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Title: The Effect of Streptococcus lactis Bacteriophage c2 DNA on the Growth of Bacillus cereus

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Dorothy K. Fraser

A lysate of Streptococcus lactis phage c2 was capable of killing 95 percent of the vegetative cells of Bacillus cereus 569R, as determined by plate count. Detailed studies were undertaken of this phenomenon. Optical density (OD_{650}) measurements of infected cultures showed that maximum inhibition of growth was obtained in brain heart infusion broth (BHI).

The phage lysate was separated by CsCl density centrifugation into fractions containing free DNA, whole phage, and protein. Only the fraction containing free DNA proved to be inhibitory for the growth of B. cereus cultures. Preparations of DNA from B. cereus, S. lactis, B. cereus phage R-1, or calf thymus were not inhibitory for growth of B. cereus cells at the concentrations used, nor were the four bases. Pretreatment of phage c2 DNA with DNase destroyed its activity.

Pure phage c2 DNA was prepared from purified phage. A mixture of phage c2 DNA and heat-treated spores in BHI (static conditions; 30° C) was followed by OD_{650} measurements at ten minute intervals, in comparison with a non-infected culture and a culture to which DNase-treated DNA had been added. Only the DNA-infected culture showed growth inhibition. When this DNA was added to one of two cultures of BHI inoculated with heat-treated spores, the viable titer of the infected culture after 300 minutes of incubation at 30° C was one percent of the titer of the uninfected culture.

Phage c2 labeled with ^{3}H thymine was used to prepare labeled DNA. When this was added to a static BHI culture inoculated with heat-treated spores, samples taken at one hour intervals, and the cells tested for ^{3}H uptake, maximum incorporation of label was found between 1-2, 3-4, and 5-6 hours after the addition of DNA. The cells were lysed, and lysates submitted to dialysis and CsCl centrifugation. The label was found to be associated with high molecular weight DNA, in one major and three lighter minor peaks.

DNA isolated from cells of B. cereus labeled with ^{32}P and the ^{3}H labeled phage c2 DNA proved to have the same density. Heat-treated unlabeled spores were grown in BHI to which ^{32}P had been added, and the culture infected with ^{3}H phage c2 DNA. Samples were removed at intervals of one hour, the cells washed and lysed, and the lysates centrifuged in CsCl. Membrane filtration of the

fractions was used to remove labeled RNA. The major peak of ^{3}H label corresponded exactly to the major peak of ^{32}P label. Therefore it was not possible to tell whether the ^{32}P peak represented only cell DNA or both cell and replicated phage DNA. Three minor peaks of ^{3}H label were found higher in the gradient and a small amount of ^{32}P was also associated with these peaks. One of the minor peaks was close to, but not at, the top of the gradient and may represent an association of DNA with a protein component, which could be either new phage protein or cellular in origin.

The Effect of *Streptococcus lactis* Bacteriophage c2 DNA on the Growth of *Bacillus cereus*

by

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INTRODUCTION

During a routine plating of *Streptococcus lactis* phage c2 on its normal host, several colony-centered plaques were observed. Bacteria from the center of these plaques were recovered and proved to be Gram positive spore forming rods, tentatively identified as *Bacillus cereus*.

Attempts were then made to infect pure *Bacillus cereus* cultures with a phage c2 lysate. Since *B. cereus* 569R (McDonald *et al.*, 1963) and *S. lactis* C_2S^s (Knittel, 1965) have the same G+C content, *B. cereus* 569R was used for this study, as well as several other *B. cereus* strains. Although it was not possible to grow phage c2 on any of these strains, it was found that the phage lysate was inhibitory for the growth of *B. cereus* 569R. Detailed studies were then undertaken to investigate this phenomenon.

HISTORICAL

Hershey and Chase (1952), using ^{32}P and ^{35}S labeled bacteriophage, demonstrated that during phage infection only the phage DNA was injected into the host cell. This work led to the idea that the complete phage particle was not required to initiate infection. Spizizen (1957) and Fraser et al. (1957) found that disrupted preparations of E. coli phage T2 could infect spheroplasts of both E. coli B and E. coli B/2 to form mature phage particles. E. coli B/2 cells are resistant to infection by phage T2, demonstrating that the phage sensitivity or resistance is a character of the cell wall. This work demonstrated that the complete phage particle was not required for infection.

Spizizen (1957) also found that spheroplasts of A. aerogenes 417 could be infected with a disrupted preparation of phage T2. A. aerogenes cells cannot be infected with the whole phage, thus these experiments indicated that phage infection was controlled by the cell wall of the bacteria. Both workers found that if their disrupted phage preparation was treated with deoxyribonuclease (DNase) before being added to the spheroplasts, no mature phage particles could be recovered, showing that the phage DNA was probably responsible for infection. Since in both cases infection occurred at a low frequency, the number of mature phage particles recovered being less than the

number of phage equivalents added, it could not be determined if the phage DNA replicated in the spheroplast or was simply wrapped up in a protein coat.

When better techniques for isolating phage nucleic acid were worked out, it was found that when phenol extracted DNA from the single-stranded E. coli phage ØX174 was added to spheroplasts of E. coli, mature phage particles could be recovered after a suitable incubation period (Guthrie and Sinsheimer, 1960; Sekiguchi et al., 1960; Hofschneider, 1960). Sekiguchi et al. (1960) were able to infect spheroplasts of A. aerogenes 1033 with DNA extracted from ØX174 and recover mature phage particles. A. aerogenes cells cannot be infected with phage ØX174. The infection of E. coli with DNA extracted from ØX174 was the first demonstration of transfection, defined by Foldes and Trautner (1964) as "infection of cells by the isolated nucleic acid from a virus resulting in the production of complete virus". This definition has been extended to include spheroplasts. In all cases, if the DNA was treated with DNase prior to addition to the spheroplasts, no mature phage particles could be recovered. The DNA preparations were not sensitive to the action of ribonuclease (RNase) or trypsin, showing that RNA and protein were not involved.

Meyer et al. (1961) were the first to demonstrate transfection using a temperate phage. They found that DNA extracted from

E. coli phage λ was infectious for spheroplasts of E. coli W-1485. Brody et al. (1964) found that λ DNA was infectious for spheroplasts of E. coli 3350/λ but not for E. coli B. Both organisms are resistant to infection by phage λ. These results demonstrated, for the production of mature phage particles, more than entry of the phage DNA into the spheroplast was required.

Brody et al. (1964) using E. coli phage T1, found that DNA extracted from phage T1 was infectious for spheroplasts of E. coli W-1485. Brody, Makal and Evans (1964) also demonstrated that DNA extracted from E. coli phage T7 was infectious for spheroplasts of E. coli B and E. coli 3350/λ. These experiments resulted in the first reports of infection of E. coli spheroplasts using double-stranded DNA from a virulent phage.

Hofschneider (1963) isolated three small phages (M12, M13, and M20) for E. coli K12 and found that the nucleic acid from all three phages was infectious for spheroplasts of their host. He found that M12 contained RNA while M13 and M20 contained single-stranded DNA. The infection of E. coli with M12 RNA was the first report of transfection with an RNA phage.

Transformation may be defined as a genetic change introduced by incorporation of free DNA into a recipient cell and subsequent integration of the DNA into the cell chromosome. In order for recipient cells to be transformed they must be in a competent state.

Competence is a transitory state of bacterial cells defined by the fact that they are able to incorporate free DNA.

After the discovery of transformation of Bacillus subtilis (Spizizen, 1958) interest was renewed in B. subtilis phages. Romig (1962) using DNA extracted from B. subtilis phage SP3, was the first to demonstrate transfection in B. subtilis. For transfection to take place it was necessary to use competent cells. The transfection system was sensitive to DNase and insensitive to RNase, trypsin and phage SP3 antiserum showing that DNA was responsible for infection.

Since the initial discovery of transfection in B. subtilis by Romig (1962), transfection of B. subtilis has been reported using B. subtilis phages SP50 (Foldes and Trautner, 1964), SPO-1 (Okubo, Strauss and Stodolsky, 1964), SP8 and SP82 (Green, 1964), 2C (Pene and Marmur, 1964), Ø1, Ø25, and Ø29 (Reilly and Spizizen, 1965) and SP18 (Gwinn and Thorne, 1966).

Transfection was observed in Hemophilus influenzae by Harm and Rupert (1963) and Goodgal (1964). Tokunaga and Sellers (1963) and Sellers and Tokunaga (1966) have been able to show transfection in Mycobacterium smegmatis using DNA extracted from M. smegmatis phages D4, D29, and D32. DNA from M. smegmatis phage D28 was non-infectious. The non-infectious activity of D28 has not yet been explained. Competent cells were not apparently required for transfection of M. smegmatis, but Ca^{++} was necessary to initiate infection. Attempts by Sellers and Tokunaga (1966) to transfect

staphylococci, Nocardia, streptococci, pneumococci, Bacillus sp. and HeLa cells with DNA from M. smegmatis phages were unsuccessful.

In 1964 Bayreuther and Romig reported that they could successfully transfect competent B. subtilis with DNA extracted from polyoma virus. They calculated that the polyoma virus DNA must have replicated in the infected cells. The mature polyoma virus particles obtained from transfection had identical properties with the normal virus as shown by their infectivity of chick embryo cells. Abel and Trautner (1964) reported infection of competent B. subtilis with DNA extracted from vaccinia virus and subsequent recovery of mature virus particles. The results of both groups have yet to be confirmed. If these reports prove to be true, the finding is extremely important because it shows that the genetic code in animal and bacterial cells is sufficiently similar so that the animal virus protein synthesizing mechanism can use bacterial sRNA and rRNA.

Transfection of mammalian cells with animal viruses was first demonstrated in 1959 when Holland et al. reported that non-primate cells could be infected with RNA extracted from polio virus with recovery of mature virus particles. The whole virus does not infect these cells. When the virus particles recovered from DNA infection of non-primate cells were added to the original host it was found that the virus caused polio, showing that these particles were

identical to those produced in the normal manner.

Orth et al. (1964) reported infecting hamsters with DNA extracted from polyoma virus (polyoma virus induces tumor formation in hamsters). They found that mature virus particles could be recovered from the hamsters, and that tumor formation was induced. From their work they were unable to conclude whether the DNA induced tumor formation or whether the complete virus was formed first, which in turn induced tumor formation. If DNase was added to the DNA before inoculating the hamster, neither mature virus particles nor tumors were found.

In 1966 Adldinger et al. found that DNA extracted from African swine fever virus was infectious for pig kidney cells. The mature virus particles recovered from the pig kidney cells were identical to virus particles propagated in the normal manner.

MATERIALS AND METHODS

Bacterial Cultures

Bacillus cereus 569R was obtained from the culture collection of Dr. Curtis B. Thorne, Department of Microbiology, University of Massachusetts. *Escherichia coli* C600 was obtained from the culture collection maintained by Dr. Dorothy K. Fraser, Department of Microbiology, Oregon State University. *Streptococcus lactis* C_2S^s was obtained from the culture collection maintained by Dr. William E. Sandine, Department of Microbiology, Oregon State University.

Phage Strains

Streptococcus lactis C_2S^s phage c2 was obtained from Dr. William E. Sandine. *Escherichia coli* phage λ was obtained from Dr. Dorothy K. Fraser. *Bacillus cereus* 569R phage R-1 was isolated from soil.

Media

Table 1. Composition of λ broth

Ingredient	Amount per liter
Tryptone	10.0 gm
NaCl	2.5 gm
Agar[1]	

[1] For hard agar 15 gm/l were added; for soft agar 7.5 gm/l were added

Reconstituted milk consisted of 13.04 grams of Matrix non-fat dry milk (Galloway West Company) per 100 ml of distilled water.

Table 2. Composition of S. lactis semi-defined medium (SLM)

Ingredient	Amount per liter
Vitamin Free Casamino Acids	5.0 gm
K_2HPO_4	4.0 gm
Sodium Citrate	2.0 gm
NH_4Cl	3.0 gm
Sodium Acetate	1.5 gm
NaCl	2.0 gm
Glutamine	0.1 gm
Asparagine	0.1 gm
Glucose[1]	5.0 gm
Riboflavin	1.0 mg
Calcium Pantothenate	1.0 mg
Nicotinic Acid	1.0 mg
Pyridoxine	0.1 mg
Thiamine HCl	0.1 mg
Xanthine	5.0 mg
Adenine	5.0 mg
Guanine HCl	5.0 mg
Uracil	5.0 mg
$MgSO_4 \cdot 7H_2O$	80.0 mg
$FeSO_4 \cdot 7H_2O$	4.0 mg
$MnCl_2$	1.2 mg
Biotin	1.0 μg
Yeast Extract[1]	0.6 gm
$CaCl_2$[1]	0.1 gm
pH 7.0	

[1]Added aseptically after autoclaving

Nutrient agar (NA) consisted of 8.0 grams of Difco nutrient broth and 15 grams of Difco agar per liter of distilled water.

2X brain heart infusion (2X BHI) consisted of 74 grams of Difco brain heart infusion per liter of distilled water.

Table 3. Composition of phage assay broth (PAB)

Ingredient	Amount per liter
Nutrient Broth	8.0 gm
NaCl	5.0 gm
$CaCl_2 \cdot 2H_2O$	0.15 gm
$MgSO_4 \cdot 7H_2O$	0.20 gm
$MnSO_4 \cdot H_2O$	0.05 gm
Agar[1]	
pH 5.9-6.0	

[1] For hard agar 15 gm/l were added; for soft agar 7.5 gm/l were added

Table 4. Composition of SMCA medium

Ingredient	Amount per liter
Vitamin Free Casamino Acids	5.0 gm
KH_2PO_4	0.25 gm
K_2HPO_4	0.25 gm
$MgSO_4$	0.1 gm
NaCl	5.0 mg
$FeSO_4 \cdot 7H_2O$	5.0 mg
$MnSO_4 \cdot 4H_2O$	5.0 mg
Concentrated HCl	2.0 ml
Biotin	25.0 μg
Nicotinic Acid	0.25 mg
Calcium Pantothenate	0.27 mg
L-tryptophan[1]	5.0 mg
Glucose[1]	5.0 gm
pH 6.2	

[1]Added aseptically after autoclaving

Table 5. Composition of enriched lactic broth (ELB)

Ingredient	Amount per liter
Tryptone	20.0 gm
Glucose	5.0 gm
NaCl	4.0 gm
Sodium Citrate$\cdot 5H_2O$	1.5 gm
Ascorbic Acid	0.5 gm
Yeast Extract	5.0 gm
$CaCl_2 \cdot 2H_2O$	0.15 gm
$MgSO_4 \cdot 7H_2O$	0.20 gm
$MnSO_4 \cdot H_2O$	0.05 gm
Agar[1]	
pH 6.8	

[1]For hard agar 15 gm/l were added; for soft agar 7.5 gm/l were added

Scintillation Fluid

Table 6. Composition of scintillation fluid for non-aqueous material

Ingredient	Amount per liter
POPOP[1]	0.1 gm
PPO[1]	3.0 gm
Toluene	1.0 liter

[1]Obtained from Packard Instrument Company

Table 7. Composition of scintillation fluid for aqueous material[1]

Ingredient	Amount per liter
POPOP[2]	0.2 gm
PPO[2]	4.0 gm
Naphthelene	60.0 gm
Methanol	100 ml
Ethylene Glycol	20 ml
p-Dioxane[3]	

[1]Bray, 1960

[2]Obtained from Packard Instrument Company

[3]Bring volume up to one liter with p-dioxane

Preparation of B. cereus Spores

A 500 ml Erlenmyer flask containing 100 ml of SMCA, supplemented with 1×10^{-3} M $CaCl_2$, was inoculated with 1.15×10^{7} B. cereus spores/ml. The culture was incubated in a 37° C rotary

shaker for 18 hours. The spores were harvested by centrifuging for 15 minutes at 5000 × g and were washed six times with sterile distilled water. The spores were resuspended in 10 ml of distilled water, heat-shocked for 60 minutes at 65° C, washed with distilled water, and stored at 4° C.

The viable spore count was determined by diluting the spores in physiological saline and plating appropriate dilutions on nutrient agar plates that had been dried for 48 hours at 37° C. The colonies were counted after 24 hours incubation at 37° C.

Maintenance of S. lactis Stock Cultures

S. lactis was maintained by transferring 0.5 ml of a milk culture to 10 ml of reconstituted dry milk every month. The new culture was incubated for six hours at 30° C before storing at 4° C.

Assay of Phage

The titer of phages c2, R-1, or λ was determined by adding 0.1 ml of an appropriate dilution to 2.5 ml of soft agar seeded with approximately 1×10^7 sensitive indicator cells/ml, and the mixture poured over hard agar plates. ELB agar was used for phage c2, PAB agar was used for phage R-1, and λ agar was used for phage λ. Assay plates were incubated overnight at 30° C for phages c2 and R-1 and at 37° C for phage λ.

Optical Density Growth Curve of S. lactis

A 500 ml Erlenmyer flask containing 100 ml of ELB was inoculated with 1.0 ml of a 16 hour culture of S. lactis. The culture was incubated in a 30° C rotary shaker. Three ml samples were removed every 30 minutes for six hours. The optical density at 650 mμ was recorded using a Beckman Model DU spectrophotometer equipped with a Gilford Model 2000 Multiple Sample Absorbance Recorder (Figure 1).

Propagation of S. lactis Phage c2 Stock

A 250 ml Erlenmyer flask, containing 50 ml of ELB, was inoculated with 0.5 ml of a 16 hour culture of S. lactis. The culture was incubated for 2.5 hours in a 30° C rotary shaker (Figure 1). Phage c2 was added to a final concentration of 3.0×10^7 phage/ml and incubation was continued until complete clearing of the flask occurred (approximately 75 minutes). The crude lysate was centrifuged for ten minutes at 6000 × g to remove cellular debris. The supernatant was filtered through a Millipore HA membrane filter and the filtrate was collected in a sterile 18 × 150 mm screw cap tube. The phage titer was determined and the filtrate was stored at 4° C.

Isolation of B. cereus Phage R-1

A 250 ml Erlenmyer flask containing 50 ml of PAB was seeded with 1×10^7 B. cereus spores/ml and inoculated with 5.0 grams of a soil sample. The culture was incubated in a 30° C rotary shaker for 16 hours. After incubation the soil debris was allowed to settle out and the supernatant, containing phage, was decanted into a centrifuge tube and centrifuged for 15 minutes at 6000 × g to remove cells and cellular debris. The supernatant was filtered through a Millipore HA membrane filter and the filtrate was collected in a sterile 25 × 150 mm test tube. Five ml of the filtrate was inoculated into 50 ml of fresh PAB seeded with 1×10^7 B. cereus spores/ml. After 16 hours incubation the phage lysate was centrifuged, filtered, and assayed for phage as described.

A single clear plaque was picked and inoculated into 50 ml of PAB seeded with 1×10^7 B. cereus spores/ml. After incubation in a 30° C rotary shaker for 12 hours the phage lysate was centrifuged, filtered, and assayed for phage as described. A single clear plaque was picked from the second assay plate and grown as described. The phage lysate obtained was treated as described and stored at 4° C. The phage was designated as phage R-1.

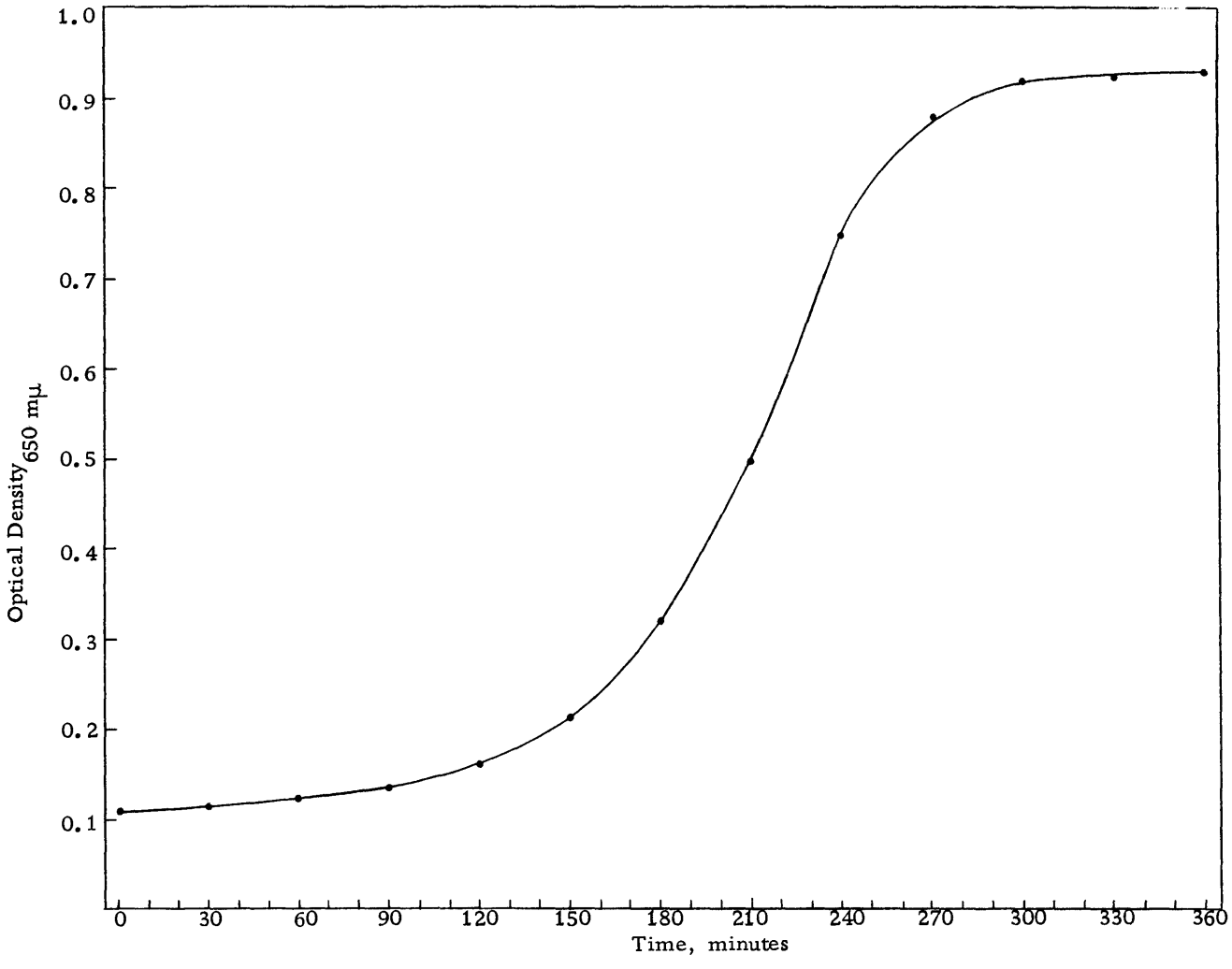

Figure 1. Growth curve of S. lactis C_2S^s

Growth of Phage for DNA Extraction

Growth of Phage c2

A one liter Erlenmyer flask containing 200 ml of ELB was inoculated with 2.0 ml of a 16 hour ELB culture of S. lactis. The culture was incubated for 2.5 hours in a 30° C rotary shaker. Phage c2 was added to a final concentration of 3.0×10^7 phage/ml and incubation was continued until complete clearing of the flask occurred. One liter of phage was prepared for DNA extraction.

Growth of ^{3}H Labeled Phage c2

A 500 ml Erlenmyer flask containing 100 ml of SLM, supplemented with 0.5 μC/ml of ^{3}H-thymine (New England Nuclear) was inoculated with 1.0 ml of a 16 hour ELB culture of S. lactis that had been washed once with saline. The culture was incubated in a 30° C rotary shaker for four hours. Phage c2 was added to a final concentration of 1×10^7 phage/ml and incubation was continued for eight hours. Two liters of phage were prepared for DNA extraction.

Growth of Phage R-1

A 500 ml Erlenmyer flask containing 100 ml of PAB was inoculated with 1×10^7 B. cereus spores/ml and incubated in a 30° C

rotary shaker for four hours. Phage R-1 was added to a final concentration of 5×10^7 phage/ml and incubation continued for 12 hours. One liter of phage was prepared for DNA extraction.

Removal of Cells from Phage Lysates

The crude lysates were centrifuged for 15 minutes at 5000 × g to remove cellular debris and resistant cells. The supernatant was filtered through a Millipore HA membrane filter and the filtrate collected in a one or two liter flask. Phage titers were determined by the procedure previously described. The phage filtrates were stored at 4° C until the purification step.

Purification of Phage

The procedure for purifying the phage is outlined in Figure 2. The phage lysates were centrifuged for 75 minutes at 23.000 × g in a Beckman Model L-2 ultracentrifuge. The supernatant was decanted into a beaker and the viable phage titer was determined by assaying as described. The phage pellets were resuspended by adding 1/10 the original volume of Tris-NaCl buffer (1×10^{-2} M Tris, 1×10^{-1} M NaCl, pH 7.2) and allowing the pellets to stand overnight at 4° C. The resuspended pellets were pooled and centrifuged for ten minutes at 5000 × g to remove cellular debris. The supernatant was decanted into a sterile tube and the Mg^{++} concentration was adjusted to

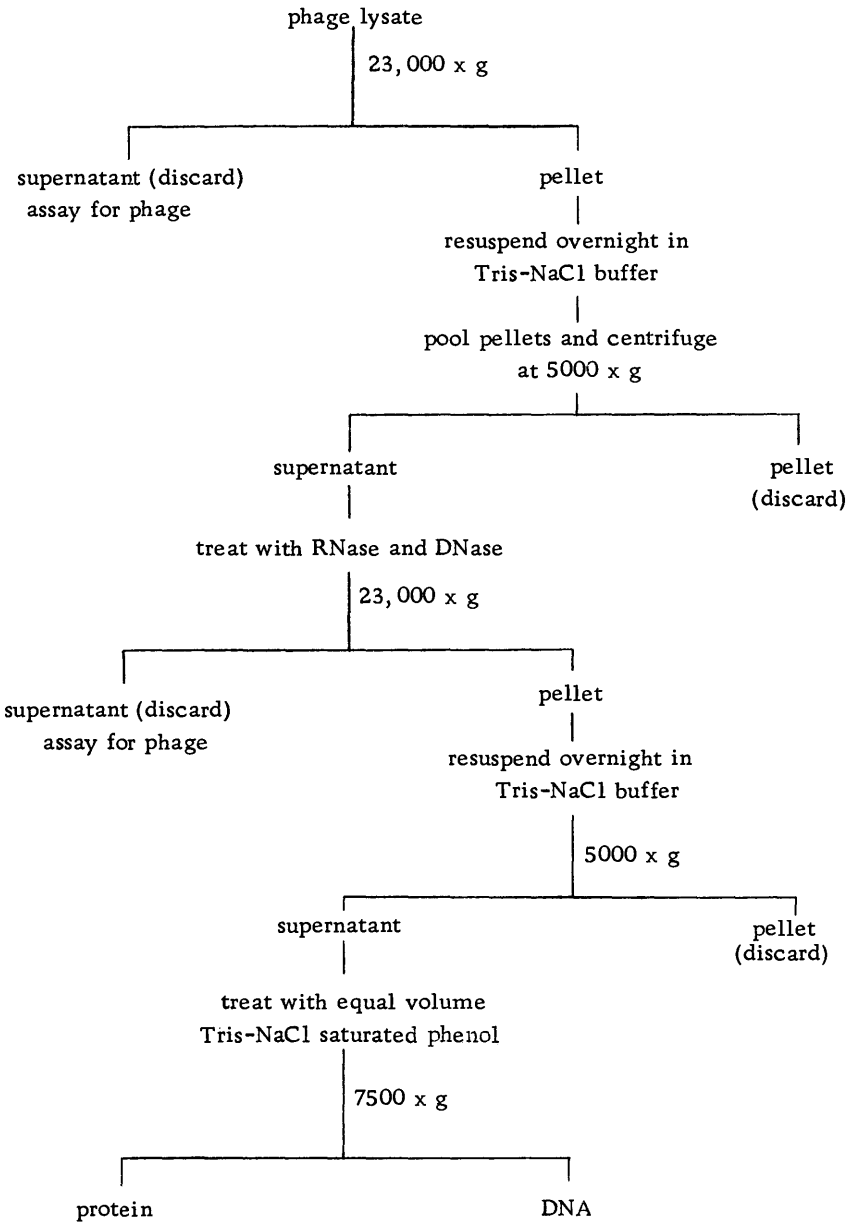

Figure 2. Isolation and purification of phage DNA

5×10^{-3}M with $MgCl_2$. Fifty μg/ml of RNase (Ribonuclease A, 5X crystallized, Sigma Chemical Company) and 50 μg/ml of DNase (B Grade, CalBioChem) was added and the phage suspension incubated for 60 minutes at 37° C. After treatment with RNase and DNase the phage suspension was centrifuged at 23,000 × g for 75 minutes. The supernatant was decanted and assayed for viable phage. The phage pellet was resuspended in 5.0 ml of Tris-NaCl buffer as described above. After resuspension, the phage was centrifuged for ten minutes at 5000 × g to remove any remaining cellular debris. The purified phage suspension was stored at 4° C until the DNA extraction step.

Extraction of Phage DNA

An equal volume of freshly distilled phenol, saturated with Tris-NaCl buffer, was added to the phage suspension and the tube gently agitated at room temperature for ten minutes. The resulting emulsion was transferred to a Corex glass centrifuge tube and centrifuged for 15 minutes at 7500 × g to separate the phenol and aqueous layers. The aqueous layer, containing DNA, was removed with a capillary pipet and transferred to a clean 15 × 125 mm screw cap tube. The aqueous layer was repeatedly treated with an equal volume of Tris-NaCl buffer saturated phenol until no more protein was observed at the interface. Usually three of four extractions were

sufficient. After the final phenol treatment the aqueous layer was transferred to dialysis tubing (size 8, Union Carbide Company) and dialyzed for 24 hours against four 500 ml changes of SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0) at 4° C. After dialysis the DNA was transferred to a sterile 15 × 125 mm screw cap tube and stored at 4° C.

Sterility of the DNA was determined by adding 0.1 ml of the DNA sample to 50 ml of media and incubating for 48-60 hours at 30° C. ELB was used for phage c2 DNA and PAB was used for phage R-1 DNA.

Residual free phage was determined by the phage assay procedure previously described.

Extraction of *B. cereus* DNA

A 500 ml Erlenmyer flask containing 100 ml of BHI was inoculated with 1.15×10^7 *B. cereus* spores/ml and incubated for six hours in a 37° C rotary shaker. The cells were harvested by centrifugation (5000 × g for ten minutes) and were washed once with distilled water. The cells were resuspended in 10 ml of Tris buffer (0.05 M Tris, pH 8.0) and incubated at 30° C. Lysis occurred by 180 minutes (Figure 3). This is a modification of the procedure by Mohan *et al.* (1965).

The DNA was extracted by a modification of Marmur's

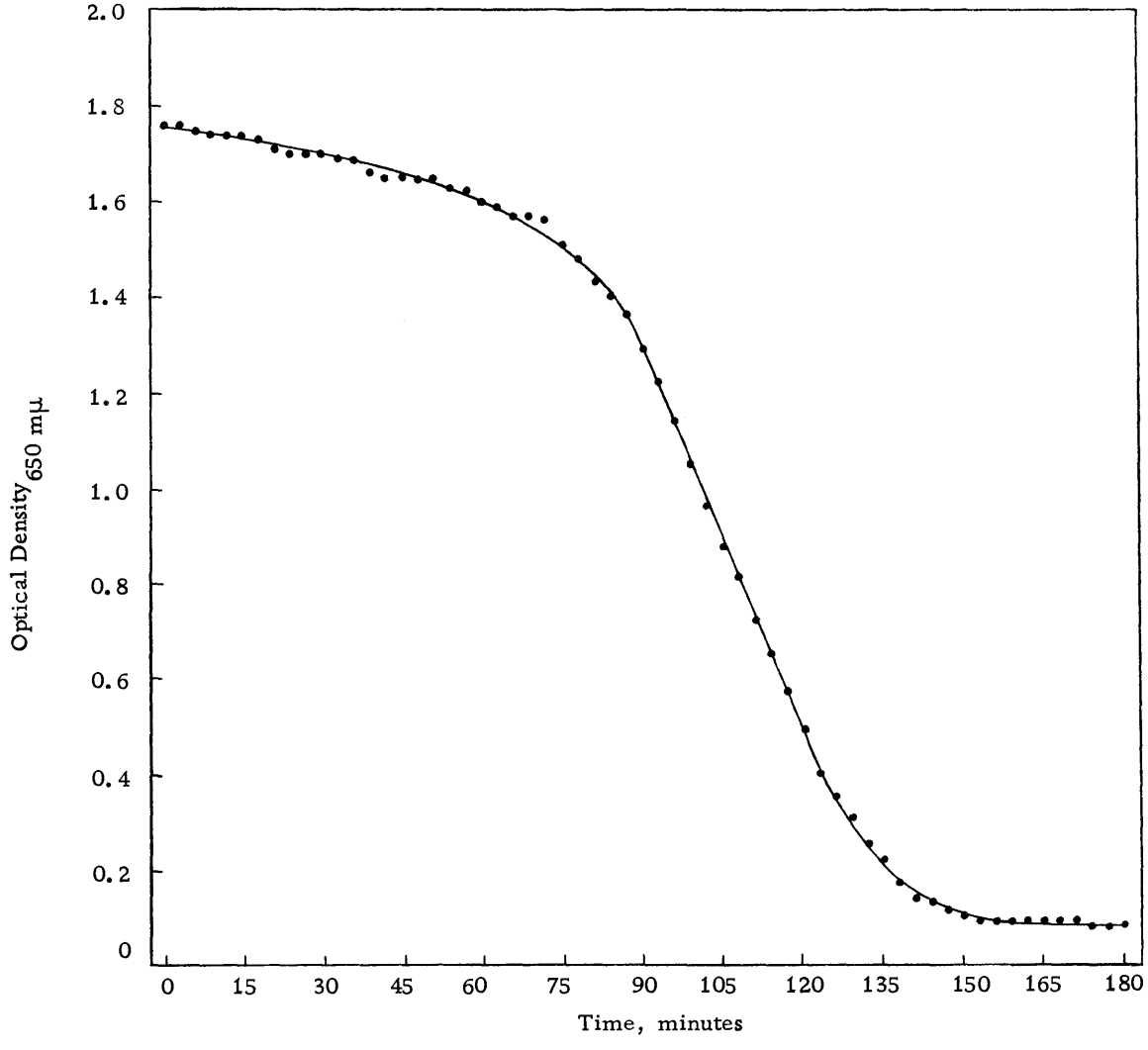

Figure 3. Lysis of B. cereus 569R in Tris buffer

procedure (Marmur, 1960). The lysed cell suspension was transferred to a 125 ml ground glass stoppered flask and the Na^+ concentration was adjusted to 1.0 M with 5.0 M $NaClO_4$. An equal volume of chloroform-isoamyl alcohol (24:1 v/v) was added and the contents agitated on a rotary platform for ten minutes at 160 revolutions/minute. The resulting emulsion was transferred to a Corex centrifuge tube and centrifuged at 7500 × g for ten minutes to separate the layers. The aqueous phase was removed with a pipet and transferred to a clean 125 ml ground glass stoppered flask. The DNA was repeatedly deproteinized with chloroform-isoamyl alcohol until no more protein could be seen at the interface after centrifugation. After the final deproteinization the aqueous layer was transferred to a 25 × 150 mm test tube and layered with two volumes of cold 95 percent ethanol. A glass stirring rod, wet with SSC, was inserted into the tube and the DNA was wound up on the rod as it precipitated out of solution. The DNA was dissolved in 10 ml of dilute SSC (0.015 M NaCl, 0.0015 M Na citrate, pH 7.0) and treated with RNase, which had been heated to 80° C for ten minutes to inactivate any contaminating DNase, to digest any free RNA. The DNA was precipitated with ethanol as described and redissolved in 10 ml of dilute SSC. The DNA was taken up in a 10 ml pipet and blown into four volumes of cold 95 percent ethanol. The resulting DNA clump was removed with a sterile loop and dissolved in 9.0 ml of dilute SSC. The SSC

concentration was adjusted to standard SSC concentration by adding 1.0 ml of concentrated SSC (1.5 M NaCl, 0.15 M Na citrate, pH 7.0).

The DNA was checked for sterility by adding 0.1 ml of the DNA to PAB and incubating 48-60 hours at 37° C. The DNA was stored at 4° C over a drop of chloroform.

Extraction of S. lactis DNA

S. lactis DNA was extracted by a modified procedure described by Knittel (1965). A one liter Erlenmyer flask containing 200 ml of ELB was inoculated with 2.0 ml of a 16 hour culture of S. lactis and incubated in a 30° C rotary shaker for six hours. The cells were harvested by centrifugation at 6000 × g for 15 minutes. The cells were washed with 50 ml of saline-EDTA (0.15 M NaCl, 0.1 M ethylenediamine tetra acetate, pH 8.0). The cells were resuspended in 25 ml of saline-EDTA in a 250 ml ground glass stoppered flask and four mg of lysozyme (egg white, 3X crystallized, CalBioChem) were added. The cells were incubated with occasional shaking at 37° C until the resulting mixture became viscous. Two ml of a 25 percent solution of sodium lauryl sulfate were added and the mixture was heated at 60° C for ten minutes. The contents of the flask were cooled to 0° C and the Na^+ concentration was adjusted to 1.0 M with 5.0 M $NaClO_4$. The mixture was shaken for ten minutes at room temperature with an equal volume of chloroform-isoamyl alcohol.

The resulting emulsion was centrifuged at 7500 × g for ten minutes to separate the layers. The aqueous layer was removed with a pipet and transferred to a clean 250 ml ground glass stoppered flask. The deproteinization step was repeated twice more. After the final deproteinization the aqueous phase was transferred tc a 25 × 150 mm test tube and layered with two volumes of cold 95 percent ethanol. The DNA was collected by winding the DNA on a glass stirring rod. The DNA was dissolved in 10 ml of dilute SSC and treated with 50 μg/ml of RNase, preheated to 80° C for ten minutes, for 30 minutes at 37° C. The DNA was precipitated as described and dissolved in 10 ml of dilute SSC. The DNA was taken up in a 10 ml pipet and blown into four volumes of cold 95 percent ethanol. The resulting DNA clump was removed with a sterile loop and dissolved in 9.0 ml of dilute SSC. The SSC concentration was adjusted to standard SSC by adding 1.0 ml of concentrated SSC.

The DNA was checked for sterility by adding 0.1 ml of the DNA to ELB and incubating for 48-60 hours at 30° C. The DNA was stored at 4° C over a drop of chloroform.

Determination of DNA Concentration

The optical density of the DNA sample was recorded at 260 and 280 mμ using a Beckman Model DU spectrophotometer. The DNA and protein concentration were determined with a Nomograph based on the

extinction coefficients for enolase and nucleic acid (Warburg and Christian, 1942) and distributed by CalBioChem.

Killing of B. cereus by a Crude Lysate of Phage c2

One-tenth ml of a phage c2 lysate (1×10^{10} phage/ml) was added to 0.9 ml of a log culture of B. cereus (1×10^{8} cells/ml) that had been washed once with saline. The phage treated cells were incubated for 30 minutes at 30° C, after which time they were diluted in saline and appropriate dilutions were spread on PAB agar plates that had been dried for 48 hours at 37° C. A control culture, substituting 0.1 ml of peptone diluent (0.5% peptone) in place of the phage lysate, was treated in the same manner. The plates were incubated for 24 hours at 30° C before counting.

CsCl Density Gradient Centrifugation of Phage c2

Twenty grams of CsCl (99%, K & K Laboratories) were dissolved in 10 ml of distilled water at 45° C. The CsCl solution was filtered through a Millipore GS membrane filter and the filtrate collected in a 25 × 150 mm test tube. The CsCl was stored at 4° C to allow the excess CsCl precipitate out of solution. One and eight-tenths ml of saturated CsCl (4° C) was mixed with 0.1 ml of phage c2 (9×10^{9} phage/ml) and 1.1 ml of distilled water in a 1/2 by 2 inch cellulose nitrate tube. One-hundredth ml of phage λ (1×10^{7}

phage/ml) was added as a reference marker. The mixture was layered with 2.0 ml of mineral oil and the tube placed in a bucket of the SW-39 rotor. The phage was centrifuged for 20 hours at 29,000 RPM in a Beckman Model L-2 ultracentrifuge.

The gradient was collected by piercing a small hole in the bottom of the tube with an insect pin (size 00) and collecting the drops in 1.0 ml of peptone diluent. Each drop was spotted against a sensitive lawn of S. lactis and E. coli to determine the phage peaks of phage c2 and phage λ, respectively. After determining the drops that contained active phage, these drops were assayed as described.

The first five drops of the gradient were pooled and designated as Fraction I. The five drops containing the active phage c2 peak were pooled and designated as Fraction II. The last five drops of the gradient were pooled and designated as Fraction III. The three fractions were transferred to dialysis tubing (size 8) and dialyzed against three 100 ml changes of SSC to remove the CsCl. After dialysis the three fractions were transferred to sterile screw cap tubes and stored at 4° C until tested for their effect on the growth of B. cereus.

Treatment of B. cereus Spores

B. cereus spores were heat-shocked for 15 minutes at 65° C. Ten volumes of L-alanine (2.5 mg/ml), prewarmed to 37° C, were

added and the spores were incubated for 30 minutes at 37° C. For all experiments, treated spores were added to a final concentration of 1.15×10^7 spores/ml.

Optical Density Growth Curves of B. cereus

Method

One and two-tenths ml of heat-treated spores were added to six ml of 2 X BHI and 1.8 ml of the spore culture was transferred to 1 cm^2 Pyrex cuvettes. The culture volume was adjusted to three ml with sterile distilled water. All cultures were incubated in the cuvette sample well of the spectrophotometer. The temperature was maintained at 30° C with a thermostatically controlled Haske circulating water bath.

Optical density changes were measured with a Beckman Model DU spectrophotometer (wave length = 650 mμ) equipped with a Gilford Model 2000 Multiple Sample Absorbance Recorder. Optical density readings were recorded every ten minutes for six hours. An untreated culture was run to determine the growth curve of B. cereus.

Effect of Additions

Phage c2 lysate. To test the effect of phage c2 lysate on the growth of B. cereus, 0.1 ml of phage c2 lysate (1×10^{10} phage/ml)

was added to each of two cultures. A non-infected culture was run as a control.

Phage c2 CsCl gradient fractions. The effect of the three dialyzed CsCl gradient fractions were tested for their effect on the growth of B. cereus. Five-tenths ml of each fraction was added to each of two cultures. A third culture was run as a control.

Phage c2 DNA. Thirty μg of DNA was added to one culture; 30 μg of DNA pretreated with DNase was added to the second culture; 0.1 ml of SSC was added to the third culture.

DNA from B. cereus 569R, S. lactis C_2S^s, B. cereus phage R-1 and calf thymus. One-tenth ml of SSC was added to the control culture; 0.1 ml of DNA (30 μg) was added to the second culture; 0.1 ml of DNA pretreated with DNase was added to the third culture.

Free bases. The four bases, adenine, guanine, cytosine, and thymine, were dissolved in SSC to give a mixture with a final concentration of 300 μg/ml of each base. One-tenth ml of SSC was added to the control culture and 0.1 ml of the free base solution was added to each of the other two cultures.

The Effect of Phage c2 DNA on the Growth of B. cereus when Grown under Aerobic Conditions As Measured by Optical Density Change

Ten ml of heat-treated spores were added to each of three 500 ml Erkenmyer flasks containing 50 ml of 2X BHI. Phage c2 DNA was

added to one flask (final concentration = 10 μg/ml); phage c2 DNA pretreated with DNase was added to the second flask; an equal volume of SSC was added to the third flask as a control. The final volume of each culture was adjusted to 100 ml with sterile distilled water. The cultures were incubated in a 30° C rotary shaker and three ml samples were removed at 15 minute intervals. The optical density of each sample was recorded at 650 mμ using a Beckman Model DU spectrophotometer.

"Competence" of B. cereus

To determine if B. cereus during growth develops a stage similar to that of competence in transformable strains, a modified procedure of Jensen and Haas (1963) was used.

Growth Conditions

Growth in SMCA. Ten ml of heat-treated spores were added to 50 ml of 2X SMCA that was prewarmed to 37° C. The final volume was adjusted to 100 ml with sterile distilled water and the culture was incubated in a 37° C rotary shaker. Three ml samples were taken every 15 minutes beginning at 5.5 hours and continuing through 8.5 hours.

Growth in BHI. Ten ml of heat-treated spores were added to 50 ml of 2X BHI and the final volume adjusted to 100 ml with sterile

distilled water. The culture was incubated at 30° C under static conditions and three ml samples were taken every 15 minutes through six hours. The culture volume was maintained at 100 ml by adding cells from a second culture.

Method for Determining Competence

The samples were centrifuged at 5000 × g for 15 minutes and washed once with an equal volume of buffer. The cells were resuspended in three ml of methylene blue (83% dye content) which had been diluted in water to an $O.D._{660}$ of approximatedly 0.200 ($O.D._I$). The cells were shaken for ten minutes in a 37° C reciprocal shaker, centrifuged, and the $O.D._{660}$ of the supernatant was recorded ($O.D._F$). The $O.D._I - O.D._F$ (uptake of methylene blue) was plotted versus time. In the procedure of Jensen and Haas (1963), periods of competence, as shown by transformation, correlated with periods of maximum methylene blue dye uptake.

Effect of Phage c2 DNA on "Competent" B. cereus

B. cereus cells that were grown in SMCA for seven hours, showing a peak of "competence" by the test given above, were harvested by centrifugation and washed once with an equal volume of buffer. The cells were diluted to an $O.D._{650}$ of approximately 0.1 in BHI. To one culture 0.1 ml of phage c2 DNA (10 μg/ml) was added; 0.1 ml of

phage c2 DNA pretreated with DNase was added to the second culture; 0.1 ml of SSC was added to the third culture. The final volume was three ml. The cultures were incubated in the cuvette chamber of a Beckman Model DU spectrophotometer and optical density readings were recorded every ten minutes.

Effect of Phage c2 DNA on "Non-competent" B. cereus

B. cereus cells that were grown for 6.5 hours in SMCA, showing low "competence" by the methylene blue technique, were harvested by centrifugation. The cells were washed and treated as described above.

Viable and Total Cell Counts of B. cereus Infected with Phage c2 DNA

Three ml of heat-treated spores were added to 15 ml of 2X BHI. The spores were divided into three parts in 50 ml Erlenmyer flasks. One ml of SSC was added to the control culture; one ml of phage c2 DNA (100 μg) was added to the second culture; one ml of phage c2 DNA pretreated with DNase was added to the third culture. The final volume of each culture was adjusted to 10 ml with sterile distilled water. The cultures were incubated at 30° C under static conditions. One-tenth ml samples were removed every 30 minutes through six hours. The total cell count was made using a Petroff-Hauser counter. The

viable cells count was made by diluting the cells in saline and plating on nutrient agar plates that had been dried for 48 hours at 37° C. The plates were incubated overnight at 30° C before counting.

Uptake of ^{3}H Phage c2 DNA by B. cereus

Five ml of heat-treated spores were added to 25 ml of 2X BHI. Five hundred μg of phage c2 ^{3}H-DNA were added and the volume of the culture was adjusted to 50 ml with sterile distilled water. The culture was incubated under static conditions at 30° C and four ml samples were removed at one hour intervals up through six hours. The cells were filtered through a Millipore HA membrane filter and washed with five volumes of saline. The cells were resuspended in four ml of SSC, supplemented with 5×10^{-3} M Mg^{++}. The samples were divided into two parts of 2.0 ml each. One part was treated with 50 μg/ml of DNase for 30 minutes at 37° C. The other part was untreated. After incubation both parts were filtered and washed with five volumes of saline. The samples were resuspended in two ml of Tris buffer and the cells lysed by incubating for three hours at 30° C. Five-tenths ml of each sample was transferred to a scintillation vial and the radioactivity was measured. The balance of the untreated sample was dialyzed for six hours against three 100 ml changes of SSC. After dialysis, 0.5 ml of each sample was transferred to a scintillation vial and the radioactivity measured.

CsCl Density Gradient Centrifugation of Cell Lysates of *B. cereus* Cells Infected with ^{3}H Phage c2 DNA

Ten ml of heat-treated spores were added to 50 ml of 2X BHI. One thousand μg of phage ^{3}H-DNA and 100 μC of ^{32}P phosphoric acid (New England Nuclear) were added and the volume adjusted to 100 ml with sterile distilled water. The culture was incubated in a 30° C incubator under static conditions. Ten ml samples were removed at one hour intervals up through seven hours. The cells were filtered through a Millipore HA membrane filter and washed with five volumes of saline. The cells were resuspended in two ml of Tris buffer and lysed by incubating for three hours at 30° C. Six-tenths ml of each cell lysate was mixed with 2.4 ml of saturated CsCl (4° C) in a 1/2 × 2 inch cellulose nitrate tube. Duplicate samples were made. The contents of the tubes were layered with two ml of mineral oil and placed in buckets of a SW-50 rotor. The samples were centrifuged for 50 hours in either a Beckman Model L-2 or L-2 65 ultracentrifuge.

After centrifugation a small hole was pierced in the bottom of the tube and two drop fractions were collected in 13 × 100 mm test tubes. One ml of 0.1 N NaOH was added to each fraction and the tubes were incubated at room temperature for ten minutes. The tubes were cooled to 0° C in an ice bath and one ml of 0.1 N HCl and two ml of 6X SSC were added. Each fraction was filtered through a nitrocellulose membrane (Type B6, Carl Schleicher and Schuell

Company) and both sides of the membrane were washed with 10 ml of 6X SSC. The membranes were transferred to scintillation vials and dried overnight at 37° C. Ten ml of toluene scintillation fluid were added to each vial and the radioactivity measured.

Gradients of cell ^{32}P-DNA alone and a mixture of cell ^{32}P-DNA and phage c2 ^{3}H-DNA were run and treated in the same manner for comparison.

Measurement of Radioactivity

A Packard Model 300 EXS Liquid Scintillation Spectrophotometer was used for measuring radioactivity. When ^{3}H was counted separately, a window setting of 50-1000 was used at a 50 percent gain. For counting ^{3}H and ^{32}P together, ^{3}H was counted at a 55 percent gain with a window setting of 100-500 and ^{32}P was counted at a 3.5 percent gain with a window setting of 300-1000. At these settings three percent of the ^{32}P counts overlapped into the ^{3}H window and none of the ^{3}H counts appeared in the ^{32}P window.

After subtraction of the background, radioactivity of each sample was expressed in counts per minute per ml. The ^{32}P counts were corrected for decay before plotting.

RESULTS

Effect of Phage c2 Lysate on B. cereus

When log phase cells of B. cereus were incubated with a lysate of phage c2 for 30 minutes and plated for viable count, only five percent of the cells were able to form colonies (Table 8). The number of cells lost was found to be dependent on the amount of phage lysate added to the cells. If heat-treated spores (see Materials and Methods) were substituted for log phase cells similar results were obtained; therefore in subsequent studies heat-treated spores were used to standardize the experiments.

Table 8. Effect of phage c2 lysate on the viability of B. cereus

	Initial plate count	Plate count after 30 minutes incubation
Infected	1×10^8	5×10^6
Control	1×10^8	1×10^8

Since a lysate of phage c2 "killed" the majority of cells, the growth rate of "infected" and "non-infected" cultures was followed by optical density change. In preliminary studies several types of media were tested: ELB, which is used for growth of phage c2 in S. lactis; PAB, which is generally used for B. cereus phages; BHI, which is a very rich medium. The maximum inhibitory effect, as followed by optical density, was found when BHI was used as the growth medium.

The growth of B. cereus, starting with heat-treated spores, is shown in Figure 4. Log phase growth began after about 60 minutes and the stationary phase was reached after 300 minutes. Since growth kinetics could not be followed after the culture reached the stationary phase of growth, the experiments were terminated after six hours.

The effect of phage c2 lysate on the growth of B. cereus is shown in Figure 5. Growth inhibition began approximately 75 minutes after addition of the phage lysate and maximum inhibition was found after 240 minutes. From these results it was not possible to determine whether the phage lysate caused an increase in generation time or whether the "infected" cells failed to grow and the optical density increase observed was due to the growth of "non-infected" cells.

Effect of CsCl Density Gradient Fractions on Growth of B. cereus

Since it was found that a filtered phage c2 lysate caused growth inhibition of B. cereus, the following question was asked: Is growth inhibition caused by (1) the whole phage, (2) free nucleic acid in the lysate, or (3) free protein in the lysate? To determine the responsible agent(s), a phage c2 lysate was subjected to CsCl density gradient centrifugation to separate the three components. Fraction I, at the bottom of the gradient (nucleic acids), Fraction II, in the center of the gradient (whole phage), and Fraction III, at the top of the gradient (protein) were dialyzed to remove CsCl and tested for

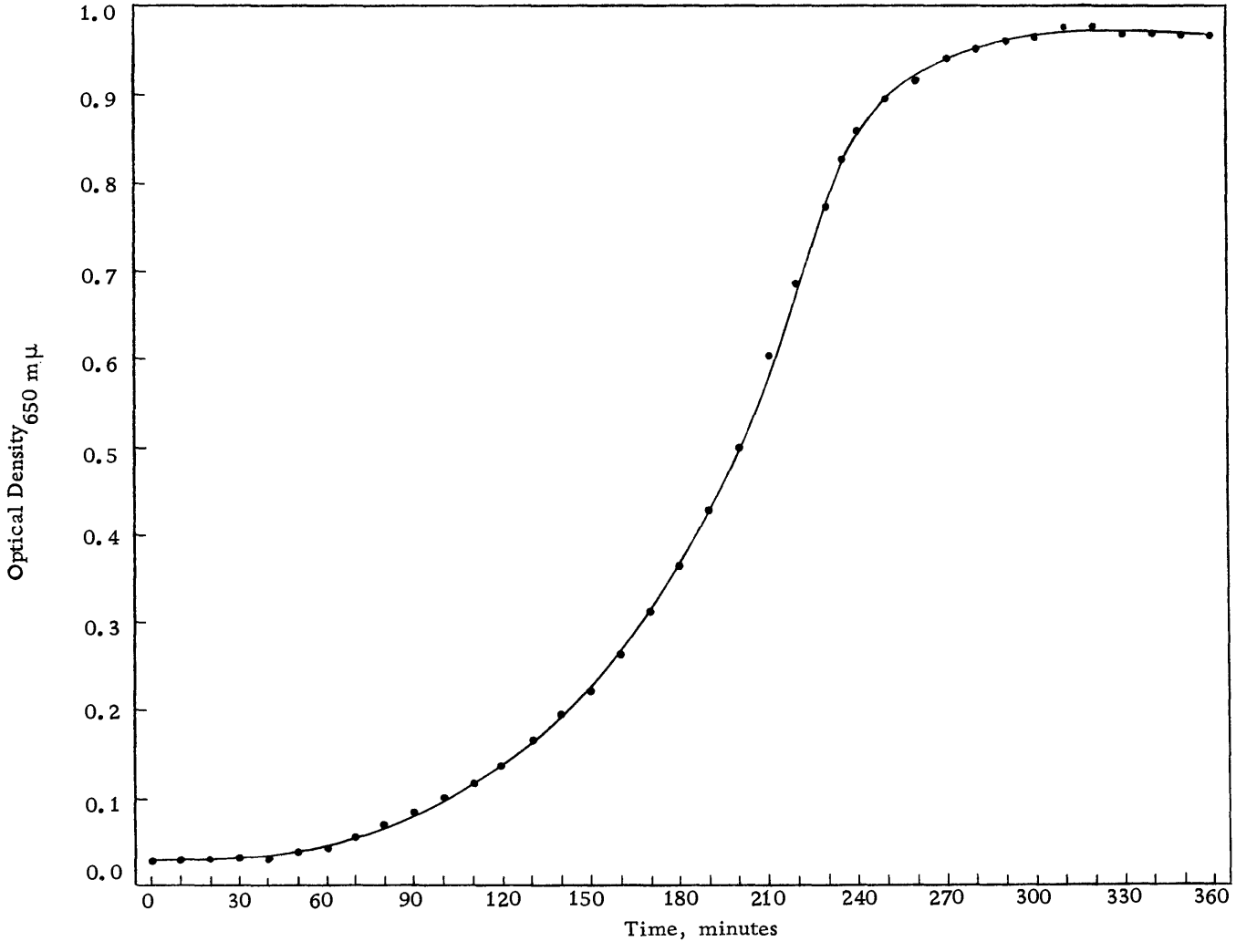

Figure 4. Growth curve of B. cereus 569R

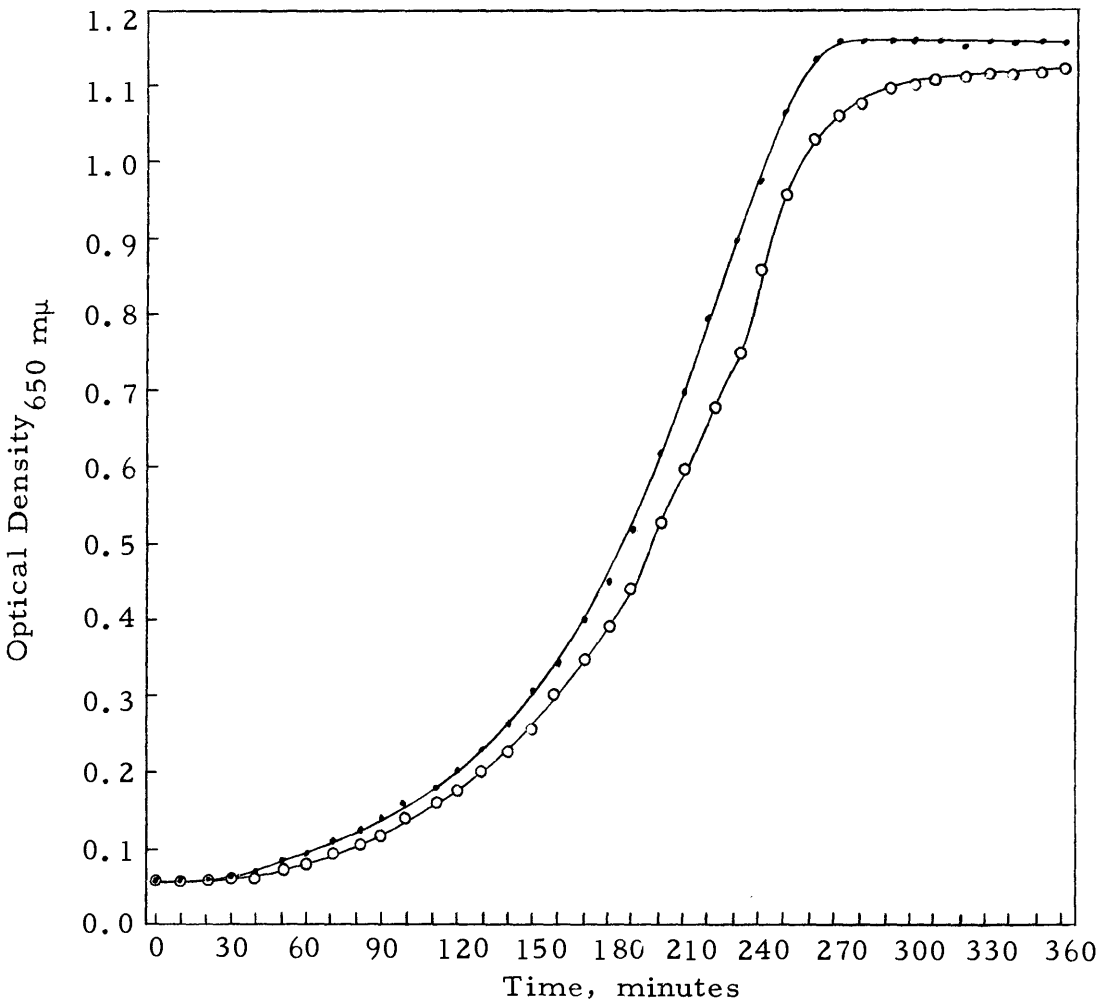

Figure 5. Effect of phage c2 lysate on the growth of B. cereus 569R. —•— B. cereus; —o—o— B. cereus plus phage lysate.

their effect on the growth of B. cereus (Figure 6). Fractions II and III had no effect on growth, but Fraction I showed a marked inhibitory effect on growth of the culture. During growth of the culture treated with Fraction I, two or possibly three discontinuities in the increase of optical density were observed.

Effect of Phage c2 DNA on the Growth of B. cereus

These results suggested that free nucleic acid of phage c2 and/or S. lactis caused growth inhibition. To test this hypothesis, DNA was extracted from phage c2 and S. lactis and tested for growth inhibition of B. cereus.

The effect of phenol extracted phage c2 DNA on the growth of heat-treated B. cereus spores is shown in Figure 7. After 240 minutes of growth the optical density of the DNA infected culture was 50 percent lower than that of the non-infected control culture. When phage c2 DNA was treated with DNase before adding to the heat-treated spores, no inhibitory effect was observed.

During growth, three discontinuities in the optical density of the DNA infected culture were found. The first was between 150 and 160 minutes after the addition of phage c2 DNA; the second between 230 and 240 minutes; the third between 270 and 280 minutes. This observation was found to be reproducible. Later experiments suggested that these discontinuities may present periods of lysis.

Since phage c2 DNA was found to be inhibitory for growth of B. cereus, total and viable cell counts of infected and non-infected

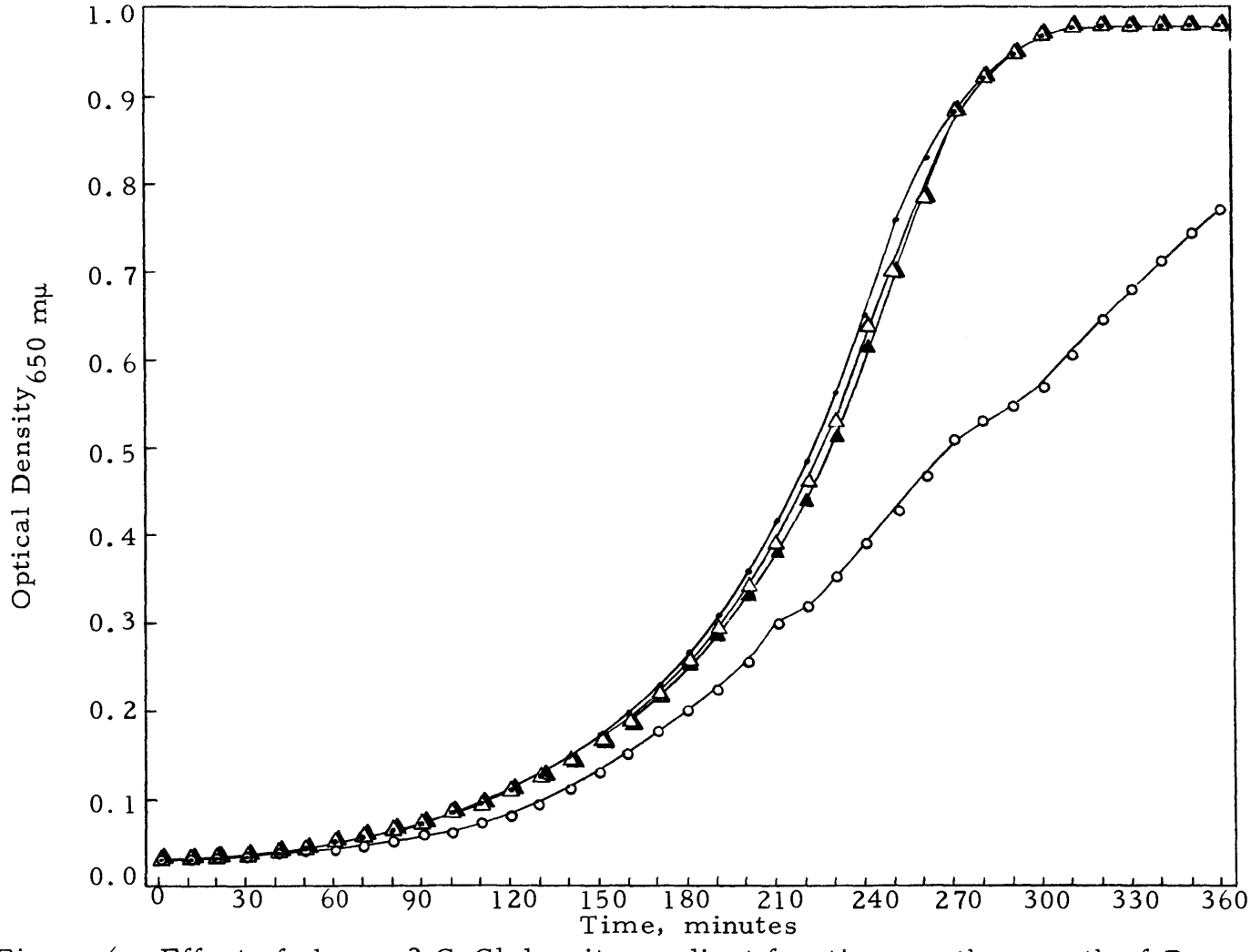

Figure 6. Effect of phage c2 CsCl density gradient fractions on the growth of B. cereus 569R. —•—•— B. cereus; —o—o— B. cereus plus Fraction I; —▲—▲— B. cereus plus Fraction II; —△—△— B. cereus plus Fraction III.

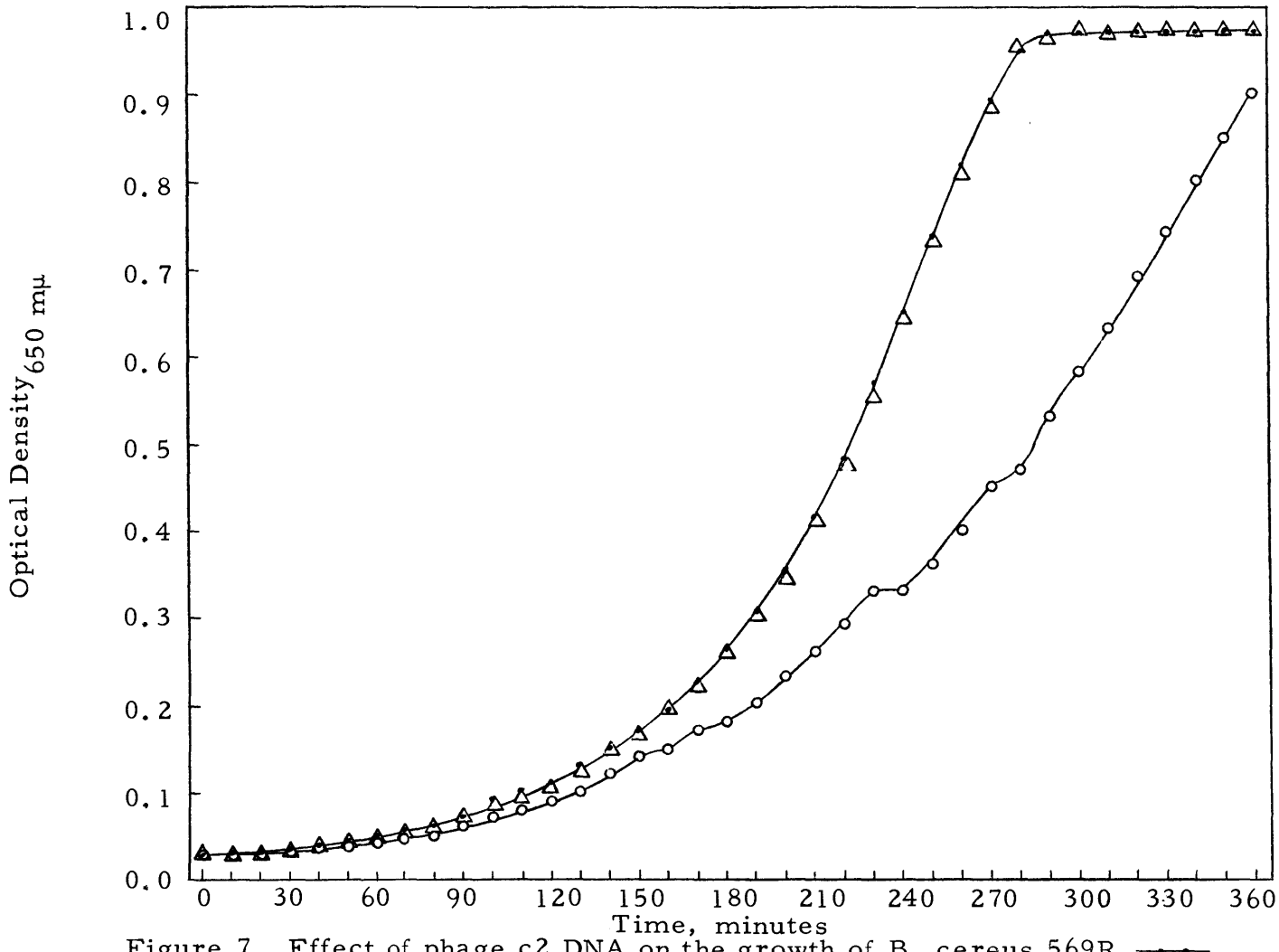

Figure 7. Effect of phage c2 DNA on the growth of B. cereus 569R. —•— B. cereus; —o—o— B. cereus plus phage c2 DNA; —△—△— B. cereus plus phage c2 DNA pretreated with DNAse.

cells were made to determine whether the cells were killed or whether the generation time of DNA infected cells was lengthened. The results of this experiment are shown in Figure 8.

The total and viable counts in the control (non-infected) culture were parallel with one another, the total count being approximately twice that of the viable count. Since B. cereus normally forms short chains this was to be expected.

During the first 150 minutes of growth the viable counts of the infected and non-infected cultures were identical. Beginning at 50 minutes the viability of the infected culture decreased as compared to the control culture, and five hours after infection the number of viable cells in the infected culture was only one percent of the number of viable cells in the non-infected culture. At 180 minutes the number of total cells in the infected culture was only about one-third of the total number of cells in the control culture. The drop in viable count indicated that actual cell killing did occur.

Since growth inhibition had been observed when the cells were grown under static conditions, a similar experiment was carried out using shaken cultures. Heat-treated spores were infected with phage c2 DNA and grown in a rotary shaker. Growth was followed by optical density change. The results of this experiment are shown in Figure 9. It was found that under these conditions that the inhibitory effect of phage c2 DNA was lost.

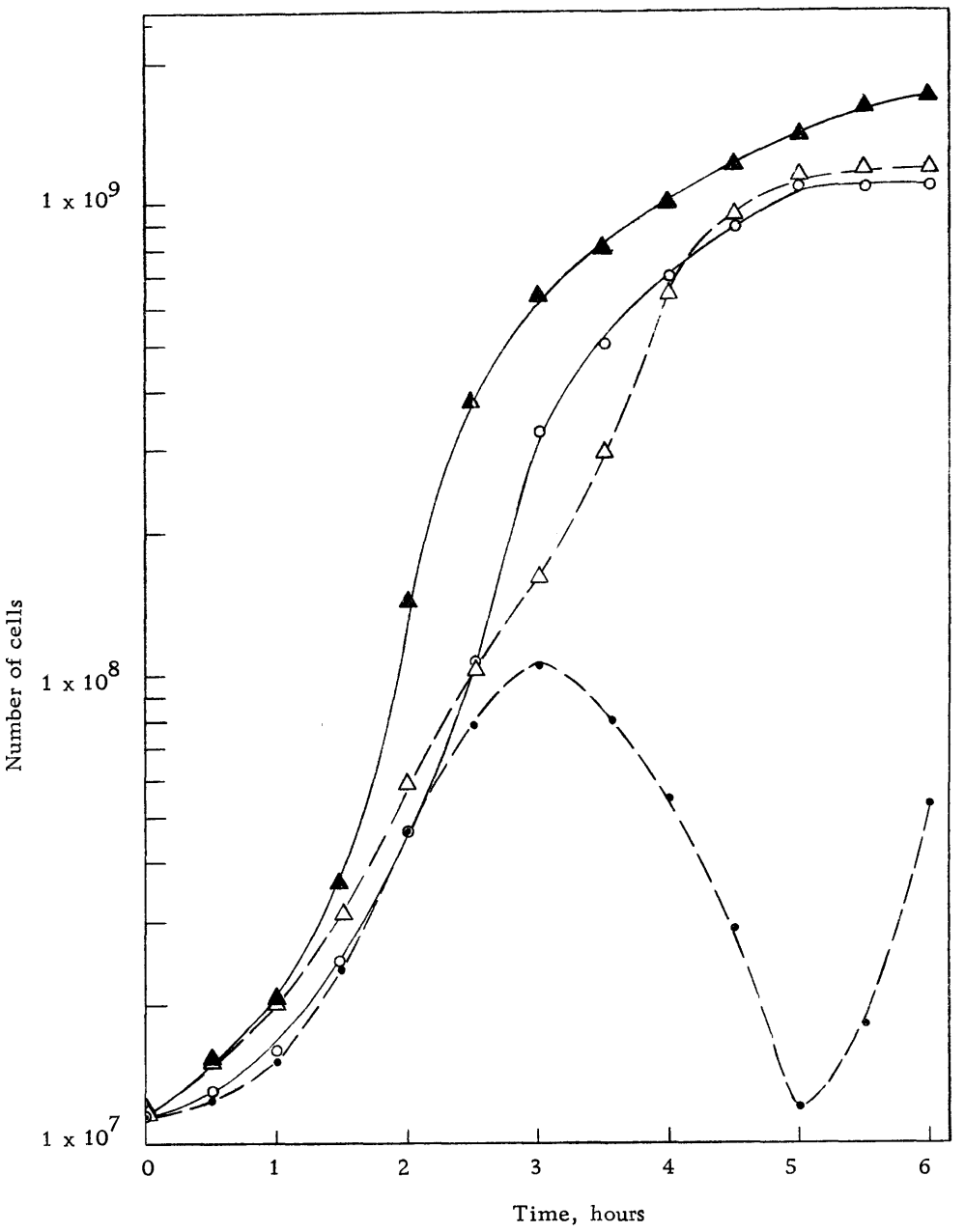

Figure 8. Total and viable cell counts of B. cereus 569R infected with phage c2 DNA. (—•—•—Viable cell count of B. cereus infected to phage c 2 DNA; △–△–△Total cell count of infected B. cereus; —o—o—Viable cell count of B. cereus; ▲–▲–▲ Total cell count of B. cereus).

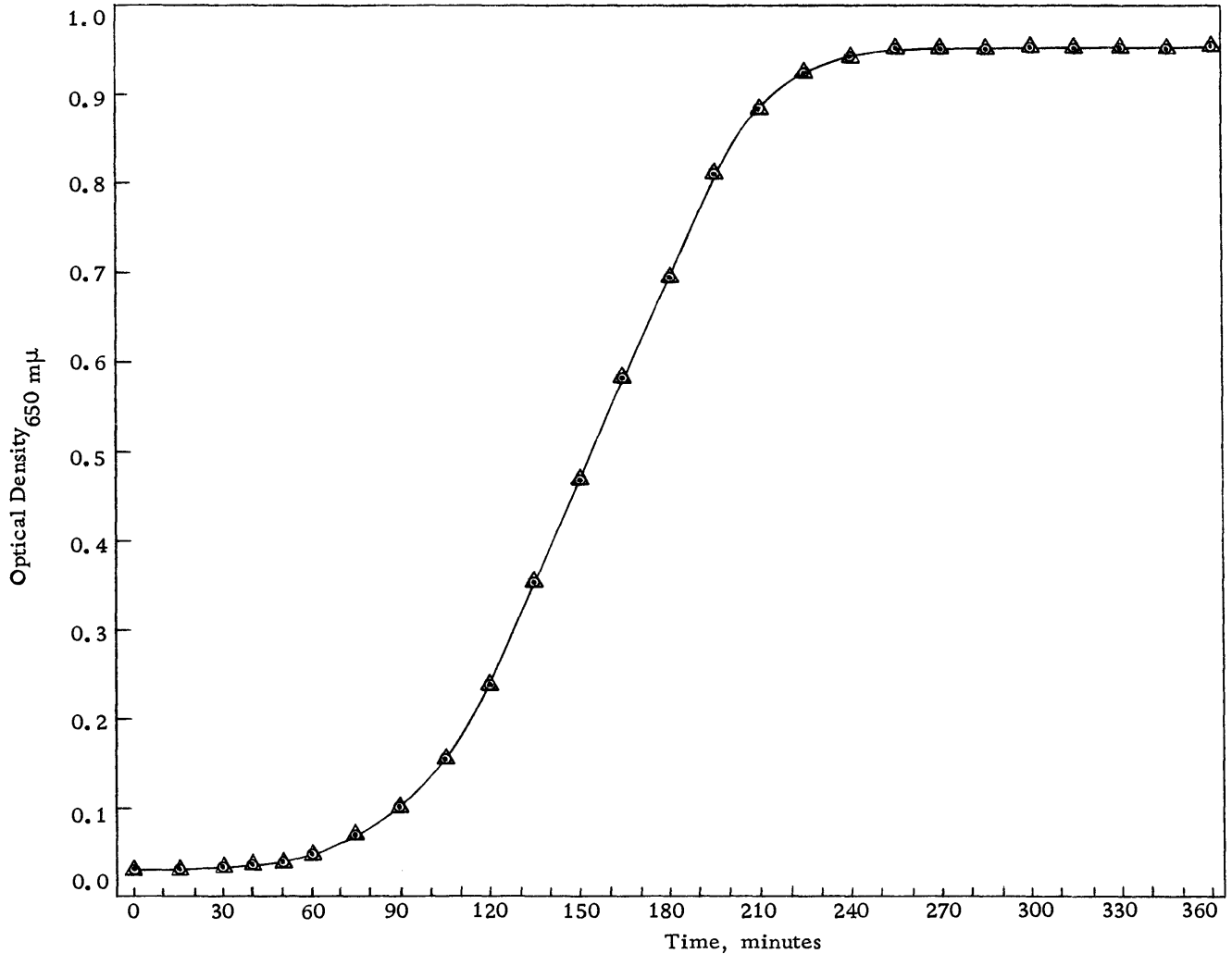

Figure 9. Effect of phage c2 DNA on the growth of B. cereus 569R under aerobic growth. —•— B. cereus; —o—o—B. cereus plus phage c2 DNA; —△—△—B. cereus plus phage c2 DNA pretreated with DNAse.

Effect of Various Types of DNA on the Growth of B. cereus

To determine if the inhibitory effect observed with phage c2 DNA was specific for this DNA or whether any foreign DNA or DNA components could cause this effect, S. lactis, B. cereus, calf thymus, B. cereus phage R-1 DNA, and the free bases were tested on the growth of B. cereus. Growth was followed by optical density change. The results of these experiments are shown in Figures 10, 11, 12, 13, and 14. It was found that none of these types of DNA nor the free bases was inhibitory at the concentration used for the c2 DNA experiments. Calf thymus DNA exerted a stimulatory effect under the conditions tested. The reason for this is not known. So far growth inhibition has only been observed using phage c2 DNA.

Incorporation of Phage c2 DNA

To determine whether phage c2 DNA was incorporated into the cells, ^{3}H labeled DNA was added to heat-treated spores and the radioactivity of washed cells was measured after the cells were lysed. During the course of the experiment it was necessary to decrease the volume of the culture, due to sampling. In every experiment the final volume was at least 50 percent of the initial volume in order to eliminate alteration of growth conditions as much as possible. Fraser and Baird (1967b) have found that decreasing

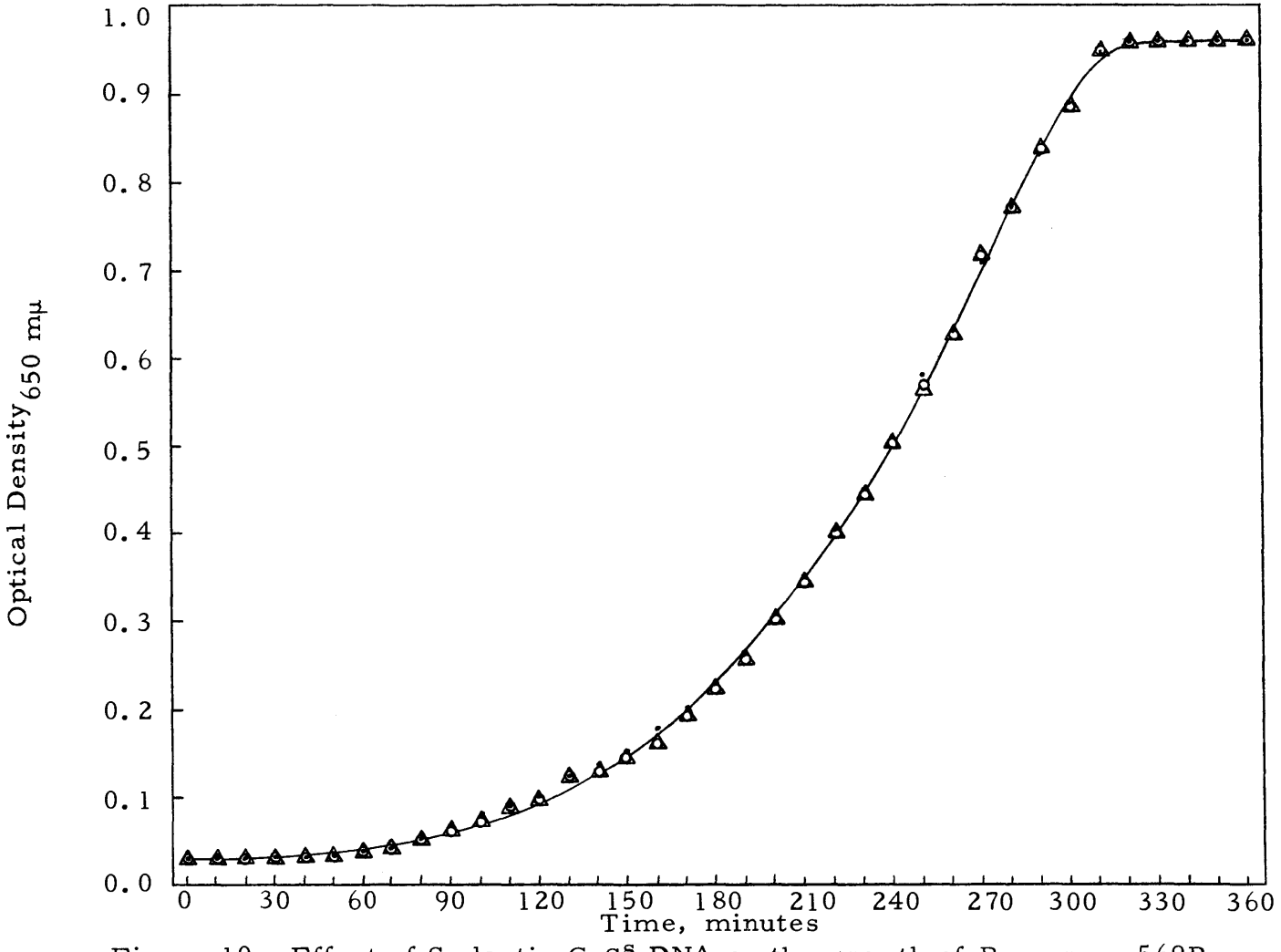

Figure 10. Effect of S. lactis C_2S^s DNA on the growth of B. cereus 569R.
—•— B. cereus; —o—o— B. cereus plus S. lactis C_2S^s DNA;
—Δ—Δ— B. cereus plus S. lactis C_2S^s DNA pretreated with DNAse.

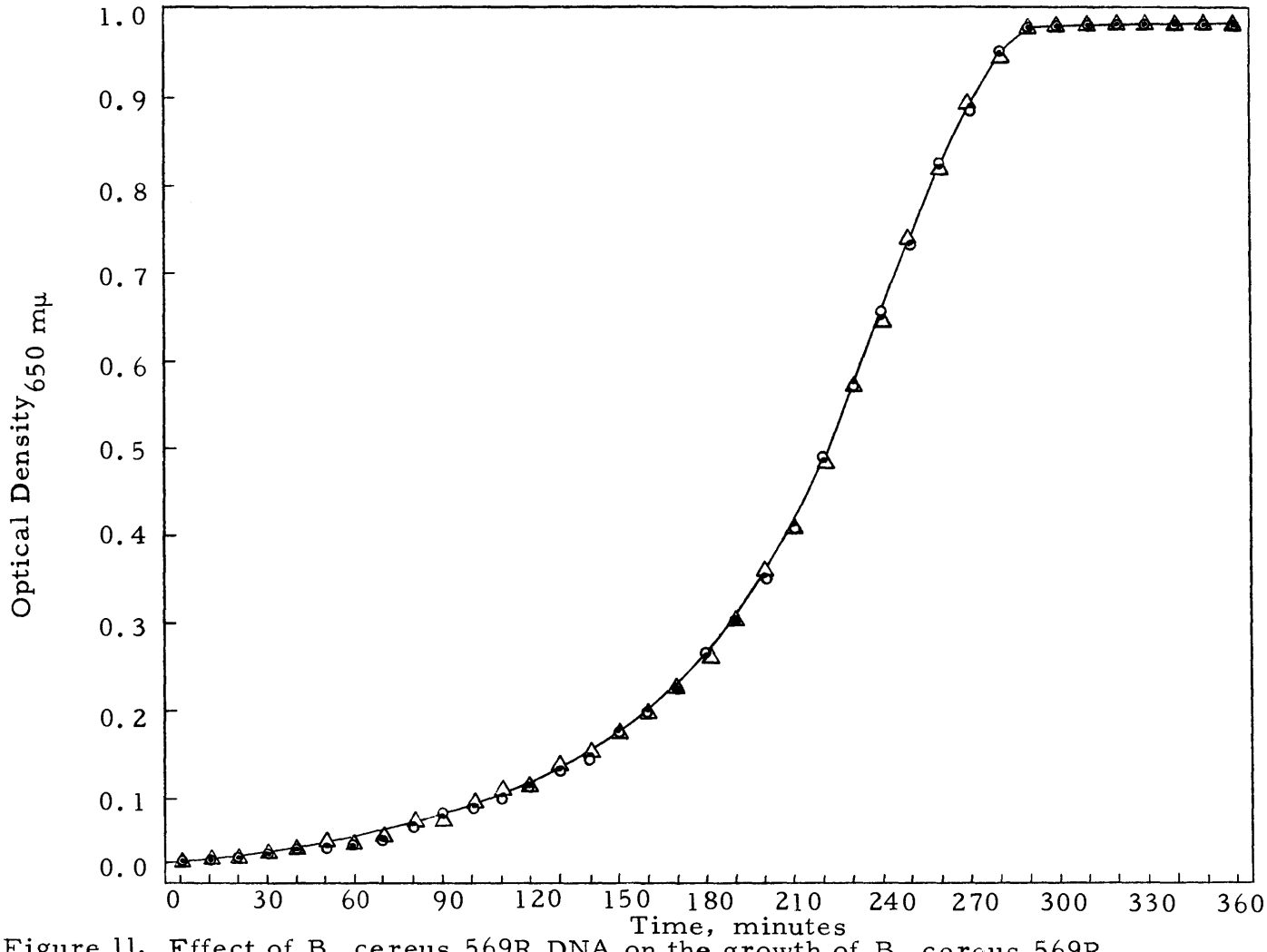

Figure 11. Effect of B. cereus 569R DNA on the growth of B. cereus 569R.
—•— B. cereus; —o—o— B. cereus plus B. cereus 569R DNA;
—△—△— B. cereus plus B. cereus 569R DNA pretreated with DNAse.

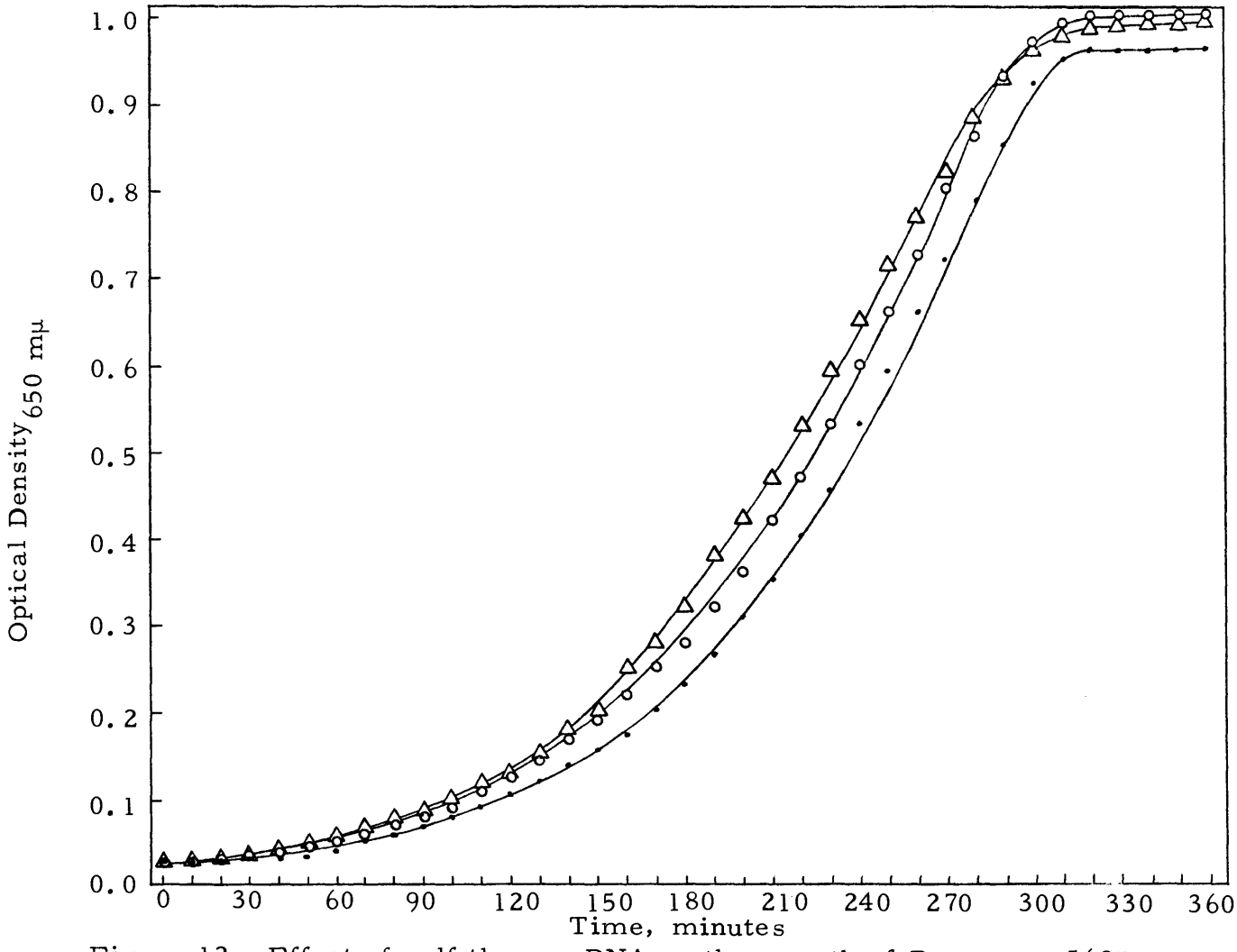

Figure 12. Effect of calf thymus DNA on the growth of B. cereus 569R. —•— B. cereus; —o—o— B. cereus plus calf thymus DNA; —△—△— B. cereus plus calf thymus DNA pretreated with DNAse.

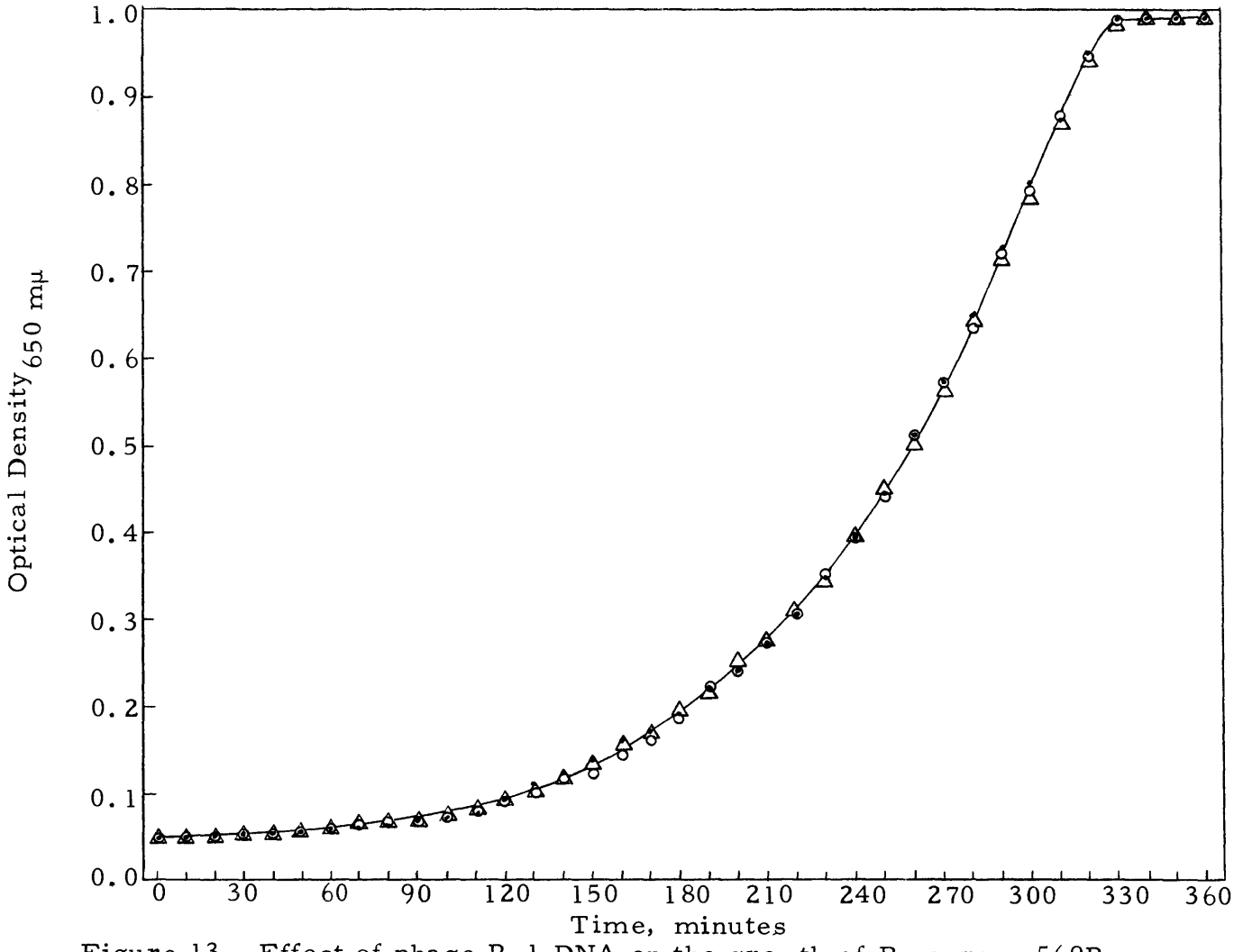

Figure 13. Effect of phage R-1 DNA on the growth of B. cereus 569R. —•— B. cereus; —o—o— B. cereus plus phage R-1 DNA; —Δ—Δ— B. cereus plus phage R-1 DNA pretreated with DNAse.

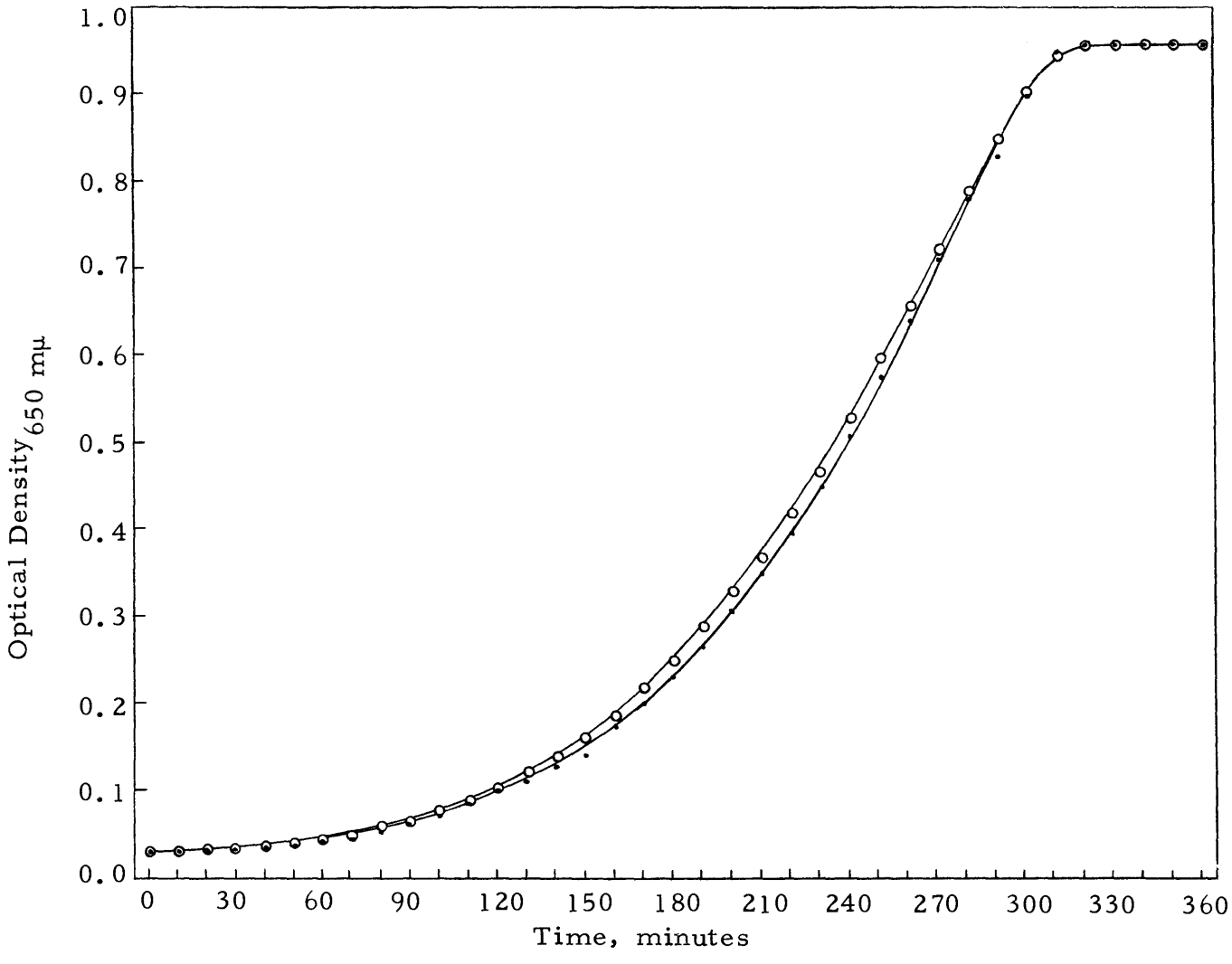

Figure 14. Effect of free bases on the growth of B. cereus 569R.
—•— B. cereus; —o— B. cereus plus free bases.

the culture volume modifies growth conditions. The results of this experiment are shown in Table 9.

Table 9. Incorporation of ^{3}H labeled phage c2 DNA by B. cereus cells

Time, hrs	Counts per minute per ml		
	untreated cells	DNase treated cells	dialysate
1	207	9	184
2	184	200	196
3	1108	1115	1099
4	2031	1515	2008
5	5131	4679	5088
6	6315	6203	6273

From these results it was concluded that the DNA was incorporated into the cell because most of it was insensitive to the action of DNase. It was also concluded that the DNA was not broken down into nucleotides or oligonucleotides, as the counts of the dialyzed preparation were nearly identical to those of the untreated preparation. Later experiments confirm this conclusion. The difference in counts between untreated and DNase treated cells at 1, 4, and 5 hours may mean that the DNA is still DNase sensitive during its incorporation into the cells. Subsequent experiments indicated that the DNA was incorporated into the cells at different times of growth and that incorporation was not linear.

Effect of Phage c2 DNA on "Competent" B. cereus

Since both log phase cells and treated spores were susceptible to infection by phage c2 DNA it was of interest to determine whether the cells developed competent period(s) during growth. It has been found with B. subtilis that competent cells are required for incorporation of either cell or phage DNA. B. cereus is considered to be non-transformable and therefore no attempt to demonstrate competent periods had been made. Since Jensen and Haas (1963) have shown that there is a correlation between transformation and uptake of methylene blue dye in B. subtilis, this method was employed to determine whether B. cereus exhibited periods of methylene blue dye uptake. The results of this experiment are shown in Figure 15. When B. cereus was grown in SMCA, three peaks of methylene blue dye uptake were observed between 5.5 and 8.5 hours.

B. cereus cells, grown for seven hours in SMCA, corresponding to a peak period of methylene blue dye uptake, were diluted and infected with phage c2 DNA. The growth of the culture was followed by optical density change (Figure 16). Inhibition of growth was found to begin almost immediately after infection. A plateau, or an actual drop, in the optical density was found between 130 and 140 minutes after the addition of the DNA. It seems possible that this drop represents lysis of about one-fifth of the cells. In this experiment,

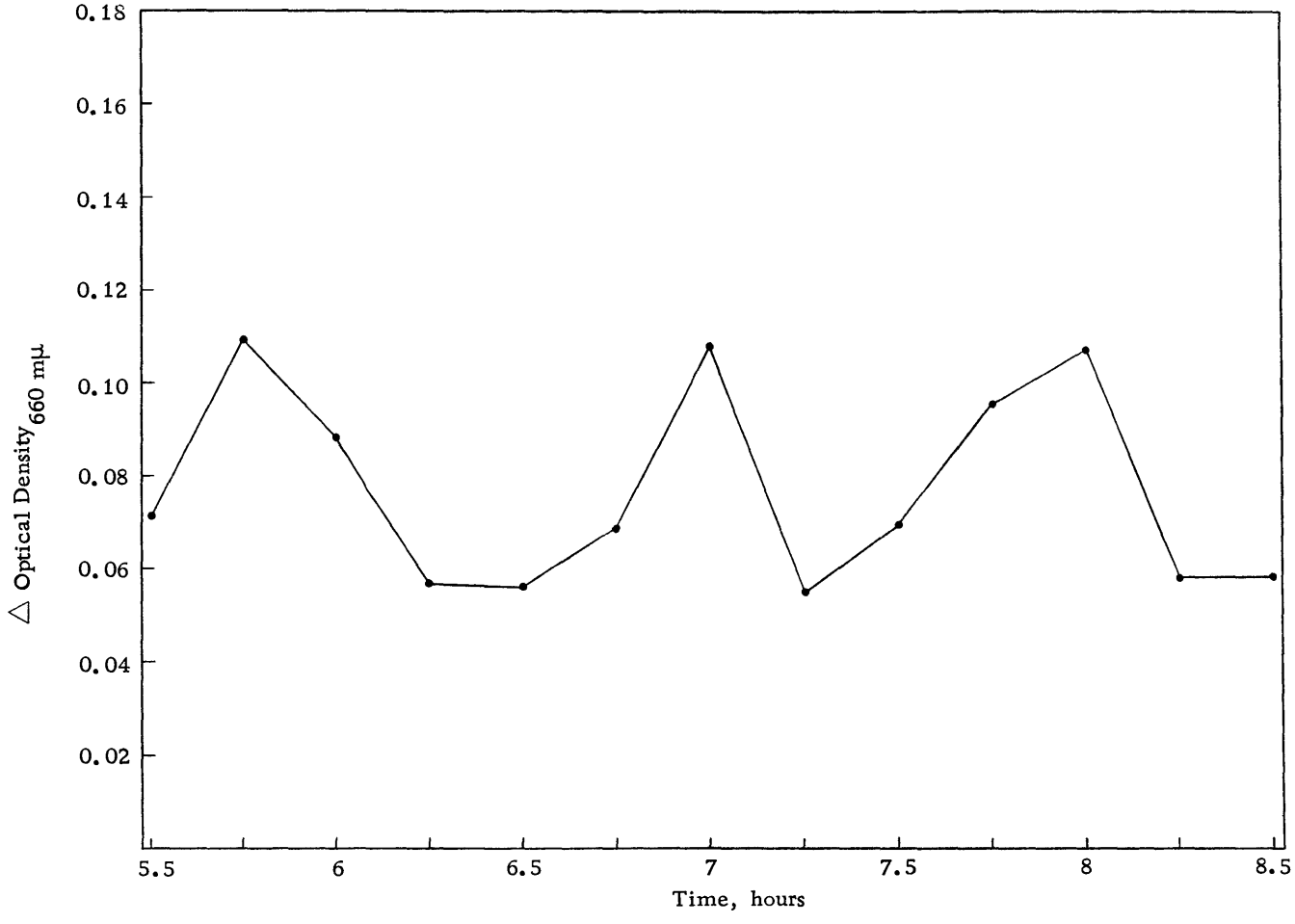

Figure 15. Uptake of methylene blue by B. cereus 569R when grown in SMCA. Initial optical density of methylene blue = 0.223.

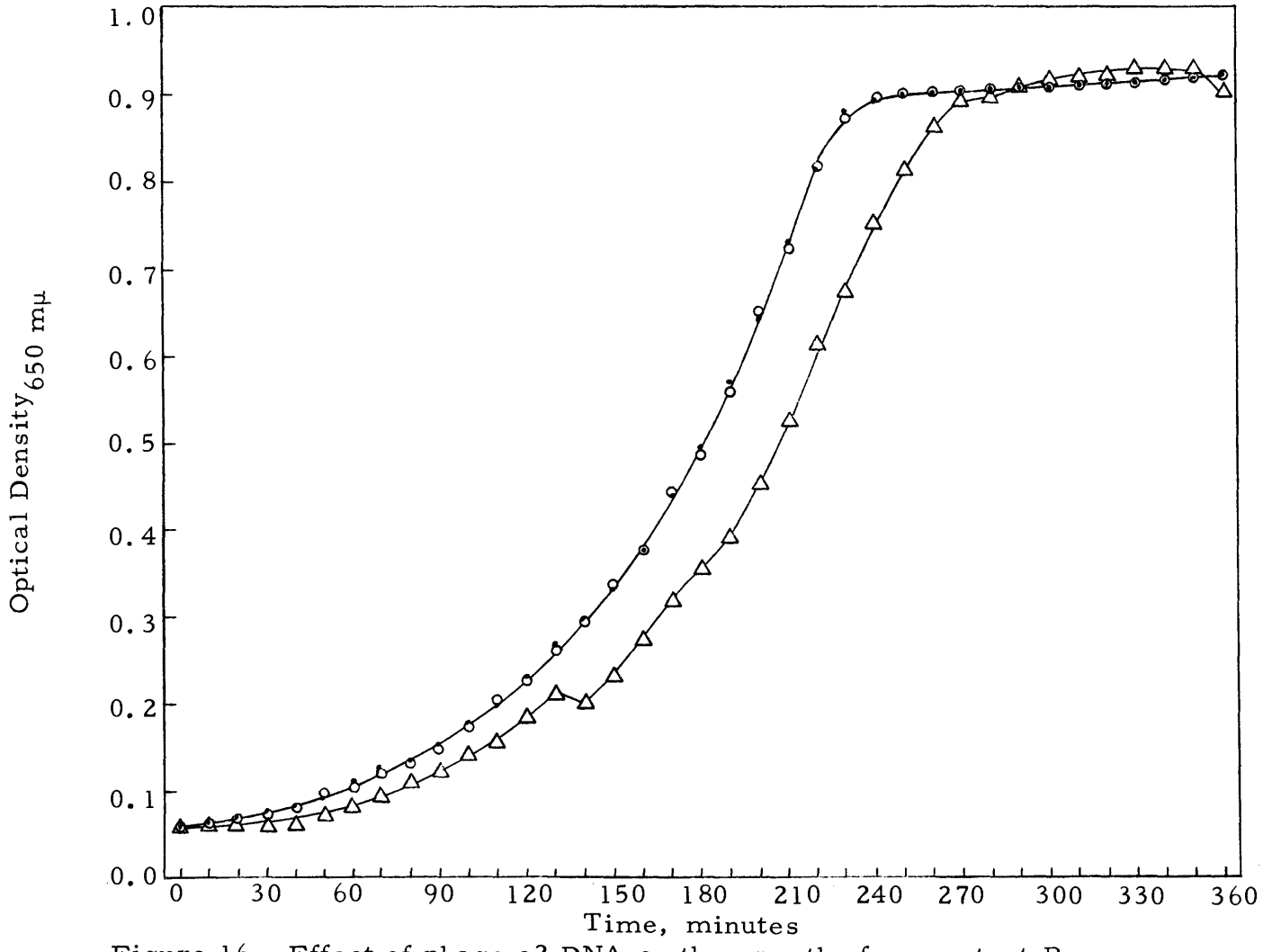

Figure 16. Effect of phage c2 DNA on the growth of competent B. cereus; —•— B. cereus; —△—△— B. cereus plus phage c2 DNA; —o—o— B. cereus plus phage c2 DNA pretreated with DNAse.

using "competent" cells, only one discontinuity was found in the optical density as compared to three when heat-treated spores were used. It may be that the cells do not become "competent" at later stages in fresh medium, and only one period of lysis is observed.

Figure 17 shows the effect of phage c2 DNA on "non-competent" cells (as determined by methylene blue dye uptake). The curve of infected cells began to fall below the curve of the uninfected cells about 70 minutes after infection. This time corresponds to the time that normal cells in a BHI culture change from low methylene blue dye uptake to peak uptake. Therefore it is possible that the cells have become "competent" at this time, and that the DNA slows their growth.

Methylene blue dye uptake was also studied with cells grown in BHI. The results of this experiment are shown in Figure 18. Four periods of dye uptake were observed; between 0 and 30 minutes; between 90 and 120 minutes, between 150 and 180 minutes; between 240 and 270 minutes.

Attempts to Recover Mature Phage

During the course of this investigation numerous attempts were made to recover mature phage particles which would infect S. lactis. Cells were lysed 2, 4, 8, and 16 hours after the addition of phage DNA. Cell lysates were centrifuged to remove debris and the

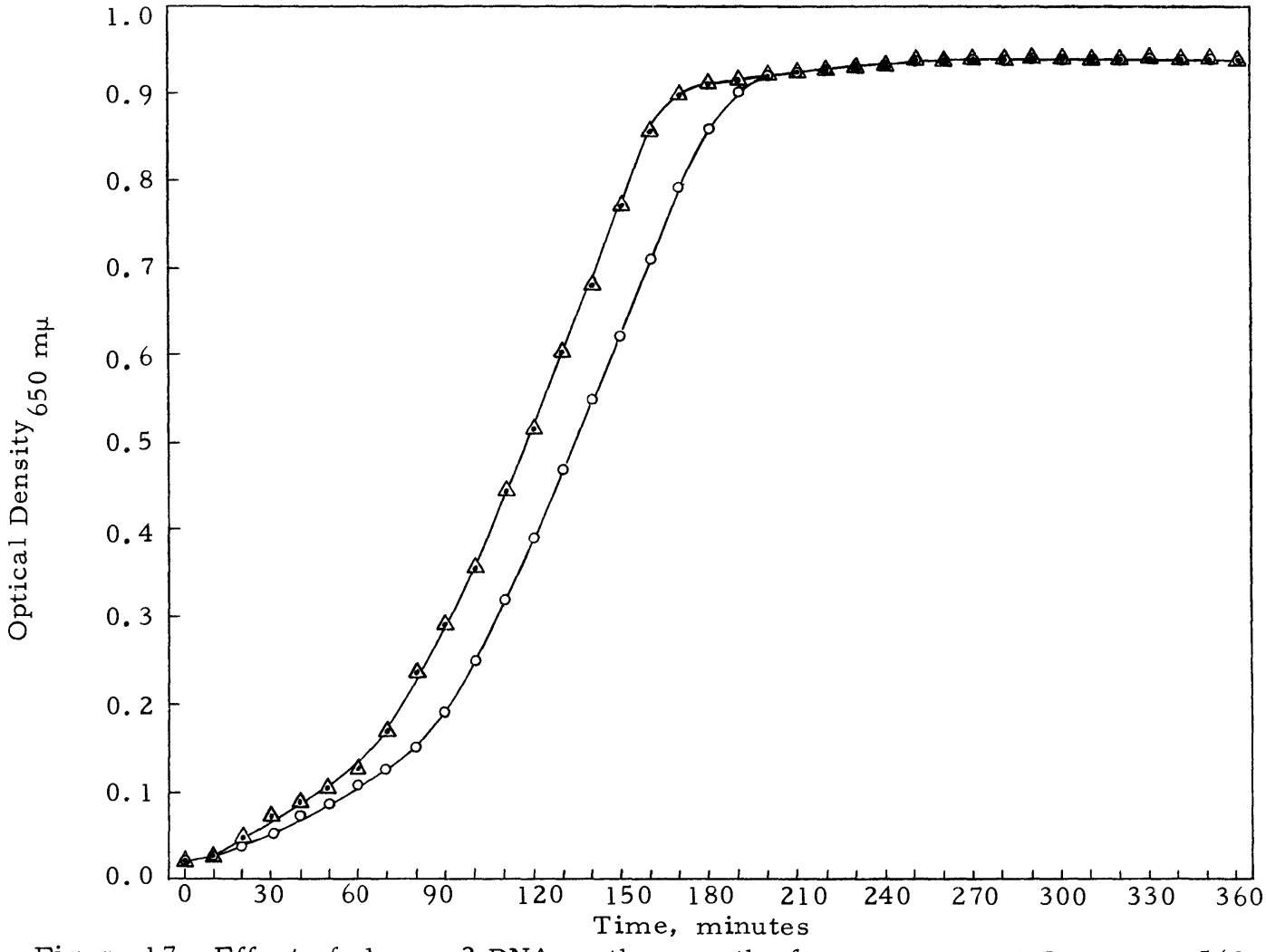

Figure 17. Effect of phage c2 DNA on the growth of non-competent B. cereus 569R. —△—△— B. cereus; —○—○— B. cereus plus phage c2 DNA; —•—•— B. cereus plus phage c2 DNA pretreated with DNAse.

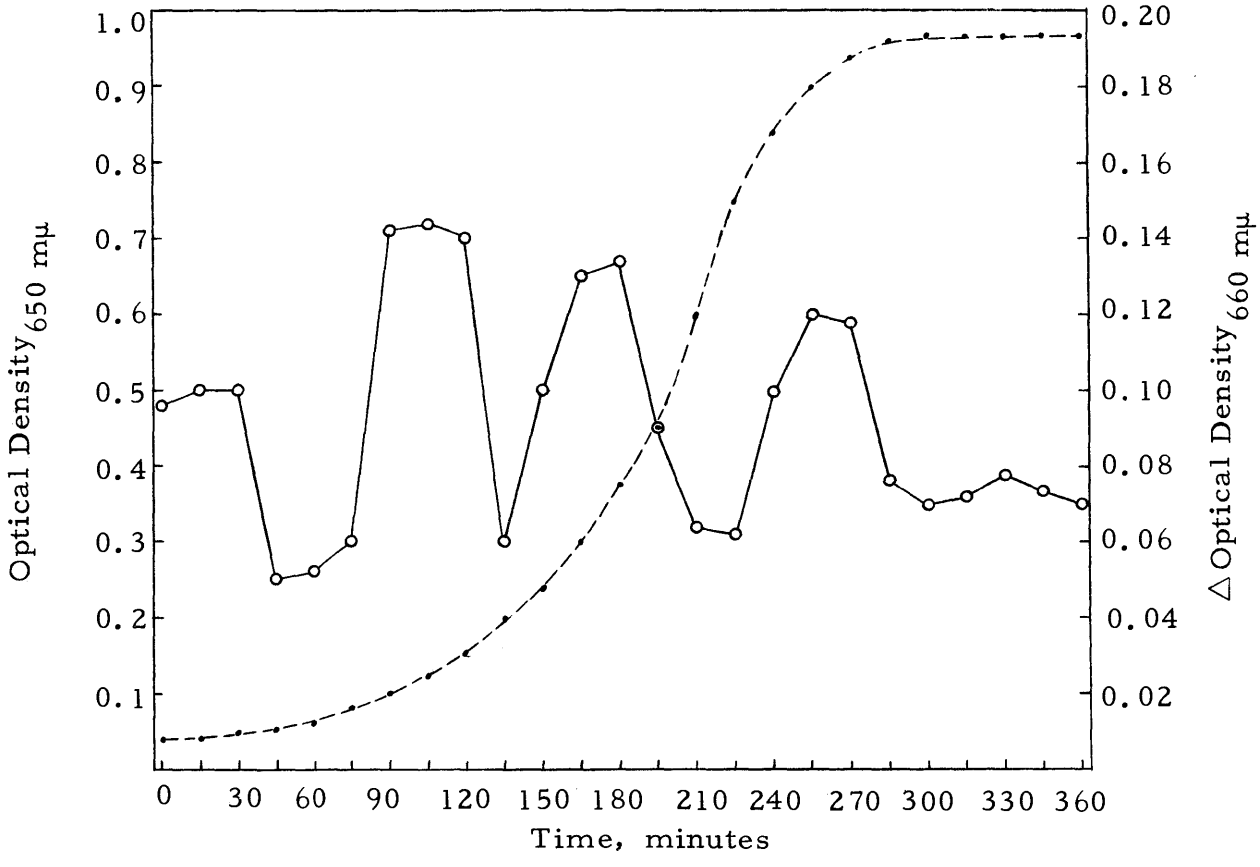

Figure 18. Uptake of methylene blue by B. cereus 569R when grown in BHI. —o—o— uptake of methylene blue; —•—•— growth curve of B. cereus.

supernatants were assayed versus S. lactis by the soft agar overlay method. BHI, PAB, and ELB were used as growth media for the infected B. cereus cells. When no phage particles could be recovered under these conditions, the infected cultures were aerated after 60 minutes incubation under static conditions. The cells were treated as above and assayed for phage. All attempts to recover phage particles were unsuccessful.

The inability to recover mature phage particles could be due to several factors: (1) Breakage of the infecting DNA so that it could not replicate or be transcribed, (2) inability of the phage genome to replicate inside the B. cereus cell, (3) failure of synthesis of coat protein in the unrelated host, (4) failure of the phage genome to be wrapped up in a protein coat, or (5) the production of altered phage particles which would not form plaques on S. lactis.

CsCl Density Gradients of Cell Lysates of B. cereus Infected with Phage c2 DNA

The results of the CsCl density gradient profiles are shown in Figures 19 through 27. When ^{32}P labeled B. cereus DNA and ^{3}H labeled phage c2 DNA were artificially mixed and subjected to CsCl density centrifugation it was found that the phage and cell DNA banded in the same position (Figure 19). Since the cell and phage DNA were found in the same position it was not possible to determine

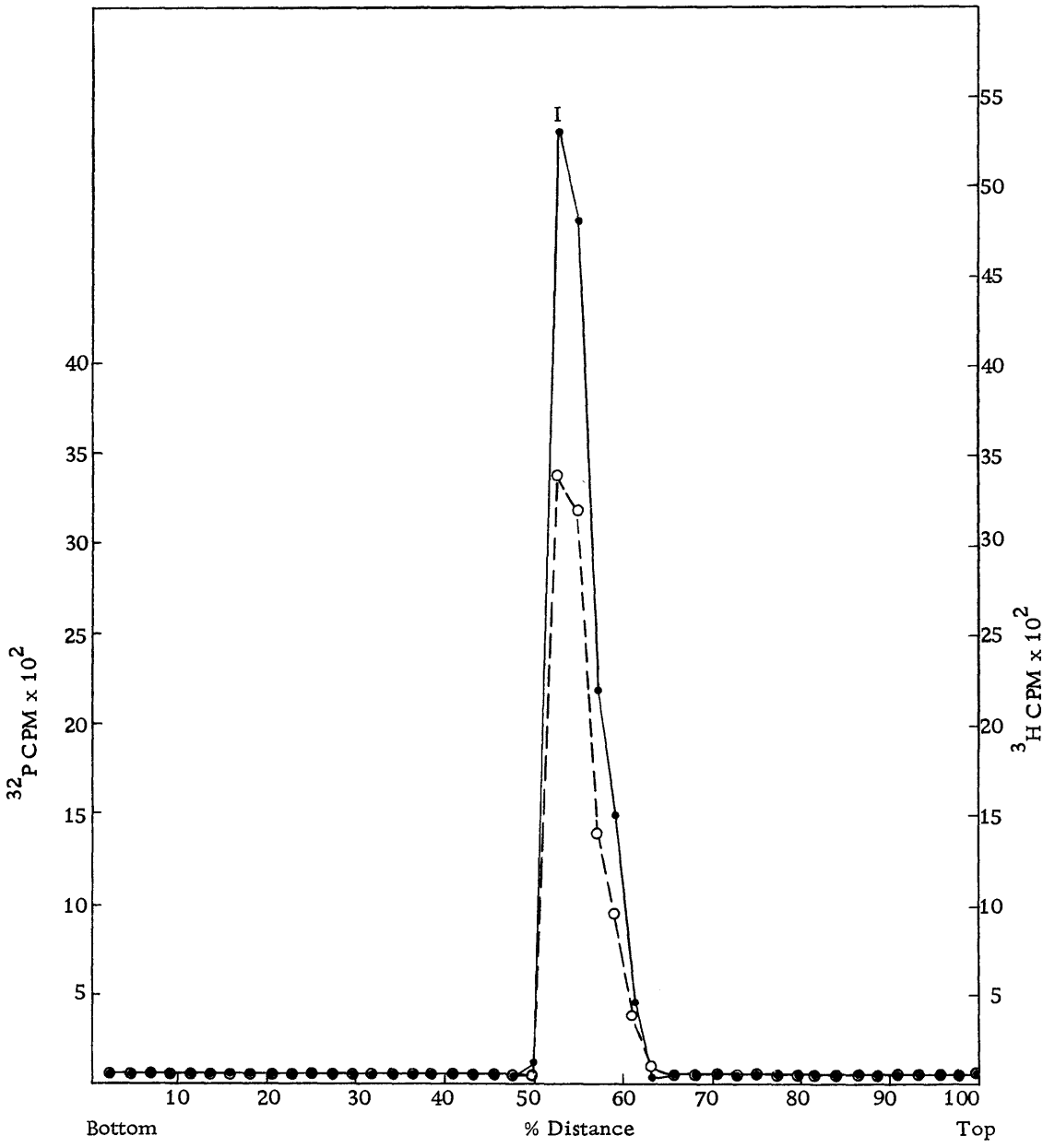

Figure 19. CsCl density gradient profile of a mixture of ^{32}P cell and ^{3}H phage c2 DNA. —•— ^{3}H; —o—o— ^{32}P.

conclusively whether or not the phage DNA replicated inside of the B. cereus cell.

In the following experiment, phage c2 DNA, labeled with ^{3}H, was added to unlabeled heat-treated spores in medium containing ^{32}P. The culture was incubated at 30° C under static conditions and aliquots were removed at one hour intervals, and handled as given under Materials and Methods.

Figure 20 shows the density profile of an infected culture lysed one hour after the addition of phage DNA. The ^{3}H showed one sharp band indicating that the DNA was not extensively broken down. There were no ^{32}P counts at this time, indicating that there was negligible DNA synthesis during the first hour.

The density profile of a two hour lysed culture showed a single peak of ^{3}H, and a single ^{32}P peak at the same position (Figure 21). This probably represents new cell DNA.

Figure 22 shows the density profile of a three hour lysed culture. The ^{3}H label gave two distinct bands in the gradient. Under the second peak (II) there was a ^{32}P peak. It is not certain whether the second ^{32}P peak represents replication of the phage DNA or whether it is a complex between cell and phage DNA.

The density profile of a four hour lysed culture is shown in Figure 23. A major ^{3}H peak was found in position I. Peak II had disappeared and two minor peaks (III and IV) appeared. A small

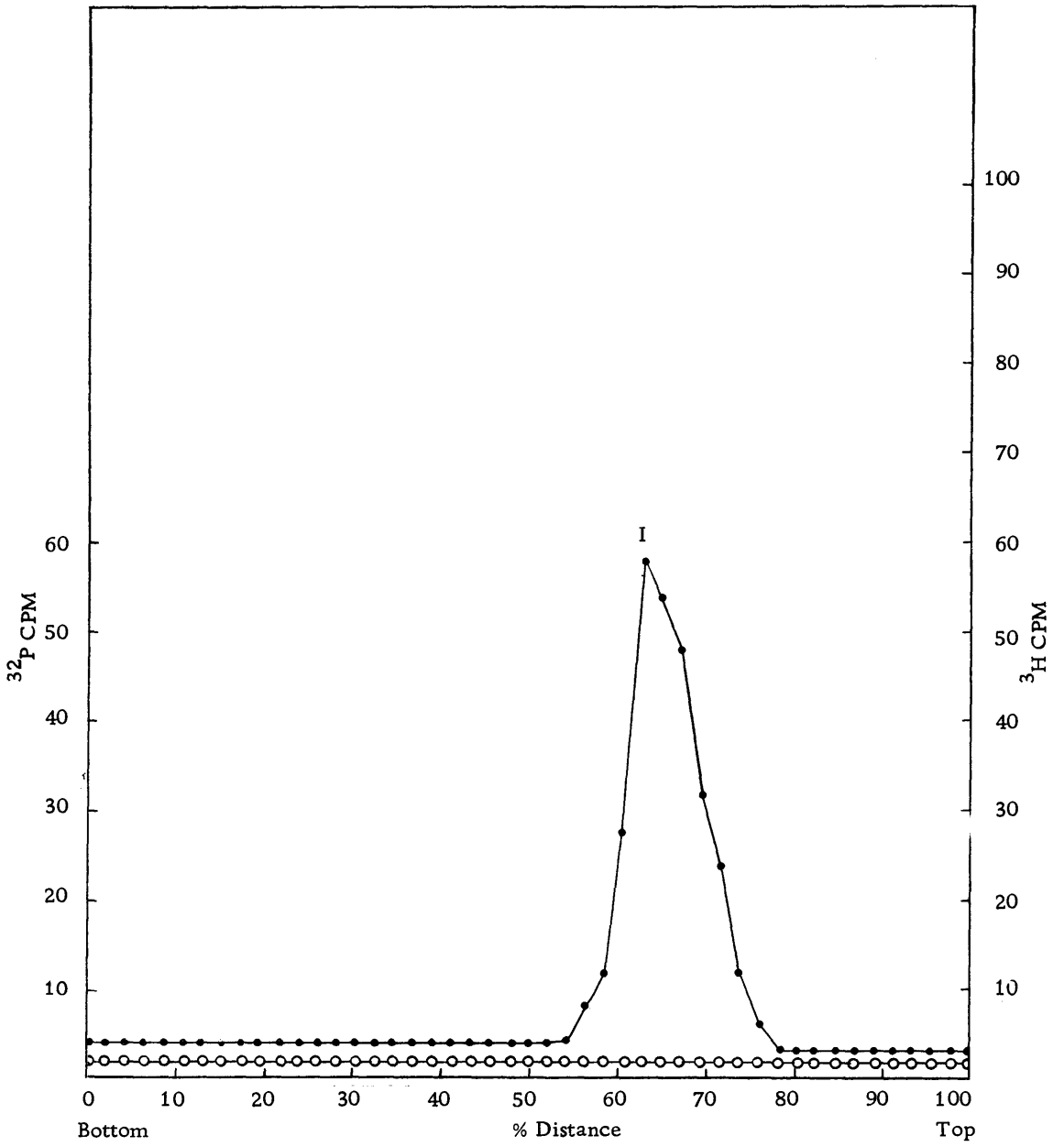

Figure 20. CsCl density gradient profile of DNA from a culture of B. cereus infected with ^{3}H phage c2 DNA, grown in the presence of ^{32}P, and lysed one hour after infection. —•— ^{3}H; —o—o— ^{32}P

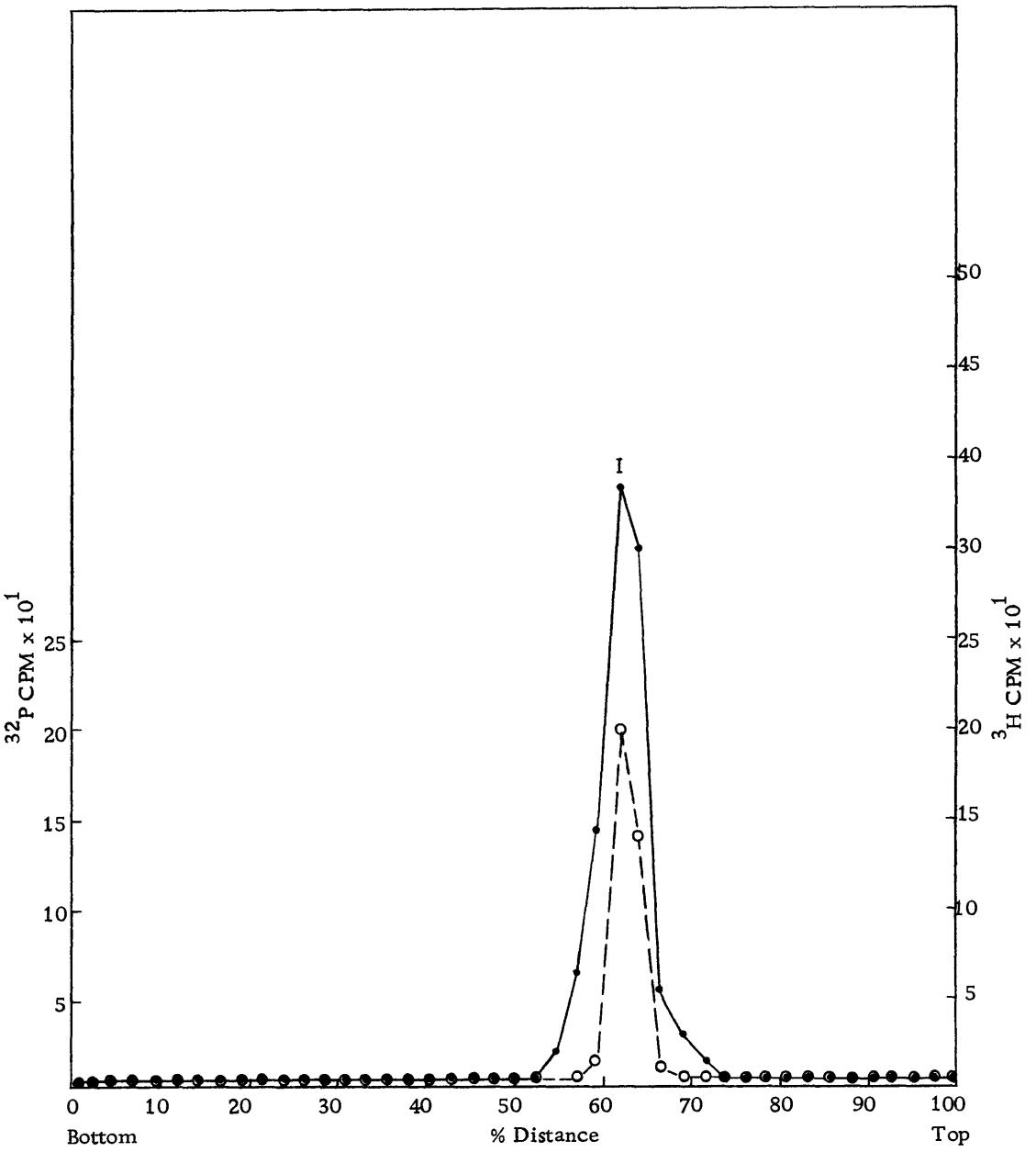

Figure 21. CsCl density gradient profile of DNA from a culture of B. cereus infected with ^{3}H phage c2 DNA grown in the presence of ^{32}P, and lysed two hours after infection. —•—•— ^{3}H; —o—o— ^{32}P.

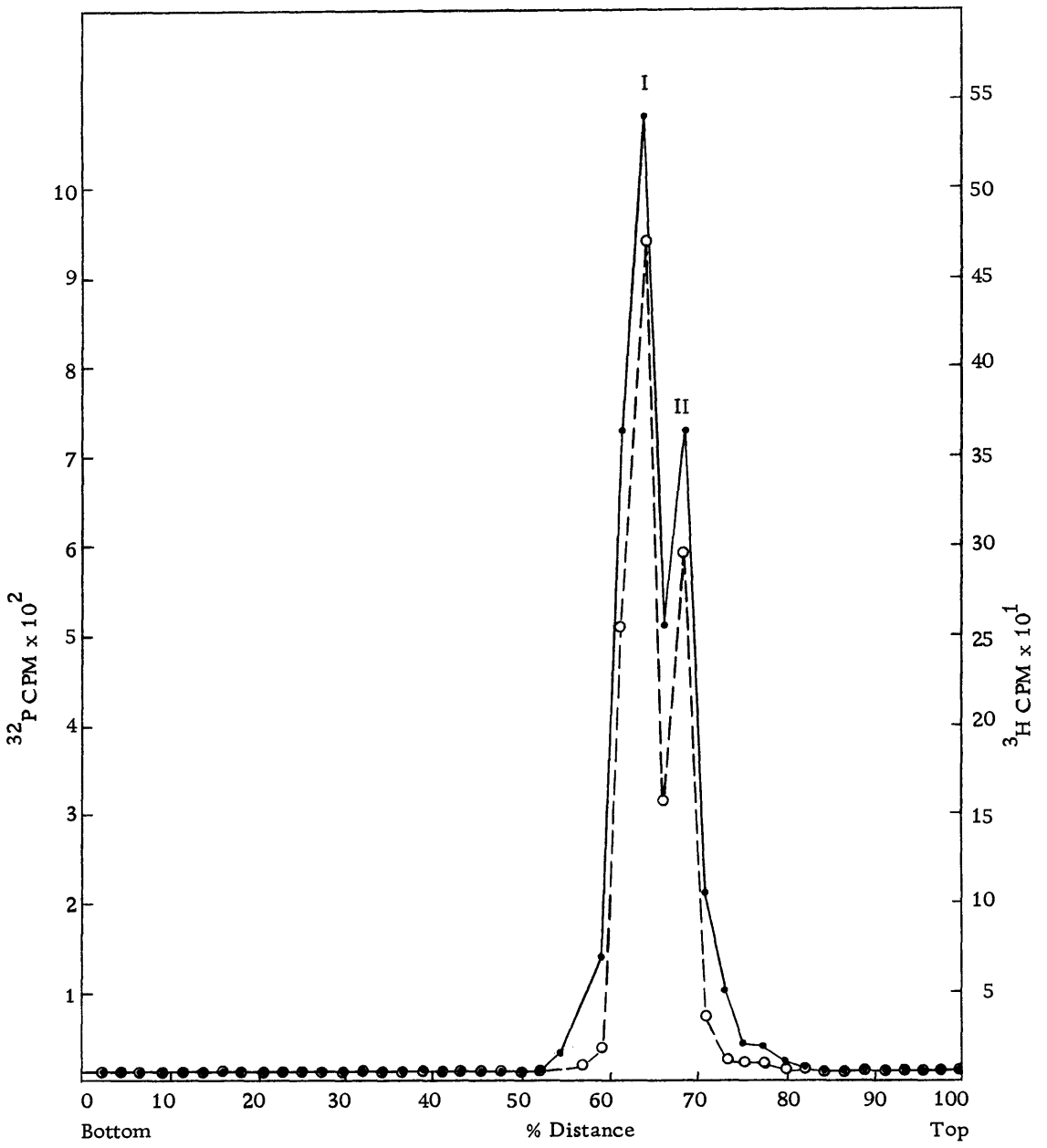

Figure 22. CsCl density gradient profile of DNA from a culture of B. cereus infected with ^{3}H phage c2 DNA grown in the presence of ^{32}P, and lysed three hours after infection. —•—^{3}H; —o—o—^{32}P.

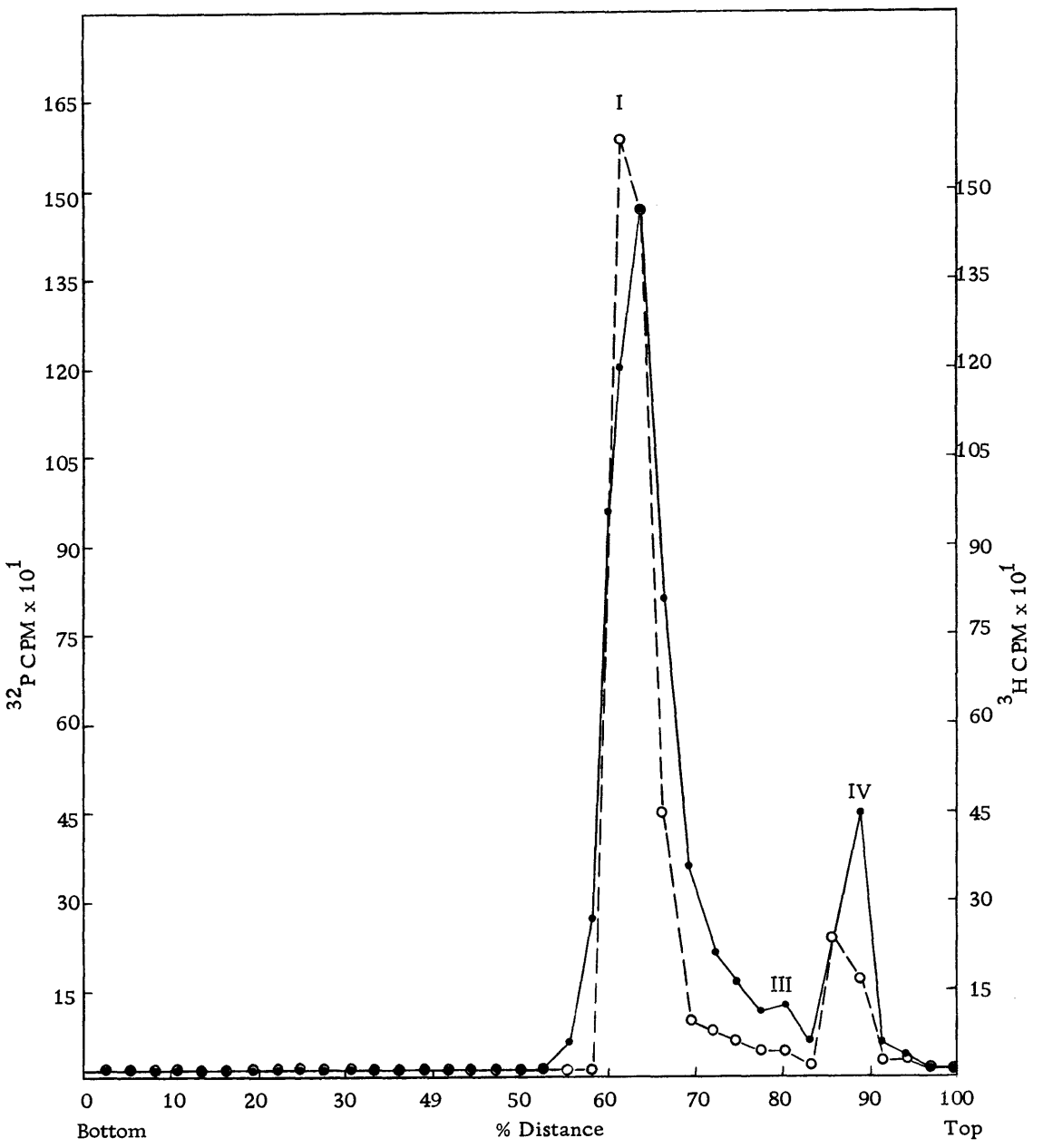

Figure 23. CsCl density gradient profile of DNA from a culture of B. cereus infected with ^{3}H phage c2 DNA, grown in the presence of ^{32}P, and lysed four hours after infection. —•— ^{3}H; —o— ^{32}P.

amount of ^{32}P was found associated with peaks III and IV.

Figure 24 shows the profile of a five hour lysed culture. At this time four ^{3}H peaks were observed. Peak III appeared as a shoulder, while peaks II and IV were very pronounced. In all four peaks, ^{32}P was associated with the ^{3}H peaks.

In the six hour density profile (Figure 25) ^{3}H peaks I and IV were prominent while peak II appeared as a shoulder and peak III as a small ridge. ^{32}P was found associated only with peaks I and IV.

In the seven hour density profile (Figure 26) the ^{3}H label showed four major peaks with ^{32}P associated with peak I and possibly peak IV. The possible significance of the density patterns found at the different time intervals will be considered in the discussion.

Figure 27 shows the density profile of ^{32}P labeled cell DNA from an unfected culture. No ^{32}P was found at the top of the gradient, thus supporting the idea that the small ^{32}peak under the ^{3}H peak IV, especially in the four hour lysate (Figure 23) may be replicated phage DNA, or an association between cell and phage DNA.

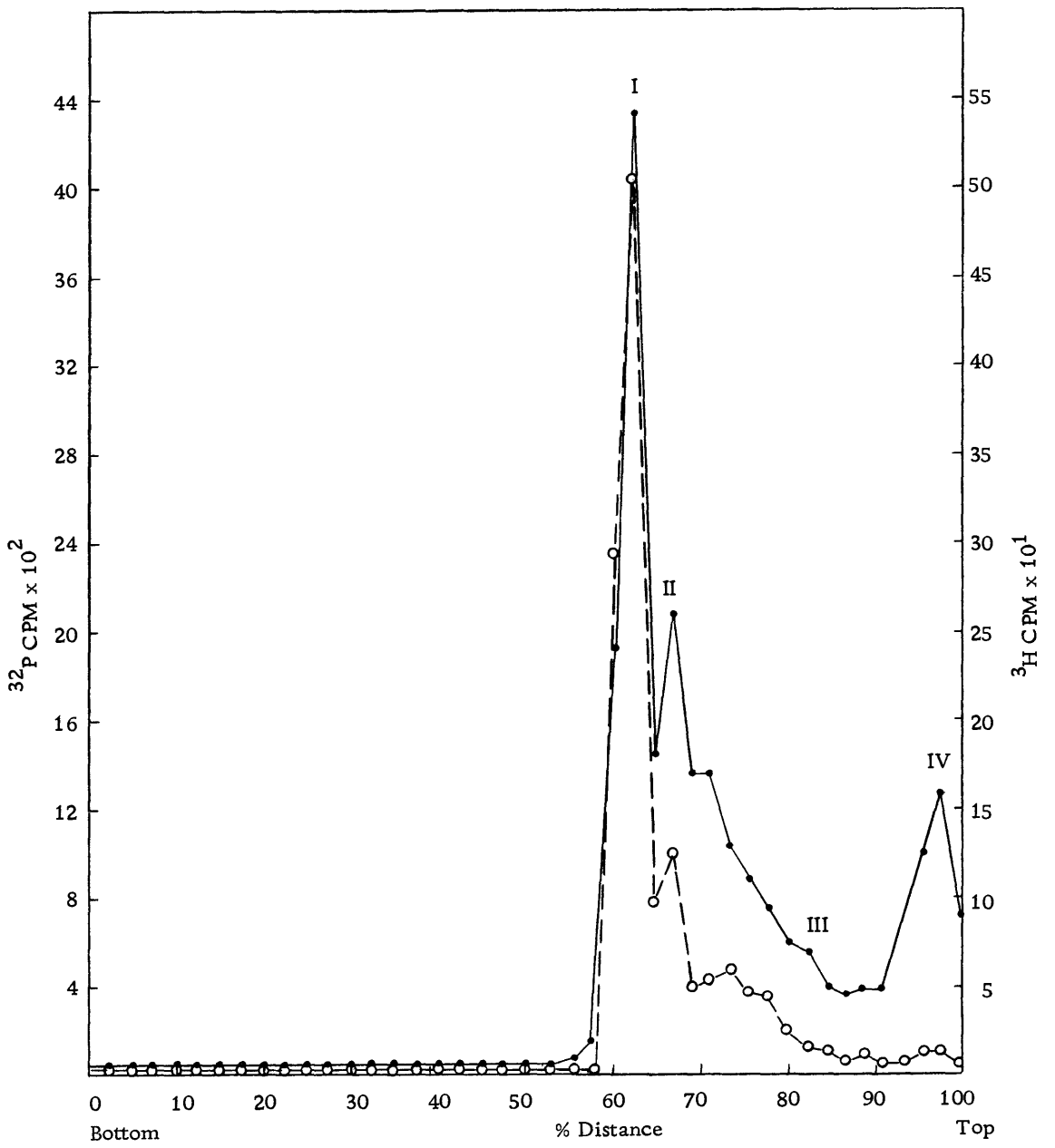

Figure 24. CsCl density gradient profile of DNA from a culture of B. cereus infected with ^{3}H phage c2 DNA, grown in the presence of ^{32}P, and lysed five hours after infection. —●—●— ^{3}H; —o—o— ^{32}P.

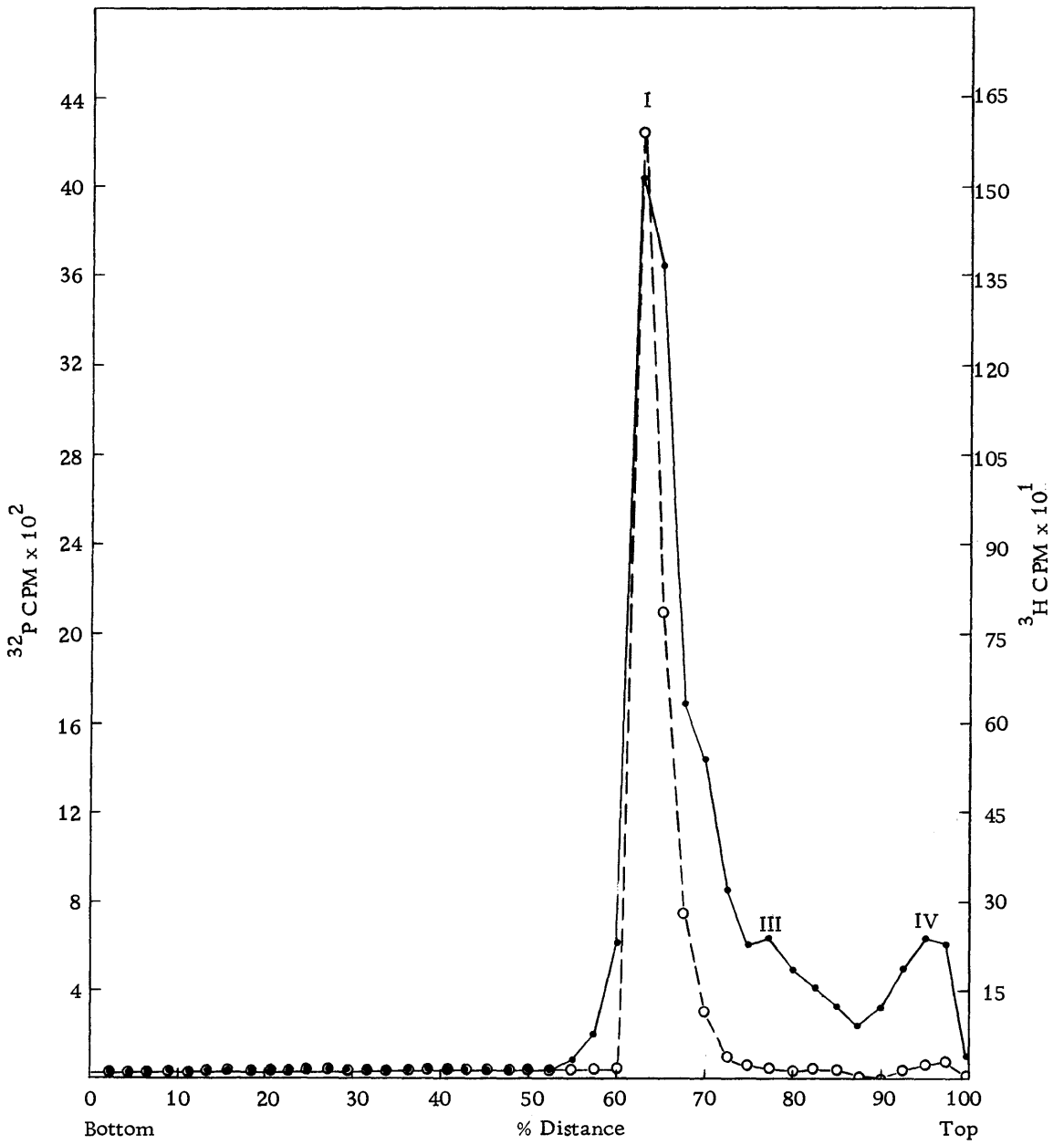

Figure 25. CsCl density gradient profile of DNA from a culture of B. cereus infected with ^{3}H phage c2 DNA, grown in the presence of ^{32}P, and lysed six hours after infection. —•—•— ^{3}H; —o—o— ^{32}P.

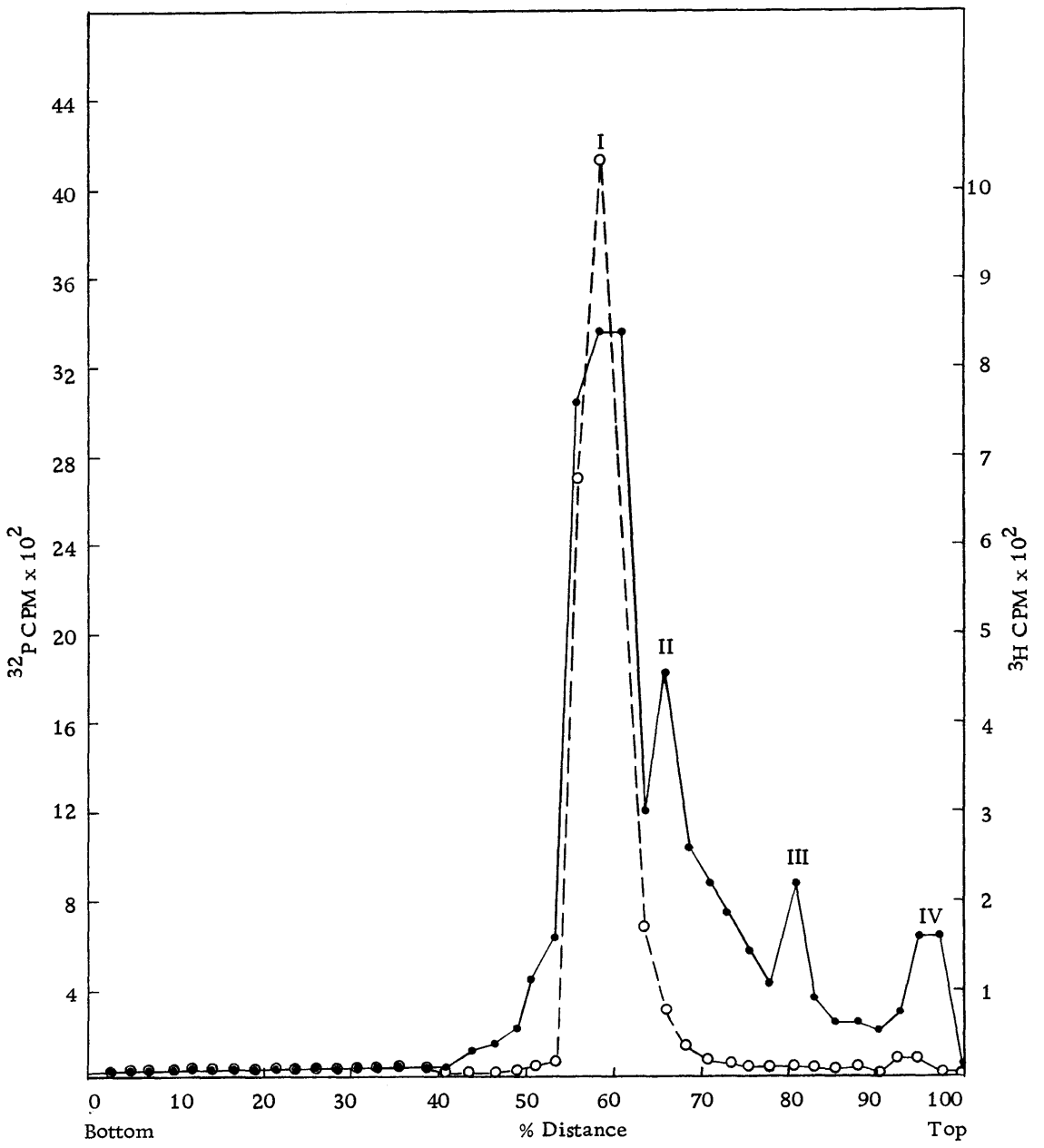

Figure 26. CsCl density gradient profile of DNA from a culture of B. cereus infected with ^{3}H phage c2 DNA, grown in the presence of ^{32}P, and lysed seven hours after infection. —•—•— ^{3}H; —o—o— ^{32}P.

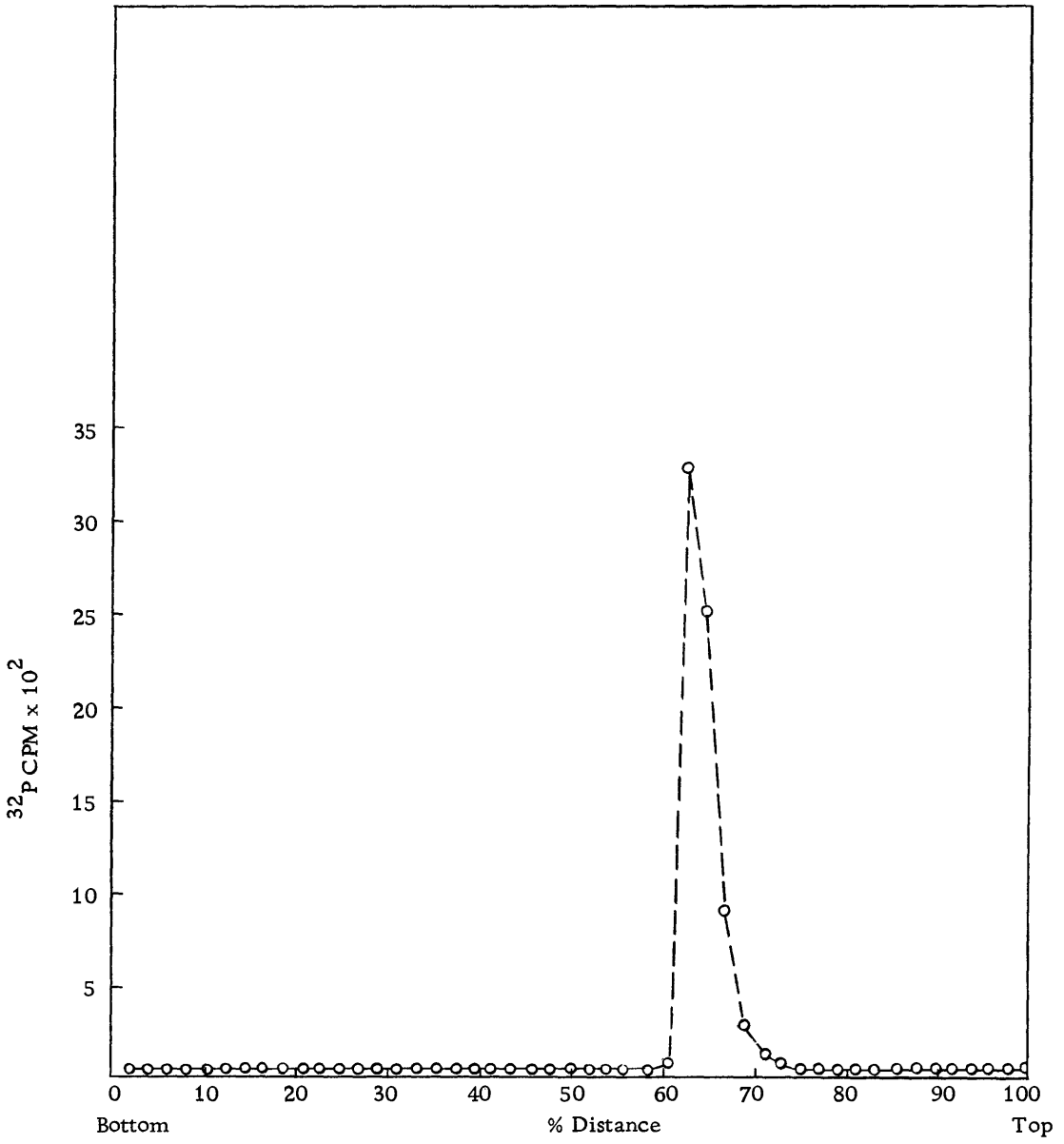

Figure 27. CsCl density gradient profile of DNA from a cell lysate of B. cereus. —•—•— ^{3}H; —o—o— ^{32}P.

DISCUSSION

Competence in B. cereus

Jensen and Haas (1963) showed that methylene blue dye uptake could be used as a measure of the proportion of competent cells in a bacterial culture. We have shown that when B. cereus was grown in SMCA (Figure 15) and BHI (Figure 18) the culture exhibited waves of methylene blue dye uptake. Apparently phage c2 DNA uptake also occurs periodically during growth (Figures 20 through 26). Fraser and Baird (1967b) have shown that when B. cereus cells were stained with neutral red and observed with phase contrast optics that a subpopulation of the culture appeared bright at definite times during growth. The times at which the bright cells appeared corresponded with the times of methylene blue dye uptake (Figure 15). Fraser, Baird and Richter (1967) have correlated neutral red and methylene blue staining of B. licheniformis cells with transformation. They found that maximum transformation took place when the cells exhibited methylene blue staining. When the cells were stained with neutral red, the bright cells appeared approximately 15 minutes ahead of the methylene blue staining.

Although B. cereus is considered to be a non-transformable strain, the results of various workers in our laboratory strongly

suggest that it does, however, have periods of competence at somewhat regular intervals. If an autolytic enzyme is involved in competence, as suggested by Dr. Frank Young (1966), B. cereus does at least possess this enzyme.

B. cereus cells at times of peak methylene blue dye uptake also were able to take up not only DNA prepared from phage c2, as shown in this research, but also homologous DNA (Fraser, Baird and Richter, 1967). Thus it seems likely that the failure of many attempts to transform B. cereus must be due to a failure to carry out a step following the uptake of high molecular weight DNA, possibly the integration step.

Growth Inhibition of B. cereus by Phage c2 DNA

Figures 7, 16, and 17 show that the growth of B. cereus cells infected with phage c2 DNA lags behind that of non-infected cultures. There appear to be two reasons for this, first, that the infected cultures actually increase in optical density more slowly (Figure 7, 16, and 17) and, second, the infected cultures usually show short periods at which there are discontinuities in the growth curve (Figure 7), sometimes an actual drop in the optical density (Figure 16).

We do not know whether an increase in generation time might account for the slowing of growth. Pieghowska and Shugar (1967) have found similar results using competent cells of S. Challis. They

found that when homologous DNA was added to competent cells, the growth of the culture was markedly slowed and 240 minutes after the addition of DNA, the number of viable cells in the infected culture was only 1/14 of the number of viable cells in a control culture, with a three fold difference in the optical density. The authors apparently did not look for lysis of the DNA treated cells. DNA from E. coli, phage T2, and the poly A-T complex of Cancer magister DNA also caused growth inhibition. In contrast, B. cereus DNA did not cause inhibition of growth in our system, nor did other species of DNA tested.

In the work presented here it seems probable that cell lysis is responsible for the periodic lags in the growth curves of infected cells. When Figures 23 and 24 for the four and five hour lysates and Figures 25 and 26 for the six and seven hour lysates are compared it is clear that there is an actual decrease in the ^{3}H label from the infecting phage DNA, but not in the ^{32}P label during these periods. The volume of culture used for each of the uptake experiments was the same. The simplest explanation of these results is that the ^{3}H labeled infected cells were preferentially lysed.

Intracellular Forms of Phage DNA

Material labeled with ^{3}H from the infecting phage c2 DNA was found in the gradients (Figures 22-26) distributed in several distinct

peaks at or higher in the gradient than the free phage DNA. Peak I was in the same band as the cell DNA, and therefore it has the same density as the DNA isolated directly from phage c2 particles (Figure 19). Apparently newly adsorbed DNA appeared in this band. Adsorption was greatest during the periods 1-2, 3-4, and 5-6 hours after the addition of the DNA. Peaks II and III were found to vary in proportions at different times. It is probable that these two peaks represent physical changes in the infecting DNA, perhaps intermediates in its replication. Peak IV appeared near the top, and perhaps represents phage DNA associated with protein. The fact that peaks II, III, and IV have a lower density than directly isolated DNA is remarkable. Single-stranded DNA is higher in density than double-stranded DNA. Little work has been done on the density of replicating phage DNA and therefore the significance of these intermediate bands is not known at this time. Kozinski and Lin (1965) found that replicating phage T4 DNA was associated with protein and appeared at the top of a CsCl gradient. Perhaps peak IV might be comparable to this type of protein-DNA complex.

The experiments of Figures 20-26 were designed to determine whether phage c2 DNA was able to replicate in B. cereus cells. It was not possible to obtain a clear answer to this question from the data, because the cell DNA had the same density as phage c2 DNA and of peak I for the infected cells. However, there is a slight

indication that replication may occur to a limited extent as ^{32}P was found under the ^{3}H peaks of the lower densities. This label might, however, mean that there is some form of association between the cell and phage DNA in these peaks.

SUMMARY

The data presented here has led to certain conclusions but also has given rise to many questions which are unanswered at this time. The results of this work can be summarized as follows:

1) When log phase B. cereus cells were incubated with a lysate of Streptococcus lactis phage c2 for 30 minutes, only five percent of the cells survived.

2) The growth of an infected culture was slower than that of a non-infected culture. The effect was found to be maximum when BHI was used.

3) When the phage c2 lysate components were separated by CsCl density centrifugation only the nucleic acid fraction was found to inhibit growth.

4) Phage c2 DNA was found to kill 99 percent of the cells 300 minutes after addition of DNA.

5) When growth of a phage c2 DNA-infected culture was followed by optical density, the growth rate of the infected culture was slower than that of a non-infected culture. DNase inactivated the inhibitory effect. Three discontinuities in the growth curve were found in the DNA-infected culture which probably represent periods of lysis.

6) When B. cereus was grown in SMCA, three periods of methylene blue dye uptake were observed between 5.5 and 8.5 hours.

When cells from a period of peak methylene blue dye uptake were infected with phage c2 DNA, inhibition of growth began immediately and a period of lysis was observed after 130 minutes of growth.

7) When methylene blue dye uptake was measured in BHI grown cells, four periods of dye uptake were found.

8) All attempts to recover mature phage particles from DNA infected cells were unsuccessful.

9) B. cereus, S. lactis, calf thymus, and B. cereus phage R-1 DNA had no effect on the growth of B. cereus.

10) Infecting ^{3}H phage c2 DNA becomes DNase resistant soon after uptake by the B. cereus cells. Dialysis of cell lysates showed that the DNA was still in a high molecular weight form.

11) When a mixture of phage c2 and B. cereus DNA were centrifuged together in CsCl it was found that both types of DNA had the same density and therefore it was not possible to determine whether the phage DNA was capable of replication in the infected cells.

12) When ^{3}H c2 DNA was added to B. cereus and the culture grown in ^{32}P, cell lysates showed four peaks of ^{3}H when subjected to CsCl density centrifugation. The three intermediate peaks were found to be lighter in density than that of the infecting DNA. Peak IV was always found near the top of the gradient. It may be that the DNA in peak IV was associated with protein. The two peaks II and III were found to vary in amount with time. Whether these two peaks are forms of replicating phage DNA is not known at this time.

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