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Parasitization Of Spirillum Serpens By Bdellovibrio Bacteriovirus

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PARASITIZATION OF SPIRILLUM SERPENS

BY

BDELLOVIBRIO BACTERIOVORUS

by

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ABSTRACT

A Gram-negative bacterium, Bdellovibrio bacteriovorus strain 6-5-S which parasitizes other Gram-negative bacteria was isolated from a sample of sewage collected at London, Ontario. This organism is an obligate bacterial parasite since it develops only in the presence of susceptible host cells.

Five stages in the growth cycle of Bdellovibrio 6-5-S in Spirillum serpens VHL have been illustrated microscopically: (1) attachment of the parasite to the host cell; (2) penetration of the host cell wall by the parasite; (3) change of the morphology of the living host; (4) development of the parasite in the host cell; and (5) release of mature parasite progeny.

In the early stage of attachment of Bdellovibrio to S. serpens, a bulge sometimes develops on the cell wall of the host after contact between the two organisms. A combination of factors, including damage to the host cell wall and turgor pressure were likely contributes to the development of the bulge.

The parasite penetrates the host cell wall and develops intracellularly by thickening and elongation to form a ribbon-like structure. The ribbon, upon maturation, segments into individual parasite units,

presumably by constriction. Release of Bdellovibrio progeny and fragmentation of the host cell occur simultaneously.

Bdellovibrio requires cations, such as Ca^{++} and Mg^{++}, for parasitization of S. serpens VHL. The cations very likely facilitate neither the attachment of Bdellovibrio to nor the invasion of the host cell by the parasite. The growth of Bdellovibrio in heated or untreated cells of S. serpens VHL is accompanied by a loss of turbidity of the two-membered culture and a release into the medium of UV-absorbing materials.

The host range of Bdellovibrio 6-5-S was assessed directly (1) by determining the increase in the number of Bdellovibrio in the presence of a potential host organism suspended in broth or buffer; (2) by the ability of the parasite to form plaques or confluent lysis in semi-solid agar seeded with a lawn of the test organism. The host range was also assessed indirectly by determining the loss of turbidity of the two-membered culture established in broth or in buffer. No relationship between the results obtained by any of the methods tested was evident. Washed cell suspensions of the potential host culture in buffer and inoculated with Bdellovibrio gave a consistently wide host spectrum.

The structured layer on the cell wall of S. serpens MW1 and MW11 appears to protect the host cells from parasitization by Bdellovibrio. Removal of the structured layer by treatment of host cells with EDTA and sodium lauryl sulfate and by heating rendered them susceptible to parasitization.

During parasitization of S. serpens by Bdellovibrio the latter produced lytic enzyme(s) which degraded the host cell with the consequent release into the suspending medium of soluble reducing sugars and amino sugars. A crude enzyme preparation from Bdellovibrio lysate dissolved cells of Micrococcus lysodeikticus. Amino sugar was not detected in the culture supernatant during attachment to and penetration of the host cell by the parasite but was detected following conversion of the Spirillum from the normal to the spheroplast form and also after its eventual disruption.

INTRODUCTION

In 1962, Stolp and Petzold found a new bacterium which parasitized and lysed susceptible bacterial cultures. Stolp and Starr (1963) further characterized the organism and named it Bdellovibrio bacteriovorus ("Bdello" in Greek means "leech"; vibrio represents the comma shape of the parasite; bacteriovorus means bacterial eater).

The parasitic strains of B. bacteriovorus are widely distributed in nature and may be important in the ecology of microbial populations including that of animal and plant pathogens. It is reasonable to assume that additional information about microbial interrelationships in general could be obtained if host-parasite relationships were better understood. The studies of host-parasite relationships of host-dependent protista, including malaria (Trager, 1960; Moulder, 1962), rusts (Shaw, 1963), other fungi (Barnett, 1963), Mycobacterium (Hanks, 1966), rickettsiae and Psittacine bacteria (Moulder, 1962, 1964, 1966), and bacteriophage (Adams, 1959) are all pertinent to this aim. B. bacteriovorus merits extensive study because it is the only bacterium known to parasitize other bacteria.

Little information on the interaction between B. bacteriovorus and its susceptible bacteria was available in September, 1965, when

the present study was initiated. Prior to that time, investigations were directed toward identification and characterization of isolates mainly for descriptive purposes. Electron microscopy revealed the superficial morphology of B. bacteriovorus and the attachment of the parasite to the host cell. Phase-contrast micrographs provided no additional information. Bdellovibrio-induced bacterial lysis was noted, but its mechanism was not investigated.

The present study was undertaken to establish the steps involved in the multiplication of parasitic B. bacteriovorus by observations of cell preparations by microscopy and to determine the specific biological and environmental factors essential for establishment of parasitization. The part played by the bacterial cell wall in susceptibility to parasitization by B. bacteriovorus and the production of lytic enzyme(s) by B. bacteriovorus during parasitization of Spirillum serpens were considered.

HISTORICAL REVIEW

Bdellovibrio bacteriovorus was accidentally discovered by Stolp and Petzold (1962) when they were screening soil samples for bacteriophages active against plant pathogens.

B. bacteriovorus resembles bacteriophages in its ability to lyse host bacteria. The bacteriolysis is characterized by the clearing of host bacteria in broth culture, and by the development of plaques or confluent lysis in a lawn of host bacteria in solid media. The lysis was also assessed microscopically (Stolp and Starr, 1963). Stolp and Petzold (1962) were able to differentiate B. bacteriovorus from bacteriophages by the rate of plaque development and the manner of its spreading. Phage plaques usually appear within 24 hr, while B. bacteriovorus plaques develop after two to three days and enlarge with continued incubation.

Three reasons why B. bacteriovorus escaped early discovery may be cited: (1) Phage isolation plates were discarded as negative before B. bacteriovorus plaques could develop; (2) Late development of the plaques was attributed to contamination; and (3) Conventional methods for isolation and identification of new bacterial species depend on the isolation of pure cultures. B. bacteriovorus, however, could not

be found by this technique since it grows only in the presence of a host cell.

B. bacteriovorus is widely distributed in nature. It is found in sewage (Stolp and Starr, 1963; Wood and Hirsch, 1966; Dias and Bhat, 1965; Scherff, DeVay and Carroll, 1966; Huang, Robinson and Murray, 1966; Guélin, Lepine and Lamblin, 1967; Burger, Drews, and Ladwig, 1968), in river water (Guélin, et al, 1967), in sea water (Mitchell, Yankofsky and Jannasch, 1967; Wood and Hirsch, 1966; Shilo, 1966) and in soil (Stolp and Petzold, 1962; Stolp and Starr, 1963; Wood and Hirsch, 1966; Klein, Dennis and Casida, 1966; Sullivan and Casida, 1968).

Klein and Casida (1967) found that the number of Bdellovibrio varied between 100 to 100,000/g of soil; Stolp and Starr (1963) found 40 to 50 cells of Bdellovibrio in a gram of soil or a milliliter of sea or pond water in California. Dias and Bhat (1965) found that the Bdellovibrio content varied between 10 and 1000 cells/ml of Indian sewage. Guélin, et al (1967) found 100,000 cells of Bdellovibrio in a milliliter of sewage from France.

The importance of Bdellovibrio in the biological control of pathogenic microflora in nature was suggested by Stolp and Petzold (1962) and Stolp and Starr (1963). This has been confirmed in river water (Guélin, et al, 1967), and in sea water (Mitchell, et al, 1967), and in soil (Klein and Casida, 1967).

Isolation of B. bacteriovorus

Stolp and Petzold (1962) isolated Bdellovibrio from a soil suspension after centrifugation followed by successive filtration of the suspension through a set of membrane filters with pore sizes 3μ to 0.45μ. The filtrate from the 0.45μ filter was plated with prospective host cells in semi-solid agar using the double agar-layer technique (Adams, 1959). Enrichment of the Bdellovibrio population with susceptible bacteria was unnecessary (Stolp and Starr, 1963). The culture thus obtained was subsequently purified three times by the single plaque isolation technique. Varon and Shilo (1968) developed a sucrose gradient centrifugation technique which allowed a clear and quick separation of Bdellovibrio from other bacteria. The culture was checked microscopically for the presence of Bdellovibrio. The strains of B. bacteriovorus isolated and studied are listed with appropriate references in Appendix I.

Culture media (see Appendix II)

Stolp and Petzold (1962) isolated Bdellovibrio in nutrient broth (N.B-A) using the double agar-layer technique. The top and bottom layers contained in addition to N.B-A broth, 0.9% and 1.8% Bacto-agar, respectively. Another nutrient broth (N.B-HP) was also used to cultivate obligate and saprophytic strains of Bdellovibrio. Sullivan and Casida (1968) used the Campbell-Hoffer medium (1943) or a modification of Brown's medium (1962) to cultivate their host and

parasite. Burger, et al (1968) grew Bdellovibrio in bacteria cultivated in five different culture media.

Shilo and Bruff (1966) found that a ten-fold dilution of the N. B-A (Stolp and Petzold, 1962) or a four-fold dilution of the yeast peptone (YP) medium (Stolp and Starr, 1963) were most satisfactory. The YP broth was found most widely employed (Mitchell, et al, 1967; Klein and Casida, 1967; Dias and Bhat, 1965).

Starr and Baigent (1966) incorporated THAM (tris-(hydroxy-methyl) aminomethane) buffer (0.05M, pH = 7.5) into the YP broth to obtain a constant pH. Scherff, et al (1966) cultivated host bacteria separately in a complex medium and then grew Bdellovibrio in distilled water containing host bacteria to avoid physiological change of the culture medium. Guélin, et al (1967) employed the same concept but suspended host bacteria in distilled water containing salts (KCl, $CaCl_2$, MgSO4 and NaCl, pH = 7.2).

The survey of culture media leads to the conclusion that the complex media used by many have some limitations in connection with the metabolism of the host bacterium and the accumulation of products possibly inhibitory for the parasite. These problems could be avoided by using living bacteria in the absence of any nutrient.

Maintenance of cultures

Burger, et al (1968) found that the number of Bdellovibrio decreased from 10^8/ml to 10^1/ml when kept as a lysate for 36 days. Stolp and Starr (1963) maintained their Bdellovibrio isolates by

depositing a drop of fresh Bdellovibrio lysate on the top layer of YP agar containing host bacteria in a flask. Subculture was made monthly. Lyophilization of Bdellovibrio lysate in skim milk proved satisfactory. The viability of the parasite preserved in milk is influenced by the composition of the medium employed for propagation of host and parasite. Media which limited growth of the host bacterium usually gave maximal recovery of the Bdellovibrio. Sullivan and Casida (1968) reported that slime produced by Azotobacter preserved Bdellovibrio in the frozen state.

General morphology

Bdellovibrio is a small curved gram-negative rod, 0.3-0.4μ wide and 0.8-1.2μ long (Stolp and Petzold, 1962). Vibrios of slightly different proportions have also been described. The variation depends on the stage in the life cycle at which the cells are measured (Starr and Baigent, 1966). The Bdellovibrio possesses an extremely thick flagellum which consists of a sheath (28mμ) and a core (18mμ) (Murray, 1964; Huang, et al, 1966; Abram and Shilo, 1967; Shilo, 1966; Seidler and Starr, 1968a; Burger, et al, 1968). The sheath which is continuous with the cell wall may swell, break and separate from the core in the presence of 6M urea (Seidler and Starr, 1968a).

Typical appendages or extrusions were frequently observed in phosphotungustic acid (PTA) preparations in the electron microscope (Huang, et al, 1966; Shilo, 1966; Abram and Shilo, 1967). Shilo (1966)

found a fine filament, 45-55Å in diameter and 0.8μ in length, located at the polar end distal to the flagellum. These structures may be artifacts formed during fixation or staining. Murray (1968) noted that zinc ions in the fixative for Bdellovibrio were required for the preservation of the cell wall and the cell membrane. In the absence of zinc, the cell wall may become loosened.

Shilo (1966) and Burnham, Hashimoto and Conti, (1968) found a hold fast at the anterior end of some Bdellovibrio. Scherff, et al (1966) suggested that this structure facilitates penetration of Bdellovibrio into the host cell. However, Starr and Baigent (1966) were unable to find such a structure in their preparations.

Burger, et al (1968) observed that in the lysed host cells of Rhodospirillum rubrum, some Bdellovibrio were encysted. The morphology and the fine structure of these cells are entirely different from those of normal virulent strains.

Life cycle of B. bacteriovorus

Stolp and Petzold (1962) observed bacteriolysis of Xanthomonas and Pseudomonas in the presence of Bdellovibrio using phase-contrast microscopy and shadowed preparations in the electron microscope. They found that the parasite attached to the host cell surface within a few minutes after establishment of the two-membered culture. The host cells subsequently lysed. These observations led Stolp and Starr (1963) to describe Bdellovibrio as a predatory, ectoparasitic and bacteriolytic microorganism.

Stolp and Starr (1963) noted formation of spheroplasts after establishment of the two-membered culture. The sequential intermediate steps between attachment and bacteriolysis, however, were not discovered.

Huang, et al (1966), Starr and Baigent (1966), and Scherff, et al (1966) reported simultaneously that several distinct steps are involved in this particular host-parasite interaction. These steps may be described as follows: (1) attachment of Bdellovibrio to its host cell, (2) invasion of the host cell by the parasite, (3) rounding up or other morphological change of the host cell, (4) multiplication of the parasite within its host, and (5) disruption of the host cell and release of Bdellovibrio progeny.

These observations definitely show that Bdellovibrio is an endoparasite, not an ectoparasite as described by Stolp and Starr (1963), which has a distinctive parasite life cycle similar to that of other parasites of protista.

Abram and Shilo (1967) observed two distinctive types of interaction in the two-membered culture: (1) multiple attachment which led to rapid disintegration of the susceptible host cell by "lysis-from-without," and (2) penetration by a single parasite resulted in intracellular reproduction. This finding may account for the discrepancy between the observations made by the earlier workers and the more recent ones.

Recently released Bdellovibrio have a short generation time (1 hr. according to Scherff, et al, 1966), while cells from inocula that had been stored have a much longer generation time.

Attachment

Active attachment of Bdellovibrio to the host cell occurs in seconds. Bdellovibrio hits a host cell with force and pushes it for several cell-lengths according to Stolp and Starr (1963). These workers suggested that the impact may be necessary for successful attachment. As against this, Wood and Hirsch (1966) suggested that attachment of Bdellovibrio to Hyphomicrobium is due to physical forces of attraction.

Starr and Baigent (1966) found that in the early stages of parasitization attachment is reversible. Infection of a host cell by several Bdellovibrio has been noted (Stolp and Starr, 1963; Stolp, 1967; Scherff, et al, 1967; Shilo and Bruff, 1965) and this depends upon the number of host and parasite cells in the two-membered culture (Stolp, 1964).

More Bdellovibrio became attached to host cells when one of the former were added to 10 of the latter than under any other conditions tested (Varon and Shilo, 1968). This proportion of parasite to host is equivalent to a multiplicity of infection (M.O.I.) of 0.1. Unattached and attached Bdellovibrio in this case were enumerated after differential filtration of the two-membered culture.

Many other physiological factors are known to affect the attachment of Bdellovibrio to the host cell in addition to the M.O.I. including

composition and pH of the medium, and incubation temperature (Varon and Shilo, 1968). The following inhibitors of motility of Bdellovibrio prevented attachment: chelating agents, NaN_3, low pH and anaerobiosis. Ultra-violet-killed host cells retained their attractiveness for attachment of Bdellovibrio, while heat-killed cells completely lost this property. Bdellovibrio does not attach to gram-positive bacteria (Stolp and Starr, 1963).

Generally the non-flagellated end of the parasite attaches to the host cell (Stolp and Petzold, 1962). Murray (1964), however, observed that the opposite can occur.

Stolp and Starr (1963) hypothesized that actual collision netween parasite and host resulted in physical damage to the latter which was a prerequisite for attachment to and lysis of the host cell wall. It has been suggested that possible drilling action of the motile Bdellovibrio against the host cell is a necessary factor which leads to mechanical damage, attachment and eventually lysis of the host cell wall. Observations of Varon and Shilo (1968) and Burnham, et al (1968) seem to support this hypothesis. Shilo (1966) favored this concept primarily because he was unable to detect cell wall lytic enzymes other than protease in the Bdellovibrio lysate.

Starr and Baigent (1966) postulated that host and parasite were held together by a strong "surface bonding" since a device which could explain the physiological attachment is lacking. Burnham, et al (1968)

also postulated that enzymes for Bdellovibrio possibly associated with the anterior mesosome, serve to release the attachment bonds of the parasite. Wood and Hirsch (1966) suggested that enzymatic host-parasite interaction, which does not involve attachment to the host cell, exists, since Bdellovibrio attached non-specifically to glass surfaces or debris.

Abram and Shilo (1967) showed the existence of "lysis-from-without" in cultures containing B. bacteriovorus and Escherichia coli or Pseudomonas. This implies that an enzyme is produced by the Bdellovibrio which can degrade the host cell wall. Scherff, et al (1966) favored this hypothesis since they could not find host cell debris in the Bdellovibrio lysate.

Burnham, et al (1968) observed bulge formation in the host cell wall as a result of the collision and attachment of the parasite. Stolp and Petzold (1962) suggested that Bdellovibrio may possess a suction disc, like that possessed by a leech. Similarly, Scherff, et al (1966) found that Bdellovibrio attached to the host cell wall by a small infection cushion. Guélin, et al (1967) reported that bulge formation during the early stages of attachement was induced by the internal pressure of the host cell.

Penetration

Burger, et al (1968) reported that Bdellovibrio breached the host cell wall within 3 to 20 min. and completed the penetration of the host cell in 10 to 60 min.

Varon and Shilo (1968) measured the penetration of Bdellovibrio into the host cell by preparing C^{14}-labelled Bdellovibrio and determining radioactivity in the host cell after severe mechanical agitation. These workers found that penetration is completely blocked by inhibitors of protein synthesis such as streptomycin, puromycin and chloramphenicol. They interpreted their data as suggesting that inducible enzymes have to be formed by the parasite before it is able to penetrate the host cell. This induction requires a direct contact between Bdellovibrio and its host cell or between Bdellovibrio and its inducers. The inducer is masked by the host cell wall and is exposed to the Bdellovibrio only after attachment of the latter. It would be of interest to see whether the inducible enzymes are formed in the system containing disrupted host cell and parasite.

The mechanism for the formation of a pore in a host cell wall is not immediately clear. Stolp and Starr (1963) postulated that the violent collision of the parasite with the host cell and the subsequent rotation of the parasite are responsible for pore formation. Guélin, et al (1967) and Burnham, et al (1968) supported this concept since the mucopeptide,

which is responsible for rigidity of the cell wall during the early stages of the host-parasite interaction, was observed unchanged in electron micrographs.

Burnham, et al (1968) postulated that Bdellovibrio penetrated through a bulge preformed on the host cell wall. At the point of attachment, the Bdellovibrio flattened against its host cell and became constricted at the center of the bulge. The Bdellovibrio then pierced the host cell wall to make a hole. Further constriction of the Bdellovibrio was observed as it advanced into the host cell. This may be described as an intimate "lock and key" interaction at the macromolecular level. Thus, Bdellovibrio after primary penetration is not readily separated from the host cell by a violent shaking or mixing (Burnham, et al, 1968).

Scherff, et al (1966) reported that the infection cushion which is an integral part of the parasite becomes enlarged and it is this part of the parasite which penetrates the host cell wall first. Bdellovibrio pushes into the host cell. Once inside its host cell wall, the Bdellovibrio is located between the cytoplasmic membrane and the cell wall where it develops and produces vacuolated areas. Scherff, et al (1966) observed that as many as six Bdellovibrio entered and reproduced within a host cell.

A single mesosome is always observed (Scherff, et al, 1966; Burnham, et al, 1968) near the infection cushion or hold fast of the Bdellovibrio. The function of the mesosome in penetration is not yet clear.

Intracellular multiplication

Stolp and Starr (1963) suggested that Bdellovibrio multiplied by binary fission. Guelin, et al (1967) reported that cell division started with elongation and then binary fission.

Scherff, et al (1966), with two dimensional pictures, concluded that Bdellovibrio grew by forming a long spiral structure within the host cell which then constricted to produce individual cells which were capable of rapid movement within the host cell. Separation of the individual parasite units which constitute the intracellular spiral form is presumably facilitated by the motility of the Bdellovibrio. Growth was observed to occur from both ends of the intracellular Bdellovibrio. The end which entered the host cell usually was the first to grow. The number of Bdellovibrio produced within cells of Pseudomonas fluorescenes varied between 6 and 30 although a more usual range was 8 to 12. The latter observation has been confirmed by Starr and Baigent (1966) and by Abram and Shilo (1967).

Disruption of host cell and release of Bdellovibrio progeny

The manner in which the host cell was disrupted with the consequent release of mature Bdellovibrio was not understood.

The size and shape of Bdellovibrio cells has been reported to vary according to their age. Varon and Shilo (1968) found that newly released cells of Bdellovibrio were usually motile and small; older cells were immotile and relatively large.

Scherff, et al (1966) found occasionally that the host cell wall collapsed before division of the long intracellular form of the Bdellovibrio, a factor which could explain the appearance of spiral forms of the parasite in the medium.

Effect on the host cell

Stolp (1964) found that host cells remained viable five minutes after the attachment of Bdellovibrio. Shilo (1966) observed that Rhodospirillum rubrum lost its motility 5 sec after attachment.

Shilo (1966) showed, using PTA negatively stained and shadow casted preparations in an electron microscope, that holes or pits on the host cell wall appeared after attack by Bdellovibrio. The damage caused leakage into the medium of cellular contents of the host.

Scherff, et al (1966) observed that spheroplast formation of the host cell occurs within a few minutes after attachment of Bdellovibrio. These authors suggested that this morphological change in the host cell is caused mainly by damage to the rigid component of the cell wall by Bdellovibrio. This spheroplast-like body is related morphologically to spheroplasts produced by either lysozyme or penicillin in media containing the supporting agents, sucrose and cations. The term "spheroplast" is used to designate the spherical form of the bacterial cell in which cell wall has been modified (McQuillen, 1960; Martin, 1963). Under optimal conditions, these host cells lyse within 1/2-1 hr according to Shilo (1966) or in a few min according to Stolp and Petzold (1962).

Morphological alteration of the living host cell during parasitization by Bdellovibrio was observed although 5 days old of Pseudomonas as described by Scherff, et al (1966) and Starr and Baigent (1966) and filamentous forms of the same organism remained unchanged (Stolp, 1964). Starr and Baigent (1966), however, observed that some cells of Pseudomonas are only partially converted to the spheroplast form. The non-spheroplasted portion of the cell wraps around the sphere. These infected host cells eventually disintegrated completely.

Three points are particularly pertinent for this discussion:

(1) Formation of the spheroplast-like body suggests that the pore formed on the host cell wall during penetration by Bdellovibrio may be closed. Guélin, et al (1967) noted that host cells tended to agglutinate during spheroplast formation. This may be explained in part by the production of extracellular material which may seal the pore.

(2) The mechanism of spheroplast formation and the role of this structure in the host-parasite relationship is still unknown.

(3) The presence of spheroplasts in non-osmotically supported media such as quarter-strength-YP or one-tenth strength-NBA (Stolp and Starr, 1963) requires further investigation.

Another effect of Bdellovibrio on the host cell is the disorganization of its cellular content. Starr and Baigent (1966) and Scherff, et al (1966) found cytological evidence that the contents of the host cell became disorganized. Nuclear materials dispersed and disappeared. Ribosomes and cytoplasmic materials became granular and unevenly

distributed and the murein layer of the host cell was partially digested. Although enzymatic degradation of the host cell wall is evident, the effect may be either caused by exoenzymes produced by Bdellovibrio or by autolytic enzymes produced by the damaged host cell.

Shilo and Bruff (1965) reported that an exposure of host cells to Bdellovibrio for 5 to 10 min., rendered the host cell sensitive to the action of the exoenzymes produced by Bdellovibrio. These authors concluded that lysis of the host cell by Bdellovibrio involved two steps:
(1) Host cells are damaged during attachment by the parasite;
(2) Exoenzymes, presumably proteases produced by the Bdellovibrio lyse the damaged host cell.

Host specificity

The measures used by many to determine the host range are (1) attachment of Bdellovibrio to the host cell as observed in a phase-contrast microscope, (2) clearing of a host culture in broth, and (3) plaque formation or confluent lysis.

The host microorganisms which have been tested for parasitization by Bdellovibrio are listed in Appendix III.

Stolp and Starr (1963) divided their 12 strains of Bdellovibrio into the following five groups on the basis of host specificity:
(1) active only against the host cells used for isolation; (2) activity restricted to fluorescent Pseudomonas and Xanthomonas; (3) active against Pseudomonas including Pseudomonas salanacearum and Pseudomonas caryophyll (two strains which are not generally parasitized);

(4) active against some enterobacteria and pseudomonads but not against Proteus; and (5) the same as type 4, but in addition, active against Proteus.

The spectrum of host specificity of Bdellovibrio strains was dependent upon the conditions provided during host-parasite interaction. Stolp and Starr (1963) found that lysis of host bacteria in liquid culture was paralleled by the results of lysis on a semi-solid agar surface. However, Wood and Hirsch (1966) observed that the strain of Bdellovibrio with which they worked failed to lyse Hyphomicrobium in semi-solid agar although lysis occurred in liquid media. Shilo and Bruff (1965) found that host range was wider in the liquid media than in an agar medium. Stolp and Starr (1963) found that some strains of Bdellovibrio lysed only proliferating host bacteria in agar plates. Robinson and Huang (1967) noted that Bdellovibrio permitted multiplication but did not lyse E. coli B/r in a complex medium supplemented with Ca^{++} and Mg^{++}, while the same host-parasite system suspended in THAM buffer containing the essential cations permitted multiplication of Bdellovibrio and dissolution of the host cell.

Bdellovibrio isolated by Stolp and Petzold (1962) parasitizes only Pseudomonas and Xanthomonas. Of 126 strains of Pseudomonas tested, 23% were unaffected by Bdellovibrio. Those which were susceptible showed variable lysis, although Bdellovibrio resistant mutants from the susceptible host cell were not described (Stolp and Starr, 1963). The

lysis is not related directly to the action of extracellular enzymes nor did sonic extracts of Bdellovibrio have a detectable effect on host bacteria (Stolp and Starr, 1965).

Stolp and Starr (1963) reported that Bdellovibrio lysed only the gram-negative bacteria tested. Agrobacter, Caulobacter, Rhizobium, and Alcaligenes according to Dias and Bhat (1967) were not parasitized.

Guélin, et al (1967) found that their Bdellovibrio strain X Ty parasitized gram-negative bacteria and Clostridium perfringens. Burger, et al (1968) isolated Bdellovibrio strain W which parasitized in addition to some gram-negative bacteria, Streptococcus faecalis and Lactobacillus plantarum, but it failed to parasitize Pseudomonas aeruginosa or Spirillum serpens.

Many factors affect the development of Bdellovibrio and consequently, bacteriolysis of the host cell. These factors include: number (Stolp and Starr, 1963), age (Wood and Hirsch, 1966; Stolp and Petzold, 1962), and metabolic activity of the host cell (Stolp and Starr, 1965), composition of medium, accumulation of host metabolic products, and presence of Ca^{++} and Mg^{++} (Robinson and Huang, 1967; Huang, 1968), and also physiological age and numbers of the parasite (Stolp and Starr, 1965).

Sullivan and Casida (1968) failed to isolate Bdellovibrio which parasitizes Azotobacter directly from soil but the Bdellovibrio isolated from the soil using E. coli as the host cell parasitizes Azotobacter chroocaccum.

Burger, et al (1968) expressed host specificity in terms of

plating efficiency. The efficiency of their Bdellovibrio varied from 0.0001 with Serratia marscesens and 12 with Proteus vulgaris. The plating efficiency of Rhodospirillum rubrum was arbitrarily designated as 1.

Host specificity of Bdellovibrio was determined using various serotypes of E. coli (Klein and Casida, 1967). They observed that Bdellovibrio strain OX9-1 and strain 167-1 parasitized all E. coli serogroups tested and the S and R forms of E. coli strain MGH 6. Host activity spectrum of Bdellovibrio strain OX9-1, however, was less broad than that of strain 167-1. Variations were noted in the appearance of the Bdellovibrio plaques and the time required for plaque formation. These observations suggested to Klein and Casida that Bdellovibrio may vary in its characteristics.

General Physiology

Stolp and Starr (1963) reported that host cells killed by heat, $CHCl_3$, toluene or disinfectant failed to support growth of Bdellovibrio. This failure was not related to differences of pH or redox-potential in media containing dead host bacteria. They also found that carry-over of nutrients from the Bdellovibrio lysate was essential for lysis of heat-killed cells. However, Robinson and Huang (1967) and Burger, et al (1968) observed that Bdellovibrio developed and lysed heat-killed host cells extensively in broth culture.

Host bacteria that are inactivated by streptomycin or by ultraviolet (UV) irradiation are found to support growth of Bdellovibrio (Stolp and Starr, 1963). Burger, et al (1968) found that Bdellovibrio strain W failed to grow in synthetic medium but it multiplies in cell free extract of Rhodospirillum rubrum. Bdellovibrio strain 128 parasitizes only living cells of Aerobacter cloacae.

Bdellovibrio strains 100, 321 and A 3.12 developed plaques in THAM buffer agar (pH = 7.5, 0.05M) in the presence of the host cell used for its isolation, but the degree of bacteriolysis varies with the strain employed (Stolp and Starr, 1963).

Stolp and Starr (1963) noted consistent increase in the number of Bdellovibrio after all host cells were killed. These authors suggested that Bdellovibrio probably developed at the expense of nutrients made available by dissolution of the host cell. This concept, however, could not explain their observations that sonic extracts and phage lysates of host bacteria failed to support growth of Bdellovibrio.

Bdellovibrio failed to lyse a streptomycin-resistant host in the presence of streptomycin, an observation which suggests that the parasite is sensitive to the antibiotic.

Robinson and Huang (1967) reported that a washed cell suspension of E. coli B/r harvested from complex and from synthetic media and suspended in 0.025M THAM buffer (pH = 7.5) supported the growth of Bdellovibrio only in the presence of Ca^{++} or Mg^{++} or both ions, but not in their absence. Huang (1968) noted that specific divalent cations affected

neither the attachment of the parasite to the host, nor maintenance of host spheroplasts formed through parasitization by Bdellovibrio. Bdellovibrio isolated from the Mediterranean Sea (Shilo, 1966) or the Atlantic Ocean (Mitchell, et al, 1967) form plaques on plates containing high salt concentration (3% NaCl).

Stolp and Starr (1963) reported that Bdellovibrio grows aerobically. Simpson and Robinson (1968) confirmed this statement. Burger, et al (1968) observed that Bdellovibrio grew and infected the host cell only under conditions of 0_2 pressure of 4 to 5 mmHg and more.

Some strains of Bdellovibrio are rich in catalase (Simpson and Robinson, 1968; Seidler and Starr, 1968) while others lack it (Stolp and Starr, 1963).

Bdellovibrio possesses a cytochrome system (cytochrome c, b, a, and a_2) which is believed to couple to phosphorylation (Simpson and Robinson, 1968; Seidler and Starr, 1968). Bdellovibrio contains the enzymes of the tricarboxylic acid cycle in soluble form and a bound succinic dehydrogenase in the particulate fraction. The presence of aldolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and lactic acid dehydrogenase suggests that Bdellovibrio can obtain energy from the substrate-linked phosphorylation of the glycolytic system. The absence of the glyoxalate cycle suggests that Bdellovibrio may be able to utilize proteins, amino acids and nucleic acids (Simpson and Robinson, 1968). Mitchell, et al (1967), on the other hand, observed that Bdellovibrio was also capable of utilizing cell walls of E. coli as a sole source of organic carbon.

In the presence of an appropriate host bacterium, Bdellovibrio shows a strong proteolytic activity on gelatin and casein (Stolp and Starr, 1963).

Saprophytic mutants from parasitic strains of Bdellovibrio

Stolp and Petzold (1962) selected saprophytic mutants from their cultures of Bdellovibrio. Shilo and Bruff (1965) isolated host-independent (HI) strains from Bdellovibrio strain A 3.12 and found that the HI strain retained its motility, predatory and lytic activity. Stolp (1968) found that parasitic strains were invariably present with the HI strains. The saprophytic mutant is an important tool for studying nutritional requirements of Bdellovibrio, mechanism of bacteriolysis and its intracellular mode of growth, and chemical analysis of Bdellovibrio cells, although no work of this nature has been reported.

The saprophytic strains isolated by Stolp and Petzold (1962) were less homogeneous in appearance than the parental type. They were non-motile, displayed no lytic activity and possessed as many as three flagella at either one or both ends (Stolp, 1968). Fresh isolates may exhibit some lytic activity, but lose this property entirely after several transfers.

The saprophytic isolates possess strong proteolytic activity which causes lysis of host cells killed by heat or treated with various organic solvents, alkali and acids. Intact host cells are resistant to degradation by the proteolytic enzymes. The enzymes lyse also heat-

killed gram-positive bacteria.

The mutation frequency from parasitic to saprophytic strains was found to be $1:10^9$ (Stolp and Starr, 1968; Stolp, 1967, 1968). Stolp and Starr (1963) suggested that the variants are recognizable by specific markers, including requirement for growth factor(s) present only in living host cell (r^+), ability to attach to host cells (a^+), ability to penetrate the host cell wall (w^+), possession of protease (p^+) and motility (m^+). Thus the wild type of parasite is designated as $r^+ a^+ w^+ p^+ m^+$. Since saprophytic strains are still proteolytic, but the other factors may vary, the possible genotypes for saprophytes are $r^\pm a^\pm w^\pm p^+ m^\pm$.

Two methods have been used for isolating saprophytic mutants. The first method (Stolp and Starr, 1963) involves the inoculation of a concentrated parasite suspension (over 10^9 cells) on a YP agar. The second method requires an enrichment technique. HI mutants are concentrated by the successive addition of host cells to Bdellovibrio and separation of host cells and host-dependent Bdellovibrio (those attached to the host cell) from the HI mutants by filtration. The HI mutants in the filtrate were transferred to NB or 1/10 NB broth containing heat-killed (120^0C, 15 min) host cells and shaken at 30°C. This second method is based on the working hypothesis that (1) saprophytic strains may develop as a result of mutation and selection; that (2) HI mutants do not parasitize the host cell and therefore remain unattached in the two-membered culture and that (3) heat-killed host cells provide the growth factors for HI mutants although they are not parasitized by

Bdellovibrio.

Saprophytic strains have the following characteristics:

(1) Optimum temperature for Bdellovibrio strain S.P.19, derived from Bdellovibrio strain 321, is 22°C. No growth occurred at 35°C.

(2) The same strain develops within the pH range of 6 to 8.5.

(3) All saprophytic strains tested are aerobic and catalase-negative.

(4) Some are proteolytic.

(5) All fail to produce H_2S, indole, acid (as measured by the Methyl red reaction) and acetyl methyl carbinol (as measured by the Voges-Proskauer test). Some reduce nitrate.

(6) All fail to utilize carbohydrates, alcohols or organic acids oxidatively or fermentatively.

(7) All possess dehydrogenase and cytochrome c-oxidase activity.

(8) Some form a yellow pigment (a xanthophyll carotenoid).

Back mutation from saprophyte to parasite is restricted to proteolytic saprophytes (Stolp, 1964).

Seidler and Starr (1968b) found that their isolates of HI strain morphologically resembled the parasite. The nucleic acids and cell proteins of the HI strains possessed no unusual features. The DNA and protein content were 5% and 60-65% of the dry weight, respectively. The HI strain and the parent strain from which it was derived contained 50-52.4% G+C% (Molar percentage of guanine plus cytosine in Aeoxyribonucleic acid). They were catalase-positive or negative. Cytochromes a and c were present.

Systemic position of Bdellovibrio

The systemic position of Bdellovibrio is still in doubt. It is procaryotic. A simple polar flagellum permits Bdellovibrio to be placed in the order Pseudomonadales. Since Bdellovibrio is not photosynthetic, it is to be referred to the suborder Pseudomonadineae. Its vibrio-like shape suggests that it be placed in the genus Vibrio. However, the predatory and parasitic characters separate Bdellovibrio from other members of that genus. Stolp and Starr (1963) proposed the name Bdellovibrio bacteriovorus which describes the organism according to its shape and behavior.

Seidler and Starr (1968b) found that a vibriostatic compound, designated 0/129 inhibits the growth of all strains of saprophytic and parasitic Bdellovibrio. These authors concluded that Bdellovibrio is more closely related to Spirillum than to Vibro because of its inability to utilize carbohydrates and its G+C%.

MATERIALS AND METHODS

Media

The following media were prepared in glass distilled water and adjusted to pH 7.5 with NaOH. Wherever possible, reagent grade chemicals were employed. The amounts were indicated in percentages (w/v).

1. Yeast-peptone-sodium acetate-cysteine (YPSC)

   Bacto-yeast extract, 0.1; Bacto-peptone, 0.1; sodium acetate, 0.05; L-cysteine, 0.005; $MgSO_4$, 0.025 and $CaCl_2$, 0.025. Solid or semi-solid agar contained respectively 1.5 or 0.6 Bacto-agar.

2. Yeast-peptone (YP)

   Bacto-yeast extract, 0.3; Bacto-peptone, 0.06 and $CaCl_2$, 0.1.

3. Modified N. B-A (Stolp and Petzold, 1962)

   Bacto-casamino acids, 0.5; Bacto-nutrient broth, 0.8 and Bacto-yeast extract, 0.1.

Cultures

B. bacteriovorus 6-5-S (*Bdellovibrio*) was isolated from a sample of sewage collected at London, Ontario, using the following technique with *S. serpens* MW5 as host culture. A 200 ml portion of sewage was supplemented with 50 ml of the nutrient medium; the

mixture was shaken for 30 min at room temperature and then centrifuged (3000 x g, 10 min) to eliminate solid matter and large cells. An enrichment culture was then established using the clear supernatant containing bacteriophage particles and cells of Bdellovibrio to inoculate 150 ml of a 12 hr host culture. This culture was incubated 48 hr at 30°C on a reciprocating shaker during which time the cells of the host culture were almost entirely destroyed. The few remaining intact host cells and much of the fragmented material from host cells destroyed by the Bdellovibrio were removed from suspension by centrifugation at 7000 x g for 10 min. Smaller fragments of the host cell, cells of Bdellovibrio and bacteriophage particles remained in suspension. Further separation of the components of the supernatant on the basis of size was accomplished by passage through a membrane filter (0.6μ). Bdellovibrio were present in the filtrate and these were isolated and purified after three successive plaque isolations employing the double agar layer technique (Gratia, 1936; Adams, 1959). The upper semi-solid agar layer in this case contained 0.1 ml of a 10 to 20 fold concentration of a broth culture of S. serpens VHL and 0.1 ml of an appropriately diluted suspension of B. bacteriovorus. Plaques were identified as those developing from B. bacteriovorus following microscopic examination. B. bacteriovorus, 6-5-S, was one of several strains isolated by this procedure.

Stock cultures of B. bacteriovorus were prepared by inoculating YPSC broth simultaneously with the Bdellovibrio and S. serpens VHL

and by incubating the two-membered cultures 48 hr at 30°C. S. serpens was completely destroyed during this period. The Bdellovibrio survived for several months at 4°C in lysate or for at lease one year in a lyophilized lysate. Lyophilized stock cultures were prepared by horse serum-nutrient broth plus 7.5% glucose (final concentration) as a protective medium. The lysate which contained Bdellovibrio at its stationary phase of growth was retained at 4°C for several months and was used as the sourse of inoculum for subsequent experiments.

Bdellovibrio strain 100 was isolated by Stolp and Starr (1963) using Erwinia amylovora as the host cell.

Spirillum serpens VH was isolated by Dr. C.F. Robinow of this Department in 1957. This culture possessed a surface layer of hexagonally arranged units similar to the external "macromolecular monolayer" of a Spirillum species described by Houwink (1953). The surface pattern observed in S. serpens VH was published by Murray (1963). However, the strain selected by subculture in this laboratory, S. serpens VHL, permanently lost the surface pattern. A culture of S. serpens VH maintained by Dr. S. Maier in Athens, Ohio, S. serpens VHA still possessed the surface component. A taxonomic study of the genus Spirillum has been presented by Williams and Rittenberg (1957).

S. serpens MW strains and other gram-positive and -negative bacteria used for assessment of host specificity were received from the culture collection in this Department.

Stock cultures of S. serpens were subcultured monthly on YPSC agar.

Micrococcus lysodeikticus, received from Dr. F. Simpson of the Prairie Research Labor atory of the National Research Council of Canada, was used for the qualitative assessment of lysozyme activity.

Host cultures were cultivated for 12 to 16 hr on a shaker at 30°C using YPSC broth or modified N. B. -A broth (Stolp and Petzold, 1962). Host cells were harvested (3, 000 x g, 5 min, 4°C), washed three times and resuspended in distilled water. On subsequent dilution (10- to 50-fold) in 0.025M THAM-HCl (tris-(hydroxymethyl) amino-methane) or HEPES-NaOH (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) buffer (pH 7. 5) unless specified otherwise supplemented with 0.002M each of CaCl2 and $MgSO_4$, the cell suspensions had a turbidity which corresponded to approximately 150-350 Klett units (No. 66 filter).

Washed cell suspensions of Bdellovibrio were used to inoculate preparations of S. serpens in THAM-HCl or HEPES NaOH buffer. The washed cell suspensions of Bdellovibrio, inevitably contained fragments of the host culture, S. serpens, although no viable cells of the latter were detectable.

The two-membered culture was cultivated on a shaker at 30°C for a certain time depending on the experiment.

The cell preparations for cytological work were slightly modified. B. bacteriovorus 6-5-S and S. serpens VHL were inoculated simultaneously into YPSC broth and incubated 2 to 3 days at 30°C on a

receiprocating shaker. Freshly prepared cells of S. serpens VHL in YPSC broth was added to this culture and sampled at time intervals for the study of the early stage of parasitization. Erwinia amylovora was grown for 2.5 hr. in YP broth with aeration and then infected with B. bacteriovorus 100. Samples for embedding were taken from the cultures which were held without shaking for 10 min at room temperature.

Turbidity

Either the Spectronic 20 (Bausch & Lomb) with wave length 520μ or the Klett-Summerson photoelectric colorimeter, with No. 66 filter (660mμ) was used for measuring turbidity of cultures.

Enumeration of bacterial cells

Viable cell counts of Bdellovibrio were performed using the double-agar layer technique (Adams, 1959) with a lawn containing S. serpens VHL. Cultures other than this particular strain of S. serpens were employed for specific experiments. The Petroff-Hausser bacteria counter was also employed for assessing total numbers of host or parasite.

Specific treatment of cells of S. serpens strain VHL

Washed cells of S. serpens VHL suspended in distilled water to a final concentration of 3.4×10^9/ml of cells were divided into four portions:

(1) The first portion was heated for 5 min at 121°C. (2) The second portion was mixed with an equal volume of 6% glutaraldehyde in distilled water and shaken gently for 1 hr at room temperature. The

fixed cells were washed 3 times and left in distilled water for 15 hr at 4°C. These cells were then washed an additional 4 times to remove the last traces of glutaraldehyde and resuspended to the original volume of 10 ml. (3) The third portion (10 ml) was dispensed in a Petri dish, 14 cm diameter, and was placed 30 cm from the source of an ultra-violet light. It was illuminated with a germicidal lamp, 20 watts, and 2537 Å wave length for 20 min. (4) The fourth portion was retained as an untreated control.

The viability of the cells of S. serpens VHL treated by each of the procedures was determined by plating 1 ml portions onto YPSC agar. No colonies developed from preparations of cells which had been autoclaved, treated with glutaraldehyde or by ultra-violet irradiation: 3.4×10^9 cells per ml were detected in the untreated control preparation.

A washed cell suspension of S. serpens VHL was also subjected to sonic oscillations for 7 sec in a 20 KC MSE ultra-sonic unit using ice for cooling. A similar preparation of cells autoclaved 5 min at 121°C was also subjected to sonic oscillation at 30 sec intervals to a total time of 10 min.

Cell walls of S. serpens were prepared according to a modification of the method of Salton (1964). A washed cell suspension of the test organism was subjected to sonic oscillation for 5 min in the MSE ultrasonic unit. Large cell particles were removed from suspension by centrifugation at 2500 x g for 5 min. The supernatant solution

containing cell walls and cytoplasm, was then centrifuged again (7000 x g, 20 min). The sedimented material contained crude wall fractions and was washed twice in 1 M NaCl, 3 times in distilled water and centrifuged again (2500 x g, 5 min) to remove large cell particles which remained in suspension. The supernatant contained the wall preparations of S. serpens, a typical example of which is shown in Fig. 1.

Preparation of crude enzyme preparation from the Bdellovibrio lysate

S. serpens VHL was grown 16 hr in modified N. B-A broth with shaking, and the cells sedimented, washed and finally resuspended into 500 ml of THAM buffer supplemented with Ca^{++} and Mg^{++} to a final concentration of 1 x 10^9 cells/ml. This quantity of cells was normally obtained from 3 liters of culture. The preparation was heated for 5 min at 121°C to kill the organisms and to inactivate the cellular enzymes and was then inoculated with 5 x 10^9 Bdellovibrio as a washed cell suspension. During the 3 day period of incubation, the Bdellovibrio multiplied and the heat-killed host cells disintegrated. Cells and cell debris were removed by centrifugation (10, 000 x g, 15 min) and the supernatant treated with 0. 38M ammonium sulfate (50 g of ammonium sulfate was added for each 100 ml of supernatant solution), stirring at 4°C for 20 min. The precipitate was collected by centrifugation (10, 000 x g, 15 min), resuspended in distilled water (100 ml), and dialysed for 15 hr at 4°C against distilled water (21, 5 changes).

FIGURE 1

A Phosphotungustic acid (PTA) negatively stained preparation of cell wall of S. serpens VHL prepared by the modified method of Salton (1964). (See Materials and Methods).

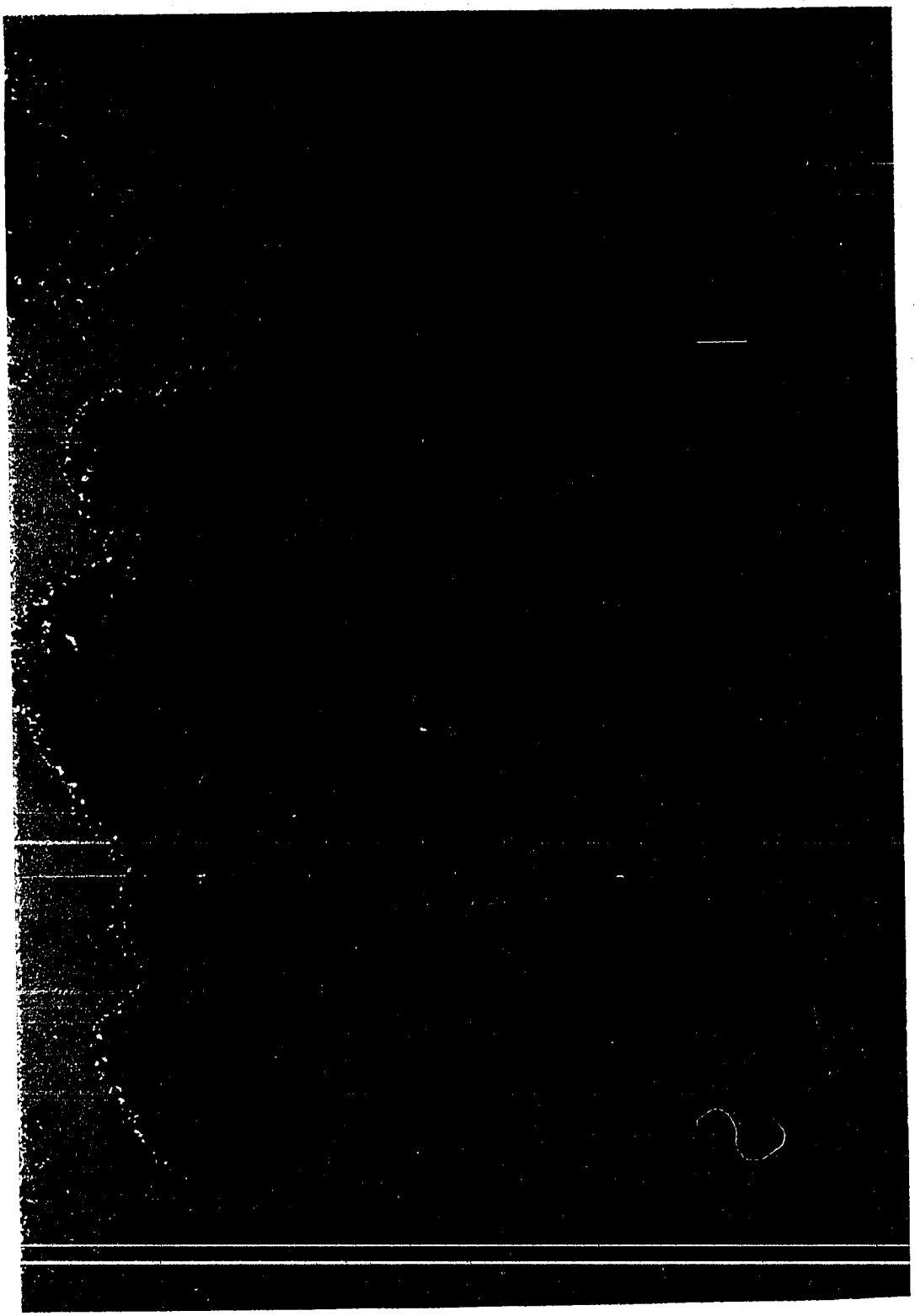


Figure 1

The dialysed enzyme preparation was lyophilized and retained at -18°C as the crude enzyme preparation.

Prior to use, the entire sample of crude enzyme derived from 3 liters of culture was dissolved in 4 ml distilled water and clarified by centrifugation at 48, 200 x g, 30 min. The supernatant solution was sterilized by filtration through a 0.45μ Millipore filter.

Light microscopy

The fixation and staining technique of Robinow and Murray (1953) was employed. A mixture of host and parasite was centrifuged (8700 x g, 5 min) and washed once with 1% neutralized formalin to halt further host-parasite interaction. The formalinized suspension was spread evenly on a layer of surface-dried water agar. A small block (1 x 1.5 cm) was cut from the agar and transferred surface down to a coverslip and fixed through the agar with Bouins' fluid. Impression films obtained by this procedure were washed with H_2O, 75% ethyl alcohol, and subsequently stained for 45 to 60 sec with Ziehl-Neilsen's carbol-fuchsin diluted 30-fold in distilled water. The stained cells were mounted wet on slides and sealed with wax.

Optical equipment (Ernst Leitz Wetzlar microscope) consisted of an achromatic condenser N.A. 1.4 and an achromatic oil immersion objective N.A. 1.32 in conjunction with a compensating eye piece 15x. Photographs were taken on Kodak RS Pan sheet film using Zeiss Jena photograph apparatus. Green light illumination was produced

with Kodak Wratten filter (B No. 58) using a tungusten ribbon light.

Electron microscopy

The standard fixation method employed by Ryter and Kellenberger (1958) was used with slight modification. Broth cultures were prefixed with 5% glutaraldehyde prepared in 0.1M phosphate buffer (pH 7.1)(Chrispeels and Vatter, 1963), using 10 parts by volume of broth to 1 part of the glutaraldehyde. The fixed cells were centrifuged immediately and the pellet suspended in 2% Bacto-Noble agar dissolved in Kellenberger's buffer (pH 6.5). Small blocks (approximately 1 mm^3) were trimmed from the agar and fixed for 2 hr at 4°C in 3% glutaraldehyde dissolved in the same buffer. The blocks were washed successively in a solution containing 0.2M sucrose dissolved in phosphate buffer at pH 7.1 for 2 to 3 hr and left overnight in the sucrose buffer at 4°C. The blocks were then fixed in Kellenberger's buffer containing 1% OsO_4, 0.1% $CaCl_2$, 0.05% NaCl and 0.1% tryptone for 15 to 18 hr, and in 1% uranyl acetate for 2 hr. The blocks were dehydrated in graded acetone, embedded in Vestopal W and polymerized at 60°C for 2 days. Thin sections were cut with glass knives held in a Porter-Blum microtome and further stained with Reynolds lead citrate (Reynolds, 1963). In case of the Erwinia and Bdellovibrio (100) system, glutaraldehyde prefixation was omitted.

Negatively stained cells were prepared by centrifuging a quantity of culture, resuspending in a fresh portion of broth and staining

with a solution containing 2% phosphotungstic acid (PTA) at pH 6.5 and 0.2% sucrose. The cell suspension and stain were mixed in the proportion of 2 to 1. Preparations were made on the grids by following the loop-film technique developed by Murray (1963).

Specimens were examined using a modified Philips EM 100 at 60 KV, fitted with 1.8 mm pore piece, a Ladd anode, mechanical astigmatism correction and accessory voltage stabilizer and photographed using Kodak fine grain positive film.

UV absorbing materials

Liquid cultures were centrifuged at 30,000 x g, for 30 min at 4°C and the supernatant diluted with distilled water to the point which showed a maximum sensitivity in the spectrophotometer (usually a 3- to 10-fold dilution). Absorbance at 260 mμ was read in a quartz cuvette (3.5 ml, 1 cm light path) in a Hitachi-Perkin-Elmer 139 UV - VIS spectrophotometer.

Determination of amino sugar

Amino sugar was determined by the method of Cessi and Piliego (1960). The soluble amino sugars released into solution during the growth of Bdellovibrio in S. serpens (Bdellovibrio lysate) was determined directly without acid hydrolysis (sometimes the preparations were acid hydrolyzed for comparative purposes).

Cell preparations for acid hydrolysis, obtained from (1) the two-membered culture, (2) host cell alone, (3) Bdellovibrio alone, or

(4) standard glucosamine-HCl, were hydrolyzed at 105°C for 6 hr in a sealed tube. The standard curves were obtained, either with or without acid hydrolysis using a concentration range of glucosamine-HCl between 0 and 50 μg in distilled water. The acid concentration, 4 N and the time, 6 hr at 105°C were found to give consistently the highest reading of amino sugars from the standard solution and the host cell preparation.

The amino sugars were acetylated in boiling water for 20 min using a 1% solution of freshly redistilled acetyl acetone in a stoppered flask. After cooling, 2 ml of distillate from the acetylated sample was collected in a 10 ml volumetric flask containing 8 ml of p-dimethyl-aminobenzaldehyde in acidic ethanol. The absorbancy (wave length, 545 mμ) was measured with a Hitachi-Perkin-Elmer UV-Vis spectrophotometer (3.5 ml, 1 cm light path) after the mixture was allowed to react at room temperature for at least 30 min. The standard deviation, ± 5% from the mean, was estimated from four concentrations (2.5, 5, 25 and 50 mg) of glucosamine-HCl and three treatments of the standard solution (with or without acid hydrolysis at 105°C for 6 hr using 4 N HCl.

Determination of Reducing Sugars

Carbohydrate was determined by the ferricyanide method of Park and Johnson (1949) using D-glucose as the standard, and a ferric iron reagent containing 0.15 N H_2SO_4 as recommended by Ghuysen, Tipper

and Strominger (1966). For the test, 1 ml each of sample, ferricyanide and carbonate-cyanide reagent were combined in the order indicated and heated 15 min in a boiling water bath. After cooling 5 ml of ferric iron solution was added, allowed to stand 15 min before reading at 690mμ. The confidence limit of the procedure was in excess of 90% with a standard deviation from the mean of ± 9%.

Determination of free amino groups

Free amino groups were determined by the fluorodinitrobenzene method described by Ghuysen, et al, 1966. The sample (1 ml) was mixed with 1 ml of 2% potassium borate and 0.03 ml of fluorodinitrobenzene reagent, and placed immediately in a water bath at 60°C for 30 min. The reaction mixture was then acidified with 1.2 ml of 2N HCl and read at 420mμ. L-glutamic acid was used as standard.

RESULTS

GROWTH CYCLE OF BDELLOVIBRIO BACTERIOVORUS IN SPIRILLUM SERPENS

B. bacteriovorus appeared generally as a comma-shaped organism (Figs. 2, 3, 4), although a variety of other forms and sizes have been observed. Numerous protrusions of diverse form were observed associated with the surface of B. bacteriovorus. This has also been observed by others (Shilo, 1966; Starr and Baigent, 1966).

The flagella were sheathed (Figs. 6, 7B, 7C) and had a diameter of 28mμ and an average length of 2.7μ, with variable amplitude and wave length. The sheath is a continuation of the cell wall of Bdellovibrio (Fig. 6). The core measured 14 mμ in diameter and often was visible at the distal end (Figs. 2, 7B, 7C). These values of sheath and core agree with the observations made by others (Seidler and Starr, 1968a; Burnham, et al, 1968; Burger, et al, 1968). Bdellovibrio has a flagellum with a diameter approximately twice that of S. serpens (Fig. 7A, 7B). That the protrusions arose from the flagellar sheath is seen in Figs. 2, 7C. Thin sections of Bdellovibrio resembled typical gram-negative bacteria in possessing plasma membrane and outside this a "double track" cell wall continuous over the surface (Fig. 5). Nuclear material (N), ribosomes (R), mesosome (M)

FIGURES 2 and 3

PTA stained preparations of Bdellovibrio strain 6-5-S (Figs. 2 and 3) propagated in YPSC broth supplemented with Ca^{++} and Mg^{++} (see Materials and Methods) with S. serpens VHL.

Fig. 2 x 45,500; Fig. 3 x 57,400.

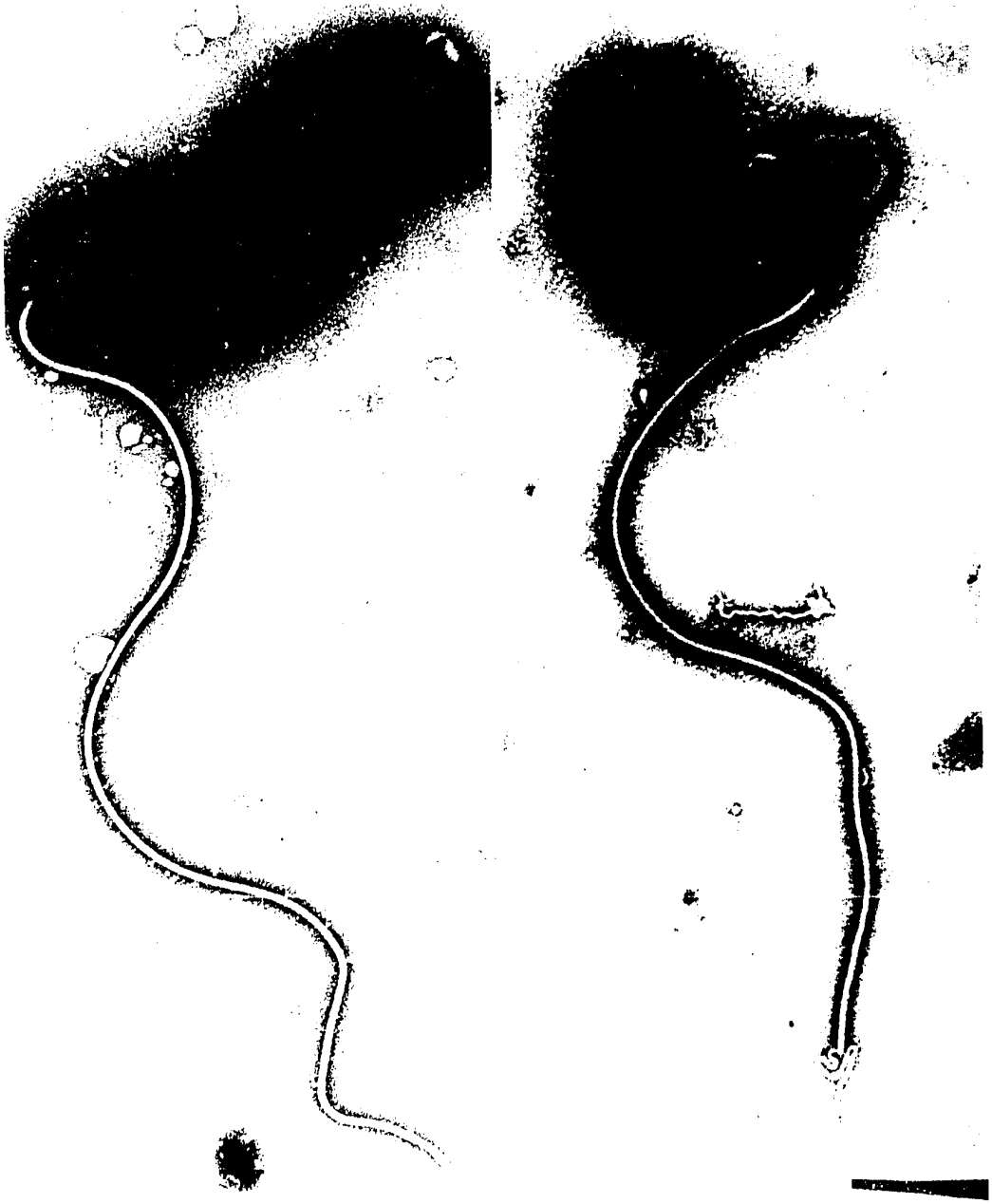


Figure 2

Figure 3

FIGURE 4

A PTA stained preparation of Bdellovibrio strain OX9-1 (obtained from Dr. D.A. Klein of Oregon State University, Corvallis, Oregon) propagated in YPSC broth with E. coli strain OX-9.

x 39,040.

Figure 4

FIGURES 5 and 6

Uranyl acetate (1% Uranyl acetate dissolved in distilled water) and lead citrate (prepared by Reynold's Method) stained preparations of a longitudinal and cross section of a mature Bdellovibrio 6-5-S (Fig. 5). Cell wall (CW), plasma membrane (PM), a hold fast (HF), mesosome (M), nuclear material (N), and ribosomes (R) are identified.

Fig. 5 x 145,500.

Fig. 6 x 194,000.

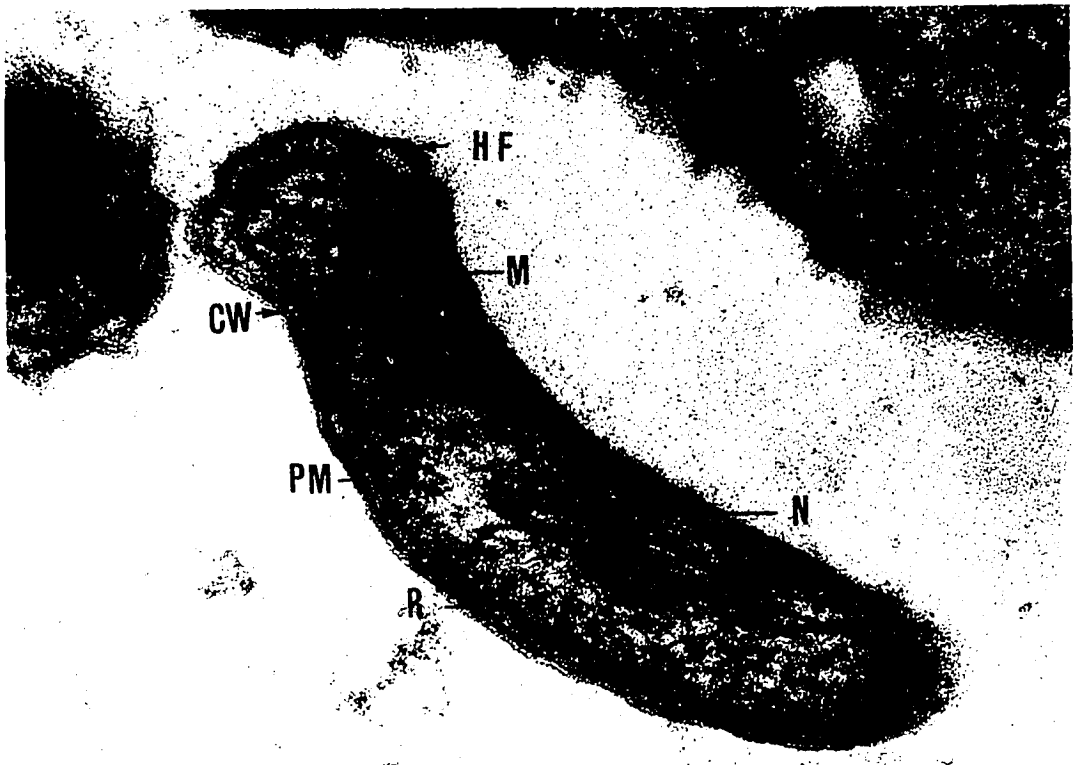


Figure 5

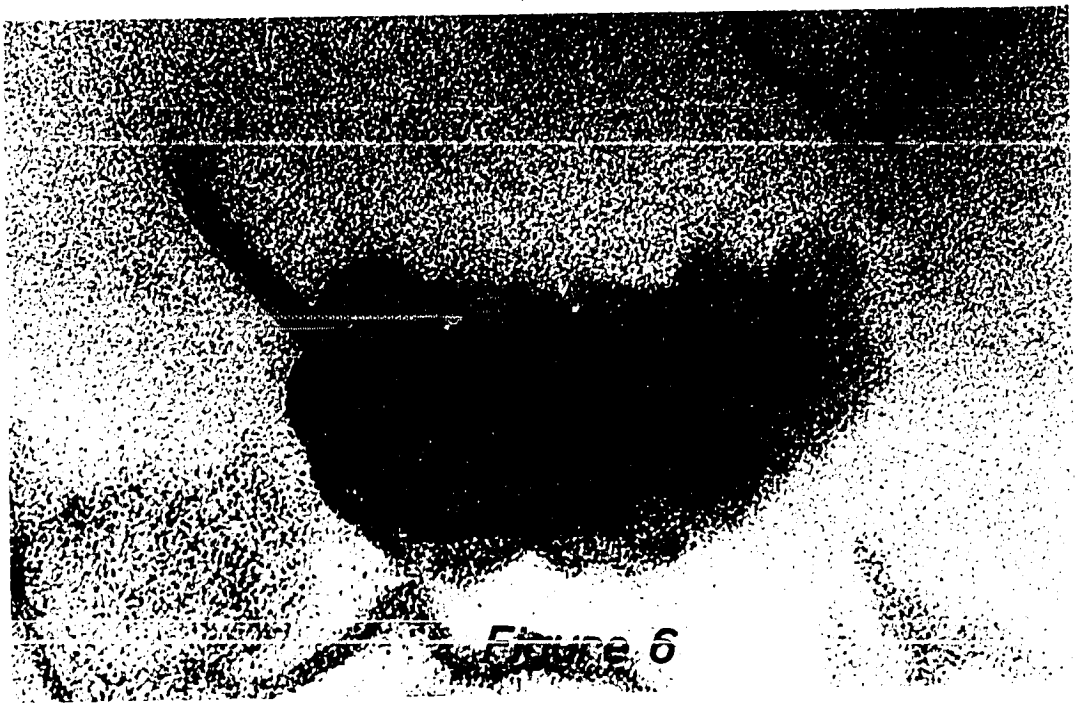
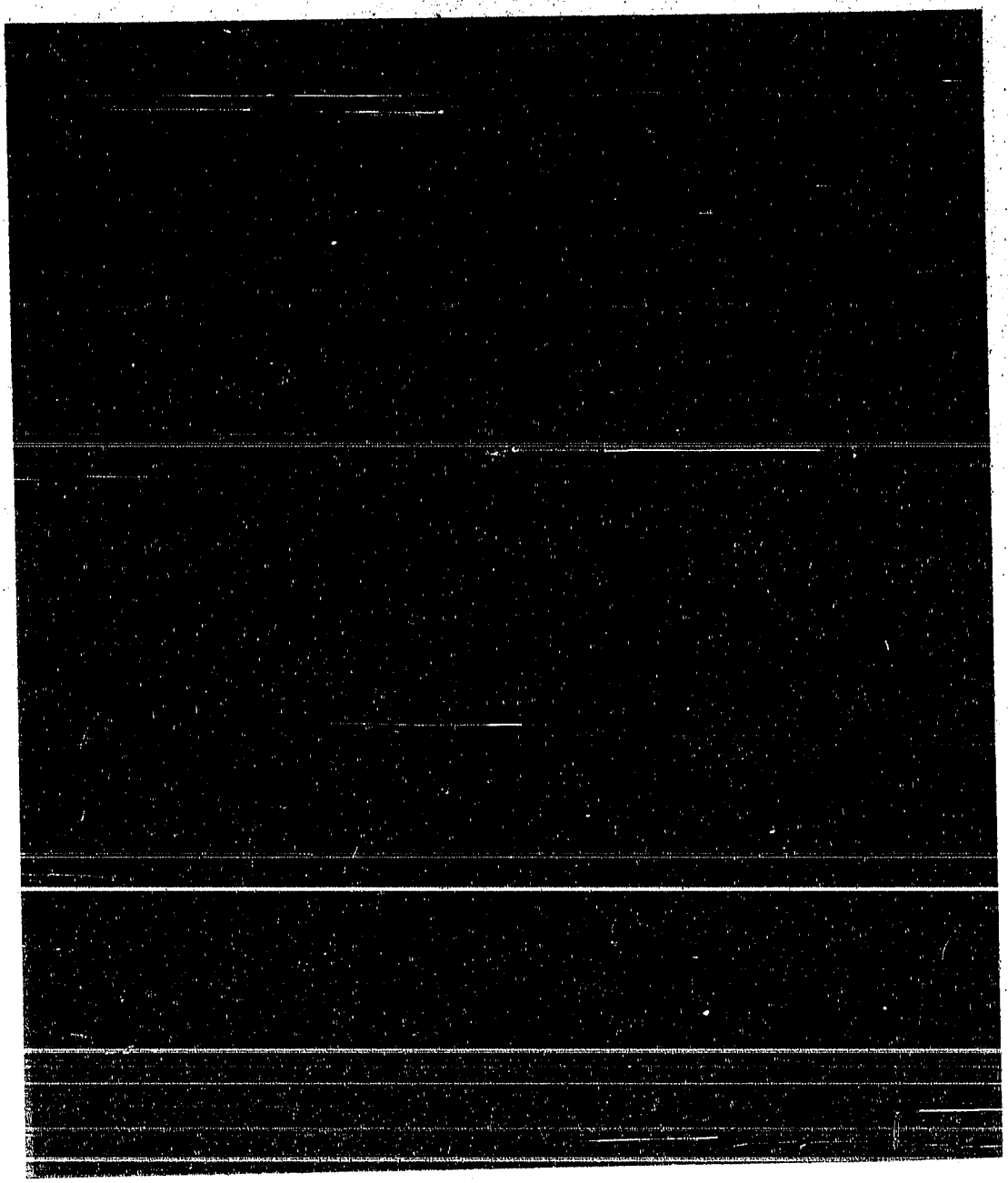


Figure 6

FIGURE 7

A PTA stained preparation of the flagella of S. serpens VHL (A) and Bdellovibrio 6-5-S (B and C). A light micrograph (D) showing monotrichous flagellum of Bdellovibrio prepared by a silver staining technique (Blenden and Goldberg, 1965). x 136,000. Magnification of D; x 3,200



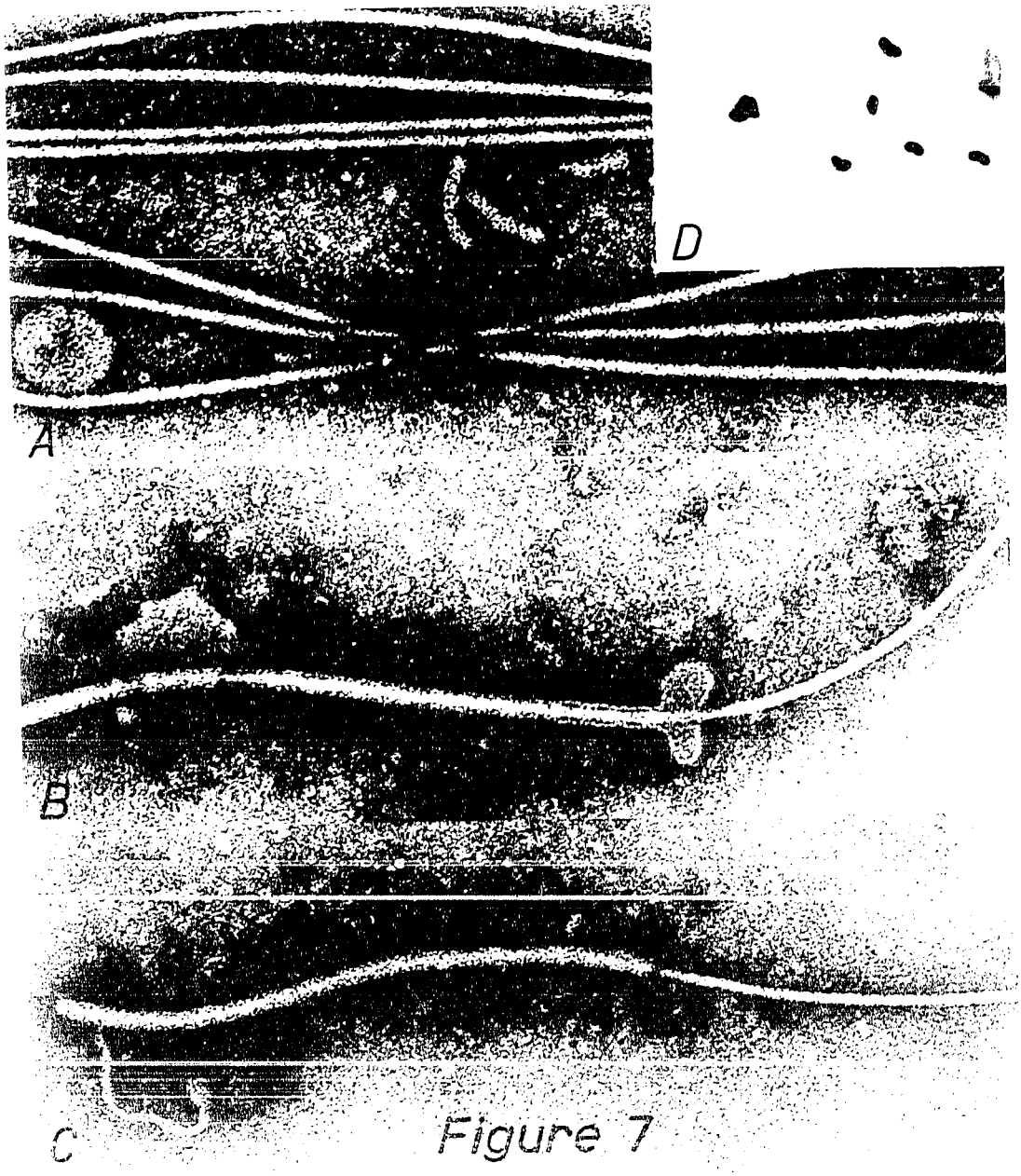


Figure 7

and a hold fast (HF) are also shown in Fig. 5, but the mucopeptide layer was not visible. This agrees with the observations of Murray and Maier (1965). Mesosome-like structures (electron dense bodies) were seen in negatively stained preparations (Fig. 4).

During the establishment of the host-parasite relationship, several Bdellovibrio were observed to attach to a host cell. Usually only one, but sometimes two or three Bdellovibrio penetrated a host cell.

That living host cells and spheroplasts were "parasitized" is indicated in the light photomicrographs (Fig. 23). The dense staining of rod-shaped cells was mainly due to the basophilic nature of the living cytoplasm. More lightly stained cells were considered to be damaged, possibly dying cells. It will be evident that both types of cells were parasitized by the Bdellovibrio. The cells of S. serpens heated for 75 min at 65°C were no longer viable although they too were susceptible to parasitization by Bdellovibrio. These observations are contrary to those reported by Stolp and Starr (1963).

When a young culture of B. bacteriovorus 6-5-S (10^7 cells/ml) is inoculated into a YPSC broth culture of S. serpens VHL (10^8 cells/ml), attachment is completed in less than 10 min as observed by phase contrast microscopy. Non-motile Bdellovibrio remained unattached to the Spirillum. Motility of S. serpens ceased 12 min after attachment of Bdellovibrio while that of Erwinia ceased immediately after attachment.

Thin sections and negatively stained preparations of a mixture of host and parasite were prepared in an attempt to show some of the steps of the parasitization of S. serpens by Bdellovibrio. The first visible step in the establishment of the host-parasite relationship is the actual contact between the two organisms (Figs. 8, 9). In the early stage, the attachment may be reversible. In many host cells there is no evidence of either bulging of the host cell wall as suggested by Burnham, et al (1968), an infection cushion (Scherff, et al, 1966), or a suction disc (Stolp and Petzold, 1962) (Figs. 8, 9). Bdellovibrio subsequently degrades or pierces the host cell wall but not the cytoplasmic membrane (Fig. 9). The same process may be seen in negatively stained preparations (Fig. 8) Bulge formation is, however, evident in a later stage in the parasitization process (Figs. 10 and 11).

The Bdellovibrio seen in Figs. 13, 14 and 15 has gained access to its host by way of an opening in the cell wall of the latter. The Bdellovibrio was constricted at the point of contact with the host cell and this observation suggested that the cell wall of Bdellovibrio is flexible. From the same illustration, it appears that although the cell wall was breached, the underlying plasma membrane was pushed inward but otherwise unaffected. The appearance of the host cell wall adjacent to the point of entry suggests that its rigidity is retained.

Figs. 16 and 17 show that size of the opening in the host cell wall (see arrow) has been reduced from that observed in Fig. 13,

FIGURE 8

A PTA stained preparation of Bdellovibrio 6-5-S in contact with S. serpens. Evidence for attachment without bulge formation is evident.

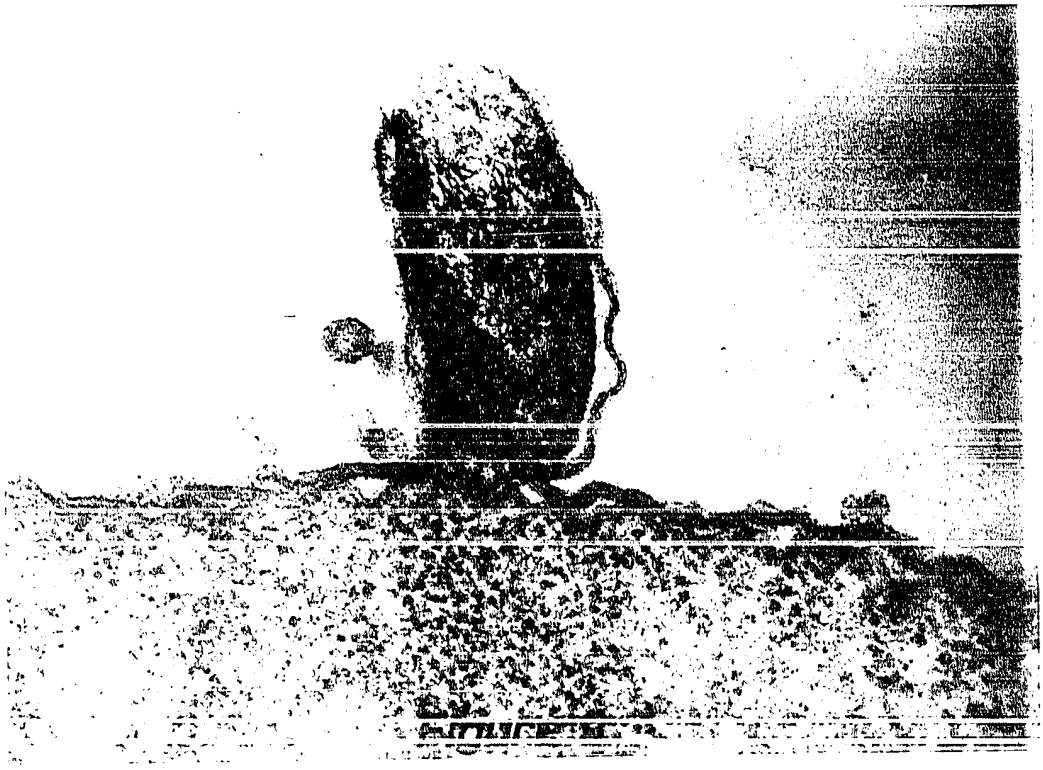
x 117,000.

FIGURE 9

A section of B. bacteriovorus strain 100 piercing the cell wall of Erwinia amylovora. The cell preparation was grown in YP broth. Evidence for the penetration of Bdellovibrio without a bulge formation is apparent.

x 93,900.

Figure 8



FIGURES 10 and 11

Sections of Bdellovibrio strains 6-5-S (Fig. 10) and 100 (Fig. 11) attached respectively to S. serpens VHL and Erwinia amylovora giving evidence of bulge formation.

Fig. 10 x 97,000.

Fig. 11 x 86,070.

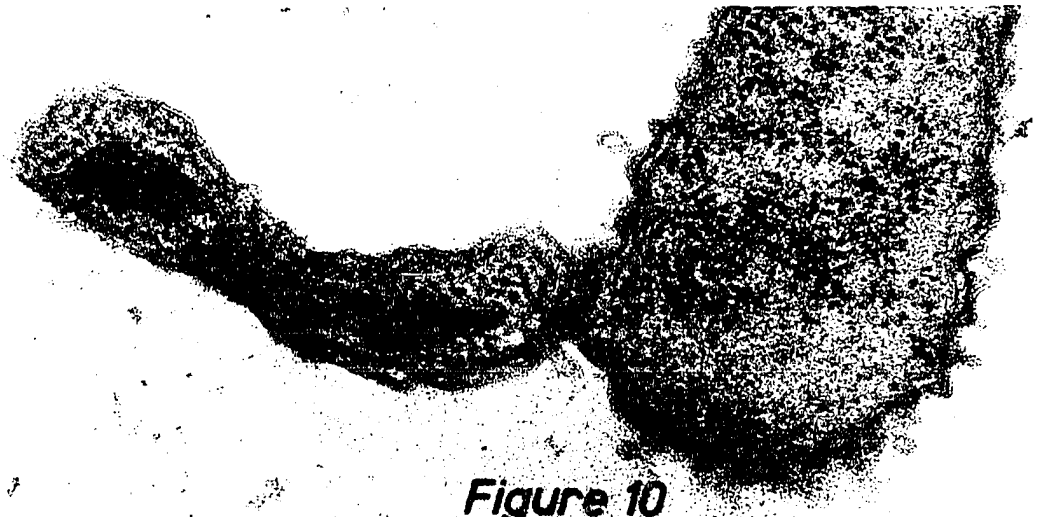


Figure 10

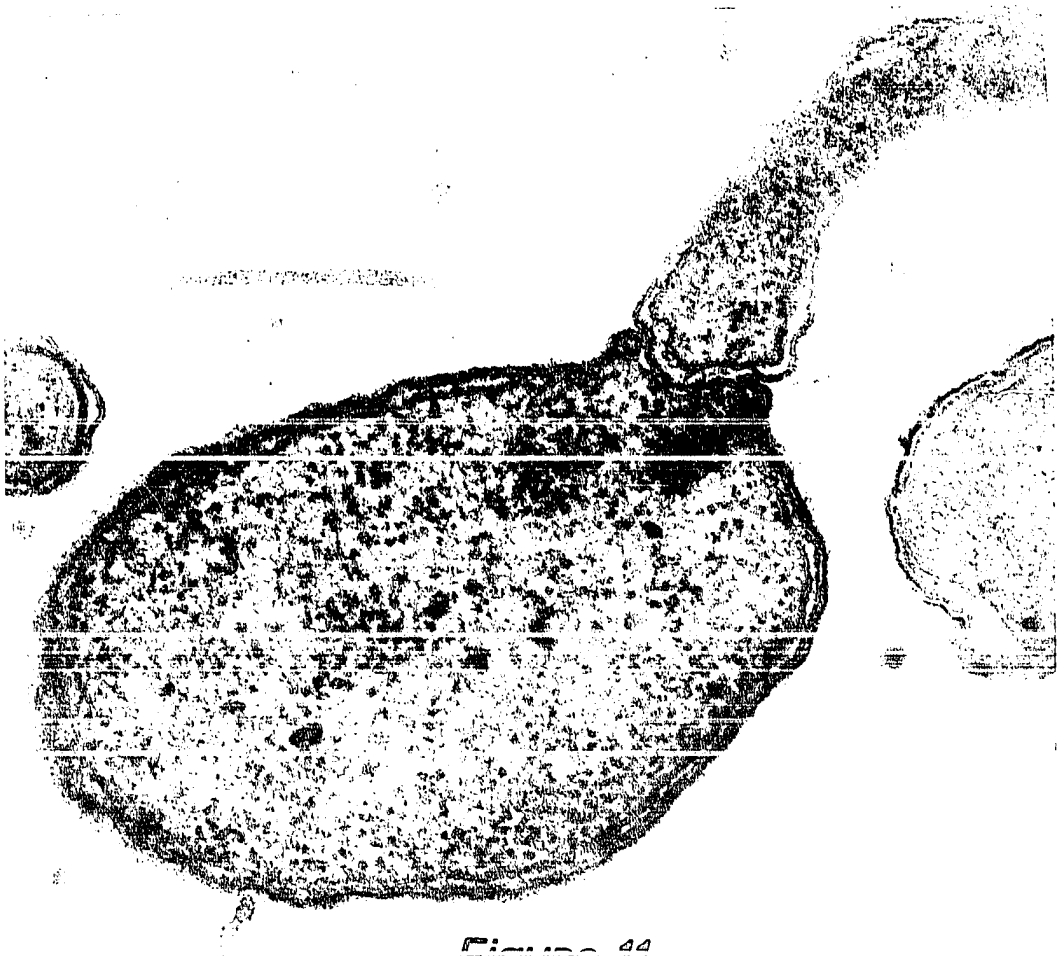


Figure 11

FIGURE 12

A section of Bdellovibrio 100 and Erwinia amylovora suggesting that bulge formation is caused by turgor pressure within the host cell.

x 109,550.

FIGURE 13

A section of Bdellovibrio 6-5-S which has penetrated S. serpens VHL.

x 108,800.

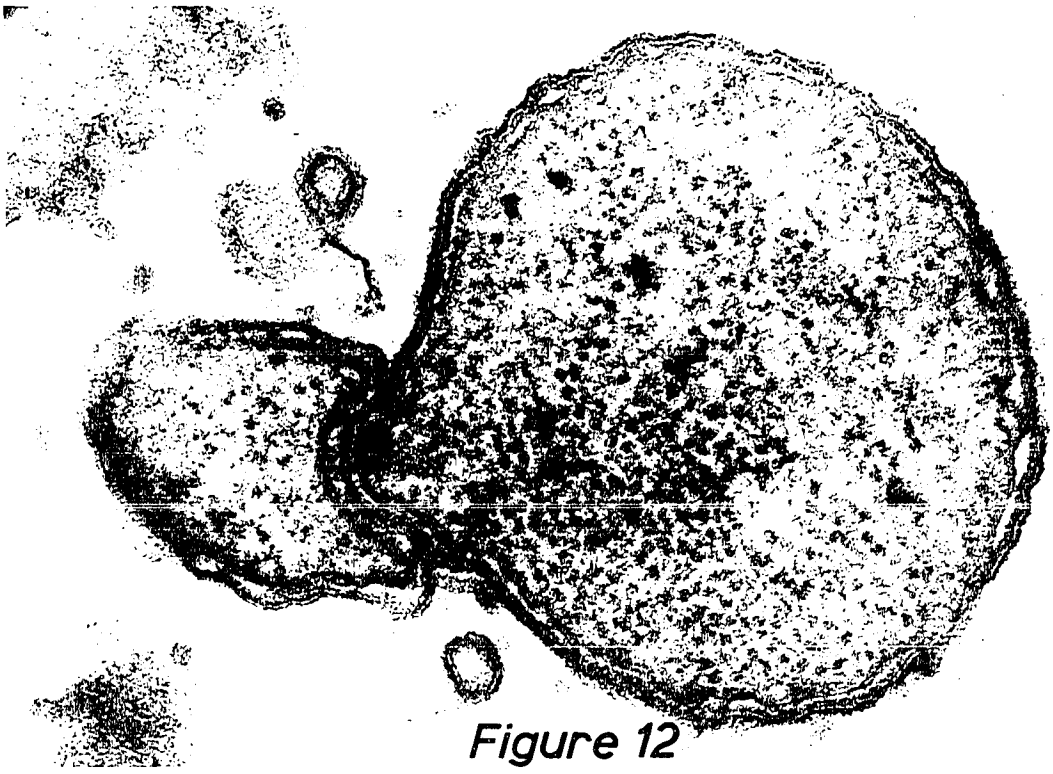


Figure 12

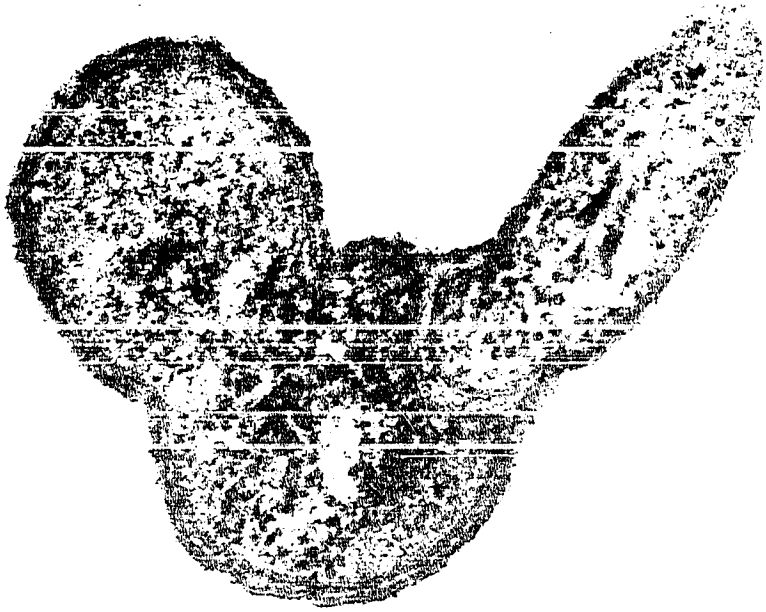


Figure 13

FIGURE 14

A section of Bdellovibrio 6-5-S which has penetrated S. serpens VHL.

x 94,400.

Figure 14

FIGURE 15

A PTA stained preparation of Bdellovibrio 6-5-S which has penetrated S. serpens VHL.

x 45,500.

Figure 15

Figure 15

FIGURE 16

A section of Bdellovibrio 6-5-S showing further penetration into S. serpens VHL.

x 95, 200.

FIGURE 17

A section of Bdellovibrio 6-5-S which has completed penetration of S. serpens VHL. Arrow indicates opening in cell wall.

x 67, 320.

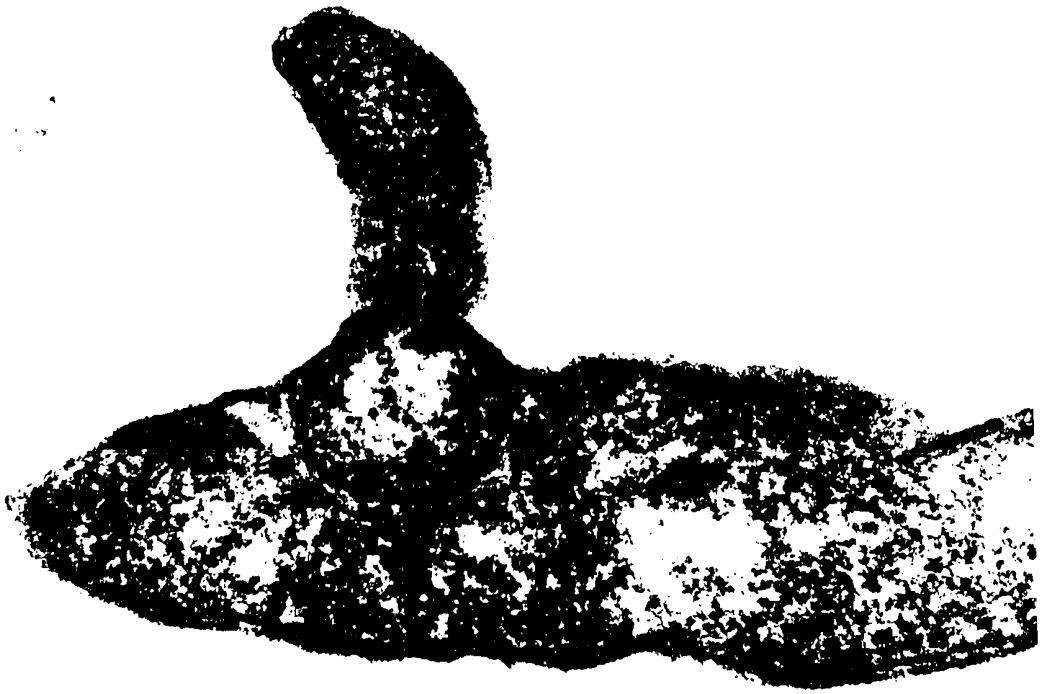


Figure 16

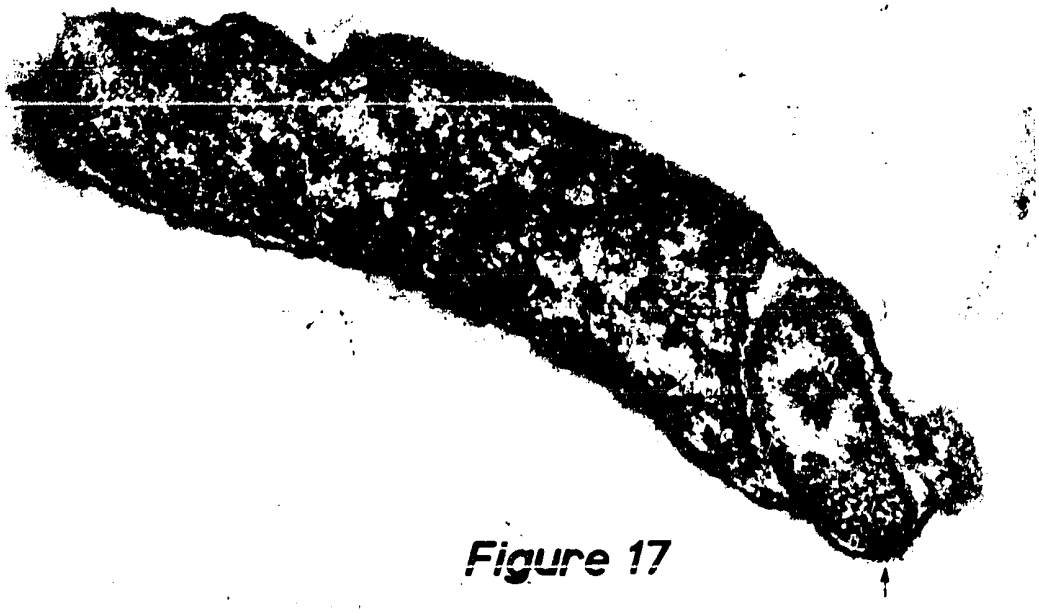


Figure 17

possibly by resynthesis of the cell wall at the damaged site. This phenomenon is shown clearly in Fig. 17 where the width of the opening is smaller than the diameter of the Bdellovibrio although this may be an artifact. Electron dense materials are also visible in the Spirillum at the opening.

The Bdellovibrio grew extensively in thickness and in length within the host cell before division was evident. Long coils of the parasite developed in situ, usually at one end of a parasitized cell but within the space between cell wall and cytoplasmic membrane (Figs. 18, 19, 22). However, some cells appeared to grow inside the cytoplasm (Fig. 20). The coiled filament (Figs. 19, 20) reached the maximum length without signs of septa, then divided (Figs. 21, 22, 23, 24) with the subsequent formation of progeny. On the average 16 Bdellovibrio units developed from one parent cell although as many as 25 have been observed in a cell of S. serpens (Fig. 24). Only two or three progeny developed in one parasitized E. coli B/r cell. The size of the host cell, therefore, appears to influence greatly the number of progeny which may develop from one parasite unit. No flagellated Bdellovibrio have been seen within a host cell. Figs. 22 and 24 suggest that the cytoplasm and cell wall of the host cell are destroyed partially prior to the time when the Bdellovibrio were released.

Occasionally, long ribbon-like structures or chains of vibrios were free in a two-membered culture (Fig. 23). The possibility exists that these units represent host-independent variants of Bdellovibrio, although such variants have not been isolated from preparations

FIGURE 18

A section of Bdellovibrio 6-5-S which has completely penetrated S. serpens VHL. The Bdellovibrio is located between the cell wall and the cytoplasmic membrane.

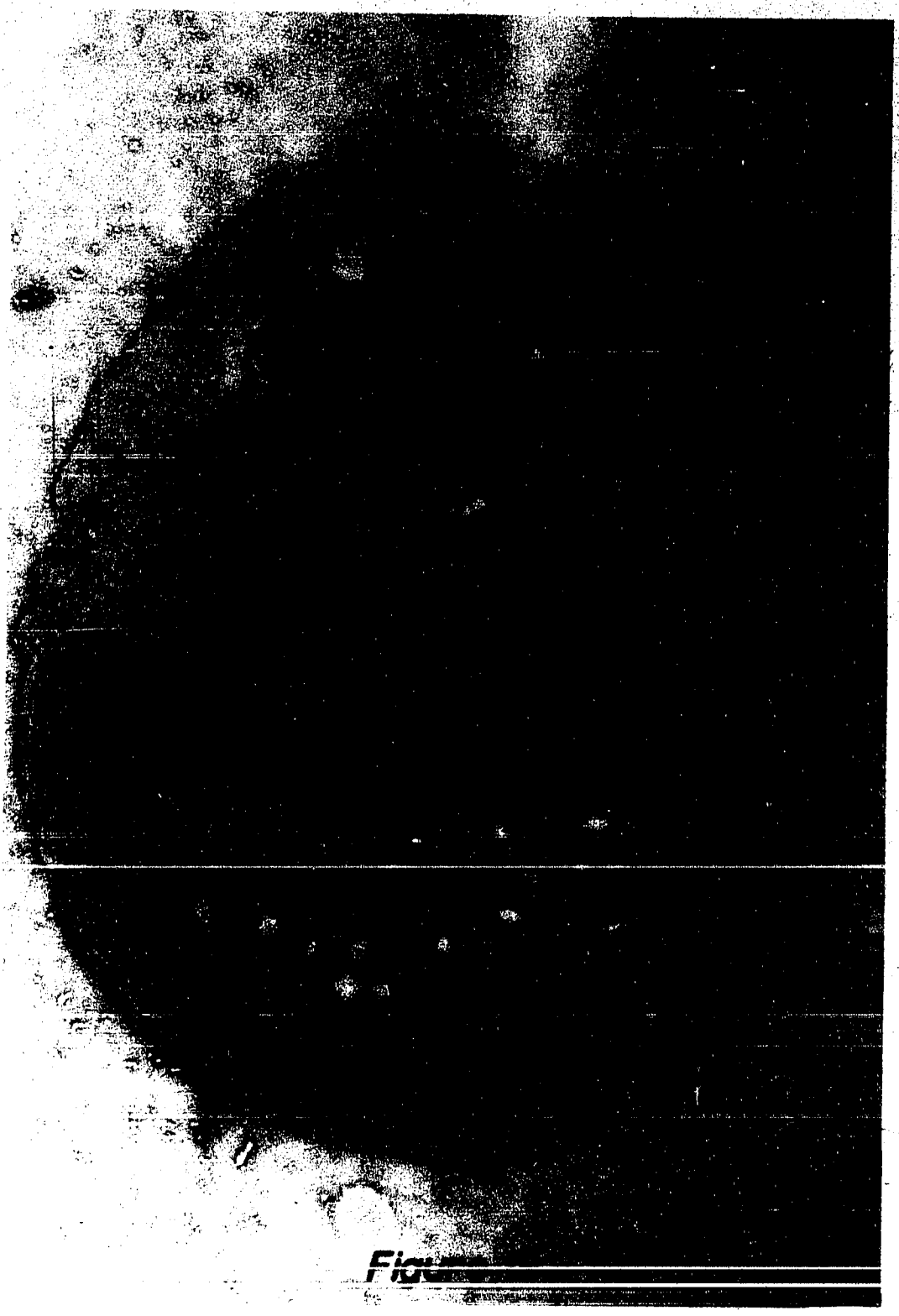
x 82,600.

Figure 18

FIGURE 19

A PTA negatively stained preparation of the spheroplast-like host cell with Bdellovibrio developing intracellularly.

x 65,600.



Fig

Figure 19

FIGURE 20

A PTA negatively stained preparation of the spheroplast-like host cell with Bdellovibrio developing intracellularly.

x 18, 560.

FIGURE 21

A PTA negatively stained preparation of S. serpens VHL containing Bdellovibrio 6-5-S progeny.

x 37, 500.

Figure 20

Figure 21

FIGURE 22

A section of S. serpens VHL with an enclosed and partially broken cytoplasmic membrane and with Bdellovibrio 6-5-S progeny.

x 68, 320.

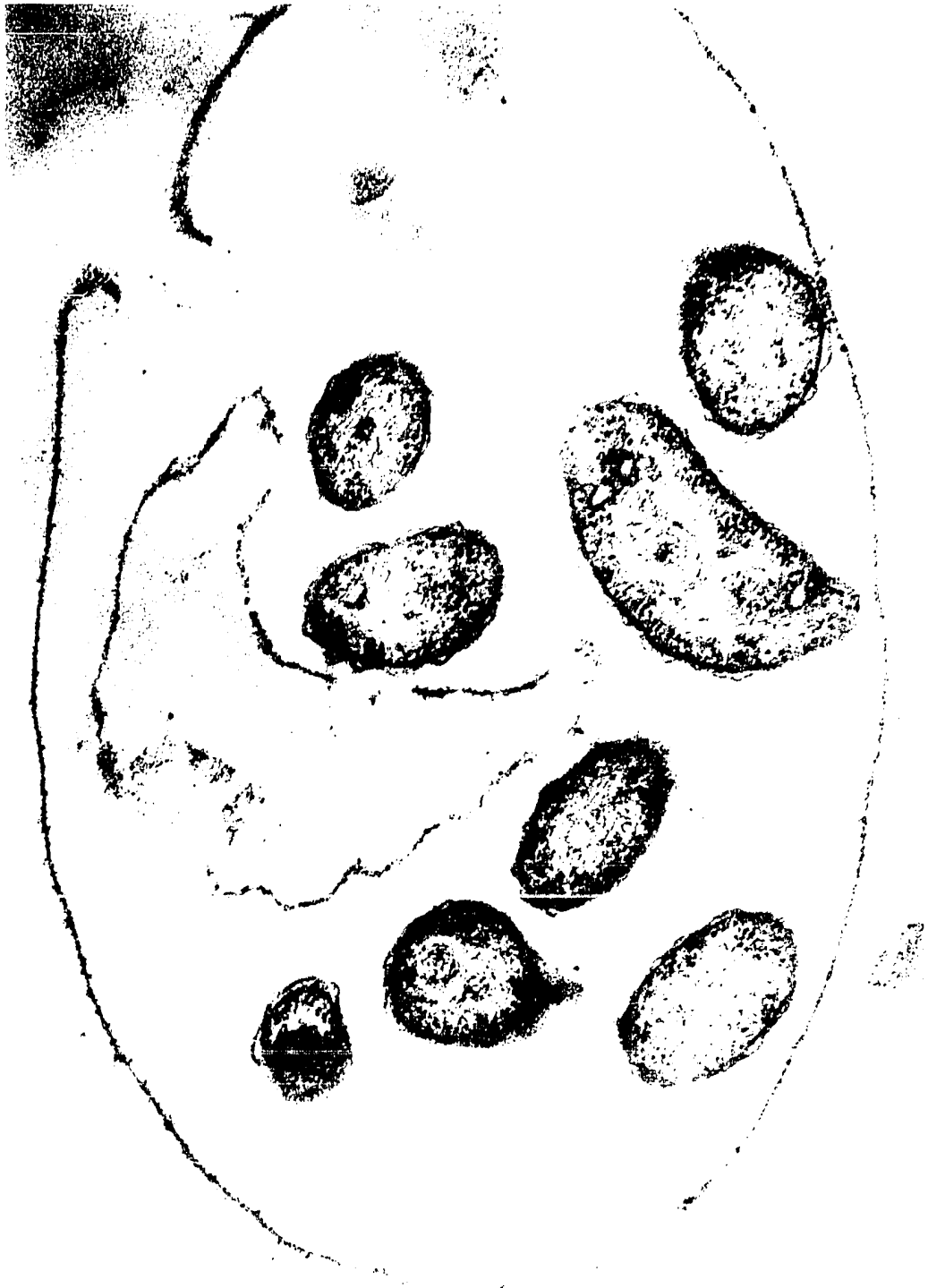


Figure 22

FIGURES 23 and 24

Light micrographs showing attachment, growth of Bdellovibrio 6-5-S within the host cell of S. serpens VHL and release of Bdellovibrio progeny. Intact and spheroplasted cells of S. serpens VHL are also shown.

x 3, 200.

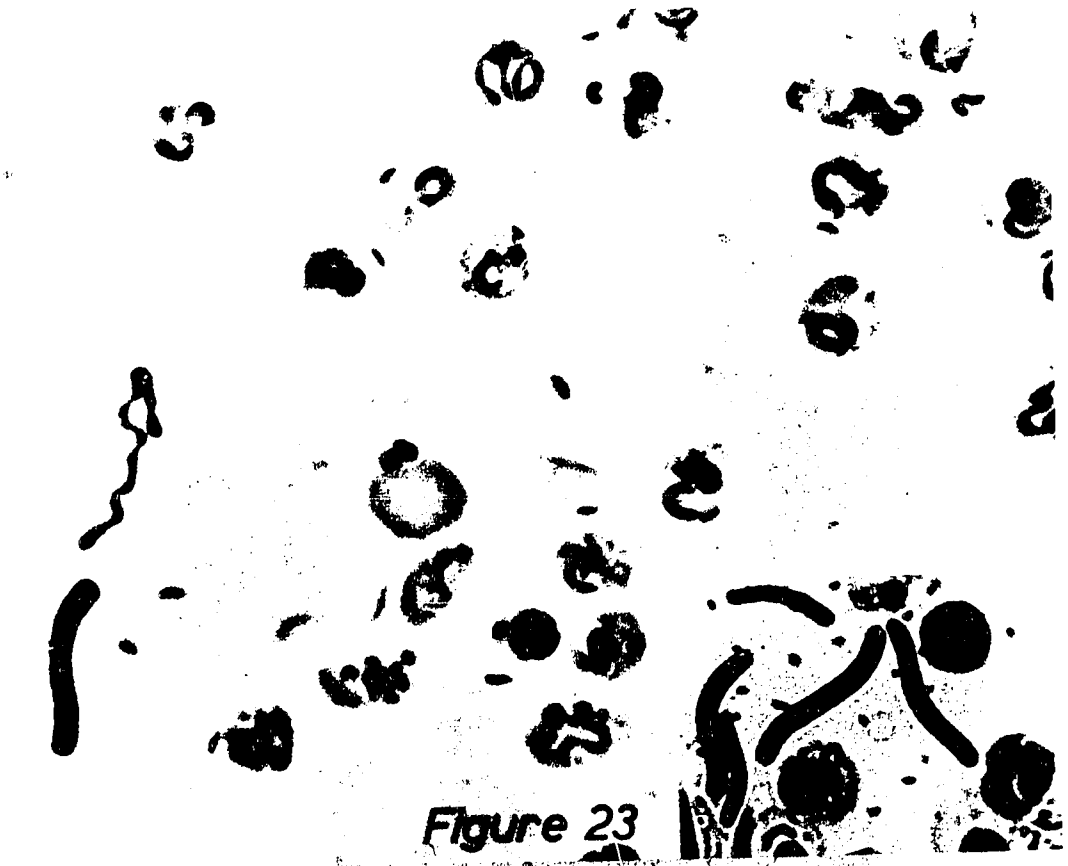


Figure 23

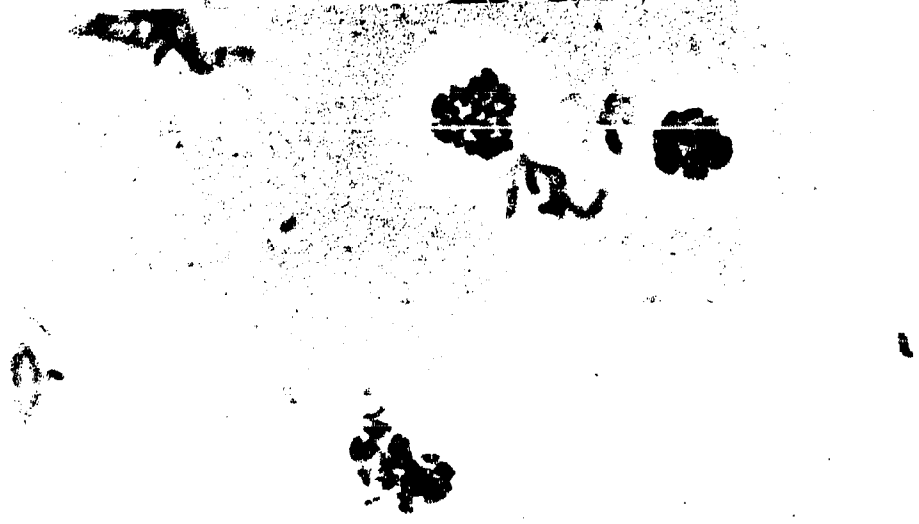


Figure 24

of Bdellovibrio 6-5-S. These structures may be derived from immature coiled filaments released into the environment during mechanical or enzymatic damage to the host envelope.

The wall of viable cells of S. serpens lost its rigidity and was converted to a spherical (Fig. 18) or pear-shaped (Fig. 23) unit 2 hr after parasitization had been initiated. Osmotically fragile spheroplasts were observed in the early stage of host-parasite interaction. The degradation of host cytoplasm by Bdellovibrio gradually reduces the internal turgor pressure of the host cell and the spheroplasts become non-fragile as the Bdellovibrio develop. These spheroplast-like units were not produced in a two-membered system containing Bdellovibrio and heat-killed cells of S. serpens although the dead host cell eventually disintegrated. Heat-killed cells of S. serpens contain large numbers of intracellular spaces and it is in these that the Bdellovibrio appears to develop.

Attachment, growth of Bdellovibrio within a host cell of S. serpens VHL and release of the Bdellovibrio progeny are illustrated in Fig. 23 and 24. The relative sizes of host a parasite are also evident.

THE INFLUENCE OF CATIONS ON THE GROWTH OF BDELLOVIBRIO BACTERIOVORUS

Washed cells of S. serpens VHL maintained in distilled water, 0.025M THAM or 0.025M HEPES buffers, or the YPSC medium in

the absence of added divalent cations were inoculated with approximately 10^4 Bdellovibrio/ml. B. bacteriovorus were enumerated unless specified otherwise after three days incubation at 30°C. The results (Table I) show that Bdellovibrio failed to develop in a two-membered culture in the absence of added calcium and magnesium. Addition of the two-divalent cations to the various suspending media at a final concentration of 0.002M resulted in the development of 60 to 1200 x 10^6 cells/ml of B. bacteriovorus in the presence of S. serpens VHL. The divalent cations, Mg^{++} and Ca^{++}, therefore are required by this strain of B. bacteriovorus for parasitization of S. serpens. A higher yield of B. bacteriovorus in the YPSC medium was probably due to an increased number of host cells. HEPES buffer containing cations was used in the subsequent experiments. Optimal concentration of HEPES buffer ranged from 0.025 to 0.25M and optimal pH from 6.5 to 9.0. Optimal growth temperature ranged from 20 to 35°C. Growth was favored by aeration but inhibited by anaerobiosis.

Data presented in Fig. 25 show that B. bacteriovorus multiply in HEPES buffer containing a washed cell suspension of S. serpens, B. bacteriovorus and cations. Development of Bdellovibrio is accompanied by (1) an increase in transmittance of the two-membered culture and (2) a release into the medium of UV-absorbing materials (260 mμ). The transmittance and release of UV-absorbing materials from S. serpens alone does not change greatly during the entire incubation period. No significant release of UV-absorbing materials

TABLE I

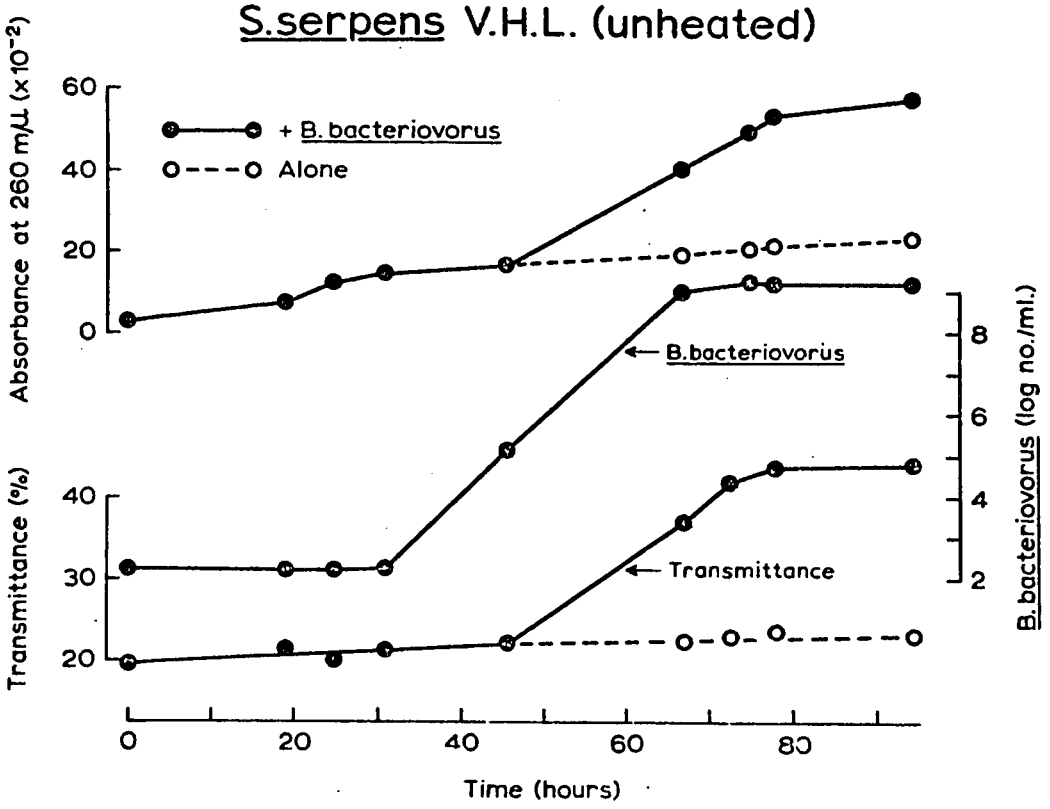
Effect of $CaCl_2$ and $MgSO_4$ on the growth of *B. bacteriovorus* in a washed cell suspension of *S. serpens*

Suspending Media	Supplement to Suspending Media $CaCl_2 + MgSO_4$	
	None	0.002M each
	(*B. bacteriovorus* No/ml x 10^6)	
Distilled water (initially, pH 6.8)	0.009	200
THAM-HCl buffer (0.025M, pH 7.5)	0.015	60
HEPES-NaOH buffer (0.025M, pH 7.5)	0.002	300
YPSC Medium (initially, pH 7.5)	0.003	1200

Multiplicity of infection: 1×10^{-4}

FIGURE 25

The development of B. bacteriovorus in a washed cell suspension of S. serpens in HEPES buffer (0.025M, pH 7.5) supplemented with 0.002M levels each of $MgSO_4$ and $CaCl_2$ as assessed by several parameters, including release of UV absorbing compounds (absorbance at 260 mμ), change in transmittance (% at 520 mμ) and determination of viable cell counts of the parasite. Multiplicity of infection: 2×10^{-6}


S.serpens V.H.L. (unheated)
Absorbance at 260 mμ (x10⁻²)
60
40
20
0
+ B. bacteriovorus
Alone
B.bacteriovorus
Transmittance (%)
40
30
20
Transmittance
B.bacteriovorus (log no./ml.)
8
6
4
2
0
20
40
60
80
Time (hours)

was noted when either 10^2 or 10^6 Bdellovibrio/ml were incubated in HEPES buffer supplemented with cations. No correlation was found between time lag (or latent period) and the ratio of Spirillum per number of Bdellovibrio cells inoculated.

Several hypothesis have been considered to account for the growth of Bdellovibrio in buffer supplemented with Mg^{++}, Ca^{++} and cells of S. serpens.

(1) S. serpens provides all the nutrients including divalent cations required for growth of the parasite. This possibility was considered by incorporating several levels of sonic extract obtained from 10^2 to 10^9 washed cells of S. serpens into buffer with or without supplement of Ca^{++} and Mg^{++} and inoculated with Bdellovibrio. None of the concentrations of extract tested supported the growth of Bdellovibrio.

(2) The divalent cations, Mg^{++} and Ca^{++}, support the integrity of the spheroplasts of S. serpens formed during parasitization by Bdellovibrio. This possibility was tested by providing an environment in which the integrity of the spheroplasts may be maintained in the absence of divalent cations, with results which are presented in Table II. It will be seen that Bdellovibrio numbers increase in all two-membered cultures in the absence of sucrose and to a greater extent in the presence of 0.1M sucrose in THAM HCl buffer and YPSC broth. The Bdellovibrio also multiplied readily in a two-membered culture in HEPES NaOH buffer containing 0.1 M sucrose. Divalent cations were required in all cases. The Bdellovibrio failed to multiply

TABLE II

Effect of Sucrose on the Growth of B. bacteriovorus in S. serpens suspended in buffers or in a culture of S. serpens in YPSC broth

Sucrose Concentration (M)	HEPES buffer without or (with)* Ca^{++} and Mg^{++}		THAM buffer Without or (with)* Ca^{++} and Mg^{++}		YPSC broth without or (with)* Ca^{++} and Mg^{++}	
	S. serpens + B. bacteriovorus	B. bacteriovorus alone	S. serpens + B. bacteriovorus	B. bacteriovorus alone	S. serpens + B. bacteriovorus	B. bacteriovorus alone
	B. bacteriovorus (log No/ml) (Initial value was 4.14)					
0	3.36 (8.47)	<0.30 (<0.30)	4.17 (7.78)	3.65 (3.65)	3.74 (9.07)	1.95 (2.90)
0.1	3.59 (8.14)	<0.30 (0.30)	3.61 (8.30)	3.50 (3.57)	4.34 (9.20)	3.95 (4.14)
0.4	3.70 (3.60)	<0.30 (0.30)	3.82 (3.77)	3.53 (3.62)	3.94 (4.23)	3.63 (4.23)
0.7	3.78 (<0.99)	<0.30 (<0.99)	3.64 (3.67)	3.00 (3.66)	3.72 (3.81)	3.75 (3.82)

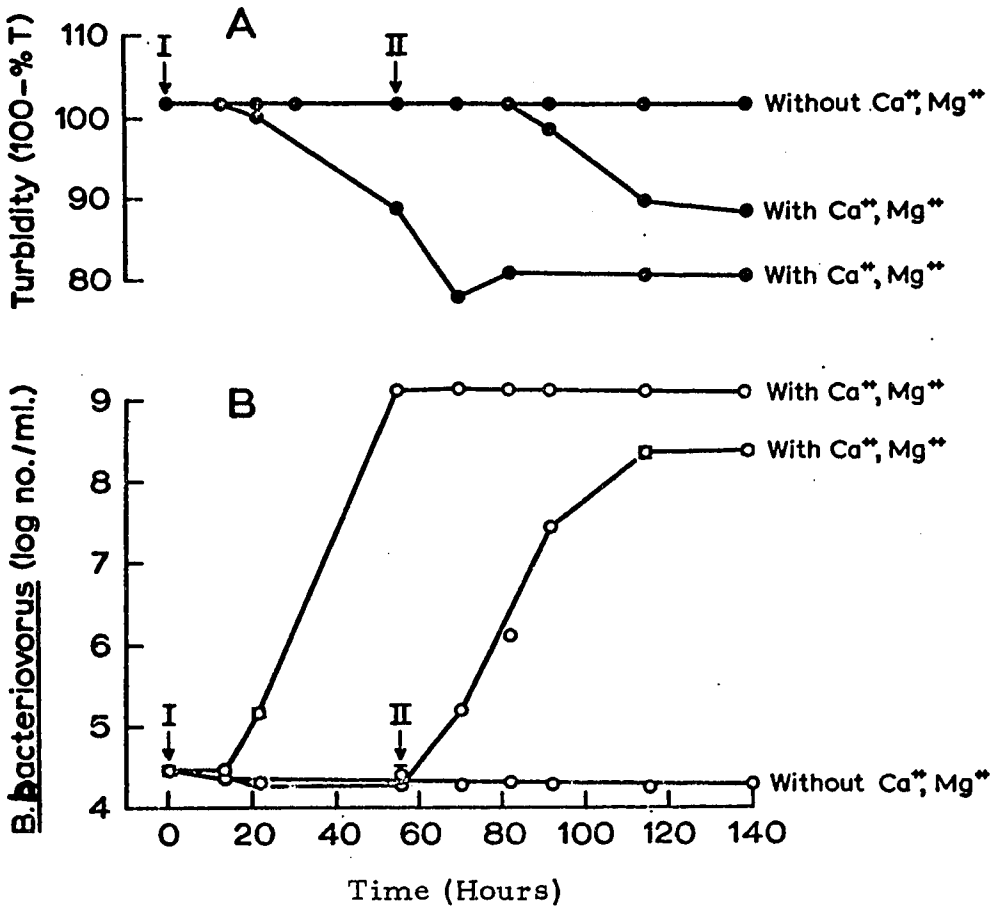
* Supplemented with 0.002M levels of $CaCl_2$ and $MgSO_4$. The pH of the two-membered cultures in YPSC broth after five days incubation varied between 7.7 to 8.9. Sucrose was sterilized separately by filtration and mixed with heat-sterilized buffers or broth prior to inoculation. Multiplicity of infection: 1×10^{-4}

in all suspending media containing 0.4M or higher levels of sucrose. Bdellovibrio alone in YPSC broth and THAM buffer was unaffected as far as cell numbers are concerned by the presence of sucrose at any of the levels tested. The data may indicate that levels of sucrose which support the integrity of the spheroplast (0.2-0.4M sucrose) does not substitute for Ca^{++} and Mg^{++} in the growth of Bdellovibrio although this does not rule out the possibility that sucrose supports spheroplast in the presence of Mg^{++} but not without it.

(3) Divalent cations are required for replication of Bdellovibrio. If Ca^{++} and Mg^{++} are essential for the replication of Bdellovibrio, the length of the lag phase and the growth rate of Bdellovibrio should be similar when the two-membered culture established in buffer devoid of the cations was supplemented with Ca^{++} and Mg^{++} initially or sometime later. This possibility was considered by establishing a two-membered culture in HEPES buffer and supplementing it with the cations initially and after 48 hr incubation. Changes in turbidity and in the viable count of Bdellovibrio were determined as a function of time. These parameters measure the rate of appearance of new Bdellovibrio. Data presented in Fig. 26 show that a lag or latent period of 18 hr is followed by rapid growth of the parasite in a two-membered culture when Ca^{++} and Mg^{++} were present initially. Addition of Ca^{++} and Mg^{++} 48 hr after the establishment of the culture stimulated immediate growth of Bdellovibrio and appeared to eliminate the time lag. In another experiment which is not shown here, Ca^{++} and Mg^{++} were added at time intervals up to 60 hr after the establishment of the culture, the results confirm that delayed addition of cations shorten the lag time.

FIGURE 26

Effect of delayed addition of Ca^{++} and Mg^{++} on the growth of Bdellovibrio. A washed cell suspension of S. serpens VHL in HEPES buffer was inoculated with washed cells of Bdellovibrio (5×10^4/ml). The arrows indicate the time of addition of $CaCl_2$ and $MgSO_4$ (0.002M each). Multiplicity of infection: 5×10^{-4}


A
B
I
II
Turbidity (100-% T)
110
100
90
80
Without Ca++, Mg++
With Ca++, Mg++
With Ca++, Mg++
B. bacteriovorus (log no./ml.)
9
8
7
6
5
4
With Ca++, Mg++
With Ca++, Mg++
Without Ca++, Mg++
0
20
40
60
80
100
120
140
Time (Hours)

The possibility was considered that cations other than those of calcium and magnesium might support the growth of the Bdellovibrio. Ten-fold dilutions of salts in concentrations which varied between 2×10^{-1}M and 2×10^{-7}M were introduced into HEPES buffer pH 7.5. Data presented in Table III show that 16 salts support the growth of Bdellovibrio to a variable extent in cells of S. serpens suspended in HEPES buffer. Monovalent cations and the divalent cations calcium and magnesium appear generally to be less toxic and therefore better able to support growth of the parasite than is the case with trivalent cations and the remaining divalent cations. Ca^{++} or Mg^{++} can act independently. Growth of Bdellovibrio proceeded as a function of the concentration of Ca^{++} or Mg^{++} added between the levels of 2×10^{-6}M and 2×10^{-2}M. The requirement for cations is clearly non-specific.

Growth of Bdellovibrio in washed cells of S. serpens VHL suspended in buffer containing 0.002M each of $CaCl_2$ and $MgSO_4$ was halted by addition of 0.01M EDTA (pH 7.5). Bdellovibrio alone was not affected by the chelating agent (Fig. 27). EDTA, therefore, appears to sequester the cations required for the multiplication of Bdellovibrio.

Results presented in Fig. 28 show that Bdellovibrio strain 6-5-S grew in and dissolved heat-killed cells of S. serpens VHL maintained in buffer in the presence of the appropriate cations. Development of the Bdellovibrio in the heat-killed suspension of S. serpens is accompanied by a modest increase in the transmittance of the two-membered culture and a simultaneous release into the medium of UV-absorbing materials.

TABLE III

Development of B. bacteriovorus in a washed cell suspension of S. serpens VHL in HEPES buffer supplemented with specific salts (Results expressed as log no. Bdellovibrio/ml after 94 hr incubation).

	Salt Concentration 2 x M							
Salt	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
LiCl	2.6*			3.9	4.8	6.0	3.6	
KCl	4.7**				6.6	7.9	8.0	1.0
NaCl	4.7				4.8		7.6	1.0
NH_4Cl	4.7				1.5	2.3	7.3	1.0
$MgCl_2$	4.7	7.8	7.8	7.3	8.3	8.8	9.0	3.0
$CaCl_2$	4.7	5.0	7.3		8.6	8.9	9.3	1.0
$MnCl_2$	2.6			2.3	3.0			
$CoCl_2$	2.6		4.6	1.0				
$CuCl_2$	2.6		4.8	6.0				
$ZnCl_2$	4.7		5.8	1.0				
$SrCl_2$	2.6			8.9	4.5	4.8		
$CdCl_2$	4.7		7.8	1.0				
$BaCl_2$	2.6				2.8	8.8		
$PbCl_2$	2.6	3.9	4.7	1.8				
$AlCl_3$	2.6		6.3	7.0	9.0			
$FeCl_3$	2.6		5.3	5.5				

Multiplicity of infection: * 4×10^{-6}
** 5×10^{-5}

FIGURE 27

Effect of EDTA on the growth of B. bacteriovorus in a washed cell suspension of S. serpens VHL in HEPES buffer (0.05M, pH 7.5) containing 0.002M each of $CaCl_2$ and $MgSO_4$. Neutralized EDTA was included with the buffer at the levels indicated. Bdellovibrio were enumerated after 4 days incubation.

Multiplicity of infection: 5×10^{-3}

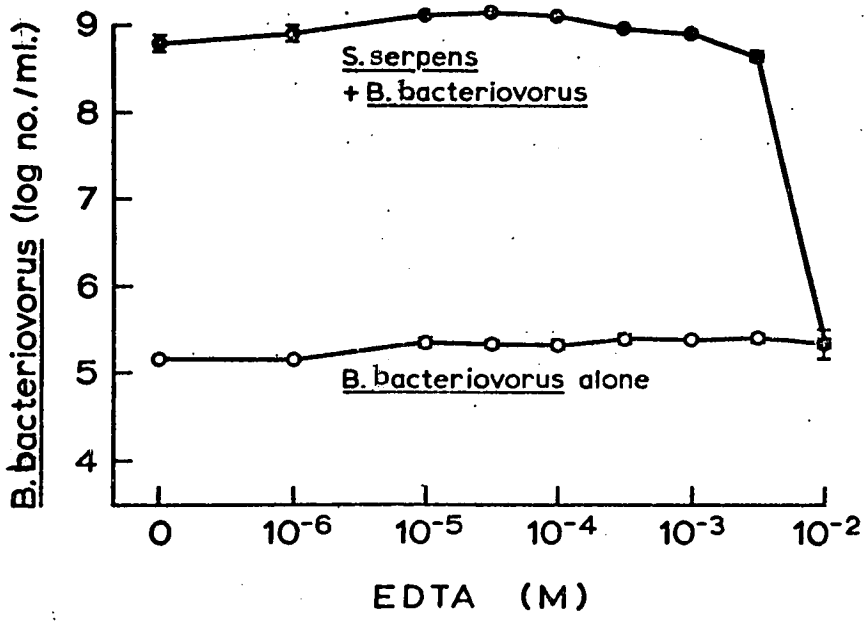
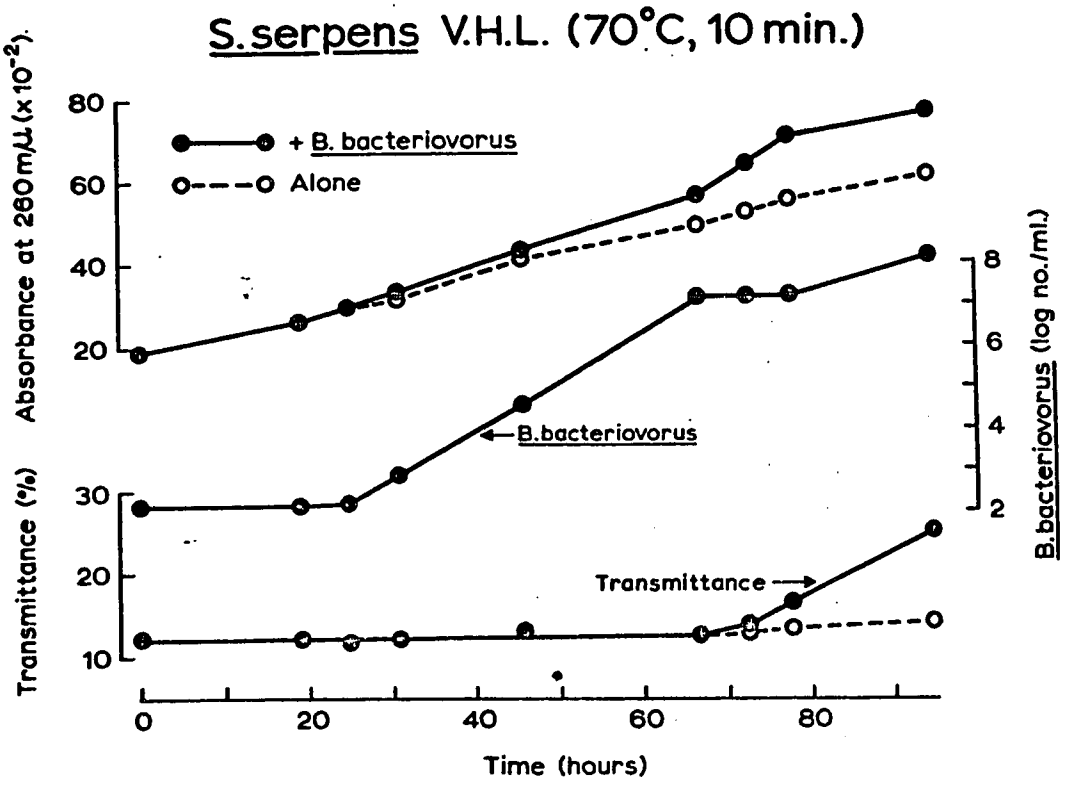

B. bacteriovorus (log no./ml.)
9
8
7
6
5
4
S. serpens
+ B. bacteriovorus
B. bacteriovorus alone
0
10-6
10-5
10-4
10-3
10-2
EDTA (M)

FIGURE 28

The development of B. bacteriovorus in a washed cell suspension of S. serpens in HEPES buffer (0.025M, pH 7.5), heat-killed at 70°C for 10 min, and supplemented with 0.002M levels each of $MgSO_4$ and $CaCl_2$ as assessed by the same parameters shown in Fig. 25. Multiplicity of infection: 3×10^{-6}


S. serpens V.H.L. (70°C, 10 min.)
Absorbance at 280 mμ (x 10⁻²)
+ B. bacteriovorus
Alone
B. bacteriovorus
Transmittance
Transmittance (%)
B. bacteriovorus (log no./ml.)
Time (hours)
80
60
40
20
30
20
10
8
6
4
2
0
20
40
60
80

ALTERATIONS IN THE SUSCEPTIBILITY OF CELLS OF SPECIFIC GRAM-NEGATIVE BACTERIA TO PARASITIZATION BY BDELLOVIBRIO BACTERIOVORUS

HOST RANGE OF B. BACTERIOVORUS 6-5-S

The ability of a selected group of bacteria to support the growth of Bdellovibrio was assessed under four conditions which included the use of (1) a washed cell suspension, (2) a growing culture in YPSC, of each of the potential host cultures and a determination of the ability of Bdellovibrio (3) to establish plaques in a lawn or (4) to cause confluent lysis of each growing on YPSC agar slant. Results from this series of experiments are presented in Table IV and show that the extent of the lysis induced by Bdellovibrio varied with the species of the host cultures tested and the conditions under which host and parasite were combined. The Bdellovibrio failed to multiply in all gram-positive organisms examined, in Lineola longa or in Rhizobium japonicum under any of the conditions examined. The parasite multiplied to only a limited extent in a washed cell suspension of Salmonella typhimurium or Erwinia amylovora. Growing cultures of these gram-negative organisms, conversely, supported growth of the parasite. Growing cultures of Aerobacter aerogenes, S. paratyphi B, Serratia marcescens, Kiebsiella pneumoniae and Proteus vulgaris failed to support growth of Bdellovibrio, although washed cell suspensions of the same organisms allowed the multiplication of the latter. A suspension of Bdellovibrio which contained 30 plaque forming units as assessed on a lawn of S. serpens VHL was equally effective in the production of plaques in a lawn of each of the

TABLE IV

Host range of B. bacteriovorus 6-5-S

Cultures Tested	Suspension of test organisms in THAM buffer, pH 7.5, 0.025M with 0.002M each of $CaCl_2$ and $MgSO_4$	
	Decrease in Turbidity (Klett units)	B. bacteriovorus No/ml, x 10^8 *
Gram-Negative		
Aerobacter aerogenes	16	6.8
Shigella sonni	45	13
Salmonella typhimurium	34	0.7
Salmonella para B	30	3.3
Serratia marcescens	36	36
Pseudomonas aeruginosa	37	35
Klebsiella pneumoniae	25	7.2
Proteus vulgaris	79	10
E. coli B/r	63	12
Erwinia amylovora	2	0.7
Spirillum serpens VHL	76	5
Lineola longa	0	0.1
Vibrio percolans	47	10
Rhizobium japonicum	0	0.2
B. bacteriovorus (initial)		0.2
Gram-Positive		
Bacillus subtilis	0	0.1
Bacillus megaterium	0	0.1
Staphylococcus aureus	0	0.2
Arthrobacter globiformis	0	0.1

* After 4 days incubation at 30°C.

TABLE IV

Host range of *B. bacteriovorus* 6-5-S

Two-membered culture in YPSC broth		Lawn of test organisms in YPSC semi-solid agar	
Decrease in Turbidity (Klett units)	*B. bacteriovorus* No/ml, x 10^8 *	Plaque formation by 30-3000 cells of *B. bacteriovorus*	Confluent lysis established by 10-1000 cells of *Bdellovibrio*
0	0.2	+	+
32	13	+	+
9	8.3	+	+
0	0.2	+	+
0	0.5	+	+
3	0.4	+	+
0	0.2	+	-
0	0.1	+	+
23	11	+	-
0	4.5	+	-
48	3.4	+	-
0	0.07	-	-
32	14	+	-
0	0.02	-	-
	0.2		
0	0.003	-	-
		-	-
0	0.03	-	-
0	0.01	-	-

* After 4 days incubation at 30°C.

** Multiplicity of infection: 2×10^{-2}

other gram-negative bacteria tested, with the exception of the two already mentioned, L. longa and R. japonicum. No relationship existed between these results and the ability of Bdellovibrio to cause confluent lysis on an agar slant of each of the test organisms. No relationship existed either between the ability of the parasite to develop in a two-membered culture and the change of turbidity of those cultures as assessed with the Klett-Summerson colorimeter.

Preliminary data showed that numbers of Bdellovibrio could be determined with a high degree of accuracy by the double agar layer method described by Adams (1959) and Gratia (1936). The confidence limit of the procedure was in excess of 90% with a standard deviation from the mean of ± 8%. The efficiency of the plate counting is equivalent with various host cultures used.

Other preliminary data showed that the maximum number of plaques of the Bdellovibrio in a lawn of S. serpens VHL developed after five days incubation at 30°C (Fig. 29).

HUMIDITY

Plaque size was related to the relative humidity of the environment in which the plates were incubated (Fig. 30). Small, clear plaques developed at 10-30% relative humidity and large turbid plaques with a clear center at relative humidities greater than 40%. The concentration of agar in the medium was also a factor which influenced plaque size. Plaque size is inversely related to agar content of the medium.

HEAT-KILLED SPIRILLA

Previous experiments showed that cells of S. serpens VHL subjected

FIGURE 29

Number of Bdellovibrio plaques developing in a lawn of S. serpens after designated periods of incubation. Results represent the average of counts with 15 plates. The vertical bars indicate the range.

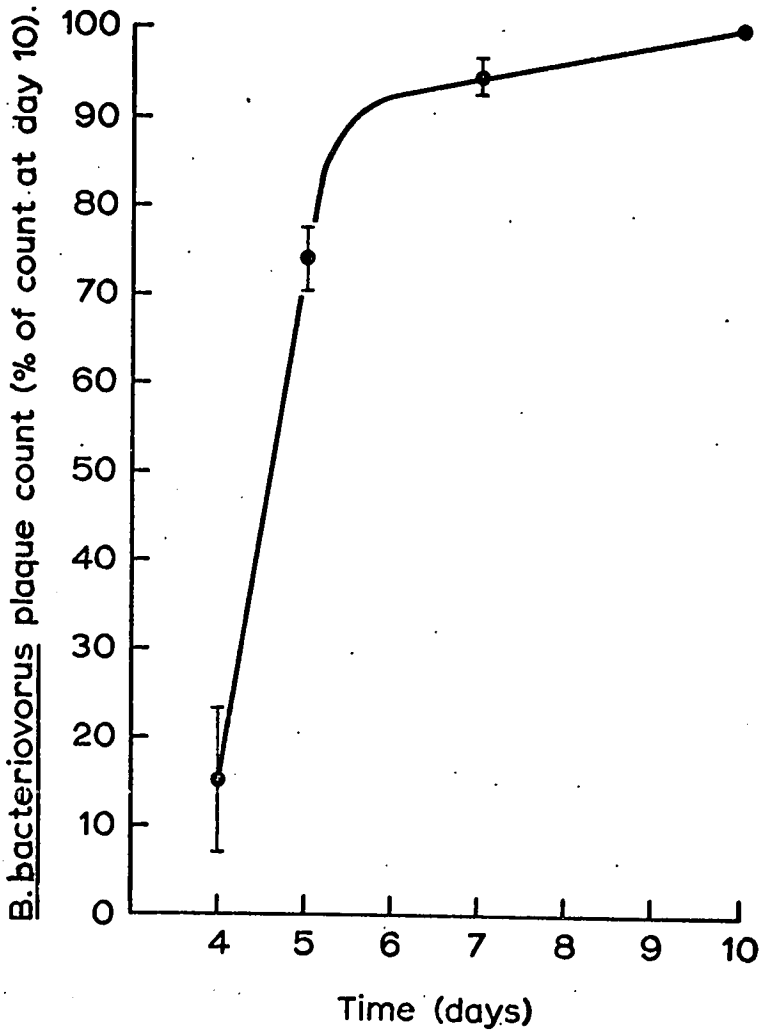
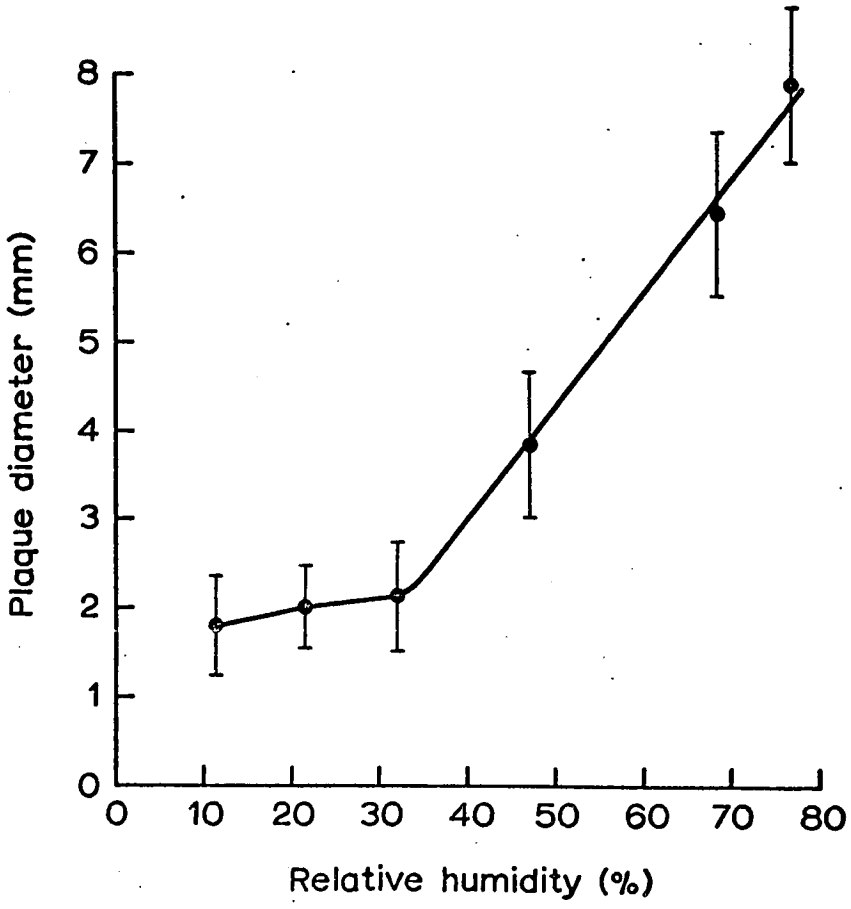

B. bacteriovorus plaque count (% of count at day 10).
100
90
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
Time (days)

FIGURE 30

Effect of relative humidity on the size of plaques produced by Bdellovibrio in a lawn of S. serpens VHL. Relative humidities were provided by saturated salts in sealed battery jars (2L) (Hedlin and Trofimenkoff, 1965). The plaque diameter was the average of 200 plaques in five plates. The vertical bars indicate the range.


Plaque diameter (mm)
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
70
80
Relative humidity (%)

to temperature of 70°C for 10 min supported the growth of Bdellovibrio 6-5-S. Autoclaved cells of S. serpens VHL subjected to sonic oscillation were added aseptically to THAM buffer (pH 7.5) supplemented with Ca^{++} and Mg^{++}. The cell suspension was then inoculated with the Bdellovibrio and the resultant culture was shaken for 5 days at 30°C. Growth of Bdellovibrio was measured by increase in numbers of Bdellovibrio. Results presented in Fig. 31 show that cells subjected to a temperature of 121°C for 5 min also support the growth of Bdellovibrio. The ability of such heat treated cells to support growth of the parasite is rapidly destroyed by sonic oscillation. The results show that failure of the parasite to develop in autoclaved host cells subjected to sonic oscillation for 30 sec coincided with the complete loss of integrity of these host cells.

GLUTARALDEHYDE-TREATED, ULTRAVIOLET-IRRADIATED, OR AUTOCLAVED SPIRILLA

The host cell preparation was also treated by other methods which might alter the nature of its surface. Cells of S. serpens VHL treated separately with glutaraldehyde, by ultraviolet irradiation, and by autoclaving were added aseptically to THAM buffer (pH 7.5) supplemented with Ca^{++} and Mg^{++}. The suspensions of treated cells were then inoculated with Bdellovibrio and the resulting culture incubated on a reciprocating shaker for 3-6 days at 30°C. The loss of turbidity of the culture and increase in number of Bdellovibrio were measured as parameters reflecting Bdellovibrio. Data presented in Table V show that the Bdellovibrio multiplies in ultraviolet-treated cells but not in glutaraldehyde-fixed cells of S. serpens, even when the two-membered culture was

FIGURE 31

The effect of extent of sonic treatment of autoclaved cells of S. serpens VHL on the development of B. bacteriovorus. The vertical bars indicate the range. Multiplicity of infection: 1×10^{-5}

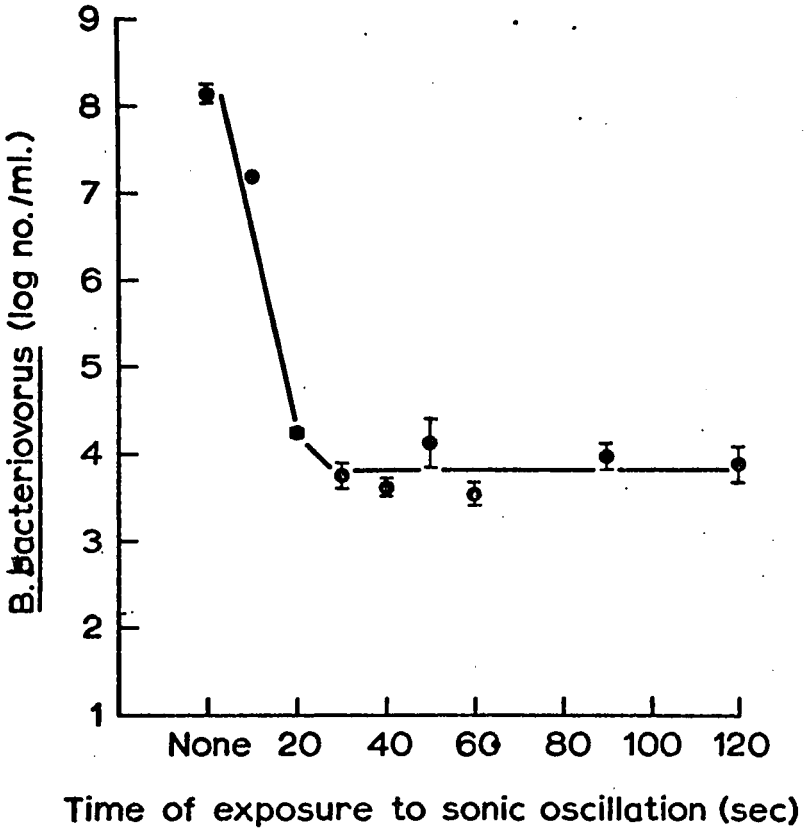

9
8
7
6
5
4
3
2
1
B. bacteriovorus (log no./ml.)
None
20
40
60
80
100
120
Time of exposure to sonic oscillation (sec)

TABLE V

Development of B. bacteriovorus in preparations of S. serpens VHL killed by autoclaving, UV-irradiation and by treatment with 3% glutaraldehyde.

Treatment	Decrease in Turbidity (Klett units)	B. bacteriovorus, No./ml, x 10^6 (days incubation)
S. serpens (3.4 x 10^8/ml)		
Autoclaved (30 min, 121°C)	194	100 (6 days)
3% glutaraldehyde (1 hr, room temperature)	0	0.4 (5 days)
UV-irradiation (30 cm, 20 min)	81	2600 (3 days)
None	60	2200 (3 days)
Bdellovibrio alone (7×10^6/ml)	1	1.0 (6 days)

Multiplicity of infection: 7×10^{-3}

incubated for five days. The Bdellovibrio incubated with the glutaraldehyde-treated cells of S. serpens remain viable during this period, an observation which lends support to the concept that the host cell, but not the parasite, has been influenced by treatment with the glutaraldehyde.

Plaque formation by Bdellovibrio was not demonstrated by the double layer technique in semi-solid agar to which autoclaved, UV-treated or glutaraldehyde-treated cells of S. serpens were added.

SPHEROPLASTED SPIRILLA

Since heat-killed cells of S. serpens VHL were parasitized by Bdellovibrio, the possibility that Bdellovibrio could also parasitize heat-killed spheroplasts of S. serpens was investigated. Spheroplasts were prepared by treating cells of S. serpens initially with EDTA and then with lysozyme. The supporting medium contained sucrose and Mg^{++}. These spheroplast preparations were heated at 70°C for 10 min to reduce activity of lysozyme, washed to eliminate added chemicals and resuspended in YPSC broth or HEPES-NaOH buffer supplemented with Ca^{++} and Mg^{++}. The preparations were inoculated with washed Bdellovibrio and shaken for four days. The results show that heated spheroplasts of S. serpens failed to support the growth of Bdellovibrio.

Nine strains of S. serpens distinguished by pattern dependability were compared for their susceptibility to parasitization by Bdellovibrio. Electron microscopy revealed a hexagonally patterned structured layer in four strains; (Group 1 of Table VI) (VHA, MWI, MW3 and MW11), (Murray, Steed and Maier, 1967); five other strains possessed an atypical linear structure, (Group 2, Table VI) (MW5, MW6), or a smooth surface,

TABLE VI

Susceptibility of nine strains of S. serpens to parasitization by B. bacteriovorus

Strains of S. serpens	Presence (+) or Absence (-) of the structured layer* on the surface of cells of S. serpens	Development of B. bacteriovorus No/ml, x 10^6, 4 days incubation at 30°C
Group 1 (VHA	+	856
(MW1	+	0.1
(MW3	+	234
(MW11	+	0.2
Group 2 (MW 5	Atypical -	1190
(MW 6	Atypical -	1230
Group 3 (VHL	Typical -	1640
(MW2	Typical -	1490
(MW7	Typical -	700
None	N/A	0.2

Cultures of S. serpens and B. bacteriovorus were inoculated simultaneously into YPS C. The structured layer was observed in sonicated preparations of the test organisms using the PTA negative staining technique. (Cells were sonicated 7 sec at 1.5-1.75 amp in a 20 KC Ultrasound, MSE Ultrasonic Apparatus). Multiplicity of infection: 2×10^{-4}

* See Murray, 1963; Murray, et al, 1967.

(Group 3, Table VI), (VHL, MW2 and MW7).

Plaques of Bdellovibrio were readily seen on semi-solid agar seeded with the strains of S. serpens in Group 2 and 3 but not with those in Group 1 harvested from 18 hr slant cultures. The data suggest that the surface-structured layer may play a role in the defence of the host cells against parasitization by Bdellovibrio.

The nine strains of S. serpens and the Bdellovibrio were then inoculated simultaneously into YPSC broth. The results in Table VI show that Bdellovibrio parasitizes and multiplies in a two-membered culture containing each of the mutant strains (Groups 2 and 3) and in similar cultures containing the two parent strains, VHA and MW3 from Group 1. However, Bdellovibrio parasitized neither strains MW1 nor MW11 under the identical condition.

The possibility that the structured layer of S. serpens VHA and MW3 protected host cells from parasitization by Bdellovibrio was further investigated. Although plaques failed to develop in plates seeded with strains VHA harvested from 18 hr slant cultures, turbid plaques, identified as of Bdellovibrio origin, developed in those seeded with a pellet of a 3-day old shaken culture of the same strain. This observation has been interpreted as indicating that S. serpens strain VHA possesses an unstable surface structure under the growth conditions used. This observation has already been made by Steed-Glaister (1967). The turbid plaques were formed probably because the lawn contained both structured and non-structured cells of strain VHA.

Removal of the structured layer of S. serpens MW1 and MW11 by chemical and physical methods was next attempted. Washed cells of S. serpens MW1 and MW11 were mixed with 1-2% EDTA (neutralized) or with 0.1% sodium-lauryl sulfate (neutralized) for 2-4 hr at room temperature. The treated cells were thoroughly washed and resuspended in HEPES buffer or in YPSC and inoculated simultaneously with washed cells of Bdellovibrio. Cells treated with these chemicals were susceptible to parasitization by Bdellovibrio although the untreated cells were not. The same treatment applied to strain VHL did not change its susceptibility to parasitization by Bdellovibrio.

The structured layer of S. serpens, strains MW1 and MW11, is removed by subjecting washed cells from these cultures to 70°C for 10 min. Structural units, separated from the cells which produced them, were identified by electron microscopy in treated cell preparations. Results (Table VII) show that the same heat treatment greatly influences the ability of several of the strains of S. serpens to support growth of the Bdellovibrio. Particularly notable is the observation that the parasite is unable to grow in the presence of untreated cells of S. serpens, strains MW1 and MW11, although it develops readily and extensively with the same strains pre-treated by exposure to 70°C for 10 min.

EVIDENCE FOR PRODUCTION OF LYTIC ENZYMES BY BDELLOVIBRIO BACTERIOVORUS STRAIN 6-5-S DURING PARASITIZATION AND LYSIS OF SPIRILLUM SERPENS

An assessment was made of the ability of Bdellovibrio growing in heat-killed cells of S. serpens to produce enzyme extracellularly which dissolved viable and heat-killed cells of M. lysodeikticus and S. serpens.

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TABLE VII

Development of B. bacteriovorus in heated and unheated cell suspensions of S. serpens* in THAM buffer supplemented with calcium and magnesium after 4 days incubation at 30°C. (Bdellovibrio number/ml, x 10^6)

Strains of S. serpens*	Heated (70°C, 10 min)	Unheated
MW1	340	0.008
MW3	25	2.2
MW11	470	0.027
VHA	500	130
VHL	300	410
None	0.012	0.012

* S. serpens were grown in YPSC, harvested, washed and adjusted to 150 Klett units in THAM buffer. Multiplicity of infection: 1 x 10^{-3}

Crude enzyme preparation from the Bdellovibrio lysate was spotted onto the surface of the semi-solid layer of YPSC agar previously seeded with approximately 5 x 10^8 host cells and overlaying a primary layer of YPSC 1.5% agar. S. serpens strains VHL, MW1 and MW11 and M. lysodeikticus, either heated (121°C, 5 min) or untreated, were employed for this test. Results presented in Fig. 32 show that a crude enzyme preparation from the Bdellovibrio lysate (e) diffused into the semi-solid agar and lysed all heat-killed cell preparations which were tested, including those of M. lysodeikticus. Heat treatment of the crude enzyme preparation destroyed its lytic capacity (he). Under the conditions of the test, the Bdellovibrio (b) failed to lyse viable cells of S. serpens strains MW1 and MW11, although it lysed heat-killed cells of the same organisms to a limited extent after a prolonged period of incubation (7 days). It formed plaques on living or heat-killed cells of S. serpens VHL. Bdellovibrio was without effect on living or heat-killed cells of M. lysodeikticus. These results suggest that the enzyme preparation may contain bacteriolytic enzyme(s). The same enzyme which dissolves M. lysodeikticus may also effect dissolution of heat-killed cells of S. serpens.

Since the Bdellovibrio lysate contained bacteriolytic enzymes, release of amino sugars into the supernatant solution of the lysate was investigated. A washed cell suspension of S. serpens, containing 3-9 x 10^{10} cells/ml in THAM buffer supplemented with cations, was inoculated with similarly washed cells of B. bacteriovorus to a final concentration of 8.5 x 10^6 cells/ml. The content of amino sugar in the culture or in the supernatant solutions obtained from

FIGURE 32

Lysis of heat-killed (top) or untreated (bottom) cells of S. serpens MW11 (left) and M. lysodeikticus (right) by the crude enzyme preparation from a Bdellovibrio lysate in buffer. The crude enzyme preparation is identified as (e), heat-treated enzyme by (he), Bdellovibrio 6-5-S by (b) and suspending medium by (c). An Asahi Pentax camera equipped with a Macro-Takumar 1:4/50 lens was used. Kodak high contrast copy film was exposed (f = 8, 2 sec). Plates were photographed against the built-in dark background of the Quebec colony counter. The clear zones of bacteriolysis appeared as dark zones on the photographic print.

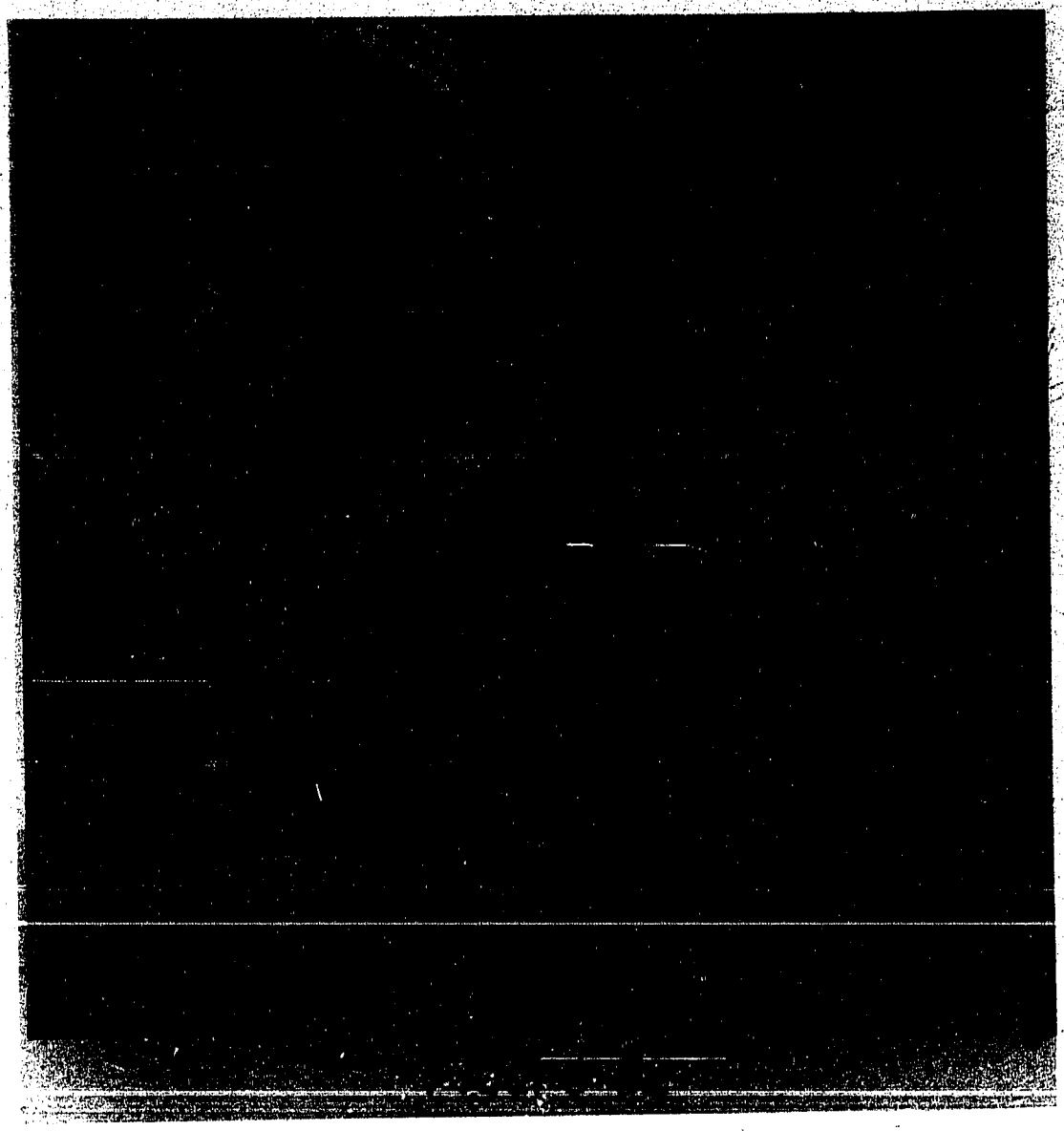

Figure 32

the two-membered culture, was determined after 2.5 days incubation with shaking. Appropriate controls were examined at the same time. The host cells were completely lysed during incubation. The results presented in Table VIII show that the amino sugar content of the supernatant of a two-membered culture increases approximately 10-fold during growth of the Bdellovibrio but remains unchanged with Bdellovibrio alone or with S. serpens VHL alone in the presence of divalent cations and viable cells of S. serpens. The total amino sugar content of the same two-membered system increased slightly, possibly due to the increase in Bdellovibrio. Results are also presented which show that during growth of the Bdellovibrio in a heat-killed suspension of S. serpens, the hexosamine content of the supernatant solution increases 4-fold. Lysis of the host culture and release of amino sugars into the supernatant solution was not detected in the absence of divalent cations.

The release of soluble reducing sugars and free amino groups from cell wall preparations was employed by Ghuysen, et al (1966) to measure respectively, muramidase and glucosaminidase activity and amidase and peptidase activity. The production of reducing sugars, amino groups and amino sugars from heat-killed cells of S. serpens VHL, as a direct result of the action of Bdellovibrio, therefore, may well provide information about the nature of the enzymes found extracellularly during the growth of the latter. The results presented in Table IX show that parasitization of S. serpens by Bdellovibrio increases the total reducing

TABLE VIII

The effect of Bdellovibrio on the release of amino sugars from heat-killed and unheated cells of S. serpens VHL in THAM buffer in the presence and absence of Ca^{++} and Mg^{++}

THAM Buffer	Amino sugars (μg/ml) Initial		After 2.5 Days	
	Total	Supernatant (10s15)*	Total	Supernatant (10s15)*
(1) With Ca^{++} and Mg^{++}				
S. serpens VHL alone	30.2± 1.2	1.4 ± 0.3	29.7± 7.0	1.5 ± 0.0
B. bacteriovorus alone	1.3± 0.2	0.5 ± 0.0	1.4± 0.1	0.8 ± 0.5
S. serpens VHL + B. bacteriovorus	31.0± 2.0	1.7 ± 0.2	40.0± 7.0	15.1± 1.3
Heat-killed S. serpens VHL	24.5± 7.0	1.9 ± 0.1	23.7± 5.9	2.4 ± 1.2
Heat-killed S. serpens VHL + B. bacteriovorus	25.3± 4.2	3.2 ± 0.6	29.8± 4.2	13.7± 2.3
(2) Without Ca^{++} and Mg^{++}				
S. serpens VHL alone	30.0± 2.5	2.8 ± 0.6	25.2± 5.6	1.6± 0.8
B. bacteriovorus alone	4.3± 2.7	1.0 ± 0.0	1.7 ± 0.6	0.2 ± 0.0
S. serpens VHL + B. bacteriovorus	32.1± 7.6	2.7 ± 0.9	27.2± 3.5	1.8 ± 0.6

Initial Bdellovibrio Count: 8.5×10^6.
Initial level of S. serpens: $3\text{-}9 \times 10^{10}$. Multiplicity of Infection: 1×10^{-4}
*Centrifuged fractions were identified according to the proposal of Alexander and Wilson (1955), e.g. 10 s 15 = supernatant obtained after centrifugation at 10,000 x g for 15 min; 100 p 120 = particles sedimented by centrifugation at 100,000 x g for 120 min.

TABLE IX

Comparison of lysis and the amounts of amino sugars, reducing sugars and free amino groups present in the supernatant solution (10 $\overset{s}{-}$ 15*** of the cultures)

Cell Suspensions in THAM buffer	Klett Units	amino sugars (μg/ml) Without acid hydrolysis	amino sugars (μg/ml) With acid hydrolysis	Reducing Sugars (μg/ml)	Free amino groups (mg/ml)
S. serpens VHL alone	850* (830)**	1 (1.5)	12 (14.5)	50 (52)	1.2 (1.45)
B. bacteriovorus alone	11 (10)	0.5 (0.5)	0.75 (0.75)	7.5 (16)	0.2 (0.2)
S. serpens VHL + B. bacteriovorus	800 (250)	1.5 (11)	12.5 (80)	55 (260)	1.4 (5.1)

* Initial
** (After 6 days incubation at 30°C)
*** Refer to Table VIII

Multiplicity of Infection: 1×10^{-2}

sugar content of the supernatant solution by a factor of 5, its amino sugar content by a factor of 5 following acid hydrolysis or by a factor of 19 when the preparation was not treated initially with acid, and the free amino groups by a factor of 3. Increases in the amino sugar and reducing sugar content of the supernatant solution lends support to the concept that a bacteriolytic enzyme is produced by Bdellovibrio, although a possibility exists that these compounds are produced directly by the Bdellovibrio.

An attempt was made to determine when, during the growth cycle of the Bdellovibrio, maximum production of amino sugars occurred. Preliminary results showed that a large proportion of the cells of S. serpens were converted to spheroplasts 36 hr after establishment of the two-membered culture. Complete disruption of host cells and the simultaneous release of Bdellovibrio occurred after 60 hr of incubation. The release of amino sugars into the supernatant solution was determined, therefore, initially and after 36 and 60 hr incubation of a two-membered culture containing Bdellovibrio and viable cells of S. serpens VHL in THAM buffer supplemented with divalent cations. The data presented in Table X show that considerable quantities of the amino sugars were detected in the supernatant solution of the 36 hr culture, at which time maximal spheroplast formation had occurred. The results show that the products of the lytic enzyme are released into solution primarily at the stage in the growth cycle when the host cells are converted to spheroplasts.

TABLE X

Release of amino sugars from viable cells of S. serpens VHL suspended in THAM buffer supplemented with Ca^{++} and Mg^{++} during three distinct phases of the host-parasite relationship

Phase	Total amino sugars (μg/ml)	Level of amino sugars in supernatant ($10 \overset{s}{-} 15$) (μg/ml)
Separate cells of host and parasite (Initial)	298	17
Spheroplast phase (36 hr)	295	105
Fragmented host cells and free Bdellovibrio (60 hr)	304	148

Multiplicity of infection: 1×10^{-2}

The distribution of amino sugars in the Bdellovibrio lysate was investigated using differential centrifugation and dialysis. The lysate was first centrifuged at 10,000 x g for 15 min to obtain a supernatant (10 $\underline{S}$ 15) and pellet (10 $\underline{P}$ 15). Similar amounts of amino sugars were detected in each fraction, determined after acid hydrolysis (Table XI). The 10 $\underline{S}$ 15 fraction was further centrifuged at 100,000 x g for 120 min. The supernatant (100 $\underline{S}$ 120) and pellet (100 $\underline{P}$ 120) contained respectively 31% and 13% of the total amino sugar of the uncentrifuged preparation. Dialysis of the 10 $\underline{S}$ 15 fraction against distilled water (1 liter, 5 changes, for 15 hr at 4°C) showed that only 9% of the total amino sugar content was dialysable and therefore had a molecular weight lower than 3500. These results show that the cell wall of S. serpens parasitized by Bdellovibrio is degraded into fragments which vary considerably in size. A large proportion of the cell (51%) is sedimentable at 10,000 x g and a much smaller portion (9%) is dialysable.

TABLE XI

Distribution of amino sugars in a Bdellovibrio lysate

Treatment of Bdellovibrio lysate	amino sugars	
	µg/ml	%
1. Lysate alone	71.3	100
2. Centrifugation of lysate		
10 $\frac{s}{}$ 15*	34.7	49
10 $\frac{p}{}$ 15	36.6	51
3. Centrifugation of 10 $\frac{s}{}$ 15 fraction		
100 $\frac{s}{}$ 120*	22.3	31
100 $\frac{p}{}$ 120*	9.5	13
4. Dialysis of 10 $\frac{s}{}$ 15 fraction		
dialyzed	5.9	9
retained	28.8	40

* Refer to Table VIII

All determinations were measured after acid hydrolysis.

Multiplicity of Infection: 1×10^{-2}

DISCUSSION

GROWTH CYCLE OF BDELLOVIBRIO BACTERIOVORUS IN SPIRILLUM SERPENS

Stolp and Starr (1963) failed to show intracellular development of Bdellovibrio and therefore classified it as ectoparasite. Our observation and those of Starr and Baigent (1966) and Scherff, et al (1966) showed that Bdellovibrio is an endoparasite. Starr and Baigent (1966) suggested that intracellular growth of Bdellovibrio could be viewed better by a stereoscopic technique than by other means.

Data are presented which show clearly the existence of a sheath and core in the flagellum of Bdellovibrio (Huang, et al, 1966), although their function is unknown. These observations were confirmed by others (Abram and Shilo, 1967; Seidler and Starr, 1968; Berger, et al, 1968; Burnham, et al, 1968). A sheath and core have also been found in the flagellum of Vibrio metchnikovii (Glauert, Kerridge and Horne,

1963).

Electron photomicrographs of intact cells of Bdellovibrio show the presence of blebs or finger-like protrusions from the cell wall and from the flagellar surface. Similar protrusions have also been noted on the cell wall of Bdellovibrio by Shilo (1966) and Burnham, et al (1968) and also on cells of E. coli transferred to distilled water from a nutrient medium (Bayer, 1967). The term "flanna" is suggested for the protruded structure on the flagellum of Bdellovibrio after the name of "granna" in grain. The significance of the finger-like structure is not immediately apparent.

Burnham, et al (1968) noted that in the initial stage of host - parasite interaction, the host cell envelope swelled and formed a bulge by collision and gyrating motion of Bdellovibrio on the host cell wall. It is at this site that Bdellovibrio will attach and eventually penetrate the host cell successfully. Formation of the prerequisite bulge on S. serpens was not observed consistently in the present work (see Fig. 9). However, our data presented in Fig. 12 and those presented by Burnham, et al (1968) strongly suggest that the bulge is formed by the turgor pressure of a host cell after the host cell wall was degraded or weakened. Gram-negative bacteria usually possess four to eight atm of internal pressure (Stolp and Starr, 1965). This pressure may cause disruption of the host cell. Bulge formation may be suppressed either by the Bdellovibrio which has penetrated and effectively sealed the damaged portion of the cell wall or by specific bonding at the same site

as suggested by Starr and Baigent (1966) and Burnham, et al (1968).

Data presented in this thesis show, for the first time, the intracellular development of Bdellovibrio using negatively stained preparations in an electron microscope and fixed, stained preparations in a light microscope. The successful illustration of intracellular growth of Bdellovibrio using negatively stained preparations depends largely on the consumption of host cytoplasm by the parasite. Successful differentiation between living cells of Bdellovibrio and dead or damaged host cytoplasm depends upon the basophilic nature of the former.

Lepine, et al (1967) reported that cells of Bdellovibrio elongated and subsequently divided by binary fission. Data presented in our Fig. 23 (bottom center of plate) show a constricted filament and this suggests that division is accomplished by multiple point constriction.

THE INFLUENCE OF CATIONS ON THE GROWTH OF BDELLOVIBRIO BACTERIOVORUS

It is apparent that addition of Ca^{++} and Mg^{++} markedly increased the population of B. bacteriovorus in distilled water, buffers and the nutrient medium inoculated with the host bacterium, S. serpens VHL. Other points not illustrated in Table I but pertinent to this discussion are: (1) In the absence of its host, Bdellovibrio failed to develop even in the presence of Ca^{++} and Mg^{++}; (2) S. serpens is independent of mineral supplements for growth in the complex nutrient medium; and (3) Growth of Bdellovibrio reaches a maximum after three days incubation at 30°C. Mamkaeva (1966) also reported that addition of 0.1% $CaCl_2$ stimulated lysis of Chlorella vulgaris by a vibrio-like bacterium.

B. bacteriovorus of marine origin required a high salt concentration (3% NaCl) for parasitization to occur (Mitchell, et al, 1967; Shilo, 1966). These findings suggest that Bdellovibrio requires cations to grow, but the amount of cations needed depends on whether the organism is of terrestrial or marine origin.

The importance of cations on growth of Bdellovibrio cannot be understood without considering each step of the life cycle of Bdellovibrio. Five distinct steps have been visualized in the life cycle of the endoparasite, B. bacteriovorus 6-5-S. These include: (1) Survival of parasite outside of host cell and maintenance of active motility for parasitization (Stolp and Starr, 1963); (2) Attachment of the parasite to the host cell and penetration of the cell wall; (3) Development of the parasite in the host; (4) Morphological change of the host; and (5) Release of mature parasite progeny.

It is unlikely that cations facilitated the locomotion of B. bacteriovorus since the parasite remained motile in the medium with or without cations. Simpson and Robinson (1968) have proposed that endogenous metabolism of B. bacteriovorus may provide the energy for the motility of Bdellovibrio.

The part played by metallic ions in host - parasite interaction was extensively reviewed by Weinberg (1966). Fifteen cations, namely, Na^{+}, K^{+}, Rb^{+}, Cs^{+}, Mg^{++}, Ca^{++}, Zn^{++}, Cd^{++}, Cr^{+++}, Cu^{++}, Mn^{++}, Co^{++}, Ni^{++}, Al^{+++} as well as NH_4^{+} activate one or more enzymes according to Dixon and Webb (1965) and many of these are reported here

(Table III) to stimulate the growth of Bdellovibrio. Two possible explanations for the wide range of cation requirement are: (1) various cations are required for the integrity of the cell membrane of S. serpens VHL which appears to be important for the intracellular development of the Bdellovibrio; (2) the two-membered culture established in buffer contains trace amounts of other essential cations. Since cells of S. serpens VHL and Bdellovibrio were previously cultivated in YPSC or modified N. B-A broth and yeast extract contains detectable amounts of Mg^{++}, Fe^{++}, Zn^{++}, Cu^{++}, Cr^{++} and Ni^{++} (Grant and Pramer, 1962), it is not surprising to find intracellular levels of these cations in the cell suspensions (Webb, 1968), even after washing in water. One would also expect that HEPES buffer, adjusted to pH 7.5 with NaOH and supplemented with specific salts would also contain trace amounts of contaminating cations originating from the individual salts incorporated in the buffer. The trace amounts of ions may act independently on the Bdellovibrio or they may act synergistically with one or more of the components of the buffer. Webb (1968) observed that Mn^{++} exerted a Mg^{++} sparing effect for a specific Bacillus.

Tables I and III show that Bdellovibrio requires certain levels of cations. The data presented in these tables do not explain the role of a specific cation properly. Multiplication occurred equally well with a variety of individual cations, including those which have normally been considered toxic, Pb^{++}, Zn^{++}, etc. have been included in this latter group.

Data presented in Fig. 26 show that delayed addition of cations to the buffer containing the two-membered culture shortens the lag time, a fact which may be explained in several ways. Webb (1968) noted that addition of Mg^{++} to a Mg^{++}-deficient medium resulted in stimulation of the growth rate of B. subtilis. Later additions of Mg^{++} to similar Mg^{++}-deficient cultures resulted in a progressively more rapid rate of growth of the test organism, although the length of the lag phase tended to increase (Webb, 1968). The author interpreted his results as indicating that the requirement for Mg^{++} was reduced by the presence of organic or inorganic products released from autolyzing cells. The shortening of the lag period in the growth of Bdellovibrio, following delayed addition of Mg^{++} to the two-membered culture, may be influenced similarly by release of organic or inorganic nutrients from S. serpens. Another way to explain the shortened lag period in the growth of Bdellovibrio following delayed addition of cations is that the attachment and penetration of Bdellovibrio to a susceptible host cell occurs in the absence of Ca^{++} and Mg^{++}, but the multiplication of the intracellular Bdellovibrio proceeds only after the addition of Ca^{++} and Mg^{++}. The long lag which follows initial addition of the cations may reflect only the time required for the parasite to become attached to and possibly also to penetrate into its host cell. Actually the time required for attachment to and penetration into the host cell by Bdellovibrio was not measured for purposes of this experiment. A greatly shortened lag period would be obtained following delayed addition of divalent cations to a washed cell suspension of S. serpens to which

Bdellovibrio were already attached, if the divalent cations are required only for multiplication of Bdellovibrio.

The study on the role of cations on the growth of Bdellovibrio in S. serpens VHL is hampered by technical problems which include such points as morphological and physiological synchronization of the host-parasite interaction, and measurement of the fate of bound or free cations intracellularly or extracellularly in the host cells parasitized at different stages.

Development of Bdellovibrio in a washed cell suspension of S. serpens VHL shows that the host cell contains sufficient organic nutrients for the Bdellovibrio to grow when supplemented with cations. Under the same conditions sonic extracts of a washed cell suspension of S. serpens VHL failed to support the growth of B. bacteriovorus. This fact may be interpreted in two ways. One is that the nutrilite, essential for the growth of the parasite and produced by the host cell, is destroyed by sonication (Kapoor, et al, 1965); another is that the intact host cell provided a micro-environment in which the parasite could develop. The latter suggestion is not unreasonable in view of the observed development of Bdellovibrio in an intracellular cavity between cell wall and cytoplasmic membrane of the host cell which has been seen in many published electron micrographs (Burger, et al, 1968; Burnham, et al, 1968; Lépine, et al, 1967; Scherff, et al, 1966; Starr and Baigent, 1966; Stolp, 1967, 1968).

Data presented earlier from this laboratory (Huang, 1968; Robinson and Huang, 1967; Huang, et al, 1966, and confirmed in

the present study show that Bdellovibrio will grow in a culture of heat-killed host bacteria. The kinetics of growth apparently indicate that an increase in the Bdellovibrio numbers coincides with lysis of the host bacteria. The increase in Bdellovibrio number was not caused by the selection of host-independent mutants. This endoparasitic growth of Bdellovibrio in heat-killed host cells, especially those which were autoclaved, suggests the following points: (1) Bdellovibrio actively parasitizes the bacterial host cell; (2) Bdellovibrio synthesizes the entire enzyme complement required for its normal growth and for lysis of the host cell.

ALTERATIONS IN THE SUSCEPTIBILITY OF CELLS OF SPECIFIC GRAM-NEGATIVE BACTERIA TO PARASITIZATION BY BDELLOVIBRIO BACTERIOVORUS

Results presented in this study show that the host range of Bdellovibrio is influenced greatly by the medium in which host and parasite are combined and by the method employed for detecting growth of the parasite. A wide spectrum of host activity was observed when Bdellovibrio was grown in host cells suspended in buffer, either THAM or HEPES, supplemented with essential cations as determined by increase of plaque-forming units or by decrease of optical density of the culture. A narrow spectrum of host activity was observed, however, when host and parasite were incubated together in YPSC. These results bore no relationship to those obtained when host and parasite were incubated together on a surface of agar slant (last column, Table IV). Suspension of Bdellovibrio and potential host cells in buffer supplemented with

appropriate cations and adjusted to a pH optimum for the growth of Bdellovibrio may well represent the ideal environment for the study of this particular host-parasite relationship.

Stolp and Starr (1963) have reported that their strains of Bdellovibrio parasitize only living host cells. For determination of host range in THAM buffer supplemented with cations, (Stolp and Starr, (1963) washed living cells of the susceptible host harvested from 12 to 15 hr cultures were employed. These washed host cells might die during the 48 hr incubation period in buffer. Death of host cells, however, would not interfere with the determination of host range because Bdellovibrio strain 6-5-S develops in living and dead host cells, provided either artificially, as by heat-treatment, or spontaneously. B. bacteriovorus strain W (Burger, et al, 1968) was also found to infect dead host cells. The buffer system therefore may be suitable for the detection of host range of other Bdellovibrio isolates.

The multiplication of Bdellovibrio in appropriate host cells is followed by an increase in the number of Bdellovibrio in the two-membered culture and usually by a decrease in the turbidity of the culture. The latter observation is a direct reflection of the lysis of host cells. Occasionally, however, Bdellovibrio develop in the two membered culture in which the optical density either remains stationary or actually increases. Stolp and Starr (1963) have observed this phenomenon which is also noted in the present series of experiments with Erwinia amylovora cultivated in YPSC (Table IV). The development of resistant or of

partially resistant host cells by genotypic change or by temporary physiological changes may explain why host cells occasionally fail to lyse despite infection by Bdellovibrio.

Indirect evidence is presented in Table VI which shows that the hexagonally structured layer described by Houwink (1953) and by Murray (1963) at the surface of cell walls of S. serpens may protect this organism from parasitization by the Bdellovibrio. This layer, produced by S. serpens strain VHA may contain protein and may be associated with a "backing" substance, mainly phosphatidyl ethanolamine as suggested by preliminary data presented by Steed-Glaister (1967). S. serpens strain VHL, produced "backing" without the hexagonal pattern. The components of the structured layer of S. serpens VH are readily stripped from the underlying layer by sodium lauryl sulfate or by water saturated with phenol (Murray, 1963). These chemicals are also known to remove at least lipoproteins and lipopolysaccharides from the cell wall of gram-negative bacteria (Martin and Frank, 1962). Data also presented by Steed-Glaister (1967) show that the structured layer on the surface of cells of S. serpens VHA is removed at low pH by ethylenediaminetetraacetic acid (EDTA) and by heat. EDTA is also known to damage the cell wall probably (1) by removing components of ionic cross-linkages, (2) by removing portions of some lipopolysaccharides (Martin, 1963; Asbell and Eagon, 1966) and mucopeptide (Eagon, Simmons and Carson, 1965; Gray and Wilkinson, 1965) and (3) by disrupting the lipoprotein layer of gram-negative cell walls (Salton, 1964). Three of the

four treatments which remove the structured layer from S. serpens VHA or VH effectively convert S. serpens strains MW1 and MW11 from resistant cells to cells susceptible to parasitization by Bdellovibrio. The structured layer found on S. serpens MW1 and MW11, therefore, may physically or chemically protect the host cell from extensive attack by the parasite or it may cover possible Bdellovibrio attachment sites at the surface of the host cell.

Our stock cultures of S. serpens may be grouped as follows according to their susceptibility to parasitization by Bdellovibrio and according to the nature of the cell surface of the organisms: (1) Those which have a smooth surface (VHL, MW2 and MW7) and are susceptible to parasitization by Bdellovibrio; (2) Those with an atypical linear structure (MW5 and MW6) also susceptible to parasitization by Bdellovibrio; (3) Those with a hexagonally patterned surface structure (VHA and MW3) are susceptible to parasitization by Bdellovibrio in liquid culture but not in semi-solid agar and finally (4) Those with a hexagonally patterned surface (MW1 and MW11) which resist parasitization by Bdellovibrio in liquid or in agar culture.

The fact that some strains from group 1 and all of group 2 are susceptible to parasitization by Bdellovibrio may be attributed to incomplete wrapping of the cells by the structured layer or by instability of this layer under certain conditions of cultivation.

S. serpens, normally parasitized by Bdellovibrio strain 6-5-S may be treated to render it susceptible to parasitization. An assessment of the possible effects of each treatment will provide

preliminary information about the nature of the factor or factors in S. serpens to be required for the establishment of the successful host-parasite relationship. The host cells (S. serpens VHL, in this case) are effectively killed by heat, and by ultra-violet treatment, also they remain as units which are susceptible to "parasitization" by Bdellovibrio. Heat denatures protein, disrupts membrane lipids but is without effect on carbohydrates. The heat denaturation of nucleic acids is reversible (Davis, et al, 1968). Ultra-violet irradiation of nucleic acids hydrates pyrimidines at the four-five linkage to form dimers which block the replication of the nucleic acid. Protein lipid, and nucleic acid of the host cell, therefore, are not likely required for the intracellular development of Bdellovibrio. Glutaraldehyde cross-links with active hydrogen amino and imino groups in protein and hydroxyl groups of polyalcohols. This cross-linking renders some but not all proteins insoluble. The activity of many enzymes is unaffected by treatment with glutaraldehyde (Sabatini, et al, 1963). Glutaraldehyde-treated cells of S. serpens VHL fail to support the growth of Bdellovibrio, an observation which suggests that the compound may modify the outer structures of the host cell, possibly by combining with or otherwise modifying the hypothetical receptor sites mentioned in an earlier paragraph.

EVIDENCE FOR PRODUCTION OF LYTIC ENZYME(S) BY BDELLOVIBRIO BACTERIOVORUS DURING PARASITIZATION AND LYSIS OF SPIRILLUM SERPENS

Many investigators (Stolp, 1968; Starr and Baigent, 1966; Varon and Shilo, 1968) have suggested that enzyme activity is involved in the

parasitization of bacteria by Bdellovibrio. Shilo and Bruff (1965) reported that saprophytic mutants of B. bacteriovorus strain A3:12 possessed a proteolytic enzyme which degraded heat-killed bacteria. Scherff, et al (1966) also reported that Bdellovibrio after it entered an appropriate host cell, gradually degraded the host protoplasm, presumably as a result of proteolytic enzyme activity. In the present study we have considered the possibility that other lytic enzyme(s) are produced by parasitic strains of B. bacteriovorus and that the enzyme is important for the establishement of successful host-parasite relationships.

Fleming demonstrated in 1922 that lysozyme dissolves cells of Micrococcus lysodeikticus, an organism that has since been used in the quantitative determination of the activity of this particular enzyme. Release from bacterial mucopeptide of soluble amino sugars and free reducing sugars has also been considered as a measure of lysozyme activity (Perkins, 1960). The possibility exists, however, that enzymes other than lysozyme such as peptidase or amidase may hydrolyse the bacterial cell wall by splitting penta-peptide chains or cross-bridge chains of the mucopeptide network of the cell wall. Strominger and Ghuysen (1967) considered that an increase in the concentration of free amino groups in a reaction mixture containing cell wall preparations and lytic enzymes as a measure of the activity of peptidase or amidase.

A number of procedures were employed: these included (1) an assessment of the ability of a crude enzyme preparation from Bdellovibrio

lysate to clear a lawn of Micrococcus lysodeikticus (Salton, 1957); (2) an assessment of the ability of viable cells of Bdellovibrio to dissolve viable or heat-killed cells of S. serpens with the simultaneous release into solution of reducing sugars and amino sugars. These observations seem to suggest that the lytic enzyme is produced by B. bacteriovorus strain 6-5-S during "parasitization" of heat-killed or viable cells of S. serpens.

The increased amino sugar content of the two-membered culture is not related to degradation of the Bdellovibrio since increases in the numbers of the Bdellovibrio parallelled the release of amino sugars and since the Bdellovibrio contained low levels of amino sugar. Additional indirect, but confirmatory, evidence was provided by Murray and Maier's (1965) observations of electron micrographs which failed to show the typical mucopeptide layer usually seen in gram negative bacteria.

The release of soluble amino sugars very likely occurs during the stage of host-parasite relationship when the host cells are transformed from their normal shape to spheroplasts. Rupture of some host cells before complete spheroplasting of all the host may occur since the two-membered culture was not synchronized. Release of the same products of the enzyme(s) activity in still greater amounts is associated with liberation of the Bdellovibrio progeny. This observation may imply a relationship between the two factors. These data suggest

that lytic enzyme(s) may be produced by Bdellovibrio only during its presence as an intracellular inhabitant. Thus, the enzyme may be responsible for weakening the host cell envelope with consequent "lysis from within."

The walls of viable cells of S. serpens are degraded by the Bdellovibrio into different-sized fragments, some of which are dialyzable while others are retained in the dialyzing bag or are large enough to be sedimented by centrifugation at 10,000 x g. An explanation of the appearance in the reaction mixture of large-sized cell fragments apparently refractory to degradation by the enzyme liberated by the Bdellovibrio would appear to be in order. This anomalous situation may indicate that all sections of the mucopeptide layer of the cell wall are not uniformly susceptible to degradation by the Bdellovibrio enzyme.

Cessi and Piliego (1960) modified the Elson-Morgan reaction for the determination of amino sugars. A positive Elson-Morgan reaction indicates that the bacterial cell wall has been disrupted to yield disaccharide fragments of N-acetyl glucosamine and N-acetyl muramic acid, either free or attached to a peptide molecule (Ghuysen, et al, 1966). The actual mechanism of the reaction is still unknown. Cornforth and Firth (1958), however, suggested that an intermediate compound is formed by condensation of hexosamine with acetyl acetone. The intermediate, in alkaline solution, subsequently loses its polyhydroxy chain and its acetyl group to yield 2-methyl pyrrole, a volatile and chromogenic substance which reacts with a para-dimethylaminobenzaldehyde

(Ehrlich's reagent). The distillation step employed by Cessi and Piliego (1960) eliminates the possibility of reaction of the Ehrlich's reagent with amino acids, carbohydrates other than amino sugars, and probably those disaccharide fragments attached to peptide moieties in the reaction mixture.

SUMMARY

1. The growth cycle of an obligatory parasite, B. bacteriovorus 6-5-S, which attacks living cells of S. serpens VHL was followed using light and electron microscopic techniques.

Five distinct steps were visualized in the establishment of the host-parasite relationship: (1) Attachment of the parasite to the host cell; (2) Penetration of the cell wall of the host by the parasite; (3) Change of the morphology of the living host; (4) Development of the parasite in the host; (5) Release of mature parasite progeny.

In the early stage of attachment of Bdellovibrio to S. serpens, a bulge sometimes develops on the cell wall of the latter after contact between the two organisms. A combination of factors, including damage to the host cell wall and turgor pressure very likely contributes to the development of the bulge.

The parasite develops intracellularly by thickening and elongation to form a long ribbon-like structure. The ribbon, upon maturation, segments into individual parasitic units, presumably by constriction.

Dead cells, including those which have been heat-killed, are also parasitized, although initially they remain unchanged morphologically. Release of Bdellovibrio progeny and fragmentation of the host

cell occur simultaneously.

2. B. bacteriovorus 6-5-S requires cations for the parasitization of S. serpens. Parasitic activity was assessed by several parameters, including increase of plaque-forming units, decrease of transmittance of the two-membered culture at 520 mμ and release of substances into the medium which absorb maximally at 260 mμ. Our indirect data may suggest that cations facilitate neither the attachment of Bdellovibrio nor the invasion into the host cells by the parasite; the prime role is probably that of a growth factor. The requirement for cations is non-specific. Addition of 0.01M EDTA halted the development of Bdellovibrio in the culture of S. serpens containing cations. Washed cells of S. serpens in THAM and HEPES buffers and in distilled water supplemented with cations supported the growth of Bdellovibrio. Bdellovibrio grows more slowly on heat killed than on living cells of S. serpens.

3. The host range of B. bacteriovorus 6-5-S was determined by a number of direct procedures including: (1) determination of the increase in numbers of Bdellovibrio in a two-membered culture established in YPSC broth or in HEPES or THAM buffer; (2) a determination of the ability of the Bdellovibrio to establish plaques in a lawn seeded with each of the potential host cultures; (3) A determination of the ability of the Bdellovibrio to form confluent lysis of potential host cultures growing on a nutrient agar slant.

The host range of B. bacteriovorus 6-5-S was also determined

by an indirect procedure which involved assessment of the decrease of turbidity of the two-membered culture in YPSC or THAM or HEPES buffer supplemented with 0.002M level of $CaCl_2$ and $MgSO_4$.

No direct relationship between results obtained by any of the methods employed was evident. For purposes of the present study, washed cell preparations of potential host cultures were suspended in buffer and a two-membered system established by the addition of appropriate numbers of the Bdellovibrio. By using this procedure, modifications of nutrient medium as a result of growth of different host cultures were maintained at a minimum.

4. The structured layer on the cell wall of S. serpens strains MW1 and MW11 (Murray, 1963) appears to protect the cells from parasitization by Bdellovibrio. Removal of the structured layers by a variety of procedures invariably rendered the cells susceptible to parasitization. The structured layer was effectively removed by treatment of the cells with EDTA and sodium lauryl sulfate and by heating (at 70°C, for 10 min).

5. A washed cell suspension of S. serpens strain VHL supports extensive growth of the test strain of Bdellovibrio in buffer. Treatment of the same cells by UV-irradiation or by heat (70°C, 30 min) does not alter the capacity of this strain of S. serpens to support growth of the Bdellovibrio. The host cell treated with glutaraldehyde is not parasitized by the Bdellovibrio.

6. During the parasitization of S. serpens by B. bacteriovorus, the latter produced lytic enzyme(s) which degraded the host cell with the consequent release into a two-membered system in buffer soluble reducing sugars and amino sugars. The crude enzyme preparation from Bdellovibrio lysate dissolved cells of M. lysodeikticus. Amino sugar was not detected in the culture supernatant during the attachment and penetration of the parasite into the host cell, but was detected readily when the host cells were converted from their typical spirillum shape to spheroplasts and also after its eventual disruption.

PROPOSAL FOR FURTHER WORK

An extensive study on the mechanism of bacteriolysis caused by Bdellovibrio lysozyme should be carried out. Possible fields of study include: (1) identification of the intermediate and final products of the lysozyme activity on the host cell in the two-membered culture; (2) isolation, purification and characterization of the lysozyme.

Host specificity of Bdellovibrio measured in complex media is influenced greatly by the composition, physical condition of both host and parasite, and the metabolites produced into the media. The buffer supplemented with essential cations as described in the present study should be a useful tool to study the host-range spectrum in defined conditions. A comparative study of host specificity in THAM-HCl and HEPES-NaOH buffer for descriptive and systematic purposes should be made. The Bdellovibrio strains now available from many workers should be systematically characterized for GC content, immunological characteristics and the host range.

Bdellovibrio growth may be synchronized by addition of Ca^{++} and Mg^{++} to the two-membered culture after the host and parasite are cultivated together in buffer or YPSC broth deprived of the added cations.

Bdellovibrio is able to cause physical damage to the host cell wall and to expose mucopeptide, a cell wall rigid component, to the action of Bdellovibrio lysozyme. This system of physical damage may be more specific than EDTA-lysozyme system for the study of cell walls of gram-negative bacteria. The isotope labelling of either the host cell or the parasite or both may enable one to differentiate the origin of chemical components in the two-membered culture.

Studies on the effect of Bdellovibrio on the metabolism, growth and chemical and enzymatic changes of the host cell will increase the understanding of the mechanism of parasitization by this microorganism.

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APPENDIX I

LIST OF B. BACTERIOVORUS STRAINS ISOLATED AND BEING STUDIES

Original Strain Number	ATCC Number	Host Bacterium	Origin	Reference
A. Parasitic Strains				
Bd100	15356	Erwinia amylovora EA 137	Soil	Stolp and Starr, 1963 Murray, 1964 Murray and Maier, 1965 Starr and Baigent, 1966
Bd101		Erwinia catovora EC 153	Soil	Stolp and Starr, 1963
Bd109	15143	E. coli B-2262	Soil	Stolp and Starr, 1963 Varon and Shilo, 1968 Burnham, et al, 1968 Seidler and Starr, 1968
Bd110	15360	Aerobacter aerogenes 2001	Soil	Stolp and Starr, 1963
Bd114	15362	Proteus mirabilis	Soil	Stolp and Starr, 1963
Bd118	15364	Serratia marcescens 2031	Soil	Stolp and Starr, 1963
Bd120		Bacterium stewartii SS12	Soil	Stolp and Starr, 1963
Bd127		Pseudomonas solanacearum PS138	Soil	Stolp and Starr, 1963
Bd128		Aerobacter cloacae 2112	Soil	Stolp and Starr, 1963
Bd321	11355	Pseudomonas phaseolicola	Soil	Stolp and Starr, 1963
Bd233	15372	Pseudomonas tabaci NRRL B877	Soil	Stolp and Starr, 1963 Starr and Baigent, 1966
Bd A. 3:12	12633	Pseudomonas fluorescens	Soil	Stolp and Starr, 1963
Bd Strains		Enterobacteriaceae	Sewage Activated Sludge	Dias and Bhat, 1965

Bd 6-5-S	Spirillum serpens MW5	Sewage	Huang, et al, 1966 Simpson and Robinson, 1968 Robinson and Huang, 1967 Huang, 1968
Bd156-2D	Pseudomonas fluorescens migula	Sewage	Scherff, et al, 1966
Bd OX9-1	E. coli OX9	Soil	Klein, et al, 1966 Klein and Casida, 1967
Bd167-1	E. coli 167	Soil	Klein and Casida, 1967 Klein, et al, 1966
Vibrio-like bacterium	Chlorella vulgaris Beijer 197 Gromov Strain	Mass algae	Mamkaeva, 1966
Bd Strain	E. coli K12	Sea Water	Mitchell, et al, 1967
Bd XTy	Salmonella typhi Vi4	River Water	Guelin, et al, 1967 Lepine, et al, 1967
BdW	Rhodospirillum rubrum	Soil	Burger, et al, 1968
Bd-p-167	E. coli B-167	Soil	Sullivan and Casida, 1968
Bd sp.	Hyphomicrobium sp. and Pseudo. fluorescens	Sewage or Soil	Wood and Hirsch, 1966
B. Saprophytic Strains			
Sp. 6			Stolp and Petzold, 1962
Sp. 8			Stolp and Petzold, 1962
Sp. 32			Stolp and Petzold, 1962
Sp. 19		Bd 321 of	Stolp and Starr, 1963
HI-Bd			Seidler and Starr, 1967
Host-Independent A. 3:12		BdA. 3:12	of Stolp and Starr, 1963 Shilo and Bruff, 1965

APPENDIX II

COMPOSITION OF MEDIA USED FOR TESTING PARASITIZATION OF MICROORGANISMS BY B. BACTERIOVORUS

Media	Composition and pH	Bdellovibrio Strains	References
N. B-a (Nutrient broth)	0.8% Bacto-nutrient broth 0.5% Bacto-casamino acids 0.1% Bacto-yeast extract 0.02% $Ca(NO_3)2$ 0.001% $FeSO_4$ 0.001% $MnSO_4$ pH = 6.8	Bd205-354 Bd607-613	Stolp & Petzold, 1962
1/10 N.B. (diluted nutrient broth)	1 vol. N.B + 9 vol. solution containing 0.02% $Ca(NO_3)_2$, 0.001% $FeSO_4$ and 0.001% $MnSO_4$	Bd. 3.12 Bd. 109	Stolp & Starr, 1963 Seidler & Starr, 1968 Varon & Shilo, 1968 Shilo & Bruff, 1965
N.B.-HP (Yeast-peptone)	1% Bacto-peptone 0.3% Bacto-yeast extract pH = 6.8	Sp. 6, Sp. 8, Sp. 17, Sp. 32, Bd strain Bd 3.12	Stolp & Petzold, 1962 Mitchell, et al, 1967 Seidler & Starr, 1968
Y.P. (Yeast-peptone)	0.3% Bacto-yeast extract 0.06% Bacto-peptone pH = 7.2	Bd. 100, 101 109, 110, 114, 118, 120, 127, 128, 321, 233, A. 3. 12 Bd strains Bd OX9-1	Stolp & Starr, 1963 Dias & Bhat, 1965 Klein & Casida, 1967

		Bd167-1 Bd W ATCC 15143	Klein & Casida, 1967 Burger, et al, 1968 Burnham, et al, 1968
1/4 Y.P. (diluted yeast-peptone)	1 vol. Y.P. + 3 vol. distilled water	Bd x Ty	Gruelin, et al, 1967
YPDA (Yeast-peptone dextrose agar)	0.3% Bacto-yeast extract 0.06% Bacto-peptone 0.3% Dextrose 0.1M THAM buffer pH = 7.2	Bd 156.2D	Scherff, et al, 1966
TYP (THAM-yeast-peptone)	0.3 % Bacto-yeast extract 0.06% Bacto-peptone 0.5% THAM buffer pH = 7.5	Bd.100 Bd.233 Bd.321	Starr & Baigent, 1966
FP	1% Bacto-protese peptone 0.5% meat extract 0.2% NaCl 0.05% K_2HPO_4	Bd. W	Burger, et al, 1968
FGP	1% Bacto-protese peptone 0.5% Meat extract 0.2% NaCl 0.05% K_2HPO_4	Bd. W	Burger, et al, 1968

APPENDIX II - continued

Media	Composition and pH	Bdellovibrio Strains	References
Mineral Medium (Medium I)	0.01% KNO_3 0.0033% $MgSO_4$ 0.00667% K_2HPO_4 0.1% (v/v) microelement solution which contains: 0.0022% $ZnSO_4$ $7H_2O$ 0.181% M. SO_4 0.0079% $CuSO_4$ $5H_2O$ 0.263% $NaBO_3$ $4H_2O$ 0.1% $(NH_4)6MO_7O_{24}$ $4H_2O$ 0.93% $FeSO4$ $7H_2O$ 0.12% $CaCl_2$ 0.002% $Co(NO_3)_2$ H_2O 1% EDTA pH = 7.0 - 7.2	Vibrio-like bacterium	Mamkaeva, 1966
Campbell-Hoffer Medium	0.1% Calcium glycerophosphate 0.02% $MgSO_4$ 0.02% NaCl 2% sauer kraut juice (v/v) pH = 7.6	Bd-P-167	Campbell & Hoffer, 1943

Modified Brown's Medium	0.05% glucose 0.02% $MgSO_4 \cdot 7H_2O$ 0.004% $FeSO_4$ $7H_2O$ 0.0005% Na_2MoO_4 0.015% $CaCl_2$ 0.08% K_2HPO_4 pH = 6.8 - 7.0	Bd-P-167	Brown, et al, 1962 Sullivan & Casida, 1968

APPENDIX III

GENERA OF MICROORGANISMS BEING TESTED FOR PARASITIZATION BY B. BACTERIOVORUS

	Genera	
Gram-negative bacteria	Aeromonas	Hyphomicrobium
	Acetobacter	Klebsiella
	Alcaligenes	Pasteurella
	Athrobacter	Proteus
	Achromobacter	Pseudomonas
	Agrobacterium	Protaminobacter
	Aerobacter	Pectobacterium
	Azotobacter	Rhizobium
	Azotomonas	Rhodospirillum
	Bacterium	Rhodopseudomonas
	Brucella	Spirillum
	Caulobacter	Salmonella
	Erwinia	Shigella
	Escherichia	Serratia
		Vibrio
		Xanthomonas
Gram-positive bacteria	Bacillus	Streptococcus
	Corynebacterium	Staphylococcus
	Clostridium	Streptomyces
	Leuconostoc	Nocardia
	Lactobacillus	Proactinomyces
	Micrococcus	
	Mycobacterium	
Others	Chlorella	