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Biological studies of Bdellovibrio bacteriovorus

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BIOLOGICAL STUDIES OF BDELLOVIBRIO BACTERIOVORUS

By

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CHAPTER I

INTRODUCTION

Bdellovibrio bacteriovorus is an aerobic, gram negative bacterium, which is obligately parasitic on other gram negative and a few gram positive bacteria. It was discovered in 1962 by Stolp and Petzold(30). It is motile, comma-shaped, and possesses an unusually thick polar flagellum, approximately 29 nanometer(nm) in diameter. The organism is 0.3 micrometer(μm) wide and 1.0μm long. So far only one species has been identified but there are many known strains. These strains appear to differ with regard to host range. Some show a limited host range while others can attack a wide variety of host bacteria(25). Shilo(25) felt that this was not an actual difference in the B. bacteriovorus strains but depended on the method used in assaying the host-parasite interaction.

Certain enteropathogenic bacteria as well as other bacteria are suitable hosts for B. bacteriovorus(Table 1). Therefore it is possible that the B. bacteriovorus plays an important role in various ecosystems. It is also conceivable that this parasite may affect bacterial pathogens in natural habitats.

B. bacteriovorus appears to be ubiquitous and has been isolated from soil, sewage, and seawater. Furthermore, it has wide geographical distribution as evidenced by the fact that it has been isolated in the United States(12), India(7), Germany(3), and Israel(24).

B. bacteriovorus is placed in the order Pseudomonadales, the suborder Pseudomonadineae, the family Spirillaceae, the genus Bdellovibrio, and the species bacteriovorus(29). The generic name is derived

Table 1

Hosts Parasitized by *Bdellovibrio bacteriovorus*

Host	Gram Reaction	Reference
Aerobacter aerogenes	-	29
Aerobacter aeruginosa	-	26
Aerobacter cloacae	-	29
Erwinia amylovora	-	28, 29
Erwinia atroseptica	-	12
Erwinia carotovora	-	29
Escherichia coli	-	26, 29, 33
Pectobacterium	-	30
Proteus mirabilus	-	29
Proteus vulgaris	-	3, 26
Pseudomonas aeruginosa	-	26
Pseudomonas fluorescens	-	23, 26, 29
Pseudomonas phaseolicola	-	22, 26, 28
Pseudomonas putida	-	19
Pseudomonas solanaceacum	-	29
Pseudomonas tabaci	-	22, 28, 29
Salmonella paratyphi	-	26
Salmonella typhi	-	10, 26
Salmonella typhimurium	-	26
Serratia kiliensis	-	19
Serratia marcescens	-	29
Shigella spp.	-	9
Agrobacterium tumefaciens	-	3, 16
Azotobacter	-	31
Bacterium stewartii	-	29
Caulobacter spp.	-	26
Pasteurella pestis	-	26
Rhizobium meliloti	-	16
Rhizobium trifolii	-	16
Rhodopseudomonas capsulata	-	3
Rhodopseudomonas gelatinosa	-	3
Rhodopseudomonas palustris	-	3
Rhodopseudomonas spheroides	-	3
Rhodospirillum rubrum	-	3
Rhodospirillum spheroides	-	26
Spirillum serpens	-	27
Xanthomonas	-	30
Bacillus megaterium	+	26
Lactobacillum plantarum	+	3
Streptococcus faecalis	+	3

from Bdello which is Greek for leech. Thus Bdellovibrio refers to a leech which has the morphology of a vibrio. The specific name, bacteriovorus, refers to "bacteria-eater"(24).

Phase-contrast or electron microscopes are useful in the study of B. bacteriovorus. The double-layer plaque technique is used to study the parasite indirectly(29). The latter technique employs two layers of media. The bottom layer contains 2.0% agar and the upper 0.6% agar and host and parasite cells. The host "lawn" develops in the top layer and consists of a confluent turbid growth of bacteria. When B. bacteriovorus attacks and lyses the host bacteria, plaques develop in an otherwise turbid lawn. This technique may be used to quantitate B. bacteriovorus particles in terms of plaque forming units(PFU/ml(It is assumed that one B. bacteriovorus cell forms one plaque). Others have used the Petroff-Hauser chamber to quantitiate parasite populations(8, 18).

Seidler and Starr(20) divided the parasitism of the host by B. bacteriovorus into the following cycle: attachment, penetration, elongation, fragmentation, and burst(Figure 1).

According to electron micrographs it appears that attachment to the host by B. bacteriovorus follows collision between the two resulting in damage to the host cell wall(4, 28). Therefore, motility presumably plays an important role in the attachment of the parasite. Electron micrographs reveal that a bulge develops on the host cell wall at the site of attachment(4). This is the initial evidence of damage of the host. Further damage to the host after attachment by B. bacteriovorus consists of separation of the plasma membrane from the cell wall and the formation of spheroplasts by the host cell. Prior to attachment

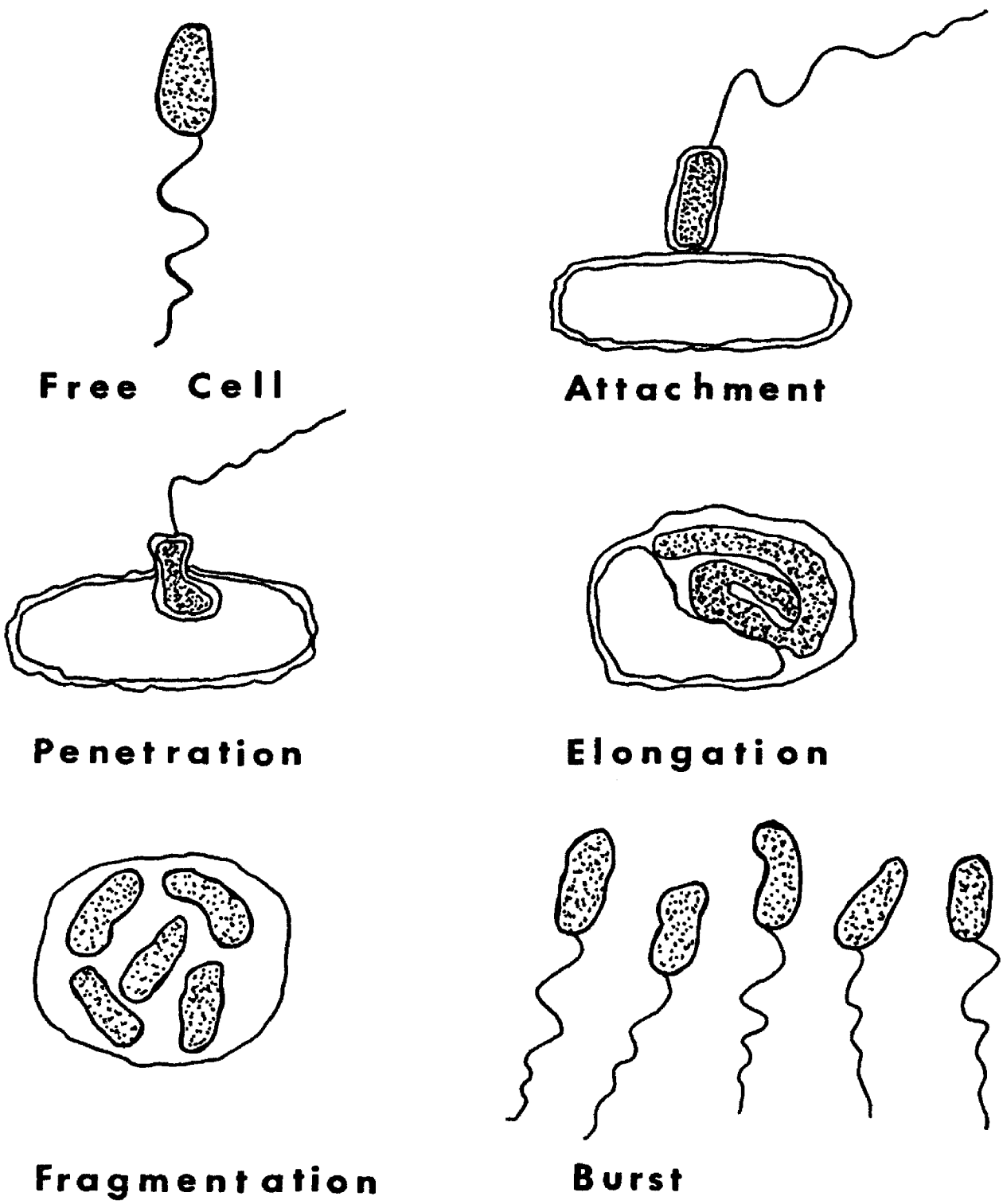

Figure 1

Parasitic Cycle of Bdellovibrio bacteriovorus

the anterior end of the B. bacteriovorus is characterized by large convolutions. Also a mesosome is closely associated with the anterior end of the cell. These structures may function in cell penetration. Long filaments present at the anterior end of B. bacteriovorus cells may be involved in the attachment process (1, 4, 28).

As many as ten B. bacteriovorus cells may attach to a single host cell at one time(29). However, under this condition the host cell lyses within a few minutes. In order for the intracellular life cycle to be completed and a new generation of parasites observed, a single host cell must be attacked by not more than two B. bacteriovorus cells.

B. bacteriovorus possibly penetrates the host cells through a pore by an evagination process(4). The pore appears to be formed by a collision process and subsquent rotating motion by the parasite rather than by an enzymatic process. This is evidenced by electron micrographs which show that the cell wall remains rigid as the B. bacteriovorus penetrates(4, 28). Once the host is penetrated by the B. bacteriovorus the periplasm begins to deteriorate, presumably due to the release of an exoenzyme(protease) by the parasite.

During the elongation stage, the parasite is between the host cell wall and the cytoplasmic membrane. Here it utilizes the host's protoplasm as its energy source and begins to elongate. When a critical length is reached, the elongated cell fragments into approximately five smaller units. Flagella are formed by the B. bacteriovorus when they are still inside the host cell, and the motile B. bacteriovorus breaks out of the host cell wall. All that remains of the host after burst is a cell ghost or cellular fragments.

The growth curve of B. bacteriovorus shows a latent period of several hours during which the organism is parasitizing the host cells. A rise in plaque forming units is seen after the latent period. In the exponential growth phase the average generation time is 1.3 hours(20). The time required to complete the growth cycle depends upon the physiological age of the B. bacteriovorus cell. When the cell is older, the growth phase is longer.

Several optimal conditions have been established for B. bacteriovorus parasitism of host cells by attachment and burst size studies. These studies revealed that the largest percentage of attachment and the greatest burst size occurred when the incubation temperature was 30-35 C(20, 33). No attachment occurred at pH 5 or below and the optimum pH for the greatest burst size was 7.5-8.1(20, 33). The ratio of host to parasite in the inoculum was also shown to affect the attachment percentage. The largest attachment occurred when the ratio of host to parasite was 10:1(33).

A few biochemical properties of B. bacteriovorus have been studied. It requires an abundance of oxygen and thus is classified an aerobe. B. bacteriovorus strain 6-5-S was shown to be catalase positive, to contain nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate oxidases, and to possess enzymes present in the tricarboxylic acid cycle and carbohydrate metabolism. Thus, they possess the necessary energy supplying mechanisms. Some strains of B. bacteriovorus produce large amounts of an exoprotease(26, 29). The function of this enzyme has not been determined. However, Shilo(25) felt that it had a part in invasion after the host cell was damaged by

the impact of the parasite.

Studies have shown that the genus Bdellovibrio can also be facultatively parasitic: mutant forms which are capable of growth on artificial medium and can remain predacious. The host-independent(H-I) form of the organism is invaluable since comparisons can be made between the biology of it and the host-dependent(H-D) forms, particularly concerning their nutritional requirements and cell physiology. Reiner and Shilo(17) have shown that the wild-type strains of B. bacteriovorus can also grow into elongated forms in the presence of cellular extracts.

The effect of chemicals on the growth of both H-I and H-D forms of B. bacteriovorus has been studied. Varon and Shilo(33, 35) established that various antibiotics would affect certain stages of the parasitic cycle. Streptomycin, chloramphenicol, and puromycin were shown to inhibit the invasion stage. Penicillin inhibited multiplication but did not affect attachment or invasion. Seidler and Starr(21) reported the effect of antimetabolites and antibiotics on H-I B. bacteriovorus strains. The results are shown in Table 2.

B. bacteriovorus has been obtained in a relatively pure(axenic) state from mixed populations in nature. Two techniques have been devised for accomplishing this. Stolp and Starr(29) used differential filtration through a series of membrane filters of decreasing pore size (3.0, 1.2, 0.8, 0.65, and 0.45 μm). Varon and Shilo(25) obtained relatively pure B. bacteriovorus by differential centrifugation of the cells in linear ficoll gradients(1%-15% w/v). They recovered 80-90% of the B. bacteriovorus cells in the upper portion of the gradient column. The cells were centrifuged at 1620 x g for 20 minutes in a

Table 2

Response to Chemicals by H-I Bdellovibrio bacteriovorus

Chemicals	Response*
altafur	S
methenamine mandelate	S
neomycin	S
kanamycin	S
novobiocin	S
polymyxin B	S
oxytetracycline	S
vibriostat	S
colistin	R
sulfisomadin sulfisoxazole	R
isoniazid	R
sulfadimethoxine mystatin	R
oleandomycin	R
sulfadiazine	R
sulfa methoxy-pyradazine	R
triple sulfa	R
vancomycin	R

*Symbols: S=sensitive; R=resistant.

The above table is modified from the data of Seidler and Starr(21).

refrigerated horizontal centrifuge.

Knowledge concerning the host-dependent form of B. bacteriovorus consists mainly of electron microscopic studies of the ultrastructure and intracellular growth and development of the parasite. Expanded knowledge about and further contributions to the biology of this organism are necessary to further characterize this unique host-parasite relationship. The role the parasite plays in various ecosystems and also possible therapeutic uses are important areas to be clarified.

STATEMENT OF PROBLEM

Although electron microscopic studies dealing with the ultrastructure and intracellular growth and development of Bdellovibrio bacteriovorus have been reported, there is a paucity of information on the basic biology of this parasite.

This investigation was undertaken to determine optimal conditions for the cultivation and preservation of B. bacteriovorus. In addition, the effect of several physical and chemical agents on the biological behavior of this organism were tested. Since B. bacteriovorus is widely distributed naturally and parasitizes many enteric bacteria, its presence in human and animal intestinal tracts was investigated. This was done to learn something about the parasite's ecological role and also possible therapeutic applications.

Studies were conducted to try to elucidate the nature of host specificity in the attachment phenomenon. This was done by serological studies and by cell envelope attachment studies.

CHAPTER II

GENERAL MATERIALS AND METHODS

(1) Organisms

Bdellovibrio bacteriovorus strain 109, American Type Culture Collection(ATCC 15143) was employed in this investigation. The host organism employed for the majority of studies was Escherichia coli (ATCC 15144). Shigella flexneri 2 was used as the host in determining the effects of antimicrobial agents on B. bacteriovorus growth. The culture was obtained from the stock culture collection of the Department of Microbiology, University of Montana, Missoula, Montana.

The facultative(host-independent) form of B. bacteriovorus strain 110 was obtained from Ramon J. Seidler, Oregon State University, Corvallis, Oregon.

(2) Culture Media

Medium for growth of B. bacteriovorus and E. coli was Tris yeast peptone(YP), consisting of Difco yeast extract, 3 grams; Difco peptone, 0.6 grams; glucose, 1 gram; and 1000 ml 0.05 M Tris[tris (hydroxymethylamino)methane] buffer adjusted to pH 7.5 with concentrated hydrochloric acid.

E. coli and S. flexneri 2 stock cultures were maintained on Difco brain heart infusion(BHI) agar slants. Stock cultures of both organisms were cultured in BHI broth. BHI broth was also used as an enrichment culture for obtaining large populations of S. flexneri 2 and E. coli.

Medium for the host-independent(H-I) form of B. bacteriovorus

was peptone-yeast Tris(PYT), consisting of Difco peptone, 10 grams; Difco yeast extract, 3 grams; and 1000 ml of 0.05 M Tris buffer adjusted to pH 7.5 with concentrated hydrochloric acid.

(3) Maintenance of Cultures

Stock cultures of B. bacteriovorus were maintained on Tris YP agar slants and in Tris YP broth. The slants were made by dripping a suspension of B. bacteriovorus-E. coli culture over the slant surface and incubating overnight before storing at 4 and -20 C. Broth cultures of B. bacteriovorus were dispensed into glass tubes and retained in the frozen state(-20 C).

E. coli and S. flexneri 2 were streaked on BHI agar slants, incubated 18-24 hours at 37 C, and stored at 4 C.

(4) Cultural Conditions

E. coli was cultured in 50 or 100 ml volumes of Tris YP medium in 250 or 500 ml erlenmeyer flasks, respectively. The inoculum consisted of 0.1 ml of E. coli stock culture. The cultures were incubated at 37 C for 16-24 hours. The E. coli cultures were inoculated with B. bacteriovorus to give a host-to-parasite inoculum ratio of 1:1 or 10:1. The two-membered cultures were incubated at room temperature for up to six days in thermal destruction and survival tests. For the rest of the experiments they were incubated at 25 C for 18-24 hours. The cultures were agitated by means of a magnetic stirring bar for all experiments except for studies dealing with thermal destruction.

When E. coli and S. flexneri 2 were grown in BHI broth for stock

cultures, they were incubated for 16-24 hours at 37 C. Cultures for experimental purposes, were incubated on a reciprocal shaker maintained at 37 C for the same amount of time.

(5) Counting Method

The size of the inoculum of E. coli was determined by plating 0.1 ml of 10^6, 10^7, and 10^8 dilutions in saline of the cell suspension on Tris YP agar plates. The organism were spread evenly over the entire surface of the agar by means of sterile bent-glass "hockey sticks". The plates were inverted and incubated for 24 hours at 37 C and colonies were then counted. The number of organisms per ml was calculated by multiplying the average number of colonies on the plates by the dilution factor x 10. Only plates containing 30-300 colonies were counted.

The "double-layer" technique described by Varon and Shilo(33) was used for enumerating B. bacteriovorus cell populations. It is assumed that one B. bacteriovorus cell forms one plaque. Thus the plaque forming units(PFU)/ml are equivalent to the concentration of B. bacteriovorus cells. The technique consisted of pouring approximately 15 ml of 2% Tris YP agar in a petri dish and overlaying it with a mixture of 2.5 ml of 0.6% Tris YP agar, 0.5 ml of a stationary (16-24 hour) culture of E. coli, and 0.5 ml of 10^5, 10^6, 10^7, and 10^8 dilutions of B. bacteriovorus-E. coli suspensions. The plates were incubated at 25 C and were observed for plaque formation in 3-5 days. Circular, clear areas were counted as plaques. These plaques resemble bacteriophage plaques. The number of organisms per ml was calculated by multiplying the average number of plaques on the plates by the

dilution factor x 2. Only plates containing 30-300 plaques were counted.

EXPERIMENTAL MATERIALS AND METHODS

(1) Thermal Destruction of B. bacteriovorus

The first test was performed to determine the thermal death point of B. bacteriovorus cells. E. coli was cultured in 100 ml volumes of Tris YP medium and inoculated with 1 ml of B. bacteriovorus stock suspension. The cultures were incubated at room temperature for at least four days. The B. bacteriovorus-E. coli cultures were pipetted into 20 x 150 mm glass, screw-cap tubes in 2 ml quantities. The tubes were placed in a temperature controlled water bath.

The temperatures tested for inactivation of B. bacteriovorus were 37, 40, 43, 46, 50, 60, 70, and 80 C. Duplicate samples were exposed to heat for a period of 10 minutes. After heat-treatment the tubes were immediately cooled under tap water, and the number of surviving B. bacteriovorus particles were assayed by the double-layer technique. The control consisted of tubes not exposed to heat-treatment.

Next the temperature killing zone for B. bacteriovorus cells was determined. Again 2 ml quantities of the B. bacteriovorus-E. coli cultures were placed in glass, screw-cap tubes and the tubes placed in a temperature controlled water bath. Samples of host-parasite culture were exposed to 49 C for 10, 15, and 20 minutes and to 52 C for 3, 6, 9, and 12 minutes. The tubes were cooled in tap water and assayed as described previously.

In addition, a qualitative assay for the presence of B. bacterio-

vorus was performed. This was done by pipetting 0.5 ml of the heat treated culture on a host "lawn"(a confluent layer of bacterial growth). The lawn was prepared by using the double-layer technique. However, the parasite was omitted from the top layer. The plates were incubated overnight at 37 C to develop the lawn.

After 4, 6, and 7 days of incubation, the formation and numbers of plaques were observed and recorded. An individual clear area of lysis was considered to be a single plaque. The diameters of the plaques were measured. The controls consisted of tubes not heated.

(2) Survival Curves

B. bacteriovorus cultures were prepared by adding 10 ml of a 24 hour E. coli culture plus 5 ml of a B. bacteriovorus stock culture to 250 ml of Tris YP broth. The cultures were incubated at ambient temperature(25-30 C) for 6 days and were stirred with a magnetic stirring bar.

For the survival studies B. bacteriovorus was stored on slant and in broth cultures. For storage, in broth, the B. bacteriovorus-E. coli cultures were dispensed into glass screw cap tubes in 5 ml samples. Tubes were stored at temperatures of 4, -20, and -78 C. The initial concentrations of B. bacteriovorus in broth were assayed by determination of PFU/ml employing the double-layer technique. This concentration constituted the control or zero day population of B. bacteriovorus before being stored at any of the experimental temperatures.

Samples of the B. bacteriovorus-E. coli cultures were also added to Tris YP agar slants in 1 ml amounts. The slants were incubated overnight at ambient temperature. The initial untreated population was

determined after the incubation period by washing duplicate samples of the slants with 5 ml of Tris YP broth and assaying for PFU/ml by the double-layer technique. These slants were stored at the previously mentioned temperatures.

Periodically duplicate samples of both the broth and slant cultures, stored at various temperatures, were assayed by the double-layer technique. Prior to assay all frozen samples were thawed at room temperature and the slants were washed with 5 ml of Tris YP broth.

(3) Growth Curves of B. bacteriovorus and Parasitized E. coli

The growth of E. coli and B. bacteriovorus was followed in a two-membered culture system. E. coli was cultured in 50 ml of Tris YP broth to which was added 5 ml of B. bacteriovorus. The number of both organism was determined and this represented zero hour concentration. B. bacteriovorus was assayed by the double-layer technique; and E. coli by plate counting. The culture was stirred by a magnetic stirring bar and incubated at ambient temperature. Readings were taken at 0, 4, 9, and 14 hours for both organisms. During the next 10 hours they were assayed at one hour intervals for the parasite and at 2 hour intervals for the host. The last sample was taken at 38 hour for both the host and parasite. Thus, samples were taken over a period of 38 hours. Growth was measured in PFU/ml for the parasite and number of organisms per ml for the host.

The culture was diluted appropriately to insure accuracy and ease of counting plaques and colonies. All dilutions were plated in duplicate for both the host and parasite.

(4) Optimal Incubation Temperature

E. coli was cultured in 50 ml volumes of Tris YP broth to which was added 5 ml of B. bacteriovorus. All cultures, in duplicate, were incubated at temperatures of 5, 10, 15, 20, 25, 30, 35, and 43 C and mixed with a magnetic stirring bar. The inoculum concentration of B. bacteriovorus was assayed for each temperature. The cultures were assayed again after the incubation period to give the final concentration of B. bacteriovorus for each temperature.

(5) Host-Parasite Ratio

Once the optimal incubation temperature was determined for the complete parasitic cycle, the effect of the host-parasite ratio on the final concentration of parasite produced was determined. E. coli cultures were prepared in 50 ml volumes of Tris YP broth and inoculated with 5 ml of various concentrations of B. bacteriovorus to give approximate ratios of host-to-parasite of 1:1, 10:1, 100:1, 1000:1, and 10,000:1. The inoculum concentration of both organisms was assayed. The cultures were incubated at 25 C for 28 hours and mixed with magnetic stirring bars. After the incubation period, the final concentration of B. bacteriovorus was determined in PFU/ml.

(6) Effect of Chemicals on the Growth of B. bacteriovorus

The effects of antibiotics and antimetabolites on the host-dependent(H-D) and host-independent(H-I) forms of B. bacteriovorus were compared. Antibiotics with different modes of action were selected. These included polymyxin B sulfate, potassium penicillin G, streptomycin sulfate, aureomycin(chlortetracycline HCl), chloromycetin(chlor-

amphenicol), and actinomycin D. The antimetabolites(sulfonamides) used were elkosin(sulfadimetine), gantrisin[N'-(3,4-Dimethyl-5-isoxazole)], sulfadiazine, sulfamerazine, sulfathiazole, thiosulfil, triple sulfa (sulfathiazole, sulfacetamide, and N'benzoylsulfanilamide), and sulfamethoxypyridazine.

Two different methods were used for testing susceptibility of the H-I forms to antibiotics. These were the agar diffusion and the tube dilution techniques. In the former paper disks saturated with various concentrations of the antibiotics were laid on the surface of PYT agar plates. These plates were previously inoculated with a suspension of the H-I strains. This was done in two manners: by using the double-layer technique and inoculating the molten top layer with a heavy suspension of the organism, and by plating 0.1 ml on the surface of a Tris YP agar plate. In the tube dilution technique, the tubes contained 8 ml of PYT broth, 1 ml of a suspension of the organism, and 1 ml of the antibiotic to make a final volume of 10 ml. Varying concentrations of the antibiotics were tested. The concentrations of the antibiotics used were: penicillin and polymyxin B, 50, 100, 250, 500, and 1000 units/ml; aureomycin, streptomycin, and chlormycetin, 50, 100, 250, 500, and 1000 μg/ml; and actinomycin, 31.25, 62.5, 156, 312, and 625 μg/ml.

The agar diffusion method was also used for testing susceptibility of H-I strains to sulfonamides. The agar medium was PYT. The sulfonamides were in the form of standardized Bacto-Unidisks. These disks were laid on the PYT plates previously inoculated with the organism The concentration of the standardized disks was 300 μg/ml.

All the tubes and the plates were incubated at 25 C for 48 hours. The readings were then made. The plates were examined for zones of inhibition; turbidimetric readings were taken of the tubes on the Klett-Summerson Photoelectric Colorimeter model 800-3. Control plates and tubes were run to compare their growth with test plates and tubes. All tests were run in duplicate.

The effect of antibiotics and antimetabolites on H-D B. bacteriovorus could only be determined if the host was not affected by the chemicals. The reason for this is that if the antibiotics inhibited the host the H-D form would probably not grow. Therefore, it would be difficult to assess the direct inhibitory activity of these agents on the B. bacteriovorus. Since B. bacteriovorus was previously demonstrated to grow on autoclaved S. flexneri 2 by Gillis and Nakamura(9), this technique was utilized.

Cultures of S. flexneri 2 were prepared in 500 ml volumes of BHI broth. The cultures were incubated on a reciprocal shaker for 18 hours at 37 C. The cells were harvested by centrifuging the cell suspension in 250 ml centrifuge bottles for 90 minutes at 2,432 x g using the Model V International Centrifuge. The pellets were resuspended in 20 ml of physiological saline and dispensed in 2 ml amounts into glass screw cap tubes. The tubes were then autoclaved(10 minutes, 121 C, 15 pounds per square inch). For testing antibiotic sensitivity of H-D strains a modified double-layer technique was used. The methods included mixing 2.5 ml of the molten top agar layer with 0.5 ml of autoclaved S. flexneri 2 and 1 ml of the antibiotic. The number of autoclaved cells present was sufficient to produce turbidity within

the top layer. The desired concentrations of the antibiotics were obtained when the total volume in the top layer was reached.

The H-D strain was transferred in agar blocks to the surface of the plates containing the antibiotics. The blocks were obtained by cutting out plaques formed by plating live S. flexneri 2 and B. bacteriovorus by the double-layer technique. The blocks were transferred aseptically by sterile spatulas. The top layer of the block was placed face down on the surface of the plates containing the antibiotics. Four blocks were transferred per plate. The concentrations of all antibiotics were the same as those employed when testing the H-I forms with one exception: the concentrations of actinomycin used were 25, 50, and 100 μg/ml rather than 31.25, 62.5, 156, 312, and 625 μg/ml.

The agar diffusion method was also used for testing the effect of sulfonamides on H-D growth. In this method 2.5 ml of molten agar were mixed with 1 ml of autoclaved S. flexneri 2 cells and poured over the bottom layer of agar medium. The Bacto-Unidisks were placed on the surface of the plates. An agar block of B. bacteriovorus was placed face down directly adjacent to the disk.

All concentrations of all antibiotics were plated in duplicate. All plates were incubated at 25 C and examined for lysis or growth after 3-5 days.

(7) Investigation of Human and Animal Fecal Material

Fecal samples from 10 human volunteers, 10 rabbits, 40 mice, and 4 dogs were investigated. One fecal sample was tested for each individual except in the case of the rabbits where samples were taken from

each rabbit every two days for two weeks.

Human fecal samples were treated in the following manner. The fecal material was placed in a Waring blender and saline was added. The mixture was then homogenized until it was liquefied and of uniform consistency. Then 0.5 ml of the homogenate was plated directly by the double-layer technique. E. coli was used as the host organism. In addition, an 18 hour Tris YP broth culture of E. coli was inoculated with 5 ml of the homogenate. This culture was incubated at 25 C for 18-24 hours and mixed by a magnetic stirring bar. After incubation the culture was assayed by the double-layer technique. This procedure was employed to serve as an enrichment process for the B. bacteriovorus, if present. Plates of all fecal homogenates and enrichment cultures were run in duplicate. The plates were incubated up to 12 days at 25 C. They were examined daily for presence of plaques.

Feces from rabbits, mice, and dogs were treated in the same manner except that the enrichment procedure was not used.

If plaques appeared on the plates, further procedures were followed to separate the organism causing lysis from other fecal contaminants. This was done by cutting out agar blocks of lysed areas and transferring them face down to lawns of E. coli. These plates were incubated at 25 C until lysis appeared, usually 2-5 days. Transfers were continued until a two-membered system was obtained. Usually four to five transfers were necessary.

To confirm the presence of B. bacteriovorus the following procedures were used. After pure cultures of the organism causing lysis were obtained, portions of the lysed area on the agar plates were mixed

with saline. The agar suspension was then examined for the presence of B. bacteriovorus by using the phase microscope(97 X phase objective). The agar suspension was also filtered through a Millipore filter with a 1.2 μm pore size. This eliminated any organism larger than 1.2 μm which might be causing lysis. The filtrate and the agar suspension were both plated by the double-layer technique. The plates were incubated at 25 C and examined daily for at least 12 days.

Finally, the agar suspension was subjected to an enrichment procedure. This was done by inoculating an 18 hour Tris YP broth culture of E. coli with the agar suspension and incubating it for 48 hours at 25 C. The culture was mixed by using a magnetic stirring bar. At 24 and 48 hours, 0.5 ml of the culture was removed and plated by the double-layer technique. Samples were also placed on a slide and examined by a phase microscope(97 X objective). Ten ml of the 24 and 48 hour incubated culture were filtered through a 1.2 μm Millipore filter and 0.5 ml of the filtrate was plated by the double-layer technique.

All plates were run in duplicate and incubated at 25 C. Plates were examined daily for lysis up to 12 days.

(8) Serological Studies

B. bacteriovorus was prepared in the following manner for production of antiserum. Tris YP broth cultures of B. bacteriovorus which had been incubated for 23 hours were filtered differentially through Millipore filters of decreasing pore size(1.2, 0.8, and 0.65 μm). The filtrate was placed in 250 ml centrifuge bottles and centrifuged at 3,178 x g for 90 minutes in a Model V International Centrifuge. The pellets were resuspended in 5 ml of saline. The cell

suspension was layered over a ficoll(MW 400,000; Pharmacia, Uppsala) gradient consisting of four layers of decreasing density from bottom to top(30, 20, 15, and 5%); the amounts used of each were 5, 10, 10, and 5 ml respectively. A 40 ml centrifuge tube was used. The tubes were centrifuged at 1000 x g for 90 minutes in a Model PR-6 International refrigerated centrifuge with a swinging bucket rotor. The B. bacteriovorus cells formed a band in the 15% layer of ficoll. This band was removed sequentially and placed in sterile tubes. Each tube was tested for the presence of host organism by plating 0.1 ml on Tris YP agar plates. The plates were incubated overnight in a 37 C incubator and examined for the presence of host colonies. Only B. bacteriovorus from those tubes whose corresponding plates did not show growth were used in the immunization procedure. B. bacteriovorus cells were separated from the ficoll by centrifuging the mixture at 27,000 x g for 10 minutes in a RC2-B Sorvall refrigerated centrifuge. The pellet was then washed three times in saline.

Two rabbits were immunized against B. bacteriovorus by injecting antigen contained in complete Freund's adjuvant into each inguinal and axillary lymph node region. The antigen was prepared by first grinding together 0.6 ml Arlacel A and 3.0 mg dried tubercle bacilli in a mortar and pestle. Then 2.4 ml of light mineral oil was added and mixed. The process was continued by adding 3 ml of live washed antigen(1.98×10^9 cells/ml), dropwise, and grinding until an even emulsion of saline antigen in oil was formed. The mixture was drawn into a 10 ml sterile syringe to which was attached a second 10 ml syringe by a double needle. The mixture was then forced back and forth through the double needle

from one syringe to the other. This was done 10 times or until a drop of the adjuvant floated two-thirds submerged without spreading when dropped into cold water.

Before immunization both rabbits were bled to obtain normal serum. The rabbits were bled again 14 days and 30 days after immunization. Each time 30 ml of blood was drawn by cardiac puncture. The blood was allowed to clot at room temperature, ringed with an applicator stick, and stored in a 4 C refrigerator overnight. The serum was decanted into a conical centrifuge tube and centrifuged for 10 minutes in a Servall angle centrifuge to remove remaining red blood cells. The serum was then pipetted into sterile plastic tubes and stored in a -20 C freezer.

After 3 months, one rabbit previously immunized against B. bacteriovorus was given a booster injection. The B. bacteriovorus antigen was prepared as before except that it was administered in a 0.2% phenol-saline solution rather than in complete Freund's adjuvant. One ml of a concentrated solution(1.7×10^3 μg/ml) of B. bacteriovorus was injected subcutaneously(SC) and intraperitoneally(IP) on day one. The cells were prepared for weighing by placing an sample of the saline cell suspension in an aluminum weighing boat and drying the boat overnight in a 75 C oven. The dry weight of the cells was obtained by subtracting the weight of the boat alone plus the weight of the saline from the weight of the boat plus the dried cells. On days 2, 4, and 7, 0.5 ml of the solution was injected intravenously(IV). On day 12, 30 ml of blood were drawn by cardiac puncture. The serum was obtained and stored.

To prepare antiserum against E. coli, E. coli cells were harvested from an 18 hour BHI broth culture. This was done by centrifuging the culture at 7,710 x g for 15 minutes in a RC2-B Sorvall refrigerated centrifuge. The pellet was washed twice and resuspended in saline. The cell suspension was boiled for 2 1/2 hours. A sample of the suspension was removed, placed in an aluminum weighing boat, and dried overnight in a 75 C oven. The dry weight was obtained as above. Phenol was added to the boiled cell suspension in an amount which gave a final concentration of 0.2%. Two rabbits were injected each with 100 μg of antigen IP and 100 μg SC on day 1 of week one. On day 2 they were injected with 50 μg IV. On day 1 of week 2 the rabbits were given 500 μg of antigen IP and 500 μg SC. On day 2 they were given 100 μg IV.

As before, normal rabbit serum was drawn before immunization. To obtain the antiserum the rabbits were bled by cardiac puncture one week after the last injection. Thirty ml of blood was drawn from each rabbit. The serum was prepared and stored.

The titer of the anti-B. bacteriovorus-serum and anti-E. coli-serum was determined by tube agglutination tests. An agglutination test was also performed to see if the anti-B. bacteriovorus-serum contained antibodies against E. coli and if the anti-E. coli-serum reacted against the B. bacteriovorus cells.

The tube agglutination test was performed by placing 14 small tubes in a rack and adding 0.5 ml saline to the first 13 tubes and 0.5 ml normal rabbit serum to tube 14. Tubes 13 and 14 were the controls. One half ml serial dilutions of the antisera in saline were prepared. Next 0.5 ml of the bacterial antigen to be tested was added to each of

the 14 tubes. The tubes were mixed by shaking and were incubated in a 55 C water bath for 1 hour. The tubes were read at this time and again after they had been left overnight in a 4 C refrigerator. The tubes were read by comparing them to the controls. The last tube in the series to give a clumpy swirl instead of a smoky one was the antibody titer.

The B. bacteriovorus antigen was prepared for the agglutination test in the same way it was prepared for injection. However, after it was washed, it was resuspended in a 0.5% formalin-saline solution. It was not used in the test until it had stood for a least 24 hours. There were 10^8 cells/ml or greater in the antigen suspension used in the test.

The E. coli antigen was also prepared as before. However, after it was washed, it was also resuspended in 0.5% formalin-saline solution. The suspension contained at least 10^8 cells/ml.

Once the antisera were made and titers determined, tests were performed to determine the effect of E. coli and B. bacteriovorus antibody on the ability of B. bacteriovorus to parasitize E. coli and also to form plaques.

The first test was run using the anti-B. bacteriovorus-serum. Cultures of E. coli were grown by inoculating 50 ml of Tris YP broth with the stock suspension. Cultures of B. bacteriovorus which had incubated 18-24 hours at 25 C were filtered through Millipore filters of 1.2 and 0.65 μm pore size. The filtrate was then centrifuged at 12,100 x g for 30 minutes in a model HT International centrifuge. The pellet was washed 3 times and resuspended in saline. The B. bacteriovorus suspension was diluted 10^2 times, and 9 ml were mixed with 1 ml

of various dilutions of antibody in glass tubes to give final antibody concentrations of 1:50, 1:100, 1:200, 1:500, and 1:1000. The antibody was diluted with saline. The tubes were incubated for 1 hour at room temperature. Each E. coli culture was then inoculated with 5 ml of one of the various antibody dilutions plus B. bacteriovorus. The controls consisted of B. bacteriovorus, E. coli, and saline; B. bacteriovorus, E. coli, and a 1:100 concentration of normal serum; and B. bacteriovorus, E. coli, and a 1:100 concentration of heat inactivated antiserum. The antiserum was heat inactivated at 56 C for 30 minutes. This was done to destroy any complement present in the serum. It was used in the controls at a concentration of 1:100 so that the effect of it on B. bacteriovorus growth could be compared to that of the 1:100 antiserum concentration in which complement was still present. Any difference observed could then be attributed to the effect of complement on the system.

The B. bacteriovorus, E. coli, saline control culture was assayed to give the initial B. bacteriovorus concentration. All cultures were incubated at 25 C for 24 hours. At 1, 5, 10, and 24 hours, slides were made of each culture and examined for morphological changes in the B. bacteriovorus plus effects on the parasitic cycle with the phase microscope(97 X objective). Drawings were made to show changes that occurred. After 24 hours of incubation, all cultures were assayed by the double-layer technique to determine the final concentration of B. bacteriovorus.

To determine the percentage of B. bacteriovorus cells not bound by antibody, a indirect fluorescent antibody technique was utilized. Following the incubation of B. bacteriovorus with antiserum, one ml of

each antiserum dilution and the B. bacteriovorus-E. coli control was removed and each mixed with 1 ml of sheep antirabbit globulin antisera conjugated with fluoroscein isocyanate(Nutritional Biochemicals Corporation, Cleveland, Ohio). The labeled antibody had a final concentration of 1:40. The mixture was allowed to react for 1 hour a 4 C and then was centrifuged for 10 minutes at 12,100 x g in the RC2-B Sorvall refrigerated centrifuge. The pellet was washed 3 times and resuspended in saline. Equal amounts of each suspension were placed on slides and covered with cover slips. The cover slips were sealed with paraffin wax. The slides were examined for fluorescence by using a standard Zeiss microscope equipped with a fluorescence apparatus. Light fluorescence with dark field illumination and excitation filter UG 5 were used. The number of nonfluorescing B. bacteriovorus cells were counted in 10 fields from each slide. An average of the nonfluorescing particles per field was calculated for each concentration of antibody. An average for the number of particles per field in the control was also determined. This gave the total number of cells per field. To determine the percentage of nonfluorescing particles per antibody concentration, the average of the nonfluorescing particles per field was divided by the average of the total number of cells per field and multiplied by 100.

B. bacteriovorus cells were also diluted 10^2, 10^3, 10^4, and 10^5 times and 9 ml aliquots mixed with 1 ml of various concentrations of antibody to give the same final concentrations as mentioned above. This time, however, each tube was assayed by the double-layer technique to determine the amount of plaque formation. Plates were pre-

pared in duplicates and incubated for 8 days at 25 C. PFU/ml were then counted. Adequate controls were included in this study.

In the previous experiments, studies were done using anti-B. bacteriovorus-serum. In the next series of experiments anti-E. coli-serum was used. Cultures of E. coli were grown in 45 ml volumes of Tris YP broth. They were incubated for 18-24 hours and then filtered sequentially through Millipore filters of 1.2 and 0.8 μm pore sizes to remove cellular debris. Each culture of E. coli was mixed with 5 ml of a particular dilution of antibody to give a final antibody concentration of 1:50, 1:100, 1:200, 1:500, and 1:1000. Five ml from each culture were removed and then each culture was inoculated with 5 ml of the B. bacteriovorus filtrate and incubated at 25 C for 24 hours.

At 4 and 20 hours, slides were made of each culture and examined for morphological changes in the E. coli and for effects on the parasitic cycle. The observations were recorded by free hand drawings. Changes in the B. bacteriovorus populations were determined after 20 hours by the double-layer technique.

Nine ml of an E. coli suspension were also mixed with 1 ml of various dilutions of antiserum in glass tubes to give the same final antibody concentrations as above. These tubes were then used as the source of E. coli in assaying B. bacteriovorus by the double-layer technique. All plates were run in duplicate and incubated for 4 days. PFU/ml were then counted.

(9) Cell Envelope Attachment Studies

Cell envelopes of E. coli were prepared by use of the RF-1 Sorvall Ribi refrigerated cell fractionator. Cultures of E. coli

were prepared and harvested by centrifugation in a model V Internatical centrifuge at 3,178 x g for 90 minutes. The pellet was resuspended in saline to a final concentration of 10 mg/ml. The cell suspension was placed in a flask and precooled along with the rest of the fractionator apparatus. After cooling to 5 C the cells were fractionated using pressure of 20,000 pounds per square inch. The temperature was maintained at 4-6 C throughout the process. The crude cell envelopes were centrifuged three times at 27,000 x g for 2, 0.5, and 0.5 hours respectively in a RC2-B Sorvall refrigerated centrifuge. Each time the pellets were resuspended in saline. To separate the cell envelopes from whole cells remaining, the pellets were resuspended in 5 ml saline and layered over a ficoll gradient in 40 ml centrifuge tubes. The technique used was the one also used in separating B. bacteriovorus from its host. The tubes were centrifuged at 1000 x g for 90 minutes in a Model PR-6 International refrigerated centrifuge with a swinging bucket rotor. The top three ficoll layers were removed to obtain the cell envelopes.

A 0.1 ml samples of this was plated to determine the amount of E. coli whole cells present. A drop was also placed on a slide, air dried, heat fixed, and stained for 2 minutes with crystal violet solution. The slide was examined with a light microscope(97 X objective) to determine the efficiency of cell rupture. The ficoll suspension of cell envelopes was centrifuged for 10 minutes at 27,000 x g in a RC-2 B Sorvall refrigerated centrifuge. The pellet was resuspended in saline for use in studying attachment by B. bacteriovorus cells.

Cultures of B. bacteriovorus were prepared and incubated for 24

hours before use in the attachment studies. The cells were filtered through a Millipore filter of 1.2 μm pore size to remove cellular debris. Five ml of the B. bacteriovorus filtrate, 5 ml of the saline cell envelope suspension, and 10 ml of Tris YP broth were put in a sterile erlenmeyer flask. The flask was incubated at 25 C for 3 hours and mixed with a magnetic stirring bar. The size of the inoculum of B. bacteriovorus was determined by PFU/ml.

To separate the attached B. bacteriovorus cells from the free cells, 1 ml of the culture was filtered through a Millipore filter of 1.2 μm pore size and washed with 99 ml saline, precooled at 4 C. This was done immediately after the B. bacteriovorus cells and cell envelopes were mixed to obtain the number of free cells at time zero(T_0) and again 8 hours(T_8) after incubation. Each time the filtrate was assayed for PFU/ml. Attached cells will not come through a 1.2 μm filter(20), and therefore, only number of free B. bacteriovorus will be measured by this method.

A sample of the culture was also examined periodically by observation with a phase contrast microscope(97 X phase objective) to see if attached cells could be observed. The observations were recorded by hand drawn diagrams.

If B. bacteriovorus cells attached to E. coli cell envelopes, their normal parasitic cycle would be inhibited, if the attachment were irreversible. If they penetrated the cell envelope, they would probably die due to lack of nutrients. In order to determine if cells which may have attached to cell envelopes could carry on their parasitic cycle or if they died, the culture was assayed directly at

T_8 without filtration. The PFU/ml at T_8 were compared with the inoculum concentration at T_0.

CHAPTER III

RESULTS

(1) Thermal Death Point and Thermal Death Time Determination

The lowest temperature at which plaques were no longer formed from a suspension of B. bacteriovorus, heat treated for 10 minutes, was 51 ± 1.0 C. This is recorded in Table 3. The size of the B. bacteriovorus population was approximately 10^4 cells/ml. Approximately 10^8 E. coli cells/ml were also present in the suspension. When a suspension of B. bacteriovorus was exposed to a constant temperature of 49 C, the shortest period of time required to inhibit plaque formation was somewhat greater than 20 minutes as given in Table 4. At 52 C the shortest time period required to inhibit plaque formation was 3-6 minutes (Table 5). Increasing periods of exposure to heat at 49 and 52 C inhibited the parasitic ability of the organism. These results are recorded in Tables 4 and 5. As the exposure period increased, the days of incubation necessary for plaques to appear were increased. Also the size of the plaques decreased as the exposure period increased.

(2) Survival of B. bacteriovorus at Several Temperatures

Figures 2, 3, 4, and 5 show survival curves of B. bacteriovorus after 60 and 120 days of storage. Figure 2 shows the survival curves of B. bacteriovorus stored in broth culture. The initial population of cells before storage was 1.67×10^8/ml. A drop was seen initially in the cell populations at all temperatures up to 10 days. After 10 days, the cell population stored at -78 C remained constant up to 60 days. The number of viable B. bacteriovorus cells maintained at 4 and

Table 3

The Thermal Death Point of B. bacteriovorus Determined by Its Plaque Forming Abilities

Temperature[a] (C)	Plaque formation[b]
37 ± 0.1[c]	+
40 ± 0.5	+
43 ± 0.2	+
46 ± 0.32	+
49 ± 0.5	+
51 ± 1.0	O
60 ± 0.5	O
70 ± 1.0	O
80 ± 1.0	O

[a]The time of exposure was 10 minutes.

[b]Symbols: +=plaques present; O-plaques absent.

[c]Indicates range of temperature.

Table 4

The Effect of Temperature(49 C)[a] on the Ability of B. bacteriovorus to Form Plaques

Exposure period (min)	Plaque formation: Days of incubation[b]			Days of incubation[c]			Diameter of plaques (mm)[d]
	4	6	7	4	6	7	
0[e]	+	+	+	5800	75,000	75,000	3.6
10	O	+	+	0	f	f	f
15	O	+	+	0	10	10	1.0
20	O	O	+	0	0	2	2.0

[a]The temperatures range for 10 minutes was 48.7 ± 0.5 C; 15 minutes, 48.8 ± 0.6 C; and 20 minutes, 48.9 ± 0.6 C.

[b]Symbols: +=plaques present; O=plaques absent.

[c]Numbers indicate PFU/ml.

[d]The diameters were determined by averaging the diameters of all plaques present on a plate after 7 days of incubation.

[e]Refers to the control which was assayed initially by the double-layer technique and by plating 0.5 ml of the B. bacteriovorus-E. coli suspension on a previously grown host lawn. This was done to determine the initial population of cells not subjected to heat treatment.

[f]Individual plaques could not be counted or measured at this time period since the lysed areas were confluent. Approximately 1/4 of the total plate area was lysed after 10 minutes exposure to heat as compared with complete lysis of the entire control plate lawn.

Table 5

The Effect of Temperature(52 C)[a] on the Ability of B. bacteriovorus to From Plaques

Exposure period (min)	Plaque formation[b] After 4 days incubation	Plaque formation[b] After 6 days incubation	Plaque formation[c] After 4 days incubation	Plaque formation[c] After 6 days incubation	Diameter of plaques (mm)[d]
0[e]	+	+	5.8×10^3	7.5×10^4	3.6
3	O	+	0	1.0×10^1	0.75
6	O	O	0	0	
9	O	O	0	0	
12	O	O	0	0	

[a]The temperature range for all exposure periods was 51.5 $\pm$ 0.5 C.

[b]Symbols: +=plaques present; O=plaques absent.

[c]Numbers indicate PFU/ml.

[d]The diameters were determined by averaging the diameters of all plaques present on a plate.

[e]Refers to the control which was assayed initially by the double-layer technique and by plating 0.5 ml of the B. bacteriovorus-E. coli suspension on a previously grown host lawn. This was done to determine the initial population of cells not subjected to heat treatment.

-20 C continued to decline during the observation period of 60 days. The population stored at 4 C declined at the fastest rate.

The survival curves of B. bacteriovorus stored on slant cultures are shown in Figure 3. The number of B. bacteriovorus cells before storage was 3.28×10^8/ml. After 15 days of storage the initial rapid decrease in cell populations subsided, and the curves generally reached a plateau up to 60 days of storage. The number of cells stored at -78 C remained slightly higher than those at 4 C; and those at 4 C remained higher than those at -20 C.

The portion of the survival curves of B. bacteriovorus in broth cultures up to 60 days illustrated in Figure 4 are similar to those in Figure 2. The initial population of cells was 1.29×10^7/ml. The cell populations stored at -78 C continued to remain steady between 60 and 120 days. The curve of the cells stored at -20 C also reached a plateau after 60 days but at a considerably lower level than that of the -78 C curve. Cells stored at 4 C continued to decrease in number from 60-90 days. No PFU were detected after 90 days of storage at 4 C.

Cells maintained on slant cultures at -20 and -78 C showed little decrease in population size through 120 days. This is illustrated in Figure 5. The initial population of cells was 2.48×10^6/ml. The cells stored at 4 C declined sharply after 90 days.

The log decreases between the initial and final concentrations of cells stored at various temperatures for 60 and 120 days are shown in Tables 6 and seven. The greatest survival of B. bacteriovorus occurred at -78 C. Both the slant and broth cultures showed only a 0.03-0.85 log decrease from the initial population. Cultures stored at 4 and -20

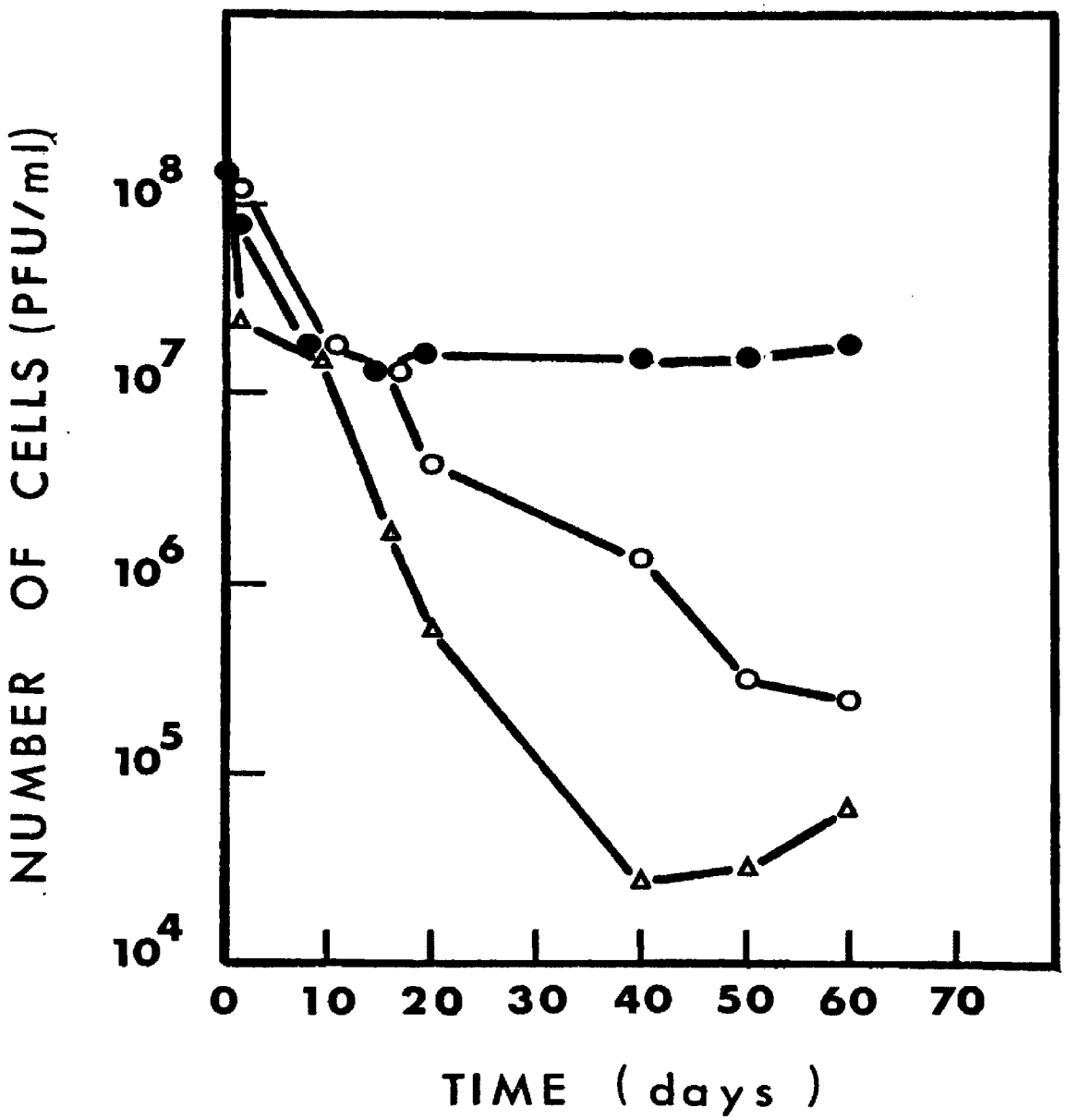

Figure 2

Survival of B. bacteriovorus at Various Temperatures in Broth Cultures. The initial concentration of the parasite before storage was 1.67×10^8/ml. Samples were stored at 4 C(o), -20 C(Δ), and -78 C(•).

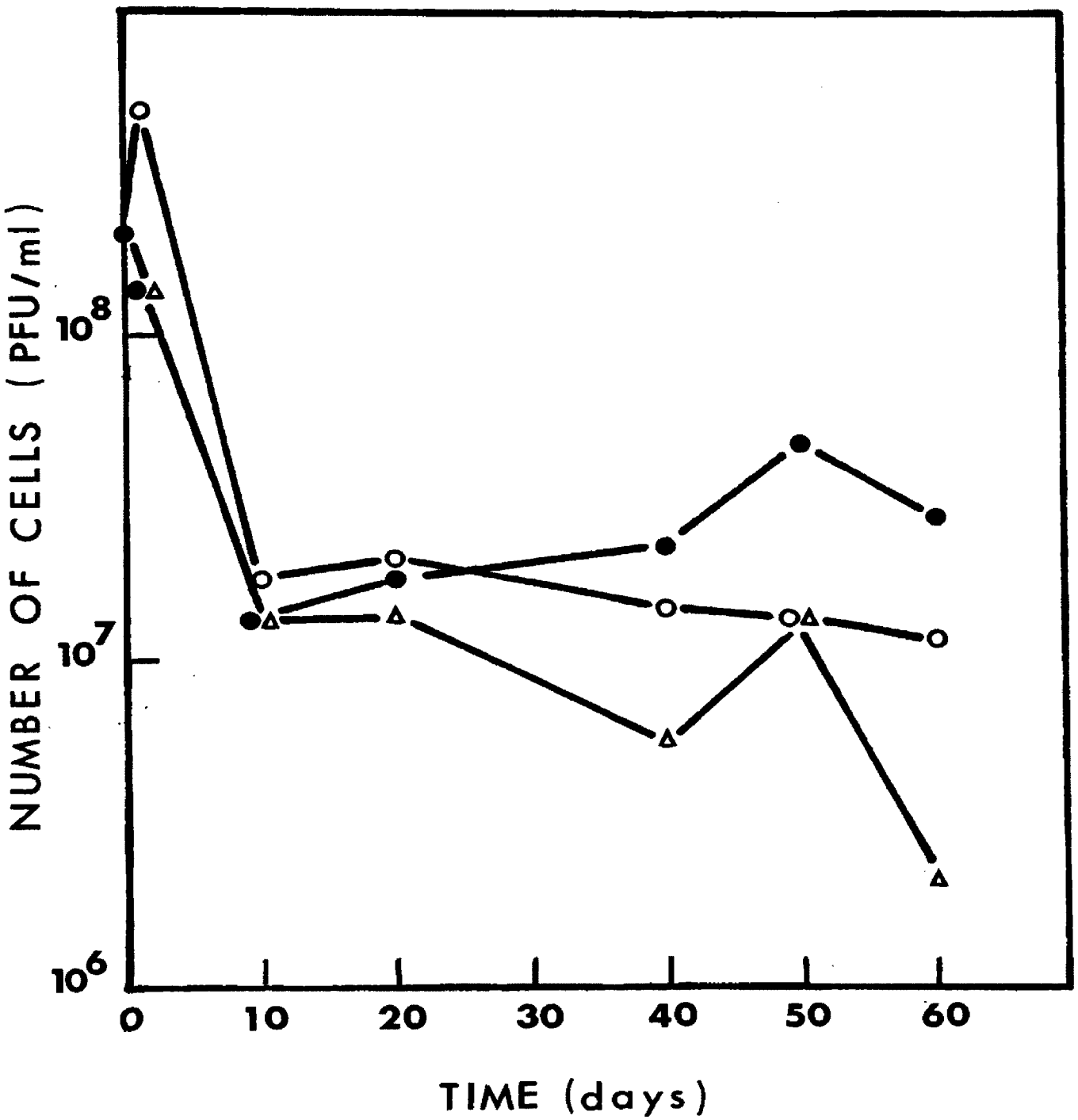

Figure 3

Survival of B. bacteriovorus at Various Temperatures on Slant Cultures. The initial concentration of the parasite before storage was 3.28 x 10^8/ml. Samples were stored at 4 C(o), -20 C(▲), and -78 C(●).

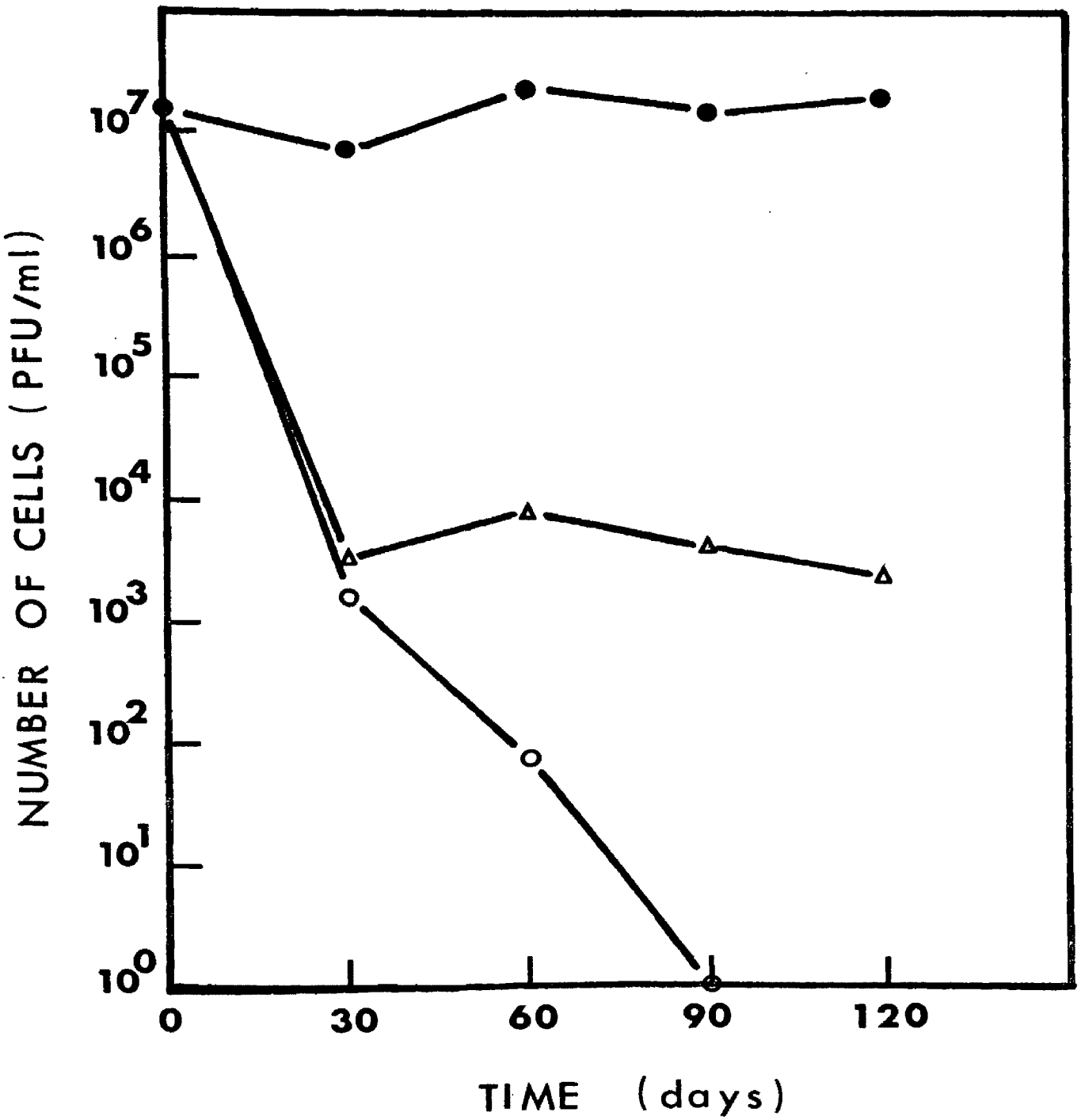

Figure 4

Survival of B. bacteriovorus at Various Temperatures in Broth Cultures. The initial concentration of the parasite was 1.29 x 10^7/ml. Samples were stored at 4 C(o), -20 C(Δ), and -78 C(•).

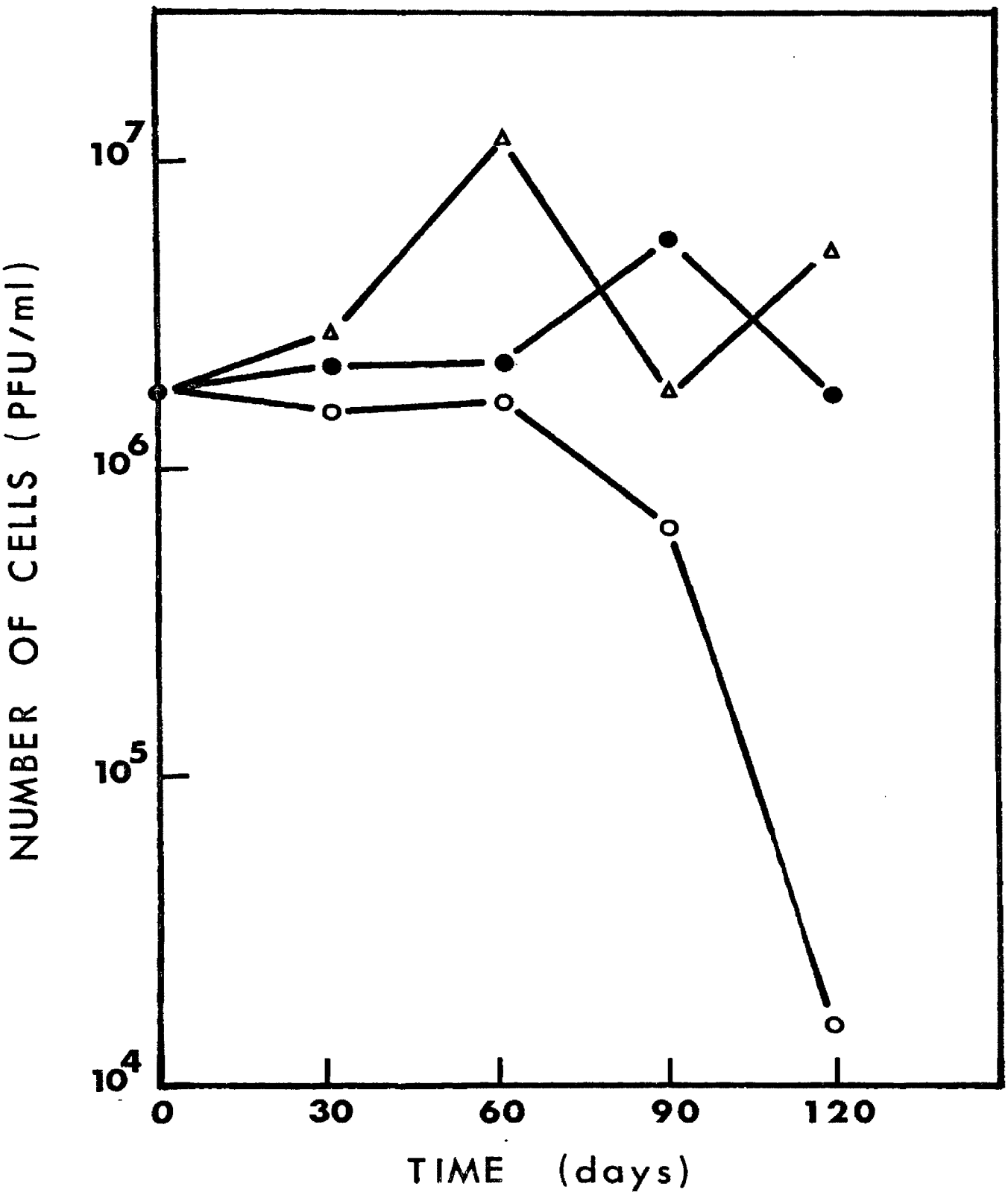

Figure 5

Survival of B. bacteriovorus at Various Temperatures on Slant Cultures. The initial concentration of the parasite was 2.48 x 10^6/ml. Samples were stored at 4 C(o), -20 C(Δ), and -78 C(•).

C showed less survival. Log decreases ranged from 0.44-7.11. Slant cultures of B. bacteriovorus showed greater survival than broth cultures did at these temperatures; log decreases ranged from 0.03-2.22 for slant cultures and 0.04-7.11 for broth cultures.

Incidentally, the ATCC cultures initially obtained were preserved on slant cultures at -78 C and were still viable after 9 months.

(3) Growth Curves of B. bacteriovorus and Parasitized E. coli

To obtain the growth curves(Figure 6) of B. bacteriovorus and parasitized E. coli a 24 hour culture of E. coli was inoculated with the parasite. Both host and parasite populations were monitored. The initial concentration of E. coli was 2.9×10^8 cells/ml and of B. bacteriovorus was 1.3×10^8 PFU/ml. The growth curves are drawn in PFU/ml for B. bacteriovorus and numbers of cells/ml for E. coli. Both populations showed a steady increase in concentration during the first hours of incubation. As the B. bacteriovorus PFU/ml rose logarithmically, the parasitized E. coli population decreased sharply. B. bacteriovorus population reached its maximum number at 9 hours, continued in the stationary phase from 9-23 hours and began the logarithmic death phase at 24 hours. Four hours after the maximum population of B. bacteriovorus was reached, the E. coli parasitized growth curve formed a plateau and remained at this level throughout the rest of the 38 hour period monitored.

(4) The Effect of Incubation Temperatures and Host-Parasite Inoculum Ratios on the Growth Rate of B. bacteriovorus

The inital populations of B. bacteriovorus used in the temperature studies were 10^8 PFU/ml(Table 8). As the temperatures of incu-

Table 6

Decrease(in logs) of PFU/ml of B. bacteriovorus after 60 Days Storage

Temperature (C)	Decrease(in logs)	
	Slant culture[a]	Broth culture[b]
4	2.03	2.6
-20	1.45	3.29
-78	0.86	0.85

[a]The initial concentration of B. bacteriovorus before storage was 3.28×10^8/ml.

[b]The initial concentration of B. bacteriovorus before storage was 1.67×10^8/ml.

Table 7

Decrease(in logs) of PFU/ml of B. bacteriovorus after 120 Days of Storage

Temperature (C)	Decrease(in logs)	
	Slant culture[a]	Broth culture[b]
4	2.22	7.11
-20	0.44	2.84
-78	0.03	0.04

[a]The initial concentration of B. bacteriovorus before storage was 2.48×10^6/ml.

[b]The initial concentration of B. bacteriovorus before storage was 1.29×10^7/ml.

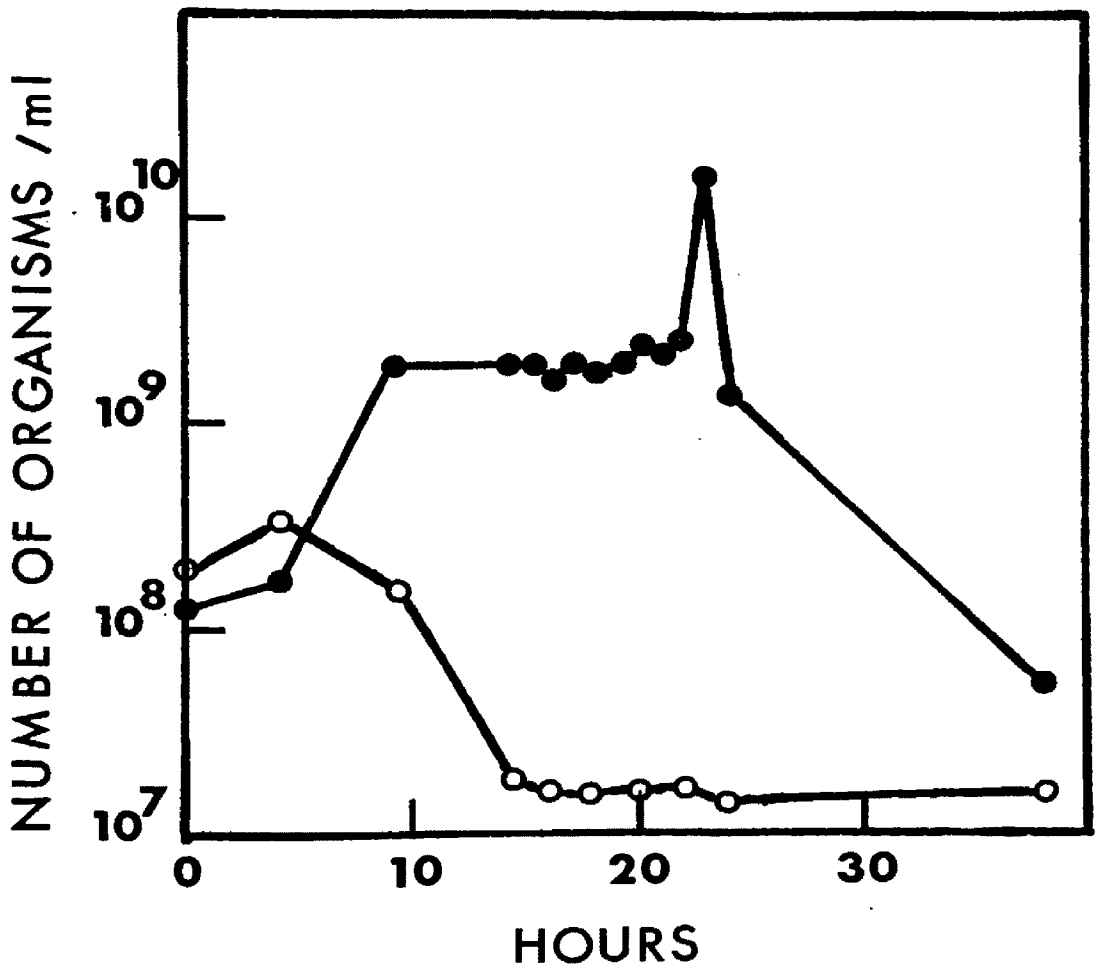

Figure 6

The Growth Curve of B. bacteriovorus and Corresponding Parasitized E. coli Growth. Symbols: ●=B. bacteriovorus; o= E. coli.

bation rose the PFU/ml of B. bacteriovorus rose through 25 C. At this point a maximum population was reached, after which the PFU/ml decreased until they could no longer be detected. Since the maximum change in population size was reached at 25 C, this temperature was chosen for the optimal incubation temperature and was employed for the remainder of the investigation.

To determine the effect of host-parasite inoculum ratios on the growth rate of B. bacteriovorus, the host population was kept constant and the parasite population was varied. The E. coli inoculum size was 3.03×10^8 cells/ml. As the host-parasite ratio increased, the change in population size also generally increased. There was one exception, namely a host-parasite ratio of 100:1(see Table 9). Growth rates were greatest at host-parasite ratios of 10:1, 1000:1, and 10,000:1. For the remainder of the study a host-parasite ratio of 10:1 was used.

(5) The Effect of Antimicrobial Agents on B. bacteriovorus

The effects of antimicrobial agents with different modes of action on B. bacteriovorus were determined.

In general, penicillin and actinomycin D inhibited the growth of the H-I strain as determined by agar diffusion and the tube dilution method(Tables 10 and 11). The lower concentrations of the antibiotics did not give complete inhibition in the agar diffusion method. Chloromycetin inhibited growth of H-I B. bacteriovorus as assayed by the tube dilution method but not when assayed by the agar diffusion method. Aureomycin, chloromycetin, penicillin, and polymyxin all inhibited H-D B. bacteriovorus at 500 μg or units/ml or above(see Table 12). Streptomycin and actinomycin D inhibited H-D growth at all concentrations.

Table 8

The Effect of Incubation Temperature on the Growth Rate of B. bacteriovorus

Temperature (C)	PFU/ml		Growth increase (in logs)
	Inoculum(I)[b]	Final(F)	
5	1.90×10^8	1.71×10^8	-1.95
10	2.35×10^8	3.09×10^8	0.12
15	3.46×10^8	3.15×10^8	-1.96
20	3.58×10^8	2.62×10^9	0.86
25	2.85×10^8	4.1×10^9	1.16
30	4.15×10^8	7.82×10^8	0.27
35	1.10×10^8	1.38×10^8	0.10
43	1.62×10^8	0.	0.

[a]Plaque forming units/ml.

[b]The ratio of host to parasite was 1:1.

Table 9

The Effect of Host-Parasite Inoculum Ratios on the Growth Rate of *B. bacteriovorus*

Host:parasite	PFU/ml[a]		Growth increase (in logs)
	Inoculum	Final	
1:1	6.45×10^8	7.3×10^9	1.04
10:1	6.45×10^7	7.8×10^9	2.08
100:1	6.45×10^6	5.2×10^8	1.91
1000:1	6.45×10^5	3.3×10^8	2.71
10,000:1	6.45×10^4	2.6×10^7	2.60

[a]Plaque forming units/ml.

Table 10

Effect of Antibiotics on Growth of H-I B. bacteriovorus[a]

Concentration[b]	Growth[c]					
	Actinomycin	Aureomycin	Chloromycetin	Penicillin	Polymyxin	Streptomycin
31.25	+					
50.		+	+	+	+	+
62.5	+					
100.		+	+	O	+	+
156.	O					
250.		+	+	O	+	+
312.	O					
500.		+	+	O	+	+
625.	O					
1000.		+	+	O	+	+

[a] The agar diffusion method was used.

[b] The concentrations of penicillin and polymyxin were in units/ml; the rest were all in μg/ml.

[c] Symbols: +=growth; O=growth inhibited. Control plates not containing antibiotics yielded growth.

Table 11

Effect of Antibiotics on Growth of H-I B. bacteriovorus[a]

Concentration[b]	Growth (Klett units)[c]					
	Actinomycin	Aureomycin	Chloromycetin	Penicillin	Polymyxin	Streptomycin
15.6	15.5					
31.25	12.					
50.		23.5	12.5	5.	29.	24.
62.5	11.					
100.		23.5	4.5	5.	25.	21.
250.		30.	6.5	9.	26.5	23.5
500.		25.	4.	6.5	22.	21.5
1000.			4.5	8.25	27.5	19.5
Control (no antibiotic)			22.5			

[a] The tube dilution method was used.

[b] The concentrations of penicillin and polymyxin were in units/ml; all the rest were in μg/ml.

[c] The number 54 filter of the Klett-Summerson Colorimeter was used.

Table 12

Effect of Antibiotics on the Growth of H-D B. bacteriovorus[a]

Concentration[b]	Growth[c]					
	Actinomycin	Aureomycin	Chloromycetin	Penicillin	Polymyxin	Streptomycin
25	0					
50	0	+	+	+	+	0
100	0	+	+	+	+	0
250		±	+	+	+	0
500		0	0	0	+	0
1000		0	0	0	0	0

[a]The agar diffusion method was used.

[b]The concentrations of penicillin and polymyxin were in units/ml; all the rest were in μg/ml.

[c]Symbols: +=growth or lysis; 0=no growth or lysis. Controls plates not containing antibiotics yielded growth.

Streptomycin inhibited H-D growth at all concentration levels but did not inhibit H-I growth at any concentration.

The effect of sulfonamides on H-I and H-D strains is shown in Table 13. In general, H-I forms were inhibited by sulfonamides and H-D forms were resistant to them.

(6) Plaque Formation by Fecal Sample Isolates

Fecal samples from rabbits, mice, and dogs produced plaques. Samples of human feces did not produce plaques(Table 14). Pure(axenic) cultures of the organims causing lysis were obtained by successive transfers to new host lawns. When agar suspensions of the pure cultures were examined with the phase microscope to determine whether or not B. bacteriovorus cells were present, no B. bacteriovorus cells were observed. Only numerous amoeba-like organisms and cysts were viewed. The agar suspension was also filtered through a 1.2 μm Millipore filter. This filter size is of large enough porosity to let B. bacteriovorus particles through but not the amoeba. The filtrate was assayed for PFU but no lysis occurred. Lysis did occur, however, when the agar suspension was plated before filtration.

Finally, an enrichment procedure was followed to try to increase B. bacteriovorus populations, if present. Cultures of the agar suspension which had incubated for 24 and 48 hours were examined by the phase microscope for presence of B. bacteriovorus particles. Again only amoeba-like organism were seen; B. bacteriovorus cells were not observed. When the culture was filtered through a Millipore filter of 1.2 μm pore size, and the filtrate assayed, no plaque formtion was observed. However, when the culture was plated without filtration,

Table 13

Susceptibility of H-I and H-D B. bacteriovorus Strains to Antimetabolites(sulfonamides)

Sulfonamides[a]	H-I[b]	H-D[b]
Elkosin	S	R
Gantrisin	S	R
Sulfadiazine	R	R
Sulfmerazine	S	R
Sulfa methoxypyridazine	S	R
Sulfathiazole	S	R
Thiosulfil	S	R
Trisulfa	S	R

[a]The concentration of all sulfonamides was 300 μg/ml.

[b]Symbols: S=sensitive; R=resistant.

Table 14

Examination of Fecal Material for Plaque Formation

Source of fecal material[a]	Plaque formation[b]	*B. bacteriovorus* confirmation[c]
Human	O	O
Rabbit	+	O
Mouse	+	O
Dog	+	O

[a]The number of samples taken were human, 10; rabbit, 10; mouse, 40; and dog, 4.

[b]Symbols: +=plaques formed; O=no plaques formed.

[c]Symbols: +=*B. bacteriovorus* present; O=*B. bacteriovorus* not present.

plaques were produced. B. bacteriovorus was not present in fecal material of any of the sources tested.

(7) Separation of B. bacteriovorus and E. coli on a Ficoll Gradient

A suspension containing a mixture of B. bacteriovorus and E. coli cells was layered on a ficoll gradient and centrifuged for 90 minutes at 1000 x g(Figure 7). B. bacteriovorus formed a band in the 15% layer of ficoll. The majority of the E. coli cells pelleted to the bottom of the tube. A slight band of E. coli cells was obtained at the interface of the 20 and 30% layers.

(8) Agglutination Tests

Anti-B. bacteriovorus-serum possessed antibodies for both B. bacteriovorus and E. coli antigens as shown in Table 15. However, the titer against E. coli antigen(1:32) was considerably lower than that against the B. bacteriovorus(1:1024). The anti-B. bacteriovorus-serum obtained from the rabbit which received a booster injection contained antibodies to a titer of 1:512 against B. bacteriovorus(Table 16).

Anti-E. coli-serum agglutinated E. coli antigen at a dilution of 1:128(Table 17). No agglutination occurred in tubes containing B. bacteriovorus antigen.

(9) Determination of the Number of B. bacteriovorus Cells not Bound to Antibody by an Indirect Fluorescent Antibody Technique

Cells bound to antibody and reacted with conjugated anti-globulin specific for the antibody, will fluoresce. This technique, therefore, provided a suitable way of measuring the number of B. bacteriovorus cells not bound by antibody. The antigen-antibody complexes formed by

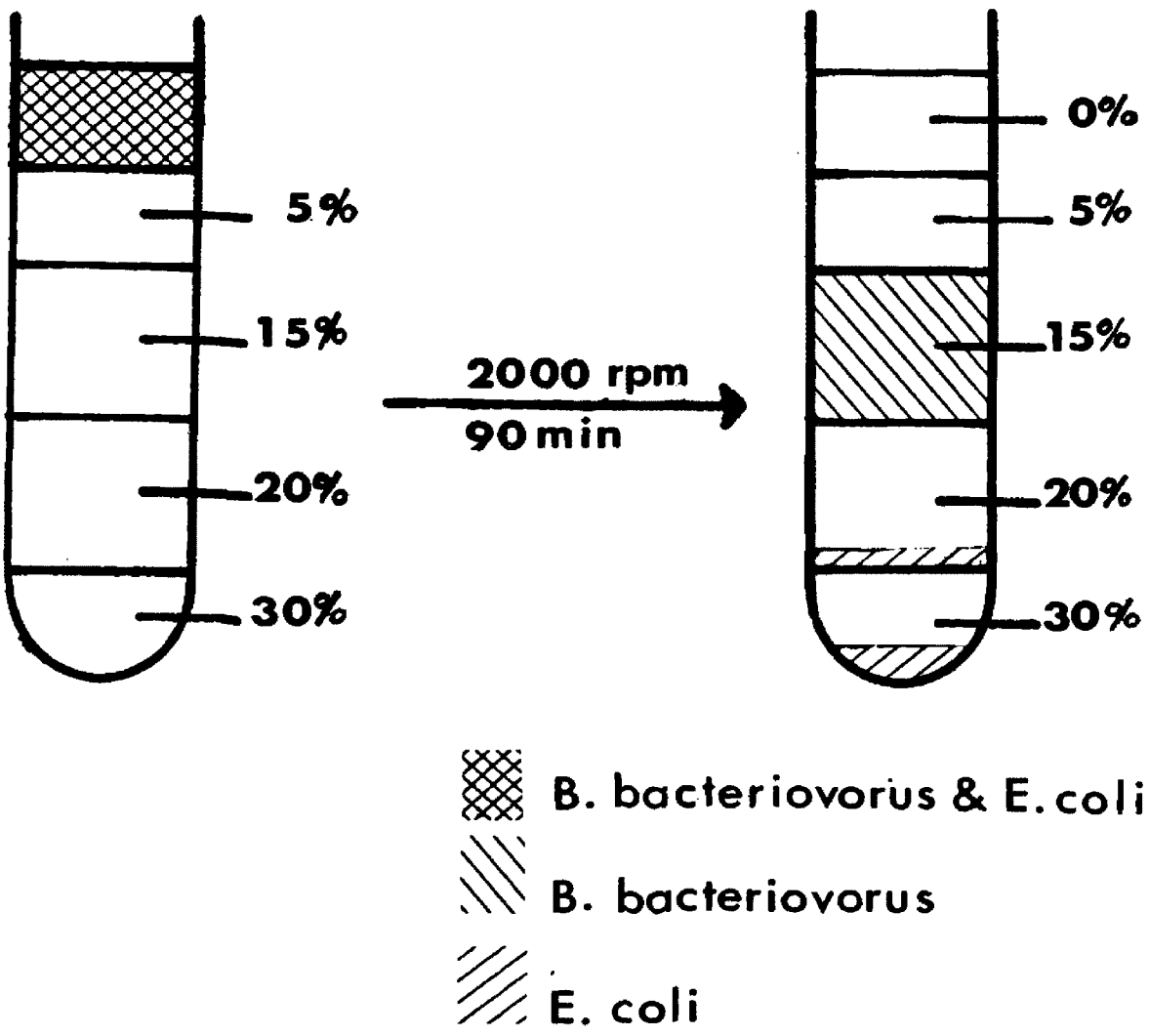

Figure 7

Separation of B. bacteriovorus and E. coli Cells on a Ficoll Gradient.

Table 15

Agglutination of *E. coli* or *B. bacteriovorus* Antigen by Anti-*B. bacteriovorus*-serum[a]

Antiserum dilution	Reaction[b]	
	B. bacteriovorus antigen	*E. coli* antigen
1:4	+	+
1:8	+	+
1:16	+	+
1:32	+	+
1:64	+	O
1:128	+	O
1:256	+	O
1:512	+	O
1:1024	+	O
1:2048	O	O
1:4096	O	O
1:8192	O	O
Saline control	O	O
Normal serum control	O	O

[a]The antiserum used was collected 34 days after immunization. Whole cell preparations of the antigens were used.

[b]Symbols: +=agglutination; O=no agglutination

Table 16

Agglutination of B. bacteriovorus Antigen
by Anti-B. bacteriovorus-serum[a]

Antiserum dilution	Reaction[b]
1:4	+
1:8	+
1:16	+
1:32	+
1:64	+
1:128	+
1:256	+
1:512	+
1:1024	O
1:2048	O
1:8192	O
Saline control	O
Normal serum control	O

[a]The antiserum used was collected 5 days after the last of a series of booster injections of B. bacteriovorus were given. The antigen used in the test was whole cell preparations of B. bacteriovorus.

[b]Symbols: +=agglutination; O=no agglutination.

Table 17

Agglutination of *E. coli* and *B. bacteriovorus* Antigens by Anti-*E. coli*-serum[a]

Antiserum dilution	Reaction[b]	
	E. coli antigen	*B. bacteriovorus* antigen
1:4	+	O
1:8	+	O
1:16	+	O
1:32	+	O
1:64	+	O
1:128	+	x[c]
1:256	O	x
1:512	O	x
1:1024	O	x
1:2048	O	x
1:8192	O	x
Saline control	O	O
Normal serum control	O	O

[a]The antiserum used was collected one week after the last injection was given. The antigens consisted of whole cell preparations in 0.5% formalin-saline solution.

[b]Symbols: +=agglutination; O=no agglutination.

[c]These experiments were not performed.

B. bacteriovorus and anti-B. bacteriovorus-serum were labeled with conjugated sheep anti-rabbit globulin. The complexes occurred singly or in aggreagates and readily fluoresced. Non-fluorescent B. bacteriovorus particles were single and motile. These were enumerated in order to calculate the percentage of the total B. bacteriovorus population not bound to antibody. The percentages of the B. bacteriovorus not bound to antibody are recorded in Table 18.

(10) Growth of B. bacteriovorus in Cultures Containing Anti-B. bacteriovorus-serum

Table 19 shows the effect of anti-B. bacteriovorus-serum on B. bacteriovorus growth. There was an inverse relationship between the growth of B. bacteriovorus and the antiserum concentration. The growth of the cultures containing 1:500, 1:1000, and 1:100 normal serum was similar to that of the saline control.

Table 20 shows the growth increase of B. bacteriovorus with the amount of B. bacteriovorus not bound to antibody used as the inoculum population. The amount of growth calculated at all antiserum concentrations is slightly higher than that of the control.

Figures 8, 9, and 10 represent diagrams of the cultures of B. bacteriovorus containing anti-B. bacteriovorus-serum. Figure 8 represents the cultures one hour after incubation. Clumping of B. bacteriovorus cells was observed in the cultures containing 1:50, 1:100, 1:200, and 1:500 antiserum dilutions. At the 1:1000 concentration of antiserum and in the control culture containing 1:100 heat-inactivated antiserum, there was no clumping. As the concentrations of antibody decreased the size of the clumps decreased, and more single B. bac-

Table 18

Percentage of the Total *B. bacteriovorus* Populations not Bound to Antibody when Mixed with Anti-*B. bacteriovorus*-serum

Antiserum dilution	% Total population[a]
1:50	3.5
1:100	12.8
1:200	7.8
1:500	20.1
1:1000	14.1

[a]This percentage was determined by dividing the average number of nonfluorescing particles per field by the total number of particles per field x 100.

Table 19

Effect of Anti-*B. bacteriovorus*-serum on the Growth of *B. bacteriovorus* in Broth Cultures

Antiserum dilution	Concentration of *B. bacteriovorus*		Increase in numbers (logs)
	Inoculum (I) (PFU/ml)	Final (F) (PFU/ml)	
1:50	2.43×10^5	1.42×10^7	1.77
1:100	"	1.32×10^8	2.73
1:200	"	2.22×10^8	2.97
1:500	"	3.40×10^8	3.15
1:1000	"	4.16×10^8	3.23
Control (saline)	"	2.58×10^8	3.03
Control[a]	"	3.02×10^8	3.09
Control[b]	"	4.16×10^7	2.23

[a]Control contained 1:100 concentration of normal serum.

[b]Control contained 1:100 concentration of heat inactivated antiserum.

Table 20

Amount of B. bacteriovorus Growth Calculated by Using B. bacteriovorus Inoculum Populations not Bound to Antibody

Antiserum dilution	Concentration of B. bacteriovorus		Increase in numbers (logs)
	Inoculum (I)[a] (PFU/ml)	Final (F)[a] (PFU/ml)	
1:50	8.5×10^3	1.42×10^7	3.22
1:100	3.1×10^4	1.32×10^8	3.60
1:200	1.6×10^4	2.22×10^8	4.15
1:500	4.9×10^4	3.40×10^8	3.78
1:1000	4.0×10^4	4.16×10^8	4.02
Control (saline)	2.4×10^5	2.58×10^8	3.03

[a]This was calculated by multiplying the percentage of total cells not bound times the total initial population of cells. See Table 18. F refers to the final concentration of B. bacteriovorus produced in the cultures. See Table 19.

teriovorus particles were present. All controls and the culture containing a 1:1000 antiserum dilution were similar. No attachment was seen in any of the cultures after 1 hour of incubation. No changes were observed between the cultures incubated for 5 hours and those incubated for 1 hour.

Figure 9 represents the cultures 10 hours after incubation. There was attachment by the B. bacteriovorus in all cultures, and also spheroplasting of the E. coli. Spheroplasts were seen attached to the clumps of the B. bacteriovorus at the 1:50, 1:100, 1:200, and 1:500 antiserum concentrations. As the antiserum concentrations decreased, the number of B. bacteriovorus particles seen per field increased. The controls and the culture containing a 1:1000 antiserum dilution appeared similar.

After 24 hours of incubation(Figure 10), all E. coli in the saline control and the control containing 1:100 normal serum were spheroplasted. There were numerous free, motile B. bacteriovorus particles. Cultures containing 1:50, 1:100, and 1:200 antiserum concentrations showed little spheroplasting. Most of the E. coli were in rod form. Some spheroplasts observed were attached to aggregates of B. bacteriovorus. In the culture containing a 1:200 antiserum dilution, single B. bacteriovorus particles were attached to E. coli forming spheroplasts. As the antiserum concentrations decreased, the number of B. bacteriovorus particles per field increased. The control containing 1:100 heat inactivated antiserum appeared the same as the culture containing a 1:100 antiserum concentration. However, the B. bacteriovorus were not in aggregates in the latter. The cultures containing 1:500 and 1:1000

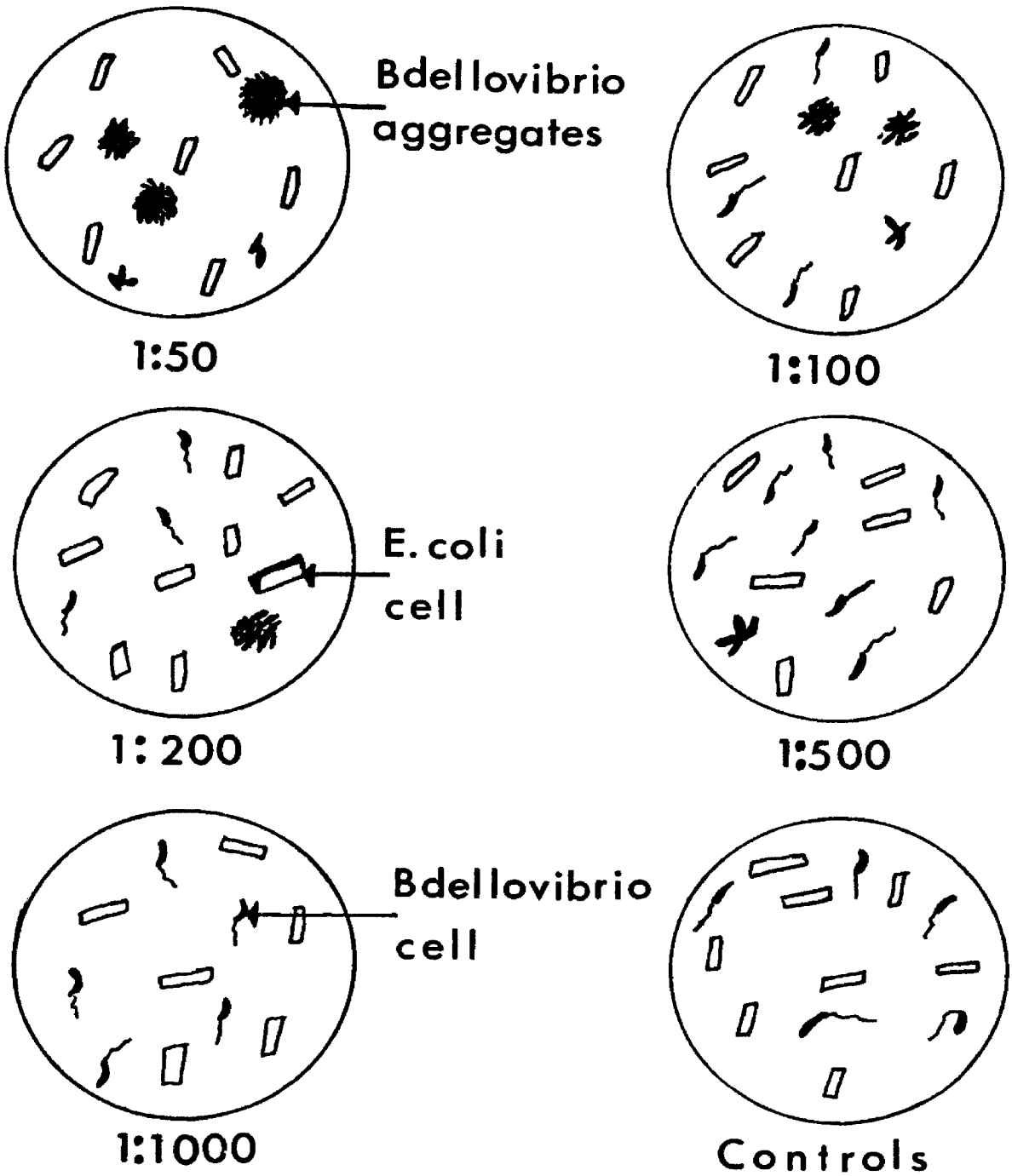

Figure 8

Schematic Diagrams of B. bacteriovorus-E. coli Cultures Exposed to Various Dilutions of Anti-B. bacteriovorus-serum for One Hour: (970 X)

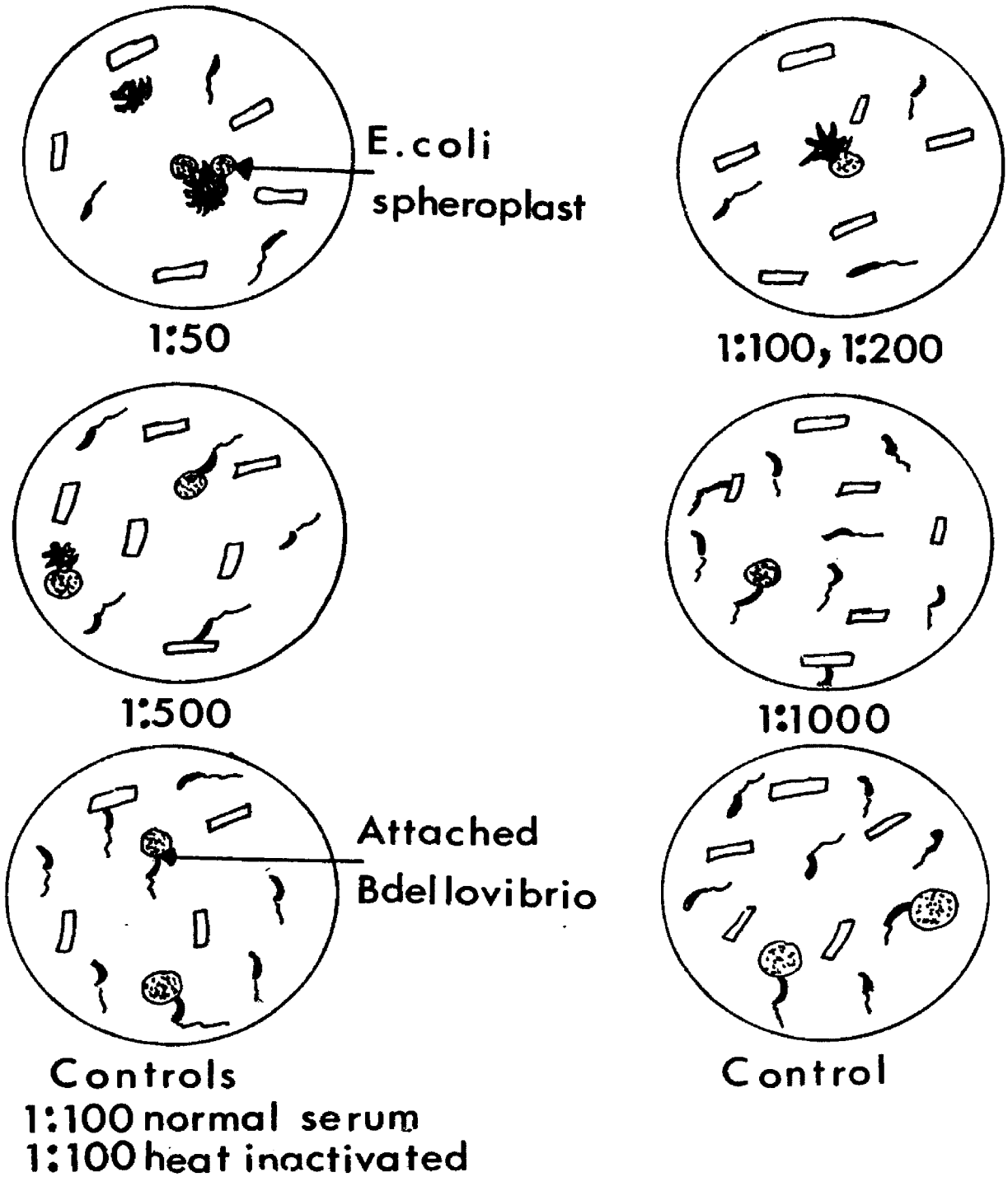

Figure 9

Schematic Diagrams of B. bacteriovorus-E. coli Cultures Exposed to Various Dilutions of Anti-B. bacteriovorus-serum for 10 Hours. (970 X)

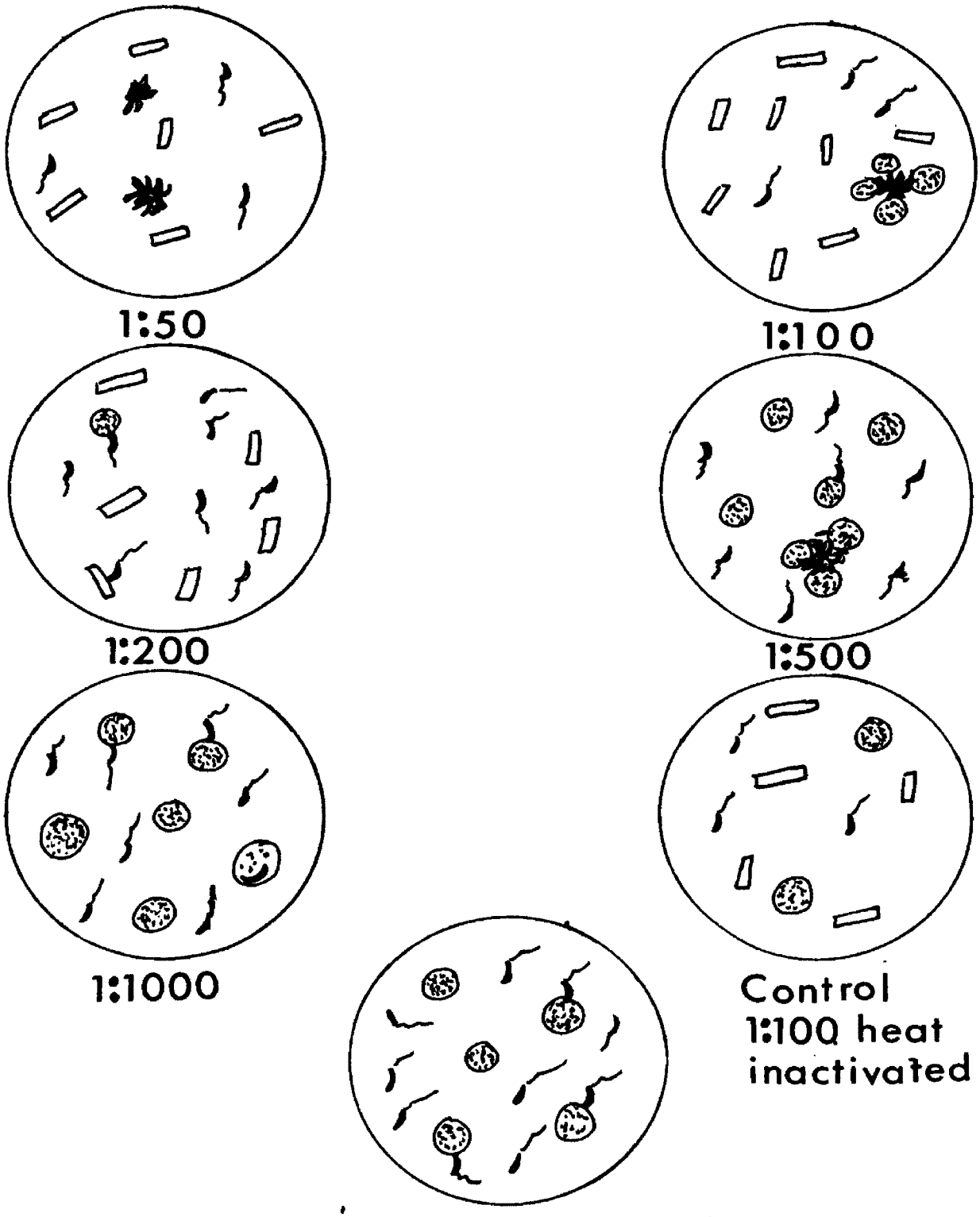

Figure 10

Schematic Diagrams of B. bacteriovorus-E. coli Cultures Exposed to Various Dilutions of Anti-B. bacteriovorus-serum for 24 Hours. (970 X)

antiserum concentrations, appeared the same as the saline control.

(11) Effect of Anti-B. bacteriovorus-serum on Plaque Formation by B. bacteriovorus

According to Table 21 there was little or no change in the number of PFU/ml formed by B. bacteriovorus compared with the saline control. The control plates containing 1:100 heat inactivated antiserum produced approximately the same number of PFU/ml as the experimental plates containing a 1:100 antiserum concentration. The only noticeable difference between the experimental plates and the controls was a qualitative one. Plaques developed in the saline controls 6 days after incubation but did not develop in the experimental plates until after 8 days of incubation. Also the size of the plaques formed in the experimental plates was smaller(approximately 1/30 of the typical plaque size).

(12) Growth of B. bacteriovorus in Cultures Containing Anti-E. coli-serum

Table 22 shows the effect of anti-E. coli-serum on B. bacteriovorus growth. There was no difference in the growth of B. bacteriovorus in cultures containing various antiserum dilutions when compared with the controls.

Figures 11 and 12 represent diagrams of the cultures of B. bacteriovorus containing anti-E. coli-serum. Figure 11 represents the cultures after four hours of incubation. E. coli are aggregated in cultures containing 1:50, 1:100, and 1:200 antiserum concentrations and 1:100 heat inactivated antiserum. The rest of the cultures contained single and paired E. coli rods. Spheroplasts of E. coli were present in all cultures with B. bacteriovorus cells attached to them.

Table 21

Effect of Anti-B. bacteriovorus-serum on Plaque Formation by B. bacteriovorus[a]

Antiserum dilution	PFU/ml
1:50	4.04×10^7
1:100	6.45×10^7
1:200	6.21×10^7
1:500	8.95×10^7
1:1000	1.55×10^7
Control(saline)	5.12×10^7
Control[b]	1.26×10^8
Control[c]	8.42×10^7

[a]Plaques in the controls developed after 6 days of incubation. Plaques did not develop on the experimental plates until after 8 days of incubation and they were 1/30 of the typical plaque size.

[b]This control contained a 1:100 normal serum concentration.

[c]This control contained a 1:100 heat inactivated antiserum concentration.

Table 22

Effect of Anti-*E. coli*-serum on the Growth of *B. bacteriovorus* in Broth Cultures

Antiserum dilution	Concentration of *B. bacteriovorus*		Growth increase (in logs)
	Inoculum (I)[a]	Final (F)[a]	
1:50	2.09×10^7	7.76×10^8	1.56
1:100	"	8.20×10^8	1.59
1:200	"	5.18×10^8	1.39
1:500	"	4.32×10^8	1.31
1:1000	"	1.03×10^9	1.69
Control (saline)	"	6.64×10^8	1.50
Control[b]	"	9.89×10^8	1.67
Control[c]	"	8.32×10^8	1.60

[a]Concentration in PFU/ml.

[b]Control contained 1:100 concentration of normal serum.

[c]Control contained 1:100 concentration of heat inactivated antiserum.

In the cultures, containing aggregates of B. bacteriovorus, spheroplasts were formed on the edge of the aggregates. B. bacteriovorus particles were seen attached to these spheroplasts.

Figure 12 represents the cultures 20 hours after incubation. Clumps of E. coli were observed in the cultures containing 1:50, 1:100, and 1:200 antiserum concentrations. Many of the E. coli in the aggregates were in spheroplast form. B. bacteriovorus particles were found to be both free and attached to spheroplasts in all cultures.

(13) Effect of Anti-E. coli-serum on Plaque Formation by B. bacteriovorus

No change, quantitative or qualitative, in plaque formation was observed in plates containing anti-E. coli-serum. The amount of PFU/ml on the experimental plates were similar to the number of PFU/ml on the control plates(see Table 23).

(14) Separation of E. coli Cell Envelopes and Whole Cells on a Ficoll Gradient

The different forms of E. coli were layered on a ficoll gradient and centrifuged for 90 minutes at 1000 x g(Figure 13). The E. coli whole cells were pelleted to the bottom of the tube. The cell envelopes formed a dispersed band in the 5, 15, and 20% layers.

(15) Cell Envelope Attachment Studies

After eight hours of incubating B. bacteriovorus with E. coli cell envelopes, 12.1% attachment occurred(Table 24). Figure 14 represents diagrams of B. bacteriovorus attached to cell envelopes that were observed with the phase microscope. Some B. bacteriovorus cells appeared to penetrate the cell envelope. The number of attached cells

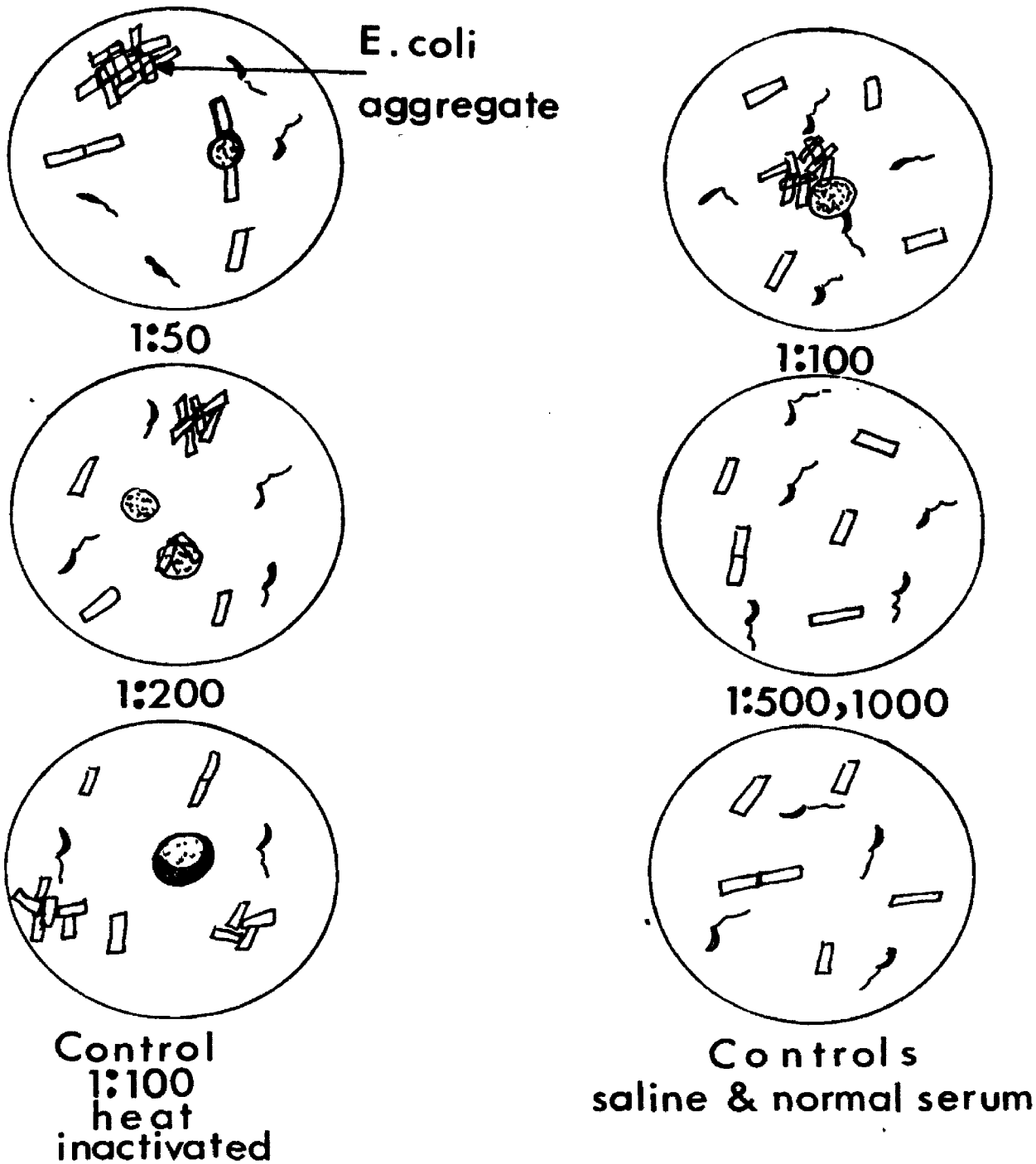

Figure 11

Schematic Diagrams of B. bacteriovorus-E. coli Cultures Exposed to Various Dilutions of Anti-E. coli-serum for 4 Hours(970 X).

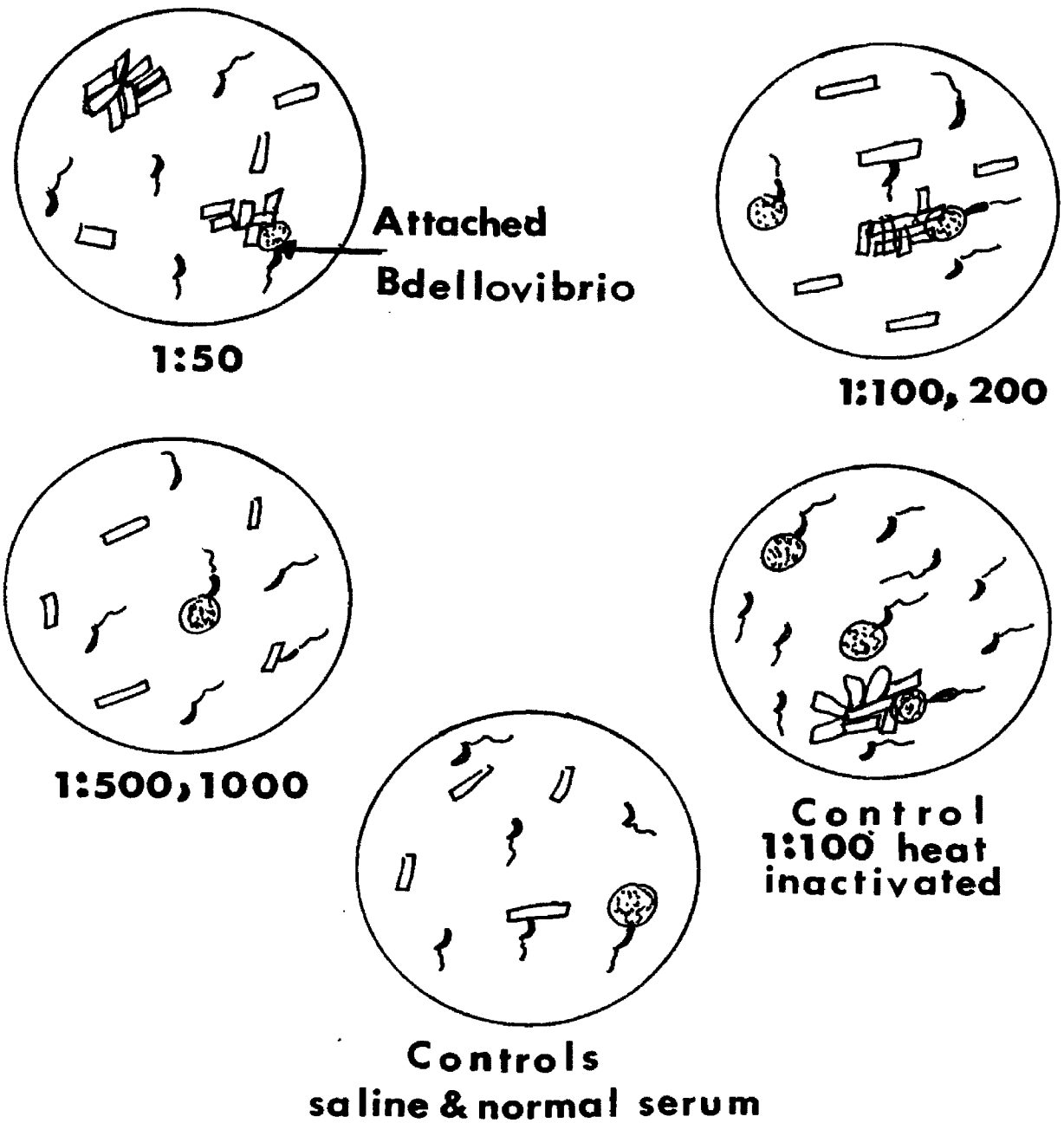

Figure 12

Schematic Diagrams of B. bacteriovorus-E. coli Cultures Exposed to Various Dilutions of Anti-E.coli-serum for 20 Hours(970 X).

Table 23

Effect of Anti-*E. coli*-serum on Plaque Formation by *B. bacteriovorus*

Dilution	PFU/ml
1:50	4.13×10^7
1:100	2.35×10^7
1:200	4.12×10^7
1:500	2.59×10^7
1:1000	2.59×10^7
Control(saline)	9.09×10^7
Control(1:100 normal serum)	1.43×10^7
Control(1:100 heat inactivated serum)	1.27×10^7

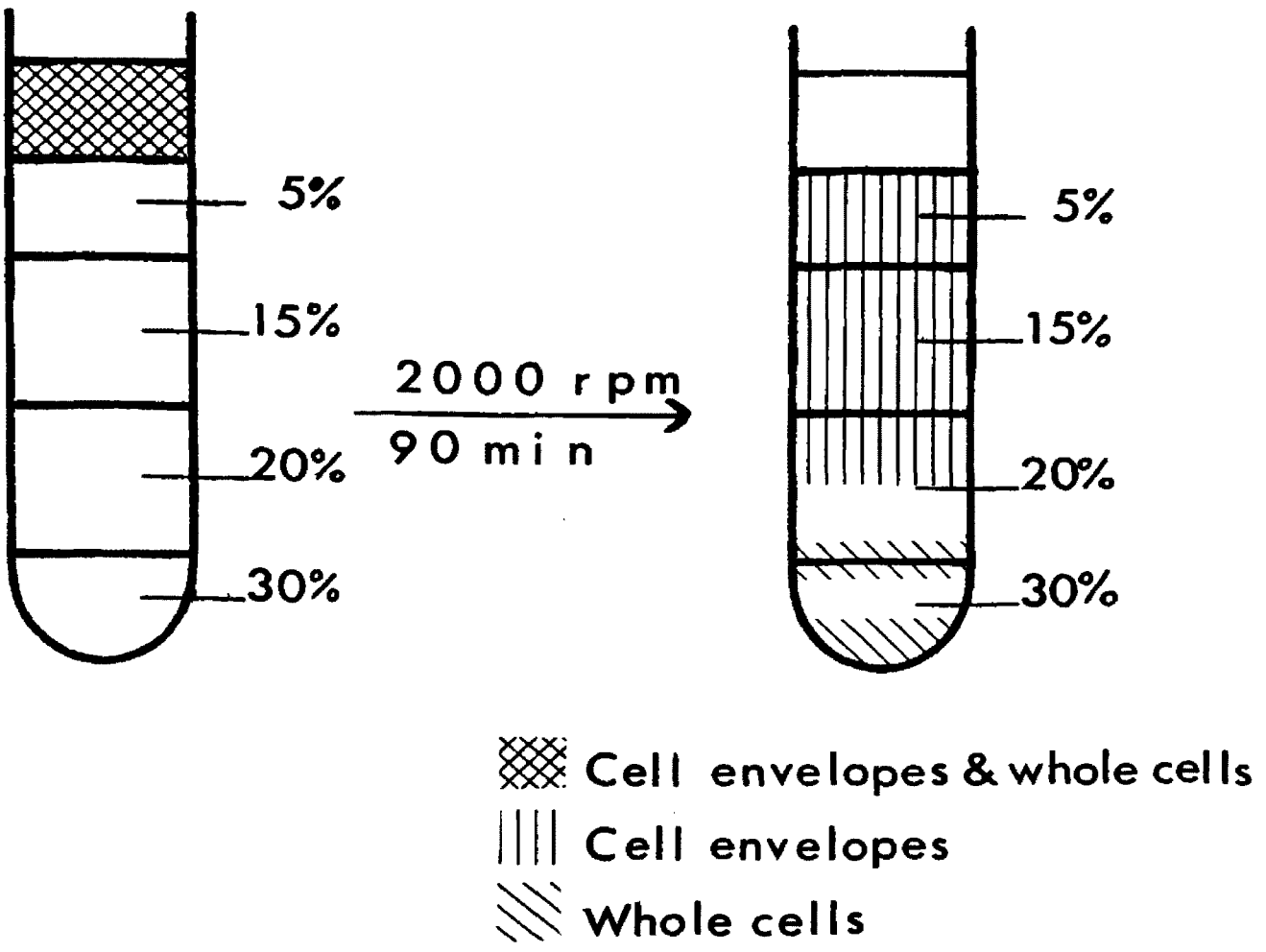

Figure 13

Separation of E. coli Cell Envelopes and Whole Cells on a Ficoll Gradient.

Table 24

Ability of B. bacteriovorus to Attach to Cell Envelopes of E. coli

Free cells[a]	PFU/ml	% Attachment[b]
T_0	7.17×10^4	0.
T_8	6.30×10^4	12.1

[a]The number of free cells were assayed, after filtration at zero time(T_0) of incubation and after 8 hours(T_8) of incubation.

[b]This was calculated by the following formula:

$$\frac{T_0(\text{PFU/ml}) - T_8(\text{PFU/ml})}{T_0(\text{PFU/ml})} \times 100 = \% \text{ Attachment.}$$

observed were not numerous.

There was a decrease(24.8%) in the number of PFU/ml of B. bacteriovorus as a result of the incubation with E. coli cell envelopes(Table 25). This percentage was determined by the following formula:

$$\frac{\text{Original inoculum(PFU/ml)} - T_8\text{(PFU/ml)}}{\text{Original inoculum(PFU/ml)}} \times 100.$$

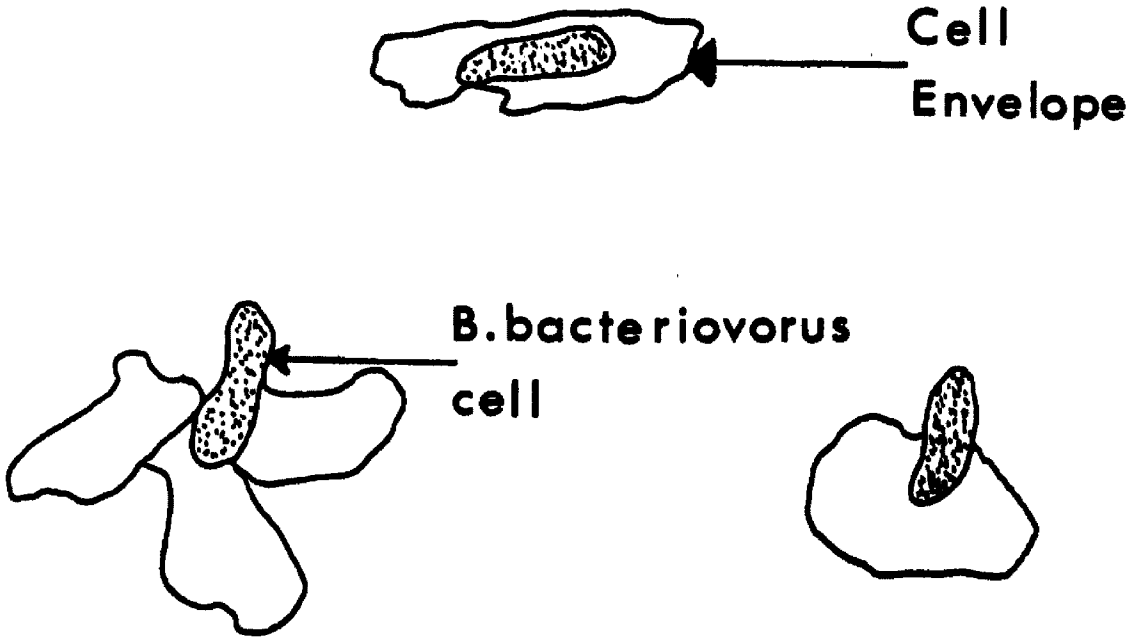

Figure 14

Schematic Diagrams of B. bacteriovorus Cells Attached to Cell Envelopes of E. coli. (970 X).

Table 25

Effect of Incubation with *E. coli* Cell Envelopes upon Plaque Fromation by *B. bacteriovorus* Cells

Population	PFU/ml
Control (inoculum)	6.98×10^4
T_8 (nonfiltered)[a]	5.25×10^4

[a] T_8 refers to the *E. coli* cell envelope and *B. bacteriovorus* culture which had incubated for eight hours.

CHAPTER IV

DISCUSSION

Seidler and Starr(20) have shown by burst size studies that the greatest burst size of B. bacteriovorus occurred when the incubation temperature was 30-35 C. Varon and Shilo(33) showed that the largest percentage of attachment of B. bacteriovorus cells also occurred at this temperature. In this study the effect of incubation temperature on the complete parasitic cycle was tested. Maximum growth occurred at 25 C. Therefore, the temperatures which were found to be optimal for the attachment and burst stages of the parasitic cycle may not be optimal for the rest of the parasitic cycle.

Seidler and Starr(33) found that no burst occurred at 42 C. In this investigation no PFU/ml could be measured in cultures of B. bacteriovorus incubated at 43 C. Conceivably, the parasites may have failed to cause bursting at this temperature and this might explain the reduction in plaque forming units seen at this temperature.

Stolp and Starr(29) found that up to 90% of the parasite population attached to the host when the ratio of host-parasite was 10:1. If more than two parasites attached to a host cell, the cell lysed and the parasitic cycle was not completed. The probability that only one parasite will attach to one host cell is greater at a host-parasite ratio of 10:1 than at a ratio of 1:1; and therefore, more growth will occur. Tests done in this study showed that increases in B. bacteriovorus growth were maximum at host-parasite ratios of 1000:1, and 10,000:1. The growth recorded at a host-parasite ratio of 10:1, however, was just slightly lower than the 1000:1 and 10,000:1 host-parasite

ratios.

B. bacteriovorus was reported to remain viable for several months at 4 and 5 C in liquid culture by Seidler and Starr(20) and Simpson and Robinson(27). Sullivan and Casida(31) reported that B. bacteriovorus survived for at least six months when maintained at -15 C in the presence of the slime from its Azotobacter host. According to studies in this investigation, B. bacteriovorus survived at -78 C with little or no loss in populations in both liquid and solid media. Since the B. bacteriovorus populations remained constant for at least four months at -78 C, it probably would remain steady for longer periods of time. ATCC stock cultures which were frozen for 9 months at -78 C were still viable when plated. Thus, this relatively simple technique is recommended as a method of preserving B. bacteriovorus stock cultures. Slant cultures could also be preserved well at -20 C.

To my knowledge, no data have been published about the effect of heat on B. bacteriovorus survival. Since B. bacteriovorus is assayed by placing 0.5 ml of the organism in 2.5 ml of molten agar kept at 47 C, the effect of heat and time exposed to heat on B. bacteriovorus is an important physical parameter to be considered. Results determined in this study indicated that the thermal death point of B. bacteriovorus was 51 C. When B. bacteriovorus was exposed to a temperature of 52 C, the thermal death time was 3-6 minutes.

Most bacterial vegetative cells are killed at temperatures of 50-70 C using moist heat. The thermal death point of B. bacteriovorus thus lies at the lower end of this spectrum. Both the nature of the medium and the number of organisms present can affect the resistance of an organism to heat. Protein can have a protective effect if it is in the

medium. If there are a large number of organisms present, this can also have a protective effect. The medium used to determine the thermal death point and death time contained 0.06% peptone. Thus, the medium probably did not provide a significant protective effect. However, E. coli organisms were also present in the suspension exposed to heat, and their presence may have increased the resistance of the B. bacteriovorus to heat.

Since the temperature of molten agar used in the double-layer technique is very near that of the thermal death point of B. bacteriovorus, one must consider the possible effects of this technique in the final PFU/ml data.

The invasiveness of B. bacteriovorus on host lawns was affected by heat as indicated by the increased time of incubation for plaques to develop and the reduced size of the plaques formed.

Studies of the growth curve of B. bacteriovorus indicated that the logarithmic growth phase began at 4 hours, the stationary phase at 9 hours, and the logarithmic death phase a 24 hours. These results corresponded to the growth curve published by Seidler and Starr(20).

The results of the parasitized E. coli growth curve were as expected. After the B. bacteriovorus reached the logarithmic growth phase, the number of E. coli began to decrease sharply. This reduction in E. coli population is a result of the lysis of these cells by the parasite. By 14 hours the parasitized E. coli curve began forming a plateau. This may be due to the presence of resistant E. coli forms, or to the fact that the B. bacteriovorus has reached the stationary phase and as many are dying as are parasitizing E. coli. This would

give the E. coli an opportunity to proliferate. Stolp and Starr(29) have reported some host populations to be completely lysed by the B. bacteriovorus. However, cultures of this strain of E. coli were never completely lysed by the parasite even when the length of incubation time was increased to 48 hours.

Shilo(25) found that relatively pure preparations of B. bacteriovorus were obtained by centrifuging a suspension of the cells on a linear ficoll gradient(1-15% w/v). Stolp and Starr(29) and Burnham, Hashimoto, and Conti(4) found that they could separate B. bacteriovorus from its host by differential filtration using Millipore filters of decreasing pore size(1.2, 0.8, 0.65, and 0.45 μm). However, when the filtration technique was tested in this study, B. bacteriovorus cells did not pass through the last pore size(0.45 μm). They were rarely recovered in the filtrate. The reason for this may be that the cultivation technique used may have produced parasite cells of larger size.

Pure cultures of B. bacteriovorus were prepared in this investigation in the following manner. The culture to be purified was always inoculated with a 24 hour culture of B. bacteriovorus stock. A young culture of the parasite caused greater lysis of the host bacteria than an older one. The culture was subjected to a combination of filtration and centrifugation on a ficoll gradient since each process alone does not produce a pure population. The filtration process removed the majority of the host cells plus cellular debris, and the centrifugation process removed the remainder of the host cells.

The effect of antibiotics on H-D strains has been studied by Varon

and Shilo(33,, 35). Since they used a live host in their studies, their technique consisted of complex procedures using sensitive and resistant forms of the host. This was done to determine whether or not the inhibition of the parasitic growth was due to the effect of the antibiotic on the host or the parasite. In this investigation autoclaved host cells were used as the source of nutrient for the H-D cells. This was done so that the host would not be a variable in the experiment; and thus, the inhibitory effect of antimicrobial agents on H-D B. bacteriovorus could be measured directly. The technique used greatly simplified the assay procedure, so that the effect of several antibiotics, with various modes of action, and antimetabolites could be tested on the H-D strain.

Streptomycin, chloromycetin, and penicillin inhibited the growth of H-D B. bacteriovorus. The results are similar to those published by Varon and Shilo(33, 35) except that higher concentrations of penicillin(500 units/ml) were necessary to inhibit growth than they recorded(100 units/ml). Since B. bacteriovorus is a gram-negative organism, it would seem probable that high concentrations of penicillin would be necessary to kill it. In addition, actinomycin and aureomycin inhibited the growth of the parasites. Polymyxin produced inhibition at a concentration of 1000 units/ml. The H-D forms of B. bacteriovorus were resistant to all the sulfonamides tested. One explanation for this is that folic acid is not a necessary growth factor. Another possibility is that metabolites that require folic acid coenzymes, are supplied directly to the parasite by the host. H-D B. bacteriovorus was mainly inhibited by antimicrobial agents which affect synthesis

of proteins, cell walls, and nucleic acids.

The effect of several antibiotics and antimetabolites on H-I B. bacteriovorus growth has been studied by Seidler and Starr(21). These are listed on Table 2, page 8. The strain used in this investigation was resistant to polymyxin and sensitive to sulfa methoxypyridazine and triple sulfa. These data differ from those obtained by Seidler and Starr. In general, this H-I strain was susceptible to sulfonamides, actinomycin, and penicillin; and was resistant to antibiotics affecting protein synthesis(aureomycin and streptomycin). Chloromycetin, which also affects protein synthesis, gave varied results depending on the technique used for assaying the antibiotics. The strains tested by Seidler and Starr were resistant to sulfonamides and penicillin; and susceptible to antibiotics affecting the cytoplasmic membrane and protein synthesis. The difference in the results recorded may have been due to a difference in strains used or the techniques employed in assaying the agents.

The H-I strain in comparision to the H-D strain is susceptible to sulfonamides. Thus, it must require folic acid as a growth factor for synthesis of compounds necessary for cell metabolism.

Studies of B. bacteriovorus in soil(12), sewage(7), and marine environments(13) have been done. Several investigators have indicated that B. bacteriovorus plays an important role in the biological food chain by eliminating or controlling populations of E. coli and other enteric bacteria in various ecosystems(25, 29). Many bacteria which are inhabitants of the soil are also present as a part of the normal intestinal flora of vertebrates. Thus, the existence of B.

bacteriovorus in vertebrate intestinal tracts, where the enteric bacteria are plentiful, is highly probable. The parasite could play a role in maintaining static conditions of the normal flora in the intestine. The parasite could also play a natural therapeutic role in the intestinal tract by acting on Salmonella and Shigella which are agents of enteric diseases in man and animals. Studies dealing with infection of experimental animals and subsequent feeding of B. bacteriovorus to determine whether infection can be alleviated might yield interesting results.

The investigation of human and animal fecal material for the presence of B. bacteriovorus has not been reported. In this study B. bacteriovorus could not be isolated from flora of humans, rabbits, dogs, and mice. The inability to detect B. bacteriovorus in the fecal material could have been due to isolation techniques, sample size, or presence of inhibitors. Further studies should be done to see if additional studies of mammals are warranted.

Fecal isolates of amoeba which lysed E. coli are possibly members of the Genera Acanthamoeba, Naegleria, and Hartmannella(2). These amoebas are found widely distributed in soil and feed on bacteria. They grow well on agar utilizing bacteria as food.

The nature of B. bacteriovorus attachment and host specificity is a mystery which remains to be resolved. Since B. bacteriovorus attach mostly to gram negative bacteria, Shilo(25) felt that the existence of a common lipopolysaccharide core was the key to the nature of host specificity. Although the physical impact of the parasites may play a large role in their attachment, receptor sites and/or surface charges

may attract the B. bacteriovorus cells. These receptor sites may be similar to bacteriophage receptor sites on the surface or within the bacterial cell wall. The somatic(O) antigen serves as a phage receptor on some gram negative bacteria. Varon and Shilo(34) used mutant strains of E. coli and Salmonella which lacked the O-specific antigen. They found that these mutant rough strains were better receptors for the B. bacteriovorus than the smooth strains. They concluded the receptors for attachment, if any, seemed to lie in the inner cell wall of the host bacteria.

Varon and Shilo determined the following factors about the attachment process(33). Ninety per cent of the B. bacteriovorus population attached within twenty minutes from the time they were mixed with the host. After this time the attachment was irreversible, as determined by agitaton treatment. They felt that physical impact of the parasite collision may be a prerequisite for attachment. It has been postulated that proteolytic enzymes have a role in the invasion process and not in the attachment process(33).

To further characterize the nature of host specificity in the host-parasite interaction, serological studies were conducted in this investigation. They were done to determine if agglutinating antibodies specific for B. bacteriovorus cells or E. coli cells would affect B. bacteriovorus growth in any way, particularly concerning its attachment capabilities. Several factors were hypothesized relative to the effect of anti-B. bacteriovorus-serum: it would cause clumping and thus make parasitism a physical impossibility, it would affect the motility of the parasite, and would possibly coat the polar end of the

B. bacteriovorus which would alter its surface charge and thus attraction to the host. The polar end is the end of the parasite which attaches to the host cells. The anti-E. coli-serum was used to possibly bind agglutinating antigens and other antigens on the surface of the host cell wall so that B. bacteriovorus receptor sites would be blocked; and hopefully, to coat E. coli cells, alter their surface charges, and affect the attraction of the parasite to the host cell.

The results showed that anti-B. bacteriovorus-serum inhibited B. bacteriovorus growth at antiserum concentrations of 1:50, 1:100, and 1:200. The control which contained the antiserum, heat inactivated to remove complement, showed the same results as its experimental counterpart. Thus complement did not function in inhibition of growth of the parasite. The indirect fluorescent antibody results indicated that not all B. bacteriovorus cells were bound to antibody. Thus the increases in growth seen could, for the most part, be attributed to the inoculum population of B. bacteriovorus not bound to antibody. Since the increases in growth due to inoculum populations as seen in Table 20, page 63 are somewhat higher than the proportionate increase of the control, some of the bound cells were probably able to parasitize E. coli. The reasons for the inhibition in growth probably was due to the clumping of the B. bacteriovorus cells and the reduction in their motility rather than an alteration of their charge reducing their attraction to host cells. This is concluded since single B. bacteriovorus particles which fluoresced, under the fluorescent microscope, lacked motility. Also, clumps were observed, when samples were examined by both fluorescent and phase microscopy. Anti-B. bacteriovorus-serum did

not affect the number of PFU/ml. This may have been due to the longer incubation period, and the type of medium used. However, the invasiveness of the parasite was affected as seen in the reduced size of the plaques when compared to typical plaque size. This again may be due to the effect of the antibody on the parasites motility and the physical blockage by clumping.

The surface charge of the B. bacteriovorus cell was probably not altered by the antibody. Furthermore, it appears that the surface charge does not play a role in attachment. The reason for this speculation is that clumps of B. bacteriovorus were seen with E. coli spheroplasts attached to their peripherary.

Anti-E. coli-serum had no effect on the growth of B. bacteriovorus nor plaque formation by B. bacteriovorus. The ineffectiveness of antibody may have been due to the following factors: the antibody did not tie up B. bacteriovorus receptor sites; there are no receptor sites; surface charges were not altered; surface charges do not play a role in B. bacteriovorus attachment.

According to the results from both experiments I concluded the following: attachment depends on motility; damage to the host is due to the collision process. If receptor sites are present, they are probably located in the inner cell wall and thus not masked by antibody.

Experiments dealing with attachment of B. bacteriovorus to E. coli cell envelopes were not conducted in great depth or detail. Therefore, additional experiments need to be performed in this area to produce more conclusive results.

In my preliminary experiments 12.1% attachment of the B. bacterio-

vorus to cell envelopes occurred after 8 hours. Using whole E. coli cells Varon and Shilo(33) reported that there was a 90% attachment of the parasite after 20 minutes of incubation. It is possible that B. bacteriovorus attaches more readily to whole cells than to cell envelopes. When samples of the B. bacteriovorus-cell envelope culture were examined, few attached B. bacteriovorus cells were found.

The attachment of B. bacteriovorus to cell envelopes was probably irreversible and thus resulted in the death of B. bacteriovorus cells, because there was 24.8% decrease in the number of B. bacteriovorus that produced plaques.

Successive stripping of the layers of the cell envelope should be performed to determine which, if any, of the layers contain specific receptor sites for the B. bacteriovorus. These studies could be performed with the electron microscope. This would help further clarify the nature of the host specificity of the gram negative bacteria.

In this investigation contributions were made to the basic biology of B. bacteriovorus. However, studies are necessary to further characterize this unique host-parasite relationship such as comparative serological studies which would help determine if there are different strains or possibly different species within the genus Bdellovibrio. Also, more biochemical studies are necessary to determine the nature of the dependence on nutrients supplied by the host cell. Genetic studies would be warranted, in addition, to analyze the nature of the H-I forms as compared to the H-D forms of B. bacteriovorus.

CHAPTER V

SUMMARY

1. The optimum incubation temperature for the complete parasitic cycle was found to be 25 C. Growth of B. bacteriovorus was maximum when the ratio of host-parasite was 1000:1 or 10,000:1.

2. B. bacteriovorus survived well at -78 C with little reduction in population size. Thus, this would be a good method of preserving stock cultures of the organism.

3. The thermal death point of B. bacteriovorus was 51 C. The thermal death time was 3-6 minutes at 52 C.

4. Exposure of B. bacteriovorus cells to temperatures of 49 and 52 C produced the following effects on plaque formation: the size of the plaques formed was reduced, and the length of the incubation time necessary for plaques to appear increased.

5. Pure preparations of B. bacteriovorus were obtained by a combined filtration-centrifugation technique.

6. H-D B. bacteriovorus was mainly inhibited by antimicrobial agents which affect synthesis of proteins, cell walls, and nucleic acids. It was resistant to sulfonamides. H-I B. bacteriovorus was susceptible to sulfonamides, actinomycin, and penicillin; and the resistant to antibiotics affecting protein synthesis.

7. B. bacteriovorus could not be isolated from the flora of humans, rabbits, dogs, and mice.

8. Anti-B. bacteriovorus-serum inhibits the growth of B. bacteriovorus in broth cultures. It reduces the invasiveness of the parasite on host

lawns by reducing plaque size to 1/30 typical plaque size and by increasing the time necessary for plaques to appear.

9. Anti-*E. coli*-serum does not inhibit the growth of *B. bacteriovorus* nor does it affect plaque formation by the parasite.

10. Preliminary experiments showed that 12.1% attachment of *B. bacteriovorus* to cell envelopes occurred after 8 hours. There was a 24.8% decrease in the number of *B. bacteriovorus* that produced plaques after being incubated with *E. coli* cell envelopes.

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