CHARACTERIZATION OF STAPHYLOPHAGE 47

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I. INTRODUCTION

Bacteriophages are viruses which parasitize bacteria. The bacteriophage-host relationship is specific; e.g., staphylophages infect only staphylococci; indeed, a given staphylophage infects only staphylococci of a given species. Diagnostic and public health laboratories customarily use phages (short for bacteriophages) to type (identify) staphylococci implicated in food poisoning. Cases of food poisoning due to enterotoxigenic strains of Staphylococcus aureus can be diagnosed and treated by similar methods, regardless of origin. The value of phage typing, then, lies in enabling a laboratory to locate the exact source of infection and to trace its spread. Healthy persons rarely suffer more than a few hours of discomfort after contracting the disease; but its effects upon the very young, the very old, and the debilitated can be devastating and even fatal. As a public health measure, it is therefore imperative that phage typing be done as rapidly and efficiently as possible in order to trace and/or prevent epidemics.

Typing phages must be readily propagated and maintained; and, above all, they must yield reproducible results in order to be of epidemiological value. At the present time, the field of staphylophage biology is a relatively unexplored one. Very few investigators have worked with staphylophages and conclusions drawn about their physical, biochemical, and immunological characteristics vary from one laboratory to

another. Many workers have been content to assume that staphylophages would behave like phages of Escherichia coli (which have been studied in detail) and have investigated little more than lytic spectra. Consequently, many laboratories have difficulty in maintaining staphylophage stocks and in deriving reproducible results. It is this investigator's opinion that, when an outbreak of food poisoning occurs, few laboratories are prepared to handle the situation with any degree of confidence.

Staphylococcus aureus strain 47 and its companion strain 6 are implicated in approximately 65% of staphylococcal food poisoning cases (19). Consequently, phages 6 and 47 are valuable epidemiological tools. Since very little is known about the growth of staphylophage 47, the purpose of this investigation was to examine its basic physiological characteristics. The areas of study included investigations of the most efficient means of propagating phage 47 and of its biochemical and physical characteristics.

II. LITERATURE REVIEW

A. Staphylophages

Staphylophages were the first lytic agents recognized to be bacterial viruses (3, 19, 46). Twort, in 1915, noticed that some colonies of staphylococci underwent a "transformation" upon prolonged incubation. The colonies changed from white and creamy to clear and watery. No viable staphylococci could be recovered from "transformed" colonies and application of the watery material to healthy colonies even after dilution caused the same change to occur. The agent which killed the staphylococci could pass through filters which retained bacteria; it survived prolonged storage in the cold but was inactivated by heat. Twort's only publication on the subject was a brief paper in which he postulated that the "transformation" might be due to: 1. a stage of the staphylococcal life cycle; 2. overproduction of a destructive enzyme of aberrant staphylococci; or 3. presence of a specific antistaphylococcal virus (47).

Twort's paper was essentially ignored until after the publication in 1917 of similar observations made by d'Herelle (3, 19, 46). While studying Shiga dysentery (now Shigella dysenteriae), d'Herelle noticed that plates inoculated with the organism occasionally showed clear circular areas where no growth occurred. He transferred filtered material from the clear areas to fresh broth cultures of the organism and found

that the cultures lost all turbidity (cleared) and contained no viable bacteria. D'Herelle's publication claimed discovery of a particulate, submicroscopic, filterable, self-reproducing virus which was an obligatory bacterial parasite; for this virus he coined the name "bacteriophage" (that which devours bacteria) (22).

In 1918 d'Herelle suggested that phages might be put to therapeutic use (23). From that time through the 1920's a widespread effort was made to treat various bacterial infections via phages. A number of phages specific for various bacteria were isolated and were administered both topically and by injection. The desired effect, that of destruction of the infecting agent without harm to the patient, rarely occurred (19). Most patients derived no benefit whatsoever; a few suffered severe allergic reactions; "successes" occurred only when other methods of treatment were employed at the same time. After phage therapy fell into disrepute, d'Herelle concentrated upon studies of the basic properties of phages. He confirmed the postulate that phages were specific for a given bacterial host and that phages must have attached to the host before lysis (disruption) could occur. He also devised a titration method in which dilutions of phage were mixed with host bacteria and spread on agar plates; the number of clear areas, or plaques, multiplied by the dilution factor gave the number of viable phage present in the original sample (assuming that each plaque arose from one parent phage) (24). This is

essentially the same technique currently in use (3).

In the 1930's and 1940's phages became popular tools in the then new fields of bacterial genetics and molecular biology. Schlesinger (46) initiated studies of phage particle size and mass (40) and of the kinetics of adsorption of phages to their hosts (41). His most important finding was that phages consisted of protein and deoxyribonucleic acid (DNA) in roughly equal proportions (42). At the same time Delbruck began using phages (the T-even E. coli phages discovered by Demerec) as a means of studying and defining the fundamental unit of heredity, the gene (46). Among other things, he found that infected bacteria showed a latent period during which few phage were found and that the end of the latent period was concomitant with lysis (16). He also developed the basis for the one-step growth experiment and laid much of the foundation for modern phage research.

An important addition to phage research was made in the early 1950's by Lwoff (31). In his work with phage lambda, he found that phages did not always lyse their hosts, but occasionally entered a state of intracellular symbiosis without causing lysis. Lytic phages were termed "virulent" and symbiotic ones "temperate"; the symbiotic state was designated as "lysogeny." The concurrent work of Watson and Crick (48), who delineated the structure of DNA, made the remaining major contribution to the basis of modern phage research; by adding to the understanding of the gene, they helped in speeding up

work with phage genetics and in tying together the observations of Schlesinger, Delbruck, and Lwoff.

Nearly all of the major advances in phage biology have been made with phages parasitizing Escherichia coli, notably the T-even phages used by Delbruck and the lambda phage of Lwoff (46). Very few investigators worked with the staphylophages originally discovered by Twort. One notable exception was Burnet (9), who defined a number of the problems encountered when working with staphylophages. However, by his own admission, he did not derive many solutions. He grouped his phages according to their growth properties: strong phages (4 types) multiplied well in broth at 37 C.; weak phages multiplied only in agar at 22 C.; one intermediate phage multiplied in broth at 37 C. if the host inoculum was very light. All 8 strains were inactivated by temperatures above 37 C. and produced tiny plaques (0.1 - 1.5 mm). He noted that the phages would adsorb to many strains of Staphylococcus aureus but did not always initiate a lytic infection; he suggested the phenomenon of lysogeny long before its demonstration by Lwoff. By preparing homologous antisera to each phage type and allowing them to cross-react with each of the other types, he found that phages fell into serological grouping corresponding to those aforementioned for growth characteristics. In addition, he noted that some phages showed changes in their characteristics over a period of time, e.g., becoming more tolerant of temperatures above 37 C. Much of his discussion dealt with

similarities between staphylophages and dysentery phages. These similarities were primarily in estimated particle size and in plaque diameter. It is unlikely that these were significant observations; truly accurate methods for estimating particle size were not available in 1935 and plaque size can vary with the host's growth rate, the temperature of incubation, and the media used (39). Burnet's major contributions were the establishment of serological groupings, the noting of acquired changes in phage characteristics, and the suggestion of lysogeny.

During the late 1940's and throughout the 1950's the possible epidemiological advantages of a staphylophage typing system were recognized; phages, since they were specific for given hosts, appeared to be an efficient means of identifying infectious carriers of those hosts (32). As antibiotic therapy became more popular, more and more resistant strains of S. aureus emerged. It was hoped that phage typing would prove a quicker and more economical means of identifying strains of S. aureus than repeated tests for resistance to a wide range of antibiotics. The finding of phage groups having restricted host ranges (25) spurred several investigators to concentrate upon deriving a typing system (3, 19, 46). If a phage were capable of lysing only a few host strains, and a suspected host was indeed lysed, indentification would be relatively simple and accurate. Antibiotic testing would have to be done only once and data would be tabulated according to host strain.

Phage typing would identify the host strain and the tabulated data could then be checked for the proper antibiotic treatment (19, 32).

Notable efforts in the typing field have been made by Rippon (36). She classified phages primarily on the basis of host range. By testing undiluted phage stocks (all derived from lysogenic cultures) on a standard set of host strains, she devised 3 categories: 1. lysing coagulase-positive hosts, restricted range; 2. lysing coagulase-positive hosts, polyvalent range; and 3. lysing coagulase-negative hosts. (Coagulase is an enzyme synthesized by pathogenic staphylococci which causes plasma to clot (19).) Subdivisions of these 3 groups were made on the basis of serological cross-reactions and further by plaque morphology and resistance to various chemical and physical agents.

Blair and Williams (6) reviewed and extended the work of earlier investigators (36, 38, 39) and derived what is now the standard procedure for propagating and maintaining typing staphylophage stocks (as approved by the International Association of Microbiological Societies, Nomenclature Committee, Subcommittee on Phage Typing of Staphylococci). They divided the phages lysing a restricted number of coagulase-positive staphylococci (cf. group 1 of Rippon) into 4 groups and added a fifth group of miscellaneous phages. The result was a set of 21 standard typing phages divided according to lytic groups (6):

I - 29, 52, 52A, 79, 80
II - 3A, 3B, 3C, 55, 71
III - 6, 7, 42E, 47, 53, 54, 75, 79
IV - 42D
V - (miscellaneous) 81, 187

Fig. 1. Staphylococcal typing phages.

Subdivisions of these 5 lytic groups were made on the basis of serological cross-reactions which have more recently been shown to depend upon phage morphology, as discussed later in this section (26). Many of these 21 basic phages required the presence of divalent cations, usually calcium or magnesium, before adsorption to the host could occur; phage 47 specifically required at least 400 ug of calcium per ml of propagating medium. They propagated all 21 phages by 2 techniques, broth and soft agar. Broth lysate yields were usually 10^8 - 10^{10} plaque-forming units (pfu) per ml. Soft agar lysate titers were usually 10^{10} - 10^{11} pfu per ml. The higher yields obtained from soft agar were offset by the extra time and effort required to produce them. In addition, soft agar was more easily contaminated and could not be used for production of phage in bulk. Most of the 21 standard phages were propagated best in broth. One exception was phage 47, which did not grow well in broth; soft agar was the method of choice for its propagation. Soft agar, however, yielded a reproducible titer of only 10^5 pfu/ml. As reported by earlier workers (9), lysogeny and acquired changes in phage characteristics sometimes interfered with studies and made reproducible results difficult to obtain.

In addition, the host cultures sometimes overgrew phage plaques, making titering difficult (6, 39).

Elek (19), in his review of staphylophage typing, stressed its uncertainties and stated that no definite correlation has yet been found between the characteristics of a given phage and the characteristics of its corresponding host. He saw phage typing as an epidemiological tool, especially in cases of food poisoning, but not as a true classification system.

A few staphylophages have been examined by electron microscopy (37, 39, 44). Most are of two morphologies corresponding to two serologically distinct groups. One group (A) had an oblong head, 60 nm by 100 nm, and a terminally "bobbed" tail, 12 nm by 290 nm. Another group (F) had a round head with a diameter of 60 nm and a tail similar to A; there were subgroups (B, L) with a shorter tail. Closer examination of a group B phage (5) has shown that the "round" head may actually be hexagonal and the tail "bob" may be relatively flat and hexagonal. It has been postulated (5) that this terminal tail structure may be a baseplate and may control adsorption to the host. (It was earlier determined (38) that adsorption did occur via the tail.) Minor groups (D, G) showed tails that were long fibers attached to a plate that was in turn attached to the head (D) or were tailless (G). It has been reported (37), but not confirmed, that some group A phages may have an inner membrane-like structure within the head. In all staphylophages thus far examined, head and tail have been protein in

composition (19); DNA was packaged within the head. The tail in the two major morphological groups (A and F) was shown (15) to be a hollow cylindrical sheath with a solid central rod. Preparations frequently showed fragmented tails, especially if they had been centrifuged at high speed (37).

Staphylophages were found to be negatively charged and were inactivated by ultraviolet light, ultrasound, heat, and high concentrations of salts or acids; they were reversibly inactivated by light, aldehydes, and $HgCl_2$ (19). Early workers (28) reported inactivation by chloroform and noted that viable phage could not be recovered from a chloroformed culture. However, the phage type and percent chloroform used were indeterminate.

Buoyant densities ranged in general from 1.45 to 1.51 g/cc (37). Ranges within a given serological (morphological) group were fairly constant. For group A, which includes phage 47, the range was 1.451 to 1.469 g/cc and the mean was 1.460 g/cc.

The process of staphylophage infection has been extensively investigated by Rountree (38, 39). Using the one-step growth curve technique (3), she found that adsorption was dependent upon phage concentration and upon age of the host. In general, young host cells (log phase) and low multiplicities of infection or the ratio of phage particles to host cells (3 - 4 phage/cell) enhanced adsorption. Some phages were noticed to adsorb to many hosts without lysing them, again (9) suggesting the possibility of lysogeny. (An alternative possibility is that the phage simply attached to the cell wall

with no subsequent reaction of any kind.) Susceptible hosts were lysed from without when multiplicities of infection (abbreviated as MOI) were very large. Varying concentrations of calcium or magnesium were needed for some phages to adsorb to their hosts, as aforementioned (6). Electron microscopy showed that adsorption occurred via the tail terminus and the protein coat did not enter the host. Rountree (39) thus concluded that infection was due to phage DNA leaving the phage head, traveling through the tail, and entering the host. Her observations were made several years prior to the classic experiments of Hershey and Chase in 1952 (25, 30), which made use of dual isotopic labeling to conclusively demonstrate that only phage DNA, and not phage protein, entered the infected host. Latent periods varied widely, ranging from 30 to 100 minutes. This was followed by a logarithmic rise in phage titer, with a plateau in titer after lysis occurred. Cultures rarely cleared, and the hosts frequently overgrew the phages. Burst sizes rarely exceeded 100 phage particles per host cell. The phages Rountree used were best propagated and maintained in broth; some were more easily propagated at 22 C. than at 37 C.; nearly all were stable when stored at 4 C., showing no drop in titer. In work dealing with group A phages (39), she noticed that adsorption should be essentially complete after 5 minutes if MOI's not greater than 3 - 4 were used. For serological studies, she recommended the use of homologous antisera which could reduce plaque titers by at least 80% after 24 hours of

incubation at 37 C.

Rountree devoted a sizeable portion of her discussions (39) to what she termed a two-fold antibody response. Rabbits injected with staphylophages showed a high initial titer of complement-fixing antibody, followed later by a high titer of neutralizing antibody. She postulated that the dual response represented two separate antibodies which must have been triggered by two separate antigens. She speculated staphylophages consisted of two structures, possibly corresponding to the head and tail, which behaved as individual unique antigens. This postulate has never been formally refuted; but it is suggested in this thesis that only one antigen is involved. Recent work (1) has shown that several subclasses of antibodies are produced in response to a given antigen. Complement-fixation and neutralization are two functional properties of antibodies defined only by the type of assay used. The former requires sequential action of a number of serum proteins ultimately resulting in a lytic event; the latter involves formation of antigen-antibody complexes which result in inactivation of the antigen. The different antibody subclasses exhibit both properties to different degrees. Immunoglobulin M (IgM) and immunoglobulin G (IgG) are two subclasses of serum gamma globulins synthesized in response to antigenic stimulation. IgM antibody appears rapidly after antigenic stimulation (the exact amount of time dependent upon the antigen/host system used); it exhibits excellent complement-fixing activity, but

little neutralizing capability. IgG antibody appears later than IgM and peaks after the IgM response has subsided; it exhibits little complement-fixing potential, but considerable neutralizing activity. In light of these properties of immune responses, it is highly probable that Rountree did not actually observe responses to two separate antigens, but rather the responses of two antibody subclasses to the same antigen. A staphylophage would then have to be considered a single antigenic unit.

More recent adsorption studies have been conducted by Lovett (29). He based his work on Rountree's studies, but used a Micrococcus phage. (Micrococci and staphylococci are both in the family Micrococciaceae.) He too used broth cultures and low MOI's, and he reported the same problem of low burst size. Furthermore, he found that host cells treated with the enzymes pronase and trypsin in concentrations sufficient to disrupt the cell membrane complex but insufficient to disrupt the cell wall could not adsorb phage. He concluded that the host cell membrane complex, in addition to the cell wall, might be involved in phage adsorption. Within the cell wall itself, N-acetyl-D-glucosamine might be required before phages can adsorb (13). In some host strains (29), resistance to certain phages (ability to block their adsorption) correlated with absence of the glucosamine residue in the cell wall. Mutant phages which could adsorb to such cells could not adsorb to other cells which, in addition to lacking the

glucosamine function, were deficient in cell wall phosphorous and ester linked D-alanine.

Several workers have investigated the phenomenon of lysis (15, 43, 45). Phage preparations contain enzymes which have activities and pH optima different from those of host enzymes, and which solubilize host cell walls after lysis. The enzyme (lysin) isolated from each phage-host system studied to date has been a peptidase. The peptidase appeared to attack and degrade the glycine pentapeptide cross-linkage of the cell wall (43, 45). For some host strains, the cell walls required exposure to viable phage (presensitization) before purified phage peptidase could degrade the walls (43), implying a need for some initial injury (disruption of cell wall integrity) to preceed enzymic action, In other strains (45) presensitization was not required for phage peptidase to degrade host cells. A curious, but as yet unexplained, observation (45) was that the cell walls from homologous hosts were not necessarily the best substrate for a given phage lysin.

After a staphylophage has adsorbed to a potential host cell, three events might occur(9, 39): 1. lytic infection; 2. lysogeny; or 3. mere attachment, reversible or irreversible, to the cell wall with no subsequent action. Steps subsequent to adsorption have been shown (35) to govern which of the three possibilities occurs. Studies (19) have shown that synthesis of lytic phage required adenosine triphosphate (ATP) and possibly nicotinamide adenine dinucleotide (NAD) and

B-complex vitamin cofactors. Infected cells continued to synthesize the same total amount of nucleic acids as prior to infection (19), but ribonucleic acid (RNA) levels decreased and DNA levels increased. Younger cells (in log phase of growth) synthesized phage more rapidly than older (stationary phase) cells (19, 39). Host DNA was thought to be degraded (19).

In addition to the virulent (lytic) phages discussed above, temperate (lysogenic) phages have been reported for virtually every strain of Staphylococcus aureus studied (6, 19, 36). Data indicating that this symbiotic state does exist have been consistent with the Campbell model for lysogeny (10, 30). The data (19) indicated that the phage genome ordinarily existed as a closed circle, but opened at a specific locus when triggered, e.g., by ultraviolet light, to undergo lysogeny. The resulting linear genome was then inserted into the host genome at a specific locus and became essentially a part of the host (prophage). As long as a host repressor prohibited transcription of the lytic enzyme coded for by the phage DNA, the prophage continued to be replicated as part of the host and caused the host no damage. If a host repressor became unavailable, the prophage separated from the host genome; it then underwent self-replication as lytic phage. Polylysogens (strains carrying several prophages) have been identified (39); but it has not yet been determined if a single host cell actually carried more than one prophage, or if a number of

host subpopulations carried one prophage apiece. In addition, lysogenic strains have been shown (39) to be capable of liberating a few prophages without lysing, readsorbing them, and reinstituting lysogeny.

Many laboratories have genetic studies currently in progress involving both host mutation (5, 11, 33) and phage mutation (21, 49). Infected susceptible host cultures could show secondary growth which was phage-resistant, but strains varied widely in their ability to acquire this character. (Host strain 47 readily acquires and loses phage-resistance (19), implying a measure of genetic instability.) Heat-treating makes some strains phage-sensitive (11), possibly either by repressing an immunity control mechanism or by derepressing control over a prophage.

Some phages can be made to transduce a number of genetic markers (antibiotic sensitivities and resistances) into susceptible hosts (33). Transducing particles do not show the same buoyant densities as infectious particles (5); the densities are greater or less than normal phage, depending upon which marker is being transduced. It was speculated (5) that transducing particles either contained amounts of DNA different from that of infectious phage, or that the DNA amounts were the same, but differed in density. Phages themselves showed host-range mutations (49), especially if stocks were maintained or propagated over a long period of time (6). Phage populations sometimes changed from virulent to temperate and back again (19)

in a single host strain. Temperature-sensitivities sometimes changed in phages (9, 21). Phage damaged by treatment with ultraviolet light can sometimes be repaired by the host (21).

It has been shown (19) that Corynebacterium diphtheriae must carry a prophage in order to be toxigenic. Searches for possible parallels in staphylococci have yielded varying, and sometimes conflicting, results (4, 51). Read and Pritchard (34) attempted to convert S. aureus strain S-6 from non-enterotoxigenic to an enterotoxin A-producer via a number of temperate phages. The attempt failed, and they concluded that no correlation existed between presence of a prophage and production of enterotoxin A. Casman, however, published a preliminary report (12) of conversion of 31 non-enterotoxigenic strains to producers of enterotoxin A after exposure to a temperate phage carried by an enterotoxin A-producer. His report has yet to be supported by any other published data, and it is possible that his conclusions were premature. In work with staphylococci producing enterotoxin B, Winkler et al. (51) reported that acquisition of certain prophages of serologic group F resulted in loss of toxigenicity. Concomitant with loss of enterotoxin B was the acquisition of ability to produce fibrinolysin. (The two characters are not adjacent on the host genome (51).) There are numerous speculations one might make about the effects of prophage at the DNA level. When enterotoxin is induced (12), it might result from repression of a host control over the gene coding for toxin or from

derepression of the gene itself. When enterotoxin is inhibited (51), the prophage might either repress the host gene coding for toxin or derepress a control mechanism. Finally, the possibility cannot be excluded that the production of enterotoxin A (12) and/or fibrinolysin (51) is directly coded for by the prophage itself.

B. Staphylococcal lipids

There is reason to suspect that staphylophage 47 is lipid-containing (see Results and Discussion). There is no real precedent for this postulate (19), other than a brief report (28) of chloroform-sensitivity. Such sensitivity is rather inconsistent with the current concept of staphylophages as composed of protein and DNA (19). Proteins are not generally affected by low concentrations of chloroform, while lipids are greatly affected (15). Staphylococcus aureus does contain lipids (50) and is as likely a starting point as any in a search for staphylococcal phage lipids.

The most thorough study of staphylococcal lipids has been made by White and Frerman (50). After disruption of the cell wall, e.g., by digestion with the enzyme lysostaphin and treatment with solvents, cells have yielded carotenoid pigments, phosphatidyl glycerol, cardiolipin, and at least 3 isoprenologues of vitamin K2. These same components were found regardless of whether lipid extraction was via chloroform-methanol, alkaline hydrolysis, or acid hydrolysis. Recovery was maximized by

acidification to pH 2 just prior to harvest, but the effect of acidification has not yet been determined. Use of the above extraction procedures plus acidification accounted for 98% of the total lipid content. The remaining 2% was recovered after prolonged refluxing in isopropanol and was shown to contain 2 glucosyldiglyceride lysyl esters of phosphatidyl glycerol. Thin layer silica gel chromatography yielded spots which were readily identified by Rf values and lipid reagent sprays. Results were consistent when checked by descending paper chromatography and silicic acid chromatography. In addition, results were not altered when methods of disrupting the cell wall were varied. The lipids were shown to be part of the cell wall-membrane complex and may have been associated with the respiratory pigments (cytochromes).

To date only one bacteriophage had been reported to contain lipids: PM2, a Pseudomonas phage (20). The report on PM2 was investigated as a possible source of ideas for approaching the analysis of staphylophage lipids. PM2 was sensitive to ether and other organic solvents and lost infectivity as a result of solvent treatment. Phage PM2 was treated with chloroform and then applied to polyacrylamide gels for electrophoresis. The gels showed two bands after staining with Sudan black, a lipid-specific stain. Chloroform extracts (27) of PM2 were also applied to thin layer silica gel plates and chromatographed in chloroform-methanol and ether solvent systems (2). Sudan black sprays revealed at least 2 lipid spots not attributable

to the host bacterium. It is thought (20) that the 2 bands appearing in electrophoresis gels correspond to the 2 spots appearing on silica gel plates. There are thus thought to be at least 2 lipids in phage PM2, one of which has been identified as phosphatidylethanolamine (20).

III. MATERIALS AND METHODS

A. Propagation

Phage propagation, plating, and terminology were primarily according to modifications of the procedures described by Adams (3). Procedures of Rountree (39) and of Blair and Williams (6) were also adapted to serve the study at hand.

1. Media

a) Dehydrated trypticase soy broth (TSB) and brain heart infusion broth (BHI) were purchased from Baltimore Biological Laboratories. They were prepared in distilled water according to the manufacturer's instructions and autoclaved before use. When TSB and BHI were used for production of broth lysates, $CaCl_2$ was added at a level of 400 ug/ml before autoclaving. In addition to uses described below, TSB was also used as a dilution fluid.

b) The agar used for placqing studies was Bacto-Agar, purchased from Difco Laboratories. Hard agar was prepared by adding 1.5% agar to TSB. Soft agar was prepared by adding 0.5% agar to TSB. $CaCl_2$ was added to both hard and soft agar at a level of 400 ug/ml before autoclaving.

c) A stock solution of calcium chloride (reagent grade, Baker and Adamson) was made up at 40 mg/ml (w/v) in distilled water. $CaCl_2$ was added to hard and soft agar and to

broths by diluting the stock solution 1:100 in the medium. The final $CaCl_2$ concentration in all media was 400 ug/ml, as recommended by Blair and Williams (6) for the production of staphylophage 47.

d) ^{3}H-(methyl)-thymidine (Sp. Act. 3C/mMole) and U-^{14}C glucose (Sp. Act. 8 mC/mMole) were purchased from the Schwarz-Mann, Orangeberg, N.Y. Thymidine was used at 0.5 uC/ml; glucose was used at 0.1 uC/ml.

e) Cesium chloride (research grade) was purchased from American Potash and Chemical Corp., Rare Earth Division. The stock solution was made by mixing equal amounts of CsCl with distilled water, e.g., 50 g CsCl and 50 ml water; density of the resulting solution was 1.6 g/cc.

f) Thin layer chromatography (TLC) silica gel sheets (Chromagrams) were purchased from Eastman Kodak. Sheets were activated by heating to 90 C. for 30 min.

g) Chloroform and other chemicals were reagent grade and were purchased from the Fisher Company.

2. Phage-host system

Staphylophage 47 was chosen because of its widespread usage in typing staphylococcal isolates implicated in food poisoning cases (6, 19)(see also introduction). Its propagator strain is Staphylococcus aureus strain 47. Staphylophage 47

has been assigned (6) to lytic group III and serologic group A. Both host and phage were obtained from the National Center for Disease Control, Atlanta, Georgia. Host cultures were maintained on slants of blood agar (TSB + 1.5% agar + 5% sterile citrated sheep's blood, pH 7.3). Blood agar was chosen because its rich nutrient base supports luxurient growth. Slants were incubated overnight at 37 C. and then stored at 4 C. Cultures to be used for lawns were obtained by inoculating TSB from a stock slant; cultures were incubated overnight at 37 C. with aeration and then stored at 4 C. until needed; maximum storage time was 3 months. Phage lysates were produced in TSB by methods described in 3c; host debris and unlysed cells were removed by passage through a millipore filter (pore size 0.45 um). The lysates were stored at 4 C.

3. Preparation of test cultures

a) Adsorption. The host propagator strain was grown in TSB + $CaCl_2$ to a turbidity reading corresponding to 1 - 2x 10^8 colony forming units (cfu)/ml (25 - 50 Klett units on a Klett photoelectric colorimeter). Phage, pre-titered by the method discussed below, was added to the cultures at MOI's (multiplicity of infection, or ratio of phages to cells) of 0.1, 0.5, and 1.0. MOI values were chosen at random, but were kept low in accordance with recommendations of Rountree (39) and Lovett (29). Samples were withdrawn at varying intervals and diluted in TSB. The subsequent dilutions were filtered to remove host cells and then further diluted for titration.

Filtering was done with a millipore filter, pore size 0.45 um, in a Swinney filter holder on a hypodermic syringe. Controls were performed to demonstrate that phage titers were not decreased by filtration.

Phage was titered by suspending 0.1 ml of a dilution of phage and 0.1 ml of a lawn culture of the indicator organism (S. aureus 47) in 3.0 ml of soft agar at 45 C. The soft agar was overlaid on hard agar (3) and incubated overnight at 37 C. Plaques were circular and clear, averaging 0.5 - 1.0 mm in diameter. Plates were made in duplicate, and the plaque counts were averaged; the average times the reciprocal of the dilution factor gave the titer of phage/ml.

No data were available on optimum temperatures for infection by staphylophage 47; consequently, it was decided to test two temperatures previously used (6, 39). The recommended temperature (chosen for convenience) was 37 C. (6), but some workers (39) report better phage growth at 22 C.

b) Preparation of antiserum. Antiphage 47 antiserum was prepared in a New Zealand White rabbit over a period of 3 months. The antigen was staphylophage 47 pelleted from a broth lysate. Host debris was removed by millipore filtration and the phage was then pelleted by centrifugation at 59,434 xg for 2 hours in a Beckman Type 30 rotor (Beckman Model L Preparative Ultracentrifuge). The phage pellet was suspended in physiological saline (0.85% NaCl) and diluted with saline to reach a titer of 10^{10} plaque-forming units (pfu)/ml. The rabbit was

inoculated with 0.5 ml of the preparation, 5 x 10^9 pfu (18), via the marginal ear vein. Two initial injections of 0.1 ml each were made to test for adverse responses; none occurred and subsequent 0.5 ml injections were made biweekly (11). The animal was bled periodically from the marginal vein of the unused ear. Serum from the final bleeding was titered and used for all subsequent experiments.

Approximately 50 ml of blood were collected and allowed to clot at 37 C. for 1 hour; the clot was then quartered and the sample was stored overnight at 4 C. The serum expressed from the clot was centrifuged at low speed (5000 x g, 5 min., Sorvall centrifuge) to remove remaining red blood cells.

Aliquots of the serum sample were diluted 1:100 and 1:1000 in TSB and tested (3) for ability to inactivate phage 47. A 0.9 ml sample of each dilution was mixed with 0.1 ml of phage of known titer. The mixtures were incubated at 37 C. Samples were withdrawn at intervals and titered by the soft agar overlay method (3). Decreases in phage titer indicated antiphage antibody activity, since serum-free controls showed no decreases. Normal serum showed very little antiphage activity (data not shown).

c) Growth curves. Propagator strain 47 was grown in TSB + $CaCl_2$ to 3 different cell concentrations: 1 x 10^8 cfu/ml, 2 x 10^8 cfu/ml, and 4 x 10^8 cfu/ml (as determined from a Klett colorimeter). The cell concentrations corresponded to cells in early, middle, and late log phase, respectively. Cultures of

varying ages were tested because, although accepted procedures (6) used relatively old cultures, there were reports (19, 39) of young cells enhancing phage growth. Phage 47 was added at MOI's of either 1.0 or 3.0. Infection was allowed to proceed at either 37 C. or 22 C. (or was started at 22 C. and shifted to 37 C. after 30 minutes). (Preliminary studies indicated 30 minutes to be the optimal adsorption time.) MOI and temperature were varied as a check on results reported above. Cultures were monitored for changes in turbidity and samples were withdrawn periodically for plaque-titering. After infection had proceeded for 4 hours, cultures were either left at the test temperature or were shifted to 4 C. (All combinations of time and temperature were tested.) A final check of turbidity and titer was made at 24 hours post-infection.

After optimal test conditions had been derived, growth curves were repeated to test for possible differences when unadsorbed phage was removed by centrifugation, filtration, or use of antiserum. The infection process was also tested with and without aeration to see if aeration increased the phage yield, as had been earlier reported (39).

When free phage was removed by antiserum, a 0.1 ml aliquot of phage of known titer was added to 0.9 ml of a host culture at an MOI of 1.0. (Adsorption studies indicated 1.0 to be the optimal MOI.) One-step growth curves were carried out by the method of Adams (3). Infection proceeded for varying times and then 0.1 ml of the mixture was added to 0.9 ml of antiserum

(1:100 dilution). Inactivation of free phage proceeded for varying times and then dilutions were made in TSB. Samples were removed and titered by standard methods (3).

B. Inactivation

1. Inactivation assays

a) Long term. Three organic solvents were tested for ability to inactivate staphylophage 47. Tests were run in TSB (pH 7.3) at 22 C. over a period of 1 hour. The input phage was of known titer; reduction of titer was interpreted as phage inactivation. Chloroform and ethyl ether were tested at concentrations of 10.0%, 1.0%, and 0.1% (v/v in TSB). Concentrations were chosen at random. Deoxycholic acid (DOC, Difco) was tested at random concentrations limited by its viscosity; it was tested at 2.0%, 0.5%, and 0.1% (v/v). Samples were withdrawn from the test cultures at intervals, diluted in TSB, and titered by standard methods.

b) Short term. Preliminary experiments indicated that 10 minutes was a sufficient test period for phage inactivation when inactivating agents were at a concentration of 1.0% (v/v). A number of agents were then tested for ability to inactivate phage 47 at 22 C. (1.0% solution in TSB) over a 10 minute period. The agents are listed in Table I. Samples were removed at the beginning and at the end of the test period and were titered by aforementioned methods.

TABLE I

AGENTS TESTED FOR ABILITY TO INACTIVATE STAPHYLOPHAGE 47

Agent*	Classification	Comments
TSB (BBL)	Broth	Control, pH 7.3
Ethanol	Alcohol	
Methanol	Alcohol	
Butanol	Alcohol	
Tween-80	Detergent	Surface-active
Nonidet-NP-40 (Shell Oil Co.)	Detergent	Surface-active
Chloroform	Organic Solvent	
Ethyl Ether	Organic Solvent	
Acetone	Organic Solvent	
DOC (Difco)	Organic Solvent	
Toluene	Organic Solvent	
Phosphate-buffered Saline (PBS)	Buffer	0.8% NaCl, 0.1 M K_2HPO_4 0.1 M KH_2PO_4, pH 7.4
Reticulocyte Swelling Buffer (RSB)	Buffer	0.01 M Tris, 0.01 M NaCl 0.0015 M $MgCl_2$, pH 7.4
TRIS (tris (hydroxymethyl) aminomethane) (Sigma)	Buffer	1 M, pH 7.2
TMA-1	Buffer	0.01 M Tris, 0.001 M $MgCl_2$ 0.03 M NH_4Cl, 0.06 M mercaptoethanol, pH 7.3
TMK-10	Buffer	0.01 M Tris, 0.01 M $MgCl_2$ 0.05 M KCl, pH 7.2

*Except where indicated, all agents used were purchased from Fisher Chemical Co.

2. Density gradient analyses

The homogeneity of phage 47 before and after solvent extraction was examined by the use of CaCl density gradients. The propagator strain was either prelabeled with radioisotope (isotope added 2 hours prior to infection, with cells washed, millipore filtered, and suspended in unlabeled medium before infection) or was colabeled (radioisotope added concurrent with infection). The isotope used was either methyl-^{3}H-thymidine at a level of 0.5 mC/ml or U-^{14}C-glucose at a level of 0.1 mC/ml. The lysates were centrifuged at 10,000 x g for 10 minutes at 4 C. to remove debris. Lysates were then spun at 59,434 x g (Beckman Model L Preparative Ultracentrifuge, Type 30 rotor) for 2 hours to pellet the phage. Pellets were resuspended by overnight elution in phosphate-buffered saline (PBS, pH 7.2) at 4 C. Samples of resuspended phage were then applied to preformed CaCl density gradients.

A stock CaCl solution with a density of 1.6 g/cc was diluted in PBS to densities of 1.50, 1.48, 1.45, 1.40, and 1.35 g/cc. Gradients were built in 6 steps (1.60 g/cc to 1.35 g/cc) of 0.5 ml per step. Samples were centrifuged in the SW 50 L rotor at 134,211 x g for 4 hours. The tubes were pierced at the bottom and 0.1 ml fractions were collected. Fractions were diluted to 1.0 ml with PBS and aliquots were removed for further testing. For each fraction, a plaque titer was done to check for viable phage; optical density (OD) was read at 260 nm (Gilford Spectrophotometer) to check for

bacteriophage DNA. In addition, each fraction was checked for presence of radioisotopes by scintillation counting (Beckman LS-150). Aliquots were suspended in 3 ml of scintillation cocktail. Per liter, scintillation cocktail consisted of 5.5 g Permablend II (Packard Instruments), 667 ml toluene, and 333 ml Triton X-100 (Packard Instruments; especially purified for scintillation counting).

Fractions containing phage (as determined from plaque titers, OD readings, and scintillation counting) were pooled and dialyzed against PBS (24 hours at 4 C.) to remove the CsCl. Half of the pooled sample was treated with chloroform (5% v/v for 5 minutes, determined in earlier experiments to be sufficient for phage inactivation; see III.A.). Gradients were prepared as described above. Samples applied to the gradients consisted of untreated phage, chloroform-treated phage, or a mixture of treated and untreated phage. The mixed gradient was run to see if treated and untreated phage could be separated in a single gradient. Centrifugation for all gradients was as described above.

The refractive indices of the phage-containing fractions, both treated and untreated, were determined from a refractometer (Bausch and Lomb). The refractive indices were then converted to buoyant densities (26) and corrected for the density of CaCl.

Aliquots of the fractions containing treated or untreated phage were also pooled and dialyzed as above. Samples of the 2 phage pools were mixed with equal volumes of 2% phosphotungstic

acid, mounted on grids, and viewed through an electron microscope (RCA, EMU-4). Samples were prepared and electron micrographs made by Mr. Scott Martin, Division of Biology, Kansas State University.

C. Lipid Analysis

1. Extraction (15)

Staphylophage was extracted by 3 methods: 1. chloroform-methanol, 3:1 v/v; 2. acetone followed by hot ethanol; and 3. methanol containing 10% HCl (v/v). In each case the phage sample was obtained from a pool of dialyzed CsCl fractions and was mixed with an equal volume of the extracting solvents. All extracts were evaporated by suction to near dryness and were stored at 4 C.

2. Chromatography

a) Thin layer. Phage extracts were spotted on TLC sheets (previously activated at 90 C. for 30 minutes) and then chromatographed in the solvent systems listed in Table II. System 1 was for polar lipids and 2 was for non-polar lipids (15). Systems 3 and 4 were those used for the lipid-containing phage PM2 (2, 20, 27). Systems 5 and 6 have been used (50) for analysis of the lipids of Staphylococcus aureus. System 7 was devised by the author specifically for use in analysis of staplylophage 47 and of lysostaphin-treated cells of S. aureus 47. Chromatograms were left in the test solvents until the

TABLE II

THIN LAYER CHROMATOGRAPHY OF STAPHYLOPHAGE 47: SOLVENT SYSTEMS

	Systems	Ratio (v/v)	Comments
1.	chloroform-methanol-acetic acid-water	250:90:19:20	polar
2.	hexane-ethyl ether-acetic acid	60:40:1	non-polar
3.	chloroform-methanol-acetic acid-water	250:74:19:3	Pseudomonas phage PM2
4.	petroleum ether-ethyl ether-acetic acid	85:15:1	Pseudomonas phage Pm2
5.	chloroform-methanol-pyridine-water	30:15:1:3	Staph. aureus
6.	chloroform-methanol-acetic acid-water	45:7:3.15:0.5	Staph. aureus
7.	chloroform-methanol-acetone-water	250:100:8:10	Staph. aureus phage 47

solvent front had passed beyond the upper edge of the sheet; sheets were then dried thoroughly at room temperature. For location of lipids, chromatograms were sprayed with Sudan black (0.3% in methanol) to look for black (lipid) spots. Alternatively, chromatograms were exposed to I_2 vapor in a closed jar to look for white and brown spots (see Discussion section).

2. Gas chromatography

Crude acid-hydrolyzed extracts (10% HCl in methanol) of phage 47 and of its host were evaporated to dryness under a stream of N_2 (not oxygen-free). The samples were then dissolved in hexane and applied to an 8 foot column of diethylene glycol succinate (DEGS) in a gas chromatograph (Barber-Colman Model 5000). The column was 7.5% DEGS on 60/80 mesh Chromasorb G (Barber-Colman). The inlet temperature was 225 C.; column temperature was 190 C.; the carrier gas was argon.

IV. RESULTS AND DISCUSSION

Since Staphylococcus sureus, strain 47 is so frequently implicated in cases of food poisoning (19), the production of staphylophage 47 in bulk lysates which remain stable over long periods of time is an essential prerequisite for typing at minimum cost and maximum accuracy. The current recommended (6) procedure for the production of phage 47 is as follows: host cells are in stationary phase; infection takes place in soft agar; the temperature throughout infection and lysis is 37 C; the MOI is 3 - 4. It had been earlier noted (19, 39), though, that some phages multiplied more rapidly in younger host cells or (39) when a temperature of 22 C. was used. Furthermore, aeration was also thought (38, 39) to aid adsorption.

A. Adsorption Studies

Very little information is available that deals specifically with the adsorption and growth of phage 47. The standard propagation procedures (6) for staphylophages had been developed for other phages; therefore, these standard conditions are not necessarily optimum for staphylophages, and, consequently, it was decided to carefully study propagation, including the adsorption of phage 47 under varying conditions. Host cells were relatively young, mid-log phase (2×10^8 cfu/ml). Various MOI's were tested (0.1, 0.5, and 1.0); the values were selected at random. Adsorption was monitored at 22 C and at

37 C., with and without aeration.

Figure 2 shows selected results from adsorption studies. Adsorption of phage 47 to host strain 47 proceeded rapidly and efficiently in broth when the MOI was 1.0, the temperature was 22 C., and the culture was aerated. The MOI of 1.0 resulted in more phage being adsorbed at either temperature than did the lower MOI's. At an MOI of 1.0, greater than 99% of the input phage adsorbed to the host within 30 minutes when the temperature was 22 C., while 90% adsorbed in 60 minutes at 37 C. It has been reported (6, 39) that host staplylococci can overgrow phages. Perhaps lowering the temperature from 37 C. to 22 C. during adsorption might retard host growth and permit infection to become better established. Non-aerated cultures became quite viscous and adsorbed essentially no phage at either temperature (when monitored by techniques developed for this study). No explanation has been offered for the aeration requirement, and the reason for viscosity is not known. It is possible, though, that the viscous material masks attachment sites on the cells and/or phages and prevents establishment of infection.

B. Growth Curves

The above studies were extended to cover the entire course of phage infection. Experimental conditions were varied as stated in the Materials and Methods section. Early attempts to use BHI broth in addition to TSB were abandoned. BHI is

EXPLANATION OF PLATE I

Fig. 2. Adsorption of phage 47.

——— MOI 1.0, 22°C.

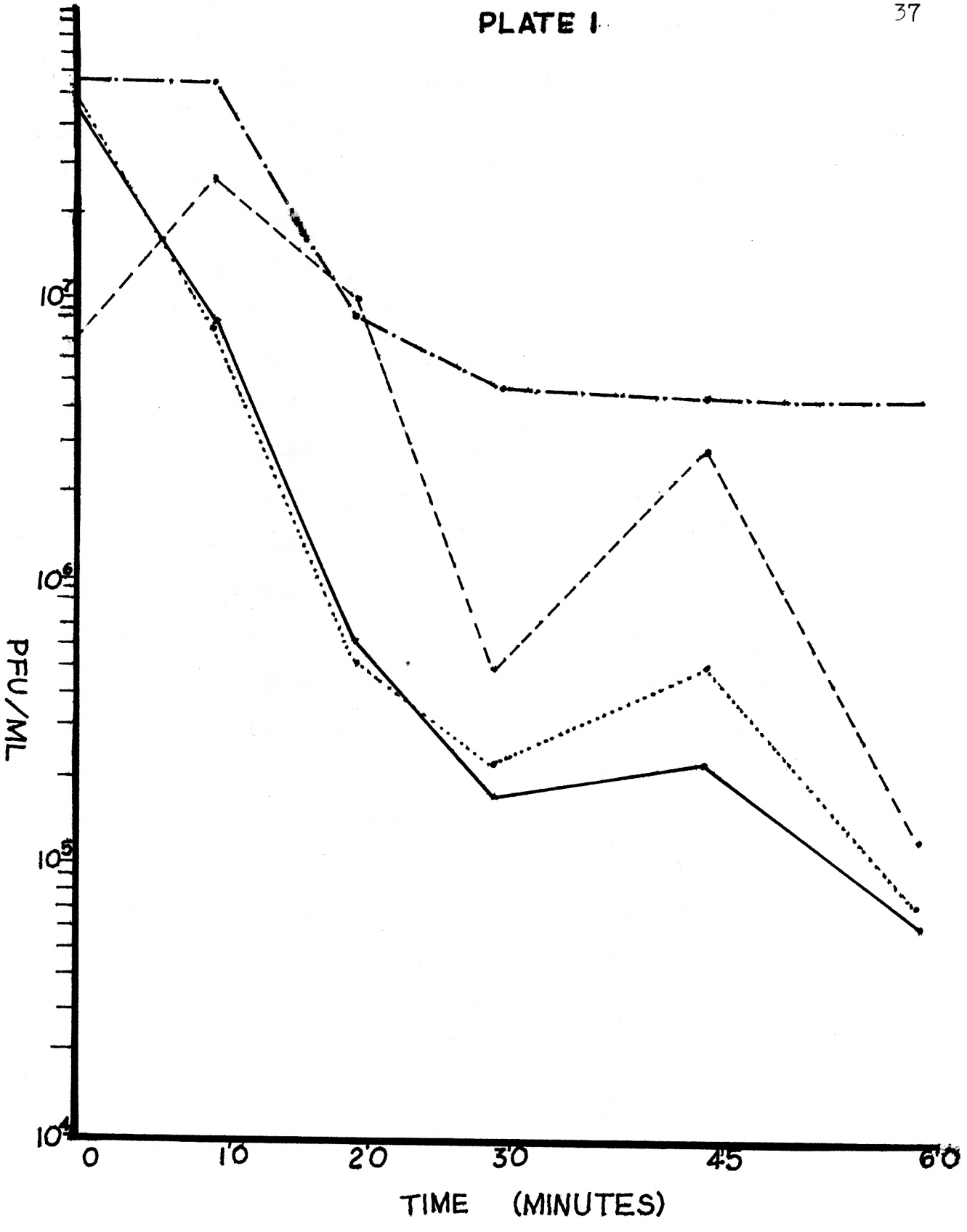
..... MOI 0.5, 22°C.

- - - - MOI 0.1, 22°C.

—.—. MOI 1.0, 37°C.

Cultures were infected at the MOI's and temperatures stated above. Pfu/ml values were determined from plaque titers.

PLATE I



a very rich medium and promoted host growth at so rapid a rate that phages were probably overgrown; BHI cultures also became extremely viscous, further reducing phage titer. Consequently, all data discussed below were taken from tests run in TSB.

Figure 3 shows a growth curve generated by conditions determined in this study to be optimal for the growth of phage 47. Optimal conditions were as follows: the host culture was grown to 1 x 10^8 cfu/ml (early log phage); MOI was 1.0; the culture was aerated; to promote adsorption the temperature was initially 22 C., then shifted to 37 C. after 30 minutes, and finally to 4 C. after 4 hours. None of the curves generated by other combinations of the variables (time, temperature, MOI, cell age, aeration) showed so large a percentage of input phage adsorbed or so great a final phage titer as in Figure 3. As in the adsorption studies, aeration was essential; the process was essentially complete after 30 minutes; and the most efficient MOI was 1.0. It is possible that, for phage 47, MOI's less than 1.0 do not allow infection of enough cells for the titer of progeny phage to be very dramatic; MOI's greater than 1.0 might result in interference phenomena, again resulting in low titer. Host cells younger than those used in the adsorption studies yielded highest titers of progeny phage (10^{10}). The cells were in very early log phase; cells in mid-log phase gave lower titers (10^8 - 10^9); cells in late log-early stationary phase gave lower titers yet (10^8). This was in accordance with earlier studies (19, 39), but did make use of host cultures

EXPLANATION OF PLATE II

Fig. 3. Growth of phage 47.

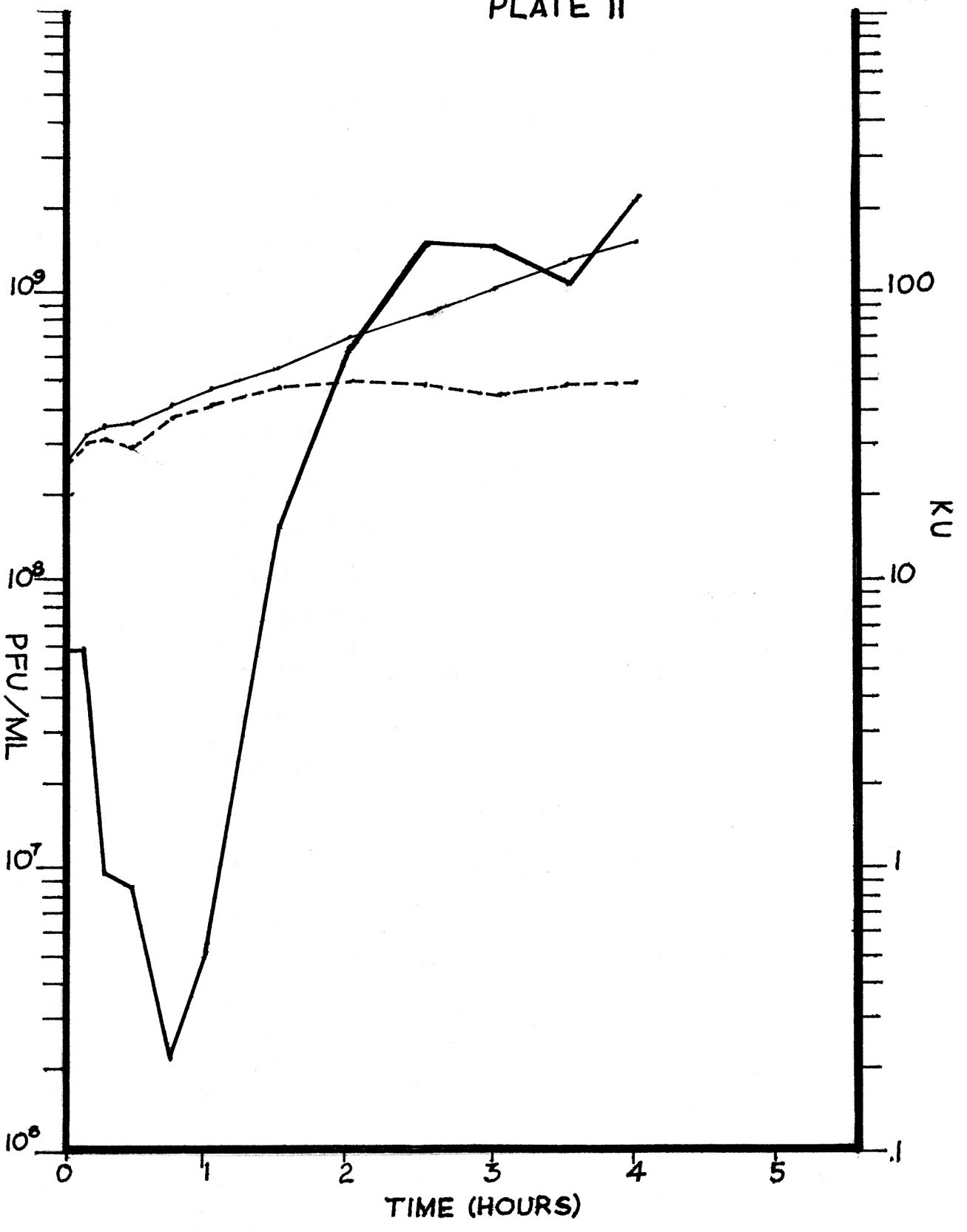
——— Titer of phage 47.

____ Turbidity of uninfected culture.

_ _ _ Turbidity of infected culture.

The culture was infected at an MOI of 1.0 at 22°C. The temperature was shifted to 37°C. at 30 minutes, and then to 4°C. at 4 hours post-infection. The final pfu at 24 hours was 2.4×10^{10}, representing a burst of 240 particles/cell.

PLATE II



even younger than those used in those studies. The cultures were much younger than those used in the standard recommended propagation procedure (6), which are well into stationary phase. The titer at 4 hours post-infection was highest if infection was started at 22 C. and then shifted to 37 C. at the end of the 30 minutes allowed for adsorption. The results confirmed those of the adsorption study and were similar to results of some earlier studies (39). If cultures were left at 22 C. throughout the test period, the titer was low; this may have been due to retardation of host growth to the extent that phage could not be rapidly synthesized (19). If the cultures were started at 37 C., adsorption was not efficient. If cultures were maintained at 37 C., host overgrowth obscured any phage produced, presumably by readsorbing new phage as soon as they were released (39). Host overgrowth has not been thought (39) to reflect host resistance, but rather a more rapid production of progeny cells than progeny phage. Overgrowth was avoided by either ending the tests at 4 hours post-infection or by shifting cultures to 4 C. at that time; shifting was done at 4 hours because that was the point of minimum turbidity of the cultures.

Under optimized conditions, the titer at post-infection was invariably on the order of 10^9 pfu/ml. The titer represented a burst size of approximately 20 phage particles per infected cell, and was not unusual for staphylophages (29, 39). Notably such titers were 4 orders of magnitude greater than the 10^5

pfu/ml yielded by 18-hour lysates produced by currently recommended methods (6). If lysis was permitted to continue at 4 C. (to minimize host overgrowth) until 24 hours post-infection, titers invariably rose to the order of 10^{10} pfu/ml. A titer of 10^{10} pfu/ml represented a burst of 240 particles per originally infected cell, roughly 2.5 times the maximum (100 particles) reported by other workers (29, 39). This titer was 5 orders of magnitude (or 100,000 times) greater than the standard (6) yield.

Even at 4 C., where lysis was much more complete, cultures never entirely cleared. Failure to clear cultures has been reported (39) for staphylophages. The final turbidity readings were usually very low and may have represented non-solubilized cell debris.

Both lysates with a titer of 10^{10} pfu/ml could be produced in bulk. Volumes of 2 liters were found to be convenient. When stored at 4 C., they remained stable in titer as long as they were kept (1 year to date). The soft agar process recommended (6) for phage 47 was found by its investigators to be time-consuming and it invited contamination. Only a few hundred milliliters of phage lysate could be produced in soft agar and lysates did not remain particularly stable in storage. Soft agar lysates yielded considerably fewer phage than the broth lysates made for this study. Finally, antigen preparation made for this study from soft agar lysates proved to be unsatisfactory for the experiments here reported because of the

difficulty in removing agar particles (and presumably host toxins) from the injection stock. Use of a soft-agar lysate antigen resulted in the loss of two rabbits, both of which displayed symptoms of staphylococcal poisoning. Agar particles could not be removed, even by filtration and centrifugation. Antigen preparations made from broth lysates seemed less likely to harm the animals used to produce antiserum.

The growth curve done under optimal conditions (Figure 3) showed a drop in phage titer over the adsorption period followed by a rise which remained essentially steady throughout the course of the experiment. Classic growth curves (3) of the E. coli/T-phage systems have shown a constant titer during adsorption and phage synthesis (latent period) followed by a very rapid rise (burst) during lysis and ending in a plateau. Under optimal conditions attempts were made to generate classic curves by removal of unadsorbed phage, in the event that free phage were obscuring the true phage. Cultures were infected and 30 minutes was allowed for adsorption. Infected cells were isolated by millipore filtration (pore size 0.45 um) or by centrifugation at 10,000 x g (Sorvall centrifuge) for 10 minutes. Free phage was left in the filtrate and the supernatant. Infected cells were resuspended in fresh medium and the lytic process was allowed to proceed. The resulting growth curves showed no essential differences from Figure 3. Apparently staphylophage 47 growth curves were not obscured by the presence of free phage.

As a check of the above tests, one-step growth curves (3) were run with antiserum used to inactivate free phage. Homologous antiserum to staphylophage 47 was prepared as described in Materials and Methods. The ability of the antiserum to inactivate phage 47 is summarized in Table III. After only 5 minutes of exposure to the antiserum (1:100 dilution), 98% of the free phage had been inactivated. This represented a more effective antiserum than that used by other workers (9, 38, 39). When growth curves were run using this antiserum (at 1:100 dilution, 5 minute exposure), essentially no differences from Figure 3 were seen. Neither the classic latent period nor a sharp burst could be generated.

It has been suggested (39, 49) that staphylophages may vary widely in their ability to penetrate the host, replicate, and lyse the cell wall. Variations, possibly age-dependent, have also been thought to occur (19) in the host cell's ability to synthesize phage, i.e. the older the cell, the fewer phages produced. In work with phages other than 47, Rountree (39) produced growth curves with a latent period and a burst, but her methods did not yield a final titer as high as those derived for this study and may not have permitted maximum contact between phage particles and host cell attachment sites (see discussion of adsorption). It is possible that a single cell phage-host system might have a true latent period and a clear burst. In multiple cell systems, however, variations in phages and hosts would cause individual curves to overlap.

TABLE III

INACTIVATION OF PHAGE 47 BY HOMOLOGOUS ANTISERUM

Time	Titer of phage stock = 1.2×10^7 pfu/ml	% inactivation
	titer, antiserum diluted 1:100	1:100
0 minutes	1.2×10^7	0
5 minutes	2.8×10^5	97.7
10 minutes	2.0×10^4	99.8
15 minutes	1.0×10^4	99.9
30 minutes	0	100.0
60 minutes	0	100.0
	titer, antiserum diluted 1:1000	1:1000
0 minutes	1.2×10^7	0
5 minutes	7.8×10^6	35.0
10 minutes	7.4×10^6	63.0
15 minutes	3.0×10^6	75,0
30 minutes	7.0×10^5	94.0
60 minutes	0	100.0

At any given time some hosts would be adsorbing input phage, some would be lysing. The curve for the phage population as a whole, then, could show neither a latent period nor a sharp burst. Another possibility, not yet studied, is that the lytic phenomena of phage 47 may resemble those of certain animal viruses. The myxoviruses and arboviruses do not show a burst (30); they are released from infected cells over a long period of time. The slow leakage of these virus particles may somehow be due in part to the lipid envelopes acquired as the virus particles leave the host.

C. Inactivation

In order to avoid contamination with extraneous airborne bacteria, several phage lysates used for inoculating stocks were treated with chloroform prior to storage. No viable phage could be recovered from treated lysates. This apparent inactivation of phage 47 was examined further by monitoring the titers of lysates exposed to various concentrations of chloroform over a period of time. The results are shown in Figure 4. Inactivation was rapid and extensive. Phage titer dropped dramatically within the first few minutes after the chloroform treatment and then leveled off. A concentration as low as 1% could inactivate nearly 90% of the input phage in as little as 10 minutes. Evaporation of the chloroform did not restore the phage titer; therefore, inactivation appeared irreversible. There is one brief report (28) of chloroform inactivation of a staphylococcal

EXPLANATION OF PLATE III

Fig. 4. Survival of chloroform-treated phage.

_ _ _ 10.0% chloroform.

▬▬ 1.0% "

_____ 0.1% "

Tests were conducted in TSB at 22°C. Cultures were aerated. Percent survival was determined by withdrawing samples at intervals and comparing the plaque titer to the titer recorded at the outset of the experiment.

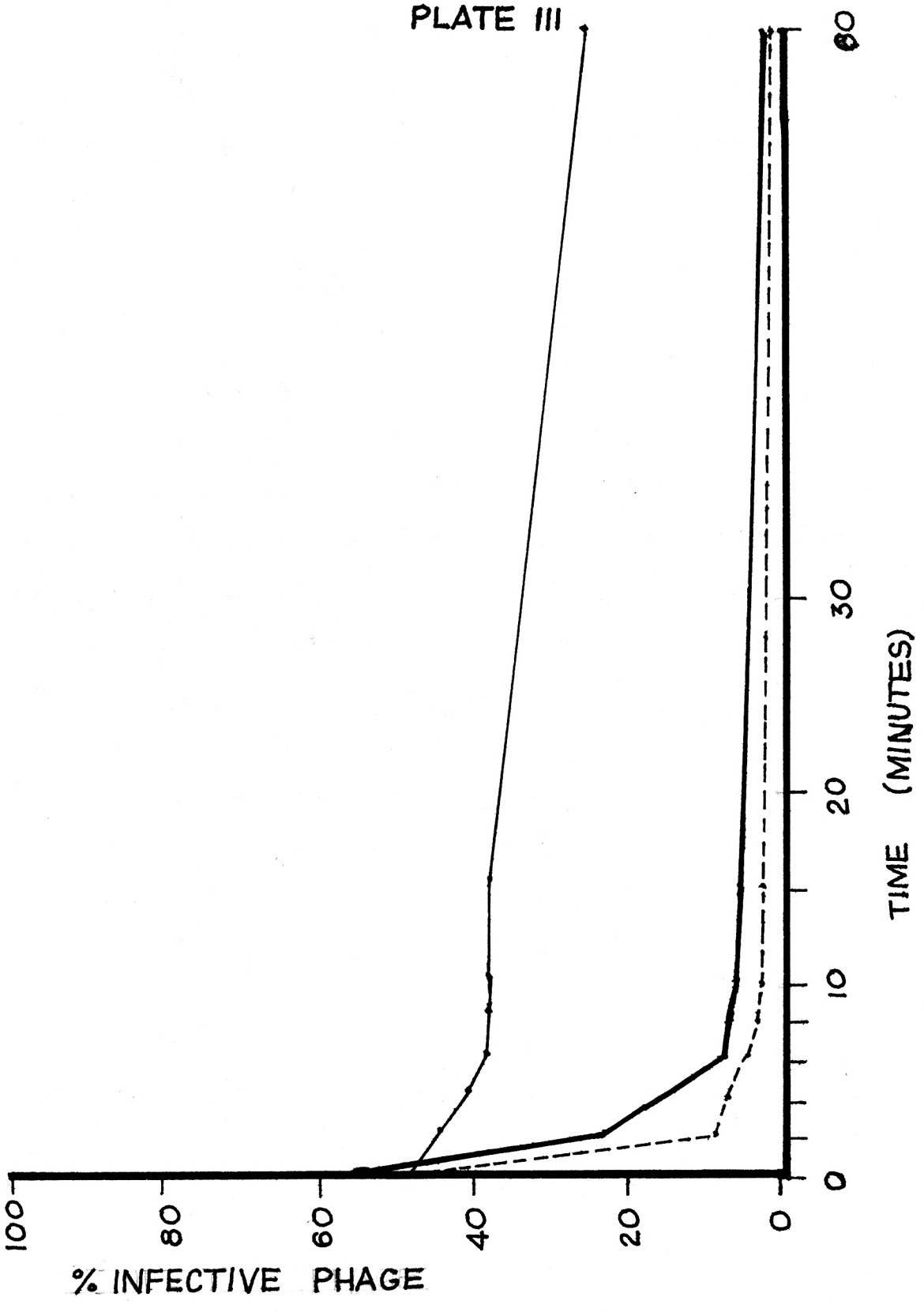


PLATE III
100
80
60
40
20
0
% INFECTIVE PHAGE
0
10
20
30
60
TIME (MINUTES)

phage (type unknown); but no study of inactivation has ever been made prior to the one herein reported. Chloroform is frequently used (15) to extract lipids; consequently, it was decided to treat phage 47 with other lipid solvents to see if they would also cause inactivation. Results were nearly identical when ethyl ether was used; DOC was approximately 25% as effective for inactivation as the other two compounds (Figure 5).

The test conditions were then standardized (see Materials and Methods), and a variety of agents (Table I) were tested for ability to inactivate phage 47. The results are depicted in Figure 5. Broth (Control, TSB) and the buffers (PBS and RSB, checked for possible ion effects) had no effect. TRIS (TRIS hydroxymethylaminomethane) buffer caused 20% inactivation, which was offset by the addition of various ions, primarily magnesium, via TMA-1 and TMK-10 buffers. Phage 47 might thus have a magnesium requirement, in addition to a known (6) calcium requirement, for infectivity. Detergents (TWEEN-80 and NP-40) did not affect phage 47. Of the alcohols, ethanol and methanol caused no inactivation; butanol caused roughly 25% inactivation. Inactivation by other organic solvents as follows: toluene, essentially none; acetone, roughly 25%; DOC, 40%. Again, inactivation by ether and chloroform was marked: 85% for the former, 90% for the latter.

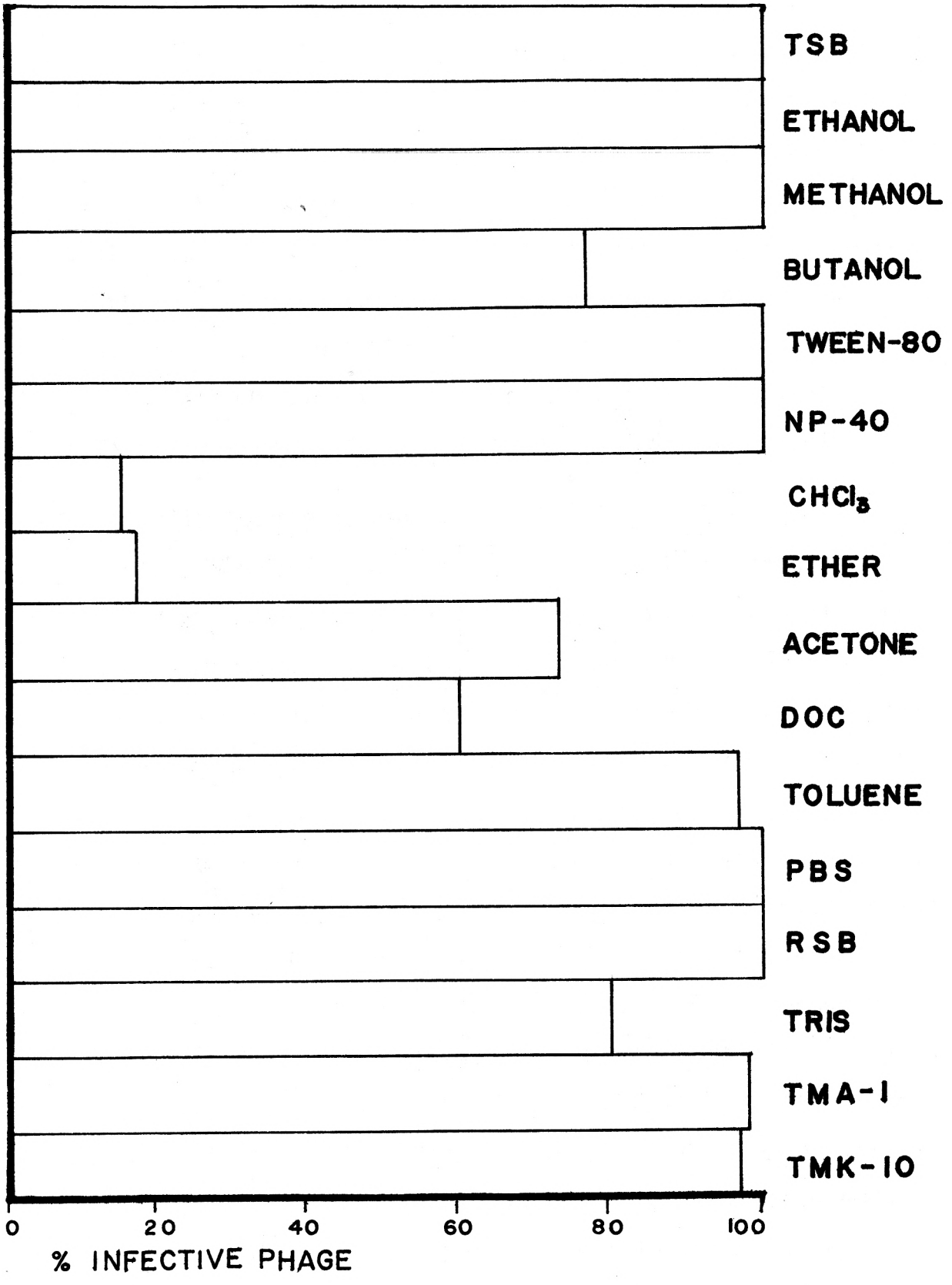
The inactivating agents should have no effect upon the protein coat of phage 47 at the 1% test concentration--if the coat were solely protein, as has been reported (19). There is

EXPLANATION OF PLATE IV

Fig. 5. Survival of phage 47.

All agents were in 1.0% solution in TSB. The tests were conducted at 22°C. with a duration of 10 minutes. Survival was determined by comparing endpoint plaque titer to initial titer.

PLATE IV


TSB
ETHANOL
METHANOL
BUTANOL
TWEEN-80
NP-40
CHCl3
ETHER
ACETONE
DOC
TOLUENE
PBS
RSB
TRIS
TMA-1
TMK-10
0
20
40
60
80
100
% INFECTIVE PHAGE

a report (Buller, personal communication) of partial chloroform denaturization of a streptococcal phage. Uncharged areas of the phage protein coat were affected by chloroform, but only when the phage was mixed with undiluted chloroform. It is highly unlikely that the marked inactivation reported here occurring in such dilute solutions of chloroform is due solely to alteration of uncharged areas in the protein coat of phage 47. It is more likely that other uncharged compounds, e.g., lipids, are present in phage 47 in addition to protein.

D. Density Gradients Analyses

As aforementioned, density gradients were used as a check of phage homogeneity. The gradients exhibited two visible bands. One band was located in the center of the gradient and corresponded roughly to fraction 15; the other occurred near the top of the gradient, roughly at fraction 25. Gradients were tested for plaque titer, OD, and radioactivity. Graphs (Figures 6, 7, and 8) of titer and OD consistently showed that both definitely peaked in fractions 15 and 16. Occasionally some infective phage or absorbance, but not both, would appear at the very top of the gradient. No true peak ever appeared at the top (fraction 25). It is most likely that the upper band and OD at the top of the gradient represented host and phage debris. The infective phage at the top of the gradient may have been small numbers of normal phage associated with host debris; it is remotely possible that they were

EXPLANATION OF PLATE V

Fig. 6A. Density gradient analysis of ^{3}H-prelabeled phage 47.

——— pfu

▬▬▬ cpm

- - - - OD (A_{260})

Label was added two hours prior to infection. Debris were removed from the lysate. Phages were pelleted, resuspended, and applied to CsCl density gradients. Fractions were collected and analyzed as indicated above. See text for details on centrifugation.

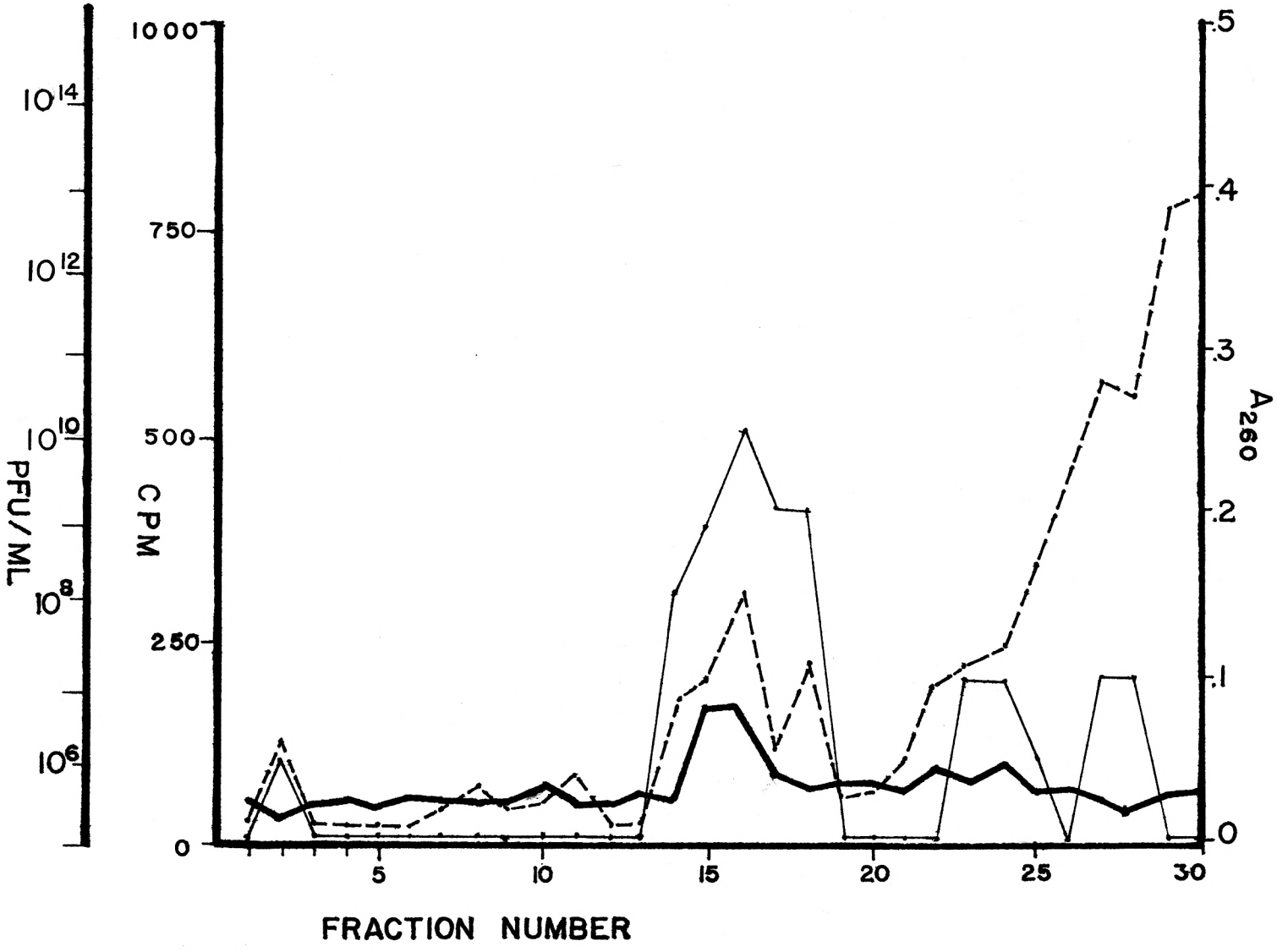

PFU/ML
CPM
A_{260}
FRACTION NUMBER
10^{14}
10^{12}
10^{10}
10^{8}
10^{6}
1000
750
500
250
0
.5
.4
.3
.2
.1
0
5
10
15
20
25
30

PLATE V

EXPLANATION OF PLATE VI

Fig. 6B. Density gradient analysis of ^{3}H-colabeled phage 47.

——— pfu

▬▬▬ cpm

– – – – OD (A_{260})

Label was added concurrent with infection. Debris were removed from the lysates. Phages were pelleted, resuspended, and applied to CsCl density gradients. Fractions were collected and analyzed as indicated above. See text for details on centrifugation.

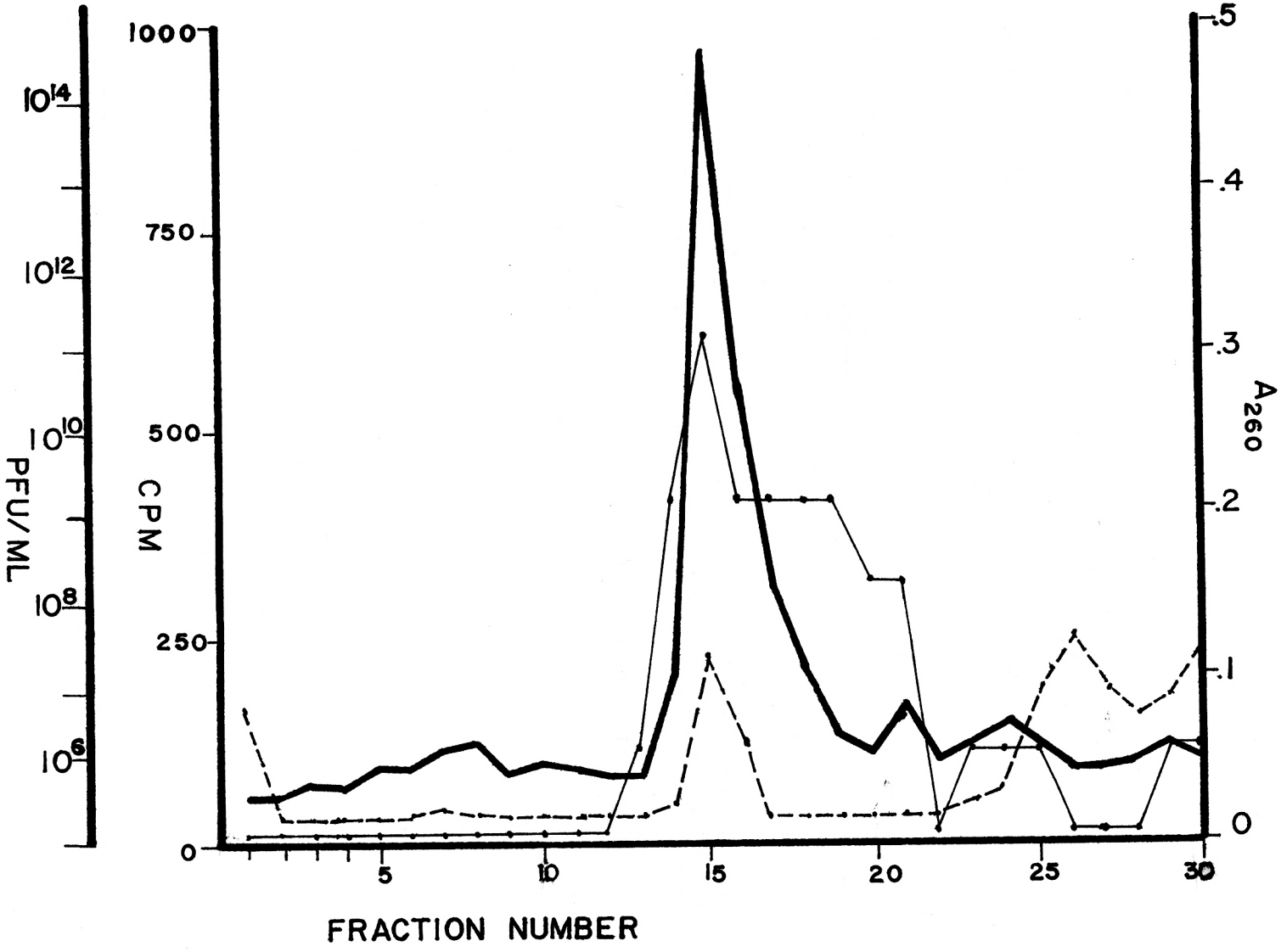

1000
750
500
250
0
CPM
10^{14}
10^{12}
10^{10}
10^{8}
10^{6}
PFU/ML
.5
.4
.3
.2
.1
0
A_{260}
5
10
15
20
25
30
FRACTION NUMBER

PLATE VI

EXPLANATION OF PLATE VII

Fig. 7A. Density gradient analysis of ^{14}C-prelabeled phage 47.

——— pfu

▬▬▬ cpm

- - - - OD (A_{260})

Label was added two hours prior to infection. Debris were removed from the lysates. Phages were pelleted, resuspended, and applied to CsCl density gradients. Fractions were collected and analyzed as indicated above. See text for details on centrifugation.

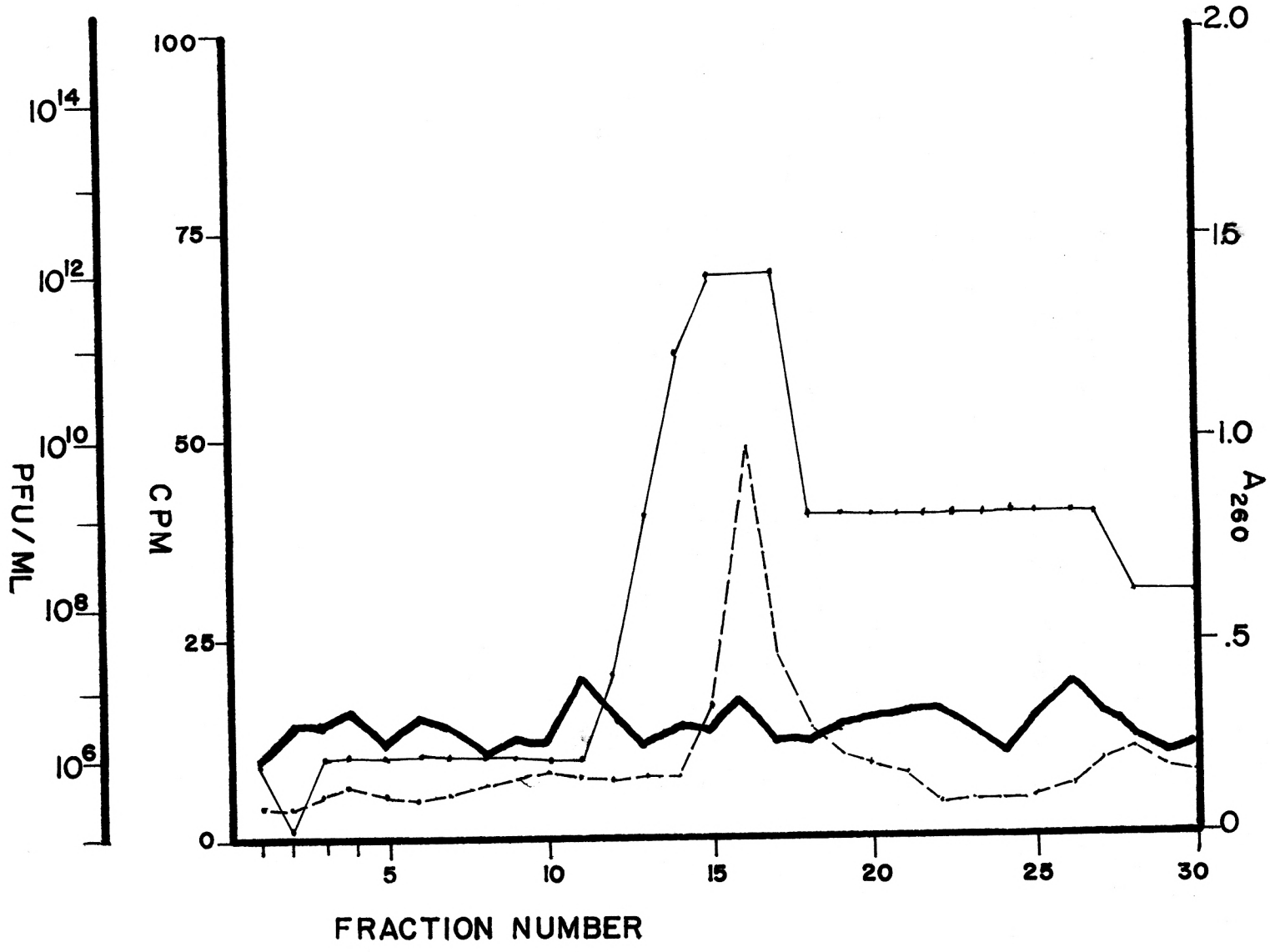

PFU/ML
10^{14}
10^{12}
10^{10}
10^{8}
10^{6}
CPM
100
75
50
25
0
A_{260}
2.0
1.5
1.0
.5
0
5
10
15
20
25
30
FRACTION NUMBER

PLATE VII

EXPLANATION OF PLATE VIII

Fig. 7B. Density gradient analysis of ^{14}C-colabeled phage 47.

——— pfu

▬▬▬ cpm

– – – OD (A_{260})

Label was added concurrent with infection. Debris were removed from the lysates. Phages were pelleted, resuspended, and applied to CsCl density gradients. Fractions were collected and analyzed as indicated above. See text for details on centrifugation.

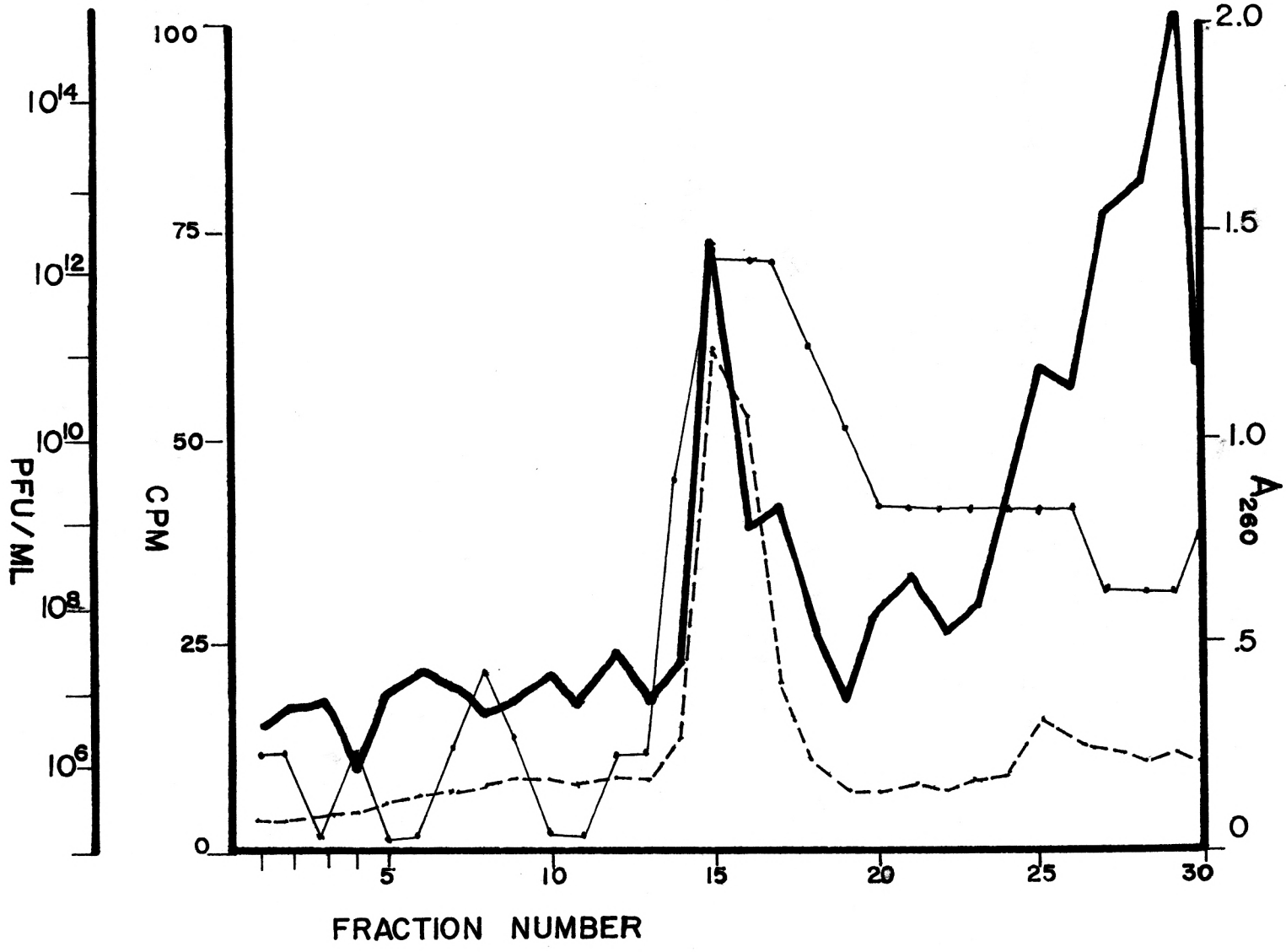

PFU/ML
10^{14}
10^{12}
10^{10}
10^{8}
10^{6}
CPM
100
75
50
25
0
A_{260}
2.0
1.5
1.0
.5
0
5
10
15
20
25
30
FRACTION NUMBER

PLATE VIII

EXPLANATION OF PLATE IX

Fig. 8A. Density gradient analysis of normal phage 47.

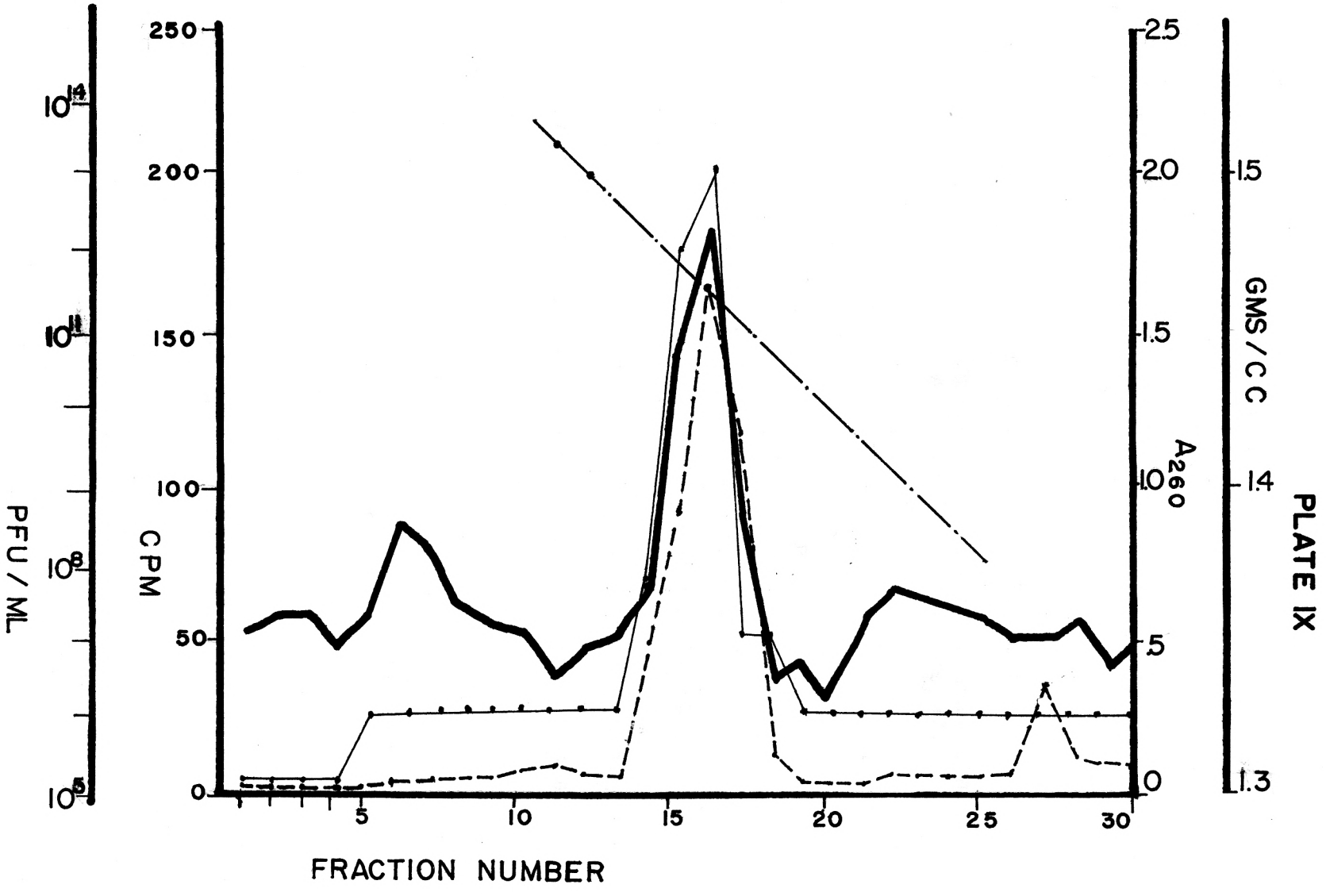
——— pfu

▬▬▬ cpm

- - - - OD (A_{260})

—.—. buoyant density

Phage samples were prepared and analyzed according to standard method detailed in the text.


PFU / ML
CPM
A260
GMS / C C
PLATE IX
FRACTION NUMBER
250
200
150
100
50
0
2.5
2.0
1.5
1.0
.5
0
1.5
1.4
1.3
5
10
15
20
25
30

EXPLANATION OF PLATE X

Fig. 8B. Density gradient analysis of chloroform-treated phage 47.

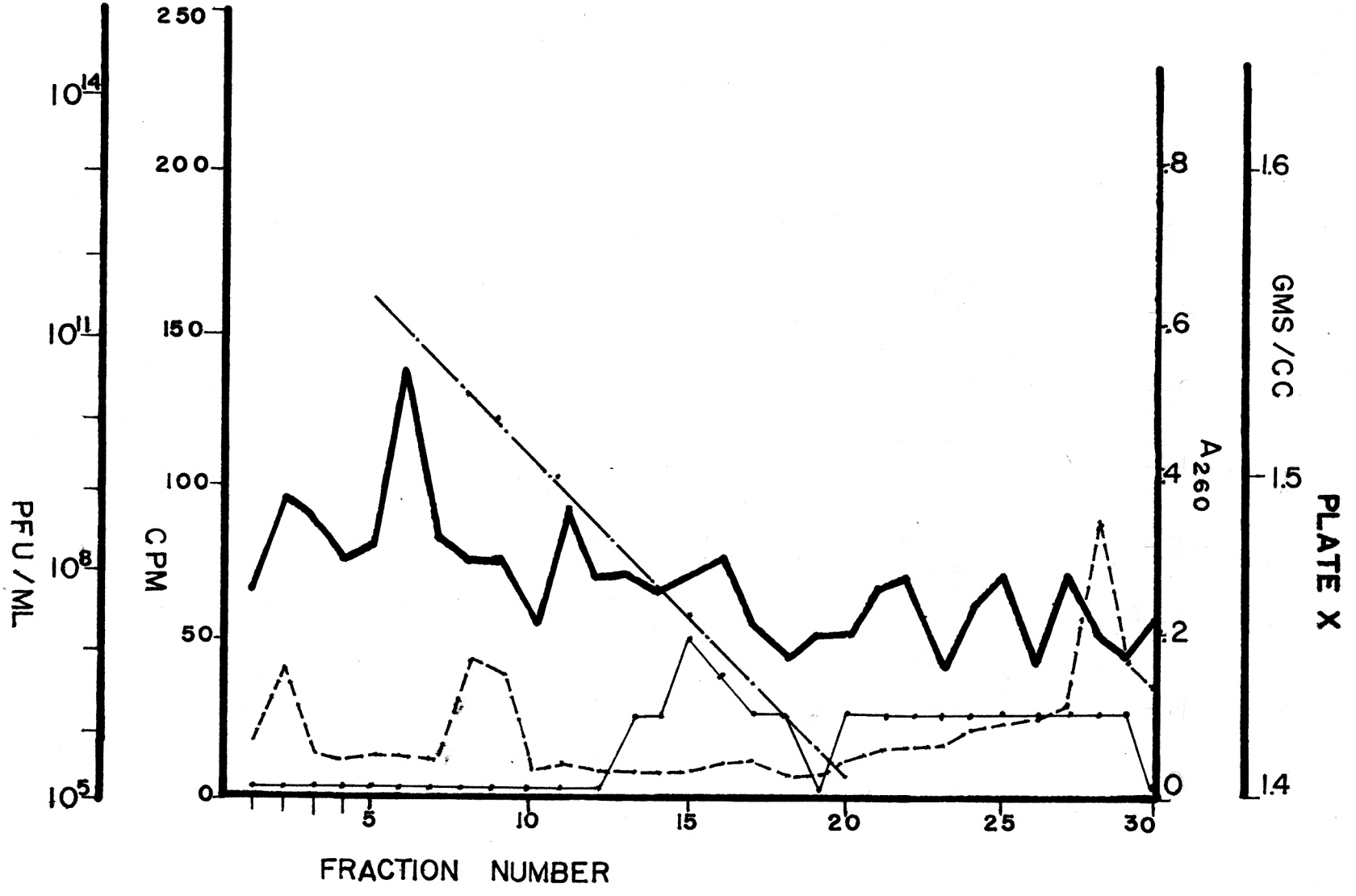
——— pfu

▬▬▬ cpm

- - - - OD (A_{260})

—.—. buoyant density

Samples were prepared and analyzed according to standard methods detailed in the text. Phages were exposed to chloroform (5% v/v) for 5 minutes.


PFU/ML
10^{14}
10^{11}
10^{8}
10^{5}
CPM
250
200
150
100
50
0
A_{260}
.8
.6
.4
.2
0
GMS/CC
1.6
1.5
1.4
PLATE X
5
10
15
20
25
30
FRACTION NUMBER

EXPLANATION OF PLATE XI

Fig. 8C. Density gradient analysis of a mixture of normal and of chloroform-treated phage 47.

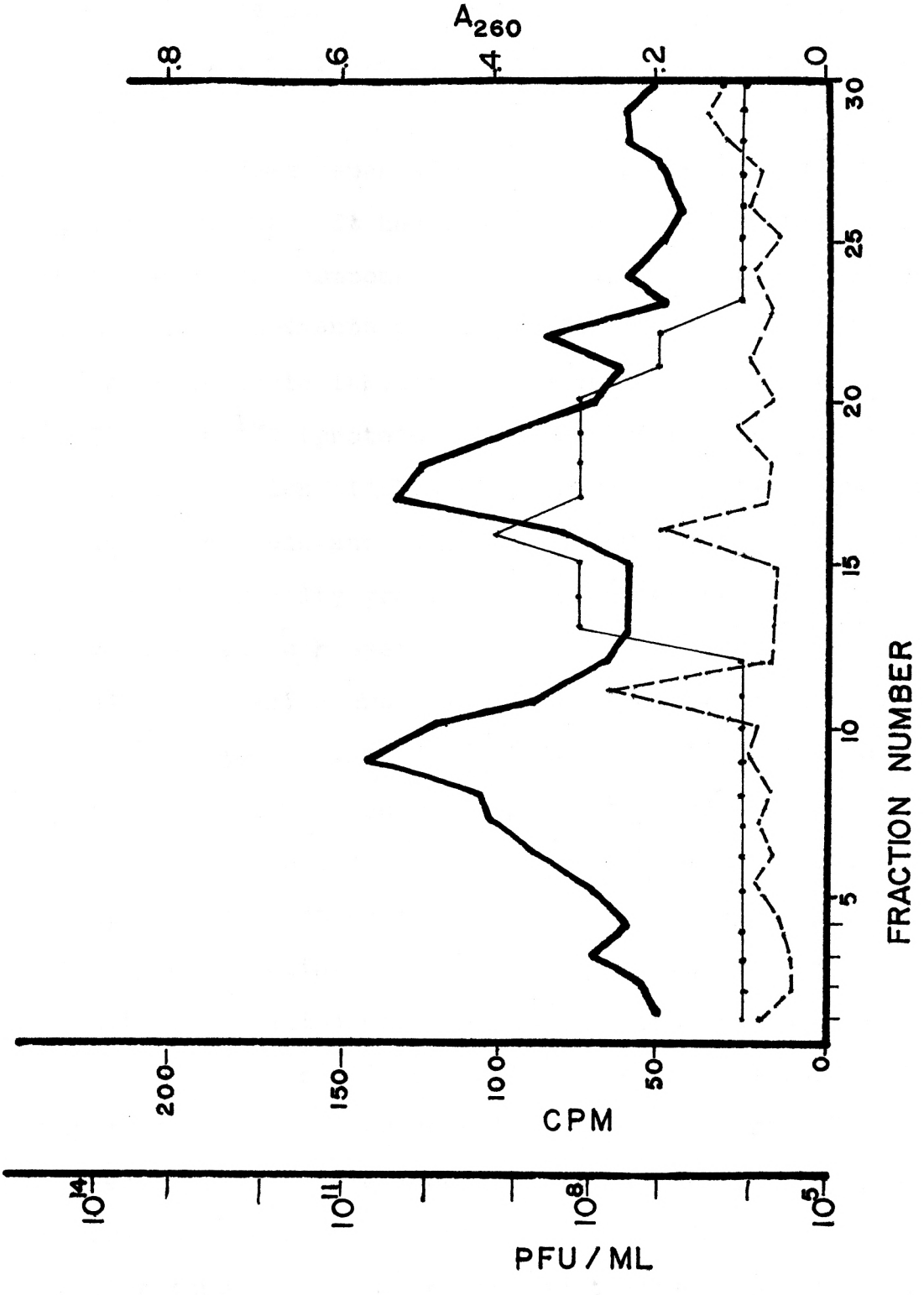
——— pfu

▬▬▬ cpm

- - - - OD (A_{260})

Samples were prepared ana analyzed according to standard methods detailed in the text. The treated portion of the mixture was exposed to chloroform (5% v/v) for 5 minutes.

PLATE XI


A260
.8
.6
.4
.2
0
30
25
20
15
10
5
FRACTION NUMBER
200
150
100
50
0
CPM
10^14
10^11
10^8
10^5
PFU / ML

particles lighter than those appearing in the main peak (fraction 15). The peak was sharp and consistent in location; consequently, it is thought to represent a homogeneous phage population.

It has been reported (19) that staphylophages degrade host components. It has not been reported whether or not phages reassemble host components (host turnover) for their own use or synthesize components de novo. The subject was examined by means of isotopic labeling. Prelabeled cells were exposed to ^{3}H (DNA) or ^{14}C (protein) for a period of time sufficient for the incorporation of a label into various host materials, including protein and DNA; excess label was removed before phage was added. Density gradients showed normal production of progeny phage in both systems; titers were comparable to those obtained in making broth lysates. The ^{3}H-thymidine gradient is shown in Figure 6A, the ^{14}C-glucose gradient in Figure 7A. If host turnover had occurred, the phages would have been heavily labeled. However, no significant peaks appeared, indicating de novo phage synthesis instead. As a check of the above finding, colabeling studies were done. Colabeling does not permit localization of label in host components; rather, it remains in host precursor pools. The results for both ^{3}H-thymidine and ^{14}C-glucose (Figure 6B and Figure 7B, respectively) showed definite peaks identifiable as phage, since they were superimposable on the peaks revealed by OD and plaque titer. Studies thus indicated de novo synthesis of phage components.

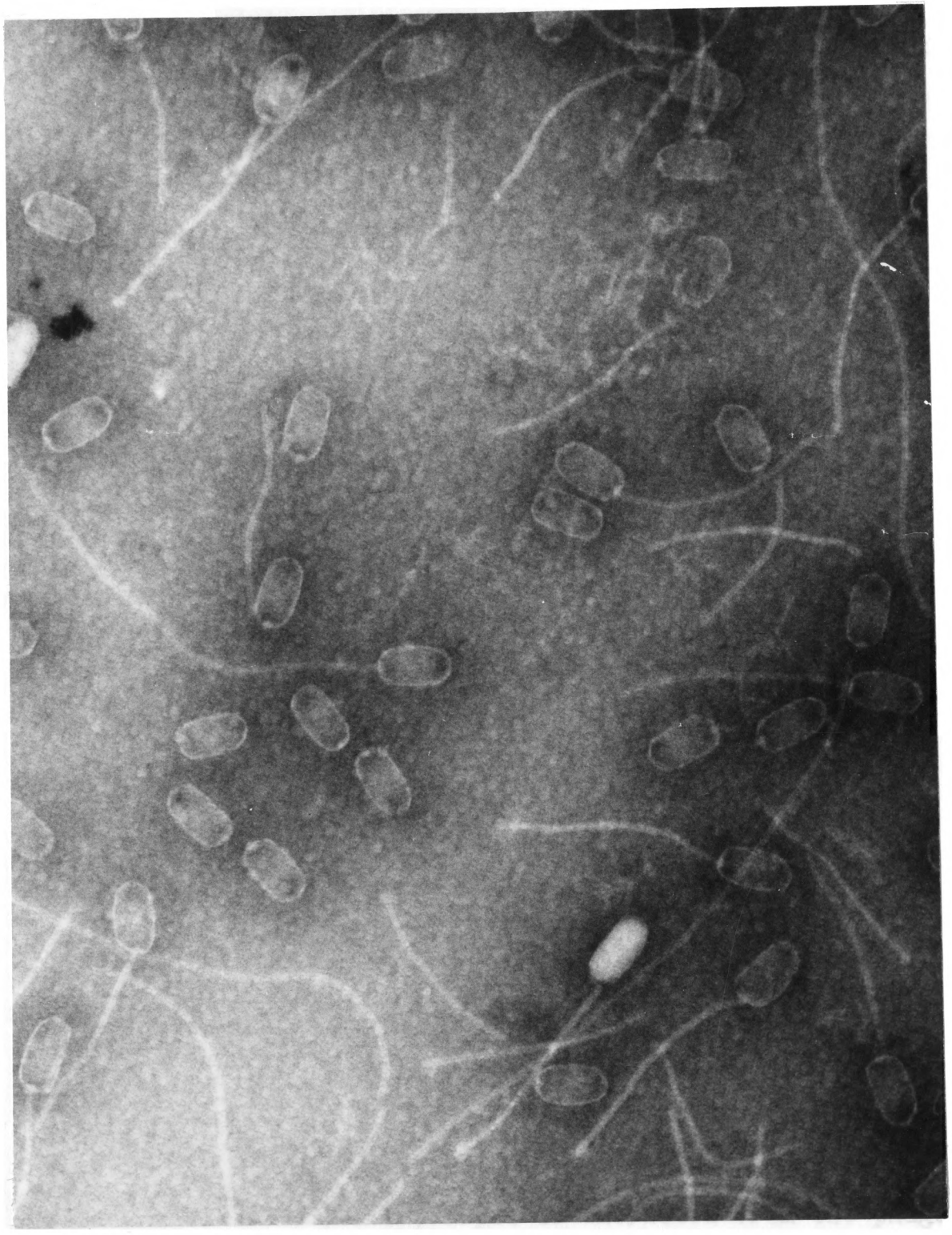
This result has also been noted for phages such as the T-even phages (30).

Additional gradient studies revealed that normal phage have a buoyant density of 1.462 g/cc as shown in Figure 8A. This figure was well within the range of 1.451 - 1.469 g/cc reported for group A phages (37). Chloroform-treated phages (Figure 8B) showed a few infective phage at an expected position in the gradient, having an acceptable buoyant density of 1.455 g/cc. However, OD and activity data showed the presence of non-infective phage in the portion of the gradient corresponding to buoyant densities of 1.530 to 1.550 g/cc. These values were higher than those characteristic for group A phages; but they were consistent with the supposition that the peak may have represented inactivated phage 47 from which lipid had been extracted. (Lipids are much less dense than protein or DNA (15); a lipid-containing phage would thus be less dense than one not containing lipid.) Other constituents being comparable, a lipid-containing phage would have a lower density than a non-lipid-containing phage. In density gradient analyses, then, a lipid-containing phage would appear higher in the gradient. The data derived from the two gradients were essentially duplicated when treated and untreated phages were mixed and applied to the same gradient, as shown in Figure 8C.

Electron micrographs were made of treated and untreated phage isolated from the peak areas of the gradients described above. Untreated phages, shown in Figure 9A, had oblong heads

EXPLANATION OF PLATE XII

Fig. 9A. Electron micrograph of normal phage 47. Magnified 144,000x. Purified phage samples were mixed with equal volumes of 2% phosphotungstic acid and mounted on grids for viewing.



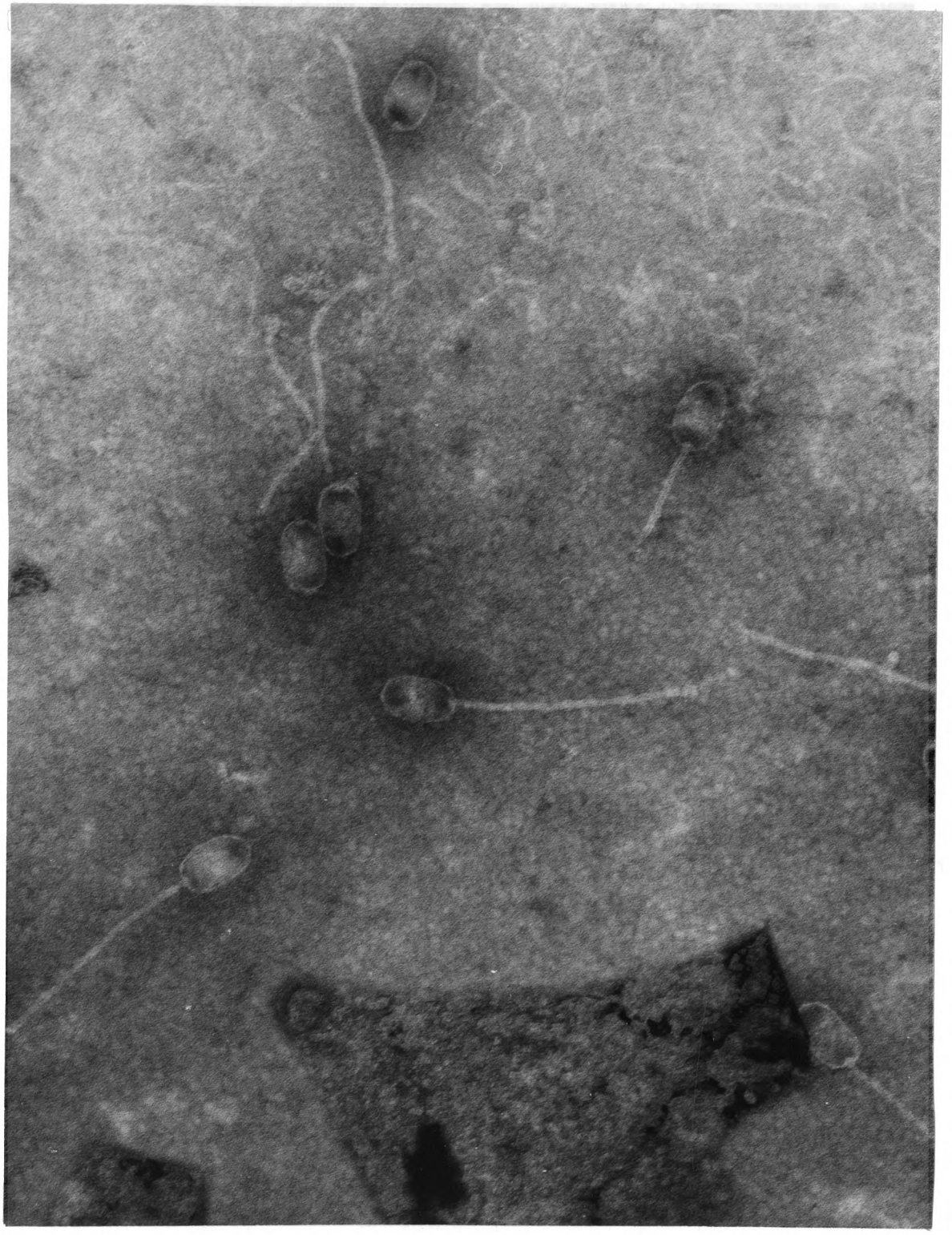
and long tails with terminal "bobs," consistent with other group A phages (37, 39, 44). Resolution was not sharp enough to tell whether the tail "bob" might be a hexagonal baseplate, as has been recently reported for a group B phage (7). Nor could it be seen if there were an inner membrane, as has been postulated for one group A phage (37). Some headless tails were seen, a phenomenon which has been reported by other workers for various phages, including phage 47 (37). Chloroform-treated phage are shown in Figure 9B. Fragmentation was more evident; many more headless tails and tail pieces were seen per unit area. Terminal tail "bobs" were less evident; many phages lacked them, and termini which did appear seemed smaller than those of untreated phage. Observations were limited by the resolution of the electron microscope; therefore, it was not possible to determine whether the phages might have contained any internal structure which chloroform treatment might have altered.

Though there were no striking differences in the morphology of untreated and treated phages, the two viruses are not the same based on gradient analysis. OD, label activity, and microscopy confirm a shift of phage to a lower level in the gradient (increased density) and a concomitant loss of infectivity. Chloroform treatment definitely alters phage 47. The mechanism of this alteration can only be postulated at the present time. Most likely, lipid is extracted from the phage. (Evidence for the presence of lipid is presented below).

EXPLANATION OF PLATE XIII

Fig. 9B. Electron micrograph of chloroform-treated phage. (5% chloroform for 5 minutes)

Magnified 144,000x. Purified phage samples were mixed with equal volumes of 2% phosphotungstic acid and mounted on grids for viewing.



Whether the alteration occurs in the tail "bob" and/or some interior structure has not yet been determined.

E. Lipid Extraction and Chromatography

1. Extraction and thin layer chromatography

The possibility that staphylophages (at least phage 47) might be lipid-containing had not been examined prior to this study. A report (28) of chloroform-sensitivity of a staphylococcal phage existed; but it was published as a curiosity and never investigated in detail. When a similar observation was made in this laboratory, it was decided to pursue the problem. Since there was no background for the study, numerous methods of extraction and chromatography had to be tested in order to develop workable procedures for the material at hand.

Staphylococcus aureus contains a number of lipids (50). Consequently, host cells are a possible source of contamination in any study of staphylophage lipids. Preparation of phage 47 used for lipid analysis was purified (filtration, centrifugation, exhaustive dialysis) as thoroughly as possible in order to avoid such contamination. Aliquots of chloroform-methanol and acetone-hot ethanol extracts of phage 47 were applied to TLC sheets and chromatographed in polar and non-polar solvent mixtures (systems 1 and 2 in Table II). The sheets were sprayed with Sudan black for location of lipids. No spots appeared on the chromatograms. Extraction was then done by acid hydrolysis (10% HCL in methanol, with the HCl evaporated from the

extract before spotting on TLC sheets). Acid hydrolysis had been previously avoided because of the severity of the process and its tendency to fragment lipids (15). Acid-hydrolyzed extracts of phage 47 yielded one large spot when sprayed with Sudan black; sheets exposed to I_2 vapor instead of Sudan black showed a spot in the same place with white and brown areas within it. Dark brown areas result from the addition of I_2 across double bonds (unsaturated lipids) and light spots appear against a faint brown background where a saturated lipid (to which I_2 cannot add) is present. Iodine vapor, apparently giving better resolution of spots, was used for all subsequent chromatographs. In the polar system the spot traveled with the solvent front; in the non-polar system the spot did not leave the origin. The solvent systems quoted for Pseudonomas phage PM2 and for S. aureus (see Table II) were relatively polar and, again, spots from acid-hydrolyzed extracts appeared at the solvent front and were not clearly distinguishable from it. A solvent system of intermediate polarity was devised, consisting of chloroform-methanol-acetone-water at 250:100:8:10. In this system, the components of the extract of phage 47 left the origin but did not travel as far as the solvent front. The components were not completely separated, but were distinguishable from one another. Two-dimensional chromatography did not aid separation. Representative one-dimensional chromatographs are shown in Figures 10A and 10B. The phage (Figure 10A) yielded a large white area (area 1) corresponding to an

EXPLANATION OF PLATE XIV

Fig. 10A. Thin layer chromatography of phage 47.

Fig. 10B. Thin layer chromatography of S.aureus 47.

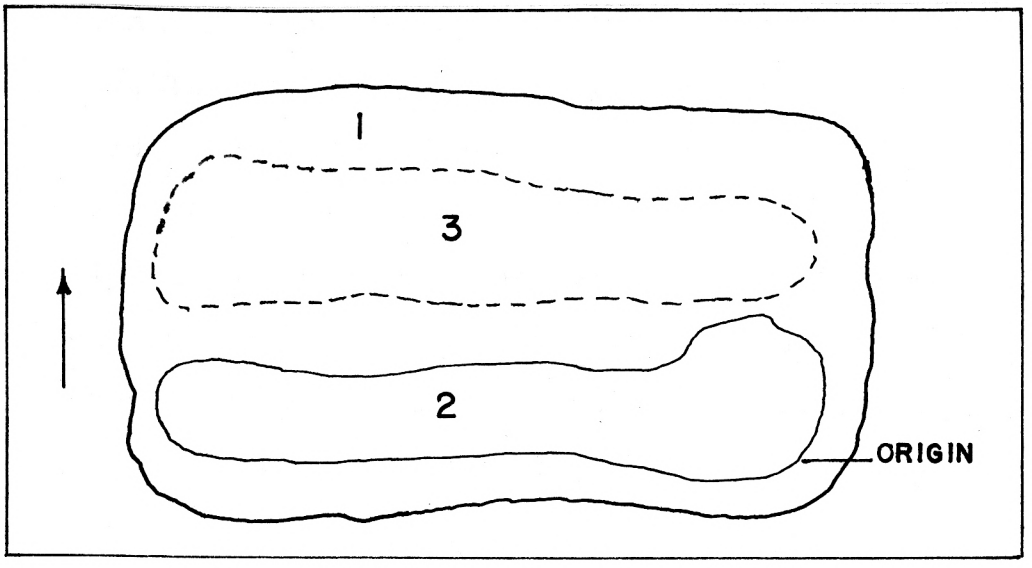
Solvent system: chloroform-methanol-acetone-water
250:100:8:10

Acid-hydrolyzed (10% HCl in methanol) were used.

PLATE XIV

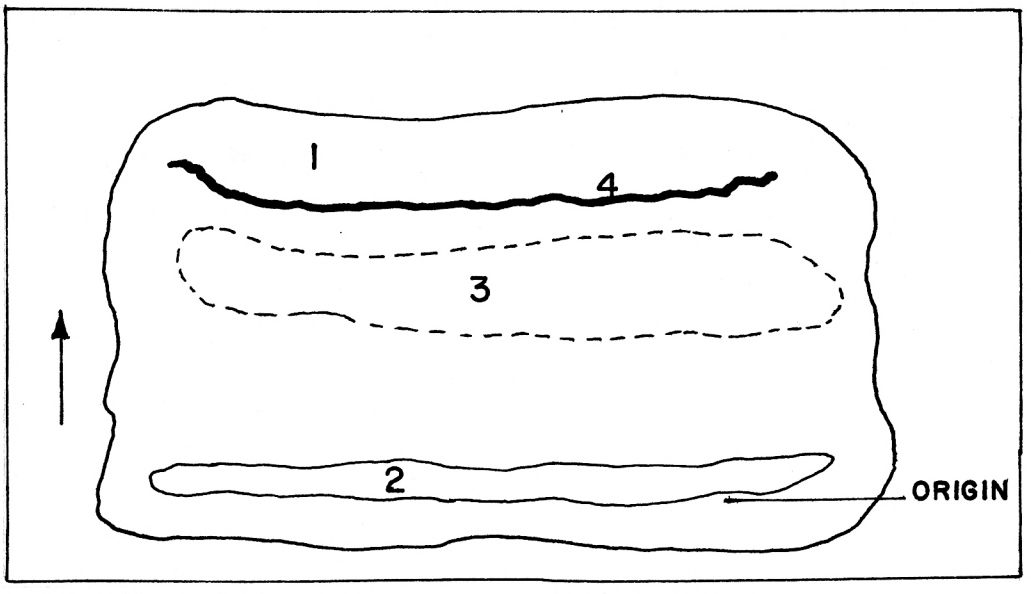
A.

STAPHYLOPHAGE 47



B.

STAPHYLOCOCCUS AUREUS 47



unsaturated lipid; an unsaturated lipid (area 2) remained near the origin; there may also have been another unsaturated component (area 3, which was very faint) between the leading edges of areas 1 and 2. Host cells were also extracted and chromatographed; cells were treated with lysostaphin (0.1% in TSB) prior to acid hydrolysis and chromatography in order to release the lipid-containing membrane complex from the cell wall. Results (Figure 10B) were similar, except that component 2 was less evident and another component (area 4) appeared. Area 4 was pigmented (yellow) prior to exposure to I_2 vapor and very likely represented the host pigments which give Staphylococcus aureus its name.

2. Gas chromatography

Since resolution of components in thin layer chromatography was not as complete as could be desired, it was decided to attempt further resolution via gas chromatography. Both phage (Figure 11A) and host (Figure 11B) showed at least 6 definite peaks. Comparison to known standards indicated that peak 3 had the same retention as palmitic acid (saturated) and that peak 5 had the same retention time as oleic acid (unsaturated); the other peaks were indeterminate. Results did not correlate with those previously reported for the host (50); but acid is a severe procedure and the components may have fragmented.

The evidence for the presence of lipids in staphylophage 47 is extensive, but is only circumstantial. A solvent system

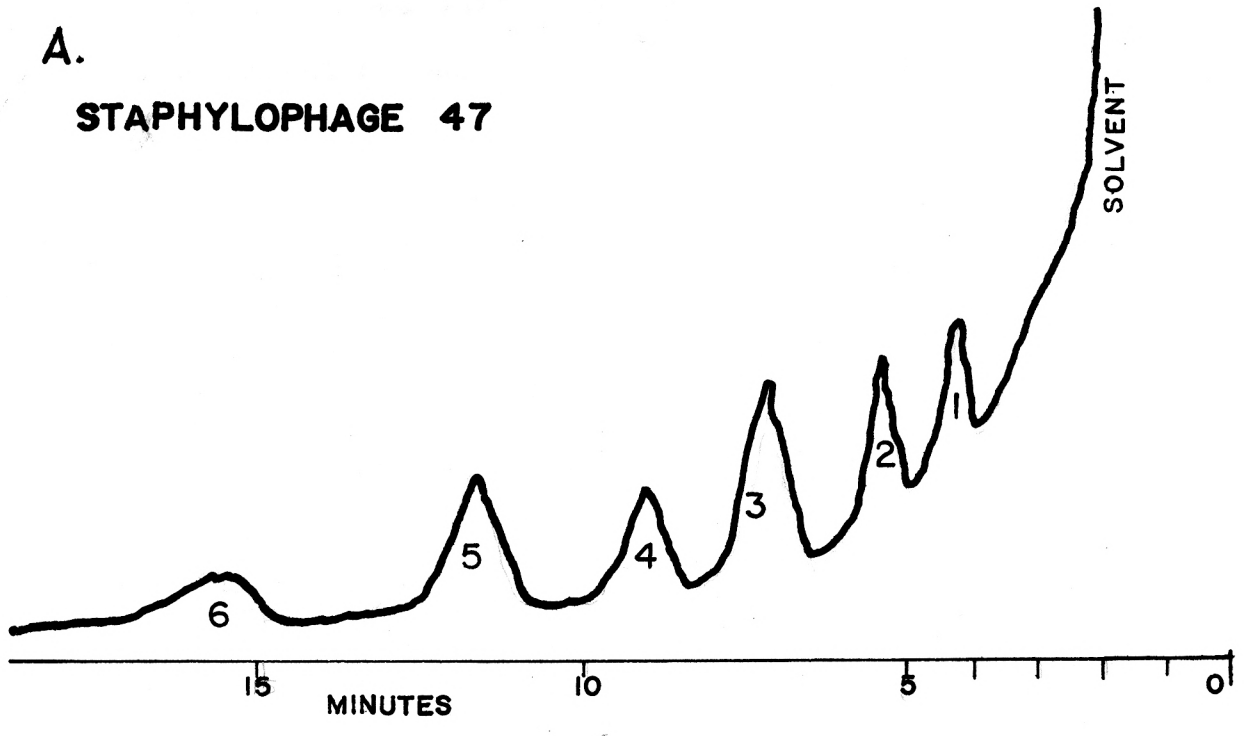
EXPLANATION OF PLATE XV

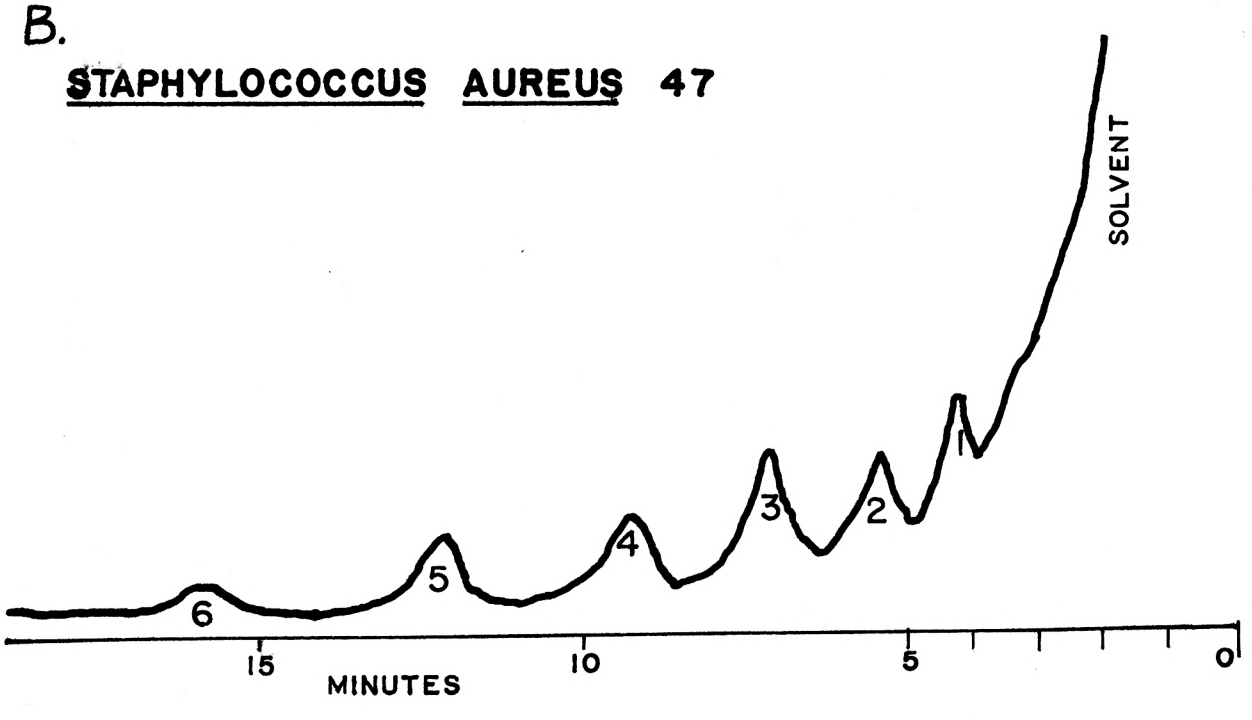
Fig. 11A. Gas chromatograph of phage 47.

Fig. 11B. Gas chromatograph of S.aureus 47.

Acid-hydrolyzed extracts were evaporated under N_2, dissolved in hexane, and applied to an 8' DEGS column. Inlet temperature was 225 C; column temperature was 190 C; the carrier gas was argon.

PLATE XV


A.
STAPHYLOPHAGE 47
SOLVENT
1
2
3
4
5
6
15
10
5
0
MINUTES


B.
STAPHYLOCOCCUS AUREUS 47
SOLVENT
1
2
3
4
5
6
15
10
5
0
MINUTES

is yet to be developed which totally separated the components so that they might be purified and positively identified.

V. SUMMARY AND CONCLUSIONS

Staphylophage 47 was most efficiently propagated by infecting early log phase host cultures at an MOI of 1.0 at 22 C. Cultures were shifted to 37 C. after 30 minutes and to 4 C. after 4 hours. Titers at 24 hours post-infection were on the order of 10^{10} pfu/ml, representing a yield of 240 infectious phage per originally infected cell. The results were 5 orders of magnitude greater than those obtainable by currently recommended methods (6) and indicated that such methods are in need of extensive revision. The results also suggest that phages may have varied widely in the length of time needed to infect and lyse the host cells and/or host cells may have required a long time to release a full complement of phages (rather than bursting and releasing them all at once).

Phage 47 was inactivated by several organic solvents which are known to extract lipids (15), but which are not known to drastically affect protein at the concentrations tested (15). Phage 47 has traditionally been thought (19) to contain only DNA and protein, neither of which should have been much affected by the solvents. Consequently, it was suggested that phage 47 might be lipid-containing.

Density gradient analyses indicated that phage populations were homogeneous and that particles had a buoyant density of 1.462 g/cc, consistent with earlier findings (37). The phage did not appear to synthesize phage-specific components by host

turnover, but rather by de novo synthesis. Chloroform-treated phage showed a marked drop in infectivity, though some infective phage did appear where expected in the gradient. A peak corresponding to non-infective phage appeared lower than usual in the gradient and showed densities of 1.530 to 1.550 g/cc. The higher densities are consistent with the supposition that non-infective chloroform-treated phage would lack the lipid present in normal phage and would thus be denser.

Electron micrographs showed that normal phage had oblong heads and long tails with a terminal "bob," consistent with earlier reports (37, 39, 44). Chloroform-treated phage was very similar in appearance. Terminal tail "bobs" were less in evidence and fragmentation was fragmentation was pronounced.

Evidence for the presence of lipid was extended by silica gel thin layer and gas column chromatography of acid-hydrolyzed phage extracts. On TLC sheets developed in I_2 vapor, phage 47 and S. aureus strain 47 showed a saturated lipid and either 1 or 2 unsaturated lipids (in addition to pigment in the host). Phage had been purified to the extent that host contamination was extremely unlikely. It is more likely that phage 47 synthesizes lipids similar to or derived from those of its host. Gas chromatography showed that both phage and host might contain as many as 6 lipids or lipid fragments, two of which had the same retention times as palmitic and oleic acids. The lipid analyses for the host did not correspond with earlier reports (50); but differences could have been due to the method of

extraction. The presence of lipids in staphylophage 47, or any other staphylophage, has never before been reported. Evidence for the presence of lipids was not conclusive, but was indeed substantial.

BIBLOGRAPHY

1. Abramoff, Peter and Mariano LaVia, 1970. Biology of the Immune Response. McGraw-Hill, Inc., New York.

2. Abranson, D. and M. Blecher, 1964. Quantitative two-dimensional thin-layer chromatography of naturally occurring phospholipids. J. Lipid. Res. 5: 628-631.

3. Adams, M. H., 1959. Bacteriophages. Interscience Publications, Inc., New York.

4. Bergdoll, M. S., 1970. Enterotoxins, pp. 265-326. In T. C. Montie (ed.), Microbial Toxins, vol. 3. Academic Press, Inc., New York.

5. Berryhill, David L. and P.A. Pattee, 1969. Buoyant density analysis of staphylococcal bacteriophage 80 transducing particles. J. Virol. 4: 804-806.

6. Blair, J. E. and R. E. O. Williams, 1961. Phage typing of staphylococci. Bull. W. H. O. 24: 771-784.

7. Brown, Dennis T., 1972. Morphology and physical properties of Staphylococcus bacteriophage P11 - M15. J. Bacteriol. 9: 664-671.

8. Buller, C. Personal communication.

9. Burnet, F. M. and Dora Lush, 1935. The staphylococcal bacteriophages. J. Path. Bact. 40: 455-469.

10. Campbell A., 1965. The steric effect in lysogenization by bacteriophage lambda. Vitology 27: 319-345.

11. Campbell, D. H., 1970. Methods in Immunology. W. A. Benjamin, Inc., New York.

12. Casman, E. P., 1965. nn. In T. C. Montie, op. cit.

13. Chattarjee, A. A., 1969. Use of bacteriophage-resistant mutants to study the nature of the bacteriophage receptor site of Staphylococcus aureus. J. Bacteriol. 98: 519-531.

14. Chun, D. and J. K. Chung, 1970. Inhibition phenomenon of staphylococci by bacteriophages. J. Virol. 5: 709-713.

15. Clark, J. M. (ed.), 1964. Experimental Biochemistry. W. H. Freeman and Co., San Francisco.

16. Delbrück, M., 1940. The growth of bacteriophage and lysis of the host. J. Gen. Physiol. 23: 643.

17. Doughty, C. C. and J. A. Mann, 1967. Purification and properties of a bacteriophage-induced cell wall peptidase from Staphylococcus aureus. J. Bacteriol. 93: 1089-1095.

18. Eisenstark, A., 1967. In Maramorosch and Koprowski (ed.), Methods in Virology. Academic Press, Inc., New York.

19. Elek, S. D., 1959. Staphylococcus pyogenes and Its Relation to Disease. E. and H. Livingston, Ltd., Edinburgh.

20. Espejo, R. T. and E. S. Canelo, 1968. Properties of bacteriophage PM2: a lipid-containing bacterial virus. Virology 34: 738-747.

21. Goering, R. V. and P. A. Pattee, 1971. Mutants of Staphylococcus aureus with an increased sensitivity to ultraviolet radiation. J. Bacteriol. 106: 157-161.

22. d'Hérelle, F., 1917. Sur un microbe invisible antagoniste des bacille dysentériques. C. R. Acad. Sci. Paris 165: 373. In G. Stent, 1963. Molecular Biology of Bacterial Viruses. W. H. Freeman and Co., San Francisco.

23. _____, 1918. Sur le rôle du microbe filtrant bactériophage dans la dysenterie bacillaire. C. R. Acad. Sci. Paris 167: 970. In G. Stent, op. cit.

24. _____, 1926. Le bactériophage et son comportement. Masson, Paris. In G. Stent, op. cit.

25. Hershey, A. D. and M. Chase, 1952. Independent functions of viral protein and nucleic acid in growth of bacteriophage. J. Gen. Physiol. 36: 39-56. In S. Luria and J. Darnell, 1967. General Virology. John Wiley and Sons, Inc., New York.

26. Ifft and Vinograd, 1961. A constant for use in conversion of refractive index to density. J. Phys. Chem. 65: 1138.

27. Kates, M. 1961. Lipids of influenza virus and their relation to those of the host cell. Biochim. Biophys. Acta 52: 455-466.

28. Liégois-Muller, A. and P. Frédérique, 1952a. Stérilisation par le chloroforme des bactériophages staphylococciques. C. R. Soc. Biol. Paris 146: 1293-1295.

29. Lovett, P. S. and G. Shockman, 1970. Characteristics of bacteriophage N1 and its attachment to cells of Micrococcus lysodeikticus. J. Virol. 6: 125-134.

30. Luria, S. and J. Darnell, 1967. General Virology. John Wiley and Sons, Inc., New York.

31. Lwoff, A., 1953. Lysogeny. Bacteriol. Rev. 17: 269. In G. Stent, op. cit.

32. Munch-Peterson, E., 1963. J. Food Sci. 28: 692. In T. C. Montie, op. cit.

33. Pattee, P. A. and J. N. Baldwin, 1961. Transduction of resistance to chlortetracycline and novobiocin in Staphlococcus aureus. J. Virol. 3: 33-37.

34. Read, R. B., Jr. and W. L. Pritchard, 1963. Can. J. Microbiol. 9: 879. In T. C. Montie, op. cit.

35. Riggs, H. G. and E. D. Rosenblum, 1969. Transfection of lysostaphin-treated cells of Staphylococcus aureus. J. Virol. 3: 33-37.

36. Rippon, Joan E., 1956. The classification of bacteriophages lysing staphylococci. J. Hyg. 54: 213-226.

37. Rosenblum, E. S. and S. Tyrone, 1964. Serology, density, and morphology of staphylococcal phages. J. Bacteriol. 88: 1737-1742.

38. Rountree, Phyllis M., 1949. The serological differentiation of staphylococcalbacteriophages. J. Gen. Micro. 3: 164-173.

39. _____, 1952. Serological studies of the multiplication of a staphylococcal bacteriophage. Aus. S. Exptl. Bio. and Med. Sci. 30: 567-586.

40. Schlesinger, M., 1930. Die Bestimmung von Teilchengrösse und spezifischem Gewicht des Bakteriophagen durch Zentrifugierversuche. Z. Hyg. Infektionskrankh. 114: 161. In G. Stent, op. cit.

41. _____, 1932. Über die Bindung des Bakteriophagen an homologe Bakterien. II. Quantitative Untersuchungen über die Bindungsgeschwindigkeit und die Sättigung-Berechnung der Teilchengrösse des Bakteriophagen aus deren Ergebnissen. Z. Hyg. Infektionskrankh. 114: 149. In G. Stent, op. cit.

42. _____, 1934. Zur Frage der chemisches Zusammensetzung des Bakteriophagen. Biochem. Z. 273: 306. In G. Stent, op. cit.

43. Schuytema, E. C., H. L. Glenn and C. C. Doughty, 1969. Relative rates of lysis of staphylococcal cell walls by lytic enzymes from various bacteriophage types. J. Bacteriol. 98: 920-923.

44. Seto, J. T., P. Kaesberg and J. B. Wilson, 1956. Electron microscopy and serology of staphylococcal phages. J. Bacteriol. 72: 847-850.

45. Sonstein, S. A., J. M. Hammer and A. Bondi, 1971. Staphylococcal bacteriophage-associated lysin: a lytic agent active against Staphylococcus aureus. J. Bacteriol. 107: 499-504.

46. Stent, Gunther, 1963. Molecular Biology of Bacterial Viruses. W. H. Freeman and Co., San Francisco.

47. Twort, F. W., 1915. An investigation on the nature of the ultramicroscopic viruses. Lancet 189: 1241. In G. Stent, op. cit.

48. Watson, J. D. and F. H. C. Crick, 1953. The structure of DNA. Cold Spring Harbor Symp. Quant. Biol. 18: 123. In G. Stent, op. cit.

49. Wentworth B. B. and W. R. Romig, 1968. The multiplication of staphylococcal phages in nonpermissive hosts. Japan J. Microbiol. 12: 309-316.

50. White, David C. and Frank E. Frerman, 1967. Extraction, characterization, and cellular localization of the lipids of Staphylococcus aureus. J. Bacteriol. 94: 1854-1867.

51. Winkler, K. C. 1965. Lysogenic conversion of staphylococci to loss of beta toxin. J. Gen. Micro. 39: 321-333.

CHARACTERIZATION OF STAPHYLOPHAGE 47

by

DORIS SODEN BUTLER

B.A., University of Kansas, 1970

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Microbiology
Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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ABSTRACT

Staphylophage 47, a bacterial virus specifically parasitizing Staphylococcus aureus strain 47, was studied. A procedure was devised for the propagation of staphylophage 47. Broth lysates yielded titers of 10^{10} pfu/ml. Such titers were 5 orders of magnitude greater than those obtainable by standard methods of propagation in soft agar. Phage were released from infected host cells over a long period of time, rather than in a single burst. Phage might have varied widely in ability to infect and lyse host cells; host cells may have varied in the rate of phage release. Phages did not specifically degrade and reassemble host components in the infection process; de novo synthesis of phage components was indicated. Density gradient analyses indicated that phage populations were homogeneous and had a buoyant density of 1.462 g/cc. Phage 47 was inactivated by a number of organic solvents which are capable of extracting uncharged compounds, e.g., lipids; such sensitivity has not before been reported for staphylophages. Chloroform-treated phages were more dense than untreated phages and were non-infective. Evidence was presented which indicated the presence of lipids (either phage-synthesized or acquired from the host) essential for normal function of phage 47. Lipids were extracted from phage 47 by acid-hydrolysis. Thin-layer chromatography yielded 3 lipids. Gas chromatography yielded possibly 6 lipids or lipid fragments; 2 of the 6 components had column retention times comparable to palmitic and oleic acids.