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CHARACTERIZATION OF GROWTH AND STRUCTURAL PROPERTIES OF THE COLIPHAGE UC-1

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For

Judy and Jennifer

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CHAPTER I

INTRODUCTION

The bacteriophage UC-1 was isolated in 1965 by the microbial genetics group at the University of Oklahoma (3). Briefly, UC-1 can be described as a double stranded DNA coliphage (DNA mol. wt. 3.2×10^7, GC % 48) (G. W. Cartwright, Personal Communication) whose host range is confined to various strains of Escherichia coli (E. coli) and having highly distinguishing morphology and plaque appearance. Thus as a result of the efforts of this author and several other workers at Norman enough data have been gathered to show that UC-1 is in all probability a newly described DNA coliphage. This phage possesses such a unique combination of unusual traits that it is surely not identical with any of the more commonly studied coliphages (5, 45).

Morphologically UC-1 is described (3) as having an icosahedral head of 450 Å diameter with an often distinctly three dimensionally curled tail of 1500 Å length.

Electromicrographs of UC-1 (3) give no evidence of a constriction or collar at the tail attachment site. Likewise there is no evidence for trailing tail fibers, end plates, knobs, etc. In several grid preparations phage have been observed with straightened tails or with tails having only a slight bend or twist.

This morphology coupled with the knowledge that UC-1 is a double stranded DNA phage would put UC-1 in either Bradley's group B or Tikhonenko's group IV. Both of these phage group titles denote double stranded DNA phages having a long and noncontractile tail (5, 45).

There are very few reports in the literature of a phage described with a tail as distinctively coiled as is UC-1's. When this does occur there are generally other properties that grossly differentiate it from UC-1. Bradley and Kay (6) report on a Salmonella typhimurium phage, S1BL, that has a curled tail and dimensions very similar to UC-1, (500 Å head diameter and 1350 Å tail length). Moreover in addition to a host range difference this phage also can be distinguished from UC-1 by its possession of a knob at the head-tail junction. The coliphage T1 is of almost identical morphology as S1BL and while it lacks the knob, its tail has a marked constriction at the top. Another coliphage similar to UC-1 is B4 reported by Bradley (4). B4 has no tail modifications that would distinguish it from UC-1 but its head diameter of 600 Å is surely a significant enough difference.

Tikhonenko in her monograph on bacteriophage ultrastructure (45) shows an Aerobacter aerogenes phage, phage 886, that has the same range of tail conformations as UC-1. This phage however has a slightly larger head diameter (470 Å vs. 450 Å for UC-1) and a somewhat shorter tail (1300 Å vs. 1500 Å). Also phage 886 shows a constriction of the tail at the point of attachment to the head. Lambda (λ) a somewhat larger phage (540 Å head diameter) does possess somewhat similar measurements but its tail is usually seen with only a slight bend and it possesses a trailing end fiber on its tail. A number of other phage belonging to Bradley's group B have tails of approximately 1500 Å in length; T5, P2 and Ø80, but like λ they all have heads larger than UC-1 (30).

The plaque morphology shown by UC-1 grown on either E. coli K-12 or E. coli C is also somewhat unusual. Typically the plaques appear as clear centered areas surrounded by one to three separate rings of dense bacterial growth with an outer halo. The overall diameter of these plaques is quite variable. Plaques ranging in size from 0.5 to 6 mm. at maturity are often seen on the same plate. When grown on E. coli B, UC-1 produces a clear centered plaque surrounded by a diffuse halo. The plaques produced on K-12 or C lawns are somewhat like those produced by T3 on E. coli B (13) or by a ds mutant of T2 on E. coli S (25).

Growth studies performed with UC-1 have revealed some other unique traits. If late log phase cells are infected at

a multiplicity of infection (m.o.i.) of 0.1 or less there appears among the surviving cells plated four hours post infection a sizeable population of small, translucent, irregularly edged colonies that fail to grow in either broth or on plates when attempts are made to subculture them. This is somewhat akin to the T3-E. coli B complexes founded by Fraser (19). She attributed these to some sort of "pseudolysis" or semi temperate phage-host association. That UC-1 is a T3 variant is unlikely in light of the great difference in tail morphology (150 Å for T3) and in DNA mol. wt. (3.2×10^7 for UC-1 [G. W. Cartwright, Personal Communication] vs. 2.4×10^7 for T3 [30]).

The intent of the studies presented herein was to aid in the initial characterization of UC-1 as to its growth properties, to devise effective batch growth and purification schemes, to study UC-1's response to various physical and chemical agents and to begin to characterize the protein constituents of the phage particle.

CHAPTER II

MATERIALS AND METHODS

Bacterial strains

E. coli strains K-12 and B were obtained from the University of Oklahoma stock collection. *E. coli* C was a gift of Dr. Thomas Matney of M. D. Anderson Hospital, Houston, Texas.

Phage strains

Two strains of UC-1, UC-1C and UC-1R, were isolated by separate repeated subcultures at the University of Oklahoma (38). Phages T1 and λ were procured from the American Type Culture Collection. UC-1R was the strain used throughout these studies except where otherwise noted.

Culture media

For the production of phage lysates Difco penn assay broth (PAB) was used. For cell studies and plaque assays Difco nutrient broth supplemented with 1.5% or 0.75% (w/v) Difco agar, for bottom and top agar respectively, and 0.5% (w/v) NaCl was used. For physiological studies the M-9 medium of Adams was employed (1).

Buffers

Phage buffer consisted of 0.01 M-phosphate buffer, pH 7.0, with 0.5% (w/v) NaCl and 0.001 M-$MgSO_4$.

Phage growth studies

The initial experiments to characterize phage growth parameters were all performed according to the methods of Adams (1). These experiments included; adsorption studies, one step growth curves, single cell burst size determinations and phage antiserum production and assay.

Studies to determine the effect of cell age at the time of infection on phage production were performed by infecting a 10 ml. aliquot of a log phase culture of E. coli K-12 at 1/2 hr. intervals with a m.o.i. of 0.1. The cell culture was initially started with a 1:100 inoculum from an overnight (ON) culture. The cell population dynamics were followed by recording the optical density (OD) at 420 mμ. with a Bausch & Lomb Spectronic 20 colorimeter. At four hours after infection the various tubes were assayed for phage production, surviving cells and total cells using standard plating technique and a Petroff Hausser counting chamber.

Phage production and purification

Phage production on a large scale was performed either in multiple 1 liter amounts or by means of a batch fermenter. For the flask method 1 liter of PAB in a 2 liter

flask was inoculated with 10 ml. of an ON culture and shaken at 37° on a New Brunswick gyrotory shaking incubator until the cell population reached 3 x 10^8 cells/ml. Then enough phage to give an m.o.i. of 1 or greater was then added and incubation with shaking was continued for 3 to 5 hr. For the batch fermenter method a New Brunswick Fermacell Model CF-50 batch unit was employed. The same general scheme of inoculations was employed but times were altered due to the greater efficiency of cell growth in the fermenter. After lysis the same path was followed through harvesting and purification for both systems. At the completion of lysis chloroform was added to a final concentration of 0.5% (V/V). For the production of a lysate without subsequent purification cell debris was removed by centrifugation and the lysate was further cleared by filtration through Hyflo Super Cell and Whatman No. 1 filter paper. These lysates were then sterilized by passage through a Millipore filter having 0.45 μ diameter pore size. For concentration and purification one of the three procedures as outlined in Figure 7 was employed. The continuous flow centrifugation of large batches was performed with either a Sharpels Super Centrifuge or the Szent-Gyorgyi and Blum continuous flow attachment for the Sorval RC-2 centrifuge. High speed centrifugations were carried out using either a model L2-50 or model L2-65B Beckman centrifuge with Spinco rotors. All dyalysis was performed in Visking tubing, overnight at 4° vs. phage buffer. The Freon 113, a

trichlorotrifluoreothane product was a gift of duPont Chemical Company. The polyethylene glycol (PEG) procedure is basically the method of Yamamato and Alberts (49). For preparative isopycnic banding of phage, biochemical grade CsCL purchased from Schwartz-Mann was used. The CsCL was added to phage in phage buffer in amounts according to the formula of Thomas and Abelson so as to achieve a mean density of 1.51 (44). Phage lysates were stored at 4° in either PAB or nutrient broth with 0.5% (w/v) NaCl and purified phage preparations were stored at 4° in phage buffer.

Gel electrophoresis of phage proteins

Purified phage were disrupted by sodium dodecyl sulphate (SDS), electrophoresed on polyacrylamide gels, and stained according to the method of Shapiro, Viñuela and Maizel (41). Some modifications as suggested by Maizel were employed: the sample and gel buffers were made 0.004% (w/v) with disodium ethylenadiaminetetraacetate (EDTA), and the samples were boiled for 1 to 5 min. just prior to electrophoresing (31). Carboxymethylation was also performed according to the method of Maizel (31). The gels were either 7.5% or 10% (w/v) with respect to acrylamide but the amount of N', N', dimethylbisacrylamide was held constant at 0.2%. Gels 8 cm. long were cast in tubes 10 cm. long and 0.5 cm. in diameter. Electrophoresis was carried out for 3.5 hrs. for 7.5% gels and 4.5 hrs. for 10% gels at approximately

10 mA/tube using a Heath-kit IP-17 constant voltage source. The position of stained protein bands was measured visually using a vernier calipers. The calculation of rates of relative mobility (M), for molecular weight determinations, was performed according to the method of Weber and Osborn (48),

$$\text{Mobility (M)} = \frac{\text{distance of protein migration (P)}}{\text{gel length after destaining (FL)}} \times \frac{\text{gel length before staining (IL)}}{\text{distance of dye migration (D)}}$$

Proteins used as molecular weight standards were bovine serum albumin, pepsin, trypsin and glyceraldhyde phosphate dehydrogenase products of Sigma chemical company, and lysozyme and ribonuclease products of Calbiochem. Ovalbumin was prepared by Dr. John Measel.

Studies of H^3 labeled amino acid uptake by phage infected cells

Cells were exposed to ultra violet radiation by swirling 10 ml. aliquots of cells in M9 media in 10 cm. glass petri plates held 20 cm. from a single 8 watt General Electric germicidal lamp. After varying periods of UV treatment, 1 ml. aliquots were withdrawn. To these was added either phage at an m.o.i. of 1 to 10 or an equal amount of phage buffer. H^3 labeled leucine (New England Nuclear, L-leucine-4-5-H^3, 5 C/mM) diluted with cold carrier L-leucine to give a final activity of 1 μC/20 μg/ml. was also added at this time. The same m.o.i. and amount of label were used in all

amino acid uptake studies. The cells so labeled were allowed to incubate at 37° for 90 min. The cell suspension was then made 5% with cold trichloroacetic acid (TCA) and held in an ice bath for 10 min. prior to filtering through a 0.45 μ Millipore filter. The filters were washed with 10 ml. of cold 5% TCA and dried. A modified Bray's scintilant was employed (7), i.e. ethylene glycol was omitted. New England Nuclear's Omnifluor was the fluor used. The samples were counted on a Beckman DPM-100 scintilation counter.

For studies of labeled leucine uptake in normal cells the procedures of phage and label addition, sampling, and counting were the same except samples were incubated for varying times from 1 to 60 min. prior to addition of TCA.

To study the effect of different levels of phage input (m.o.i.) on label uptake all conditions were the same as those used in the above two experiments except varying dilutions of phage were used to achieve m.o.i.'s ranging from 0.1 to 100.

Effect of physical and chemical agents on phage viability

Heat.--One ml. aliquots of PAB phage lysate at approximately 1 x 10^{10} p.f.u./ml. were placed in sterile 1.5 cm. x 125 cm. screw capped tubes that had been preheated to the indicated temperatures and the phage incubated for various periods of time. Samples were withdrawn and plaque assays were performed. For electron microscopic (EM) observations

the entire aliquot was diluted to 5 ml. and the phage pelletted by spinning at 39,000 r.p.m. for 1 hr. in the SW 39 rotor. The phage pellet was resuspended in a minimal amount of phage buffer, applied to fovar coated grids, stained with phosphotungstic acid, pH 7.0, and examined using a Phillips model 200 electron microscope.

Sonication.--To study the effect of sonic disruption 10 ml. of phage lysate, 1 x 10^{10} p.f.u./ml., were placed in a cooled (4^{o}), 20 ml. cup device of a Blackstone sonicator tuned to give maximum cavitation and run at 100% power output. Samples of 0.1 ml. were withdrawn and titered at various intervals. For EM observations multiple experiments were performed and from each a 5 ml. sample was withdrawn and treated in the same manner as those that had been exposed to heat.

pH.--To study the effect of various pH's on phage viability and structure 0.1 ml. of phage in phage buffer at approximately 1 x 10^{10} p.f.u./ml. was added to 9.9 ml. of a designated buffer and incubated at 25^{o} for 60 min. Aliquots of 0.5 ml. were withdrawn and diluted in either the same buffer or in 0.5% (w/v) NaCl for titering of survivors. For kinetic studies phage was withdrawn and titered after varying periods of exposure. To see if pH inactivation was irreversible samples were dialyzed against phage buffer overnight at 4^{o} and titered. The buffers used for various pH ranges were: acetic acid - sodium acetate, 3.7 - 5.8, succinic acid - sodium hydroxide, 3.8 - 6.0, potassium

dihydrogen phosphate - disodium hydrogen phosphate, 6.0 - 8.0, tris (hydroxymethyl) aminomethane - hydrochloride (tris), 8.0 - 9.4, and glycine - sodium glycinate 9.6 - 11.4. These were all made according to the tables appearing in "Methods and References on Biochemistry and Biophysics" (12) to have an ionic strength of 0.1 or less. As in previous studies EM grids of treated phage were made from pelletted material.

Effects of phage disruptive agents

Ghosting techniques.--Several methods of treating phage that result in the release of the phage nucleic acid content from the protein coat of the phage head were applied to UC-1. The methods employed included osmotic shock using 3M-Na_2SO_4 (23), osmotic shock with 60% sucrose, treatment with 5M-LiCl (16), treatment with sodium pyrophosphate (47) or treatment with $CuCl_2$ and NH_4Cl (47).

Inactivation by chemical agents.--To test the effect of trypsin on phage protein 0.5 ml of purified phage in phage buffer, 1 x 10^{12} p.f.u./ml., was added to 4.5 ml. of a solution of 0.1 M-NH_4HCO_3, pH 7.8, having 20 µg. of trypsin per ml. The samples were incubated at 37° for various time intervals and titered. To test the effect of urea, samples of 0.1 ml. of phage lysate in PAB, 1 x 10^{10} p.f.u./ml., were added to 0.9 ml. of 8M-urea and incubated at various temperatures and for various times. Other samples were likewise treated in urea made alkaline, pH 8.9 with 1N-NaOH. To study the effect of SDS treatment on phage 0.1 ml. of phage lysate,

1 x 10^{10} p.f.u./ml., was added to 0.9 ml. of either 1.0% or 10% SDS and titered either after 10 min. at 25° or after 1 min. of boiling. Phage were also subjected to varying concentrations of dimethyl sulfoxide (DMSO) according to the procedure of Cummings (10). To test the effect of guanidine hydrochloride (GuHCl), a protein denaturant, on phage structure, 0.5 ml. of phage in phage buffer, 1 x 10^{12} p.f.u./ml., was added to 3.5 ml. of either tris-HCl buffer, pH 7.0, or to 6M-GuHCl and incubated at 52° for 90 min. This material along with blanks of tris and of GuHCl were spun at 35,000 r.p.m. in a SW 65 rotor for 3 hr. The OD at 280 mμ was observed using a Gilford recording spectrophotometer.

Freezing and thawing.--A 1 ml. sample of purified phage diluted to 1 x 10^{10} p.f.u./ml. with phage buffer was subjected to repeated cycles of freezing in a dry ice-acetone solution and thawing in a 50° water bath. Samples were titered after each of 8 cycles of freezing and thawing.

CHAPTER III

RESULTS

Growth properties

Of the several alternative methods for measuring rates of phage adsorption outlined by Adams (1) only enumeration of surviving bacteria yielded meaningful data for UC-1. All attempts to look for a decrease in the number of unadsorbed phage in an adsorption mixture that had been either filtered or centrifuged to remove cell-phage complexes failed. In all such cases no loss in titer was ever noticed. This indicated that the adsorption rate was near or below the sensitivity of our phage assay system. Phage assay systems are inprecise at best, but repeated titering of UC-1 stocks typically yields very irregular results. If surviving cells are counted after the adsorption mixture had been treated with an antiserum that inactivated 99% of the free phage, the results indeed indicate a low adsorption rate. The Poisson distribution formula, $P(r) = \frac{m^r e^{-m}}{r!}$, was applied to this data with the ratio of surviving cells to total cells being P(r) and m equal to the m.o.i. The total number of adsorbed phage is taken as m x total cells. The present

adsorption then is adsorbed phage/total phage x 100. From such calculation the adsorption rate is calculated to be 1 - 10% of the input phage after 10 min. at 37°. This rate increases only 19% over the temperature range of 37° to 42° and is thus shown to be more or less insensitive to temperature effect. To see if there might not be some ion or co-factor required for fast efficient adsorption these tests were performed in the presence of Mg^{++}, NH_4^+, Ca^{++}, Fe^{++}, Na^+ and casamino acids in concentrations of 10^{-3} to 10^{-1} M. No increase of adsorption was observed in any of these assays. To test the hypothesis that perhaps the 10 min. adsorption value represented only a small portion of the phage which were capable of rapid and efficient adsorption, while a large portion of the remaining phage would adsorb at a slower rate the adsorption mixture was allowed to stand at 4° overnight, and then tested for adsorption. No appreciable increase in total adsorbed phage was noted, however.

Single cell burst size experiments provided the first indication that two strains of UC-1 had been isolated. One strain (UC-1R) was shown to have an average burst size of 196, which was approximately twice the burst size of UC-1C (Table 1). The burst size data from each separate experiment were analyzed according to Adams (1) using the Poisson distribution formula. In addition all of the experiments performed with a given strain were considered as a single population and the burst size recalculated on this basis. For each strain, values arrived at from the individual experiments,

from the average of all experiments, and by considering all experiments as one population are in fairly close agreement. Table 1 shows these averages, and the estimated distribution within each experiment of the number of singly, doubly and triply infected cells in each experimental population. The burst size values reported by Ruth (39) (186 for UC-1R and of 93 for UC-1C) are in substantial agreement with these results.

One step growth curve data averaged from four separate experiments with UC-1R at 37° shows a mean latent period of 27.5 minutes (Figures 1, 2, and 3). This data was analyzed via probit analysis as suggested by Adams and Wasserman (2). To calculate the percent yield of phage the titers of aliquots taken up to 18 min. after initiation of infection were averaged. This value, considered as 0%, was used as a correction factor and subtracted from all subsequent titers. Titers of aliquots taken after 33 min. post-infection were averaged and considered as 100% yield. Intermediate values were used to calculate % yield. The decimal equivalent of these percentages were converted to probit values using the tables of Fenney (18). A graph of phage release as a function of time approximates an integral of a normal frequency distribution. If plaque counts are plotted versus time this curve typically assumes an S shape that is approximately linear about its inflection point (of the corresponding frequency distribution) from 20 to 80% of the terminal counts. A probit plot transforms

this integrated normal distribution from an S shaped curve into a straight line (Figure 3). The probit of the proportion of phage yield is defined as the ordinate which corresponds to a probability P in a normal distribution with a mean of 5 and a variance of 1. Therefore a probit of 5 corresponds to the mean of the distribution and 99.7% of all values in the distribution fall between probits 2 and 8. The reciprocal of the slope of a probit plot is equal to the standard deviation of the distribution. Any systematic, non-random heterogeneity in a population of infected bacteria appears as a divergence of probit points from linearity.

Figure 3 shows a plot of probit points from four separate experiments run with UC-1R at 37°. From this figure a mean, latent point, which is defined as the time at which 50% of the total phage have been released, is taken at 27.5 min. The slope is 0.425 and its reciprocal, which is the standard deviation of burst time, is 2.4 min. Therefore, 68% of all bursts occur between 25.1 and 29.9 min. while 95% of all bursts occur between 22.7 and 32.3 min. There appears to be a minimal amount of scattering of points and this indicates little systematic non-randomness in the results.

A probit plot of bursts from UC-1C at 37° shows an identical mean latent period (Figure 4), but because the slope is less steep (0.333) the distribution is over a longer time period, i.e., 68% from 24.5 to 30.5 min. and 95% from 21.5 to 33.5 min. The one step growth curve data of Ruth (39) was

converted to proportion of total yield and probit plots made. Values calculated for mean latent periods from her data are 28.5 min. at 37° and 25 min. at 42° for UC-1R and 25.5 min. at 37° and 18.5 min. at 42° for UC-1C. These calculations which indicate the occurrence of shorter mean latent periods for both strains at 42° are in agreement with her conclusion (39). The values obtained through this method of calculations differ somewhat from the mean of 26-27 min. reported by her as taken from plots of plaque count vs. time. These probit points were somewhat scattered from linearity. This may be attributed to too few data points per experiment.

Figure 6 and Table 2 show the results of experiments where different age cells were infected and the process of lysis monitored for four hours. Final phage yields and surviving cells were also counted. The extent of lysis occurring during the experiment was monitored via OD measurement. A significant drop in OD is seen by 1.5 hr. post infection for cultures infected after 1 to 2.5 hours growth. No decrease in OD was noted for cultures infected after 3 to 4 hours growth. The 3 and 3 1/2 hr. cultures increased in OD until they were 4 hr. old at which time they reached a plateau. The cultures infected at 4 hrs. also reached a plateau at that time. The 1 to 2 1/2 hr. old cultures increased in OD to a maximum 1 hr. post infection, after which all except the cultures infected after 2 1/2 hours growth rapidly dropped in OD within 1/2 hr. These 3 cultures

eventually dropped to approximately the same level. The 2 1/2 hr. culture maintained its maximum OD for 1/2 hour and then it too rapidly decreased to a final OD somewhat above that of the cultures infected earlier.

A maximum phage yield of about 1 x 10^9 p.f.u./ml. was obtained from the 1 to 2 hr. cultures. Somewhat lower numbers were obtained from the cultures infected after 2 1/2 to 3 1/2 hrs. growth. The yield from 4 hr. cultures represents only a doubling of the input phage.

The final yields of viable cells for the 1 to 2 1/2 hr. cultures are low (from 1-5 x 10^4 cells/ml.) and approximately equal. The final cell count for the 3 to 4 hr. cultures shows a jump to a level of approximately 2 x 10^8 cells/ml. In all these counts only typical E. coli K-12 colonies were considered. On the 2 1/2 and 3 hr. final cell count plates, and on 3 1/2 hr. plates in another experiment, many minute ragged edged, translucent colonies appeared. These subsequently failed to grow when sub-cultured in either PAB or on nutrient agar plates. These final cell count numbers do not at all correspond to the cell population related to OD measurement as performed on the uninfected growing cells prior to phage addition (see Figure 5). In a subsequent experiment, which followed the same OD pattern of this one the final cell population was also determined by direct count using a Petroff Hausser chamber. For the 1 and 1 1/2 hr. cultures only cell debris was noted. For the 2 1/2 to 4 hr. cultures a number of

cells were counted that approximately equaled the initial population at infection. At 2 hrs. the cells counted equaled 1/4 of the initial level at infection.

Antiserum produced in rabbits for inactivation of unadsorbed phage in various growth studies was analyzed according to Adams (1) for its ability to inactivate UC-1. Phage were exposed to various dilutions of antiserum for 5 min. at 37°. Undiluted antiserum inactivated 99.93% of the phage. The levels of inactivation obtained with other dilutions were: 1:10, 99.53%; 1:100, 98.96%; and 1:1000, 91.89%. The 1:10 dilution was used routinely in these experiments. The k value of this antiserum dilution was 10.7 when calculated by the formula of Adams (1), $k = 2.3\ D/t \log P_o/P$ where D is the dilution, t is time of exposure of phage to antiserum and P_o and P represent the initial and final phage counts.

Production

As a result of trying several alternative methods of phage production and purification several observations can be made both about the process in general and about a number of the individual operations (Figure 7). The batch fermenter method is best in terms of uniformity of yield, and, because of the higher cell population achieved due to efficient aeration, the phage yield is also higher. However the use of multiple flasks is also quite efficient, and this method has the advantage of requiring very little personal attention. Twenty to thirty flasks can be readied for autoclaving in

30 min. and from then on one need only attend to inoculating with cells and phage. The fermenter requires 3 hours of constant attention to prepare it for incubation with sterile media.

The Sharples is superior to the Sorval system for continuous flow centrifugation of the lysates to either clear cell debris or to collect phage precipitate. The Sharples has a faster flow rate, is easier to set up and take down, and it has a larger capacity for precipitate collection. The various filtering materials used to clear and sterilize lysate failed to show any appreciable adsorption of phage. For small samples, such as preparations of labeled phage, collecting by ultracentrifugation is rapid and efficient. In the Spinco 19 rotor run at 19,000 r.p.m. 80% of the phage pelleted in 1 hr. and 91% pelleted in 4 hr. While not actually monitored the 42 rotor is even more economical of time even though it has only 1/2 the volume capacity.

Of the two precipitation techniques both are efficient and yield material with a high titer of phage. Ammonium sulfate at 40% saturation brings down 10^3 p.f.u./ml. of the phage present. Sixty percent would decrease the lysate by 10^7 p.f.u./ml. Freon 113 is very effective in removing a large portion of the non-phage material precipitated by the addition of $(NH_4)_2SO_4$ but it is necessary to hand shake the freon-precipitate mixture because use of a waring blender reduces the titer by 30%. The Freon itself has no noticeable

effect on the viability of the phage. The use of PEG appears to be the best procedure available for concentrating large volumes of phage lysates quickly, completely and gently. Twelve percent PEG results in 100% phage recovery in the precipitate which is easily removed by low speed centrifugation (Figure 8). It seems that PEG unlike $(NH_4)_2SO_4$ does not co-precipitate large amounts of non-phage protein from the crude lysate. Thus the need for Freon extraction is eliminated. PEG may be easily removed from the phage precipitate in a number of ways (49). Addition of an equal volume of chloroform precipitates the PEG which may then be pelleted with low speed centrifugation. The phage may be removed from the PEG by a high speed centrifugation run, e.g., 1 hr. at 42,000 r.p.m. in the 42 rotor. Direct use of the PEG-phage material in a suitably designed isopynic gradient results in banding of the phage with the PEG remaining at the top of the gradient. Finally the PEG can also be removed by applying the PEG-phage precipitate to a Sephadex G-75 column where the PEG is included by the gel and the phage comes out in the void volume.

The various phage precipitations have all proved quite stable in regard to titer over a period of at least two years. This is true for phage lysates in PAB, nutrient broth with 0.5% NaCl or concentrated phage in phage buffer. However it was found that the omission of 0.5% NaCl from either the nutrient broth or phage buffer resulted in a rapid loss of

titer. These preparations while normally kept at 4° have also proved stable at 25° for at least a week.

Physical and chemical agents

Tests involving exposure of phage to heat, sonication, varying pH's, ghosting techniques and various chemical agents were performed both to further characterize UC-1 and to gain knowledge useful in designing further experiments. In those experiments where the kinetics of inactivation were determined, the following general rate constant formula was applied: $k = (\ln N_o/N) \times 1/t$ (34).

In all cases exposure of UC-1 to temperatures up to 65° for 30 min. resulted in titer drops of little more than 10%. Treatment at 65° for further time periods resulted in a final drop of 90% by 60 min. (Figure 9). Exposure of UC-1 to 75° showed a more drastic drop in titer, 10^5 fold for 30 min. and 10^6 fold for 45 min. A graph showing the kinetics of heat inactivation of UC-1 at 75° is presented in Figure 10. One noticeable feature is the change of slope that occurs, for 1/2 min. to 5 min. the k value is 1.26, for 5 min. to 45 min. it is 0.123, and for 45 min. to 120 min. it is -0.012. This 10 fold change in k at 5 min. indicates a heterogeneity in the phage population with regard to heat resistance. The significance of the negative k after 45 min. is unknown. EM monitoring of samples under varying treatment failed to show any noticeable changes in morphology in the phage population.

Figure 11 shows the drop in phage titer vs. time for sonic treatment. As with heat treatment a discontinuity of slope and thus in k values appear. For the 0 min. to 2.5 min. period the k value is quite high, 4.23. For the period from 2.5 to 4 min. the slope is still steep but appreciably less than the initial slope. The k value here is 1.53. As in heat treatment there appears to be a change in sign of the slope for the terminal phase of the treatment 4 min. to 5 min. EM monitoring of this treatment also failed to reveal any gross morphological changes. These variations in slope may also be attributed to heterogeneity in the phage population.

Phage exposed to buffers of varying pH's showed no drop in titer from pH 10 to pH 5.0 (Figure 12). Around pH 5.0 - 4.0 a slight drop was seen and at a pH of 3.6 a drop of more than 10^5 was noticed. The same degree of inactivation was seen with the use of succinate buffer or with a neutral solution adjusted to pH 3.6 with HCl. Therefore this is probably a true pH effect and should not be attributed to any ion peculiar to one of the buffers. In another experiment the use of glycine-HCl buffer, pH 2, resulted in a drop of 10^6 in titer. The low pH effect at pH 3.6 was found to be irreversible even after overnight dialysis against pH 7.0 phosphate buffer. As in sonication studies no morphological differences were noted among the phage samples examined with the electron microscope. A graph of the kinetics of low pH

treatment, Figure 13, shows again a diphasic drop in titer. The k value for the titer drop from 0 to 1 min. was 9.78 and from 1-6 min. it was 0.052. Thus there seems to be a heterogeneous phage population with regard to pH 3.6 inactivation.

Table 3 shows the loss of titer due to treating UC-1 according to a number of different ghosting techniques. The method of Herriot and Barlow (23) which typically yields 99% ghosts when applied to the T-even phage proved quite ineffective with UC-1. Likewise osmotic shock using 66% sucrose, or treatment with LiCl failed to produce over 10% ghosts. The use of $CuCl_2$ and NH_4Cl or $Na_2P_2O_7$, both methods applied to λ (47) proved effective yielding 99.9% ghosts. Further study of $Na_2P_2O_7$ showed that a 99% yield was achieved in 1 min. and 99.9% yield in 4 min. Here EM observation confirmed the presence of a large proportion of ghosts in the population of phage showing 99% or more drop in titer.

Guanidine hydrochloride is a protein denaturant that acts by attacking hydrogen bonds (43). Table 5 gives the result of OD_{280} mμ measurement made on phage in GuHCl or in a tris, pH 7.0, control. Phage treated with GuHCl and tris as outlined in materials and methods showed twice as much 280 mμ absorbing material in the pellet from the tris treatment as in that treated with GuHCl. Correspondingly there was 1 1/2 times as much 280 mμ absorbing material in the GuHCl supernatant as in the tris supernatant. This is taken to mean that GuHCl effects a protein denaturation that results in

phage structural proteins disassociating and becoming soluble and thus non sedimentable under the conditions used to pellet whole phage.

Treatment with trypsin, an endopeptidase, did not produce any noticeable drop in titer even after a 60 min. incubation. This indicates that the native phage proteins are folded and aggregated so as not to have susceptible lysinyl or arginyl bonds exposed to the trypsin for cleavage.

As shown by the data in Table 4, 8M urea at 25° failed to denature UC-1. One hour at 37° did affect a 10^{-3} drop in titer and a like treatment with alkaline urea produced a 10^{-5} drop in titer. Urea, like GuHCl, is a protein denaturant attacking hydrogen bonds (43). The data, however, indicates that it is not as effective by itself as GuHCl and produces its full effect only at an elevated temperature and in alkaline solution.

The use of SDS, the same denaturant used to disrupt phage for gel electrophoresis, at either 1% or 10% (w/v) concentration, produced approximately a 10% drop in viable phage after 10 min. at 25° but at 100° 1 minute of treatment was sufficient to kill all phage. However 1 minute of boiling alone achieves the same result. It thus appears the SDS by itself is unable to effectively denature the phage protein subunits. However, results of gel electrophoresis of phage prepared by boiling in 1% SDS indicate that SDS does prevent reaggragation of disrupted protein structures.

Dimethyl sulfoxide is a reagent that has proved invaluable in several laboratories studying phage structural proteins. Cummings, Chapman and DeLong (10) have shown that DMSO at 67% (v/v) or greater concentrations selectively causes a disjunction of phage tails and heads. This was shown for T^4 and λ. In our hands UC-1 showed a three-fold drop in titer when exposed to 55% DMSO, a 10^2-fold drop with 60% and with 75% DMSO over a 10^7 drop. EM observation made on the phage treated with 75% DMSO showed only tailless heads. Tails were not observed in the preparation.

Repeated cycles of rapid freezing to -70° and thawing in a 50° water bath failed to produce more than a 33% drop in titer even after 8 cycles of such treatment.

Protein studies

In an effort to study individual phage structural proteins a number of variations of the basic techniques suggested by Maizel (31) were tried. Purified phage in phage buffer, 5×10^{12} p.f.u./ml., were disrupted by treatment with 1% SDS and 8M urea with 0.1% 2-β mercaptoethanol (ME) and applied to 7.5% (w/v) acrylamide gels having the same SDS and urea concentration. These gels were run at approximately 10 mA/gel for 3 1/2 hr. After staining only four distinct bands appeared. If ME was omitted the non-reduced material showed two additional bands. It was later learned that the same patterns were seen if 8M urea was omitted from the gels and the disruptive treatment of whole phage.

In order to test whether the 7.5% gel was giving a complete resolution of all of the different protein species present in the native phage structure, a 10% gel system was tried. In this case phage were disrupted with boiling in a solution 1% in SDS and 0.1% in ME. These gels were run at approximately 10 mA/gel for 4 1/2 hours and then stained. With this system ME reduced phage protein showed 8 bands. In order to test the completeness of the disulfide bond reduction method proteins were carboxymethylated (31) and run in a gel system not having ME in the buffer. With carboxymethylation the same 8 bands were seen in the same relative intensity and at the same relative positions. In order to see if there might not be a tendency for disulfide bond formation between some of the protein moieties, proteins were disrupted and run in non-reducing conditions, i.e., no ME. In this case two additional bands were noticed (Figure 14). The same patterns of stained band position and intensity were seen with several different UC-1 preparations. This was taken as evidence that what was seen truly represented only phage proteins and that all of the different proteins were being observed.

In order to characterize the phage proteins as to molecular weight the 10% gel system was standardized with a series of proteins of known molecular weights. These markers were treated in the same manner as the phage proteins. A plot of the relative mobilities of these markers vs. the log

of their molecular weights (48, 17) gave a straight line (Figure 15). RNase is consistantly slightly displaced from this linear relationship, but this is taken to mean that with a molecular weight of less than 14,000 daltons one is at the lower limit of reliability of the 10% gel system (41, 48). From this graph the molecular weights of the phage proteins were obtained by plotting their relative mobilities. Table 6 gives the molecular weight of the standards, the 8 phage proteins, and the 2 additional bands seen on non-reduced gels. This table also includes the average mobility of these proteins along with their ± % deviation in mobility as seen in 4 or more separate runs for each, except the non-reducing gels where only two runs were made. With the coomassie blue staining procedure used (31) protein bands containing as little as 0.5 µg could be seen. The standards ran with ± 3% variation in their mobilities except for BSA which had a ± 6% deviation. With several of the standards used minor bands were noticed. Because several of these were B grade preparations these were taken to represent impurities. The large variations of up to 10% seen for some of the phage proteins was probably due to either overloading or some sort of interaction between the separating proteins. In order to have enough of a minor protein band present it was often necessary to overload the gels with respect to those proteins present in larger amounts. The molecular weight in daltons for the viral proteins (VP) numbered 1 through 8 (VP-1

through VP-8) were: VP-1, 115,000; VP-2, 93,500; VP-3, 68,200; VP-4, 59,500; VP-5, 51,800; VP-6, 36,300; VP-7, 26,300 and VP-8, 15,700. The two additional bands with non-reduced protein designated VP-A and VP-B had molecular weights of 104,000 and 76,500 respectively. These could very well be dimers of VP-5 and of VP-6 i.e., VP-5+5 and VP-6+6.

Incorporation of C^{14} amino acids

Phage infected cells vs. uninfected cells.--To test the effect of phage infection on the kinetics of protein synthesis the uptake of ^{3}H labeled L-leucine was monitored. Figure 17 shows the course of label uptake into TCA precipitable material by normal cells and phage infected cells. The curve for normal cells showed a continual ^{3}H-L-leucine incorporation for a period of over 60 min. Phage infected cells showed a dramatic cessation of label uptake starting at least by 1 min. after infection. This low level of counts in the TCA precipitate remained constant throughout experiments of up to 60 min.

Experiments designed to test the effect of varying the m.o.i. on label uptake were performed. Table 7 shows the initial vs. final count of H^{3} in TCA precipitate in cells infected with m.o.i.'s of 0.1 to 100. The trend shows that the higher m.o.i.'s have an increased depressing effect on label uptake. No EM observation was made of the phage stock used so there was no idea of how many ghosts there were per

infective phage. A measure of infective phage was taken as the basis for m.o.i. calculation.

In order to try to set up a Hosoda and Levinthal (27) type experiment, where sequential differences in phage directed protein synthesis could be monitored, label uptake was monitored in UV inactivated cells. The UV dosage was sufficient to destroy all colony forming ability in the cell population employed. Figure 16 shows the H^3-TCA precipitate levels of phage infected and uninfected UV treated cells. The trend seen is typical of several such experiments reported in the literature for Ø x-174 (20). The uninfected cells show a higher level of label uptake over phage infected cells up to one minute of UV exposure. Thereafter the phage infected cells showed an increased level of label uptake. Unfortunately it was never possible to set up conditions wherein the counts from phage infected cells would be 10 times or more over the counts from uninfected cells. It was felt that such a failure precluded any further attempts to monitor differences in specific protein levels between the two cell systems.

CHAPTER IV

DISCUSSION

One of the primary purposes of much of the initial work described here was to further characterize the phage UC-1 and to differentiate it from other coliphages. Much of the data therefore is purely descriptive. However, as a result of these studies on growth and structure coupled with the results of other workers in this lab (3, 28, 39), UC-1 can now be considered as unique, at least among the more commonly studied coliphages. Much of the information gathered from these studies will be useful as a basis of further investigations.

The low adsorption level observed for UC-1 is not typical of most coliphages. Because the total percent of phage adsorbed does not increase with time, even after 12 hr. at 4^{o}, there seems to be only a small proportion of the phage population, 10% or less, that is capable of being absorbed in broth. But obviously there does exist 10 times this number of phage that adsorb because calculations of percent adsorption are based on the total phage population as determined by plaque counts of the parent phage stock. Thus it is

possible to speculate that phage adsorption may be more efficient on plates, due either to some phenotypic difference in the cells or to the physical nature of the environment. It is known from studies of cell age vs. phage yield that only log phase cells are capable of high phage yields (Table 2, Figure 6). Perhaps this too is due to a physiological state that facilitates adsorption. While no co-factors were discovered that might increase the percent of phage adsorbed, again it is possible to speculate that the agar itself or the cells in the agar environment may provide such a co-factor.

The results of the single cell burst experiments were the first indication that two strains of UC-1 had been isolated. The work of Ruth has served to further differentiate these strains as to their growth properties (4): comparison of single cell burst size, one step growth curves, rates of plaque appearance and restriction patterns on E. coli strains B, C and K-12 all conducted at 37°, 42° and 25° for both strains. While no exact information is available as to how the two strains arose it is likely that UC-1R arose as a mutant of UC-1C and that, through the course of several passages the larger burst size of UC-1R gave it a selective advantage over UC-1C, causing it to become the dominant type in that subculture. This difference in burst size is a very stable characteristic and has been seen consistently in a number of the experiments reported here, and by other workers (39).

A point of interest is the way in which these experiments were designed. Single cell burst experiments were performed prior to fruitful adsorption studies so an estimate had to be made as to the percent phage that would adsorb in 10 min. at 37° in order to set up dilutions that would result in less than 50% of the plates having plaques. An estimation of 10% or less adsorption proved to be quite a good working assumption. This value was later confirmed by the adsorption studies that use as a basis of calculation the surviving cell population.

Probit analysis of the one step growth curve data indicates that the phage populations studied were homogenous as to their mean burst time (27.5 min.). In my hands both strains showed the same mean latent periods, and the same degree of homogeneity. There was a difference between the strains, however, as to the standard deviations of their burst times. This may represent another significant difference between the two strains. When the one step growth curve data of Ruth were subjected to probit analysis, the two strains showed a difference in mean latent period. Because her experiments were designed to give minimal latent periods many data points were taken in the upper and lower plateau regions and few through the rise. It is possible that there were too few probit points to yield a valid conclusion. Another factor indicating this was the high degree of scatter of these points, especially for UC-1C.

The experiments dealing with cell age vs. viral production have provided some intriguing results. While the limited evidence at hand does not allow for a definitive explanation of all of the phenomena seen, it is nevertheless possible to suggest some likely hypothesis. It will be mandatory to further investigate this area before a full understanding of the phage-host relationship pertaining to UC-1 is grasped. As is common to many viruses, the host cells show a phenotypic change from susceptibility to resistance with progressive aging of the culture. Because the surviving cells from this experiment produce typical mucoid colonies they probably do not adsorb phage due to a masking of the adsorption site. This sort of mucoid resistant cell is seen throughout the growth cycle of the cells. What is unusual in this case is that their numbers remain fairly constant, increasing only at the very end of the growth cycle (Table 2). It would be expected that, if the resistant mucoid cell population remained a constant percent of the total population, that the absolute number of such resistant cells would increase with time.

By far the most striking result of this study was the appearance of many minute, ragged edged translucent colonies arising from some of the surviving cells taken from cultures infected after 2 to 3 hr. of cell growth. The fact that most of these colonies failed to grow either in broth or on plates, coupled with their appearance, and the time of

occurrence phage resistant cells raises some important questions. Rather they appear to have been somewhat altered either directly by interaction with the phage or by some other factor present during phage production. The occurrence of these cell types only during a well defined part of the population growth cycle argues for some manner of host phenotypic expression connected with this phenomenon. Whatever the effecting factor(s) might be the final result (inability of most cells to produce further growth upon attempts to subculture) is not seen until after several generations. That this inability of further growth occurs irregularly is evidenced by the irregular edges of the colonies. No sign of reversion to a normal cell type was seen as might be evidenced by the appearance of sectored colonies with normal or mucoid appearing areas. Of several possible explanations the most inviting is that of some sort of pseudolysogeny that eventually proves lethal. While no direct evidence of such a phenomenon is at hand, there are precedents. Fraser saw much the same sort of surviving colonies connected with the growth of T3, which she attributed to a pseudolysogeny (19, 24). That lysogeny can alter host surface properties, and thus colony appearance, is also well documented, especially for the Salmonella phage (38).

If these studies had not given such atypical results, they might be considered complete. However, now there is a need for much further work along these lines. Several recent

studies of phage production and cell growth dynamics have been made using various continuous culturing techniques (26, 33). This sort of experiment should be conducted with UC-1 whereby many parameters can be observed at once and over a long period of time. Also it may be possible to correlate some of these results with the appearance of the concentric rings of cell growth that typify the UC-1 plaque.

An analysis of Figures 10, 11, and 13 showing the kinetics of phage inactivation due to heat, sonication and exposure to pH 3.6 respectively reveals breaks in the slopes of the inactivation curves. Such a shift in sensitivity is suggestive of the presence of some sort of subpopulation with regard to sensitivity toward the particular agent used. The more pronounced shift in slope occurs in all cases after the inactivation of 10^4 fold or more of the initial population. These shifts in the slopes of the three inactivation curves all occur within a narrow range, i.e., at a point corresponding to 5-7 x 10^5 p.f.u./ml. surviving phage. This may be coincidental or this may indicate that this 5 x 10^5 p.f.u./ml. survivors are one and the same subgroup. It is quite possible that such subpopulations do exist in natural populations and, if present, might very well be co-isolated and purified along with the major fraction.

Heat can act to either denature the protein and/or nucleic acid constituents of a phage. Short of a complete denaturation of one or more phage structures, an uneven

expansion of some structural protein or of the nucleic acid in the phage head might cause enough of a structural alteration to affect infectivity (34, 36). Evidence has been found that in naturally occurring T1, T3 and T7 populations there exists a heat sensitive sub group having less DNA per phage. Such phage are still fully infective and so would not be selected against by growth and harvesting procedures such as were used for UC-1 (36).

Sonic treatment acts mainly by the effect of cavitation and direct mechanical agitation. Thus structural elements may be broken apart or even dislodged. However, no gross morphological changes were noticed with UC-1 preparations reduced 10^5 fold in titer by sonication. Therefore, the effect is either a minor structural alteration or the removal of some component, possibly an adsorption element. The fact that 10 ml. of phage were sonicated in a reservoir in these studies may allow for the presence of differences in environment during a treatment period. Such irregularities would of course be another explanation for the slope change seen in the inactivation curve.

Inactivation due to ionic environment has been observed in a number of phage, including the T- even (8, 37). In fact either a highly acidic or alkaline environment can be used to structurally disassemble some phage (8, 37). UC-1 shows almost complete resistance to pH over a range of 11 to 4, but a rapid inactivation is observed when the pH drops

below 4. This loss of infectivity is due to a true alteration of the phage. This loss of titer is irreversible and probably reflects an alteration in protein structure. Such a permanent alteration would not be unexpected in light of pH inactivation of some enzymes (14). Also it should be pointed out that conformation of the inactivation at low pH with succinate buffer, acetate buffer and HCl preclude inactivation being the effect of a specific anion.

Ghosting of a virus is achieved by any treatment that results in the release of the nucleic acid from the intact protein capsid. One common method of ghosting is to rapidly shift a phage suspension from a highly concentrated solution to a very dilute solution. Transferring T-even phage from 4M-NaCl to distilled water exerts an osmotic pressure on the phage head equivalent to 90 atmospheres (34). That such treatments do not affect attachment or lysis from without has been demonstrated by several workers (15, 22). A number of specific chemical treatments also give high ghost yields. One of these, $Na_2P_2O_7$, acts to chelate many of the divalent cations associated with the nucleic acid. The genetic material, bereft of much of the forces holding it tightly bound in the phage head, proceeds to leak out of the capsid (46). Not all phage are equally sensitive to any one particular ghosting treatment (16, 23, 47). UC-1 is quite resistant to osmotic shock, indicating either a strong structural association between head proteins, or a head structure rather

impermeable to water. The treatments most successful in producing UC-1 ghosts were exposure either to $Na_2P_2O_7$ or to $CuCl_2$ and NH_4Cl (Table 3). These are the same treatments that have been used to differentiate wild type λ from λ virulent mutants (16, 47). UC-1 responds to these treatments in the same manner as λ virulent which has less DNA per phage than wild type λ . Such a difference in response is probably due to the nature of the DNA to protein ratio which, if the protein constituents are a constant factor, results in different DNA packing arrangements and different internal pressures (47).

The high resistance shown by UC-1 to repeated cycles of rapid freezing and thawing, like its resistance to osmotic shock, points to a firmly bound, impermeable head structure. This resistance may be profitably used in certain cases to release phage from cells that have not as yet lysed. Most cells would readily be broken down by such a regime while the phage would remain intact and infective.

The susceptibility of UC-1 to the various chemical agents tested was typical of that observed in many viruses. Trypsin had no effect. This indicates that within the tightly bound phage proteins the specific lysinyl and/or arginyl bonds were not exposed in such a way as to provide peptide bonds susceptible to the action of trypsin.

UC-1 responded to the denaturants tested in much the same manner or has been reported for other phages. Urea was

effective only at an alkaline pH. GuHCl was also quite effective and SDS coupled with 1 min. of boiling proved to be the best and most completely disruptive agent employed, especially as seen by gel analysis.

UC-1 showed a sensitivity to DMSO that was typical of that reported for other phage such as T^4 and λ (10): 67% (v/v) DMSO results in the disunion of all tails and heads of a phage population. Evidently the nature of the head to tail bond is quite similar among even diverse types of phage. E M monitoring showed that, with UC-1 preparations treated with DMSO of 67% (v/v) or greater, there was almost a 100% yield of tailless phage. Preliminary studies have suggested that the use of a 20-40% (w/w) sucrose gradient might effectively separate the products of such a DMSO treatment. If DMSO treated phage are dialyzed overnight against phage buffer, applied to such gradients and centrifuged for 5 hr. at 23,000 r.p.m. in a SW 25 rotor, three distinct bands appear. Surveying these bands with the electron microscope has revealed whole ghosts in the bottom band, tailless ghosted heads in the middle band, and tails along with contaminating pili like material in the top band. These disruptive and separatory techniques, when used with more highly purified phage, may yield sufficient amounts of pure head and tail protein material to permit differentiation between tail and head proteins on SDS gels.

Disruption of a virus by boiling with SDS and separation of the released proteins by gel electrophoresis to

determine the molecular weights of the protein subunits is a powerful and now excepted technique for viral study and characterization (31). For complete resolution of all of the subunits of UC-1, the 10% gel system proved more effective. If there are a number of lower molecular weight components in a protein mixture, the more concentrated gels often give better resolution and the most reliable molecular weight data (21, 31, 41, 48). In view of the data gathered on some phage that have been studied by this method, both the number of separate proteins and the range of sizes seen for UC-1 seems to be quite acceptable for a phage of this size and morphology (9, 29, 42). While no attempt has been made to arrive at an exact quantitation of the separate bands, even a casual observation reveals that there is a distinct quantitative difference among the different proteins. Viral proteins 6, 7, and 8 appear to make up the bulk of material applied to a gel. Most prominent, based on density of staining, is VP-6 followed by VP-8 and VP-7. Next in prominence, based on relative density of staining and frequency of appearance on gels loaded with different amounts of total protein, are VP-1 and VP-5. Bands representing VP-2, VP-3 and VP-4 are the faintest in appearance. In all likelihood VP-6, 7 and 8 represent major structural proteins of the head and/or tail, with VP-6 being the major head protein unit. The fainter moieties in all probability represent rarer occurring protein units, such as corner units or certain subunits of the tail structure.

The electrophoresis of carboxymethylated proteins serves as a check on the efficacy of mercaptoethanol as a reducing agent for disulfide bands. Since no difference in the overall band pattern was observed after carboxymethylation, it may be assumed that inclusion of 0.1% mercaptoethanol is sufficient to assure the complete reduction of all disulfide bonds. Electrophoresis of non-reduced proteins, in the absence of mercaptoethanol, allows detection of the natural occurrence of interpolypeptide dissulfide bonds. When non-reduced UC-1 proteins were electrophoresed, two additional bands appeared. As already noted the molecular weights of these bands, designated VP-A and VP-B, correspond quite well with what would be expected of diners of VP-5 and VP-6 respectively. That these represent native bonds and not some manner of random aggregation is attested to by the fact that these bands appear consistently and at the same relative positions on all gels run without mercaptoethanol.

There is always the possibility of the existence of some phage proteins present in too small a quantity to be detected by visual means. To detect such a minute structural constituent and to confirm the visual data, gel runs should be made using proteins highly labeled with a mixture of C^{14} amino acids. With the use of H^3 and either C^{14} or S^{35} double labels it would be possible to further confirm the exact nature of VP-A and VP-B by comparing the count ratio of these two proteins with that of the other 8 phage proteins.

Labeling techniques would also lend themselves quite well to electrofocusing on ampholite gels (11). This would serve to further characterize UC-1 as to the number of distinct protein types and their isoelectric points.

Label studies

Studies monitoring labeled amino acid incorporation into TCA precipitable material were conducted in the hope of learning more about the kinetics of protein synthesis associated with phage infection. The results of studies on label uptake into protein using UC-1 infected cells are not what would be expected (Figure 17). While it is normal for a phage-infected cell to show a gradual decrease in amino acid incorporation into protein, this trend occurs over a period of time and uptake in infected cells may even parallel that of normal cells for a few minutes. The kinetics of label incorporation for cells infected with UC-1 are atypical in that there is almost a complete cessation of incorporation which occurs at one minute post infection and lasts for at least 60 min. This sort of kinetic pattern seems to indicate a complete cessation of protein synthesis. This is anamolous for there can be no phage production without protein synthesis. Perhaps the explanation lies in the use of a very high m.o.i. in these experiments, that resulted in lysis from without. The results could also be explained if the infecting phage preparations used contained a large proportion of

ghosted phage. It has been shown that ghosts are quite effective as protein synthesis suppressors (15).

The study of labeled amino acid incorporation by UV treated cells, both phage infected and uninfected, yielded results quite in keeping with the sort of uptake kinetics seen for other well studied phage (Figure 11) (20). Such studies typically show a higher amount of amino acid incorporation by the normal cells over that of the infected cells after short periods of UV treatment. If cells have been UV treated sufficiently to greatly suppress their own protein synthesis, phage directed protein synthesis is taken as the reason for the greater amount of label incorporation seen in these infected cells. This trend is seen in Figure 16. The studies presented in the literature, however, show typically a ten-fold greater label incorporation on the part of phage infected cells when compared with uninfected cells. With UC-1 it has never been possible to achieve more than a two-fold greater label incorporation by phage infected cells. A number of experimental parameters were altered in order to try to achieve a higher label incorporation for the infected cells but all to no avail. These included using a higher m.o.i., preincubation of UV treated cells prior to addition of phage and/or label at 37° for 30 min. and use of 2×10^{-2} M-$MgSO_4$ in the labeling medium as suggested by Ptashne (32).

The fact that any increase of label incorporation is seen in phage infected cells over that seen in non-infected

cells is somewhat at odds with the kinetics of label uptake seen in non UV treated cells. Perhaps in the system as it was designed there were insufficient numbers of infective phage adsorbing to the UV-treated cells to initiate significant levels of phage protein synthesis. It is quite likely that the low adsorption rate of UC-1 in broth is a major obstacle to this type of study.

Table 7 showing the effect of a range of m.o.i.'s on label incorporation introduces even more questions. Unlike in the initial studies, no abrupt, complete cessation of label incorporation is seen. Rather there is a constant but decreased rate of incorporation at all m.o.i.'s. This is somewhat closer to the result expected, based on reported observations with other phage (20). What is not explainable however is the final decrease in level of label incorporation seen with each 10-fold increase of m.o.i. Again it appears almost as if either the phage or some factor in the phage preparation were acting as an inhibitor of amino acid incorporation.

CHAPTER V

SUMMARY

As a result of these studies, coliphage UC-1 has been further characterized both biologically and chemically. UC-1 displays a low adsorption rate to E. coli in broth culture with only 10% of the phage being adsorbed in 10 min. at 37°. As a result of single cell burst experiments, it was learned that two strains of UC-1 had been isolated. UC-1R has a burst size of 186 p.f.u./cell while UC-1C has a burst size of 92 p.f.u./cell. Both strains exhibit a mean latent period of 27.5 min. at 37°. However, there is an indication that the majority of the bursts of UC-1R occur within a shorter time period than do those of UC-1C. When broth cultures of E. coli K-12 cells of different ages are infected with UC-1, only young or vigorously growing cells are capable of producing high titer lysates. An unusual feature of this experiment was the appearance of surviving cells that produced small, translucent, irregular colonies that could not be subcultured. This was taken to indicate that UC-1 might be capable of initiating some type of pseudolysogeny.

Studies of UC-1's susceptibility to heat, sonication and buffers of pH 3.6 revealed inactivation kinetics that point to the possibility of two subgroups of phage with regard to sensitivity toward these agents. UC-1 was shown to be resistant to 8 cycles of rapid freezing and thawing. Of the various techniques employed to produce phage ghosts UC-1 was most susceptible to the use of $Na_2P_2O_7$ or $CuCl_2$ and NH_4Cl. These were the same reagents most successfully used on a λ virulent strain. Both this ghosting susceptibility and the phage's reaction to heat inactivation may be closely related to the nature of the DNA-protein association.

The successful use of DMSO to separate the tail of UC-1 from the phage head, along with preliminary studies of a centrifugation regime to isolate these structural components, points to a possible method of differentiating the proteins and functions of these two structures.

Gel electrophesis of SDS disrupted phage protein has revealed the presence of 8 distinctive viral proteins, 2 of which may form dimers joined by disulfied bonds in the native structure. The molecular weights of these 8 proteins were determined using gels standardized with known molecular weight protein markers. The phage proteins ranged in weight from 115,000 to 15,700 daltons.

Studies using incorporation of C^{14} labled amino acids into TCA-precipitable material as an indicator of protein synthesis yielded some anomalous results when phage infected

cells were compared to uninfected cells. However, the kinetics of label uptake observed for UV treated cells were typical of the results seen for several other phage studied in this manner (20, 27). In unirradiated cells phage infection seemed to be acting more as a suppressor of total protein synthesis than as a modifier which would direct the cell to synthesize phage material instead of cellular protein. These results have not been explained and may be either an artifact of the experimental design, i.e., a high level of ghosts among infecting phage, or they may represent some unusual manner of phage host interaction.

In conclusion, UC-1 has been characterized sufficiently to indicate that it is a new and unique phage, at least among the more commonly studied coliphages.

TABLE 1

BURST SIZE OF UC-1C AND UC1-R DETERMINED BY SINGLE CELL BURSTS

Experiment	Average Number of Plaques/Sample	Total Plaques	Distribution of Infected Cells Among Samples			Burst Size
			1	2	3	
1R	111.4	22	16	5	1	192.2
2R	25.5	6	6			212.5
3R	56.6	13	11	2		187.0
4R	145.9	28	18	8	2	180.0
1C	47.7	21	16	4	1	87.3
2C	31.7	15	13	2		91.5
3C	13.9	6	6			114.5

UC-1R: Average based on four experiments 192.9
Average based on entire population 196.0

UC-1C: Average based on three experiments 97.7
Average based on entire population 92.0

Figure 1.--One step growth curve for UC-1R.

The one step growth curve experiment was performed according to the method of Adams (1). Aliquots withdrawn at various times were diluted according to three different schemes so as to have countable plates for each time interval. The data are plotted as p.f.u./ml. vs. time.

Figure 1.--One step growth curve for UC-1R.

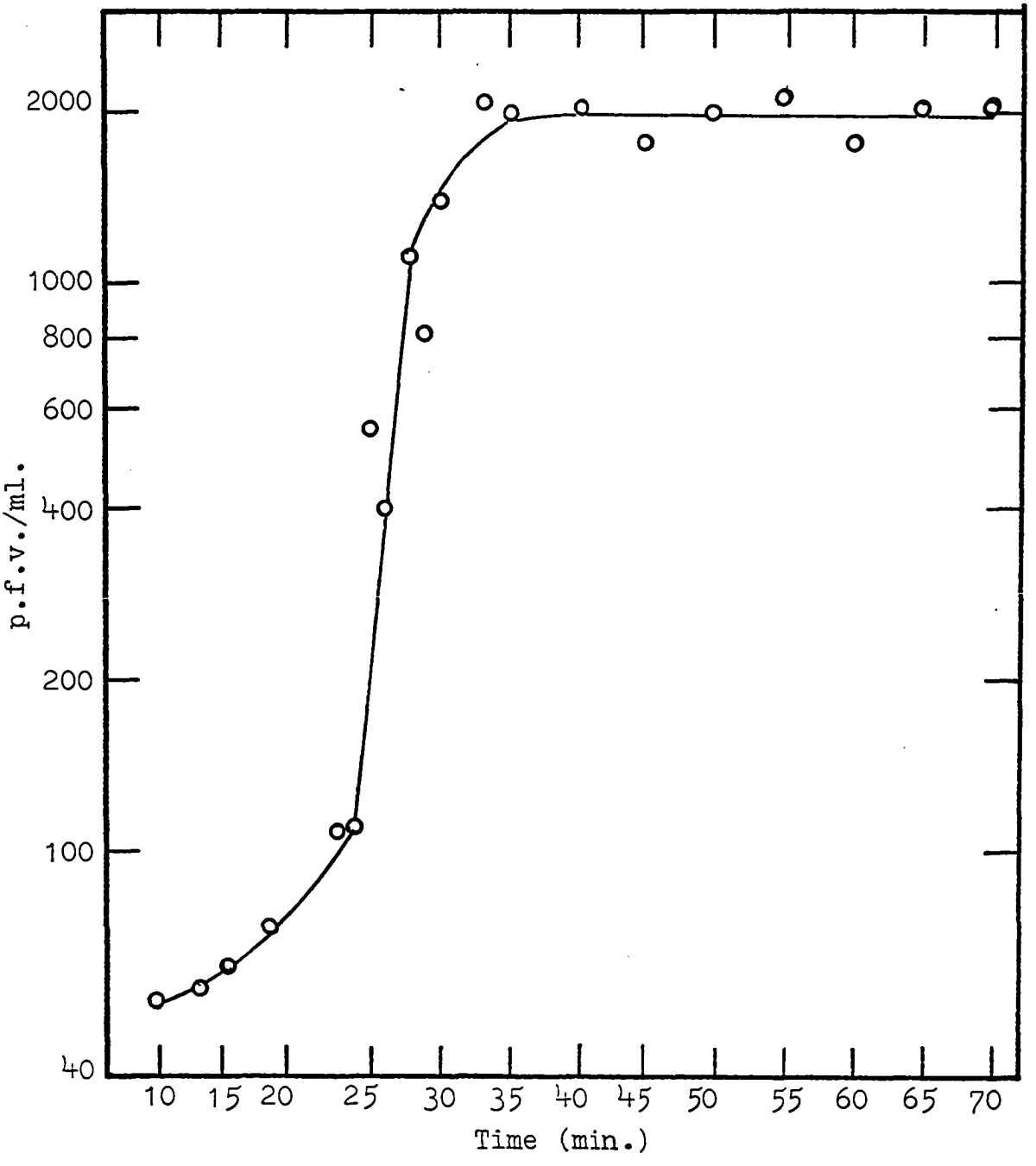


Figure 2.--Determination of mean latent period.

The data from Figure 1 were recalculated and expressed as a plot of percent phage yield vs. time in order to better determine the mean latent period (time of 50% maximum phage yield). The plaques up to 18 min. were averaged and considered as 0%. This value which represents infected cells and uninactivated free phage, was subtracted from all subsequent values. The corrected counts occurring after 33 min. were averaged and this value considered as 100% yield. The percent yield for each intervening time was calculated on the basis of this 100% yield value.

Figure 2. Determination of mean latent period.

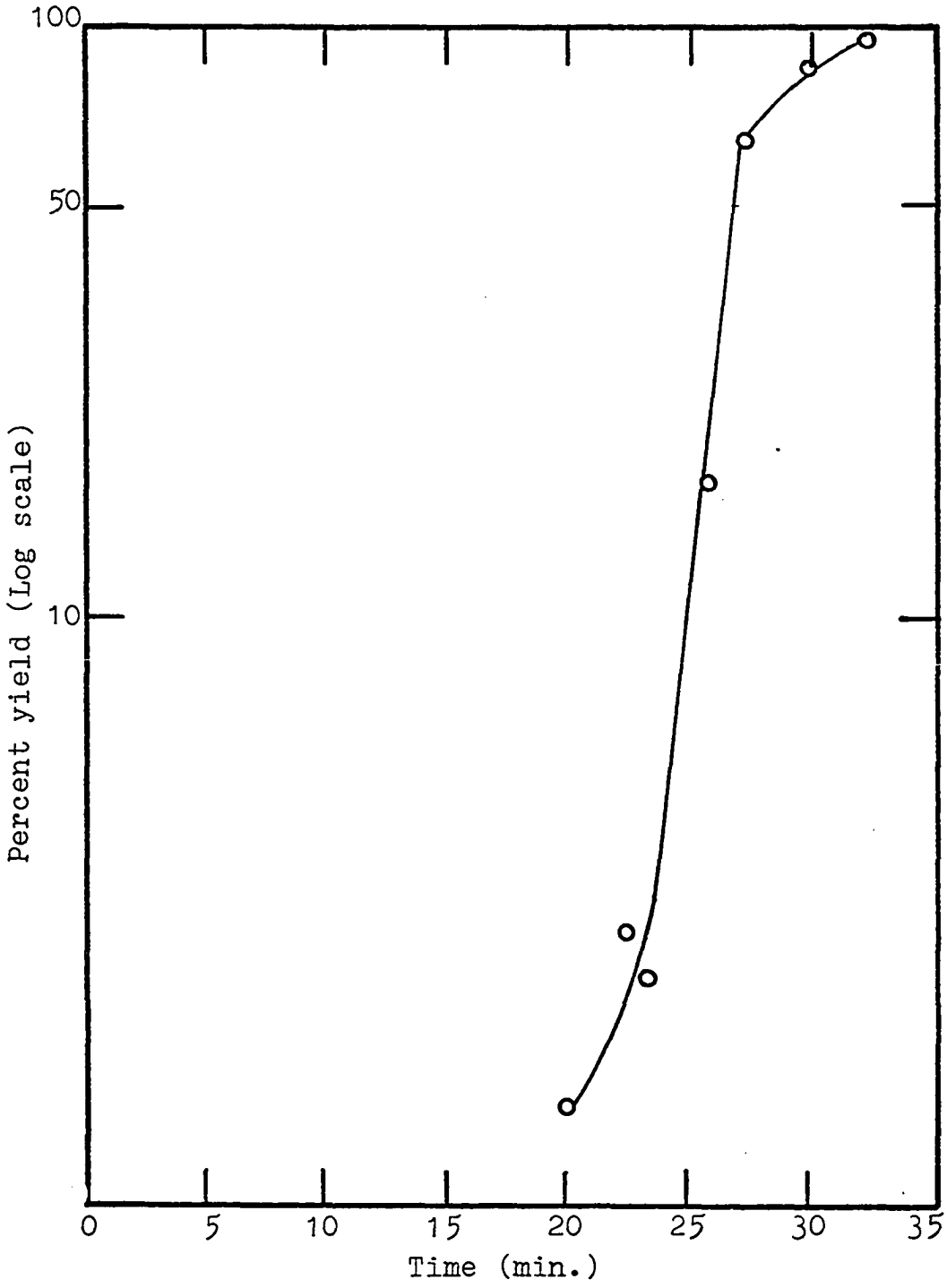


Figure 3.--Probit analysis of one-step growth data for UC-1R.

The data from four different one-step growth curves were converted to probits and expressed in a plot of probit vs. time in order to determine the presence of systematic non-randomness of probit times and to confirm the mean latent period. The probit tables of Finney were used (18). The dotted line intersects the curve at the mean latent period.

Experiment No. 1 o

Experiment No. 2 Δ

Experiment No. 3 ×

Experiment No. 4 ●

Figure 3.--Probit analysis of one-step growth data for UC-1R.

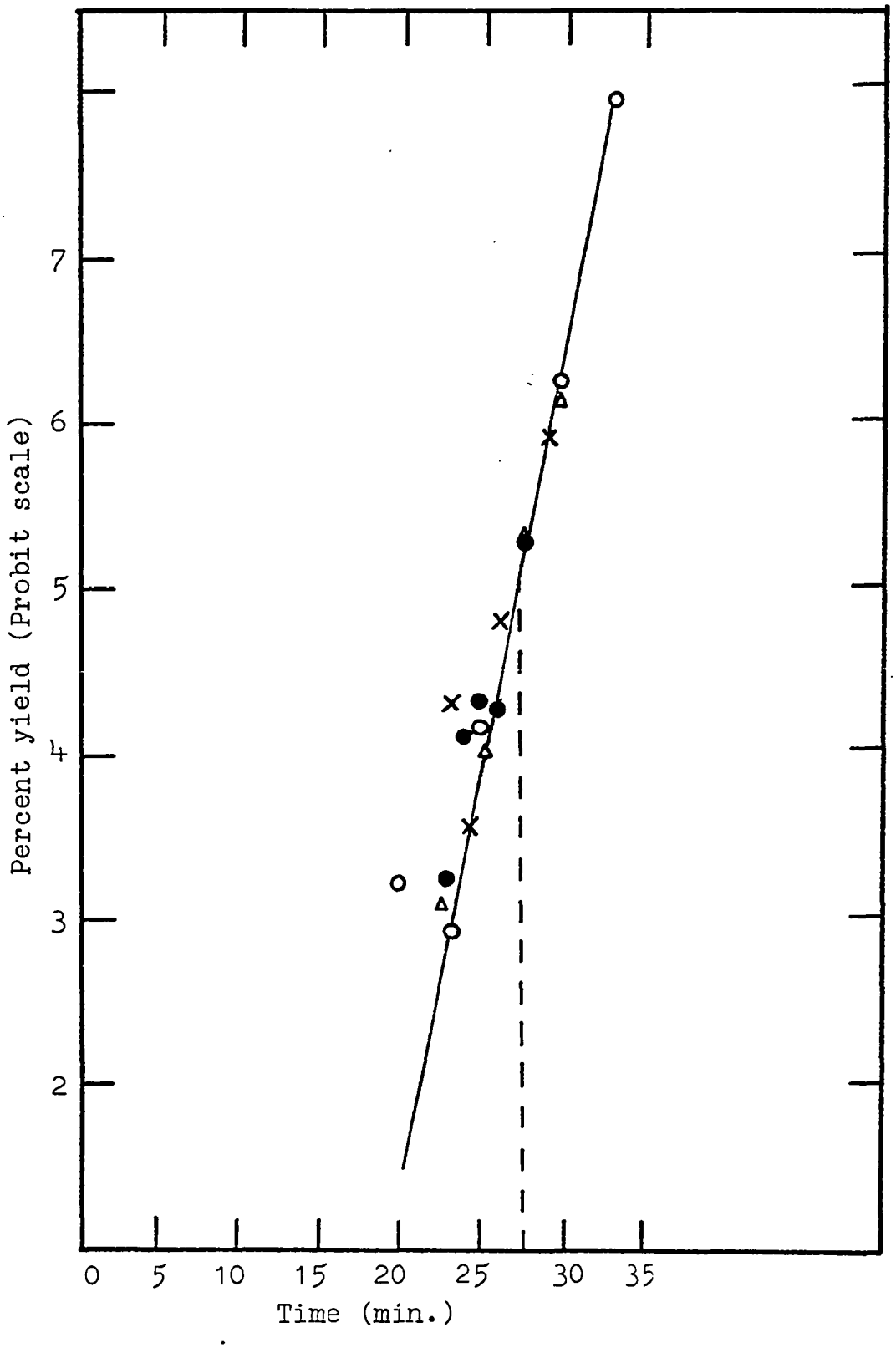


Figure 4.--Probit analysis of one-step growth data from UC-1C.

The data from four different one-step growth curves were converted to probits and expressed in a plot of probit vs. time in order to determine the presence of systematic non-randomness of probit times and to confirm the mean latent period. The probit tables of Finney were used (18).

Figure 4.--Probit analysis of one-step growth data from UC-1C.

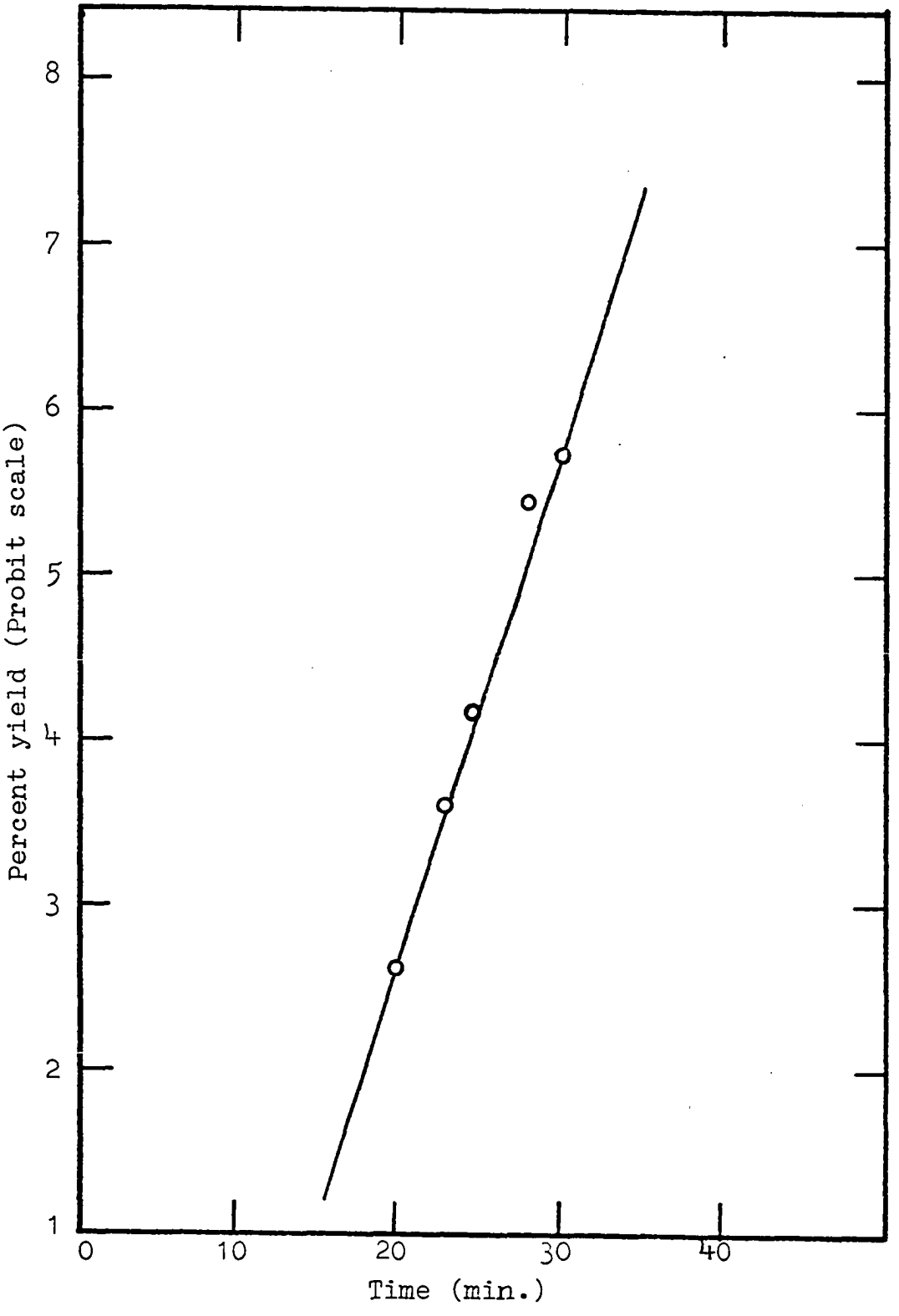


Figure 5.--Relation of viable count vs. optical density of Escherichia coli K-12.

Viable cell counts are plotted against the OD at 420 nm of broth suspensions of E. coli K-12 grown in PAB. OD measurements were made on cell suspensions in tubes 15 mm x 180mm read with a Bausch and Lomb Spectronic 20 Colorimeter.

Figure 5.--Relation of viable count vs. optical density of Escherichia coli K-12.

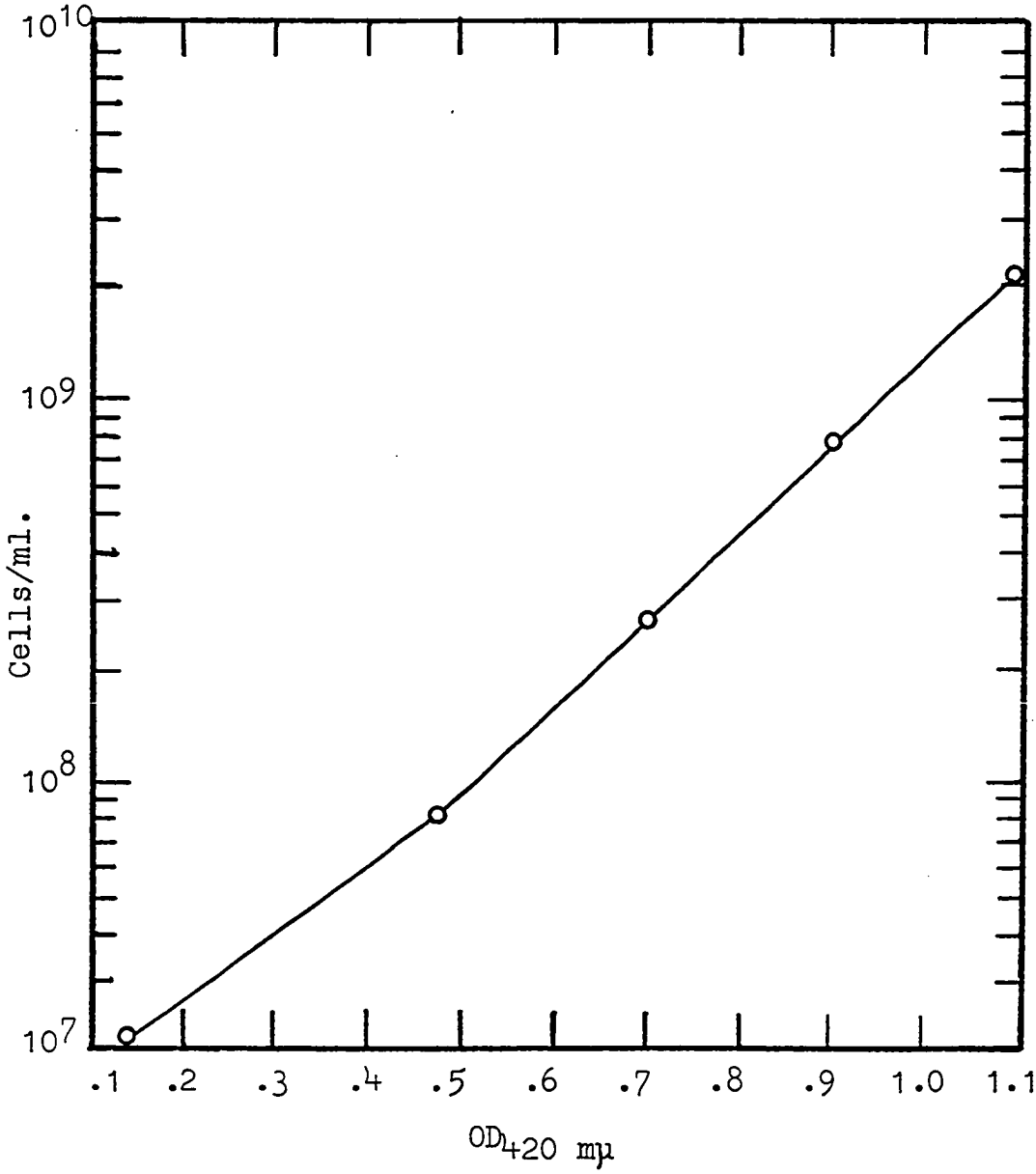


Figure 6.--Effect of culture age on lysis of E. coli K-12 by UC-1.

Studies to determine the effect of cell age at the time of infection on phage production were performed by infecting 10 ml. aliquots of a log phase culture of E. coli K-12 at 1/2 hr. intervals with UC-1 at an m.o.i. of 0.1. The cell culture was initially started with a 1:100 dilution from an overnight culture. Growth of the E. coli culture was followed by monitoring the OD at 420 mμ with a Bausch and Lomb Spectronic 20 Colorimeter.

The abscissas represent time elapsed since the start of the culture. The time unit at the end of each line on the graph represent the time of phage addition after the start of the culture.

Figure 6.--Effect of culture age on lysis of E. coli K-12 by UC-1.

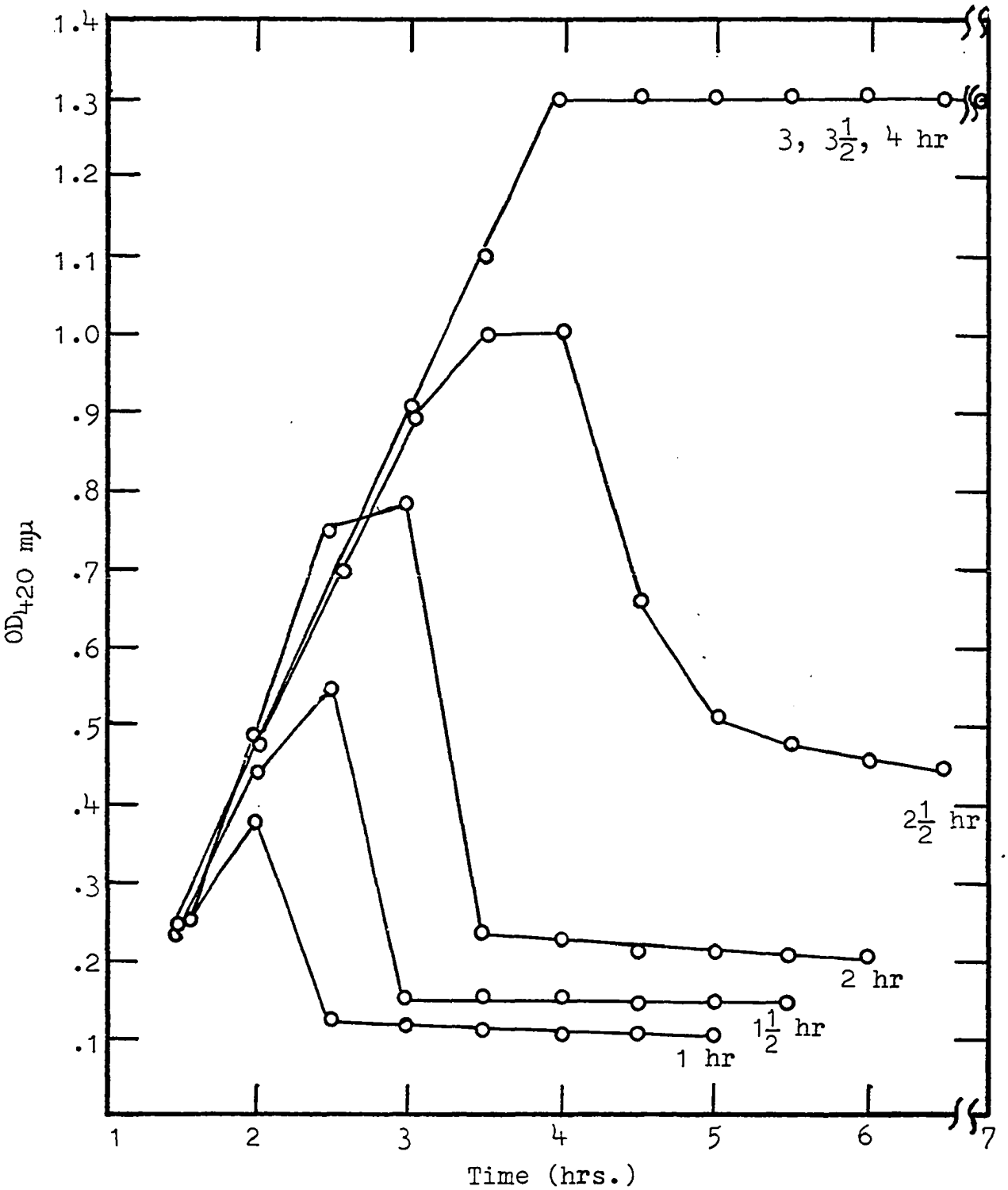


TABLE 2

EFFECT OF CULTURE AGE AT THE TIME OF INITIAL INFECTION ON CULTURE LYSIS AND FINAL PHAGE YIELD

Time (hr.) of Phage Addition	Initial Cells/ml.	Initial OD	Final Cells/ml.	Final OD	Phage Yield p.f.u./ml.	Small Colonies
1	2.2×10^7	.150	5.2×10^4	.110	1.1×10^9	-
1 1/2	4.9×10^7	.245	1.4×10^4	.145	1.2×10^9	-
2	8.3×10^7	.480	3.7×10^4	.210	1.3×10^9	-
2 1/2	2.6×10^8	.700	3.8×10^4	.450	5×10^8	+
3	7.3×10^8	.900	1.2×10^8	1.300	9×10^8	+
3 1/2	1.9×10^9	1.100	2.2×10^8	1.400	4×10^8	-
4	1.9×10^9	1.300	2.2×10^8	1.400	4×10^6	-

Figure 7.--Procedures for phage purification.

Phage lysates were prepared either in multiple 1 liter amounts in shaker flasks or in a 40 liter amount using a batch fermentor according to the procedure outlined in the materials and methods section. After lysis chloroform was added to 0.5% (v/v). For concentration and purification one of the three alternate procedures as outlined was employed. The continuous flow centrifugation of large batches was performed with either a Sharples Super Centrifuge or the Szent-Gyorgyi and Blum continuous flow attachment for the Sorval RC-2 centrifuge. All dialysis was performed against phage buffer in Visking tubing, overnight at 4°. Freon 113 is a trichlorotrifluoroethane obtained from duPont Chemical Company. The polyethylene glycol procedure is basically the method of Yamamato and Alberts (49). For isopycnic banding CsCl was added to phage in phage buffer in an amount according to the formula of Thomas and Abelson (44) to achieve a mean density of 1.51. Purified phage was stored in phage buffer at 4°.

Figure 7.--Procedures for phage purification.

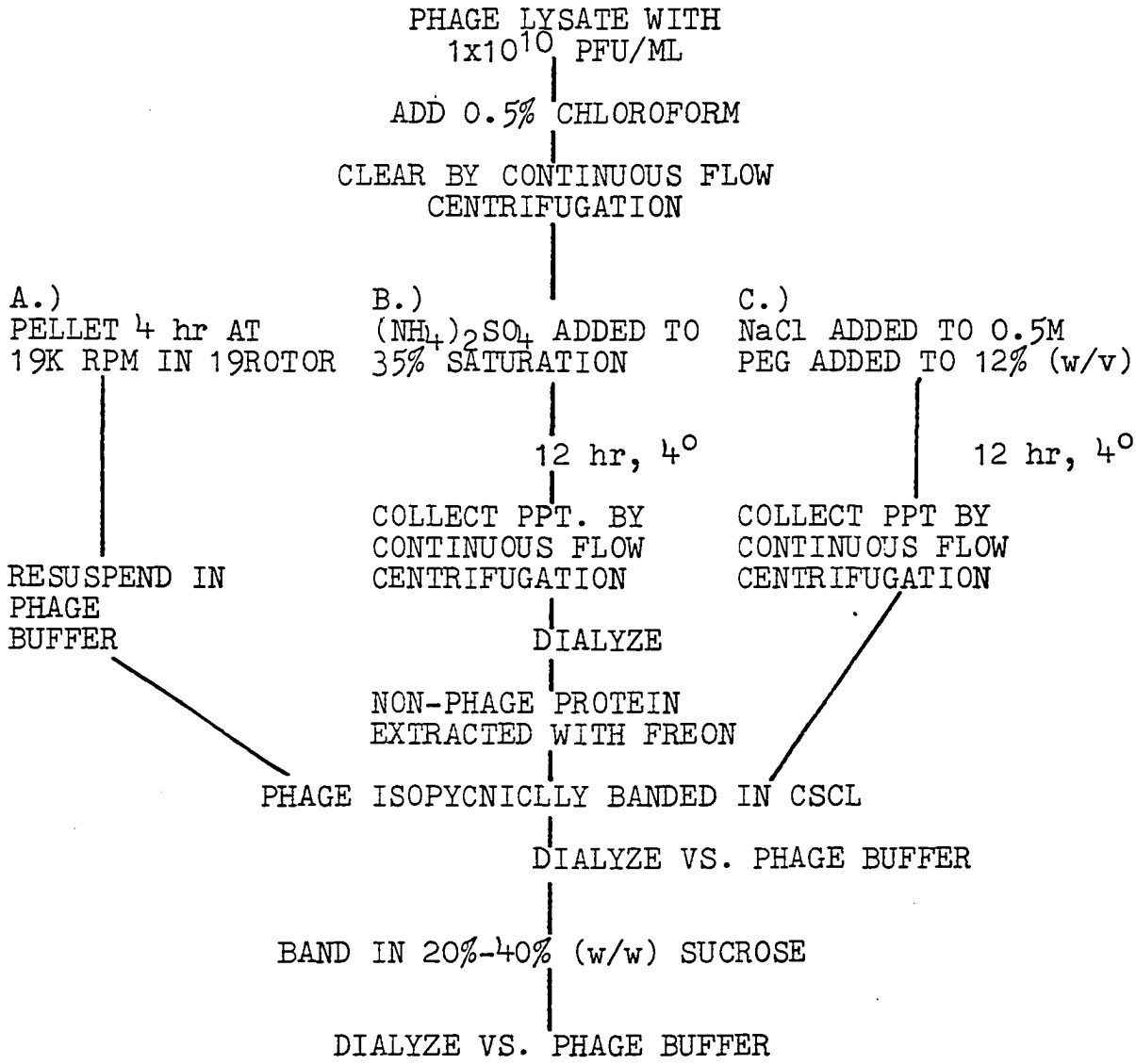


Figure 8.--Polyethylene glycol sedimentation of UC-1.

Sedimentation of phage by addition of PEG from lysates cleared of cellular debris was performed according to the method of Yamamato and Alberts (49). To arrive at the optimal concentration of PEG, varying amounts of PEG were added to 10 ml. aliquots of lysate made 0.5 M with NaCl. After setting overnight at 4^{o} the aliquots were spun for 10 min. at 5000xg. The pellet was resuspended in 1 ml. and titered. The percent phage pelleted was expressed as the ratio of p.f.u. in the pellet to total p.f.u. in the 10 ml. aliquot prior to addition of PEG.

Figure 8.--Polyethylene glycol sedimentation of UC-1.

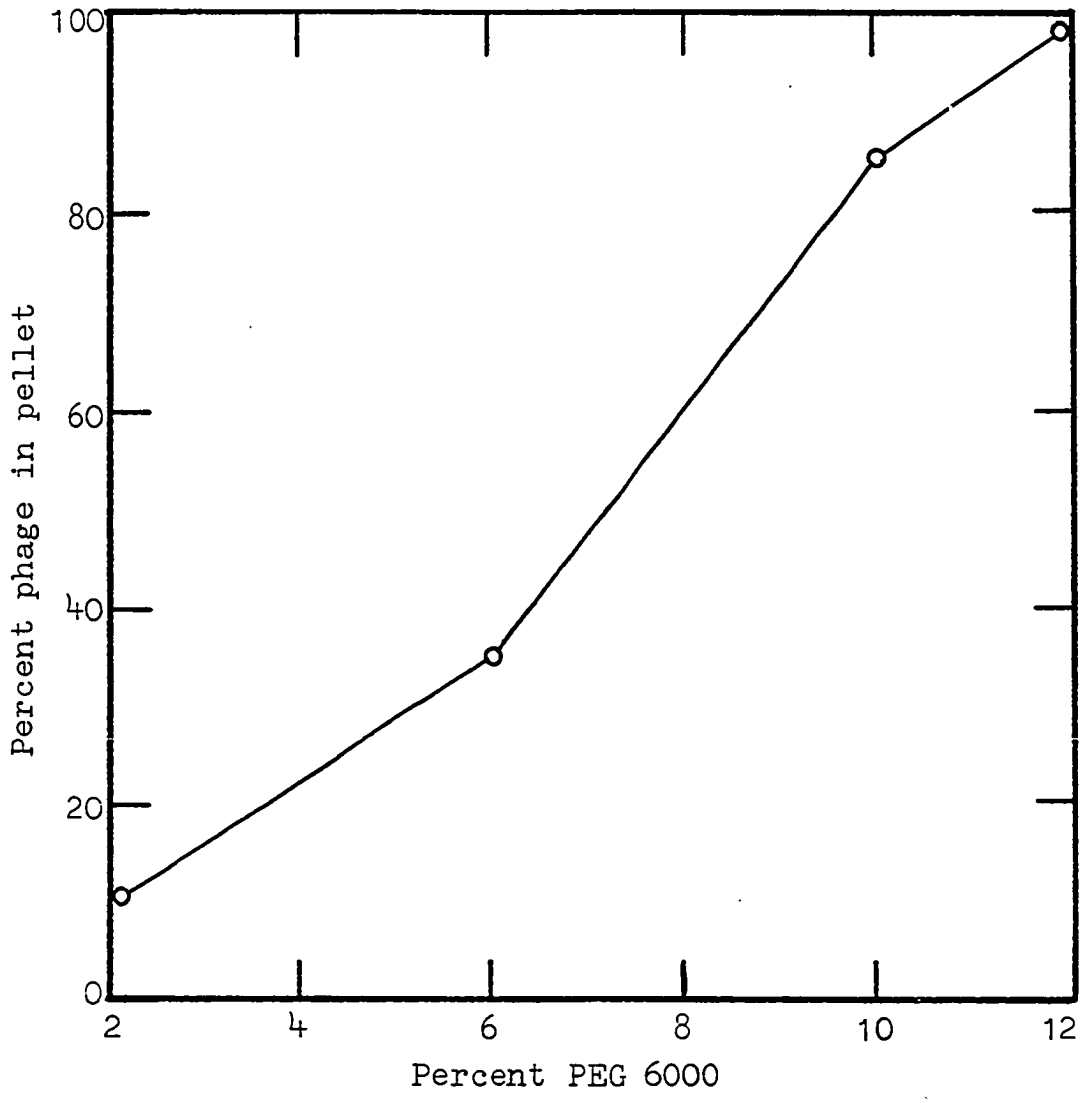


Figure 9.--Heat inactivation of UC-1.

One ml. aliquots of phage in PAB ($1x10^{10}$ p.f.u./ml.) were placed in sterile screw capped tubes (1.5 x 12.5 cm.) preheated to the indicated temperatures, and were incubated at the designated temperatures for either 30 min. or for 60 min. Samples were withdrawn at the end of the incubation period and plaque assays were performed.

30 min. ●

60 min. o

Figure 9.--Heat inactivation of UC-1.

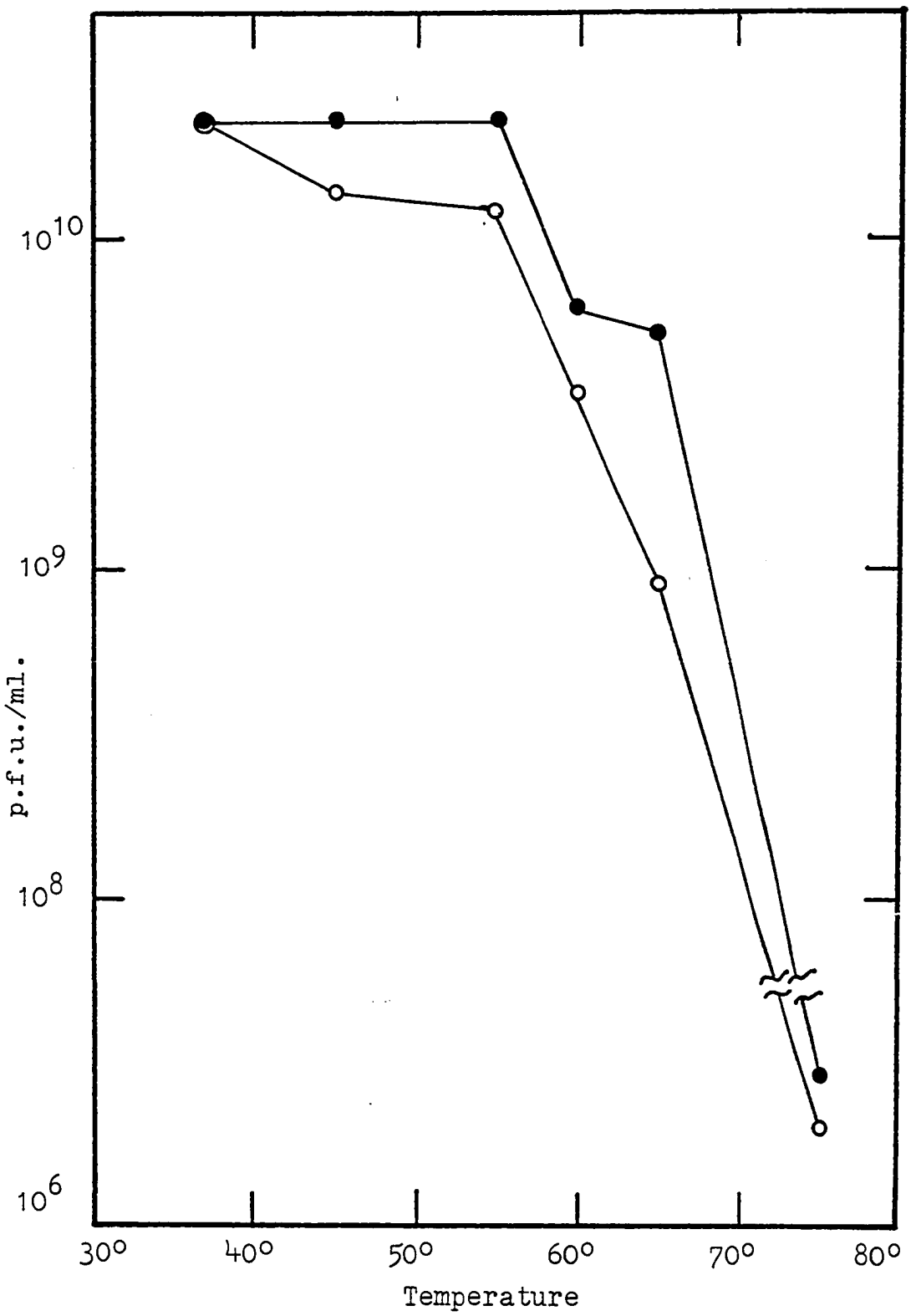


Figure 10.--Kinetics of heat inactivation of UC-1 at 75°.

One ml. aliquots of phage in PAB (1 x 10^{10} p.f.u./ml.) were placed in sterile screw capped tubes (1.5 x 12.5 cm.) that had been preheated to 75° and incubated for various time periods. Samples were withdrawn and plaque assays were performed.

Figure 10.--Kinetics of heat inactivation of UC-1 at 75°.

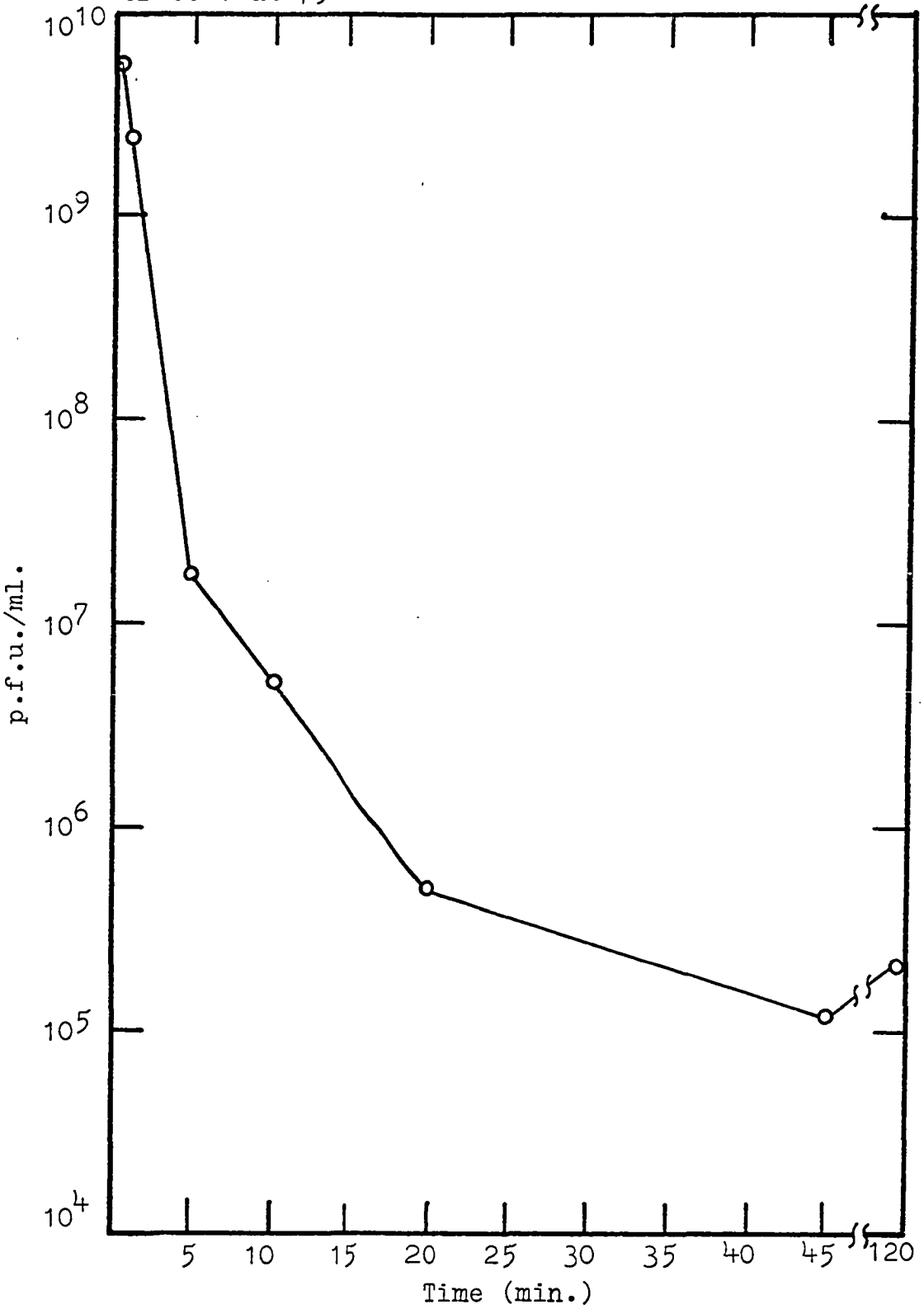


Figure 11.--Inactivation of UC-1 by sonication.

Ten ml. aliquots of phage in PAB (1×10^{10} p.f.u./ml.) were placed in the 20 ml. cup device of a Blackstone sonicator tuned to give maximum cavitation and run at 100% power output. Samples of 0.1 ml. were withdrawn at various times and titered.

Figure 11.--Inactivation of UC-1 by sonication.

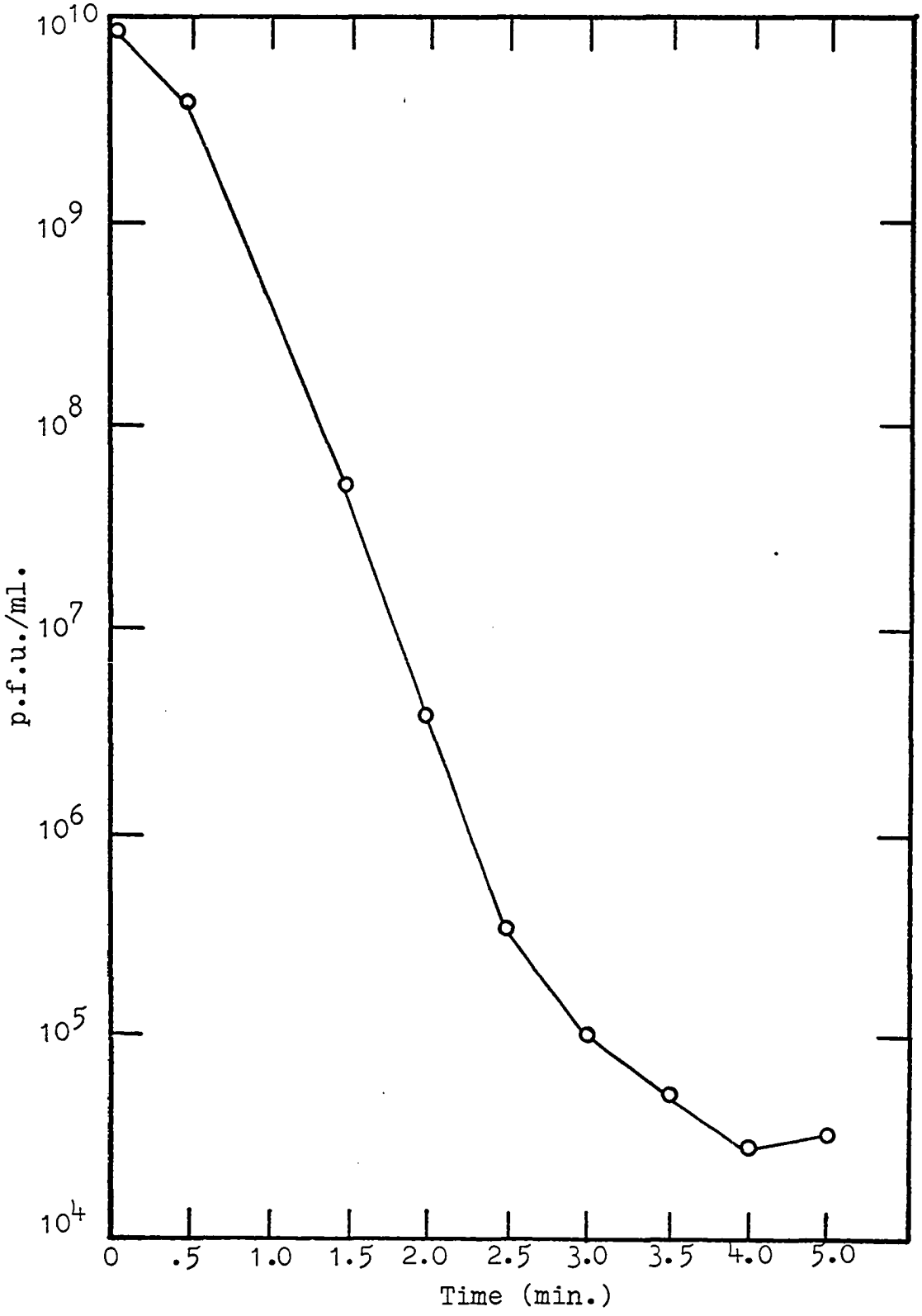


Figure 12.--pH inactivation of UC-1.

To study the effect of various pH's on phage viability 0.1 ml. aliquots of phage in phage buffer (1×10^{10} p.f.u./ml.) were added to 9.9 ml. of buffer of the designated pH and incubated at 25° for 60 min. Aliquots of 0.5 ml. were withdrawn and diluted in either the same buffer or in 0.5% (w/v) NaCl and titered. The results were the same for both dilutents. The buffers used for various pH ranges were: acetic acid-sodium acetate, 3.7-5.8, succinic acid-sodium hydroxide, 3.8-6.0, potassium dihydrogen phosphate-disodium hydrogen phosphate, 6.0-8.0, tris (hydroxymethyl) amino-methane hydrochloride-tris (hydroxymethyl) amino-methane, 8.0-9.4, and glycine-sodium glycinate, 9.6-11.4. These were all made so as to have an ionic strength of 0.1 or less (12).

Figure 12.--pH inactivation of UC-1.

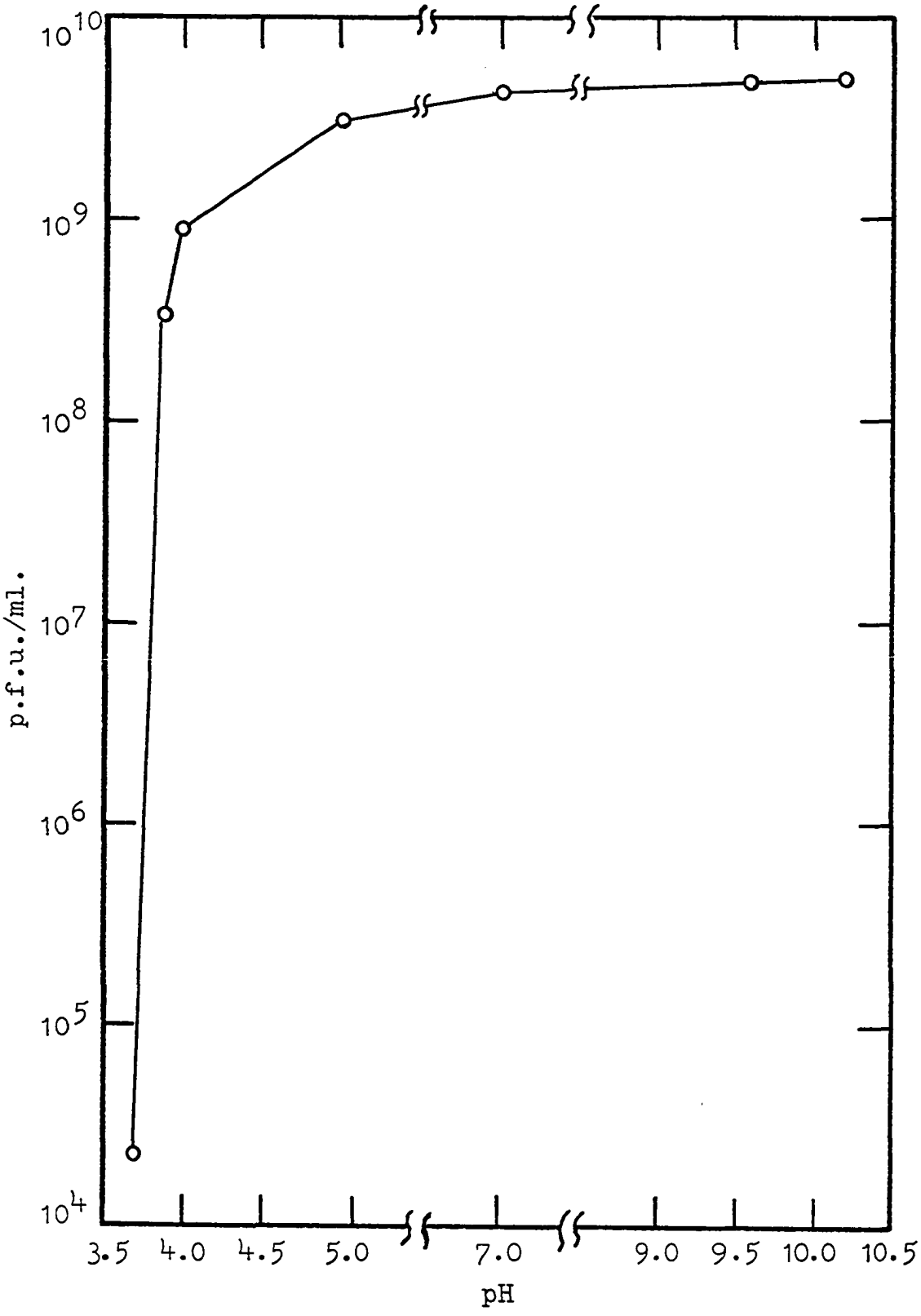


Figure 13.--Kinetics of inactivation of UC-1 at pH 3.6.

To study the kinetics of pH 3.6 inactivation of UC-1 0.1 ml. of phage (1×10^{10} p.f.u./ml.) in phage buffer was added to 9.9 ml. of acetic acid--sodium acetate buffer, pH 3.6, ionic strength 0.1. After incubating at 25° for various periods of time aliquots of 0.5 ml. were withdrawn, diluted with 0.5% (w/v) NaCl and titered.

Figure 13.--Kinetics of inactivation of UC-1 at pH 3.6.

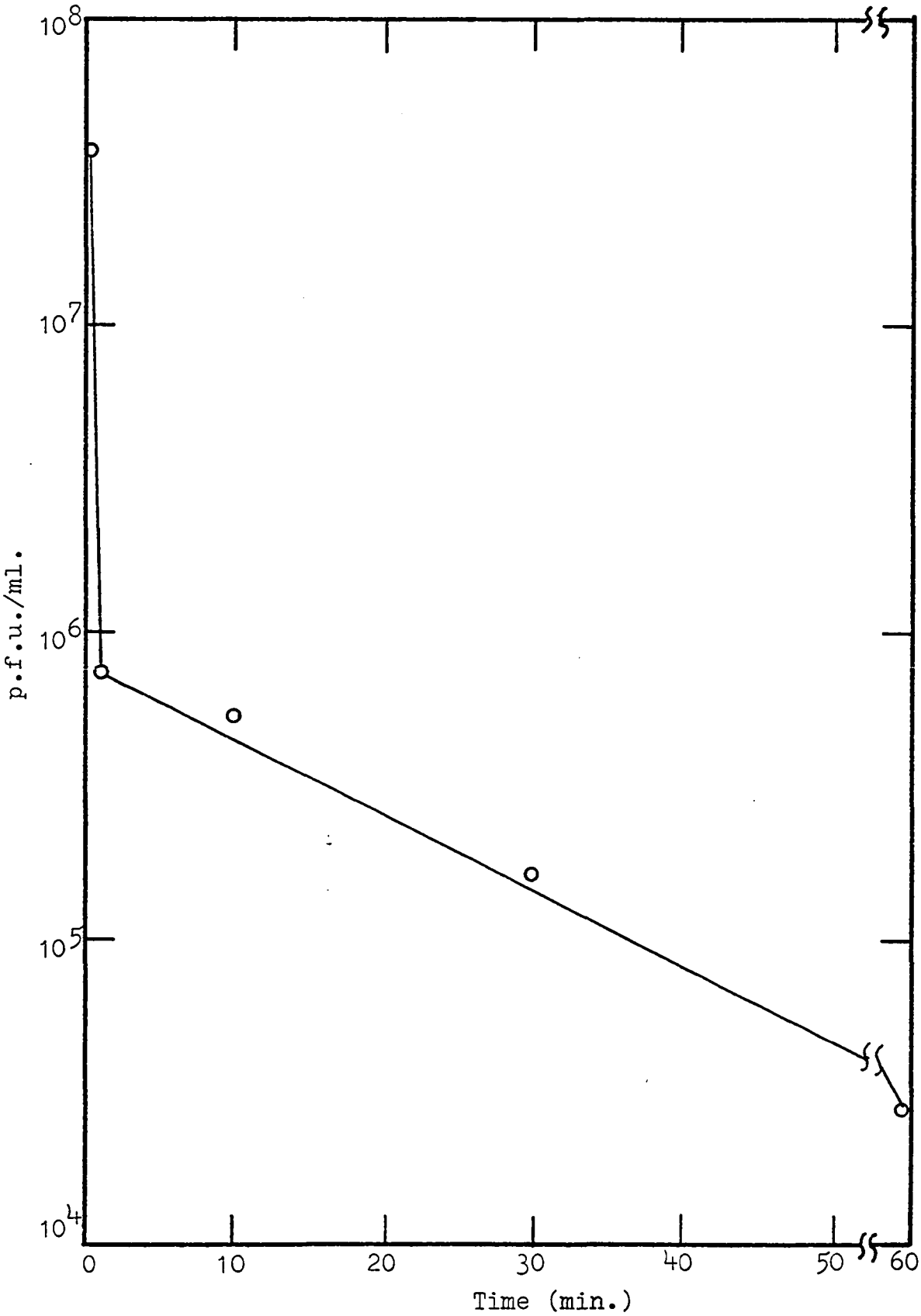


TABLE 3

EFFECT OF VARIOUS CHEMICAL AND PHYSICAL TREATMENTS ON PRODUCTION OF UC-1 GHOSTS

Technique Employed	Ghost Yield	Reference
$CuCl_2$ and NH_4Cl	99.9 %	47
$Na_2P_2O_7$	99.9 %	47
Osmotic shock from 66% sucrose (w/v) to dist. H_2O	10.0 %	23
Osmotic shock from 3 m Na_2SO_4 to dist. H_2O	10.0 %	23
LiCl	10.0 %	16

TABLE 4

EFFECT OF PHAGE DISRUPTIVE AGENTS ON UC-1

Treatment	Conditions	Effect
Urea - 8M	5 min, 25°, pH 7.0	No loss of titer
	5 min, 37°, pH 8.9	10^1 drop in titer
	1 hr., 37°, pH 8.9	10^5 drop in titer
	1 hr., 37°, pH 7.0	10^3 drop in titer
Trypsin	60 min., pH 7.8, 20 µg/ml.	No loss of titer
SDS	10% or 1%, 25°, 10 min.	10^1 drop in titer
	10% or 1%, 100°, 1 min.	Complete loss of titer
DMSO	55% (v/v)	60% drop in titer
	60% (v/v)	90% drop in titer
	75% (v/v)	10^7 fold drop in titer
Freezing and Thawing	8 cycles	10% drop in titer

TABLE 5

EFFECT OF GUANIDINE HYDROCHLORIDE

	O.D. at 280 mu					
	Tris	Tris + Phage	GuHCl	GuHCl + Phage	Tris + Phage Pellet	GuHCl + Phage Pellet
0 min.	.00	.90	.05	.90		
90 min.	.01	.90	.05	.90		
Post Centrifugation		.41 (Supernate)		.62 (Supernate)	.460	.235

Figure 14.--Electrophoretic separation and molecular weight determinations of phage structural proteins on polyacrylamide gels.

Purified phage were disrupted by SDS, electrophoresed on polyacrylamide gels and stained according to the method of Shapiro, Vinuela and Maizel (41). Some modifications suggested by Maizel were employed: the sample and gel buffers were made 0.004% (w/v) with EDTA, and the samples were boiled for 1 to 5 min. just prior to electrophoresing (31). Carboxymethylation was also performed according to the method of Maizel (31). The gels were 10% (w/v) with respect to acrylamide and 0.2% (w/v) with respect to the amount of N; N; dimethylbisacrylimide and were cast 8 cm. long in tubes 10 cm. long and 0.6 cm. inside diameter. Gels were run for 4.5 hr. at approximately 10 mA/gel.

The molecular weight markers shown in No. 3 were

A--BSA--Bovine Serum Albumin

B--OVA--Ovalbumin

C--GPD--Glyceraldehyde Phosphate Dehydrogenase

D--PEP--Pepsin

E--TRY--Trypsin

F--LYS--Lysozyme

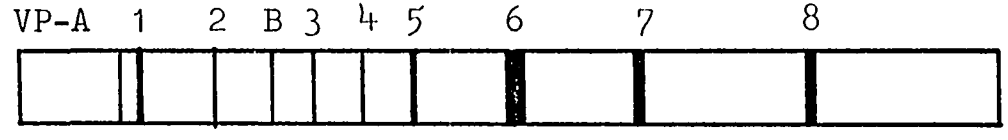
G--RNase--Ribonuclease

Figure 14.--Electrophoretic separation and molecular weight determination of phage structural proteins on polyacrylamide gels.

1.

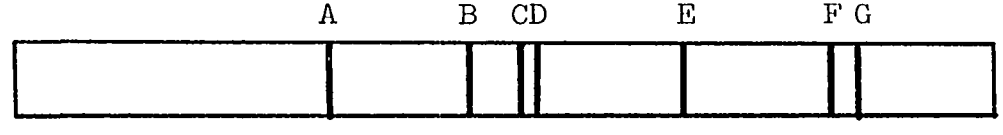
Gel: 10% acrylamide, 0.1% SDS, 0.1M pH7 PO_4 buffer, 0.1% mercaptoethanol.
Sample: Phage disrupted with 1% SDS, .01M pH7 PO_4 buffer and 0.1% mercaptoethanol.

2.



Gel: Same as No. 1 but no mercaptoethanol.
Sample: Phage treated the same as above but without mercaptoethanol.

3.



Gel: Same as No. 1.
Sample: Standard molecular weight markers treated as in No. 1.

Figure 15.--Viral protein molecular weights.

Viral proteins and molecular weight standards were prepared and electrophoresed as outlined in the materials and methods section and in Figure 14. The calculation of rates of relative migration (M) was performed according to the method of Weber and Osborn (48):

$$\text{Mobility (M)} = \frac{\text{Distance of protein migration (P)}}{\text{Gel length after destaining (FL)}} \times \frac{\text{Gel length before destaining (IL)}}{\text{Distance of dye migration (D)}}$$

Viral proteins are designated VP-1 through VP-8. Two additional proteins appearing on non-reduced gels are designated VP-A and VP-B. Molecular weight markers are designated according to Figure 14.

Figure 15.--Viral protein molecular weights.

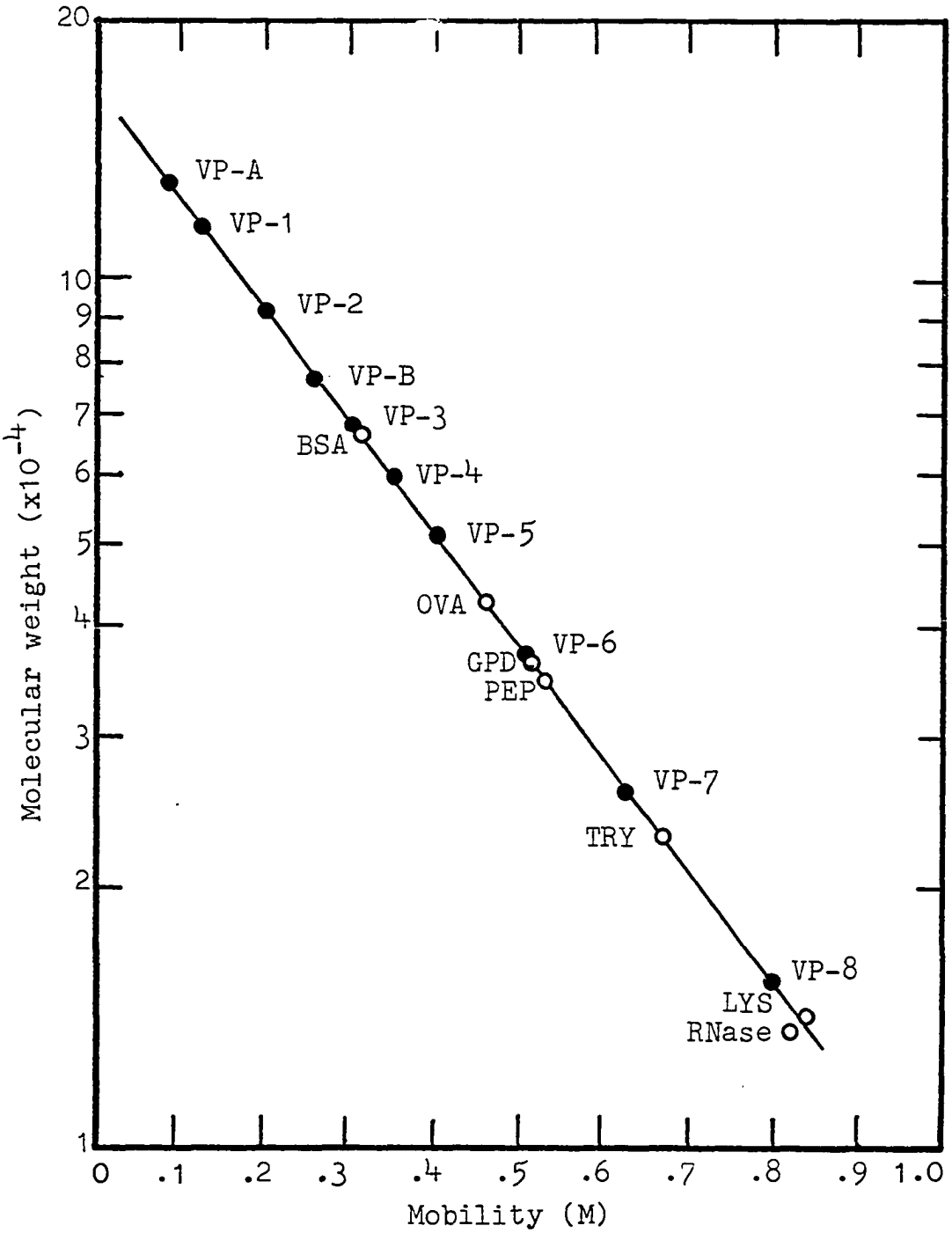


TABLE 6

MOBILITIES AND MOLECULAR WEIGHTS OF PHAGE PROTEIN AND OF MOLECULAR WEIGHT STANDARDS

Bands	Molecular Weight	Mobility (M)	±% Deviation
VP-1	115,000	.120	+ 10.0, - 5.4
VP-2	93,500	.203	+ 4.4, - 5.9
VP-3	68,200	.308	+ 10.0, - 8.4
VP-4	59,500	.355	+ 8.4, - 3.0
VP-5	51,800	.402	+ 1.9, - 6.2
VP-6	36,300	.519	+ 3.2, - 6.3
VP-7	26,300	.631	+ 2.0, - 4.9
VP-8	15,700	.806	+ 2.4, - 6.4
VP-A	104,000	.106	
VP-B	76,500	.260	
Standards			
BSA	68,000	.319	+ 5.0, - 6.0
OVA	43,000	.467	+ 3.0, - 4.0
GPD	36,000	.521	+ 1.3, - 1.3
PEP	35,000	.530	+ 1.3, - 1.1
TRY	23,300	.678	+ 2.0, - 2.0
LYS	14,300	.842	+ 1.5, - 1.5
TNase	13,700	.817	+ 2.0, - 3.0

Figure 16.--Effect of UV treatment on C^{14} amino acid incorporation by phage infected cells and non-infected cells.

After varying periods of exposure to UV irradiation, 1 ml. aliquots of E. coli K-12 cells were incubated at 37^{o} for 90 min. in the presence of C^{14} leucine. Of two aliquots used for each time of UV exposure one was infected with phage and an equal volume of NaCl was added to the other. Conditions of label level, treatment of samples after incubation and counting were as described in the materials and methods section.

o Phage Infected cells

● Uninfected cells

* These counts above 10^{6} were beyond the capacity of the scintilation counter

Figure 16.--Effect of UV treatment on C^{14} amino acid incorporation by phage infected cells and non-infected cells.

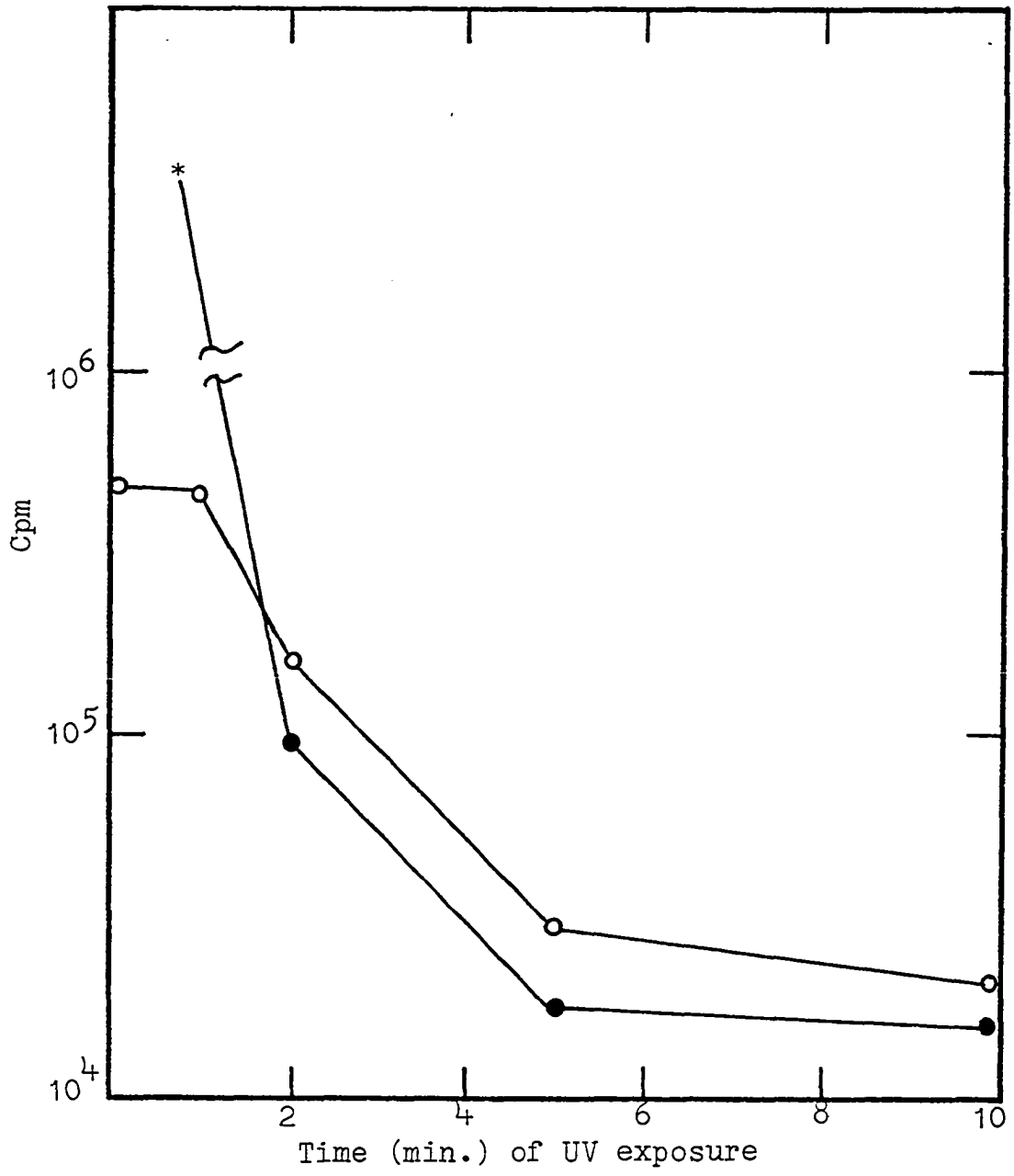


Figure 17.--Effect of phage infection on C^{14} amino acid incorporation.

For studies of C^{14} leucine uptake in normal cells all conditions were the same as outlined for Figure 15 and in the materials and methods section except samples were incubated for varying times from 1 to 60 min. prior to addition of TCA.

o Uninfected control cells

● Infected cells

Figure 17.--Effect of phage infection on C^{14} amino acid incorporation.

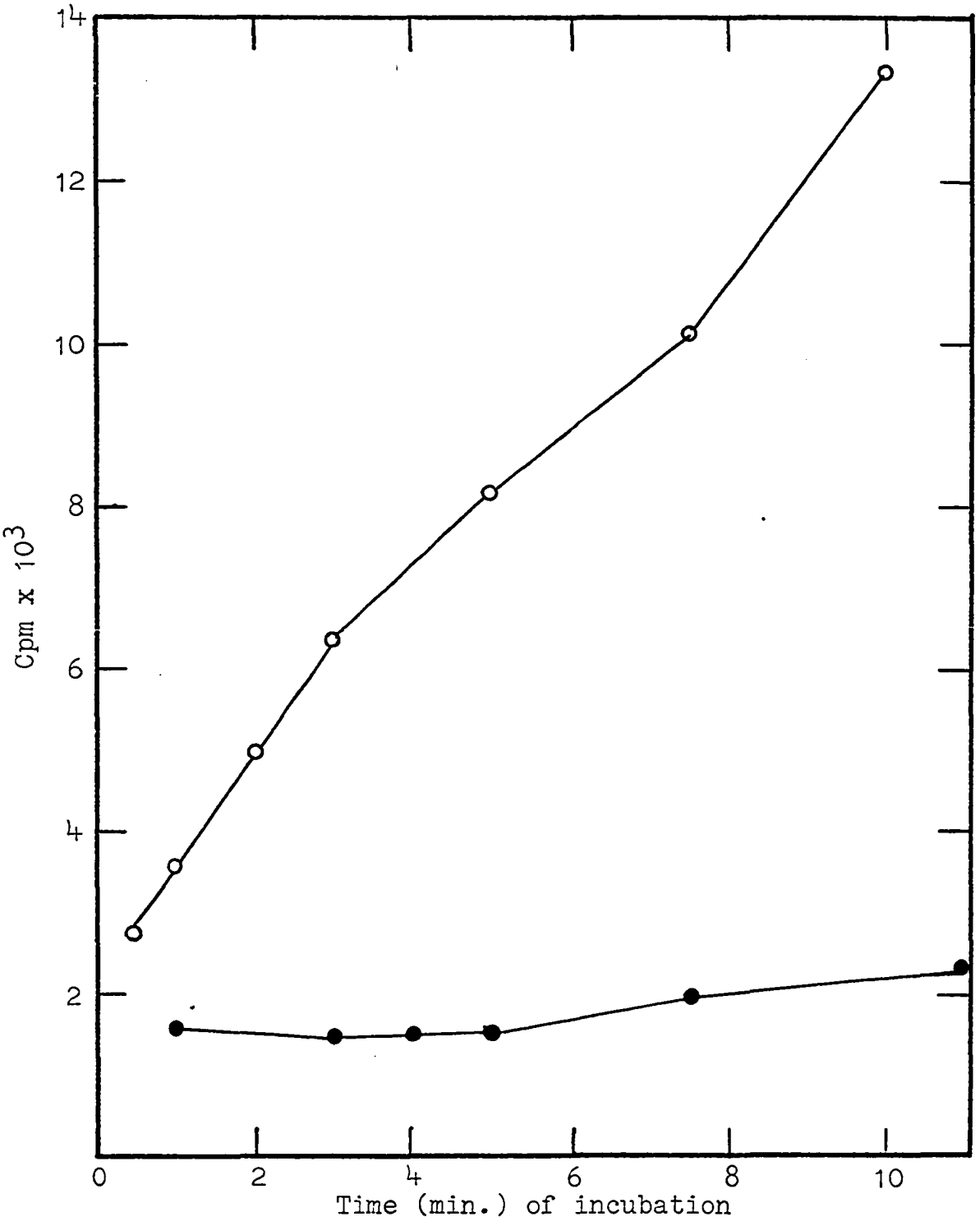


TABLE 7

EFFECT OF PHAGE MULTIPLICITY OF INFECTION ON LABEL UPTAKE

m.o.i.	C^{14} counts per minute	
	Initial (1 min.)	Final (60 min.)
0.0	1000	8600
0.1	950	8300
1.0	940	7200
10.0	750	5400
100.0	650	4200

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