. Q.

149. Zabriskie, J., 1964. The role of temperate bacteriophage in the production of erythrogenic toxin by group A streptococci. J. Exp. Med. 119:761.

150. McKane, L., 1971. Biological characterization of two temperate streptococcal bacteriophages. M. S. Thesis. The University of Oklahoma Medical Center, Oklahoma City, Oklahoma.

151. Zabriskie, J., 1966. Viral induced bacterial toxins. Ann. Rev. Med. 17:337.

152. Malke, H., 1972. Streptococci and Streptococcal Diseases: Recognition, Understanding and Management, ed. Wannamaker, L., Matsen, J., New York and London 119.

153. Stock, A., 1942. Studies on the hemolytic streptococcus, J. Biol. Chem. 142:777.

154. Stock, A., Verney, E., 1952. Properties of scarlet fever toxin of the NY5 strain. J. Immunol. 69:373.

155. Williams, H., 1955. Essential amino acid content. Cornell Univ. Agra. Expt. Sta. Mem. 337.

156. Henderson, L., Snell, E., 1947. Uniform medium for determination of amino acids with various micro-organisms. J. Biol. Chem. 172:15.

157. Leonard, C., Corley, D., Cole, R., 1967. Transformation of streptococci in chemically defined media. Biochem. Biophys. Res. Comm. 26:181.

158. Lawson, J., 1971. Growth of cariogenic streptococci in chemically defined media. Arch. Oral Biol. 16:339.

159. Wannamaker, L., Skjold, S., Maxted, W., 1970. Characterization of bacteriophages from nephritogenic group A streptococci. J. Inf. Dis. 121:407.

160. Adams, M., 1969 Bacteriophages. Interscience Publishers, Inc., New York.

161. McSharry, J., Benzinger, R., 1970. Concentration and purification of vesicular stomatitis virus by polyethylene glycol "precipitation." Virology 40:745.

162. Hershey, A., Chase, M., 1952 Independent functions of viral protein and nucleic acid in growth of bacteriophage. J. Gen. Physiol 36:39.

164. Ellis, E., Delbruck, M., 1939. The growth of bacteriophage J. Gen.Physiol. 22:365.

165. Hartmann, G., Hoikel, K., Nuesh, J., 1967. The specific inhibition of the DNA-directed RNA synthesis by rifamycin. Biochim. Biophys. Acta 145:84.

166. Golub, E., Mishell, R., Weigle, W., 1969 A modification of the haemolytic plaque assay for use with protein antigens. J. Immunol. 100:133.

167. Rose, M., Rigazzi, P., 1973 Methods in Immunodiagnosis. John Wiley and Sons, New York, London, Sydney, Toronto.

168. Fuller, A., 1938 The formamide method for extraction of polysaccharides from hemolytic streptococci. Brit. J. Exp. Path. 19:130.

169. Lancefield, R., 1933 A serological differentiation of human and other groups of hemolytic streptococci. J. Exp. Med. 57:571.

170. Oudin, J., 1946 Compt. Rend. 222:115.

171. Ouchterlony, O., 1958 Diffusion-in-gel methods for immunological analysis. Progr. Aller. 5:11.

172. McCarty, M., Lancefield, R., 1955 Variation in the group specific carbohydrate of group A streptococci. J. Exp. Med. 102:11.

173. Scheidegger, D., 1955 l'immuno-electrophorese. Int. Archs. Allergy 7:103.

174. Lowry, O., Rosenbrough, N., Farr, A., and Randell, R., 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

176. Beck, A., Bekgner-Rabinowitz, S., 1972 An absorption method for preparing anti-M typing streptococcal sera using acid hydrolized cells. J. Lab. Clin. Med. 80:834.

177. Taubman, M., Genco, R., 1971. Induction and properties of rabbit secretory gamma A antibody directed to group A streptococcal carbohydrate. Immunochemistry 8:1137.

178. Dische, Z., Shettles, L., 1948. Specific color reaction of methylpentoses and a spectrophotometric micro-method for their determination. J. Biol. Chem. 175:595.

179. Topley, W., Wilson, G., 1946 Principles of Bacteriology and Immunity. Baltimore, Williams and Wilkins Co. 1476.

180. Williams,C., Chase, M., 1967 Methods in Immunology and Immunochemistry, Academic Press, New York and London 322.

181. Caska, M., Erikson, R., Varga, J., 1971 Radioimmunosorbent assay of allergens. J. Allergy Clin. Immunol. 49:1.

182. Elwell, L., Iglewski, B., 1972 Diphtheria toxin: evidence for presence of fragment B in corynebacteriophage. Biochem. Biophys. Res. Comm. 49:609.

183. Swanson, J., and Gotschlich, E., 1973 Electron microscopic studies in streptococci II group A carbohydrate. J. Exp. Med. 138:245.