THE FEEDING OF *TETRAHYMENA PYRIFORMIS* IN THE PRESENCE OF PARTICLES

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TABLE OF CONTENTS

INTRODUCTION..... 1

LITERATURE REVIEW..... 2

Description of the Protozoan, Tetrahymena pyriformis..... 2

Nutritional Requirements..... 3

Bacteria as a Food Source..... 5

Ingestion and Digestion of Food..... 8

Phagocytic Action..... 9

Pinocytic Action..... 12

Factors Influencing Growth..... 14

METHODS AND MATERIALS..... 16

Media and Solutions..... 17

Particle Additives..... 18

Experimental..... 20

RESULTS AND DISCUSSION..... 22

Growth in Mineral Salts..... 22

Growth in Proteose-peptone..... 30

Addition of Clay Particles and Synthetic Gels..... 48

Addition of Minerals..... 53

Addition of Algae..... 53

SUMMARY..... 56

ACKNOWLEDGMENT..... 59

LITERATURE CITED..... 60

APPENDIX..... 63

THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.

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INTRODUCTION

Protozoans, as a group of microorganisms, are usually not considered as important as bacteria or fungi in the environment because of their small numbers. The variety of protozoans, classed according to their locomotion, feed upon various materials, ranging from soluble materials to the particulate matter of bacteria, algae, and other protozoans. They are found in water, soil, and at various interphases of the two; some are parasitic while others are facultatively autotrophic. Because of their universality, the protozoans encounter a complex natural environment, organic and inorganic. Other species of protozoans as well as bacteria, algae, nematodes, and rotifers are present in addition to the inanimate soil, clay, and primary minerals.

Tetrahymena pyriformis is a protozoan which is found in most fresh water lakes and because of its ubiquitous character and ease of culturing was chosen to represent the ciliates and their behavior under the experimental conditions used. Ciliates have been implicated in clearing sludge tanks of bacteria and also in the self purification of lakes. The utilization of T. pyriformis and its growth rate as the parameter for the determination of the effect of particles has the advantages of convenience, ease of duplication, and ease of handling. This specific organism has been used extensively as an experimental organism in nutritional studies. Kidder (1941) used this microorganism for studies involving a defined medium for growth, and for studies with bacteria as food sources. Seaman (1961) used this ciliate for the study of phagocytosis and determined that insufficient food entered by the mouth for maintanence of the organism; he measured the formation of the food vacuole. Mueller (1965) observed the uptake of latex particles without a selective mechanism.

The ciliate, T. pyriformis, would act as a model protozoan under laboratory conditions to indicate what responses might be expected in the natural environment. This protozoan was studied under conditions which simulated events in the natural environment, from a nutritionally deficient to a completely adequate organic medium.

The proposed study was undertaken to gain information on the feeding habits of this protozoan, especially the uptake of bacterial cells under various conditions. These conditions included variations in the suspending media, from a minimal amount of ions in distilled water to a balance of mineral salts in Ashby's Salts medium. Also observations were made in the presence of added protein to evaluate the effect of extra nitrogen on the growth of the organism.

The uptake of the bacterial cells was studied in the presence of colloidal material, such as, bentonite, other clays and primary minerals to represent "silting in" of a fresh water lake. The effect of algae upon the growth of the protozoan was also noted. The addition of the synthetic gels with varying pore sizes was used to determine whether Tetrahymena obtains its food mainly by pinocytosis or phagocytosis.

It was hoped that this investigation would yield some information about the action of protozoans in their environment and in self-purification of a lake.

LITERATURE REVIEW

Description of the Protozoan, Tetrahymena pyriformis

The protozoan which is now known as T. pyriformis has been known by many other names until Furgason coined the generic name "Tetrahymena" after a discussion of their buccal ciliature. Other authors had used "Colpidium", Glaucoma pyriformis, Leucophrys pyriformis, as well as Tetrahymena geleii for

the organism known as Tetrahymena pyriformis (Corliss, 1954).

The generic name arises from the structure of the mouth which possesses 4 membranous structures, one undulating membrane being located on the right and 3 membranelles on the left dorso-lateral wall of the buccal cavity. The ciliate measures approximately 50 x 30 microns although there is considerable variation in these dimensions under different conditions. It is typically pyriform in shape with 17 to 21 rows of cilia. It can be grown axenically in ordinary bacteriological media or in chemically defined media, although in natural habitats T. pyriformis is a bacteria-feeder. It is found in moving and standing water, small streams, ponds, and large bodies of water. It is known to exist even as a facultative parasite. It has been taken from natural habitats at temperatures ranging from 4 to 30 C, and even under laboratory conditions some strains thrive abundantly at 37 C (Elliott, 1959).

The role of protozoans in water is not confined to devouring bacteria and smaller protozoan species, but to provoke certain mutual stimulations and antagonisms between different species with all the implications for self-purifying processes in the water (Jirovec, 1963). Lilly (1965) also noticed that a consistent 50% increase in growth was obtained with T. pyriformis in response to a factor produced by Colpidium campylum. Unfortunately, our knowledge regarding the physiology and particularly the metabolism of even the very well-known protozoan species is still very limited.

Nutritional Requirements

Holz, Erwin, and Wagner (1961) utilized a strain of T. paravorax RP which required 12 amino acids, 6 B-complex vitamins, a purine, a pyrimidine, and inorganic salts. A sterol or sterol ester was necessary for optimal growth; plant and animal 3 beta - OH sterols with saturated and unsaturated ring systems were active.

Elliot (1959) stated that fatty acids are not required for growth; but lipids, supplied in the form of Tween compounds, are stimulatory. Carbohydrates are not necessary for growth, but they, too, are stimulatory. Nitrogen comprises 7 to 8% of the dry weight of the organism, of which less than $\frac{1}{2}$ is in the form of protein nitrogen while 1/3 is in the form of peptides of free amino acids. The lipid content of T. pyriformis is high, 15 to 20% of the dry weight. Glycogen also is stored in large quantities, 15 to 22% of the dry weight in non-carbohydrate media, and varies with the life cycle of the organism.

In chemically defined media at carbohydrate concentrations greater than or equal to 0.5% (w/v) T. pyriformis W multiplied more rapidly, developed larger cells and achieved greater growth as measured by optical density when the carbohydrate was provided as dextrin rather than glucose (Reynolds and Wragg, 1962). T. pyriformis grown on 1% proteose-peptone and 0.25% yeast extract utilized 9.1% of the carbon during growth with 36.5% of the carbon used going into cilicate cytoplasm and 69% into carbon dioxide by respiratory activities. These values approach those obtained when bacteria are grown on soluble substrates as determined by Curds and Cockburn (1968).

Waithe (1964) found that the rate of glucose utilization ranged from 0.23 to 1.9×10^{-7} mg/cell/hr, depending on the age of the culture. Neither differences in glucose concentrations from 33 to 100 mg/100 ml nor insulin affected the rate of utilization. The decrease in rate of glucose uptake with increasing age of the culture may involve a shift in metabolism brought about by depletion of exogenous glucose during growth. Seaman (1950) found an accumulation of fatty acids in the medium of growing cultures of T. pyriformis followed by a sharp decrease in these fatty acids as the culture reached maximum population density. Seaman (1950) stated that T. geleii synthesizes carbohydrate and fatty acids simultaneously from acetate. Carbohydrate is not a necessary precursor of lipid during the synthesis. Acetaldehyde, rather

than acetyl phosphate, is probably the active two carbon compound in the formation of lipids from acetate.

Ciliates grow better in the presence of intact complete proteins and/ or enzymatic hydrolyzates containing a variety of peptides. These proteinaceous materials may 1) correct multiple amino acid imbalances in the medium, 2) stimulate food vacuole formation and expedite nutrient translocation, 3) supply as contaminants unrecognized stimulatory or essential metabolites, 4) bind toxic materials in the medium, and 5) provide peptides which are themselves essential metabolites, or amino acid sequences that cannot be constructed from free amino acids (Hutner, 1964).

Bacteria as a Food Source

According to Kidder (1951) the holotrichous ciliate, Colpoda steinii (Tetrahymena, Corliss, 1954) was incapable of growth in various nutrient media alone or with dead bacteria when in an axenic culture. Growth was obtained when any of a number of species of bacteria was added singly in distilled water, although Aerobacter cloacae promoted the most rapid and luxuriant growth. One species of Flavobacterium was not ingested while Chromobacterium violaceum and Serratia marcescens were lethal to the ciliate. The toxicity was due to the pigment itself and acclimatization to the pigment was possible. The bacterial requirement was found to be a heat labile protein.

In the opinion of Kidder and Stuart (1939), a very important factor in the growth limitation of a population of Colpoda was the quantity of available food, with temperature, the hydrogen ion concentration, and decrease in oxygen tension in larger volumes also being important. Concentration of metabolic waste products was apparently ineffective in both the protozoa or of the food organism; otherwise, their effects would be seen by the time the ciliates reached the concentration of 20,000/0.005 ml. Single ciliates when introduced

into varying bacterial concentrations reproduced in direct relation to the bacterial count. This was also shown by Ducoff, Williams, et al (1964) using viable and killed bacteria.

Kidder (1941) later states that no species of food organism tested was as favorable for growth as the dissolved protein materials. Tetrahymena geleii W and T. vorax are both able to utilize dissolved proteins; and the addition of particles to peptone media did not increase the growth rate or the yield. He felt that if no proteolytic excellular enzymes were released into the medium that the nutritive materials, even in the dissolved state, enter food vacuoles by way of the cytostome based on the fact that five strains of Tetrahymena grow normally in dissolved casein. It seemed highly improbable that the whole protein molecule could be absorbed through the pellicle.

It was shown by the microscopic examination of Glaucoma vorax (Tetrahymena) by Kidder, Lilly, and Claff (1940) that bacteria were taken into the food vacuoles of the ciliates. Ciliates fed on washed living Aerobacter cloacae resembled the thin tailed organisms in nature. Well fed ciliates appeared quite granular, but as the bacteria were eaten out of the culture they became clear and refractive. Autoclaved Aerobacter in distilled water was not adequate to support growth in G. vorax. The ciliates remained alive for long periods of time, but they did not increase in size nor did they multiply. Baker and Huddleston (1947) also found that T. geleii reached a maximal population in the proteose-peptone as compared to the bacterialized cultures of E. coli, Pseudomonas fluorescens, and Streptococcus lactis. The protozoan population was lower in the yeast extract media than in the bacterialized cultures.

Harding (1937) determined that the rate of disappearance of P. fluorescens was a function of the concentration of Glaucoma. In low concentrations of

bacteria the rate of feeding of the Glaucoma was a function of the concentration of bacteria. In higher concentrations of bacteria, the Glaucoma were able to find almost as much food as they could take in; so that the rate of feeding tended to become independent of the concentration of bacteria. For every increase in the concentration up to at least 8×10^6 bacteria/mm^3, there was an increase in the rate of formation of food vacuoles. Between concentrations of 6 to 7×10^6 bacteria/ $mm^3$the size, but not the fission of Glaucoma, was influenced by the concentration with the generation time being 2.4 hr.

Bubanck (1942) used 27 different strains of living bacteria to determine if Colpidium colpoda were a selective feeder. His data showed that the Enterobacteriaceae; Escherichia, Aerobacter, Salmonella, Proteus, and Eberthella, supported the highest reproduction rates of Colpidium; while the Micrococcaceae and Bacillaceae gave the lowest division rates. The Rhizobiaceae, Pseudomonadaceae, and Bacteriaceae seemed to form an intermediate group, as far as acting as food for Colpidium was concerned. With the exception of the toxicity of Chromobacterium violaceus little difficulty was experienced in maintaining C. colpoda on chromogenic bacteria. Serratia marcescens and Pseudomonas aeruginosa were used with good results, although Colpidium died in drop cultures of the latter when they were allowed to run longer than 24 hr.

In a 6-month study of adaptation, Harris (1967) used washed and autoclaved cells of 9 strains of bacteria to sustain T. pyriformis. Mycobacterium lacticola did not support as dense populations of the ciliate as the strains of Aerobacter aerogenes, Azotobacter agile, Arthrobacter, Bacillus megaterium, Pseudomonas fragi, Serratia marcescens, and Thiobacillus thioparus. Ducoff, et al, (1964) found Cerophyl had to be present in concentrations above 0.1%

for the phagotrophy-inducing factor to aid in the ingestion of the bacteria. The filtrate of autoclaved bacterial cells which had been passed through 0.5 micron porosity membrane filters did not support increases in the Tetrahymena population.

Using a monoxenic culture of Klebsiella aerogenes with T. pyriformis GL, Curds and Cockburn (1968) found that the maximum growth rate at 25 C was 0.22/hr as compared to 0.20/hr in axenically grown cultures in proteose-peptone yeast extract medium. The effective yields of Tetrahymena were 9.1% (carbon to carbon) in axenic cultures and 50% (dry-weight bacteria to dry-weight ciliate) in monoxenic cultures.

Ingestion and Digestion of Food

Before a discussion of the movement of the food (soluble and particulate) can take place, a few statements should be made on the volume of liquid that can be engulfed by the Tetrahymena. The results vary depending upon the experimenter; Seaman (1963) calculated that 10^6 ciliates cleared 0.034 to 0.042 ml/ hr using trypan blue uptake for his measurements. Cox (1967) used a colorimeter to measure the amount of suspended carbon present in a culture of Tetrahymena before and after the ciliates had fed, and he found that 10^6 ciliates could remove the carbon from 6 to 10 ml of media/ hr.

Paramecium caudatus secretes a substance "P", a mucoprotein, that caused a flocculation of Indian ink particles. Curds (1963) further stated that suspended particles whose surface charge is negative will absorb some of this polysaccharide, resulting in a change of charge and the aggregation of the particles to form floccules. Chen (1965) further suggested that this slime or secretion aids in clearing the water of flocculant particles in sewage treatment tanks.

Phagocytic Action

Most studies of phagocytosis and metabolism have used leukocytes as the phagocytic cell. Metabolic energy appeared to be required for the "capture" of a particle by the phagocyte. Karnovsky(1962) found that between 1-2 x 10^{-15} moles of ATP were required to move one polystyrene spere 1.171 microns in diameter into the cell under anaerobic conditions. It is clear from innumerable studies that surface characteristics of the particle are indeed of primary importance and maybe modified by protein components of the system to permit phagocytosis or to inhibit it.

Practical application of the descriptive terms "in solution" and "particulate" to components of a culture medium is arbitrary. Ciliary metabolic activities may produce molecular aggregates of colloidal dimensions and these may themselves aggregate. Such changes in physical state may be caused by the activities of extracellular enzymes and/or changes in the hydrogen ion concentration, and have been observed in cultures of G. chattoni A in the presence of proteins (casein) and phospholipids (Holz, et al, 1961). A small amount of cell death may also yield particulate materials.

Entry for nutrients into food vacuoles at a locus on the body surface (a mouth) occurs by phagocytosis (engulfment of particles ranging in size from viruses to metazoans) and pinocytosis (cell drinking). Some pinocytosis always accompanies phagocytosis. Seaman (1963) referred to food vacuoles containing substances in solution and to vacuoles containing particulate matter as phagotrophic vacuoles, and to the process of their formation at the mouth of Tetrahymena as phagotrophy. Mueller (1965) viewed ciliate food vacuoles as lysosomes of the phagosome type and noted that food vacuoles met the criteria for lysosomes: 1) the encompassing of the vacuole in a unit membrane, presumable originating from the surface membrane at the site of

vacuole formation at the mouth, "isolating" the vacuolar contents from the cytoplasm; 2) the association with the vacuole of acid phosphatase, acid deoxyribonuclease, acid ribonuclease, acid phosphoamidase, acid glucuronidase, and neutral leucylaminopeptidase, neutral carboxylesterase, and lipase; and 3) the digestion of food in the vacuoles and passage of soluble products of digestion into the cytoplasm.

Particulate matter and substances in solution induce food vacuole formation. T. pyriformis grown in proteose-peptone media rapidly ingested the non-permeable, trypan blue; however, cells cultured in synthetic medium did not contain these vacuoles and did not ingest the dye. The active material in proteose-peptone that caused phagotrophy had a molecular weight of 12,600 by ultracentrifugation and contained only the amino acids of glutamic acid, serine, alanine, valine and leucine. No evidence has been obtained for the presence of either a lipid or a polysaccharide component (Seaman, 1963). By measurements of dye uptake and ingestion of S. marcescens by T. pyriformis (indicators of rates of food vacuole formation), and comparison of the values so obtained with figures for rates of acetate and its utilization, Seaman arrived at the conclusion that the uptake of material through the mouth region in T. pyriformis is too small for the organism to be used in the study of bound forms of required nutritional cofactors.

Seaman (1963) stated that T. pyriformis formed no food vacuoles when it was cultured axenically in chemically defined media containing only small molecules. Others have seen food vacuoles in T. pyriformis cultured in media of that description; Chapman-Andresen (1963) reported observing 5 to 15 vacuoles per individual in strain GL cultured in Kidder's medium A. Vacuoles have also been seen in ciliates washed repeatedly and left to stand in distilled water during mating experiments. There is no question, however, that

solutions of proteinaceous materials and suspensions of particulate matter greatly stimulate vacuole formation over rates seen in nonnutrient media and media composed of small molecules.

The intolerance of most ciliates for the rich media that grow tetrahymenids suggests that many may rely very little on nutrient translocation across their surfaces and depend, to varying degrees of exclusiveness, on forms of particle feeding (Hutner, 1964).

The food trapping apparatus consists of three membranelles, and undulating membrane, oral ribs, and a "valve" apparently closing the opening to the cytopharynx. Both of the latter structures are supported by microtubules. Fibers extend internally from the cytopharynx and are closely associated with food vacuoles as they form. The protozoans can form uniformly large vacuoles containing bacteria and India ink particles in 5 min with many vacuoles being formed in 15 min which are retained for 30 min. In the second state of digestion, the bacteria are compacted leaving a clear space between the bacterial mass and the membrane forming a halo. In the third stage the vacuole becomes more dense and there is condensing of the inert India ink particles. In the fourth stage, the vacuole becomes small, black, and spherical. The vacuoles are clear in proteose-peptone media; these processes were reported by Elliott and Clemmons (1966). Cytoplasmic digestive enzymes pass through the vacuolar membrane and hydrolyze the entrapped large molecules. The smaller molecular weight breakdown products are then free to pass out of the vacuoles and to serve as substrates for the cytoplasmic metabolic machinery (Seaman, 1961).

Mueller, Rohlich, and Toro (1963) observed the ingestion of non-nutritive Dow polystyrene latex particles (PLP) by T. pyriformis and the formation of vacuoles containing PLP at a rate comparable to the formation of vacuoles

containing bacteria. The particles aggregated within the vacuoles and were egested as balls of the size of vacuoles. No selection between nutritive and non-nutritive particles was revealed; the vacuole formation was induced by the mechanical action of the particles. The appearance of acid phosphatase activity in the vacuole seemed to be dependent of the vacuole formation and not on its content. In support of the view which ascribes the selection of particles primarily to physical and not to chemical factors, the ingestion of Aerobacter aerogenes and that of PLP proceeded with equal speed. The latex particles seemed to resemble strongly in their size and surface properties those bacteria which were the usual food of Paramecium.

Pinocytic Action

The formation of channels by the adhesion of the plasmalemma to the underlying plasmagel is perhaps due to the neutralization of negative surface charge by adsorption of positively charged inducer molecules at highly acidic sites in pinocytic movement. The importance of neutralization is demonstrated by the use of ferritin and methylated ferritin at various pH values, showing that the rate of pinocytosis was determined by the new charge on the protein particle (Chen, 1965).

Marshall, Schumaker, and Brandt (1959) described three stages in the uptake of protein. In the first, the protein is bound to the membrane or cell surface. This binding is reversible, is a function of the concentration of protein in the medium, is largely independent of pH or temperature, and is not greatly affected by metabolic inhibitors. A second stage begins when protein binding exceeds a certain level. This is marked by a sudden increase in binding, as though new binding sites become available. It seems insensitive to metabolic inhibitors, pH, and to cooling. Ingestion, or bound protein is no longer in equilibrium with protein in the medium and is sensitive to

inhibitors and to cooling. A basic protein is bound by a mucoid or lipoid component of the membranes, which are acidic in nature; thus, the cell appears negative in charge with the bound substance possessing a positive charge, developing a salt-like linkage. The molecular orientation of the binding sites can be demonstrated by the birefringence colors of the attached dyes. The dye colors obscure the natural surface birefringence; hence, the attachment is in immediate proximity to the major oriented component in the surface. Since proteins, which would not cross the permeability barrier, act as inducers, the binding region must be located outside the cell membrane (Rustad and Rustad, 1960). Brandt and Schumaker (1965) have also demonstrated an adsorption of the inducing solute to the outer layer of the plasmalemma due to positive and negative charges through pH changes.

Both pinocytosis and phagocytosis use plasmalemma to form channels and food vacuoles. Small vacuoles are pinched off from the food vacuoles and divided up into smaller food vacuoles during digestion. During pinocytosis, small vacuoles are pinched off from the channels, and the vacuoles are further divided into even smaller ones. These vesicles are membrane lined, but so far it is unknown whether this membrane is identical with the plasmalemma. Both phagocytosis and pinocytosis depend on changes in temperature. In pinocytosis, the contractile vacuole, which may increase to a diameter 2 or 3X that at normal diastole, is inhibited. It appears that the limiting factor for pinocytosis is the availability of the membrane. It was concluded that neither overall charge nor blockage of what are probably polyphosphate groups on the surface of bacteria are important in the actual process of phagocytosis (Chapman-Andresen, 1963).

Pinocytosis is a mechanism by which cells transport solutes across the surface membrane. Pinocytosis was found to be a discontinuous phenomenon,

lasting about 15 min, slowly decreasing after an initial burst. A fine wrinkling of the membrane was always observed prior to pinocytosis when amoebae were treated with an active agent. It was inferred that membrane tension has dropped markedly in such instances, and that the membrane tension which normally breaks the bonds of attachment between the plasmalemma and the underlying gel is now inadequate. As a result, the plasmalemma is drawn down into the cytoplasm at such points of attachment. It appears to be pulled in deeper by the plasmasol flowing forward into the pseudopod and pushing the plasmalemma ahead of it (Brandt, 1958).

The mechanisms of pinocytosis would involve decreasing the tension of the plasmalemma. The most direct would be the effect on surface tension by the adsorption of charged molecules such as salts and proteins. Also due to the complex structure of the plasmalemma, agents which are adsorbed may affect the internal bonding and structural rigidity of the plasmalemma. This would be by the indirect effect of the solutions on the mechanism by which the amoeba normally alter its surface membrane area. Since the plasmalemma may be continuously exchanging with a component of the cytoplasm, an agent which interferred with the reclamation of the plasmalemma might cause a decrease in the membrane tension. Salt solutions which caused pinocytosis were shown to cause a swelling of the outer coat of the plasmalemma. Pinocytosis may perform a double function, a nutritional one with the ingestion of protein and a defense against a concentrated ionic environment with the uptake of water and ions (Brandt, 1958).

Factors Influencing Growth

Elliott, Travis, and Work (1966) indicated that the ultrastructure of T. pyriformis was markedly influenced by the physical conditions of the medium during the growth cycle. Corbett (1958) observed that T. pyriformis reduced

its cell volume with aging of the culture, and in undergoing initial contraction of expansion in transfers from cultures or comparable growth stages to fresh medium. Initial expansion of the ciliate depended upon substrate uptake and consequent increase in intracellular water. Such cellular expansion was achieved through a conditioning of the extracellular medium by the organism.

Levy and Elliott (1968) observed that in the early stages of starvation, in the first 25 hr of starvation, ultrastructurally, numerous profiles of degenerating mitochondria were seen. The presence of oxidizable substrate such as glucose and acetate did not prevent this degeneration. Lipid reserves were apparently utilized prior to carbohydrates, as the disappearance of lipid droplets preceded glycogen utilization both in the presence of acetate and in the absence of exogenous substrate. A considerable loss of cellular protein also occurred.

The cell volume decreased from the lag phase to the logarithmic phase and reaches a minimum at the end of the log. phase according to Summers (1963). It increased again during the stationary phase. The nuclear volume also decreased from the lag to log. phase, but continued to decrease in the stationary phase until it reached the nuclear volume of the daughter cell.

T. geleii responded to the contact of a single bacterium by slowing or stopping ciliary action on the touched side (thigmotropism). When the contacting particle no longer touched the surface of the ciliate, ciliary action was immediately equalized. In a highly contaminated solution, it moved forward erratically. The protozoan is normally cathode orienting in an electric field, but when it touched a bacterium, it spiralled toward the anode. The organism altered response to gravity from positive to negative geotropism in increasing concentrations of carbon dioxide, while

simultaneously altering from thigmotropically positive to negative. In an ecological sense, the organism tended to escape upward from dense bacterial concentrations, but remained to feed upon the upper fringes of such bacterial concentrations (Browning, 1947).

Colpidium was shown to withstand a range of hydrogen ion concentration from pH 4.5 to pH 10.5 by Mills (1931). Finely ground colored substances of carmine, iron oxide, carborundum particles and aquadag--colloidal carbon, in which tannin and ammonia are employed as the dispersing agents, reduced the average number of ciliates. Browning (1949) found that osmotic pressure produced death of the organism by gelation.

METHODS AND MATERIALS

The suitability of the suspensions to support growth of the ciliate was determined by the number of Tetrahymena/ ml. The concentration of the population was determined by use of an A/O hemocytometer; counts were made on the sample which had been killed with formaldehyde to prevent clumping, multiplying, and moving of the protozoa. The counts were made with the high dry objective lens (430 X magnification) of a compound light microscope. Enumeration was also made using a Model B Coulter Counter at $\frac{1}{2}$ amplitude and $\frac{1}{2}$ aperature current with a lower threshold of 30 microns and an upper threshold of 96.5 microns. The optical density of the suspensions was measured by a Bausch and Lomb spectrophotometer in an attempt to indirectly determine the number of Tetrahymena present.

Aerobacter aerogenes cells, obtained from the Kansas State University stock culture collection, were grown in nutrient broth for two days at 30 C on a rotary shaker. The living cells (AA) were obtained by centrifugation at 5000 X g in a Serval table centrifuge under aseptic conditions, washed three

times with distilled water, and then placed in sufficient distilled water so that a 1/10 dilution gave an optical density reading of approximately 0.70 at 550 millimicrons on a Baush and Lomb spectrophotometer. The A. aerogenes cells which have been designated as ABW were autoclaved in nutrient broth at 15 lb/ sq in for 30 min before washing. The solutions were checked for sterility on nutrient agar. The cells were then washed three times with distilled water to remove any adhering medium. After washing three times with distilled water, the A. aerogenes cells, designated as AAW, were autoclaved at 15 lb pressure for 30 min. The cells, ABW and AAW, were then placed in enough distilled water so that a 1/10 dilution gave an optical density reading of 0.70 at 550 millimicrons on a Spectronic 20 spectrometer.

Media and Solutions

Nutrient broth of 0.3% beef extract and 0.5% tryptone in distilled water was prepared in 300 ml Erlenmeyer flasks in 100-ml amounts for the growth of A. aerogenes.

Soil extract medium was prepared by steaming 100 g of native pasture soil in two liters of distilled water for 3 hr; the liquid was then filtered through No. 1 Whatman filter paper and autoclaved in 100-ml amounts.

Ashby's Salts 2X solution was used to determine the tolerance of the Tetrahymena to inorganic salts. Other strengths of this solution were also prepared in 1X and $\frac{1}{2}$ X concentrations. The solution of 2X Ashby's Salts is made of the following:

K_2HPO_4	14.4 g	$CaCl_2$	1.6 ml of a 10% solution
KH_2PO_4	5.6	Microelements	0.8 ml of a 0.5% solution of Mn, Mo, Zn, and B salts with no nitrates added
$Mg\ SO_4$	1.6		
NaCL	1.6	$FeCl_3$	0.2 ml of a 20% solution
		Distilled water	4000.0 ml

Various solutions of proteose-peptone w/v (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10%) with and without particle additives were made either in side-armed 50 ml Erlenmeyer flasks or in screw capped tubes.

Potassium phosphate buffer (pH 7.0, 0.066M) was used to wash the Tetrahymena free of growth medium and to maintain them in a resting stage for an hour before experimentation.

Particle Additives

Many different types of surfaces were added to the growth media containing the Tetrahymena; among these were the following:

1) Biogel P-100, a porous polyacrylamide gel of 100-200 mesh (wet) was obtained from Biorad Laboratories, Richmond, California. This gel (3 g/ 80 ml of distilled water) was allowed to swell overnight before use; it has a pore diameter which will exclude materials with a molecular weight greater than 100,000.

2) Industrial grade glass Microballoons IG 101 were obtained from Emerson and Cuming, Inc., Canton, Mass. The hollow sodium borosilicate spheres range in size from 10 to 300 microns in diameter. A 1% stock solution in distilled water was made.

3) Polystyrene latex particles were obtained from the Dow Chemical Company; they had a diameter of 1.011 + or - 0.0054 microns with 0.015ml (3 drops) being used in 10 ml.

4) Bentonite, a colloidal hydrated aluminum silicate, produces a slightly alkaline gel in water. One gram of this material was placed in 1000 ml of distilled water and allowed to swell and come into equilibrium. The upper 20 ml of this suspension was then used in the particulate experiments.

5) Kaolinite, a native hydrated aluminum silicate, was used in a 1% stock solution in distilled water.

6) Diatomaceous Earth, a form of silica consisting of the frustules and fragments of diatoms, purified by boiling with diluted hydrochloric acid, washing, and calcining, was used in a 1% stock solution of distilled water.

7) Illite, a clay mineral of hydrated aluminum silicate with some potassium or magnesium and iron present, was used a 1% stock solution of distilled water.

8) Sephadex 200, a modified dextrin with an inner pore which will exclude molecular weights above 200,000, was hydrated and used in a 1% stock solution of distilled water. It has a range of 40 to 120 microns in diameter.

9) Primary minerals which were used in a 1% stock solution of distilled water included the feldspars, anorthite and pyroxite with anorthite; as well as the ferromagnesians, olivene and hornblende. Chlorite, silica gel, and charcoal were also used in a 1% stock solution. The particles were ground to a 2-5 micron size.

10) The following algal cultures were used after a two week period of growth in soil extract medium:

LB 143 Oscillatoria borneti

LB 796 Chlamydomonas sp.

756 Nostoc sp.

144 Pediastrum sp.

1069 Astrephomene gubernaculifera

1076 Closterium acerasum

107 Ankistrodesmus spiralis

770 Spirulina sp.

763 Gloeocystis ampla

245 Scenedesmus

437 Stigeoclonium tenue

EpC Chlorella sp.

The algae in a monoxenic culture were obtained from the Botany Department at the University of Indiana, Bloomington, Indiana.

Experimental

The protozoan, T. pyriformis W, an axenic, amicronucleate strain, was obtained from Dr. B. W. McCashland, Department of Zoology, University of Nebraska at Lincoln. This ciliate was grown and maintained in a 1% (w/v) proteose-peptone solution for three days at room temperature and in a stationary state. The organisms were then centrifuged at low speed on a Sorvall angle head centrifuge for less than a minute to concentrate the organisms without injury and washed two times with the phosphate buffer (ph 7.0), and then resuspended in 40 ml of the buffer. Counts were then made on this stock solution with the hemocytometer. Suitable dilutions were made so that the initial concentration of the Tetrahymena ranged between 0.2 to 0.5 x 10^4 organisms/ ml of solution.

Since it was a biological system, many variables were present which were difficult to control such as physiological age varying with other organisms present, temperature, food availability, etc. Because of the variability, the experiments were replicated and the results utilizing the bentonite were the values from six replications.

Solutions of Ashby's Salts in 2X and 1X concentrations were used in 13.25-ml amounts in side-armed flasks of 50-ml volume to determine the rate of growth in an inorganic medium. Tetrahymena in the stock solution was added in 0.75-ml amounts while stock solutions of bentonite and the A. aerogenes cells were added in one-ml amounts. Counts were made on a 1/10 dilution to which formaldehyde had been added on the Coulter Counter using the 100 micron orifice, at 24-hr intervals.

Growth of the Tetrahymena was followed in 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10.0% (w/v) proteose-peptone in screw capped tubes with and without the addition of the three A. aerogenes suspensions at 24-hr intervals for 90 hr. An attempt was made to follow the growth spectrometrically and to find a correlation with the counts obtained from the hemocytometer. The counts were made on solutions of Tetrahymena which had been immobilized with formaldehyde.

Coal tar pitch, illite, bentonite, kaolinite, vermiculite, olivene, chlorite, hornblende, stilbite, charcoal, albite, anorthite, pyroxite with anorthite were all used in 1-ml amounts of a 1% solution in a total of 10 ml of distilled water or proteose-peptone in screw capped tubes. ABW or AAW cells were also added to the suspension and the growth of the Tetrahymena was followed at 24-hr intervals.

The number of protozoans present was determined by sampling at 24-hr intervals for a 90-hr period in media of 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0% proteose-peptone and distilled water which contained the surfaces of Biogel-100, or latex particles, or diatomaceous earth, or bentonite in 10-ml volumes in screw capped tubes. These surfaces were also used in the presence of the three types of treated A. aerogenes cells.

Other materials with differing surface characteristics were also added to a 15-ml volume of 1% proteose-peptone in side-armed flasks; and the growth of the ciliate followed. These materials included one ml of the following suspensions; Sephadex 200, Biogel-100, Microballoons, latex particles, and bentonite. As indicated in the results, some of the materials were tested in the presence of AAW cells also.

In order to provide surfaces which are common in their natural environment, the Tetrahymena were exposed to algae in a soil extract medium. The

living algae after having grown monoxenically for two weeks had Tetrahymena added to give an initial concentration of 0.3×10^4 organisms/ ml. The growth was followed by sampling after 4 and 7 days. In another experiment using the living algae again in 10-ml volume in screw capped tubes, the Tetrahymena was added simultaneously with the algae and the growth followed.

The algae were also used after a two week growth in soil extract using a 10-hr interval of light daily; this light was produced by four fluorescent lights at a 12 inch height. This heavy growth was then autoclaved at 15 lb pressure for 15 min. This suspension in one-ml amounts was then added to 15 ml of 1% proteose-peptone in side-armed flasks. A control of 1% proteose-peptone was also used. Growth in this media was followed at 24-hr intervals.

RESULTS AND DISCUSSION

Growth in Mineral Salts

Counts of the protozoans were made by the Coulter Counter and periodically checked by the hemocytometer for comparison; both methods gave counts within a 10% range. The results showed that the maintenance of protozoans in distilled water was below the level observed in 1X and 2X Ashby's Salts media. In the distilled water, the initial rate of growth in 24 hr increased the most with AAW cells, producing 8×10^4 ciliates/ ml as compared to the 6×10^4/ ml produced with ABW cells and 3.5×10^4/ ml produced by the living cells, AA. The population appeared to cycle with the dead cells, as if the dying organisms released nutrients to allow new growth. The living cells gave a continuous upward growth curve reaching a maximum of 8×10^4 ciliates/ ml at 96 hr. The maximum population in distilled water was reached at 72 hr in AAW cells with 14×10^4 ciliates/ ml. These results are seen in Fig. 1.

The 1X Ashby's Salts medium (Fig. 2) gave a maximum population of 20×10^4 ciliates/ ml in 24 hr with the AAW cells. The ABW and AA cells gave

Fig. 1. Growth of Tetrahymena in distilled water in side armed flasks in the presence of the treated bacterial cells of AA, ABW, AAW, or alone.

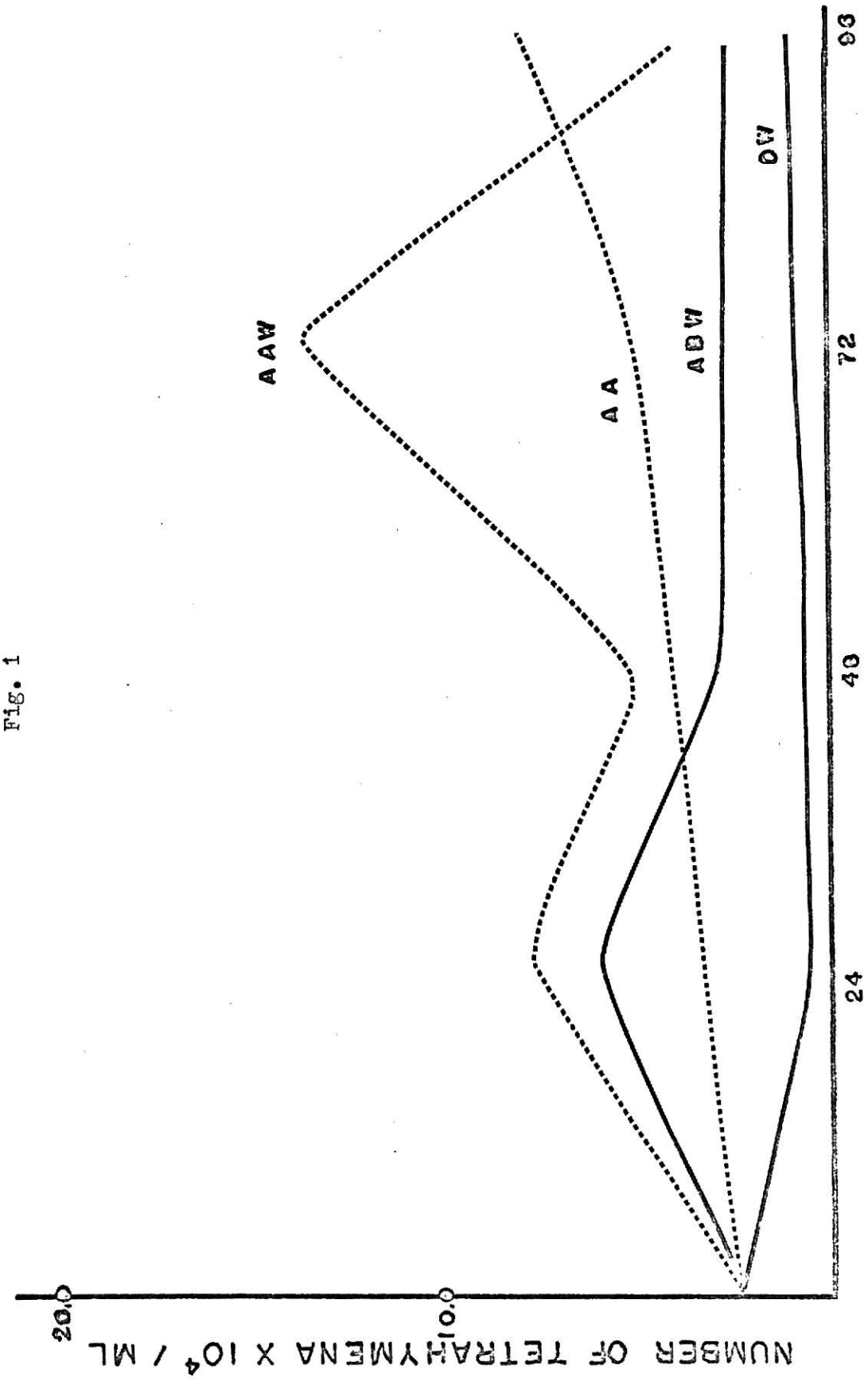
NUMBER OF TETRAHYMENA X 10⁴ / ML
20.0
10.0
HOURS OF GROWTH
24
48
72
96
AAW
AA
ABW
OW

Fig. 1

Fig. 2 Growth of Tetrahymena in 1X Ashby's Salts in side armed flasks in the presence of AAW, ABW, AA, or alone.

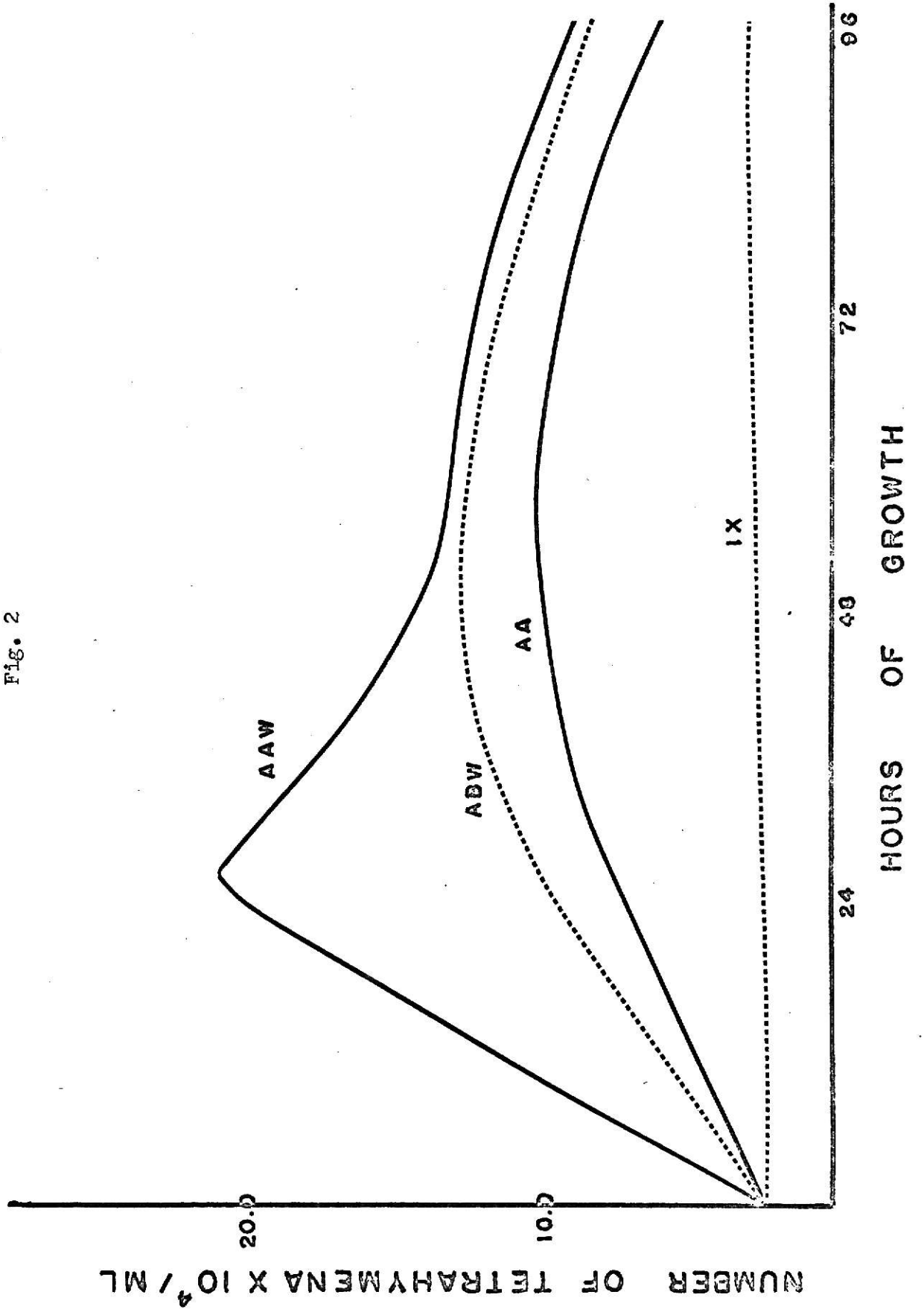
NUMBER OF TETRAHYMENA X 10^4/ML
20.0
10.0
24
48
72
96
HOURS OF GROWTH
AAW
ABW
AA
IX

Fig. 2

concentric curves with the ABW giving the greater number of protozoans. The AAW cells gave the most rapid initial rise in population, 18.5×10^4/ ml in 24 hr compared with 9.5×10^4 for ABW and 7.0×10^4 for AA. The 1X Ashby's Salts solution maintained the number of microorganisms of a period of 96 hr. The death rate appeared to be the same for all types of bacterial cells used.

The growth curves produced in 2X Ashby's Salts medium (Fig. 3) were more smooth than those obtained in distilled water and 1X Ashby's Salts medium. The 2X salt medium gave a slight increase in numbers of protozoans at 60 hr above the maintanence level. The maximum population of 52×10^4 was reached in AAW cells in 80 hr. The initial rates of fission were the greatest for AAW (22.5×10^4 ciliates/ ml in 24 hr) with ABW (18.0×10^4/ ml) and AA cells (12.0×10^4/ ml) following in that order.

The AAW cells supported the best growth of ciliates in all types of media; and in the 1X and 2X Ashby's Salts media, the ABW cells supported better growth than the living cells. This would seem to indicate that the protozoans prefer dead bacterial cells to living cells for food in a mineral salts media. Also it would indicate that they can readily utilize the material which diffuses from the bacterial cells on autoclaving; thus pinocytic action may account for most of the ingestion of food material.

Bentonite was added to the various suspending media of Tetrahymena because of preliminary results obtained using native zooplankton from Tuttle Creek Reservoir. It was found that the presence of bentonite in the media increased the uptake of living A. aerogenes and E. coli by the zooplankton. In the present case, bentonite maintained the level of the protozoans in the media used. It appeared that the Tetrahymena needed the osmotic pressure due to the ions to remain intact and maintain themselves. The bentonite may supply this requirement and also neutralize the acid produced by respiration.

Fig. 3. Growth of Tetrahymena in 2X Ashby's Salts in side armed flasks in the presence of AAW, ABW, AA, bentonite, or alone.

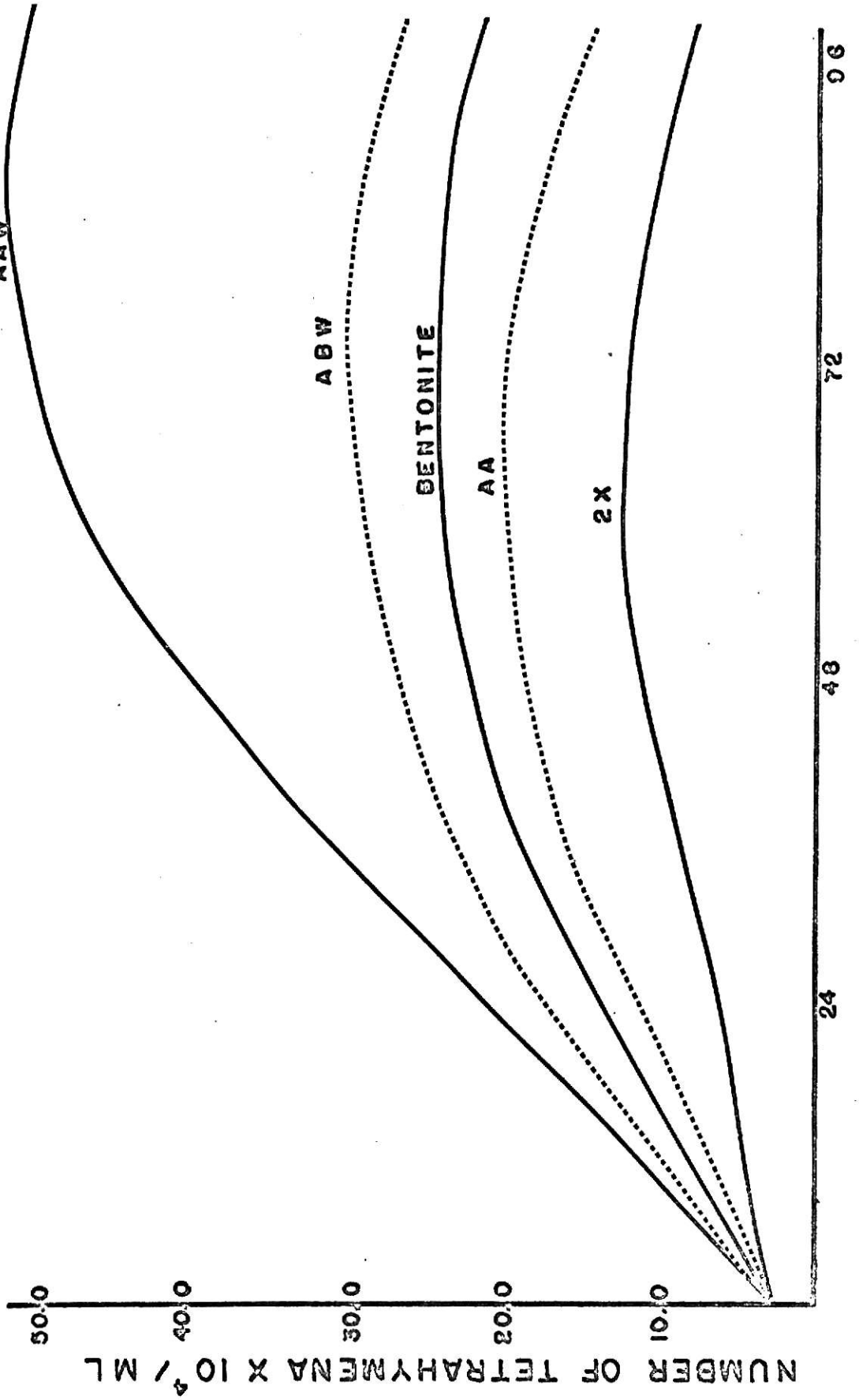
AAW
ABW
BENTONITE
AA
2X
50.0
40.0
30.0
20.0
10.0
NUMBER OF TETRAHYMENA X 10^4/ ML
24
48
72
96
HOURS OF GROWTH

Fig. 3

Since most clays are negatively charged, bentonite may attract the proteins. The Tetrahymena were seen to graze in the bentonite as if the food were surrounding or attached to the clay particle.

Growth in Proteose-peptone

To obtain the following results protozoans were enumerated with the hemocytometer after growth in screw capped tubes. This method was used as no correlation could be obtained between absorbance and the number of microorganisms present. This was in disagreement with the work of Huddleston et al, (1964), whose work used 300-ml batches rather than 10-ml samples where clumping occurred. Instead of side-armed flasks tubes were used because of the ready availability of large numbers of tubes. The tubes did not provide the surface area for the exchange of oxygen as did the flasks; thus the counts were lower in the tubes than in the flasks.

Tetrahymena, placed in screw capped tubes containing both live and dead bacterial cells suspended in distilled water, were able to grow for 24 hr (Fig. 4). The results showed that after 18 hr of growth, 6.0×10^4 ciliates/ ml were produced with AA cells; whereas 5.0×10^4 ciliates/ ml were produced with AAW cells and 0.4×10^4 ciliates/ ml were produced with ABW cells. The dead cells maintained the protozoans at the initial level throughout the experiment, while the number of ciliates supported by the living cells immediately declined after 24 hr (Table 8 in the Appendix).

A smooth growth curve was obtained in 0.5% proteose-peptone resulting in a maximum population of 5.0×10^4 ciliates/ ml at 66 hr. The AAW cells produced 11.0×10^4 organisms/ ml at 18 hr and kept the number of organisms at approximately 9.0×10^4 microorganisms/ ml for 96 hr. The growth curve for the AA cells had a short log phase which became a stationary phase at 24 hr with 1.2×10^4 ciliates/ ml. The ABW cells gave an initial growth of

Fig. 4. Growth of Tetrahymena in distilled water in screw capped tubes in the presence of AA, AAW, AAW and bentonite, or bentonite.

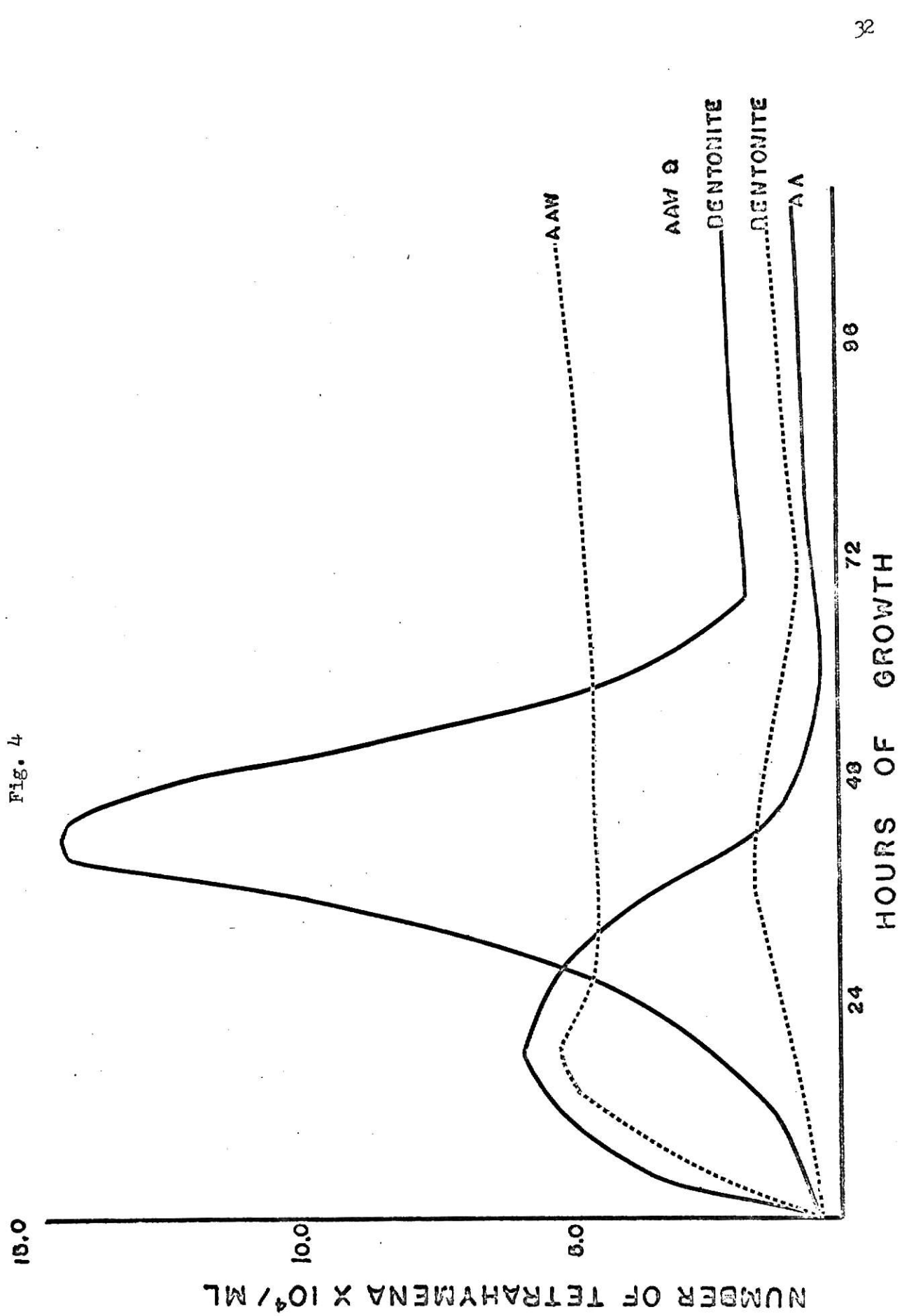
NUMBER OF TETRAHYMENA X 10⁴/ ML
15.0
10.0
5.0
24
48
72
96
HOURS OF GROWTH
AAW
AAW @
BENTONITE
BENTONITE
AA

Fig. 4

1.0×10^4 ciliates/ ml in 18 hr and a maximum population of 4.8×10^4 microorganisms/ ml in 66 hr with a smooth growth curve (Fig. 5).

The 1% proteose-peptone solution provided enough nutrients to give a smooth growth curve for the protozoan yielding a maximum population of 11.0×10^4 ciliates/ ml at 66 hr. The stationary phase began at 48 hr and the death rate increased after 72 hr. The presence of the AAW cells enabled the number of organisms to reach 12.0×10^4 ciliates/ ml in 42 hr with an initial growth of 3.8×10^4 ciliates/ ml in 18 hr. The AAW cells produced the maximum population of protozoans in 1% proteose-peptone. The ABW cells gave an initial growth of 1.4×10^4 ciliates/ ml in 18 hr, while the AA cells gave an initial growth of 0.8×10^4 ciliates/ ml. The AA cells gave a smooth curve with a stationary phase population of 2.0×10^4 protozoans/ ml in 48 hr. The ABW cells gave counts which appeared to double every 24 hr for the 90 hr observation period, reaching a count of 11.5×10^4 ciliates/ ml. These results may be seen in Table 8 in the Appendix and Fig. 6.

Protozoans in the 2% proteose-peptone gave an initial growth of 3.4×10^4 ciliates/ ml in 18 hours with the log phase ending at 11.0×10^4 ciliates/ ml at 42 hr. The growth curve of Tetrahymena with the addition of AAW bacterial cells appeared to have an inflection point at 42 hr after an initial growth of 4.4×10^4 ciliates/ ml in 18 hr. The inflection point may have been produced by the death of the microorganisms and the release of nutrients to give a sharp rise to 21.0×10^4 ciliates/ ml at 66 hr, the maximum population in 2% proteose-peptone. The AA cells produced an initial growth of 1.0×10^4 ciliates/ ml in 18 hr and a stationary phase population of 2.2×10^4 protozoans/ ml at 66 hr. A continued increase in population was

Fig. 5. Growth of Tetrahymena in 0.5% proteose-peptone in screw capped tubes in the presence of AAW, AAW and bentonite, AA, AA and bentonite, or alone.

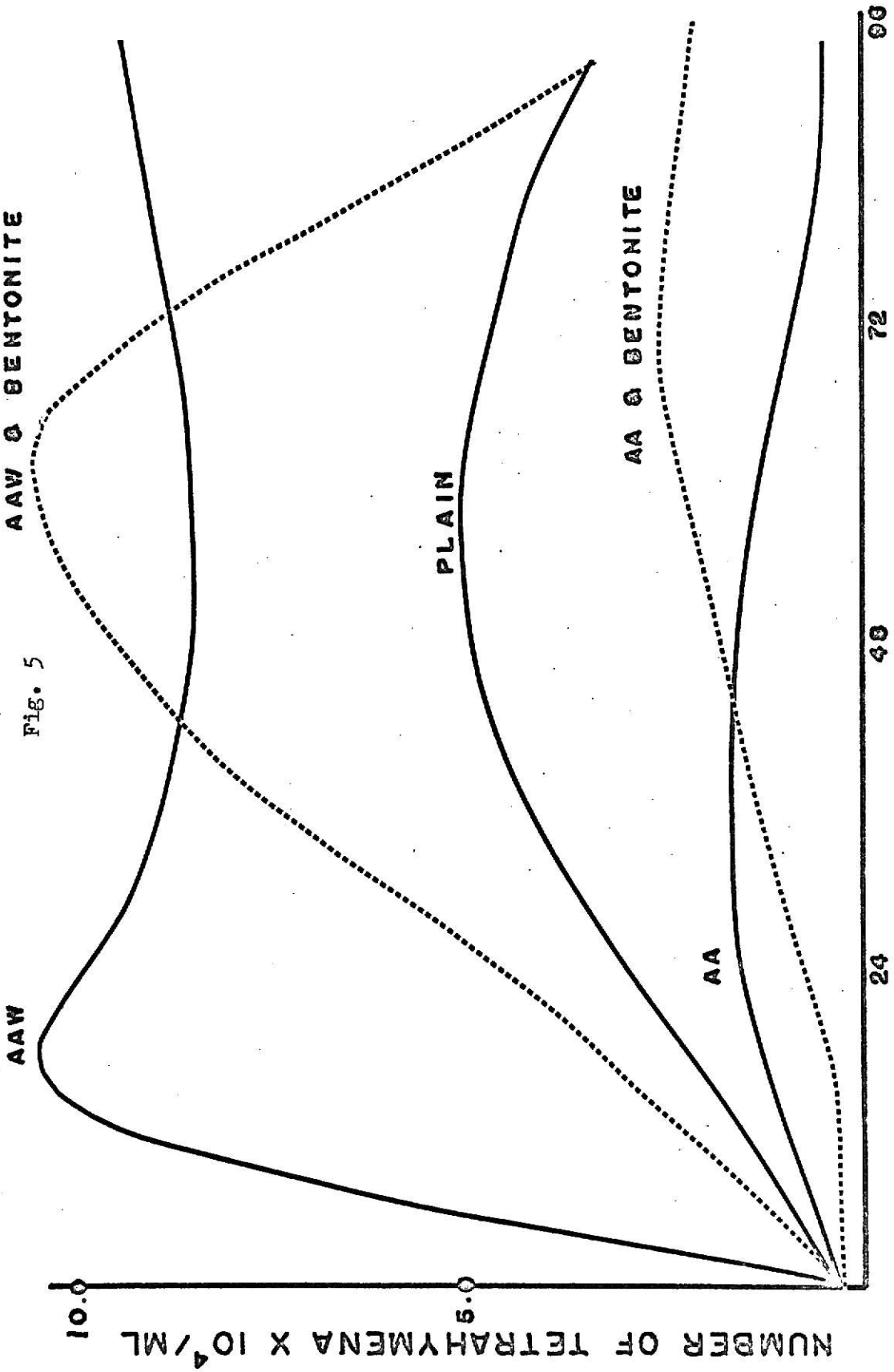
AAW
AAW @ BENTONITE
PLAIN
AA @ BENTONITE
AA
10.
5.
NUMBER OF TETRAHYMENA X 10^4/ML
24
48
72
96
HOURS OF GROWTH

Fig. 5

Fig. 6. Growth of Tetrahymena in 1% proteose-peptone in screw capped tubes in the presence of AAW, AAW and bentonite, bentonite, AA, or alone.

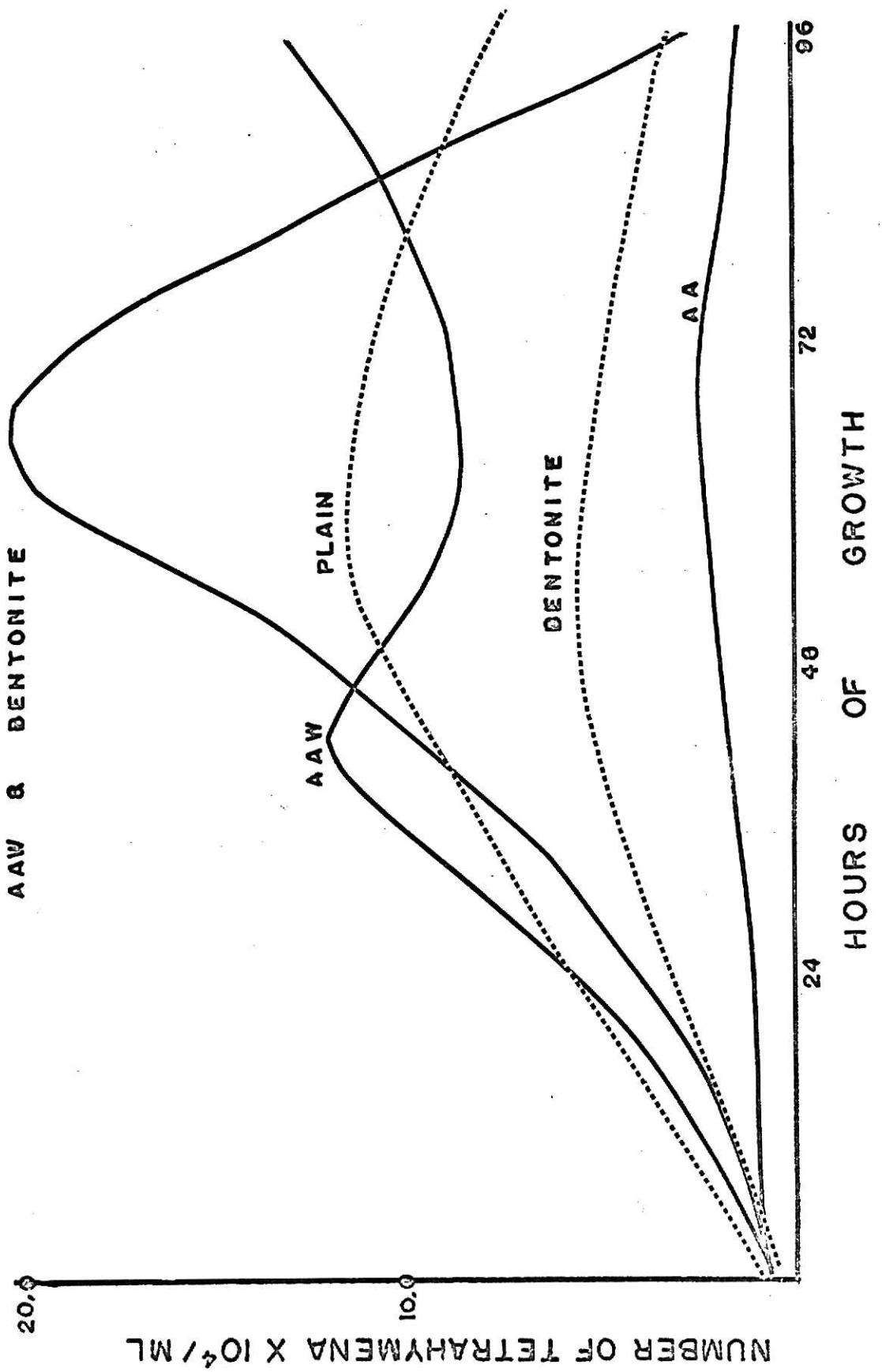
AAW & BENTONITE
PLAIN
AAW
BENTONITE
AA
NUMBER OF TETRAHYMENA X 10⁴/ML
20.
10.
24
48
72
96
HOURS OF GROWTH

Fig. 6

again produced by the ABW cells for the 90 hr observation period after a 1.3×10^4 ciliates/ ml in 18 hr initial growth (Fig. 7).

The use of 3% proteose-peptone gave an initial growth of 6.2×10^4 ciliates/ ml in 18 hr; this level was maintained until 66 hr when the organisms appeared to decline in numbers (Fig. 8). The AAW cells provided the nutrients needed to produce an initial growth of 6.2×10^4 ciliates/ ml in 18 hr and a stationary phase level of 12.2×10^4 protozoans/ ml at 42 hr, the maximum population in 3% proteose-peptone in the presence of bacterial cells only. The initial growth in AA cells produced 0.7×10^4 ciliates/ ml in 18 hr; the number of organisms then increased to 1.9×10^4 ciliates/ ml at 42 hr, before exhausting the food supply at 90 hr. The ABW cells provided food to support 1.6×10^4 ciliates/ ml in 18 hr and the organisms continued to increase until the numbers reached 6.9×10^4 ciliates/ ml at 66 hr.

The soluble materials in 4% proteose-peptone broth supported a doubling population of ciliates until the 66^{th} hour when a count of 12.4×10^4 ciliates/ ml was reached. The AAW cells appeared to provide the same support as the 4% proteose-peptone, as the initial growth of 3.1×10^4 ciliates/ ml was the same. The dead bacterial cells, AAW, provided the maximum population at 66 hr with 13.3×10^4 ciliates/ ml and the numbers of organisms/ ml declined more slowly than the control. The living cells, AA, provided an initial growth of 0.8×10^4 protozoans/ ml in 18 hr which was increased to and maintained at the level of 1.3×10^4 ciliates/ ml for the duration of the experiment. The ABW cells, on the other hand, after an initial growth which matched the living cells, attained and maintained the level of 4.0×10^4 ciliates/ ml (Fig. 9 and Table 9).

Growth in 5% proteose-peptone broth was not characteristic of the growth in the other percentages of peptone. The curve appeared to have a plateau at

Fig. 7. Growth of Tetrahymena in 2% proteose-peptone in screw capped tubes in the presence of AAW, AAW and bentonite, bentonite, or alone.

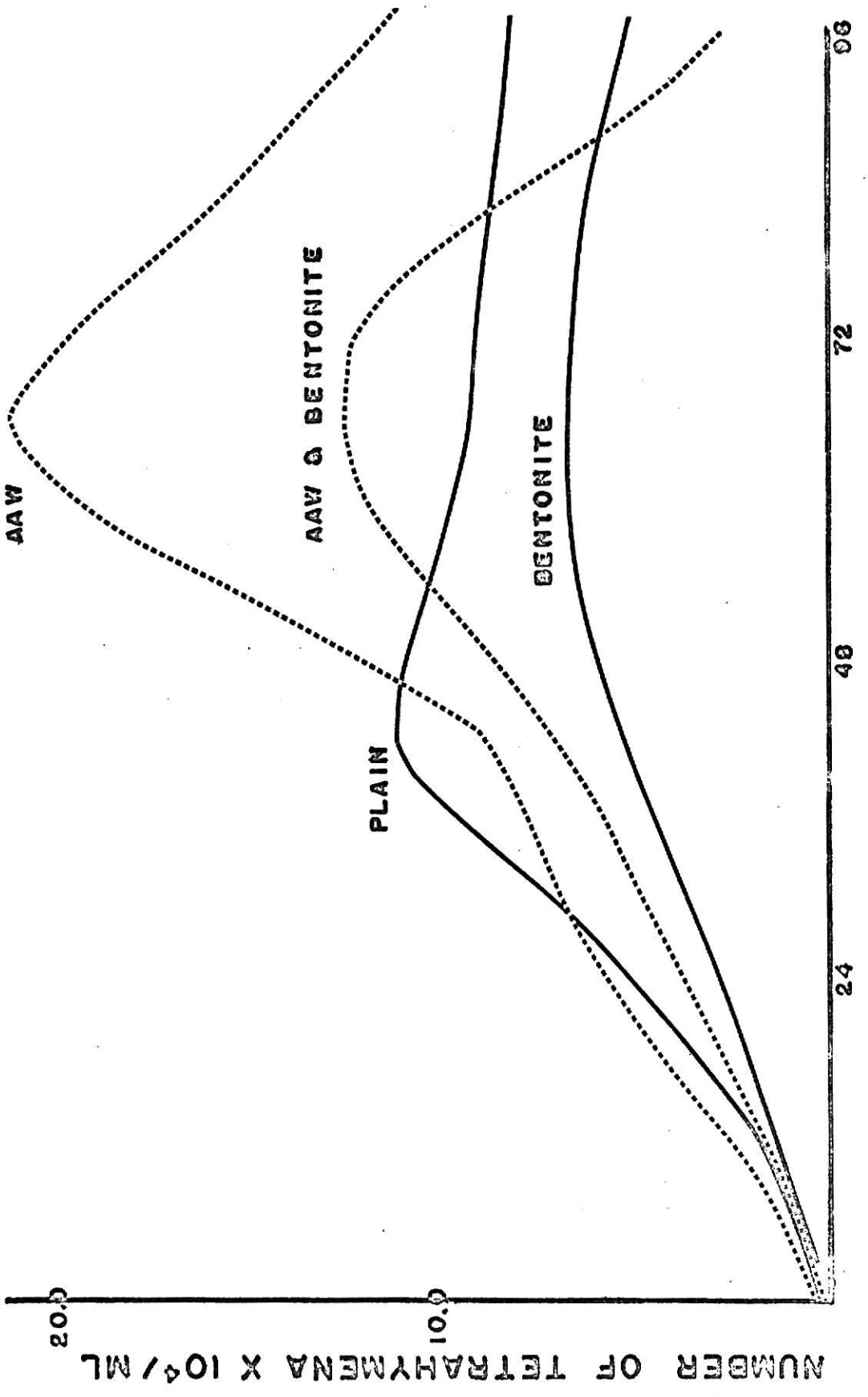
NUMBER OF TETRAHYMENA X 10⁴/ML
20.0
10.0
HOURS OF GROWTH
24
48
72
96
AAW
AAW & BENTONITE
BENTONITE
PLAIN

Fig. 7

Fig. 8. Growth of Tetrahymena in 3% proteose-peptone in screw capped tubes in the presence of AAW, AAW and bentonite, bentonite, AA, or alone.

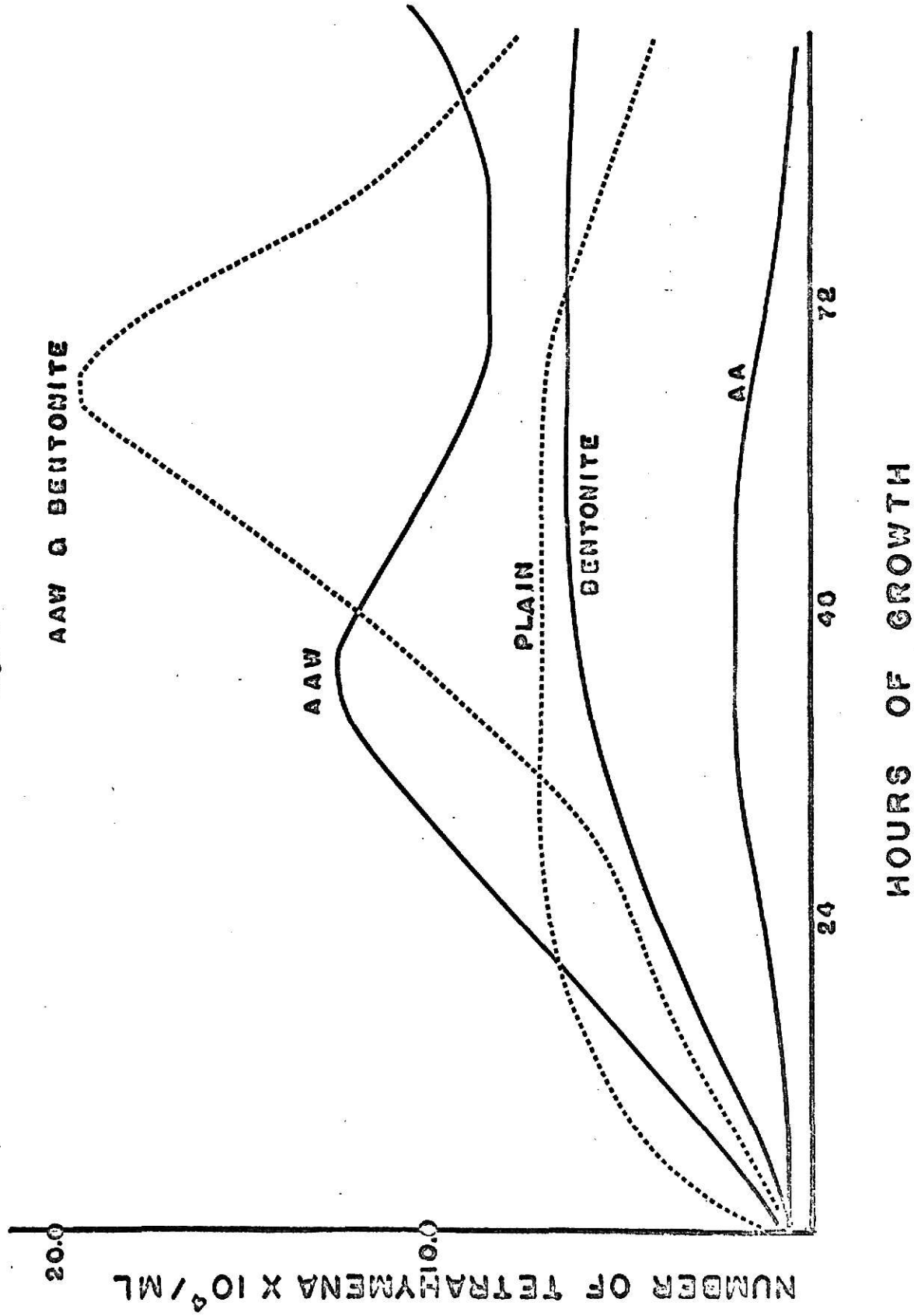
NUMBER OF TETRAHYMENA X 10⁴/ML
20.0
10.0
HOURS OF GROWTH
24
40
72
AAW & BENTONITE
AAW
PLAIN
BENTONITE
AA

Fig. 8

Fig. 9. Growth of Tetrahymena in 4% proteose-peptone in screw capped tubes in the presence of AAW, AAW and bentonite, bentonite, AA, or alone.

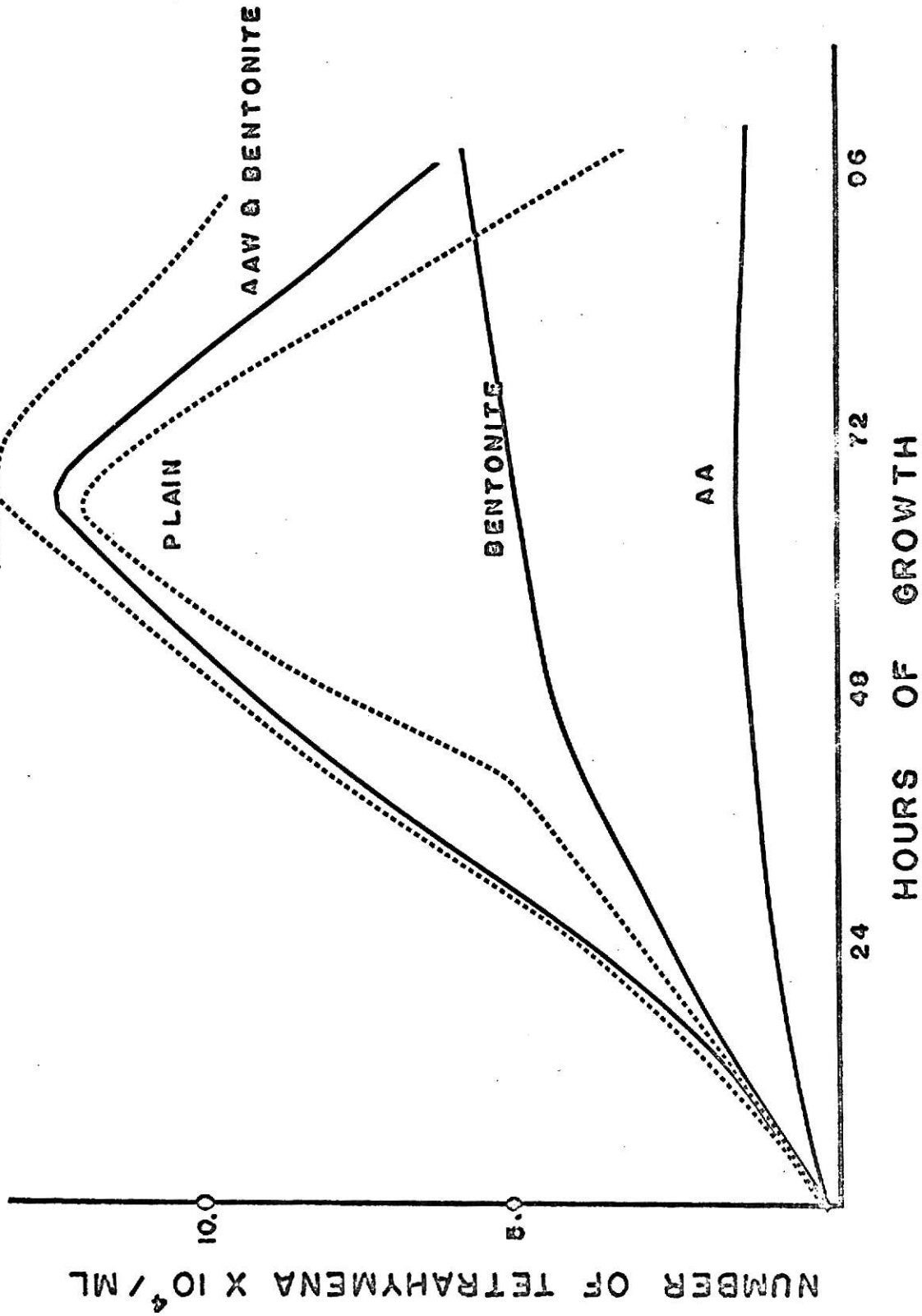
AAW
AAW & BENTONITE
PLAIN
BENTONITE
AA
10.0
8.0
24
48
72
96
HOURS OF GROWTH
NUMBER OF TETRAHYMENA X 10^4/ML

Fig. 9

42 hr and then increased at 66 hr to another level of 8.8×10^4 ciliates/ ml at 90 hr; this level proved to be the maximum population in 5% proteose-peptone. The AAW cells supported an initial growth of 2.2×10^4 ciliates/ ml which then advanced to 7.0×10^4 ciliates/ ml, the stationary phase level. The living cells supported only a population of 2.3×10^4 ciliates/ ml at 90 hr, after an initial growth of 1.2×10^4 protozoans/ ml at 18 hr. In comparison, the ABW cells supported a population of 4.3×10^4 ciliates/ ml in 90 hr with an initial growth of 1.4×10^4 ciliates/ ml at 18 hr. The population appeared to double every 24 hr until 66 hr in the presence of ABW. These results may be seen in Fig. 10 and Table 10 in the Appendix.

From the averages of eight observations of growth in proteose-peptone, the Tetrahymena appeared to grow well in concentrations from 1-5% proteose-peptone with no concentration appearing to give better growth; but the growth in 6, 8, and 10% proteose-peptone appeared to be retared. The osmotic pressure of the proteose-peptone may inhibit the advanced growth of the Tetrahymena. The results in Table 11 were subjected to a N-way analysis of variance and the following conclusions about growth in the presence of proteose-peptone and bacterial cells can be made. On the 95% level of confidence, there was no significant difference between the range of 1-4% proteose-peptone for the growth of Tetrahymena. There was a difference between 0.5% and 2% proteose-peptone, but there was no difference between 0.5% and the other concentrations of proteose-peptone. There was a significant difference between the distilled water and all concentrations of proteose-peptone.

The types of cells of A. aerogenes varied significantly on the 95% confidence level to give the conclusion that the cells could be ranked in decreasing order of food suitability; AAW, ABW, and the living cells. The decreased population in the living cells may be due to the charge repulsion

Fig. 10. Growth of Tetrahymena in 5% proteose-peptone in screw capped tubes in the presence of AAW, AAW and bentonite, bentonite, AA, or alone.

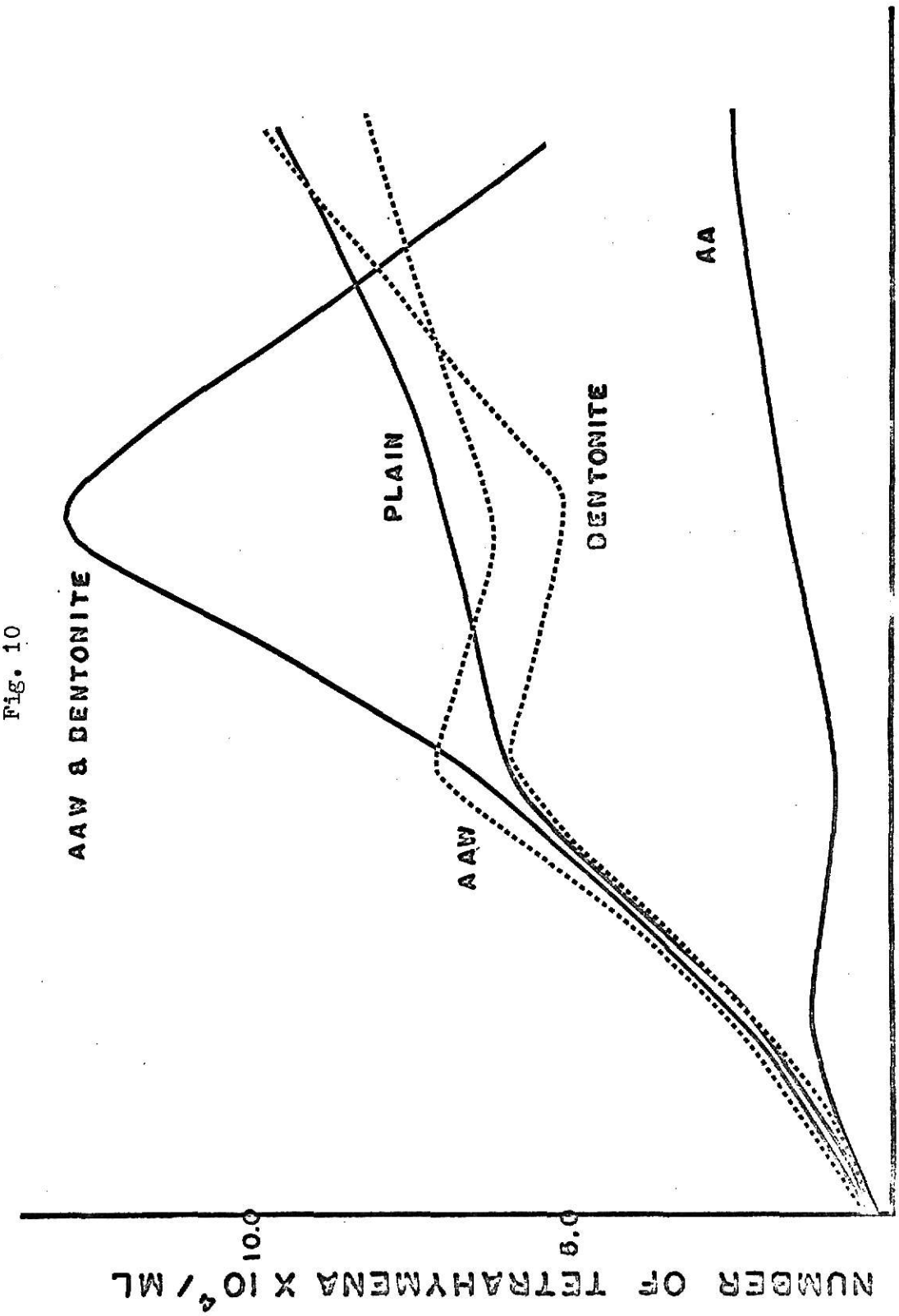
AAW & BENTONITE
PLAIN
AAW
BENTONITE
AA
10.0
8.0
24
48
72
96
HOURS OF GROWTH
NUMBER OF TETRAHYMENA X 10^3/ML

Fig. 10

between the negatively charged cells and the protozoans due to the absence of the secreted mucopolypeptide or other ions which would neutralize the charge. Also, since the AAW cells supported the most growth, in many cases, the material inside the cells may be more readily available to the ciliate after the bacteria are autoclaved, while this material has been discarded in the ABW cells. The ABW cells appeared to support growth at 66 hr; the protozoans may have used the soluble nutrients in the proteose-peptone first and then used the particulate cell wall of the bacterial cell. The ABW cell would probably contain few soluble materials, but the particles could be easily engulfed as they are pieces of a cell after autoclaving.

Addition of Clay Particles and Synthetic Gels

The presence of a colloidal clay, bentonite, appeared to enhance the growth of Tetrahymena whether by itself or in the presence of bacterial cells (Fig. 5 through 10). The effect of the bentonite became apparent in the last 24 hr of observation. It was in this time period that the maximum population was produced in distilled water, 0.5%, 1.0%, 3.0%, and 5.0% proteose-peptone in the presence of bentonite and AAW cells. These results can be seen in Tables 7, 8, 9, and 10. Bentonite may act as a concentrator of the secondary metabolites or smaller particles. The pH at this time interval was usually around 8.0, and there was no difference in the hydrogen ion concentration between the plain proteose-peptone solution and that containing the bentonite. From the N-way analysis of Table 11, there was a difference on the 90% level between the use of bentonite with AAW in proteose-peptone and the same cells without bentonite in proteose-peptone, and also a difference in proteose-peptone alone. There was a difference between all of the above and bentonite in proteose-peptone, with the bentonite being lower. In another experiment, the bentonite in proteose-peptone did not differ

significantly on the 90 and 95% level from the proteose-peptone, but both the bentonite in proteose-peptone and the plain proteose-peptone differed significantly from the washed living cell treatments with and without bentonite in proteose-peptone.

The presence of bentonite, diatomaceous earth, and kaolinite appeared to have some effect on the growth rate of Tetrahymena in 1% proteose-peptone in the presence of ABW cells. These clays vary in their base exchange capacities with kaolinite being the lowest and bentonite, the highest. These results can be seen in Table 1. Kaolinite and bentonite in 1% proteose-peptone with AAW cells supported a larger population than the control at 46 hr, while at 72 and 96 hr bentonite and vermiculite supported the greatest growth of 18.4 ciliates/ ml and 12.1 ciliates/ ml respectively.

From Tables 2 and 3, the presence of diatomaceous earth and the latex particles also appeared to give surface area for the collection of soluble protein as the counts of protozoans were higher than in the controls. The latex particles were also seen to be engulfed and remain in the vacuole of the ciliate microscopically.

Also in support of pinocytic feeding were the results obtained from samples grown in media containing Biogel-100. The proteins having a molecular weight of 100,000 and larger were excluded from the inner pore of the Biogel sphere; thus the smaller particles would be unavailable to the ciliate as a source of food. Sephadex-200 was used in a 1/1000 dilution and appeared to enhance growth in the presence of AAW cells, but not alone. This indicates the use of the dextrin gel as a surface for attachment and concentration of bacterial protein. The results with the Biogel may be seen in Tables 7, 8, 9, and 10, while the results utilizing Sephadex may be seen in Tables 2, 3, and 4. The Microballoons did not appear to have any effect on growth.

Table 1. Growth of Tetrahymena in the presence of clay and glass particles.

	48 hr				72 hr				96 hr			
	D.W.		1% pp		D.W.		1% pp		D.W.		1% pp	
	pl.	ABW	pl.	ABW	pl.	ABW	pl.	ABW	pl.	ABW	pl.	ABW
Silica gel	0.9	2.8	3.3	3.0	0.5	2.9	5.3	4.6	0.3	1.7	6.7	7.3
Illite	0.5	1.7	2.4	2.4	1.3	0.5	4.8	2.6	1.6	1.2	4.4	3.7
Microballoons	0.0	0.2	4.5	2.0	1.1	1.0	8.2	3.5	0.1	0.1	7.5	1.2
Kaolinite	1.2	1.1	3.4	1.7	1.6	1.9	25.6	2.9	1.4	2.2	3.2	2.7
Fuller's earth	1.1	1.1	3.6	2.3	1.7	4.6	5.4	12.2	1.3	1.5	2.4	9.2
Bentonite	0.3	0.7	6.9	2.4	0.5	0.1	3.8	9.3	0.3	0.2	3.6	3.4
Vermiculite	0.4	0.7	3.3	3.5	0.3	0.1	3.7	5.9	0.5	0.4	2.9	2.2
Control	0.0	0.0	5.6	1.5	0.0	0.0	6.4	2.5	0.0	0.0	7.6	2.5

Table 2. Growth of Tetrahymena in distilled water in side armed flasks.

	18 hr		24 hr		42 hr		67 hr		94 hr	
	AAW	C	AAW	C	AAW	C	AAW	C	AAW	C
Sephadex	1.5	0.2	1.7	0.7	2.0	0.0	1.4	0.2	3.6	1.5
Biogel	2.8	0.3	4.5	0.8	3.9	0.3	4.3	0.0	3.4	0.6
Microball.	1.0	0.1	2.0	0.1	2.8	1.0	2.0	0.2	1.1	0.5
Latex	3.2	0.6	2.9	0.4	2.0	0.5	2.7	0.5	7.4	0.4
Bentonite	3.1	0.3	6.6	1.7	5.7	0.4	0.0	0.8	1.9	0.8
EpC	3.2	0.3	4.8	0.6	5.8	0.6	3.1	0.4	3.0	0.0
1076	0.9	0.2	3.9	0.1	5.7	0.4	0.2	0.6	1.0	0.3
245	1.8	0.4	2.0	1.0	1.4	0.4	1.0	0.3	1.2	0.0
796	1.8	0.2	6.7	0.6	3.8	1.0	6.7	0.3	3.7	0.1
763	1.1	0.5	1.2	0.2	4.7	1.1	3.7	1.5	3.8	0.5

Table 3. Growth of Tetrahymena in 1% proteose-peptone in side armed flasks.

	22 hr		49 hr		76 hr		121 hr	
	AAW	C	AAW	C	AAW	C	AAW	C
Fuller's earth	6.5	6.2	9.5	12.2	22.2	11.8	20.4	7.6
Bentonite	11.5	5.3	8.6	7.8	17.2	5.9	24.0	2.1
Sephadex	6.4	3.3	23.6	8.0	17.9	2.8	7.7	5.2
Microball.	4.6	5.5	8.8	6.6	17.8	12.5	7.0	8.6
Latex	6.3	5.5	11.4	12.1	18.4	12.8	12.4	6.4
EpC	4.8	4.5	9.4	24.2	20.8	5.4	6.8	2.5
1076	4.0	3.5	6.9	8.4	13.0	10.2	9.8	2.6
245	6.5	7.4	9.5	9.6	1.2	10.3	6.6	5.2
796	13.5	3.7	12.4	7.2	16.1	15.2	16.4	19.0
Control	6.6	4.1	9.2	17.4	17.0	10.0	8.3	2.8

Table 4. The growth of Tetrahymena in the presence of synthetic gels and algae in 1% proteose-peptone in side-armed flasks. Number x 10^4 ciliates/ ml

	19 hr	43 hr	67 hr	90 hr	120 hr
Sephadex	2.0	17.2	14.6	14.5	4.8
Microballoons	1.7	13.5	13.7	18.8	1.9
Bentonite	3.1	21.6	18.7	23.2	8.2
Biogel 100	2.4	10.4	11.2	9.8	2.5
245	1.8	23.8	16.4	10.3	1.1
796	1.6	8.1	11.7	17.2	
Epc	1.6	14.8	11.9	13.5	0.8
1076	1.7	22.7	18.3	12.1	8.0
763	2.0	12.8	9.3	17.0	7.4
Control	2.0	17.3	15.9	20.1	8.8

The Addition of Primary Minerals

The addition of minerals did not increase the growth rate of the protozoans, but appeared to depress it; this can be seen from the results in Table 5. The minerals had been washed and suspended in the liquid media to give a neutral solution. The layers of magnesium and aluminum silicates may have been too tight to allow the trapping of organic molecules of food. It can be seen that the AAW provided food for the first 48 hr. The charcoal was observed inside the food vacuoles by microscopic examination.

The Addition of Algae

Tetrahymena were added to an algal culture which had been actively growing for two weeks in soil extract and the protozoan was maintained at a higher level than in proteose-peptone for seven days. The bacterial counts of the algae were approximately the same in all the algal cultures used, 10^7/ ml.

Table 5. Growth of Tetrahymena in the presence of minerals.

	24 hr				48 hr				72 hr			
	D.W.		1% pp		D.W.		1% pp		D.W.		1% pp	
	AAW	C	AAW	C	AAW	C	AAW	C	AAW	C	AAW	C
Olivene	2.9	0.6	5.3	5.0	6.1	0.6	6.8	4.5	8.4	2.0	34.0	10.0
Chlorite	3.5	0.8	2.9	2.0	6.7	2.3	12.8	1.0	1.1	2.6	11.9	2.2
Hornblende	2.6	0.8	3.6	1.3	4.3	1.4	16.5	7.4	0.6	0.0	12.8	9.7
Stilbite	2.2	1.4	3.6	2.8	0.0	1.1	7.9	2.1	5.6	1.7	12.1	13.6
Charcoal	1.3	2.3	4.4	2.6	0.9	1.2	8.0	1.9	0.3	0.0	12.2	7.3
Pyroxite and anorthite	3.2	0.5	6.6	3.6	0.3	2.0	5.5	4.6	6.0	10.3	37.0	12.3
Anorthite	2.6	3.8	4.8	2.3	0.5	0.8	6.1	5.0	5.5	0.8	11.7	9.7
Control	5.3	0.8	4.9	2.0	8.4	1.1	33.8	3.4	0.3	0.2	10.3	8.8

When the algae and Tetrahymena were added at the same time to the soil extract, it became more apparent that some algae support a greater population of Tetrahymena than others. There did not appear to be any difference in the counts of protozoans inoculated simultaneously with the algae and those inoculated after the algal bloom.

Table 6. Growth of Tetrahymena in the presence of algae inoculated simultaneously with the ciliate in soil extract in screw capped tubes.

	5 days	11 days
Control	3.6×10^4 T/ml	2.8×10^4 T/ml
1069	4.0	2.0
437	1.7	0.6
756	3.8	0.4
144	4.0	0.8
107	2.7	0.5
770	0.7	1.2
245	3.0	1.4
763	1.1	2.3
796	1.4	2.1
143	2.2	1.5
1076	3.0	2.3

From Table 2, the algae did not appear to supply nutrients in distilled water to the protozoans; only when they are used in conjunction with the AAW cells did they maintain or offer some form of protection for the protozoans. It was characteristic of the Chlamydomonas in these experiments to give high counts

after 67 hours. As in the case of the clay particles, the algae may provide a surface for the attachment of soluble or insoluble nutrients; thus enhancing growth by the concentration of needed nutrients.

In general, microscopic examination of the protozoan at various intervals of time enabled the observer to locate and see the stage of digestion of the food material in food vacuoles. Vacuoles were observed at all times in the body of the ciliate; these were examined closely at 1000X under oil immersion. The fewest number of vacuoles was observed in the concentrated suspension of organisms after standing for an hour in the phosphate buffer at pH 7.0. This is in agreement with the observations of Chapman-Andresen. The vacuoles became more concentrated and darker in appearance with increasing time and concentrations of proteose-peptone and the A. aerogenes cells until the 90 hr observation. At this time the ciliates no longer had an intact cellular membrane, but appeared to be a dark, highly concentrated sphere with the vacuoles ejected from the body. The vacuoles in the proteose-peptone media were lighter in appearance than those formed with bacterial cells present. The vacuoles did not become as heavily concentrated as in the cellular media. Also the shape of the ciliate in the proteose-peptone remained pyriform and did not enlarge or become oval as in the cellular medium.

SUMMARY

The growth of the ciliate, T. pyriformis, was used to determine the suitability of various solutions which may represent the many ecosystems found in the natural environment. The growth rate was determined by making counts with the hemocytometer at 24-hr intervals.

As a minimal inorganic media, Ashby's Salts medium in 1X and 2X concentrations were compared with distilled water. The maintanence of the

protozoan required the osmotic pressure of the ions present in the mineral salts solution.

Proteose-peptone solutions varying from 0.5 to 5% were added to the media to furnish additional nitrogen and organic material. All percentages gave statistically equal growth; these results were based upon three to six different samples.

A. aerogenes were used in three forms; washed and viable, washed and autoclaved, and autoclaved and then washed. These were added to the mineral salts and proteose-peptone media. Bacterial cells produced the greatest numbers of ciliates in the following order; autoclaved after washing (AAW), autoclaved before washing (ABW), and then the living cells (AA). This gives credence to the theory that most of the ciliate's food is obtained by pinocytic action, as the AAW cells contain soluble material from inside the cell which diffuses or permeates through the ruptured cell wall on autoclaving.

Colloidal materials were added in the form of hydrated clays. It was observed that the presence of bentonite prolonged the effect of the added autoclaved after washing cells. Higher counts were maintained through 72 hr with bentonite than without the colloidal clay. Bentonite when added alone to the proteose-peptone and mineral salts media gave higher counts than the control. This same effect was noticed with the use of kaolinite and diatomaceous earth, but not to the same degree of enhancement. Other clays such as illite and vermiculite had little effect. Latex particles were readily engulfed by the protozoan and higher counts than in the control were obtained. Other synthetic polymers, Microballoons and Sephadex 200, also increased the growth rate in the presence of AAW cells, but Biogel-100 appeared to only maintain the protozoan.

Primary minerals were added to the various media to determine their effect upon food uptake either by phagocytosis or pinocytosis. It was found that the minerals of anorthite, olivene, hornblende, chlorite, stilbite, pyroxite and anorthite, and charcoal did not decrease the generation time, but instead lowered the ciliate counts.

Algae, unicellular and colonial forms, illustrated their use as surfaces for concentration of nutrients by increasing the numbers of ciliates. These algae, Chlamydomonas, Astrephomene, Gloeocystis, Scenedesmus, and Closterium, produced greater numbers of protozoans than the other algae used.

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APPENDIX

Table 7. Growth of Tetrahymena in distilled water and 0.5% proteose-peptone in the presence of added particles.

	Distilled Water				0.5% Proteose-Peptone			
	18 hr	42 hr	66 hr	90 hr	18 hr	42 hr	66 hr	90 hr
Proteose-peptone	0.56	0.86	1.6	2.0	2.6	4.6	4.9	3.6
Aerobacter (living)	6.0	1.3	0.3	0.6	0.5	1.5	0.9	0.55
Bentonite	0.7	1.6	0.55	0.9	3.5	4.8	4.4	7.3
AA and Bentonite	1.2	1.4	0.5	0.6	1.4	1.4	2.5	2.1
AAW	5.2	4.1	4.7	4.7	10.5	8.6	8.6	9.1
AAW and Bentonite	2.2	14.5	2.1	2.0	2.8	9.0	10.5	3.4
ABW	0.5	0.7	0.6	0.9	1.0	2.5	4.8	2.7
ABW and Bentonite	0.2	0.4	0.3	0.2	2.3	2.8	3.6	2.8
Biogel	0.9	1.1	1.1	2.2	1.6	1.5	3.6	1.4
Latex	0.9	1.3	2.0	3.6	4.8	7.1	8.2	3.3
Diatoms	0.1	3.0	2.8	1.6	1.0	10.0	15.2	5.3
AA and Biogel	0.7	2.3	0.2	0.0	0.4	0.6	0.7	0.2
AAW and Biogel	0.8	1.8	0.0	4.3	0.9	8.7	1.4	2.4
AAW and Latex	9.6	15.2	5.5	30.6	9.8	5.7	10.0	6.0
AAW and Diatoms	4.2	3.6	4.5	6.4	9.4	16.6	19.3	25.0

Table 8. Growth of Tetrahymena in 1% and 2% proteose-peptone in the presence of added particles.

	1% Proteose-Peptone				2% Proteose-Peptone			
	18 hr	42 hr	66 hr	90 hr	18 hr	42 hr	66 hr	90 hr
Proteose-peptone	2.7	9.5	10.7	7.6	3.4	11.3	9.1	8.6
Aerobacter (living)	0.8	1.6	2.1	1.5	1.0	1.8	2.2	1.0
Bentonite	2.6	4.5	5.6	4.7	2.8	5.2	6.9	5.8
AA and Bentonite	0.6	2.2	2.6	1.8	1.4	2.8	3.6	5.0
AAW	3.8	11.9	8.3	11.5	7.7	8.4	20.8	13.5
AAW and Bentonite	4.5	9.5	20.1	6.5	2.3	6.6	12.6	5.0
ABW	1.4	2.6	5.9	11.1	1.3	3.30	3.5	4.6
ABW and Bentonite	2.8	3.8	4.8	3.2	3.6	3.3	5.1	6.6
Biogel	1.2	3.2	5.2	2.0	1.7	1.6	3.4	1.2
Latex	6.4	10.4	9.4	27.0	6.2	7.8	9.1	18.0
Diatoms	11.6	11.2	4.2	6.2	8.0	43.6	14.3	11.4
AA and Biogel	0.2	0.5	1.1	0.4	1.4	0.2	1.4	0.8
AAW and Biogel	0.9	4.1	3.5	2.3	1.3	1.3	4.6	4.9
AAW and Latex	20.8	4.3	9.0	8.0	7.9	8.6	4.2	11.0
AAW and Diatoms	5.6	13.1	12.0	20.0	7.8	7.7	10.5	13.4

Table 9. Growth of Tetrahymena in 3% and 4% proteose-peptone in the presence of added particles.

	3% Proteose-Peptone				4% Proteose-Peptone			
	18 hr	42 hr	66 hr	90 hr	18 hr	42 hr	66 hr	90 hr
Proteose-peptone	6.2	6.8	6.9	4.6	3.0	6.6	12.4	5.7
Aerobacter (living)	0.7	1.9	1.5	0.1	0.8	1.2	1.4	1.3
Bentonite	2.5	6.3	6.0	6.3	2.0	4.1	4.7	5.8
AA and Bentonite	0.8	2.8	2.6	4.0	1.3	1.7	2.7	1.3
AAW	6.2	12.3	8.3	9.3	3.1	5.6	13.3	10.3
AAW and Bentonite	3.8	9.0	19.3	8.5	2.5	8.0	11.9	7.6
ABW	1.6	2.8	6.9	5.6	0.9	3.9	4.0	4.5
ABW and Bentonite	1.9	3.0	4.6	5.3	1.4	2.7	5.8	6.4
Biogel	1.6	4.5	3.5	7.2	0.5	1.4	1.3	2.1
Latex	6.6	56.4	5.4	10.6	6.0	9.8	8.1	13.4
Diatoms	6.7	18.6	8.9	7.4	2.6	8.4	8.9	25.2
AA and Biogel	2.1	0.2	0.7	2.6	0.6	0.3	1.1	1.6
AAW and Biogel	2.3	5.0	8.3	7.7	4.4	4.7	3.0	3.4
AAW and Latex	5.5	4.9	7.4	5.2	4.8	9.8	5.3	21.6
AAW and Diatoms	7.2	7.0	8.2	12.6	4.6	15.2	14.9	18.6

Table 10. Growth of Tetrahymena in the presence of added particles in 5% proteose-peptone.

	18 hr	42 hr	66 hr	90 hr
Proteose-peptone	2.1	6.1	6.9	8.8
Aerobacter (living)	1.2	0.8	1.5	2.3
Bentonite	2.6	5.9	5.0	8.5
AA and Bentonite	1.3	1.7	1.8	2.2
AAW	2.2	7.0	6.3	7.3
AAW and Bentonite	2.5	6.4	13.2	7.5
ABW	1.4	2.3	4.3	4.3
ABW and Bentonite	1.0	2.8	4.7	4.9
Biogel	1.5	0.6	3.3	4.2
Latex	2.2	11.6	9.5	13.2
Diatoms	2.0	9.2	7.7	7.0
AA and Biogel	0.0	0.2	0.5	0.4
AAW and Biogel	1.2	2.1	0.4	6.9
AAW and Latex	5.6	3.8	5.8	6.1
AAW and Diatoms	2.9	9.3	13.2	7.1

Table 11. Growth of Tetrahymena after 66 and 90 hr in varying concentrations of proteose-peptone. These results were submitted for statistical analysis.

	66 hr							90 hr						
	D.W.	0.5	1.0	2.0	3.0	4.0	5.0	D.W.	0.5	1.0	2.0	3.0	4.0	5.0
Proteose-peptone	0.5	5.1	6.9	10.4	7.5	2.6	3.6	3.9	3.6	4.7	1.8	4.0	4.7	1.0
Aerobacter (living)	0.5	1.4	3.1	2.1	1.4	0.5	2.1	1.0	0.7	1.1	0.6	0.1	0.0	2.5
Bentonite	0.9	1.1	4.3	3.8	1.9	5.0	5.0	0.9	3.8	0.5	2.4	4.5	5.7	0.0
Bentonite with AA	0.1	2.9	4.9	4.2	3.8	0.8	0.7	0.2	4.0	3.0	6.6	5.4	0.1	1.0
Replication														
Proteose-peptone	1.7	9.0	4.3	3.1	13.8	10.0	12.0	2.3	4.9	9.5	4.9	3.2	4.0	6.3
Aerobacter (living)	0.2	0.3	1.1	2.4	1.6	2.3	0.9	0.3	0.3	1.9	1.4	0.0	2.7	2.1
Bentonite	2.1	3.0	8.6	11.2	10.6	7.1	9.9	0.3	14.4	7.3	12.3	12.0	5.4	12.2
Bentonite with AA	0.0	2.1	0.4	3.1	1.5	3.7	2.9	1.1	0.2	0.6	3.4	2.6	2.5	3.5
Proteose-peptone	2.8	5.7	3.9	11.7	5.7	6.7	6.7	0.6	1.9	2.2	5.1	6.6	7.9	1.9
Aerobacter AAW	6.5	9.0	7.0	10.7	6.0	4.8	4.9	2.9	3.4	2.3	7.2	7.0	3.6	4.4
Bentonite	2.7	5.8	4.5	8.6	9.1	7.7	9.3	0.4	3.3	2.9	2.1	7.9	0.7	7.7
Bentonite with AAW	4.3	6.3	11.8	5.7	11.8	9.1	5.9	1.8	2.7	8.3	4.8	12.1	6.6	6.3

Table 11--Continued.

	66 hr							90 hr						
	D.W.	0.5	1.0	2.0	3.0	4.0	5.0	D.W.	0.5	1.0	2.0	3.0	4.0	5.0
Replication														
Proteose-peptone	4.5	3.5	28.6	13.6	6.4	19.6	14.9	1.2	5.4	14.3	8.7	9.5	6.2	11.3
Aerobacter AAW	0.3	10.2	10.1	30.3	11.8	21.8	7.8	4.4	13.8	6.0	7.3	0.3	6.6	10.2
Bentonite	0.0	5.4	3.6	6.2	5.4	6.6	12.7	2.3	6.2	3.8	0.9	2.4	1.6	9.4
Bentonite with AAW	0.0	14.8	28.4	19.5	26.8	14.7	10.6	3.2	4.1	4.6	5.3	8.5	7.6	8.7
Proteose-peptone	0.2	5.3	6.7	7.7	2.8	2.0	1.5	0.0	4.1	5.1	10.0	5.3	3.5	2.6
Aerobacter ABW	0.4	5.0	3.9	3.6	5.0	1.4	1.5	0.4	0.4	6.6	5.1	5.3	3.8	1.2
Bentonite	0.0	8.0	6.0	6.2	6.1	2.1	0.2	0.0	5.0	10.1	9.2	13.6	5.6	2.0
Bentonite with ABW	0.0	3.5	6.8	4.4	6.2	4.2	4.1	0.3	2.1	7.1	6.7	6.3	10.6	3.9
Replication														
Proteose-peptone	0.0	3.4	3.1	2.1	1.6	5.4	4.6	0.1	3.6	3.6	9.4	2.4	20.1	5.4
Aerobacter ABW	0.4	6.0	4.1	3.8	4.2	4.1	5.0	0.2	5.4	4.3	5.5	5.2	3.8	5.2
Bentonite	1.0	2.9	4.4	4.0	0.7	4.4	3.4	0.1	4.1	6.9	5.9	1.9	3.2	6.7
Bentonite with ABW	0.4	2.8	2.6	4.4	4.3	6.3	3.4	0.0	2.4	1.5	6.9	4.3	6.1	6.2

THE FEEDING OF *TETRAHYMENA PYRIFORMIS* IN THE PRESENCE OF PARTICLES

by

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ABSTRACT

The protozoan, Tetrahymena pyriformis, was used to develop a system to determine the effect of added particles on the growth rate of this ciliate. This system was to assist in gaining information about the utilization of microorganisms in fresh water self-purification, as it has been shown that this ciliate can clear suspensions of autoclaved bacteria within 24 hr.

Using solutions of mineral salts, it was found that the protozoan maintained itself best in a 2X Ashby's Salts medium when compared to counts obtained in 1X Ashby's Salts medium and distilled water. The suitability of the solution and its additives was determined by counts obtained by the use of the hemocytometer and the Coulter counter at 24-hr intervals.

Cells of Aerobacter aerogenes, which had been washed and remained viable, or had been washed and then autoclaved, or had been autoclaved and then washed, were added to various solutions. It was observed that the autoclaved after washing cells supported the best growth with the living cells supporting the least growth in all solutions used.

A series of screw capped tubes containing varying percentages of proteose-peptone, 0.5 to 5.0, was used to represent the various quantities of nutriment that might be present in the natural environment. The solutions of proteose-peptone did not vary statistically in their ability to support growth. These solutions were augmented with particulate materials to give a 1/1000 dilution. The addition of minerals in the form of hornblende, anorthite, olivene, charcoal, stilbite, chlorite, and pyroxite and anorthite depressed the growth rate of the ciliate. Bentonite and kaolinite which are colloidal clays enhanced the growth of Tetrahymena; however, in the presence of A. aerogenes the colloids increased the numbers of ciliates more noticeably.

Synthetic polymers with varying pore sizes, Biogel-100 and Sephadex 200, were used to determine that Tetrahymena can readily utilize lower molecular weight food sources. When Sephadex was used in smaller quantities, the protein appeared to be concentrated; thus higher counts were obtained than in the control. Latex particles, Microballoons, and diatomaceous earth presented suitable surfaces for the attachment or concentration of protein material.

The presence of Chlamydomonas, Astrephomene, Gloeocystis, Scenedesmus, and Closterium produced greater numbers of protozoans than the other algae used. This did not arise from the use of the algae as food but through their use as a surface.