PHYSIOLOGICAL STUDIES OF THE BDELLOVIBRIO-HOST INTERACTION

THESIS

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By

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The purpose of this study was to focus attention on the physiology of the bdellovibrio-host interaction and to determine the metabolic requirements for this reaction. Since bdellovibrio is an aerobic organism, direct measurements of respiration, turbidity, and viable cell counts are reliable indications of the metabolic activity of the cells.

It was determined that the metabolic requirements for the parasitic interaction are constituents from either metabolically active host cells or cells which are capable of at least some metabolic activity. The nutritional requirements of host-independent bdellovibrios suspended in buffer are not met by the presence or absence of viable or nonviable Enterobacter aerogenes. Unlike the HD bdellovibrios, the HI bdellovibrios lack the ability to make economical use of their self-digesting processes.

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CHAPTER I

INTRODUCTION

Literature Review

Discovery. In 1962, two experimental accidents resulted in the discovery of a most unusual microorganism (41). The first accident occurred when Heinz Stolp and Heinz Petzold exhausted their supply of 0.2 μm filters while attempting to isolate phage from German soils. This led them to use a filter system with a pore size of approximately 1.35 μm. A soil suspension, previously inoculated with Pseudomonas phaseolicola ATCC 11355 for phage enrichment, was filtered. Bacterial lawns in petri dishes and the common double-layer technique were then employed to test the filtrate for the presence of phage. After 24 hours of incubation, no lytic zones had appeared on the bacterial lawn. Normally the cultures would have been thrown out; however, the plates were examined after three days for phage plaques. This was the second accident. This time areas of lysis were noticed. Another unusual feature was that these areas in time increased in diameter, whereas phage plaques normally remain the same size, suggesting that lysis of the host cells was caused by something other than phage. Upon examination with a phase-contrast microscope, small rapidly moving microorganisms were seen.

They appeared to be colliding with and causing the lysis of the bacteria.

Stolp and Starr named this organism Bdellovibrio bacteriovorus in 1963 (42). "Bdello-" is derived from the Greek word for leech; "-vibrio" represents the comma shape of the parasite; "bacteriovorus" means bacteria-eater (39). Bdellovibrio is a unique bacterium since, as its name implies, it is able to parasitize other bacteria. It has been observed to physically collide with, penetrate, multiply within, and devour its host. Since its discovery, Bdellovibrio has been the subject of much research.

Isolation. These parasites have been isolated from a variety of natural habitats all over the world. They have been recovered from soil and sewage (9,19,23,37,42), rivers and lakes (37) and from sea water (22,34). The two most common techniques used to isolate bdellovibrios from the environment are based on differential centrifugation and filtration. The purpose of these procedures is to separate the parasites from the larger bacteria and debris and to effectively concentrate them. Conditions suitable for propagation of the organisms are then provided.

The majority of the bdellovibrios isolated to date have been shown to attack gram-negative bacteria. However, it has been reported that some gram-positive organisms are susceptible to attack and lysis (5). Because bdellovibrios have been

isolated from a variety of sources, it has been suggested that populations of both pathogenic and saprophytic bacteria in nature are controlled by the bdellovibrios. Some researchers even predict that some day these parasites may be used to purify river and lake water and to treat sewage (1,42).

Classification. When Bdellovibrio was initially characterized, the genus was divided into two groups, parasitic and saprophytic. Stolp defined a parasitic bdellovibrio as one that "possesses the capability of attacking a living bacterium, attaching to its surface, penetrating the cell wall, multiplying inside the host, and causing lysis of the infected cell" (41). He defined a saprophytic bdellovibrio as one that "is capable of multiplying on a laboratory nutrient medium" (41). The parasitic cells were designated "host-dependent" (HD) while the saprophytic ones, "host-independent" (HI). Bdellovibrios classified as facultatively-parasitic (FP) were those capable of multiplying either parasitically or saprophytically. Many researchers have isolated and studied bdellovibrios representative of each of these three types. However, Stolp's definitions of HD, HI, and FP bdellovibrios have not been adhered to and are often misused. For example, parasites which have been cultured on media containing heat-killed cells, autoclaved cells, and/or bacterial extracts have been referred to as HI or as nonparasitic (NP) (18,25,33,44). Thus, much confusion has resulted in the literature with regard to the

nutritional requirements of the bdellovibrios. In the present paper, Stolp's definitions will be adhered to: host-independent bdellovibrios are bdellovibrios cultured on media that are entirely host-free; host-dependent bdellovibrios are those that have a definite requirement for a nutrient supplied either directly by a host cell or by products derived from host cells.

Morphology. Host-dependent bdellovibrios are small, curved, gram-negative rods (Fig. 1). Outside the host, they vary in width from 0.25 to 0.4 μm and in length from 0.8 to 1.2 μm. Inside the host, they elongate into rather large spiral cells. Segmentation of this spiral entity produces daughter cells that are morphologically identical to free-living bdellovibrios outside the host. Each cell has a thick polar flagellum approximately 21-45 nm in diameter enclosed by a sheath 7.5 nm thick which surrounds a core 13 nm in diameter (31). The sheath originates from, and is continous with, the cell wall. The parasites are highly motile and are capable of moving at speeds of 2.0 mm/min (about 2,000 times their length) (35). Some researchers have found spike-like filaments or "holdfast" structures (4.5-5.5x0.8 nm) on the end of the cells (29,34). Other researchers suggest that these are merely fixation artifacts (3,7). An encysted "resting stage" has been reported in one strain (15).

Many morphological characteristics of host-independent

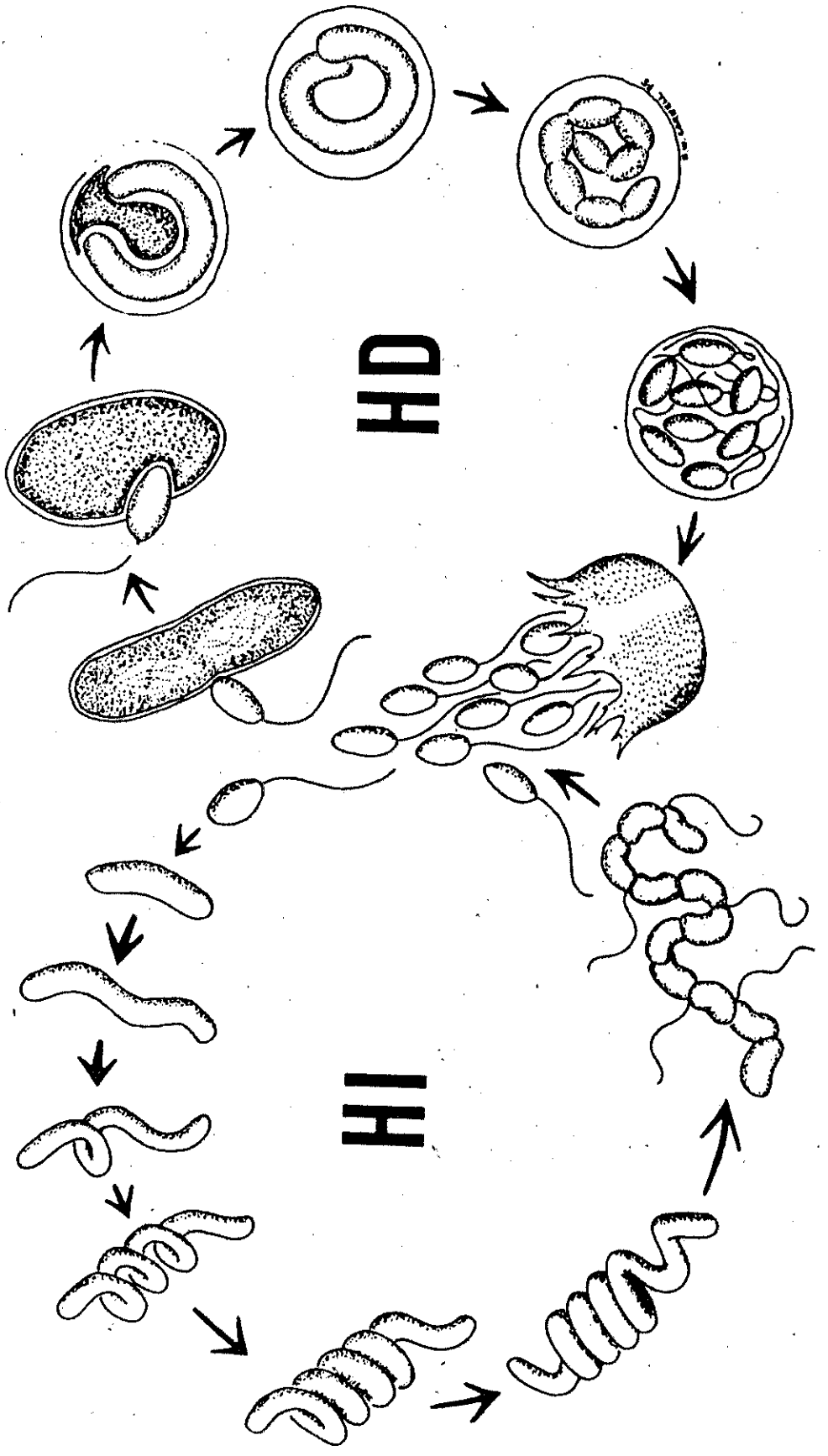
bdellovibrios are similar to those of host-dependent ones. However, two principle differences exist. One of these is that long, spiral filaments (Fig. 2), up to 50 μm in length, are regularly observed in HI cultures (16,25,33). Secondly, some HI cells have been reported to have three flagella at either or both ends of the cells while others have only one or no flagella. When HI cells are repeatedly grown on media without living host cells, they become nonmotile (33). HI cells, compared to HD cells, have greater variation in cell structure.

Life Cycle. The life cycle of a host-dependent bdellovibrio has been arbitrarily divided into five stages for purposes of study and discussion: primary contact, attachment, penetration, multiplication in the host cell, and release of the progeny (41). These are illustrated diagrammatically in Fig. 3. Primary contact occurs when actively motile bdellovibrios are brought together with susceptible host bacteria. This interaction occurs within seconds and may be violent enough to move the host cell several cell lengths (42). The ability of bdellovibrios to hunt down host cells by some means of chemotaxis is still questioned; however, chemotaxis toward yeast extract is demonstrated in obligately and facultatively parasitic strains (43). The result of this primary collision is attachment of the parasite to the surface of the host cell. Attachment occurs at the nonflagellated end of the parasite.

Fig. 1. Phase-contrast micrograph of Bdellovibrio bacteriovorus ATCC 15364 (c) and Enterobacter aerogenes (b). Bdelloplasts are visible (a). Total magnification 3,520x.

Fig. 2. Phase-contrast micrograph of Bdellovibrio bacteriovorus ATCC 25630. Total magnification 3,520x.

Fig. 3. Diagrammatic Illustration of the Life Cycles of Host-Dependent (HD) and Host-Independent (HI) Bdellovibrios [Adapted from Concepts Originally Proposed by Stolp and Starr (41, 42) Concerning the Life Cycle of Parasitic Bdellovibrios and by Seidler and Starr (33) Concerning Nonparasitic Bdellovibrios].


HD
HI

Attachment is reversible in that the bdellovibrio is able to detach from one host and attack another. The composition and pH of the medium, oxygen tension, and incubation temperature affect the ability of the parasite to attach to the host cell (45).

Penetration of the bdellovibrio into the host cell requires a hole. Researchers disagree about the formation of this portal of entry. Some believe that the bdellovibrio possesses a spike which it rams into the cell wall of the host (29,34). This may seem logical since motility is a requirement for attachment. Another theory is that the hole is formed as a result of enzymatic action. Evidence against this theory is that the hole is considerably smaller than the parasite, as may be recognized from the constrictions in the parasite seen to occur during the process of penetration (2,6). If enzymatic action is responsible, it would seem that, because of diffusion, the enzyme(s) would produce a hole larger than the area of direct contact. However, protein synthesis-inhibiting antibiotics, such as streptomycin, chloramphenicol, or puromycin, prevent penetration but not attachment. This suggests that some inducible enzymes are synthesized after the parasite has attached to the host. It has been demonstrated that bdellovibrios produce lysozyme-like enzymes, azocollases, peptidases, and lipases (17). All of these enzymes are utilized by the bdellovibrios to facilitate lysis of the host cells. Possibly the formation of the pore may be due

to a combination of both mechanical and enzymatic damage.

Some researchers believe that after the bdellovibrios attach to the host, they rotate about their long axis and in this "corkscrew-like" manner, penetrate the cell. A speed of 100 revolutions per second has been reported (41). Another belief is that the bdellovibrios swivel the posterior end of the cell with no rotation of the attachment tip (6). In any event, penetration into the host cell according to either of these two methods is accomplished by the physical efforts of the parasite.

Another theory of penetration has been proposed that involves a passive mechanism (2). The structural changes in the infected host envelope cause fluxes of water and solute. These fluxes generate forces which cause a differential expansion of the host protoplast and cell wall and their separation from each other around the entry pore. The parasite remains anchored to the host protoplast and achieves penetration by a passive force. Complete entrance into the host cell as measured from primary attachment takes anywhere from five to sixty minutes. After completion of the pore, only seconds are required for the actual invasion. The parasite invades the periplasmic zone. At some time during penetration and before the bdellovibrio completely enters the host, it loses its flagellum. After the bdellovibrios have entered the periplasmic zone, the penetration pore closes leaving a scar on the surface of the cell wall. After invasion, the

host cell is transformed into a globular body resembling a spheroplast (29,32,38)

One hour or more is generally required for intracellular growth. During this period of growth, the host cell constituents are degraded and utilized as nutrients by the developing bdellovibrios (12,13,20,21,24,26,36,41). The parasite elongates into a spiral cell that may be five to ten times the length of the infecting cell (7,29). Segmental fragmentation of this cell occurs and is followed by genesis of flagella. This fragmentation was first thought to be by constriction alone, but mesosome formation does occur indicating that the normal process of cell wall division takes place (7). At this point in the cycle, the daughter cells are motile and can be seen actively moving within the host cell ghost. Finally, the progeny are released from the parasitized cell and are capable of starting new infection cycles. The number of daughter cells produced depends primarily on the size of the host cell. For example, approximately 5.7 bdellovibrios are released from Escherichia coli (32) and 8-12 are released from Pseudomonas fluorescens (38). No detailed explanation has been given on how the progeny escape or how they "know" when it is time to escape.

The life cycle of host-independent bdellovibrios is very similar to that of the host-dependent ones, and is also included in Fig. 3. They form long spiral filaments, and daughter cells are produced by segmental fractionation. However, HI cells

live longer under ordinary culture conditions than do HD cells (41). Also, the HI cells exhibit a strong tendency to form spheroplasts in older cultures (33).

Effects on the Host Cell. The host cell undergoes a progressive morphological and biochemical disorganization during the infectious process. The first visible effect is that motile host bacteria rapidly lose motility following the attachment of the bdellovibrio. Depending on the cell, this can occur as soon as 5 seconds after attachment (42). Ribonucleic acid (RNA) synthesis of the host is inhibited within 3 minutes after infection by bdellovibrio (41) and host ribosomes and ribosomal RNA are almost completely degraded by 90 minutes (12). Protein synthesis is inhibited within 8-9 minutes after infection (41); destruction of host deoxyribonucleic acid (DNA) is complete within 45-60 minutes (21).

Host cells are transformed into spherical bodies (spheroplasts or bdelloplasts) within a matter of minutes. Localized damage of the host's cytoplasmic membrane has been demonstrated to occur within 20 to 30 minutes after the beginning of the host-parasite association (36). The damage to the membrane probably alters its permeability and allows the passage of metabolites and degradation products from the cytoplasm into the periplasmic zone, thereby satisfying the nutritional requirements of the parasite. The increase in permeability of the host's membrane, as well as the destruction

of the respiratory activity within the first hour of bdellovibrio development has been demonstrated (27).

Metabolism. Bdellovibrio is an aerobic organism, and it has been shown that both HI and HD bdellovibrios possess a tricarboxylic acid cycle (30,35). Cytochrome systems have also been demonstrated in HI and HD bdellovibrios (33,35). It has been suggested that bdellovibrio obtains energy by both oxidative- and substrate-level phosphorylation (35). This idea is in accordance with the observation that the host's respiratory potential is destroyed soon after bdellovibrio attack. Further experimentation has demonstrated that the parasite obtains its energy primarily by oxidative phosphorylation (11). Intraperiplasmically growing bdellovibrios utilize amino acids derived from host protein breakdown (13) and host fatty acids as major energy sources (20). This, together with the fact that polysaccharides are generally not involved in energy metabolism, suggests that substrate-level phosphorylation is utilized only for the generation of small amounts of energy (13).

The fatty acids of HD bdellovibrios are largely derived from those of the host. While the majority of the fatty acids are not altered, bdellovibrio does have the capacity to alter, degrade, and synthesize lipids (20).

Synthesis of bdellovibrio DNA does not begin until complete destruction of the host DNA has been accomplished.

Approximately 73% of the DNA of progeny bdellovibrios is derived from the DNA of the host bacteria (21); 20-30% of the DNA is derived from the host RNA-uracil, provided that the host is the sole source of nutrients (24). If exogenous nutrients are available, they can be utilized to form up to 20% of the bdellovibrio DNA (21). The majority of the bdellovibrio RNA is derived from the host RNA (12). Intraperiplasmic growth is not inhibited by compounds which prevent the biosynthesis of essential monomers (24) which indicates that bdellovibrio has to synthesize very few compounds de novo. Since bdellovibrio does not have to completely synthesize its nucleic acids, fatty acids, etc., it has a very high energy efficiency (Y_{ATP}). Energy efficiency is expressed as grams dry weight of cell material formed per calculated mole of adenosine triphosphate obtained from added substrate. For single growth cycle experiments, a Y_{ATP} value of 18.5 was determined (26). Bdellovibrio could be compared to a highly efficient machine in that it has the ability to hunt down, attack, penetrate, and devour another bacterium in a very efficient manner.

Purpose for the Investigation

This investigation focused on the physiology of the host-parasite interaction and attempted to determine the metabolic requirements for this reaction and how these requirements are met. Because of the scope of such a problem, this study was

limited to the development of a functional assay system for determining the respective contributions and requirements of the host and the parasite. This work was based on the observation that the parasitic cycle can be completed with dead as well as living host cells. Thus, monitoring physiological aspects of both situations should provide useful information on this interaction. Furthermore, the parasite should mutate and form a cell capable of growth independent of a host bacterium. Therefore, by measuring the physiological differences between host-dependent and host-independent cells, additional information should be obtained.

Another underlying purpose for initiating this investigation was to briefly analyze the feasibility of treating sewage and coliform contaminated water with these bacterial parasites. It seemed that Bdellovibrio would be an ideal agent in the treatment of water since it attacks and destroys gram-negative bacteria and the majority of the coliforms are gram-negative.

The most common methods of sewage treatment involve sedimentation and filtration. Provided the initial bacterial count be high, the bactericidal agent is then added. Chlorine is a reliable and rapidly acting disinfectant when water supplies are clean. However, when high levels of organic materials which rapidly react with the chlorine are present, more chlorine must be added to meet the "chlorine demand" of the water supply. Organic materials and chlorine often react with each

other to form chlorinated hydrocarbons. If the coliform count in sewage could be reduced by treatment with Bdellovibrio and the resultant bdellovibrios removed, then the amount of chlorine needed could be lessened. This in turn would lower the quantity of chlorinated hydrocarbons produced. The treatment of sewage by bdellovibrios would be a safe and natural process resembling a natural purification system.

Moreover, these parasites have been repeatedly isolated from natural environments such as rivers, lakes, seas, sewage, and soil. The report of the ability of bdellovibrios to clarify turbid water further justifies this hypothesis. Another aspect to be included in this study was the isolation and cultivation of Bdellovibrio sp. and the development of methods which enable the production of large numbers of bdellovibrios in the laboratory. A discussion on the ecological importance of bdellovibrios in an aquatic environment will also be given.

CHAPTER II

MATERIALS AND METHODS

Organisms and Cultural Conditions

The organisms used in this study were Enterobacter aerogenes, Escherichia coli B, Staphylococcus epidermidis, Salmonella typhosa, Salmonella paratyphi A, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens, Shigella paradysenteriae (all obtained from the stock culture collection, North Texas State University), Sarcina sp. (isolated as a laboratory contaminant), Proteus mirabilis derived from ATCC strain 15363, Bdellovibrio bacteriovorus derived from ATCC strain 15364, and B. bacteriovorus derived from ATCC strain 25630. All the organisms, except the bdellovibrios, were maintained on plates (1.9% agar) of modified Tris-YPG Medium (Table I-See Appendix). The plates were stored at 5°C and the organisms were subcultured monthly. Verification of the purity of the cultures was assured by biochemical tests, gram stain, visual observation of colonial morphology, and by phase-contrast microscopy.

Bdellovibrio bacteriovorus ATCC 25630 is host independent and was maintained on plates (1.9% Noble Agar) of YEP No. 3 Medium (Table II-See Appendix). Plate cultures were stored at room temperature and subcultured at weekly intervals.

Culture purity was assessed by periodic monitoring of the organism's gram-staining characteristics and by phase-contrast microscopy.

Bdellovibrio bacteriovorus ATCC 15364 is host-dependent and therefore cannot be grown in the absence of host bacteria. It was maintained using E. aerogenes as the substrate organism according to the modified method of Staples and Fry (37). One ml of the bdellovibrio suspension along with 0.5 ml of an 18 h host culture were added to 5 ml of liquefied, soft Tris-YPG agar (0.6%), poured over the surface of Tris-YPG plates, and incubated in an inverted position at 30°C. Within 24 h, a smooth, homogenous host lawn covered the plate. After 48 h, small areas of lytic activity or plaque forming units (pfu) began appearing. This process is similar to the double layer technique used for the isolation of phage (4). After 3 days of incubation, the plates were stored at 5°C. The organism was subcultured at monthly intervals. Subculturing was achieved by cutting out the upper layers of agar containing areas of lysis, homogenizing them in Tris-MgCa Buffer (Table III-See Appendix), and filtering the homogenate through a filter (pore diameter of 0.45 μm) to remove host organisms and agar. The filtrate was used as the bdellovibrio suspension as described above. Purity of the HD bdellovibrios was assured by the repeated determination of its host range, using the methodology described elsewhere in this chapter.

Bdellovibrios were isolated from activated sludge from the city of Denton's sewage treatment plant. Fresh samples were transported to the laboratory and filtered (filter pore diameter of 0.45 μm) to remove particulate matter and larger microbial cells. Methods described in the prior paragraph were employed in order to isolate bdellovibrios from the filtrate.

The pH of Tris-YPG Medium and Tris-MgCa Buffer were adjusted to 7.5 with 0.5 M HCl. Tris-YPG Medium was stored at 5°C in a 5x concentration and appropriately diluted before use. Sterilization was achieved by autoclaving at 121°C for 15 min.

Host Range

The host range of the HD bdellovibrios was determined by the double-layer technique previously described with the following modification: 1 ml aliquots of 24 h host cultures were added with the bdellovibrio suspension in the 5 ml agar overlay. Whether host bacteria were susceptible to attack and lysis by the bdellovibrios was determined by the appearance of pfu on the host lawns after 7 days of incubation at 30°C. The host range of *B. bacteriovorus* ATCC 15364 is given in Table IV (See Appendix).

Preparation of Cell Cultures

The primary organisms in this investigation were *E. aerogenes* and the HD and HI strains of *B. bacteriovorus*. The

preparation of E. aerogenes is described below. One hundred ml of Tris-YPG Broth were inoculated with 1 ml of a 9 h culture and then incubated for 19 h at 37°C with shaking at 150 rpm (½ inch circular orbit stroke). Cells were harvested by centrifugation at 16,000xg for 20 min at 5°C. The pellet was washed twice by resuspension in cold, sterile buffer followed by centrifugation. After washing, the cells were resuspended in buffer and the suspension was adjusted to the proper concentration by reference to a standard curve relating viable cell counts to turbidity. The reference curve was prepared by making serial dilutions of the washed cells, measuring the turbidity of each dilution, and counting the number of viable cells in each dilution by plating portions of serial dilutions of a 1 ml aliquot taken from the original washed cell suspension.

Some of the experiments required nonviable cells of E. aerogenes. This was accomplished by exposing them either to heat or ultraviolet (UV) radiation. Heat killing was achieved by autoclaving the washed cells at 121°C for 15 min. Ultraviolet killing was performed by exposing the washed cells to an UV lamp. Aliquots (4 ml) of the washed cells were placed in sterile, glass petri dishes and exposed to the light at a distance of 15 cm for 4 min. The cells were constantly agitated to minimize exposure differences. The energy output of the lamp at this distance was 8.25×10^5 ergs/cm^2-sec as measured by a radiometer. According to the specifications on the lamp,

the total radiant energy output was 61.3 watts, approximately 6.5% of this being at 2537 Å.

The HI bdellovibrios were prepared for the experiments by inoculating five 250-ml flasks each containing 50 ml of YEP No. 3 Broth with a loopful of cells from a 4 day plate culture. The flasks were incubated at room temperature with shaking for 48 h. Cells were harvested by centrifugation at 27,000xg for 20 min at 5°C. The pellet was washed once with cold, sterile buffer and resuspended in buffer after centrifugation. The suspension was adjusted to the proper concentration by reference to a reference curve relating viable cell counts to turbidity. The reference curve for HI bdellovibrios was prepared in the same manner as that for E. aerogenes.

The HD bdellovibrios were prepared for the experiments by inoculating two 500-ml flasks each containing 100 ml of Tris-YPG Broth with 10 ml of an 18 h E. aerogenes culture and with 1 ml of a 51 h B. bacteriovorus plus E. aerogenes culture. The flasks were incubated at 30°C and 150 rpm for 51 h. Cells were harvested by centrifugation; first at 2,000xg for 5 min at 5°C and then the supernatant at 27,000xg for 20 min at 5°C. The pellet was washed once with cold, sterile buffer and resuspended in buffer after centrifugation. The suspension was adjusted to the proper concentration by reference to a reference curve relating viable cell counts to turbidity. The reference curve for the HD bdellovibrios was prepared in the same manner as that for E. aerogenes except that aliquots

were plated with host cultures using the double-layer technique described above.

Viable Cell Counts

Viable cell counts of the E. aerogenes and HI bdellovibrios were made by the standard spread plate technique. Tenfold dilutions were made of the cultures by transferring 1 ml of a bacterial suspension to test tubes containing 9 ml cold, sterile buffer. The dilutions were homogenized using a vortex mixer to achieve even dispersal of the cells. One tenth ml of each dilution was then pipetted onto the surface of petri plates containing Tris-YPG Medium (E. aerogenes) or YEP no. 3 Medium (HI bdellovibrios) and spread evenly over the surface of the agar with glass spreaders. Each dilution was plated in triplicate and incubated 48 h at 37°C (E. aerogenes) or 8 days at room temperature (HI bdellovibrios) before colony counts were made. The average number of cells per dilution was determined and used to calculate the concentration of viable cells in the original culture.

Viable cell counts for the HD bdellovibrios were made by the double-layer technique as previously described using washed bdellovibrio suspensions. Each dilution was plated in triplicate and incubated 4 days at 30°C before pfu were counted. The average number of plaques per plate when multiplied by the reciprocal of the dilution yielded the viable cell count of the original culture.

Optical Density Measurements

The optical density (OD) of liquid cultures was measured with a photoelectric colorimeter (Klett Summerson, New York) equipped with a green filter No. 54 (500-570 nm). The instrument was calibrated with sterile broth or buffer (depending on the experiment) to zero absorbance. Cultures for which optical density was determined were contained in 500 ml sidearm flasks.

Respiration

Respiration or the uptake of oxygen from solutions of cell suspensions was measured polarographically with an oxygen electrode covered with a 0.001 inch thick Teflon membrane and maintained at 0.8 v. The instrument was standardized using sterile Tris-MgCa buffer saturated with air (211.6 nmoles O_2/ml buffer). The baseline of the instrument was readjusted between samples. The electrode was inserted into a 2 ml water jacketed sample compartment maintained at 30°C with a constant temperature water circulator. The compartment was mounted on a magnetic stirrer and the contents were continuously mixed by a miniature magnetic stirring bar to prevent the formation of oxygen concentration gradients.

Calculations of the rates of oxygen consumption were accomplished by use of the following methods and constants (14). The solubility of air in water at 30°C and 760 mm pressure is

15.54 ml air/1000 ml H_2O (a)

Since the concentration of oxygen in air dissolved in water at 30°C and 760 mm pressure is 33.6%, then there are

5.26 ml O_2/1000 ml H_2O (b)

Since 1 mole of oxygen occupies a volume of 24.9 l at 30°C and 760 mm pressure, then

5.26 ml O_2 = 211.6 nmoles O_2 (c)

Therefore, the solubility of oxygen in water at 30°C and 760 mm pressure is 211.6 nmoles O_2/ml. Using these calculations, each 2.0 ml in the sample compartment contain approximately 423.2 nmoles of oxygen. The total deflection of the recorder represents the difference between 0 and 100% oxygen concentration; therefore, oxygen concentration per recorder division per unit of time gives nmoles O_2/min. Therefore, the results of the respiration measurements will represent the rate of change of rates [d(dx/dt)/dt or (O_2/min/min] since the individual points are themselves plots of rates (dx/dt or O_2/min) (27).

CHAPTER III

RESULTS

The medium of choice in these studies was Tris-YPG containing magnesium and calcium. These cations were added to the medium because they appeared to promote completion of the parasitic cycle. This enhancement was demonstrated by comparing the turbidities over a period of days of two flasks inoculated with equal numbers of host cells and HD bdellovibrios. One flask contained Tris-YPG Medium supplemented with magnesium and calcium while the other contained only Tris-YPG Medium. The changes in turbidity of the contents of the two flasks were similar during the first 30 h of incubation. However, at that time the supplemented culture began clarifying while the turbidity of the other culture remained fairly stable. After an additional 40 h, there was a difference of 75 Klett Units between the two cultures. Phase-contrast microscope observation of both cultures after 70 h of incubation demonstrated that the culture supplemented with magnesium and calcium contained large numbers of bdellovibrios while the other culture contained numerous bdellovibrios as well as host cells.

In order to obtain the large numbers of HD bdellovibrios needed for these studies, batch cultures of E. aerogenes were used as the host organism. To monitor the progress of the

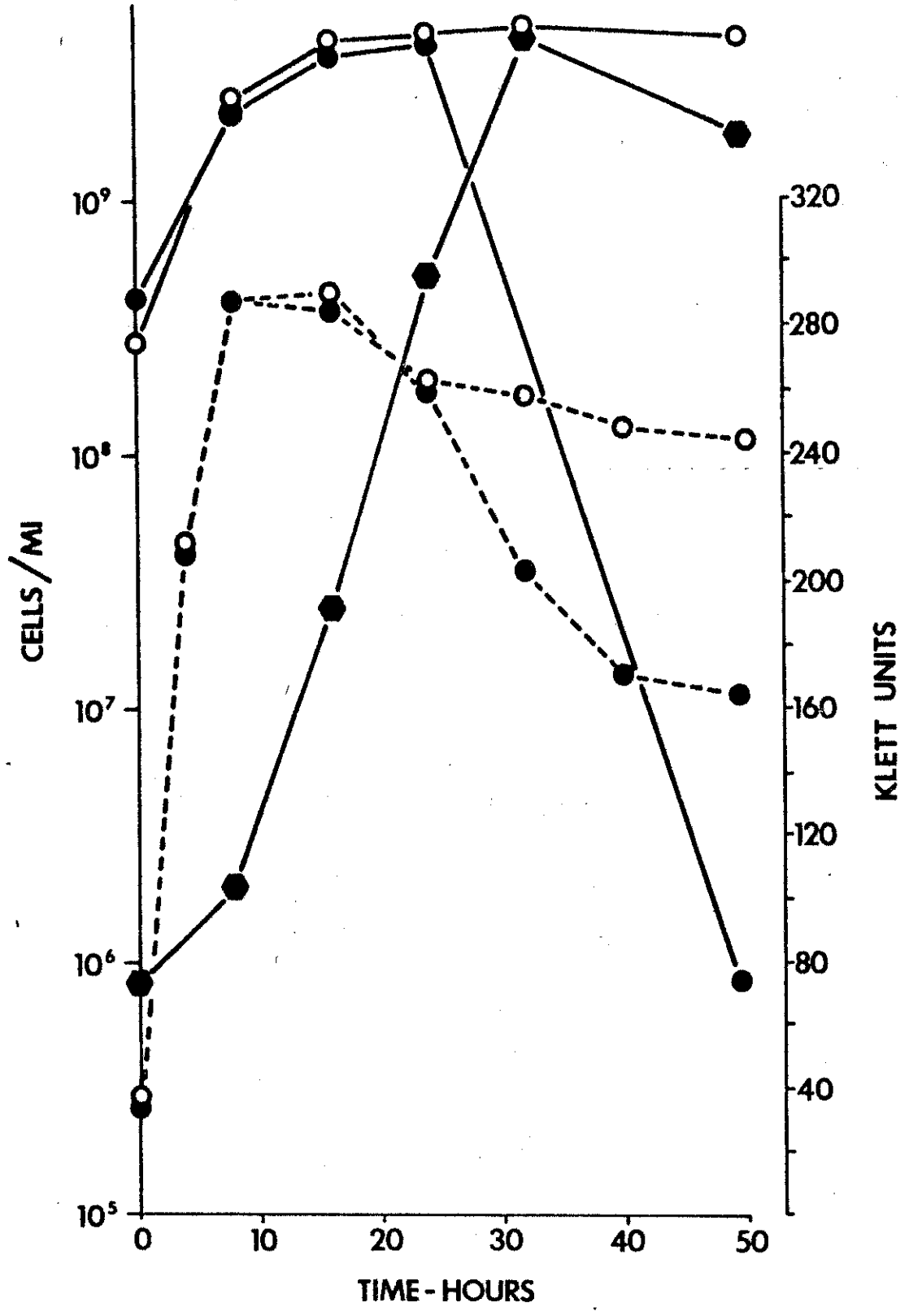
infection cycle, both host and parasite numbers were determined at eight hour intervals. As illustrated in Fig. 4, few host cells remained after 51 h of incubation while the bdellovibrios were present in large numbers. The bdellovibrios were harvested by centrifugation at that time.

In order to determine whether the physiological state of the host cell affects the number of pfu recovered and in turn the viable cell count, aliquots of a bdellovibrio suspension were plated with suspensions of host cells differing in age. The results indicated that as long as a homogenous host lawn was able to form, accurate and reliable pfu counts could be made.

In an attempt to correlate pfu with the actual number of bdellovibrios present in the original suspension, direct phase-contrast microscope counts of the bdellovibrio cells were made using duplicates of samples plated onto host lawns. Consistent direct counts were difficult to obtain, presumably because Bdellovibrio has the ability to penetrate host cells and not be readily visible. Therefore, one may see several rounded host cells or bdelloplasts and no bdellovibrios and yet anywhere from one to ten pfu per host cell may develop. Although accurate microscopic counts could not always be made, it is felt that one pfu indicates one bdellovibrio since a direct correlation between visible cells and pfu was obtained on one occasion.

In view of the reports that bdellovibrio is an aerobic

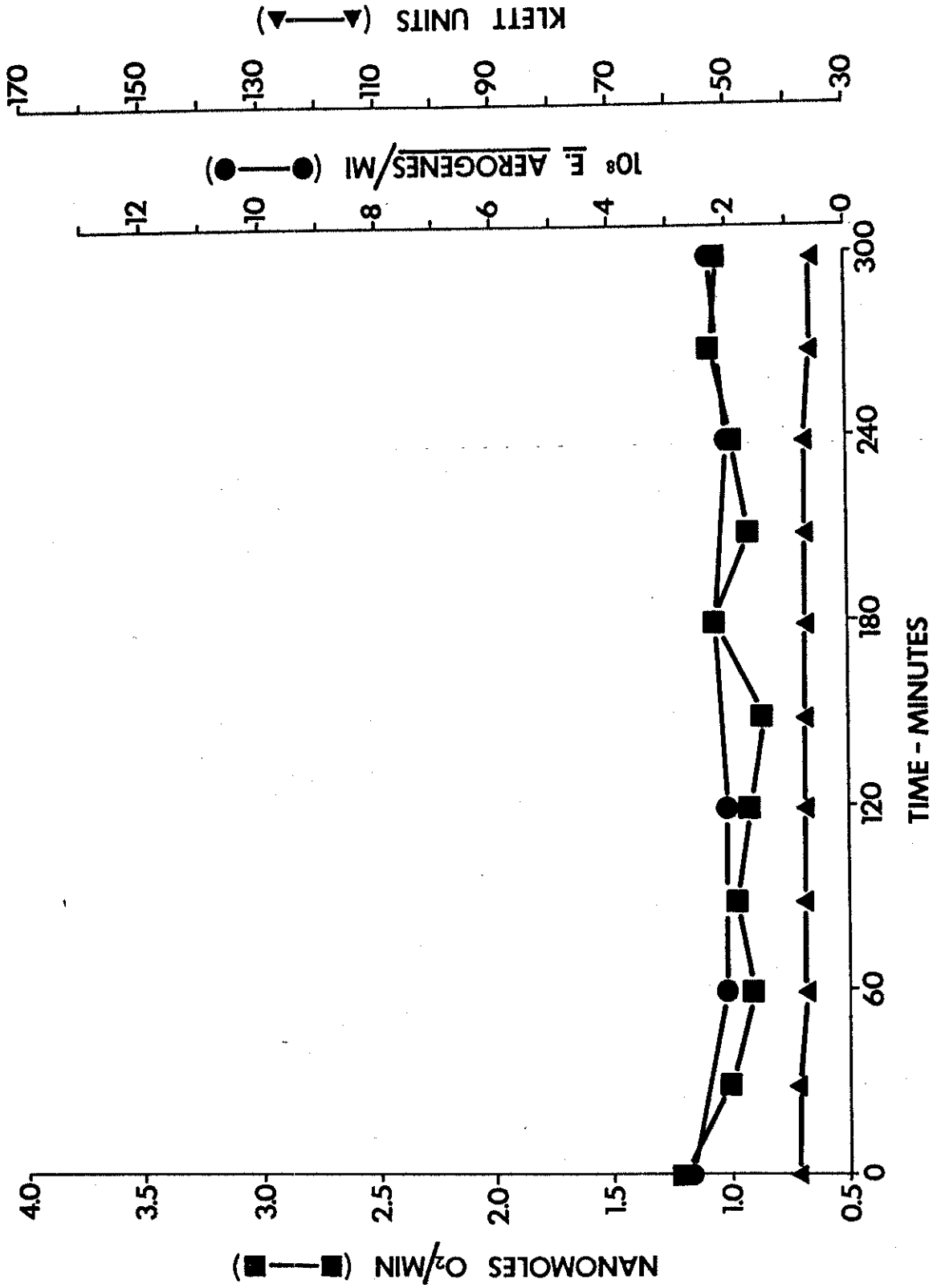
Fig. 4. Comparison of the growth of Enterobacter aerogenes in the presence and absence of Bdellovibrio bacteriovorus ATCC 15364. Symbols: (○) Enterobacter aerogenes uninfected, (●) Enterobacter aerogenes infected, (●) Bdellovibrio bacteriovorus. Solid lines represent cell numbers and broken lines represent turbidity reading.


CELLS / Ml
10^9
10^8
10^7
10^6
10^5
320
280
240
200
160
120
80
40
KLETT UNITS
0
10
20
30
40
50
TIME - HOURS

organism, it was thought that direct measurements of respiration as well as turbidity and viable cell counts would be a reliable indication of the metabolic activity. The experiments were performed in Tris-MgCa Buffer with E. aerogenes as the sole source of nutrients for the bdellovibrios. A ratio of approximately three bdellovibrios per E. aerogenes cell was used to insure that the majority of host cells were infected by the parasites within a short period of time. This was the smallest ratio of parasites to host which would give rise to changes in the parameters being studied. Time zero was designated as the time at which the two populations were combined. Flasks containing the host-parasite combinations were incubated with shaking to provide aeration. Periodically, aliquots were removed for the measurement of respiration and cell numbers.

Figure 5 illustrates the results obtained using a viable E. aerogenes suspension as a control experiment. It can be seen that turbidity, endogenous respiration, and cell numbers remained virtually unchanged over the 300 min period. Turbidity maintained an average of 37 Klett Units. Respiration remained fairly constant with a mean of 1.0 nmoles O_2/min. The cells appeared to be in the same physiological state throughout the experiment since the number of cells at the end of the 300 min time period was only slightly lower than at the beginning. An average of 2.2×10^8 viable cells was maintained throughout the experiment. Differences of the

Fig. 5. Respiration patterns and changes in turbidity and cell numbers of Enterobacter aerogenes suspended in Tris-MgCa Buffer.


NANOMOLES O2/MIN (■——■)
KLETT UNITS (▲——▲)
10⁸ E. AEROGENES/ML (●——●)
TIME - MINUTES
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
170
150
130
110
90
70
50
30
12
10
8
6
4
2
0
0
60
120
180
240
300

magnitude described can be attributed to experimental error. Suspensions of both UV-killed and autoclaved E. aerogenes showed no change in turbidity and no respiration.

In order to determine whether the oxygen demands of E. aerogenes suspended in Tris-MgCa Buffer were a reflection of endogenous-respiration or the utilization of Tris as a source of nutrients, viable cell counts were made of similar suspensions over a period of four days. In this situation, the population decreased steadily throughout the course of study. Moreover, when various quantities of Tris were added to cells suspended in deionized water, no increase in oxygen consumption could be recorded.

Figure 6 illustrates the measurements of the same parameters of the HD bdellovibrios. Turbidity remained constant at 149 Klett Units indicating that the cells were remaining intact. Respiration dropped from 3.4 to 2.2 nmoles O_2/min during the first 30 min and then leveled off fluctuating between 1.7 and 2.3 nmoles O_2/min for the last 270 min. Within the first 180 min, approximately 58% of the bdellovibrios died. However, for the final 120 min the cell numbers remained fairly constant at 5.2×10^8 cells/ml. Respiration appeared to reflect the changes in cell numbers; the bdellovibrio population decreased concurrently with respiration, and when the population leveled off, respiration became constant with respect to time. Respiration did become more stable but demonstrated slight oscillations.

Fig. 6. Respiration patterns and changes in turbidity and cell numbers of host-dependent bdellovibrios suspended in Tris-MgCa Buffer.

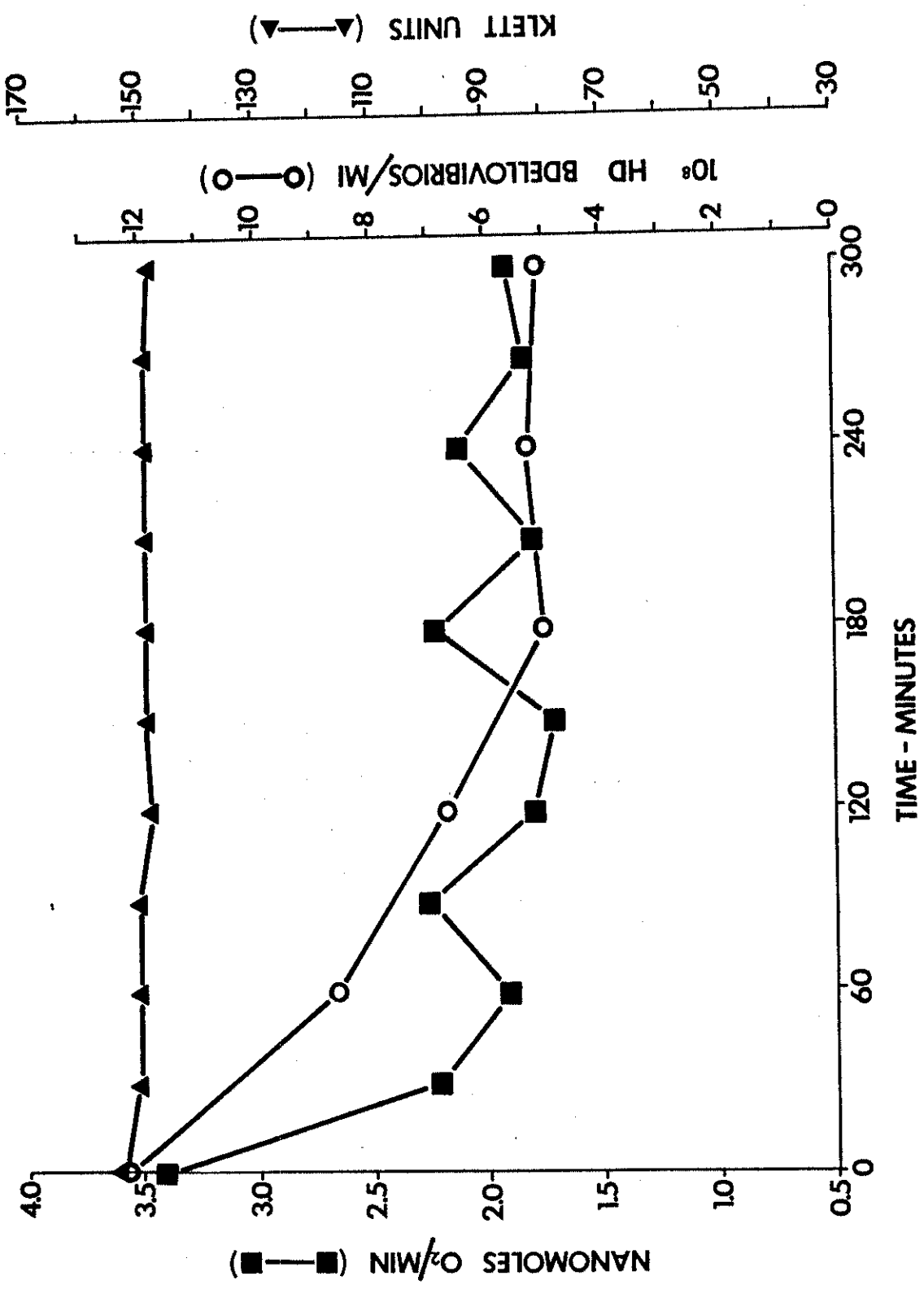
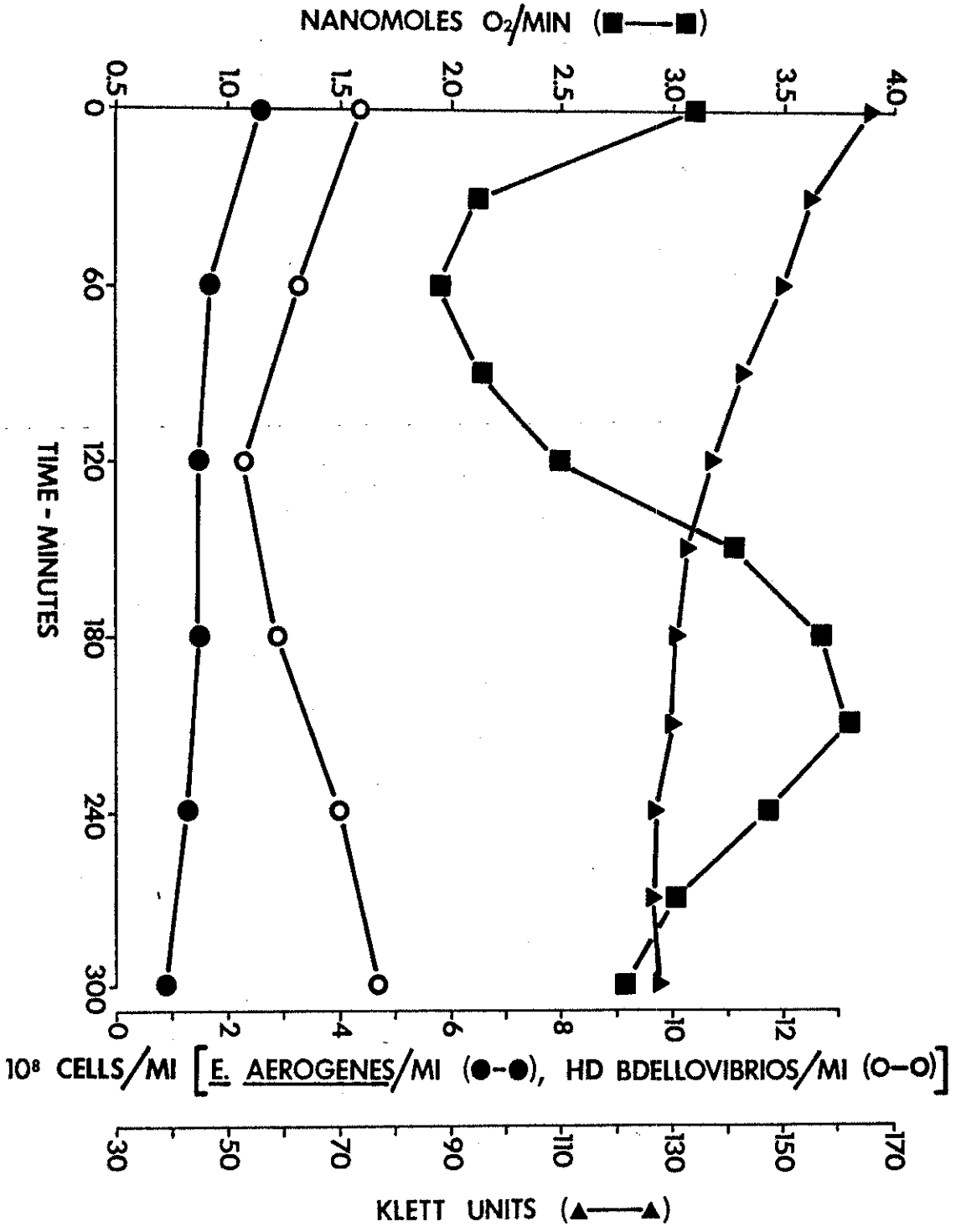

NANOMOLES O₂/MIN (■——■)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
60
120
180
240
300
TIME - MINUTES
10⁸ HD BDELLOVIBRIOS/ML (O——O)
12
10
8
6
4
2
0
KLETT UNITS (▲——▲)
170
150
130
110
90
70
50
30

Figure 7 illustrates the HD bdellovibrios with live E. aerogenes. The maximum turbidity was at time 0 and indicated by a reading of 166 Klett Units. During the experiment, the cell suspension gradually cleared until at 300 min it was 128 Klett Units. This was the greatest change in turbidity of any of the experiments and was indicative of the lysing of cells. Respiration during the parasitic interaction fluctuated up and down and was similar to that determined by previous workers (27). During the first 60 min, it dropped from 3.1 nmoles O_2/min to 2.2 nmoles O_2/min, after which, it gradually increased to a maximum of 3.8 nmoles O_2 at 210 min. Respiration for the remaining 90 min slowly decreased to 2.8 nmoles O_2/min at 300 min. The HD bdellovibrio population appeared to decrease from 4.5×10^8 cells/ml at time 0 to 2.4×10^8 cells/ml at 120 min. However, during the last 180 min of the experiment the number of bdellovibrios increased, reaching 4.7×10^8/ml at 300 min. The host population which was 2.7×10^8 cells/ml at the start of the experiment steadily declined to 9.7×10^7/ml at the end of the experiment.

The next series of experiments were designed to estimate the capability of HD strains to complete the parasitic cycle with nonviable, as well as viable host cells. The following analyses were performed. To each of four flasks containing either buffer alone, viable host cells suspended in buffer, UV-killed host cells in buffer or autoclaved hosts in buffer, approximately equal numbers of HD bdellovibrios were added.

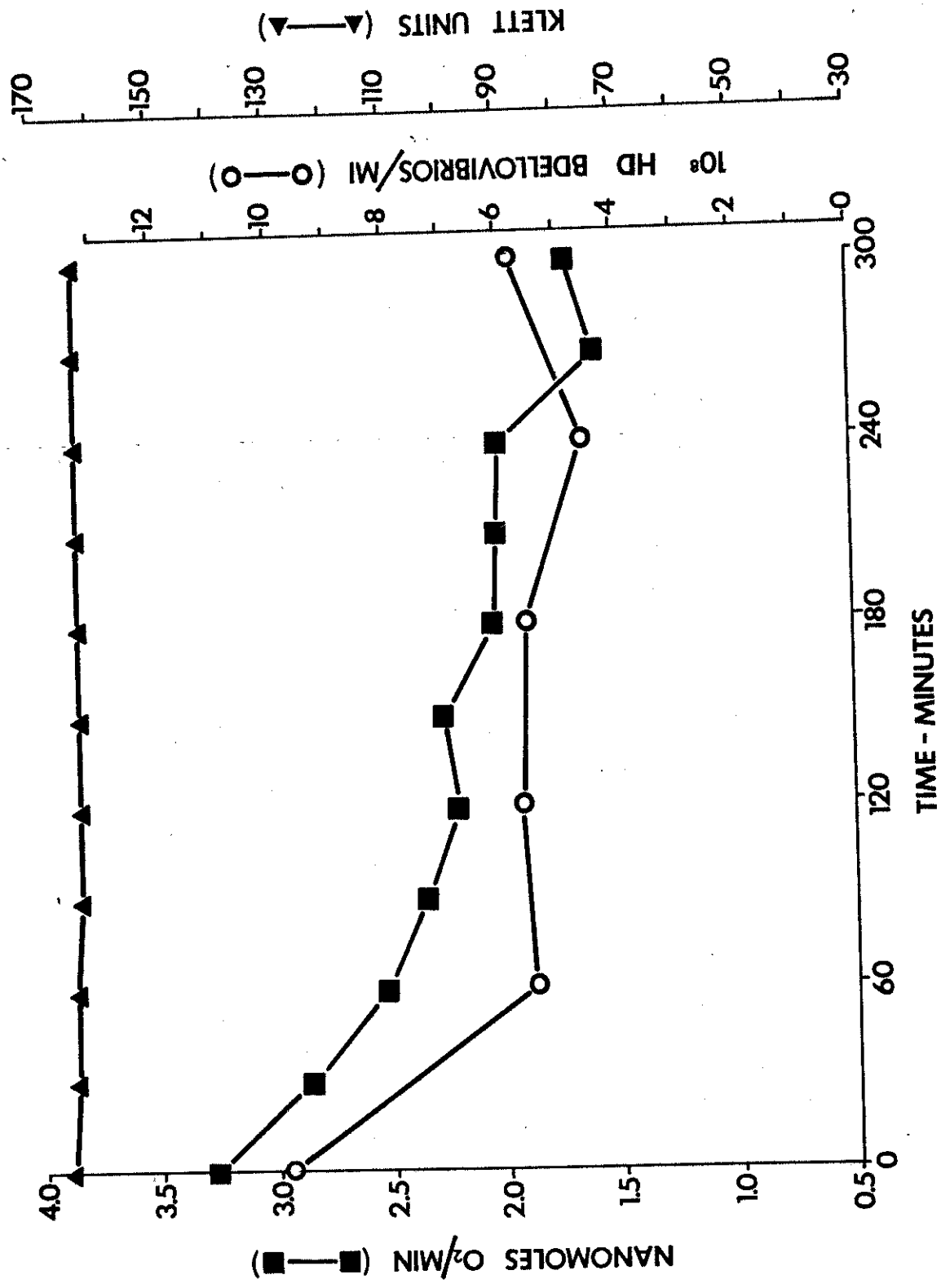
Fig. 7. Respiration patterns and changes in turbidity and cell numbers of host-dependent bdellovibrios combined with viable Enterobacter aerogenes.


NANOMOLES O_2/MIN (■—■)
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
TIME - MINUTES
0
60
120
180
240
300
0
2
4
6
8
10
12
10^8 CELLS/MI [E. AEROGENES/MI (●-●), HD BDELLOVIBRIOS/MI (O–O)]
30
50
70
90
110
130
150
170
KLETT UNITS (▲——▲)

A ratio of three bdellovibrios to one host cell was used. The flasks were incubated under identical conditions and samples were withdrawn periodically for microscopic observation. Phase-contrast microscope observation revealed that the HD strains were able to complete a true parasitic cycle with viable and UV-killed hosts but not with autoclaved hosts. A true parasitic cycle has previously been defined (41) as the ability of the parasite to attack, penetrate, and parasitize the host cell with the release of progeny bdellovibrios. The bdellovibrios were not seen to attach to the autoclaved host cells.

Host-dependent bdellovibrios were combined with autoclaved E. aerogenes to determine whether constituents of the host cells could substitute for viable cells. The results are illustrated in Fig. 8. Throughout the experiment, turbidity remained stable at 163 Klett Units indicating that neither bdellovibrio nor remaining intact host cells were lysing. Respiration decreased steadily from a high of 3.3 nmoles O_2/min at time 0 to 1.7 nmoles O_2/min at 300 min. Approximately 56% of the viable bdellovibrios died in the first 60 min of the experiment. During the remaining 240 min, the bdellovibrio population stabilized with an average of 5.4×10^8 cells/ml. Respiration reflected the decline and stabilization of the bdellovibrio population. During the 300 min time period of this experiment the bdellovibrios were not seen attached to the autoclaved host cells. However, some

Fig. 8. Respiration patterns and changes in turbidity and cell numbers of host-dependent bdellovibrios combined with autoclaved Enterobacter aerogenes.


NANOMOLES O2/MIN (■—■)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
60
120
180
240
300
TIME - MINUTES
10⁸ HD BDELLOVIBRIOS/ML (O—O)
12
10
8
6
4
2
0
KLETT UNITS (▲—▲)
170
150
130
110
90
70
50
30

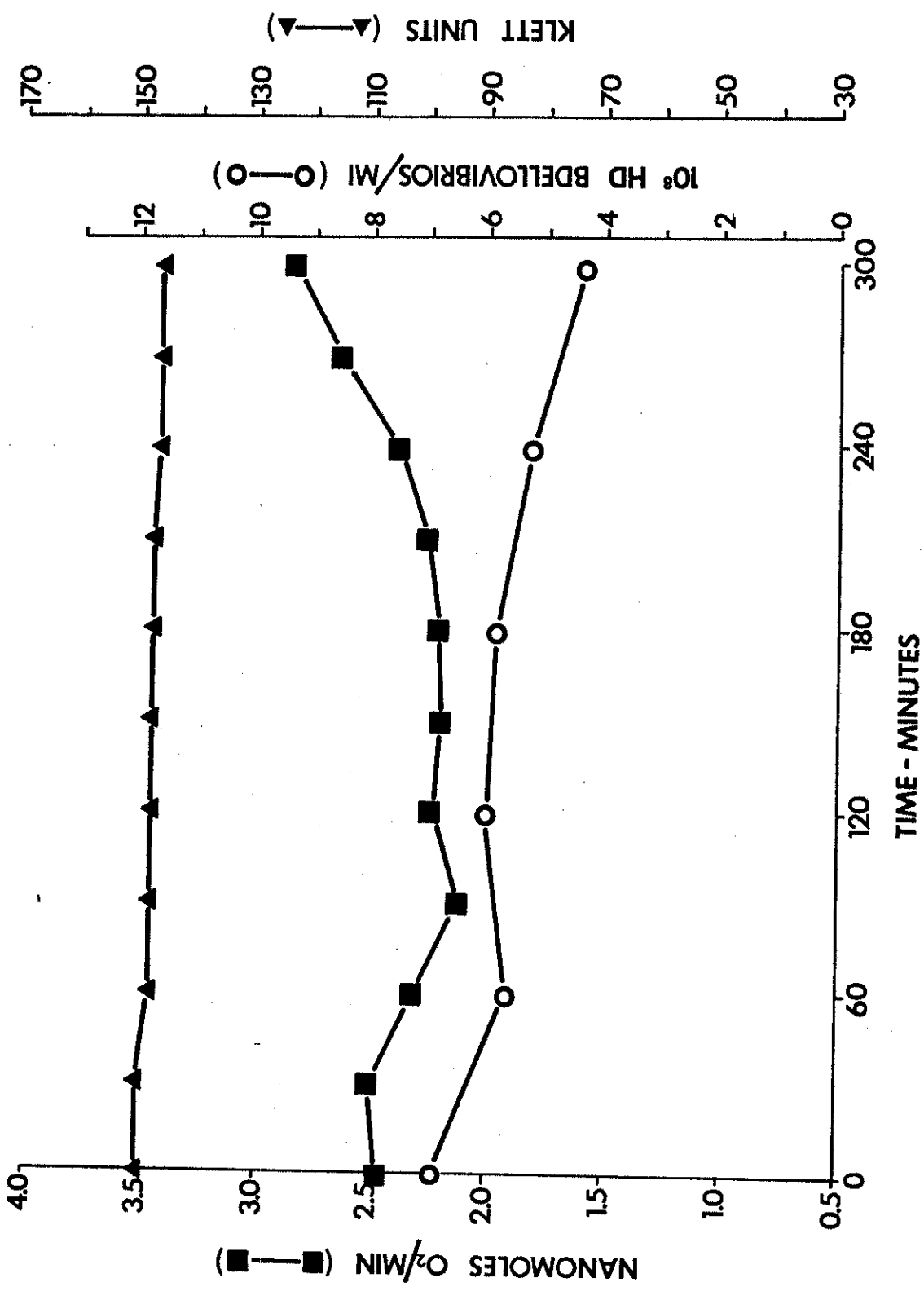
of the parasites did appear to elongate, indicating that they were undergoing an atypical development unlike the normal intraperiplasmic growth cycle.

The previous experiments were repeated using UV-killed host cells. Figure 9 illustrates the situation when HD bdellovibrios were combined with UV-killed E. aerogenes. The turbidity of the suspension remained rather constant at 148 Klett Units indicative of minimal cell lysis. Respiration dropped from 2.5 nmoles O_2/min at time 0 to 2.1 nmoles O_2/min at 90 min. However, it gradually increased during the remainder of the experiment to a maximum of 2.9 nmoles O_2/min at 300 min and was presumed to reflect the metabolism associated with initiation of bdellovibrio formation. An increase in the number of HD bdellovibrios could not be demonstrated by viable bdellovibrio cell counts at this point. In fact, a decrease in numbers was observed.

Phase-contrast microscope observation supported the concept that the bdellovibrios could at least initiate the infection cycle with UV-killed host cells. The parasites could be seen attaching to the host cells within 60 min. After 120 min, bdelloplasts had appeared and were seen for the duration of the experiment. At 180 min, both small and large bdellovibrios were seen.

This series of experiments were repeated using a host-independent strain of bdellovibrio. Changes in respriation, turbidity, and viable cell numbers of the HI bdellovibrio

Fig. 9. Respiration patterns and changes in turbidity and cell numbers of host-dependent bdellovibrios combined with ultraviolet-killed Enterobacter aerogenes.


NANOMOLES O2/MIN (■—■)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
60
120
180
240
300
TIME - MINUTES
10⁸ HD BDELLOVIBRIOS/ML (O—O)
12
10
8
6
4
2
0
KLETT UNITS (▲—▲)
170
150
130
110
90
70
50
30

control experiment are illustrated in Fig. 10. The turbidity of the suspension dropped steadily, although slowly, throughout the entire experiment. Phase-contrast microscope observation revealed the long, spiral cells to be fractionating into smaller fragments and eventually lysing. This fractionating and lysing of cells occurred concurrently with the decrease in turbidity. Respiration at time 0 was 59.2 nmoles O_2/min. However, at 30 min, it peaked to its maximum of 68.0 nmoles O_2/min. When a comparison of nmole O_2/min/cell was made between HI and HD bdellovibrios, it was noted that the HI strains were respiring at a rate ten times that of the HD ones. After reaching this maximum, respiration steadily dropped off until at the end of the 300 min interval it was 26.9 nmoles O_2/min. The number of viable HI bdellovibrios decreased steadily. At time 0, there were approximately 2.6×10^9 cells/ml and at 300 min there were 4.1×10^8 cells/ml. This indicates that about 84% of the bdellovibrios died during the 300 min time period.

The results obtained from the experiments which involved combinations of HI bdellovibrios with viable, autoclaved, and UV-killed E. aerogenes are illustrated in Fig. 11, 12, and 13, respectively, and were very similar to those of the HI bdellovibrio control. The presence or absence of the E. aerogenes cells appeared to have little effect on the HI bdellovibrios with one exception. This exception is described below.

Fig. 10. Respiration patterns and changes in turbidity and cell numbers of host-independent bdellovibrios suspended in Tris-MgCa Buffer.

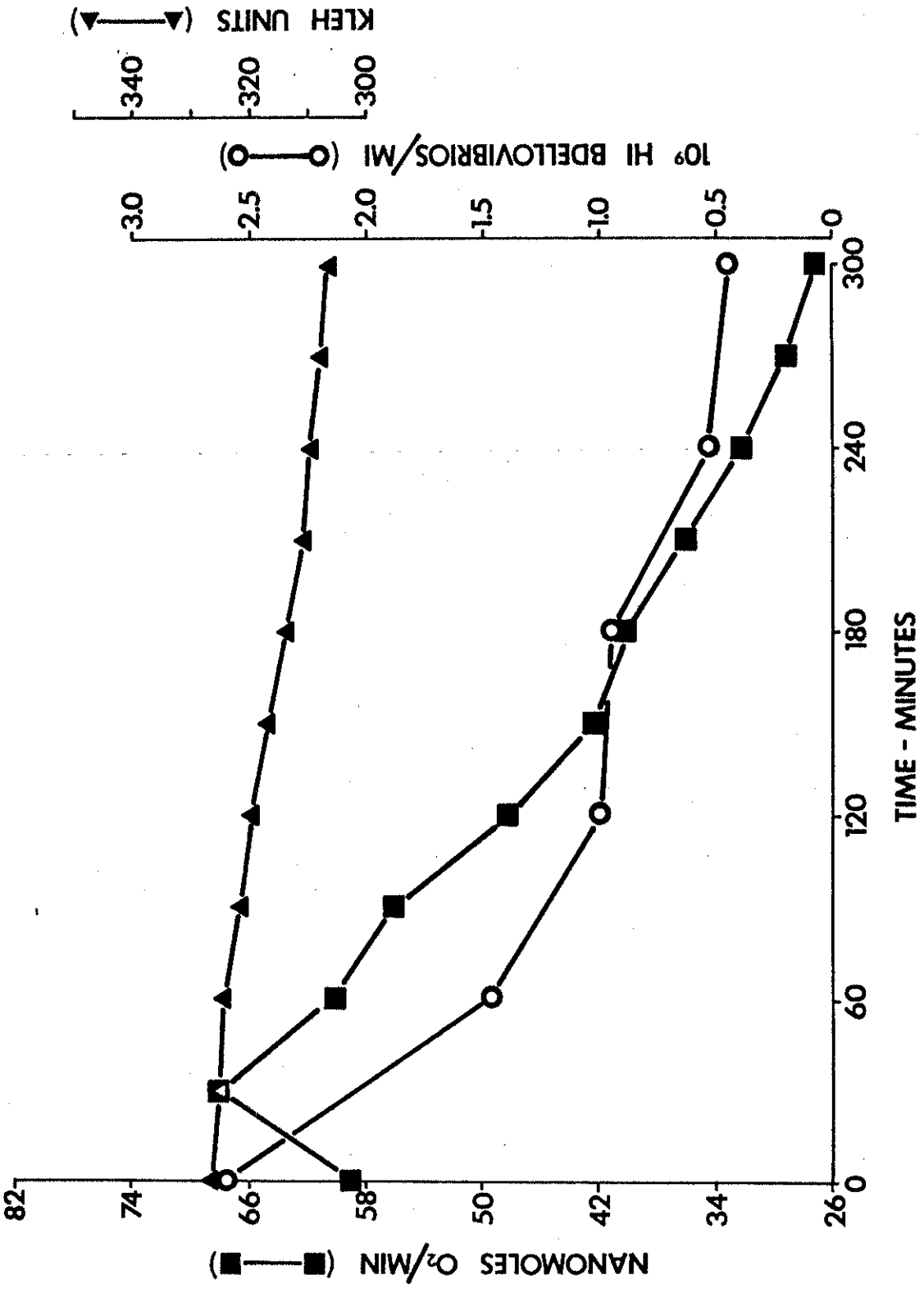

NANOMOLES O2/MIN (■——■)
82
74
66
58
50
42
34
26
0
60
120
180
240
300
TIME - MINUTES
10⁹ HI BDELLOVIBRIOS/MI (○——○)
3.0
2.5
2.0
1.5
1.0
0.5
0
KLEH UNITS (▲——▲)
340
320
300

Fig. 11. Respiration patterns and changes in turbidity and cell numbers of host-independent bdellovibrios combined with viable Enterobacter aerogenes.

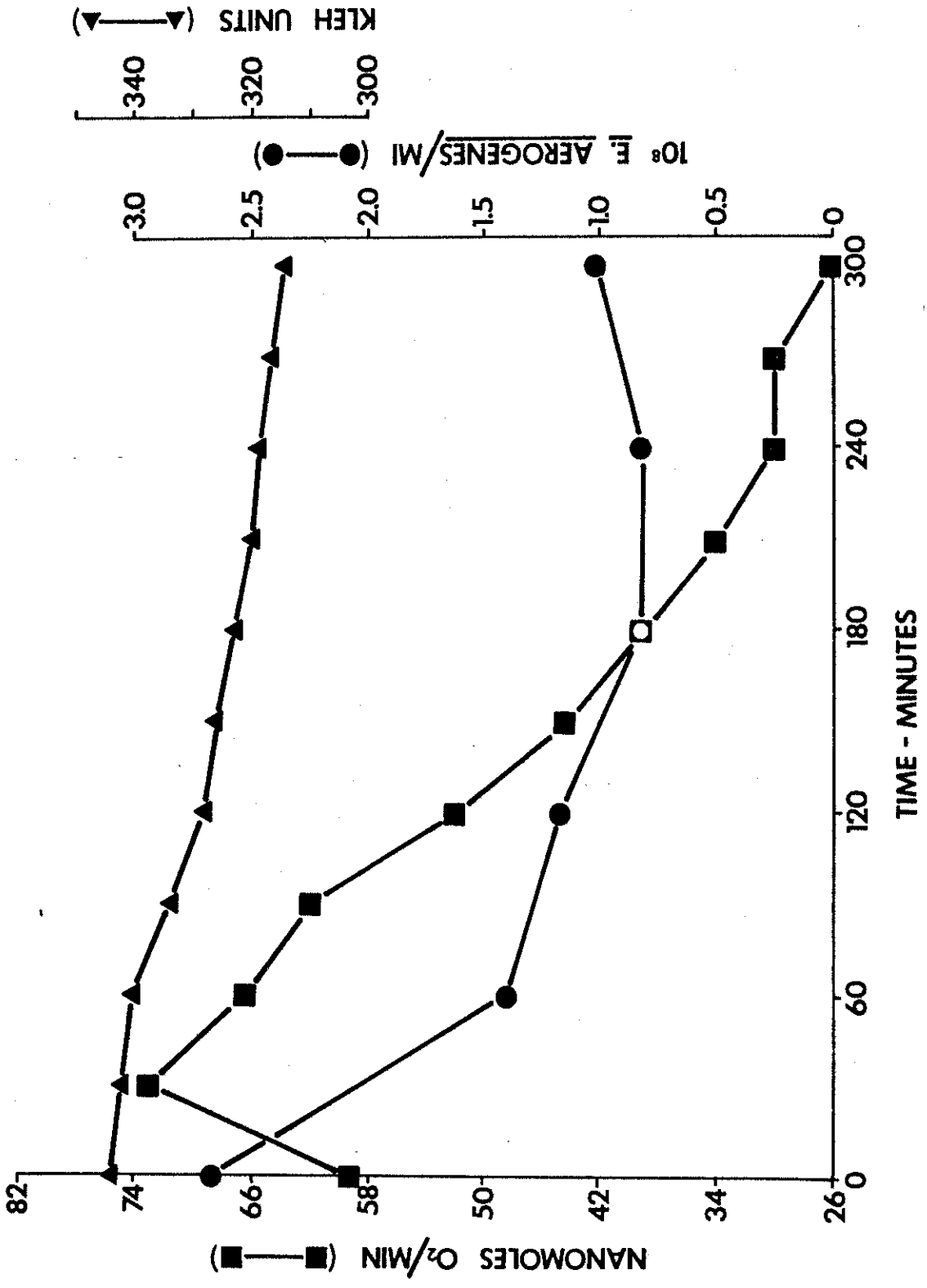

NANOMOLES O2/MIN (■——■)
82
74
66
58
50
42
34
26
0
60
120
180
240
300
TIME - MINUTES
10⁸ E. AEROGENES/ML (●——●)
3.0
2.5
2.0
1.5
1.0
0.5
0
KLEH UNITS (▲——▲)
340
320
300

Fig. 12. Respiration patterns and changes in turbidity and cell numbers of host-independent bdellovibrios combined with autoclaved Enterobacter aerogenes.

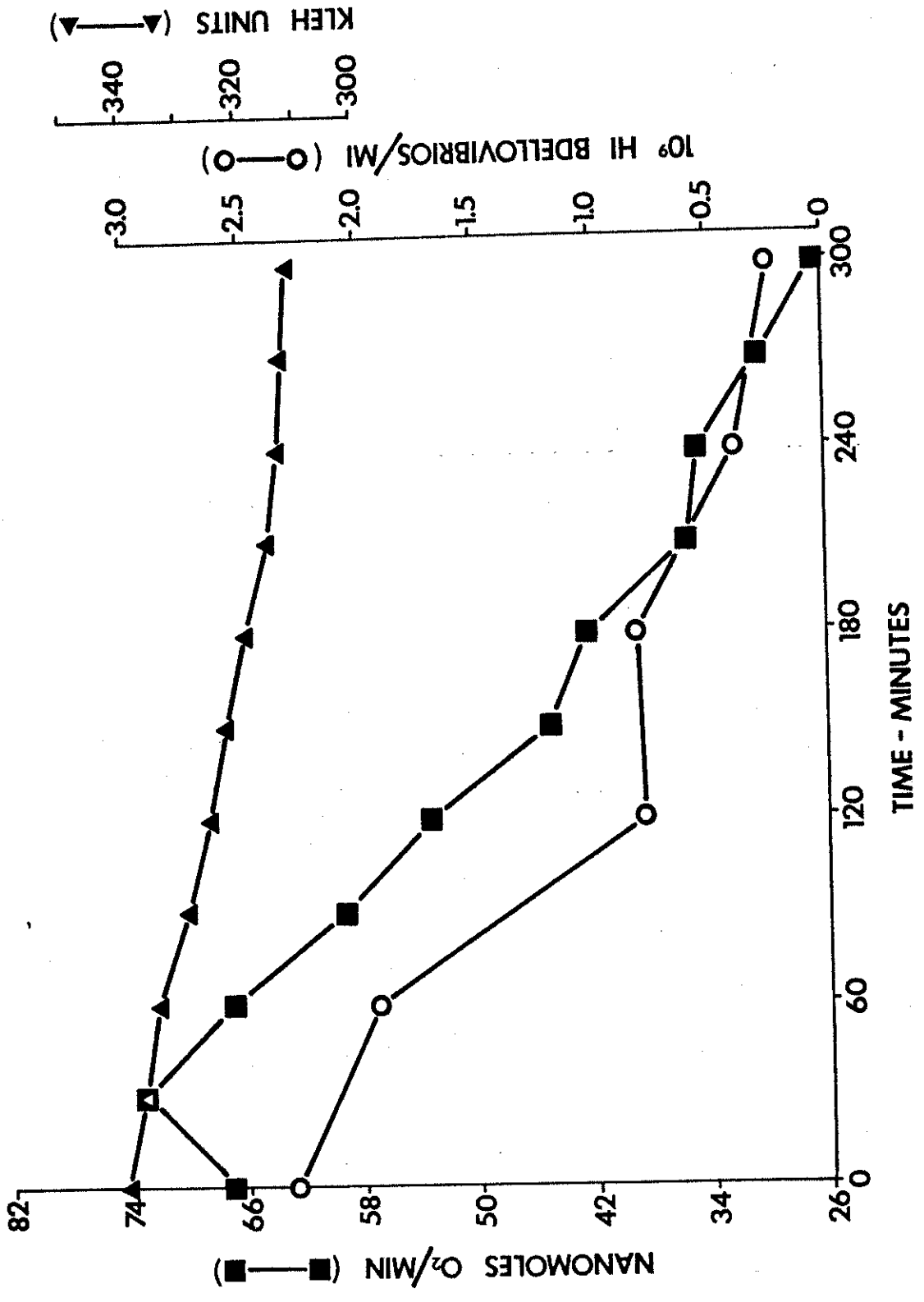
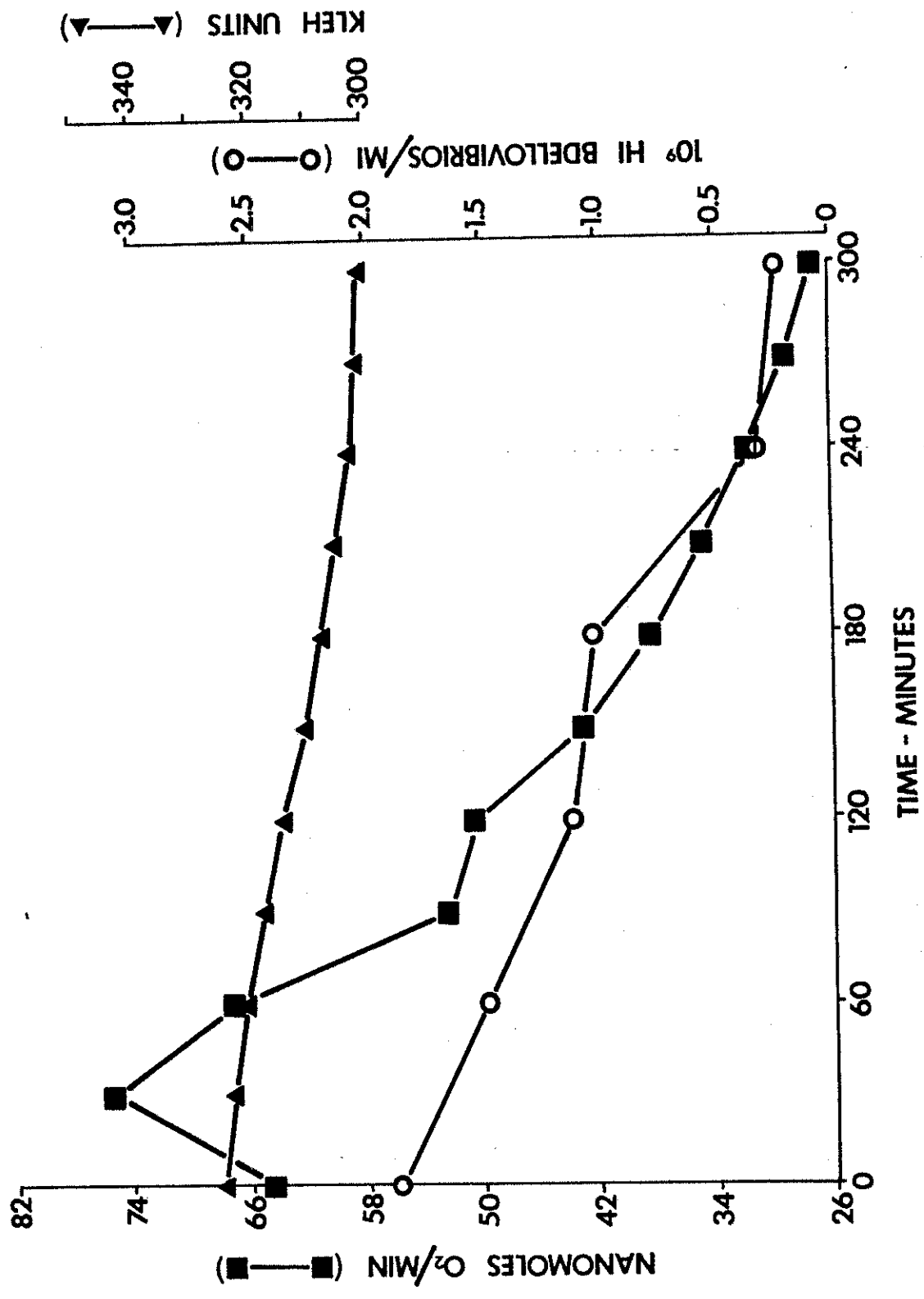

NANOMOLES O2/MIN (■——■)
82
74
66
58
50
42
34
26
0
60
120
180
240
300
TIME - MINUTES
10⁹ HI BDELLOVIBRIOS/MI (O——O)
3.0
2.5
2.0
1.5
1.0
0.5
0
KLEH UNITS (▲——▲)
340
320
300

Fig. 13. Respiration patterns and changes in turbidity and cell numbers of host-independent bdellovibrios combined with ultraviolet-killed Enterobacter aerogenes.


NANOMOLES O2/MIN (■——■)
82
74
66
58
50
42
34
26
0
60
120
180
240
300
TIME - MINUTES
10⁹ HI BDELLOVIBRIOS/MI (O——O)
3.0
2.5
2.0
1.5
1.0
0.5
0
KLEH UNITS (▲——▲)
340
320
300

The E. aerogenes cells appeared to exert an inhibitory effect upon the bdellovibrios since the bdellovibrios were not shown to form colonies on plates which contained colonies of E. aerogenes. Alternatively, they formed too few colonies to be detected by the methodology used. Since the parameters of turbidity and respiration were nearly identical whether or not E. aerogenes was present, the HI bdellovibrio population probably decreased at a rate similar to that in the HI control. It can also be seen in Fig. 11 that the number of viable E. aerogenes decreased. Since this decrease (almost 60%) was much greater than that in the control (Fig. 5), it appears as if the HI bdellovibrios had an inhibitory effect on the E. aerogenes population.

Several experiments were performed which involved bdellovibrios and domestic sewage. The purpose of these experiments was to compare the properties of bdellovibrios in sewage to those of bdellovibrios in controlled laboratory tests. Another underlying purpose was to verify that all of the requirements for the parasitic interaction are met and that conditions are permissive for it to occur. When Tris-YPG Medium was inoculated with raw sewage, the cultures became turbid in a matter of hours and remained turbid for two to three days. At this time, the turbidity regularly decreased steadily during a two- to three-day period. This clarification of the culture was likely due to the lysis of bacteria by bdellovibrios and/or bacteriophage. When various combinations of

known strains of bdellovibrios and hosts were added to media containing either raw or sterilized sewage, consistent patterns were not obtained, suggesting fluctuations in sewage constituents. However, on each of eight occasions over a three month period, bdellovibrio isolates were recovered from sewage samples, suggesting that the parasites are regularly present in sewage of the local treatment plant. It also suggests that the bdellovibrios have an important ecological role in aquatic environments. Occasionally bacteriophage-like plaques were noticed i.e. clear areas which remained a constant size as compared to bdellovibrio plaques which continually enlarge in size.

CHAPTER IV

DISCUSSION

Enterobacter aerogenes appears to possess a stable metabolic rate during periods of endogenous-respiration or starvation. This was indicated by the results obtained when viable E. aerogenes were suspended in Tris-MgCa Buffer.

Results indicate that endogenously-respiring HD bdellovibrios possess a metabolic rate unlike that of endogenously-respiring E. aerogenes. It was found that the number of viable bdellovibrios decreased rapidly when host cells or exogenous nutrients were absent. It is logical to assume that such a rapid loss of viability would result from the absence of nutrients. Since bdellovibrios are highly motile and motility is a requirement for their parasitic interaction, large quantities of energy must be necessary to meet these demands. Thus, if an organism having a high metabolic rate is without nutrients for any length of time, it would, in the absence of metabolic control mechanisms starve to death.

The fluctuations in respiration rates of the HD strains are similar to the fluctuations of the ATP pool of Bdellovibrio 109 Pa. observed during periods of endogenous-respiration (26). Since these organisms reportedly rely on the TCA cycle and oxidative-level phosphorylation for the generation of

energy, the oxygen patterns presumably are a reflection of the ATP pool level. These fluctuations are thought to be due to a special regulatory mechanism which enables the bdellovibrios to periodically produce energy for motility during periods of starvation by degrading their cellular components (26). These data appear to support the concept that the HI bdellovibrios lack the ability to regulate their self-digesting processes and maintain a high rate of respiration concomitantly with a rapid rate of cell death.

The combination of HD bdellovibrios plus viable host cells led to results unlike those of the previous experiments. The initial decrease in the bdellovibrio population reflected in the numbers of pfu was likely due to multiple attachment of the parasites to host cells. Although not highly accurate, pfu counts are a crude reflection of cell numbers. The attack and lysis of the host cells was reflected in decreases in turbidity and in the host population. The release of progeny, i.e., completion of the parasitic cycle, was evidenced by the increase in bdellovibrios beginning at about 120 min.

At the beginning of the experiment, oxygen was shown to be respired by the HD bdellovibrios and by the host cells. As the parasites began attacking the host cells, a process which would result in obvious damage to the host's cytoplasmic membrane, respiration decreased. Electron micrographs have adequately illustrated this damage (6,36). Since the cytoplasmic membrane is an important site of respiratory enzymes,

damage to it would affect respiration. As the parasitic process ensued, respiration increased, presumably reflecting utilization of host cell constituents for growth and development of progeny cells. The amount of oxygen respired during the last minutes of the experiment was due entirely to bdellovibrio respiration, since at this point the host was found to be dead, as defined by its inability to resume multiplication and colony formation when placed in a suitable environment. Upon lysis of the host cells, a further decrease in respiration rate was recorded.

When HD bdellovibrios were combined with autoclaved E. aerogenes, a decrease in viable cells was observed to occur indicating that the parasites were unable to readily utilize the autoclaved cells as a source of nutrients. However, in the latter part of the experiment, the bdellovibrio population appeared to stabilize, suggesting either a buffering effect due to the autoclaved host cell constituents or utilization of these constituents by the bdellovibrios. Possibly the thermal injury to the host cell wall or other constituents was so extensive that attachment by the bdellovibrios was impossible. These results were anticipated since the process of autoclaving would be expected to denature many, if not all host cell proteins.

The UV-killed cells appeared to satisfy to some degree the nutritional requirements of the HD bdellovibrios since the population and respiration rates were fairly stable

throughout the experiment. The large and small cells observed were probably parent and progeny cells, respectively, indicating that the parasites could utilize UV-killed cells as a nutrient source. However, very few of these UV-inactivated host cells were parasitized by the bdellovibrios since the turbidity changed only slightly. These data appear to favor the theory that autolytic enzymes produced by the host are necessary for the complete and total lysis of the host cells when they are attacked by bdellovibrios. In the latter stages of the experiment, it appeared that the parasites were adapting to the UV-killed cells as a nutrient source since the respiration increased while the parasite population decreased. The results obtained from the last hour of the HD bdellovibrio plus UV-killed host experiment are similar to those of the second hour of the HD bdellovibrio plus viable host experiment. This suggests that the parasites metabolize UV-killed host less efficiently than they do viable host, thus delaying onset of the parasitism.

The steady decrease in the number of viable HI bdellovibrios suspended in buffer may be taken as an indication that they were unable to utilize components of the buffer as nutrients and that they stored little if any reserve materials during their normal growth and development. The maximum respiration rate at 30 min coincided with the fragmentation of the spiral bdellovibrio cells. Results nearly identical to those described above were obtained for the following

combinations of cells: HI bdellovibrios plus viable E. aerogenes, HI bdellovibrios plus autoclaved E. aerogenes, and HI plus UV-killed E. aerogenes. The repeated decrease in viable HI bdellovibrios indicated that the presence of viable and/or nonviable E. aerogenes did not appear to exert a buffering effect upon the bdellovibrio population.

Since bdellovibrios are ubiquitous and natural bacterial parasites, it would seem that they would play a very important role in the regulation of microorganisms in microbial environments. Many investigators have assigned an ecological role to these organisms. Some suggest that bdellovibrios actively control their bacterial neighbors (1,42) while others imply that they do not (10). The present investigation is of the opinion that HD bdellovibrios do indeed help to regulate bacterial populations in nature, especially those in sewage. It would seem that Bdellovibrio would be an ideal agent in the treatment of water and sewage since it attacks and destroys gram-negative bacteria that are abundant in sewage.

Sewage obtained from a local plant appears to normally contain bdellovibrios. Moreover, sewage-inoculated broth cultures were shown to clarify within a few days, provided conditions permissive for bdellovibrio growth were provided. Environmental conditions such as temperature, humidity, sunshine, etc. as well as the general characteristics of the raw sewage itself including pH, dissolved oxygen, concentration of organic matter, etc. greatly influence the composition

of the sewage that is being treated. Any changes which occur in the sewage directly cause fluctuations in the populations of microorganisms present. These constant fluctuations make it difficult to maintain a reliable treatment system which would reduce or eliminate the pathogenic bacteria present in the sewage. Therefore, close monitoring of these fluctuations would have to be achieved.

In order to develop a sewage treatment system which would be able to utilize bdellovibrios, certain conditions would have to be maintained. The sewage must be kept aerobic and it should contain calcium and magnesium as demonstrated here and elsewhere (32,45). Once these conditions are provided, the bdellovibrios should be able to effectively reduce the levels of pathogenic organisms present in the sewage. Consequently, less chlorine would need to be added to the water to achieve a reduction of the bacterial counts to meet public health standards. Lower levels of chlorinated hydrocarbons would be a result. The treatment of sewage by Bdellovibrio would be a safe and natural process resembling a "purification from within" system.

CHAPTER V

SUMMARY

Populations of HD and HI bdellovibrios were combined with viable, autoclaved, and UV-killed E. aerogenes in Tris-MgCa Buffer. Direct measurements of respiration, turbidity, and viable cell counts were made.

In order for HD bdellovibrios to grow and reproduce, they require nutrients derived from intact host cells. Atypical development of progeny cells occurs if autoclaved cell constituents are present. However, a true parasitic cycle occurs when either viable hosts or UV-killed hosts are available. Therefore, the metabolic requirements for the parasitic interaction are constituents from either metabolically active host cells or cells which are capable of at least some metabolic activity.

The nutritional requirements of host-independent bdellovibrios, suspended in buffer, are not met by the presence or absence of viable or nonviable E. aerogenes. Unlike the HD bdellovibrios, the HI bdellovibrios lack the ability to make economical use of their self-digesting processes.

Provided sewage is aerobic, contains calcium and magnesium, and is closely monitored for environmental-caused fluctuations, HD bdellovibrios may be effectively used to reduce the bacterial populations.

APPENDIX

TABLE I

COMPOSITION OF TRIS-YPG MEDIUM

Component	Amount
0.05 M Tris-Buffer (pH 7.5)	1000 ml
Yeast Extract	3 g
Peptone	0.6 g
Glucose	1 g
$MgSO_4 \cdot 7H_2O$	0.74 g
$Ca(NO_3)_2 \cdot 4H_2O$	0.47 g

TABLE II

COMPOSITION OF YEP No. 3 MEDIUM

Component	Amount
Proteose Peptone No. 3	10 g
Yeast Extract	3 g
Distilled Water	1000 ml

TABLE III

COMPOSITION OF TRIS-MgCa BUFFER

Component	Amount
0.05 M Tris-Buffer (pH 7.5)	1000 ml
$MgSO_4 \cdot 7H_2O$	0.74 g
$Ca(NO_3)_2 \cdot 4H_2O$	0.47 g

TABLE IV

HOST RANGE OF BDELLOVIBRIO BACTERIOVORUS ATCC 15364

Host	Lysis*
Enterobacter aerogenes	+
Escherichia coli B	+
Staphylococcus epidermidis	-
Salmonella typhosa	+
Salmonella paratyphi A	+
Klebsiella pneumoniae	+
Pseudomonas aeruginosa	-
Serratia marcescens	+
Shigella paradysenteriae	+
Sarcina spp.	-
Proteus mirabilis ATCC 15363	+

*(+) indicates lysis, (-) indicates no lysis

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