THE ACCUMULATION, UTILIZATION, AND SIGNIFICANCE OF POLYSACCHARIDE IN *TRITRICHOMONAS AUGUSTA*

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THE ACCUMULATION, UTILIZATION, AND SIGNIFICANCE OF POLYSACCHARIDE IN TRITRICHOMONAS AUGUSTA

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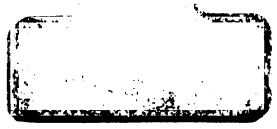

INTRODUCTION

Tritrichomonas augusta is a pyriform protozoan flagellate about 15-27u long with three unequal anterior flagella and a fourth posteriorly directed flagellum which forms part of an undulating membrane. A thick axostyle containing dark staining granules protrudes posteriorly. This organism is a common commensal of the colon of frogs and toads.

Because its hosts are poikilothermic, this organism would encounter a highly variable environment with regard to temperature and the availability of nutrients. In general this environment would provide nearly complete anaerobiasis, a slightly alkaline pH, and an intermittent source of nutrients. Although the rate of growth and metabolism of this trichomonad is reduced at lowered temperatures, Trit. augusta could be expected to require a reserve carbon and energy source in order to survive during extended periods of over-winter hibernation of its host.

Since it does not appear to form a "cyst-like" stage during its life cycle, Trit. augusta would be expected to be especially susceptible to the hazards of its host's external environment during the process of transmission. The aquatic habitat, which all amphibians frequent during part of all of their life cycles, would appear to be the most opportune route for transmission of this organism. However, such an environment probably lacks essential nutrients, and may possess a high or low pH and possibly high oxygen tensions:

all conditions unfavorable for the survival of this trichomonad. Trit. augusta is believed to follow an oral route in infecting a new amphibian host. Thus, this trichomonad must be able to resist the low pH encountered during its passage through the stomach of a prospective host. In order to survive all the adversities listed above long enough to encounter an optimal location in a new host, Trit. augusta must be capable of considerable resistance to unfavorable environmental conditions. For this resistance it certainly must require a reserve carbon and energy source such as glycogen.

Polysaccharides have been found to occur in varying amounts in the cytoplasm of nearly all microorganisms. Most of the investigations of carbohydrate storage have been attempts at characterization of the pattern of accumulation and utilization of reserve polysaccharides by organisms in vitro. These reserve compounds were accumulated by microbial cells when the exogenous carbon and energy source was in excess of metabolic requirements. When the exogenous energy source was limiting, the cells utilized previously stored polysaccharides. Although the data on these basic aspects of carbon and energy storage are steadily increasing, no information is available which explains the regulatory mechanisms for the accumulation or utilization of storage polysaccharides, if such mechanisms exist. Neidhardt (1963) made this quite clear when he stated, "To be brutally exact, not a single

mechanism has been established as physiologically responsible for the overall rate of synthesis of one class of macromolecules with another-..."

The stored carbohydrate occurring in a closely related trichomonad, Tritrichomonas foetus, has been identified as an alpha-1,4-glucosan which is quite similar to animal glycogen. The storage polysaccharide of Trit. augusta has been reported to give a reddish-brown color reaction with iodine solutions and appears to be a glycogen-like polysaccharide. For the sake of brevity and clarity the carbohydrate found in Trit. augusta has been referred to as glycogen throughout this manuscript.

The purpose of this investigation on the stored polysaccharide of Trit. augusta may be stated as follows: 1) to demonstrate the accumulation and utilization of this glycogen-like polysaccharide as a carbon and energy reserve, 2) to determine how the pattern of glycogen storage is affected by cultural age and the availability of glucose, and 3) to determine the significance of the polysaccharide reserve in the life cycle of this organism.

LITERATURE SURVEY

Accumulation and Utilization of Polysaccharides in Microorganisms:

Wilkinson (1959) suggested that the basic requirements which stored compounds must fulfill in order to serve the cell as functional carbon and energy reserves are as follows:

> "a) The compound is accumulated under conditions when the supply of energy from exogenous sources is in excess of that required by the cell for growth and related processes at that particular moment of time, b) the compound is utilized when the supply of energy from exogenous sources is insufficient for the optimal maintenance of viability and other processes, and c) that the compound is broken down to produce energy in a form utilizable by the cell, and that it is, in fact, utilized for some purpose which gives the cell a biological advantage in the struggle for existance over those cells which do not have a comparable compound."

He stated that, in general, storage compounds in bacterial cells were of high molecular weight, thereby enabling the cell to accumulate large quantities of these materials without increasing the internal osmotic pressure. The three major groups of storage compounds proposed by Wilkinson (1959) were: volutin, lipid, and polysaccharide.

The most intensive investigations on the influence of environmental conditions upon the accumulation and utilization of storage polysaccharides have involved bacteria belonging to the Enterobacteriaceae, especially *Escherichia coli* (Wilkinson and Duguid, 1960). Polysaccharide accumulation was found to reach a maximum of 20-50% of the total dry cell weight. These maximum polysaccharide levels coincided with the attainment of maximal populations in

cultures of a wide variety of enteric bacteria (Lankford et al., 1951; Levine et al., 1953; and Duguid and Wilkinson, 1953). The polysaccharide stored by these bacteria during early phases of culture was utilized in late phases of culture. Lankford et al. (1951) noted that addition of glucose to aging cultures of Salmonella oranienburg and E. coli deficient in glucose resulted in the resumption of polysaccharide accumulation. Storage of polysaccharide in a variety of enteric bacteria was found to vary with temperature by Levine et al. (1953). They noted that a greater accumulation of stored carbohydrate occurred in organisms incubated at 15°-20°C than in organisms incubated at 37°C.

Palmstierna (1955 & 1956) reported on the pattern of accumulation of a glycogen-like polymer in cells of E. coli B. Polysaccharide storage occurred during the first 30 minutes of culture, which corresponded to the lag phase; and reached a maximum just prior to the first cell division. Palmstierna (1955) stated that the stored polysaccharide appeared to be utilized during the logarithmic phase, since the concentration dropped to an intermediate level. The concentration of stored polysaccharide compound remained constant during the logarithmic phase of growth. When the rate of growth decreased polysaccharide accumulated, reaching a maximum level during the maximum stationary phase of growth. On the basis of these data as well as results from radioactive

labeling experiments, Palmstierna (1956) proposed: 1) that the small residual polysaccharide which was always present in the cells of E. coli B, behaved as a primer for the synthesis of this glycogen-like polymer, 2) that in regard to the energetics of the cells, the lag phase could be described as endergonic while the logarithmic phase appeared to be exergonic in nature, and 3) that during the break down of reserve polysaccharide, some of the carbon of the resultant intermediates was incorporated into protein synthesis. E. coli and Aerobacter aerogenes accumulated more polysaccharide when the ammonium chloride nitrogen source was deleted from a chemical defined medium than when the medium was complete. Glucose was in excess of requirements under both conditions (Holme and Palmstierna, 1956 a, b, c, and d; Holme and Cedergren, 1961; and Duguid and Wilkinson, 1953). Furthermore, the addition of NH_4Cl to cultures lacking a nitrogen source resulted in a decline in the level of glycogen reserves and a concomitant resumption in the growth of E. coli B (Holme and Palmstierna, 1956b). Following the exhaustion of the added nitrogen source, cell growth and division ceased and the level of glycogen reserves again began to rise. Holme and Palmstierna proposed that the increased amounts of polysaccharide stored by E. coli B in a medium lacking a nitrogen source was an indication that both the stored polysaccharide and the exogenous glucose were utilized as a source of carbon and energy for protein synthesis and growth. These authors

concluded that, an "inversely coupled relationship" existed between synthesis of reserve polysaccharide and synthesis of protein components required for growth. This conclusion is supported by Sols (1961) presentation of a metabolic scheme involving separate enzymatic pathways for the synthesis and degradation of glycogen.

Knowledge of the carbon and energy reserves of protozoa has accrued at a much slower pace than that of the bacteria. Fennell and Marzke (1954) compared the ratios of stored glycogen to total dry cell weight of Tetrahymena gelleii during various phases of in vitro growth. The percentages of stored glycogen followed the same general pattern described for E. coli by Palmstierna (1955 & 1956)--high concentrations in the lag phase, diminished but constant concentrations in the logarithmic phase, and increasing concentrations during the phase of maximal growth. Fennell and Marzke (1954) also noticed that the glycogen content of organisms from 288 hour cultures decreased nearly 40% within 30 minutes after organisms were suspended in saline. Armer (1944) demonstrated that, during periods of starvation of its host, the paraglycogen reserves in Nyctotherus ovalis decreased to a base level within 4 weeks. Hungate (1943), Oxford (1951), Masson and Oxford (1951), and Sugden and Oxford (1952) demonstrated rapid increases of stored polysaccharide in the rumen ciliates of sheep immediately following feeding of the host. Six to nine hours after host feeding, or after a similar period of suspension in saline and rumen

juice, this reserve polysaccharide disappeared.

A cytochemical study on the reserve glycogen in the various life cycle stages of the coccidian Eimeria tenella was reported by Gill and Ray (1954). They demonstrated the presence of glycogen in all the life cycle stages except the sporozoites. The highest glycogen reserve was found in the gametocytic stages. The authors suggested that this high level of storage was required for sporulation and prolonged survival outside the host. Gill and Ray found that the relative amount of glycogen stored by the various life cycle stages of this parasite varied according to requirements for the successful transition from one stage to the next during the life cycle. Thus they concluded that the glycogen of E. tenella played a significant role in the perpetuation of its parasitic existance.

Changes in Cell Volume of Microorganisms:

Neidhardt (1963) suggested two basic groups of environmental conditions which influenced the size of bacterial cells as; 1) "deleterious agents or conditions . . ."; and 2) "a general ... and simple relation of size to the growth-supporting ability of the chemical environment, . . ."

In their studies on E. coli, Salmonella gallinarum and S. pullorum grown in a medium with excess glucose, Huntington and Winslow (1937) found that mean cell volume increased during the lag phase of growth but then decreased upon entering the logarithmic phase. The mean cell volume then increased again during late phases of culture. Ormsbee (1942)

found that the dry weight and the length and width of individual cells of Tetrahymena galleii varied considerably during the lag phase of growth. However, the cells showed less variation during the logarithmic phase when the average dry weight, length and width was less. At the onset of the maximal stationary growth phase, the dry weight and dimensions of the organism began to increase. From these data Ormsbee proposed cell size to be directly related to cultural age. Loefer (1952) and Summers (1963) also reported that the mean cell volume of T. pyriformis, calculated from average length and width determinations, increased to a near maximum during the late logarithmic phase. The cell volume then remained constant or increased slightly during the maximal stationary phase. Scherbaum (1956) reported that the calculated cell volume of T. pyriformis during the logarithmic phase decreased to 50% of that during the lag phase and followed a normal distribution curve. Harding (1937) reported that when a species of Glaucoma (Tetrahymena) was subjected to starvation, the cells assumed a slender form which became more pronounced as long as cell division continued. Upon cessation of division the organisms assumed a more spherical form. This author proposed that "rounding-up" after extended periods of starvation was the result of a decrease in cellular surface area due to the utilization of pellicular substances for energy metabolism. Mast and Hahnert (1935) reported that Amoeba proteus decreased in volume by 95% when subjected to starvation conditions.

Movement became progressively more sluggish and pseudopods became fewer and smaller as starvation progressed. The amoebae became spherical just prior to death. Kimball et al. (1959) reported that starved cells of Paramecium aurelia continued to divide and decreased in size. These authors proposed that the production of more organisms at the expense of less nutrient was a survival mechanism. They further noted that the return of P. aurelia to excess nutrient resulted in a rapid increase in cell size as they continued their normal division rate.

Carbon and Energy Reserve in Trichomonads:

The trichomonad flagellates parasitic in homeothermic vertebrates utilize a wide variety of hexose sugars (Lwoff, 1951; von Brand, 1952; Read, 1955 & 1957; and Lindblom, 1961). Likewise, Tritrichomonas augusta a commensal of poikilothermic vertebrates, has a similar ability to utilize hexose sugars with the resultant production of H_2, CO_2 and acid end products (Twohy, 1959 & 1961; and Concannon, 1959). In addition to nutritional studies on carbohydrate utilization, a number of investigations have demonstrated the occurrence of nearly all the enzymes and intermediate compounds of the Embden-Meyerhof glycolytic pathway in Trichomonas vaginalis and Trit. foetus (Ryley, 1955; Wirtschafter and Jahn, 1956; and Wellerson and Kupferberg, 1962). An examination of the available data on the acid end products of carbohydrate metabolism and evolution of carbon dioxide and hydrogen suggests that this enzymatic pathway can be the

main route of carbohydrate metabolism in trichomonads (Suzuoki and Suzuoki, 1951 a & b; Ninomiya and Suzuoki, 1952; Doran, 1958 & 1959; and Wellerson et al., 1959). The terminal carbohydrate metabolism of trichomonads follows anaerobic pathways and most forms investigated survive and multiply only under anaerobic conditions (Bishop, 1934; Johnson, 1942; Lwoff, 1951; Hutner and Provasoli, 1955; Ryley, 1955; and Wellerson et al., 1959). To date glycogen-like polysaccharides have been the only form of reserve carbon and energy compounds considered in investigations on trichomonads. The knowledge of the composition and occurrence of lipids in trichomonads is extremely limited. However, in a recent report on the chemical analysis of lipids extracted from cells of Trit. foetus, Halevy (1963) found no evidence that compounds of this nature were stored in the cytoplasm in quantities sufficient to serve as energy reserves.

Stewart (1938) and Lyford (1941) proposed the presence of glycogen reserves in Trit. foetus on the basis of the reddish-brown color reaction of the stored polysaccharide in organisms stained with D'Antoni's iodine solution. Wantland et al. (1962) cited the occurrence of a "glycogen vacuole" in T. tenax which was demonstrable by cytochemical techniques. Feinberg and Morgan (1953) isolated and characterized a reserve carbohydrate from Trit. foetus which they considered to be glycogen. On the basis of chemical analyses and enzymatic degradation, Manners and Ryley (1955)

found that a purified polysaccharide isolated from Trit. foetus and T. gallinae resembled but was not identical to animal glycogen.

Stewart (1938) and Lyford (1941) reported the pattern of glycogen storage by Trit. foetus. Glycogen content was determined by the color reaction obtained when organisms from various cultural phases were stained with D'Antoni's iodine solution and expressed as the percentage of organisms containing glycogen. These authors found that the accumulation of glycogen reached a maximum along with attainment of the maximal population. As the cultures aged the level of glycogen reserves decreased steadily. Stewart (1938) stated that "young" organisms low in glycogen reserves were first to die in culture. Ryley (1955) reported that Trit. foetus appeared to maintain motility and metabolism through fermentation of its glycogen reserves when maintained under non-nutrient conditions. Thus far no other aspects of trichomonad physiology have been studied in respect to possible correlation with the glycogen reserves.

MATERIALS AND METHODS

Strain T-12[1] of Tritrichomonas augusta was used throughout this investigation. A clone of the parent strain (Clone 5b) was used for most of the experiments involved in this work. Since there were no consistent differences between the parent strain and the clone with respect to the phenomena studied, the results for both have been combined in this presentation.

All cultures of this organism were maintained at 29°C in Diamond's (1957) medium modified with the addition of 10% 0.11 M Na_2HPO_4 to give a pH of 6.6 and by the substitution of either 2 mM, 4 mM or 20 mM glucose for maltose.

Inocula for all experimental cultures were obtained from steady state continuous-flow cultures described by Twohy (1961). In each experiment the inoculum was adjusted to give an initial population of approximately 1 x 10^5 organisms per ml.

The experimental cultures to be used for growth studies were maintained in 500 ml "Tissue Culture Spinner Flasks"[2] under a nitrogen atmosphere. Cultures used for survival studies were maintained in 10 ml of culture medium contained in 15 x 150 mm screwcap tubes.

Samples to be used for glycogen analysis, size determinations, and population counts were taken at varying time

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[1] Isolated by Dr. D. W. Twohy from Rana catesbiana on November 12, 1959, at Oklahoma State University.

[2] Belco Glass, Inc., Vineland, New Jersey.

intervals after inoculation. All samples were withdrawn with a sterile syringe attached to a 3-inch, 22-gauge needle which was inserted through a rubber serum bottle stopper fitted in the sidearm of the flask. The organisms in all samples were killed and preserved in a final concentration of 1% formalin, added as either a 2% or 5% solution in 80% Krebs-Ringer solution (Umbriet et al., 1957).

Preserved samples to be counted were diluted ten-fold with 80% Krebs-Ringer solution. Population counts were made on a Model-A Coulter Counter[3] fitted with a 100u aperture and set at an aperture current setting of 2 and a threshold value of 15. The counts were corrected for coincidental passage of more than one organism at a time through the aperture prior to the computation of the total number of organisms per ml of sample (Mattern, et al., 1957).

The sizes of the organisms were determined on the Coulter Counter according to the methods described by Mattern et al. (1957) from samples prepared in a manner similar to that used for population counts. Threshold values were converted to cubic micron volumes by multiplying by a constant derived empirically by comparing average threshold values and packed cell volumes on each of several samples.

Organisms to be used for glycogen analyses were collected in 15 ml centrifuge tubes, killed and preserved as described for population samples, and stored at 4°C until

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[3]Coulter Electronics, 5227 N. Kenmore, Chicago, Illinois.

analyzed. In preparation for analysis the samples were centrifuged twice, once to free the organisms of medium and again to wash the organisms after suspension in 80% Krebs-Ringer solution. After each centrifugation, the supernatant was discarded. Glycogen was extracted from the washed organisms by the method of Good et al. (1933) and assayed directly by the anthrone method for blood sugar described by Roe (1955). Glycogen values were expressed as micrograms per 10^5 organisms based on population counts taken with each series of samples.

To test the survival of Trit. augusta in both aging cultures and under non-nutrient conditions a series of 15 x 150 mm growth tubes containing 10 ml of modified Diamond's (1957) medium with either 2 mM or 20 mM glucose were used. At each sampling interval after peak populations were attained, three wet mount preparations from each of four tubes were examined microscopically. Fifty organisms per wet mount were examined for motility as evidenced by movement of either the flagella or undulating membrane. Survival was expressed as the average per cent of motile forms from the twelve preparations.

The organisms in each set of four tubes were then killed and preserved. A population count was made and glycogen was analyzed. Population counts were corrected by use of the previously determined survival percentages in order to express the amount of glycogen in terms of viable cells.

Calculation of the glycogen reserves on the basis of viable organisms involved the assumption that: 1) glycogen reserves in dead organisms were nearly negligible and, 2) the fragile, dead organisms would rupture and any remaining glycogen would be removed in the supernatant while separating organisms from medium by centrifugation.

Survival of Trit. augusta under non-nutrient conditions was tested using organisms from 48 hour tube cultures similar to those used as a source of organism in the previous survival experiment. The organisms in all samples were centrifuged free of medium, and the organisms in each tube were resuspended in 10 ml of 80% Krebs-Ringer phosphate solution per tube (Umbriet et al., 1957). At each sampling interval the organisms in each of 4 tubes were examined for motility and the population and glycogen were determined by the methods previously described for aging cultures.

An approximation of the mass of organisms was obtained by multiplying mean cell volumes by 1.038; the value reported by Wichterman (1953) for the specific gravity of live Paramecium species. Use of this value for specific gravity was based upon the assumption that there is a close similarity in the specific gravities of protozoa.

RESULTS

I. A Comparison of Glycogen Content with the Cultural Age of *Tritrichomonas augusta* in the presence of Limiting and Non-Limiting Concentrations of Glucose.

The growth curves and corresponding intracellular glycogen concentrations for *Trit. augusta* are shown graphically in Figures 1 through 4. Figures 1 through 3 show that the *in vitro* growth of *Trit. augusta* followed a classical growth curve. Due to the impossibility of distinguishing between live and dead organisms by the method of counting employed in this investigation, growth curves were followed only as far as the maximum stationary phase. Separate experiments were run for each of two concentrations of glucose. A comparison of Figures 1 and 3 shows that the 4 mM glucose concentration did not reduce the maximal populations below that attained with 20 mM glucose.

Glycogen constituted between 0.8 and 7.3% of the total wet cell weight. The respective nitrogen contents were 3.9 and 3.0%. Although dry weights were not determined, glycogen probably constitutes a high percentage of the non-aqueous constituents of the cells.

The results of the series of glycogen analyses of organisms in various phases of culture are given in Figures 2 and 4. During the lag and early logarithmic phases of growth in 4 mM glucose, glycogen increased quite rapidly to a 35- to 100-fold increase over the low glycogen content of organisms used as the inoculum (Figure 2). The level of glycogen reserves began to decrease during the remainder of

the logarithmic phase. The decrease in the level of reserve glycogen was more pronounced during the deceleration phase and continued through the maximal stationary phase of growth.

In the presence of excess glucose (20 mM) the organisms showed a similar glycogen increase during the lag and early logarithmic phases of culture, followed by a rapid drop to an intermediate level during the middle of the logarithmic growth phase. The drop in glycogen reserves was more pronounced with growth in the high glucose concentration than in the low glucose concentration. The concentration of stored glycogen per organism either continued to decrease or remained at an intermediate level until the decelerating growth phase. At that time it again began to increase until the peak glycogen concentration equaled or exceeded the amount accumulated during the lag phase.

Figures 5 and 6 show the pattern of total glycogen buildup in cultures of Trit. augusta in the presence of a 4 mM and a 20 mM glucose concentration. The rate of total glycogen synthesis in both concentrations of glucose was greatest in the first 15 hours, during which time the organisms had passed through the lag phase and were beginning the logarithmic phase of growth. During the remainder of the logarithmic growth phase, glycogen accumulated at a slightly slower rate. No significant differences in the pattern of total glycogen synthesis were observed in cultures with different glucose concentrations during the first 25-30 hours after inoculation. However, the maximum level of

glycogen reserves attained during early growth varied between experiments when a 4 mM glucose concentration was used, indicating that other factors must influence the maximum level of glycogen under these conditions.

In late phases of cultural growth in medium with the 4 mM glucose concentration glycogen synthesis ceased, and there was a decline in the level of total glycogen reserves during the decelerating growth phase and the maximal stationary growth phase respectively (Figure 5). On the other hand, Figure 6 shows a continued increase in the level of total glycogen during the phase of decelerating growth when glucose was in excess in the medium. During the maximal stationary growth phase the total amount of glycogen reached its maximum level which was maintained for the remainder of this phase.

In summary, a comparison of growth and glycogen storage in the presence of 4 mM and 20 mM glucose showed that in both types of culture the glycogen reserves reached a peak during the lag and early logarithmic phase of growth. The level of stored glycogen decreased during the logarithmic phase. In the presence of 20 mM glucose glycogen again began to increase during the decelerating growth phase and reached a maximum when cultural growth ceased. In the presence of 4 mM glucose glycogen reserves continued to decrease until they reached a very low level during the maximum stationary growth phase. However, the peak population attained by organisms with low glycogen reserves in the presence of the

limited glucose concentration was the same as that attained with excess sugar.

Comparison of the total glycogen reserves in these two types of culture indicates that glycogen was accumulated in all phases of culture in the presence of excess glucose. However, in the presence of limited glucose the level of total glycogen decreased during late phases of culture.

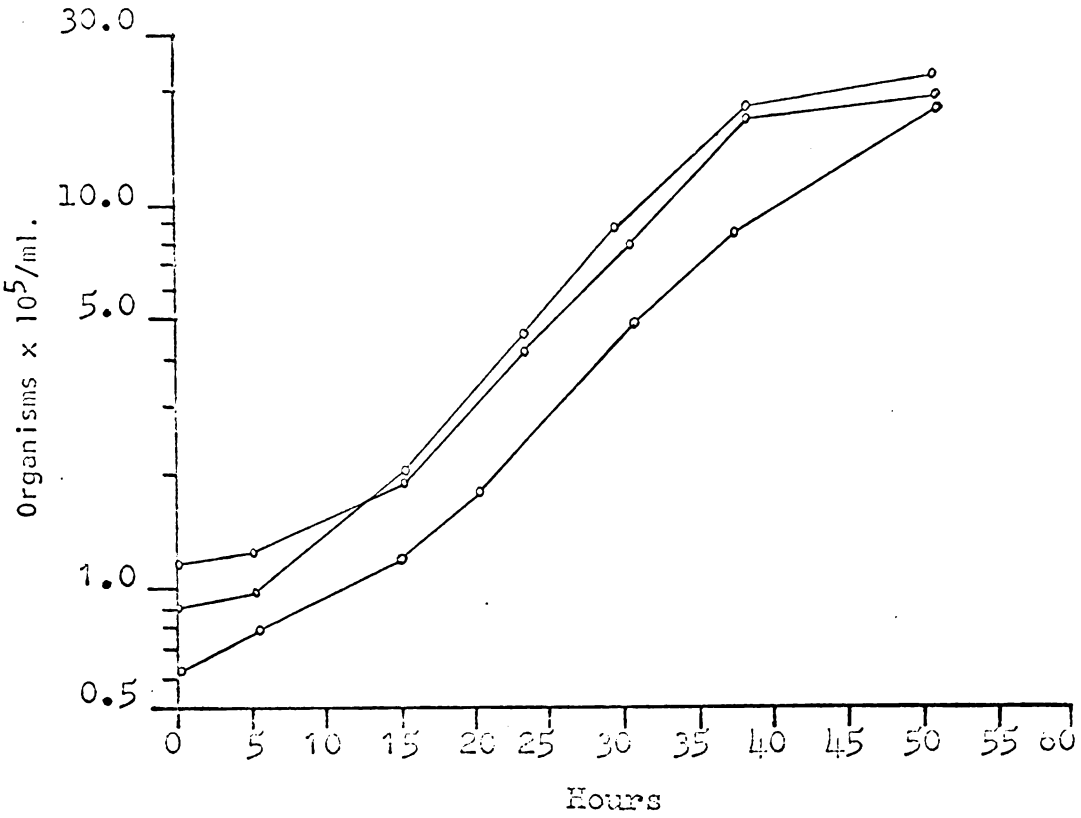

Figure 1. Growth of Trit. augusta in Diamond's medium with 4 mM glucose.

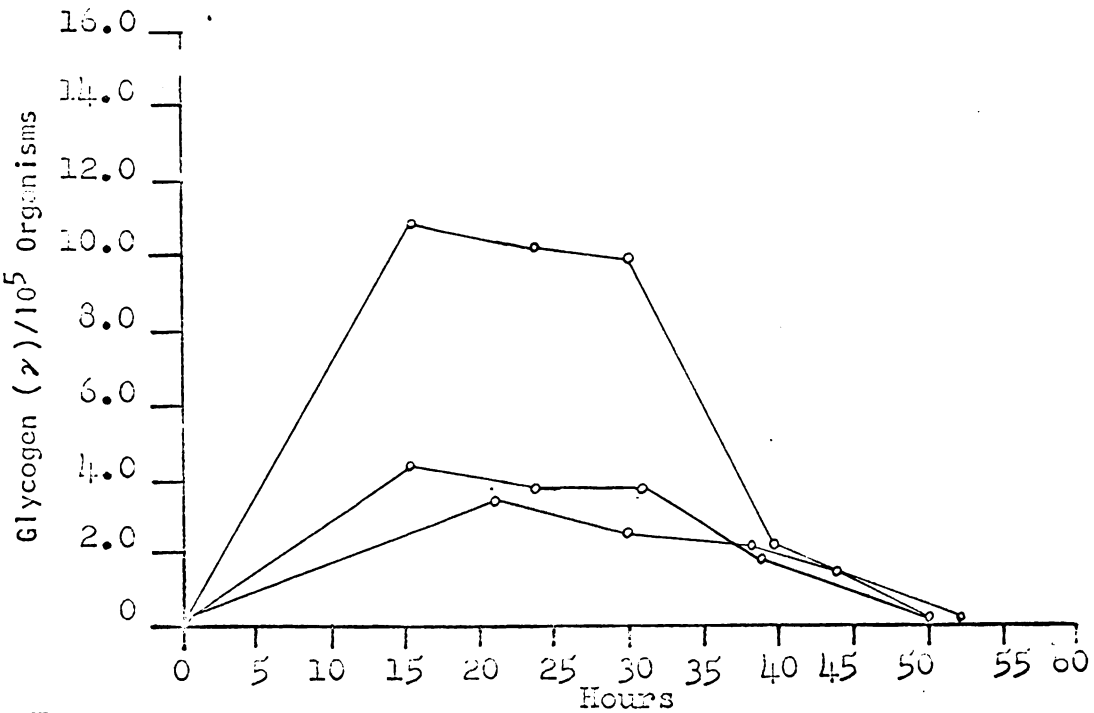

Figure 2. Variations in glycogen in Trit. augusta during culture in Diamond's medium with 4 mM glucose.

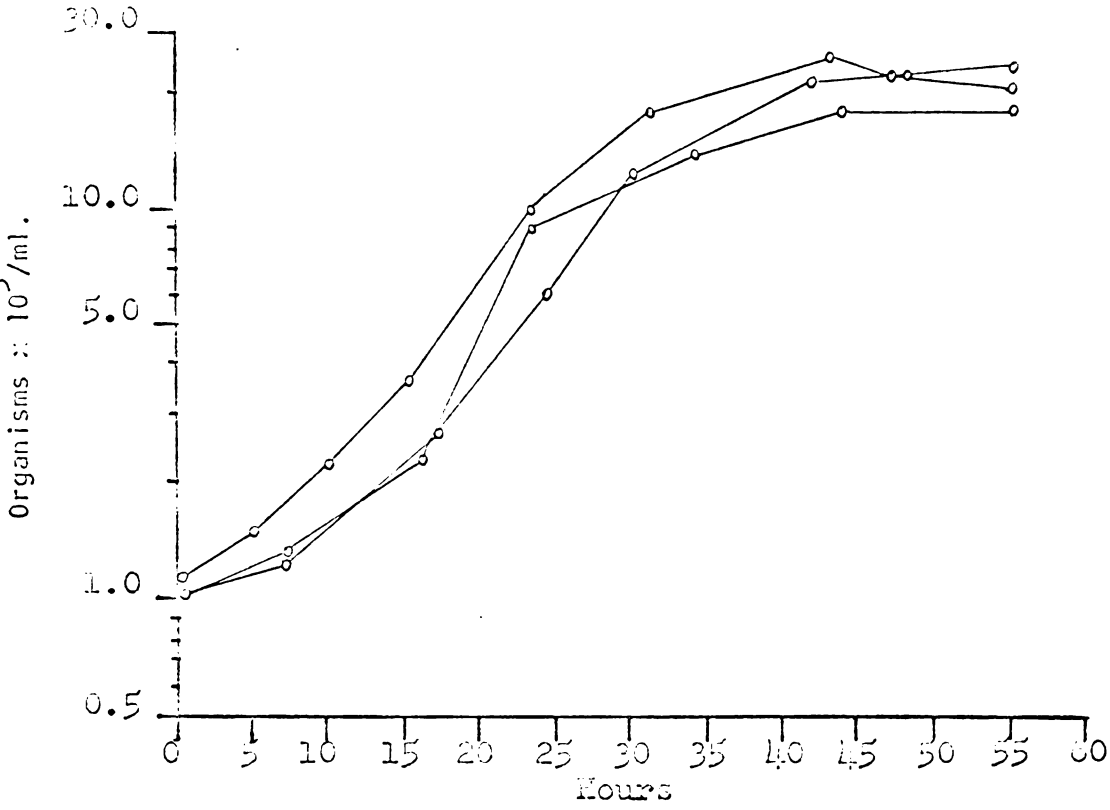

Figure 3. Growth of Trit. augusta in Diamond's medium with 20 mM glucose.

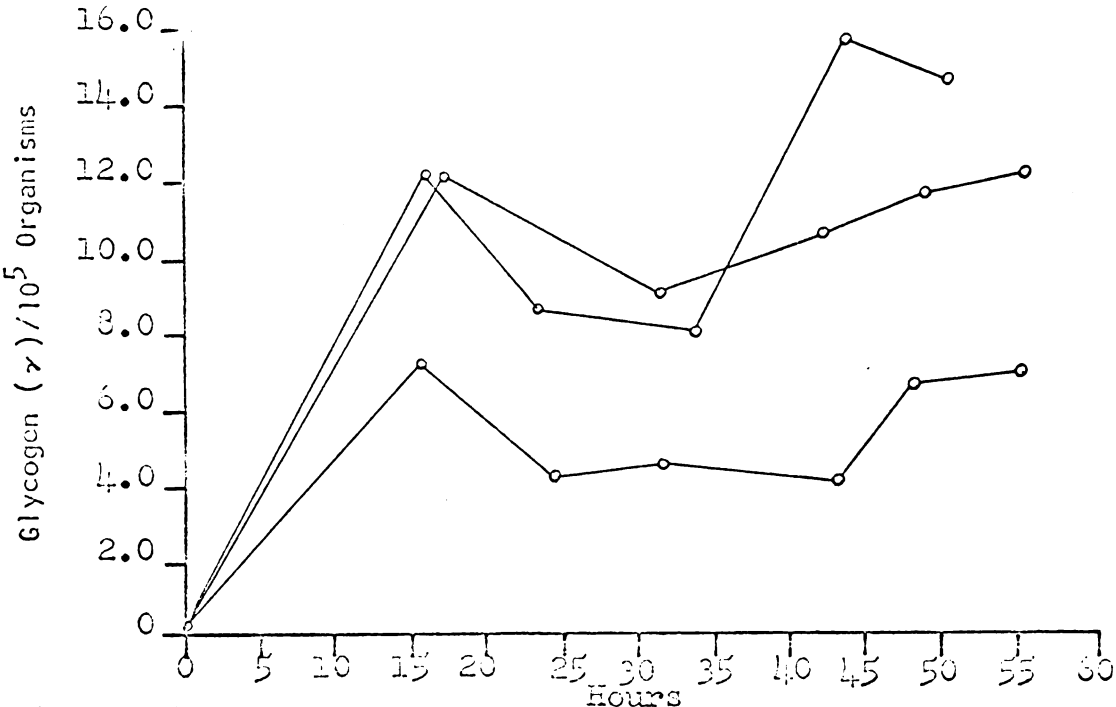

Figure 4. Variations in glycogen in Trit. augusta during culture in Diamond's medium with 20 mM glucose.

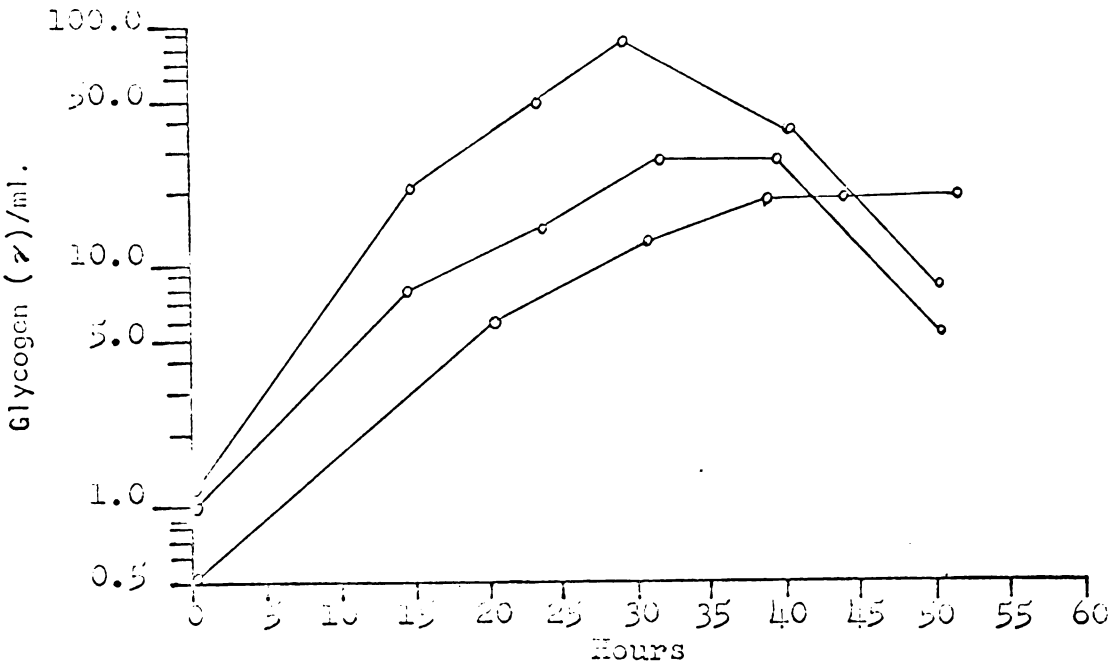

Figure 5. Total glycogen in Trit. augusta per ml of culture employing Diamond's medium with 4 mM glucose.

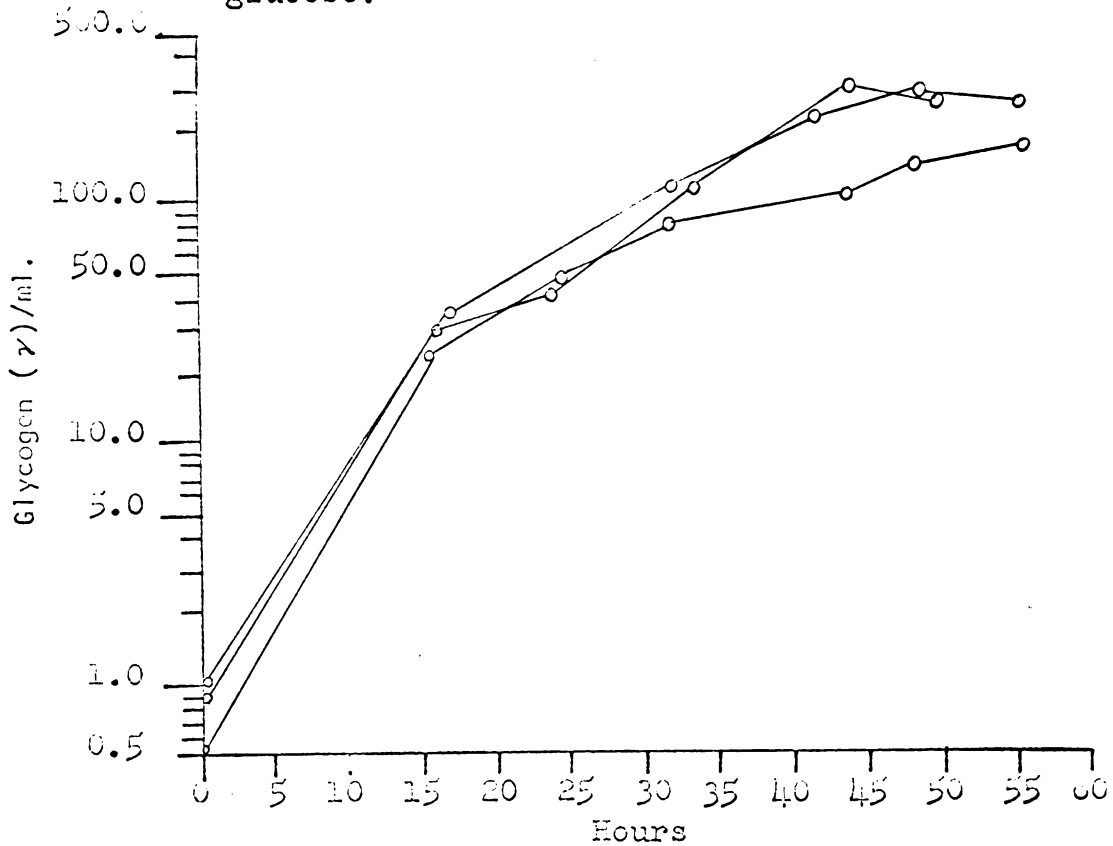

Figure 6. Total glycogen in Trit. augusta per ml of culture employing Diamond's medium with 20 mM glucose.

II. A Comparison of Cell Volume with the Cultural Age of Tritrichomonas augusta in the presence of Limiting and Non-Limiting Concentrations of Glucose.

There was a considerable range in size, expressed as cell volume, of individual forms of Trit. augusta at all stages of culture. Size frequency curves for samples taken from cultures at varying time intervals after inoculation are given in Figure 7. Figure 7a. shows the characteristic preponderance of very small organisms present in the inocula for all experiments.

The mean cell volume of organisms raised in medium containing a 4 mM concentration of glucose was found to increase during early phases of growth (Figure 8). This was followed by a plateau or decrease in mean cell volume during the logarithmic growth phase. During the decelerating and maximum stationary growth phases the mean cell volume steadily decreased until the organisms attained a mean volume very similar to that of the organisms used in the inoculum (Figures 7h and 8). Mean cell volumes and glycogen content followed similar increases and decreases over the course of the growth curve in medium containing 4 mM glucose (Figure 2 and 8). The lower concentration of glucose appears to have exerted a limiting influence on both the glycogen storage and mean cell volume of Trit. augusta during late stages of culture.

The pattern of variation of the mean cell volume of organisms raised in 20 mM glucose differed among experiments (Figure 9), and showed few common tendencies after the

initial increase in size during the lag phase. However, there was a general tendency toward maintenance of larger cell volumes during late phases of culture.

In summary, the 4 mM concentration of glucose appears to be insufficient to meet the metabolic requirements of Trit. augusta during late phases of culture. The result was a decrease in cell volume. Conversely, the availability of excess glucose in the medium enabled Trit. augusta to maintain large cell volumes during late phases of culture.

The similarity of the fluctuations in mean cell volume and glycogen content suggested a more meaningful interpretation of glycogen content might be obtained by basing glycogen concentrations on a unit of cell volume. However, a comparison of glycogen levels per unit of cell volume at various phases of culture resulted in essentially the same patterns as those based on the population (Figures 2 & 4).

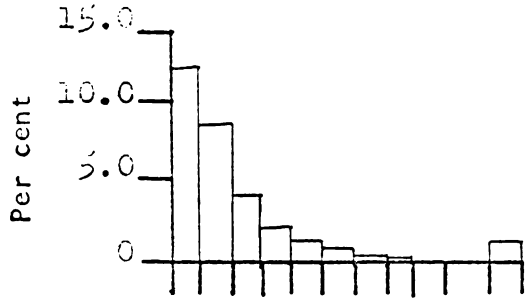

Fig. 7a - Common Inoculum

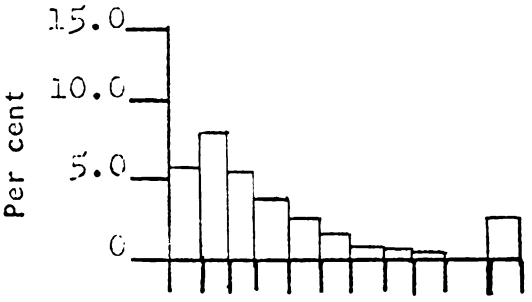

Fig. 7e - Max. Stationary Phase

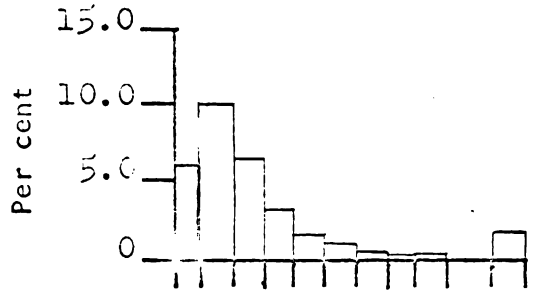

Fig. 7b - Lag Phase

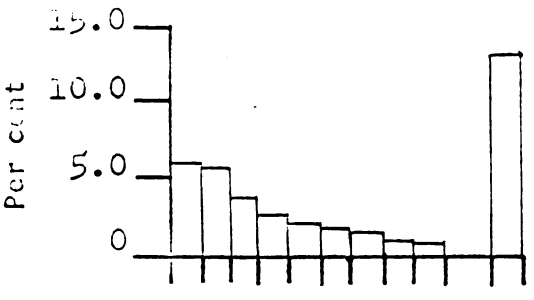

Fig. 7f - Logarithmic Phase

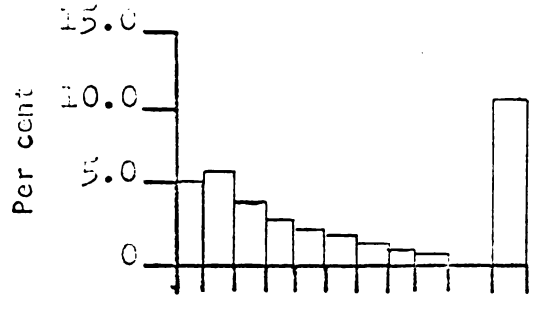

Fig. 7c - Logarithmic Phase

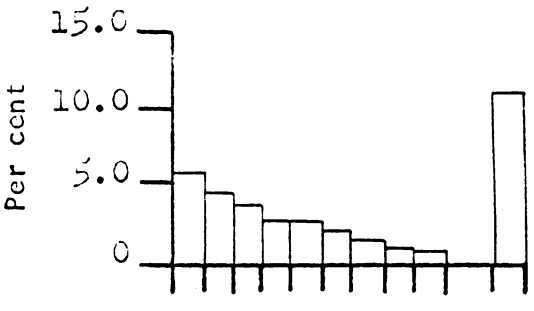

Fig. 7g - Deceleration Phase

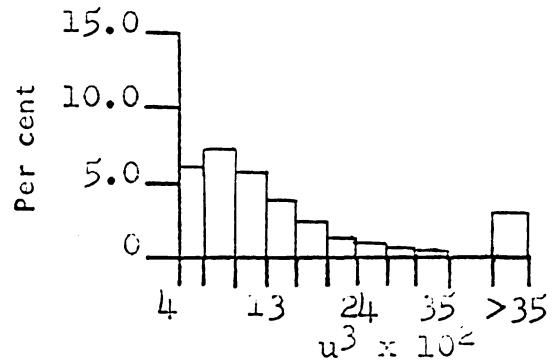

Fig. 7d - Deceleration Phase

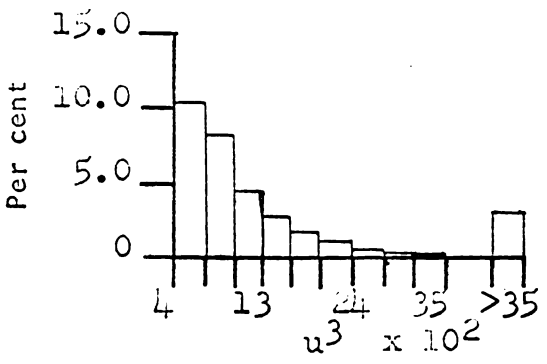

Fig. 7h - Max. Stationary Phase

Figure 7. Cell volume frequency of Trit. augusta grown in Diamond's medium. Figures (7b) through (7e) are from growth in 20 mM glucose and figures (7f) through (7h) in 4 mM glucose.

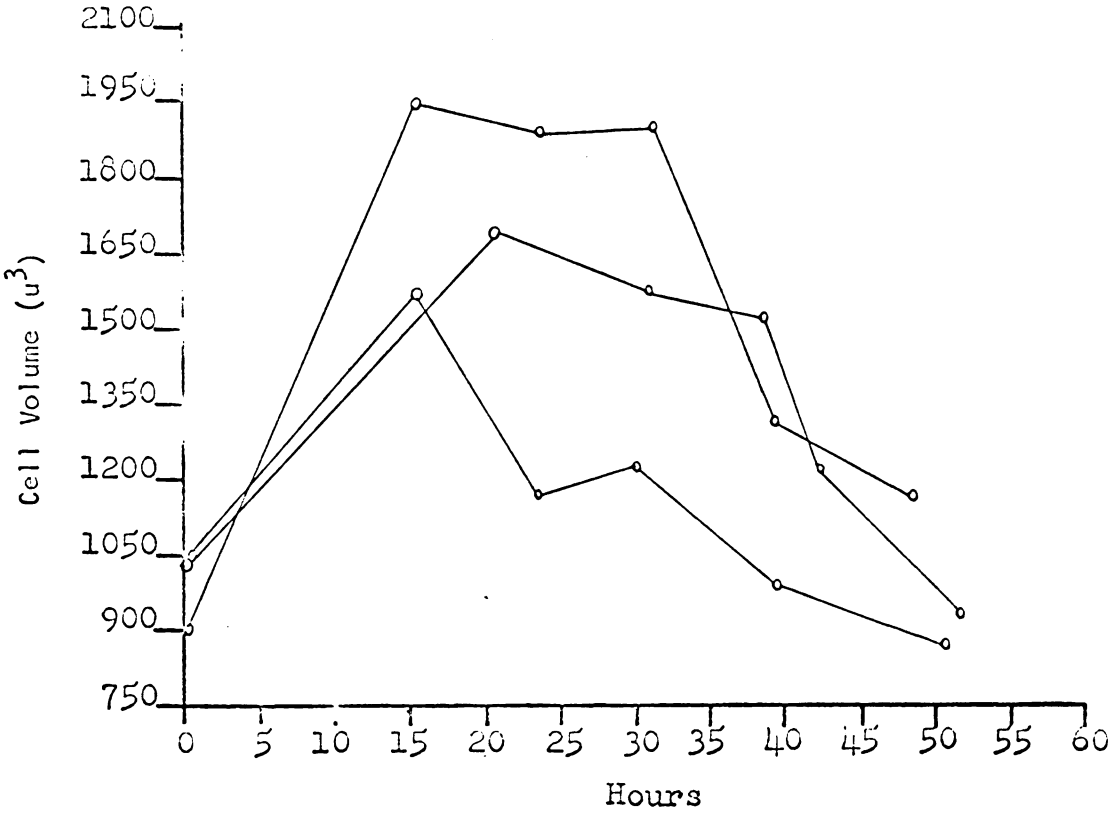

Figure 8. Mean cell volumes of Trit. augusta in Diamond's medium with 4 mM glucose.

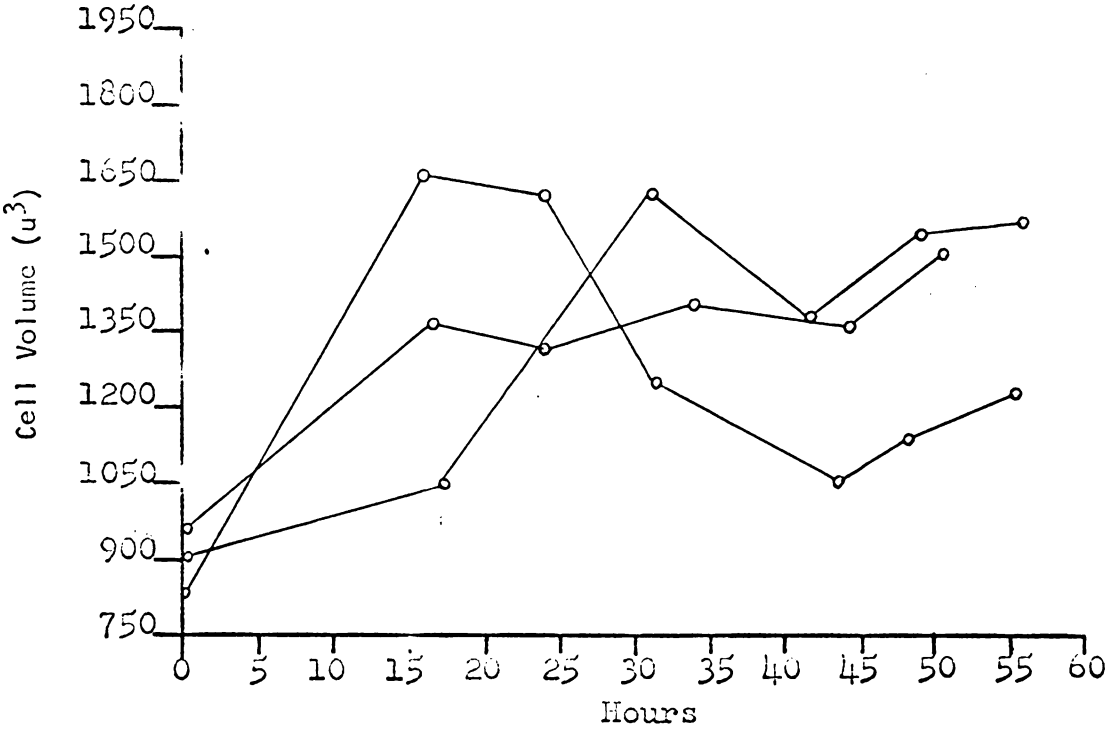

Figure 9. Mean cell volumes of Trit. augusta in Diamond's medium with 20 mM glucose.

III. The Effect of Glycogen Reserves on the Survival of Tritrichomonas augusta in Culture Medium.

A series of 48 hour cultures of Trit. augusta in tubes of Diamond's (1957) medium with 2 mM glucose were used as a source of organisms containing a low level of glycogen reserves during the maximal stationary phase of growth. Organisms high in glycogen content were obtained from similar 48 hour cultures containing 20 mM glucose.

In the presence of 2 mM glucose the percentage of motile organisms gradually began to decrease after approximately 62 hours in culture. When the last sample was examined at 168 hours after inoculation, 44% of the organisms were found to be motile (Figure 10). All microscopic examinations made during this time interval revealed these organisms to be small, slender and active. The glycogen reserves of the organisms in this series of cultures were minimal, remaining at a level which approached the limitations of the method of analysis.

The percentage of motile organisms in cultures with excess (20 mM) glucose declined sharply following the 71 hour sample. Less than 9% of the organisms remained motile 122 hours after inoculation (Figure 11). Microscopic observations showed these organisms to be spherical in form throughout this period. Although the level of glycogen reserves was high in 48 hour cultures, the amount of glycogen continued to increase until approximately 71 hours after inoculation. After 71 hours, the glycogen reserves decreased rapidly. Upon the termination of this experiment

122 hours after inoculation, a small reserve of glycogen still remained in the few motile organisms present.

Results of the previous experiments showed that in medium containing excess glucose Trit. augusta stored large quantities of glycogen. Synthesis continued during the maximum stationary growth phase. At a given point (approximately 71 hours of culture in this investigation) both glycogen reserves and motility started a rapid parallel decline. On the other hand, organisms maintained in a medium with a limiting concentration of glycose contained very low glycogen reserves during the maximum stationary phase of growth. After this period a large percentage of these organisms remained motile in spite of their low level of glycogen reserves.

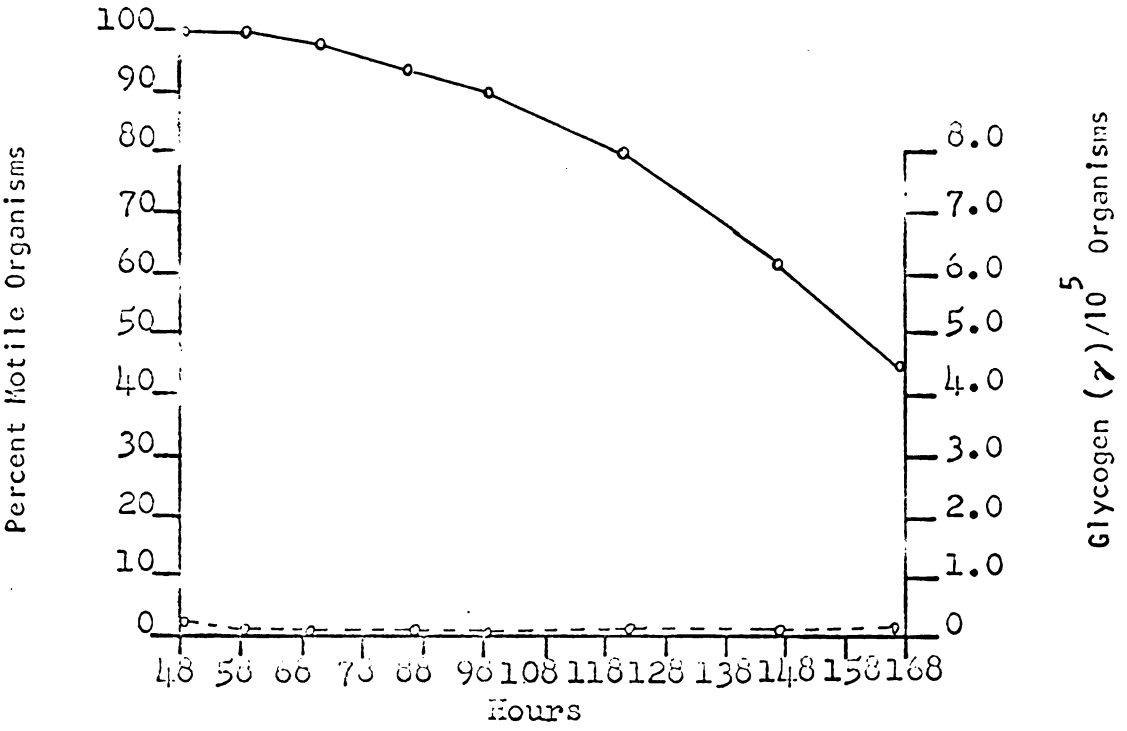

Figure 10. Glycogen content and motility of Trit. augusta after varied periods of time in aging conditions following growth in 2 mM glucose. ——— = per cent motility. - - - = glycogen / 10^5 organisms.

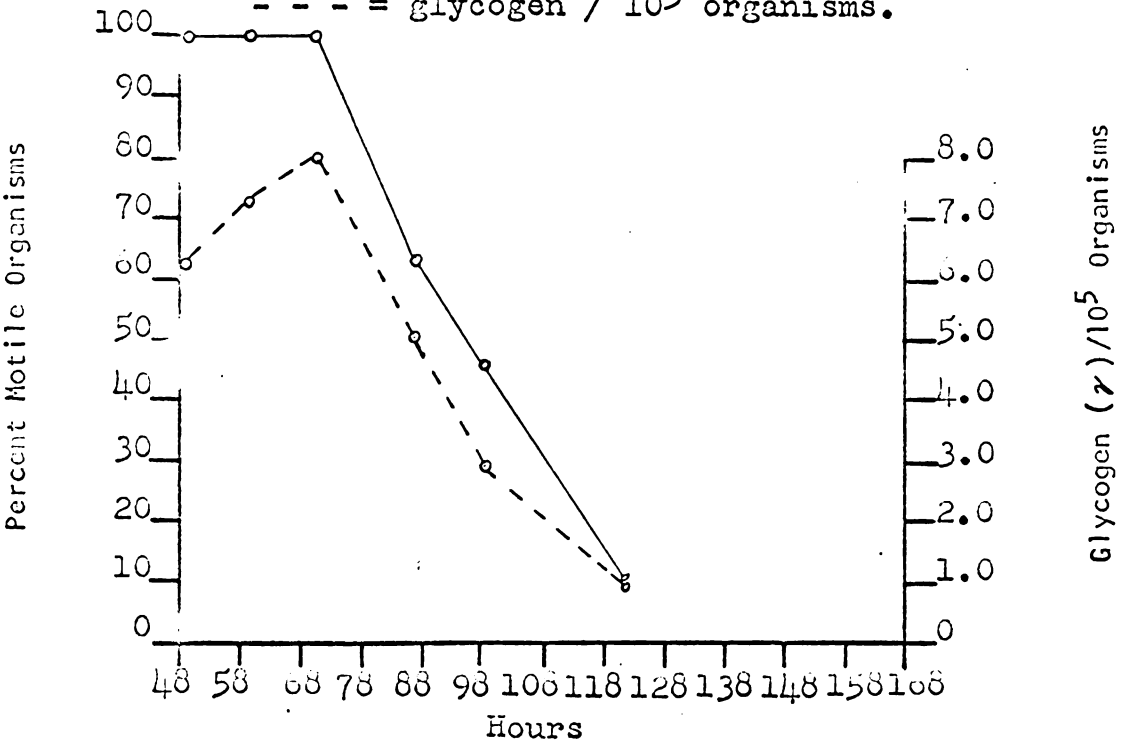

Figure 11. Glycogen content and motility of Trit. augusta after varied periods of time in aging conditions following growth in 20 mM glucose. ——— = per cent motility. - - - = glycogen / 10^5 organisms.

IV. The Effect of Glycogen Reserves on the Survival of *Tritrichomonas augusta* under Non-Nutrient Conditions.

Organisms possessing very low glycogen reserves were obtained from 48 hour tube cultures grown in a medium with 2 mM glucose as described in the previous section. Following suspension of these organisms in 80% Krebs-Ringer saline, the percentage of motile forms decreased rapidly (Figure 12). Microscopic examinations 12 hours later revealed only 26% of the organisms were motile. These surviving organisms were slender and quite active. The very few organisms remaining motile after 24 hours under these conditions were spherical and exhibited very sluggish movement.

Organisms possessing relatively high glycogen reserves were obtained from 48 hour tube cultures containing 20 mM glucose in the medium. After 24 hours under non-nutrient conditions 96% of these organisms were still motile (Figure 13). Microscopic examination revealed these organisms to be very active and spherical to moderately slender in form. During the succeeding 24 hour period the percentage of motile forms declined to 32% after 48 hours under non-nutrient conditions. The relative size and motility of the surviving individual organisms appeared to decrease progressively. At 48 hours the remaining viable organisms were small and quite sluggish. The dead organisms were usually smaller than the living organisms. The intracellular glycogen content declined in a linear fashion during the first 24 hours to what may be considered a base level at this time. The level of glycogen reserves 48 hours after suspension in

saline did not differ from the 24 hour values.

A comparison of Figures 12 and 13 shows a correlation between the level of glycogen reserves in Trit. augusta and survival under non-nutrient conditions. Nearly 100% of the organisms possessing low glycogen reserves died in 24 hoursm and the majority were dead in 12 hours. However, organisms with a high glycogen content showed substantial survival after 48 hours under non-nutrient conditions.

The data of this section present a survival pattern which is the reverse of that of the preceding section. Organisms possessing low glycogen reserves appear to have been adapted to survival in old cultures, but died rapidly when subjected to non-nutrient conditions. Conversely, the organisms containing high glycogen reserves survived for a much shorter period in old cultures, but utilized their glycogen and survived under non-nutrient conditions over twice as long as those possessing low glycogen reserves.

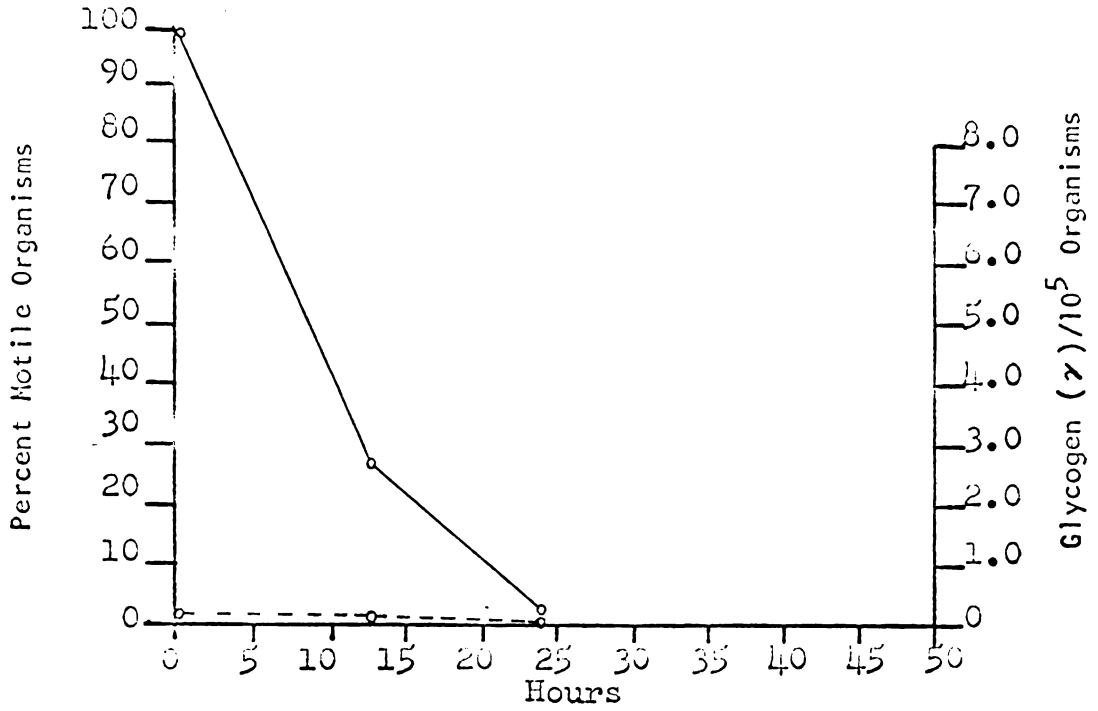

Figure 12. Glycogen content and motility of Trit. augusta after varied periods of time in non-nutrient conditions following growth in 2 mM glucose. ——— = per cent motility. - - - = glycogen / 10^5 organisms.

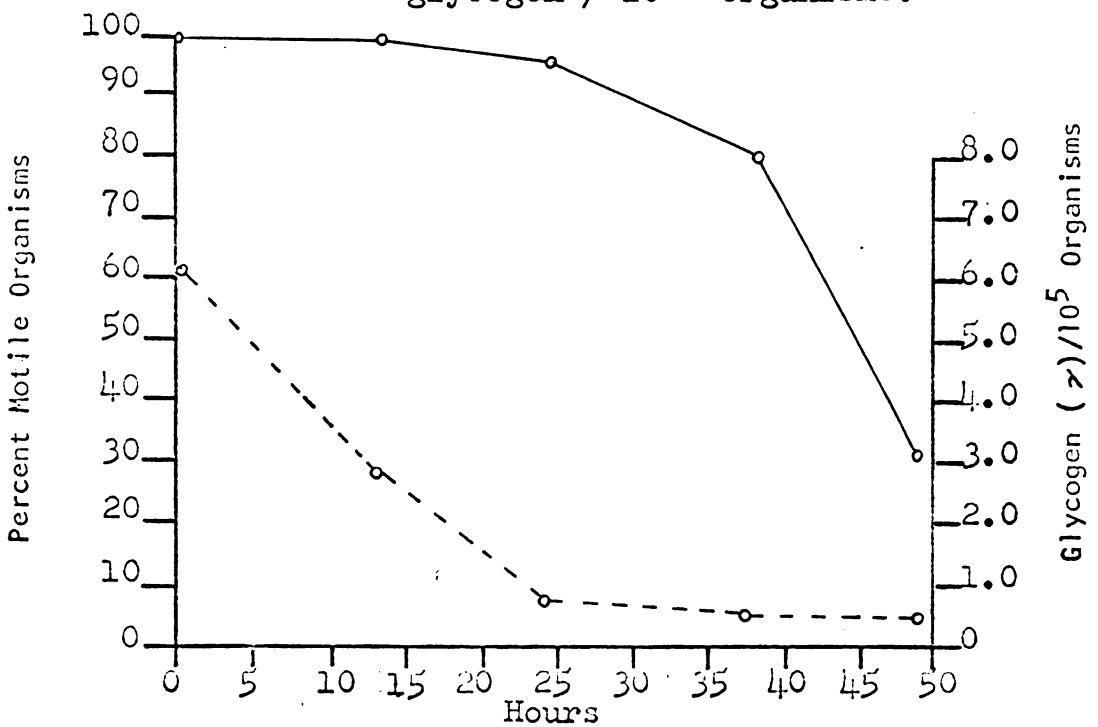

Figure 13. Glycogen content and motility of Trit. augusta after varied periods of time in non-nutrient conditions following growth in 20 mM glucose. ——— = per cent motility. - - - = glycogen / 10^5 organisms.

DISCUSSION AND CONCLUSIONS

A polysaccharide assumed to be glycogen has been extracted from cells of Tritrichomonas augusta by a technique specific for high molecular weight polysaccharides. Two possible explanations for the presence of this carbohydrate must be considered (Wilkinson, 1959). A storage polysaccharide may be accumulated as either an excess by-product which serves no function other than as a reserve energy source, or as a functional endogenous energy reserve which is utilized by the cell. With regard to the glycogen stored by Trit. augusta, the latter explanation, that this storage polysaccharide serves as a functional carbon and energy source, appears to be more correct in view of the results of the experiments and the interpretation of these results to follow.

According to Wilkinson (1959), the intracellular accumulation of a carbon and energy reserve compound must occur at a time when the exogenous carbon and energy sources are in excess of the immediate metabolic requirements of the cell. When Trit. augusta was grown in a medium containing excess glucose, the environmental conditions necessary for the accumulation of a reserve polysaccharide were present throughout the experiment. Under these conditions, the organisms responded by accumulating glycogen during all phases of growth. This can be seen by comparing the total glycogen per unit volume of culture with cultural age in the presence of 20 mM glucose (Figure 6). Although the rate

of total glycogen accumulation varied, showing the most rapid increases in the lag and logarithmic phases of growth, there was no period when glycogen synthesis ceased or when the net glycogen reserves were being utilized for growth. Thus Wilkinson's first criterion for the existence of a functional carbon and energy reserve has been satisfied by considering the culture in toto.

The above discussion does not take into consideration the glycogen concentration per organism. The glycogen content per 10^5 organisms in Figure 4 shows considerable variation in glycogen levels at different phases of culture with excess glucose. Glycogen reserves were highest during the lag, decelerating, and maximum stationary growth phases -- the periods of less than optimal rates of growth. During the logarithmic growth phase the glycogen content dropped to an intermediate level between that of organisms in the inoculum and the decelerating and maximum stationary growth phases.

As organisms increase in number the proportion of enzymes or sites for glycogen synthesis also increases and the ratio of these enzymes or sites to the total mass of protoplasm can be assumed to be constant regardless of the size of the population. Thus, theoretically, the potential rate of glycogen synthesis per unit of protoplasm would be equal at all stages of cultural growth and consequently the total potential for glycogen synthesis would increase at the same relative rate as the increase in total protoplasm.

However, other incompletely known factors probably limit the actual rate of synthesis so that it frequently falls short of this potential. If the rate of glycogen synthesis were proportional to the rate of growth, the rate of glycogen accumulation in culture would equal the rate of protoplasmic increase in culture. In this case the glycogen per organism (or more exactly the glycogen per unit of protoplasmic mass that most ideally represents growth) would remain constant and the curves for the total population plotted against time would be similar. If, on the other hand, glycogen synthesis ceased and growth continued, the amount of total glycogen in culture would remain unchanged; but the concentration per organism would decrease. Conversely, if glycogen synthesis occurred at a constant rate but growth ceased, glycogen would accumulate and the total glycogen in culture as well as the amount per organism would increase. The glycogen concentration is clearly dependent on both the rate of glycogen synthesis and the rate of growth.

From the above assumptions a comparison of the growth curve in Figure 3 with the curve for total glycogen in Figure 6 allows a prediction of the effect of the rate of growth upon the level of glycogen reserves. Initially during the lag phase of growth, the rate of glycogen synthesis was high while the rate of growth was slow to moderate. Glycogen should accumulate in the cells during this period. As the organisms entered the logarithmic phase of growth,

the rate of glycogen synthesis decreased as depicted by the slope of the curve for total glycogen values (Figure 6). Glycogen concentrations should then gradually decrease as the excess glycogen is diluted to a level that can be maintained by the new rate of synthesis. During the deceleration phase of growth, which probably started shortly after the 31 hour samples, glycogen synthesis seemed to continue at the same rate, but the rate of growth decreased. The result would be an increase in glycogen concentration. These predictions follow very closely the observed changes in glycogen concentration shown in Figure 4.

The pattern of variation in glycogen concentration at various cultural ages (Figure 4) resembles that reported for Escherichia coli B by Palmstierna (1955 & 1956) and for Tetrahymena gelleii by Fennell and Marzke (1954). However, Palmstierna's (1956) statement that glycogen is utilized by E. coli B during the logarithmic growth phase, based on a decrease in total glycogen during that phase, did not hold true for Trit. augusta.

The rate of glycogen synthesis showed some variation over the course of cultural growth (Figure 6). The factors that influence the rate of synthesis are not known (Neidhardt, 1963). Above minimal levels the concentration of glucose in the medium is not usually a limiting factor. Twohy (1963) has shown that the rate of growth of Trit. augusta is independent of the concentration of glucose in the medium and thus it would seem unreasonable to assume that the glucose

concentration would influence the rate of glucose uptake or glycogen synthesis. It is quite possible, however, that the rate of glucose uptake may have some upper limit set by the transport mechanisms, enzyme capacity or ATP pool of the cells. If most of the glucose is channeled into energy metabolism essential to the growth of the organism, less of the sugar may be available for glycogen synthesis. Therefore, if the amount of glucose utilized is directly related to the rate of growth as proposed by Monod and Tessier (1936), Monod (1942 & 1950) and Novick and Szilard (1950), and shown to be generally true for many species of bacteria if small energy requirements for cell maintenance were discounted (Herbert et al., 1959), the amount of growth would serve as an index of the amount of glucose utilized as an energy and carbon source. Thus the lower rate of total glycogen accumulation during the logarithmic phase of culture may reflect a limiting rate of glucose uptake and the conversion of most of the assimilated glucose into energy, but it would not account for the continuation of the lower rate of glycogen synthesis that seems to occur during the decelerating phase of growth. The data of this investigation are insufficient, however, for the calculation of rates of glucose uptake. In summary, polysaccharide accumulation appears to be effected by the rate of growth and the rate of glycogen synthesis and both are shown to vary over the course of cultural growth.

Fulfillment of Wilkinson's second requirement that "the compound is utilized when the supply of energy from exogenous sources is insufficient for the optimal maintenance of viability ..." has been tested by following the levels of glycogen reserves in organisms grown in cultures having limiting concentrations of glucose and in organisms held under non-nutrient conditions. When the exogenous supply of glucose became limiting in cultures containing 4 mM glucose both the mean glycogen concentration per cell and the total glycogen in the population decreased showing that the glycogen reserves were being utilized faster than they were being synthesized (Figures 2 & 5). Thus the organisms appeared to continue to multiply under these conditions at the expense of their reserves. The amount of total protoplasm may not have increased, however, to the same extent as the number of organisms since there was a decrease in mean cell volume during this period (Figures 7h and 8). Calculating the glycogen per unit of cell volume, however, did not change the general tendency for a decrease in glycogen reserves (Figure 2). The ability to use glycogen was also shown when the organisms were suspended in non-nutrient buffered saline. The glycogen reserves decreased during the period that the organisms remained viable (Figure 13).

No attempt was made to obtain direct evidence for the breakdown of glycogen to produce energy according to Wilkinson's third requirement, "that the compound is broken down

to produce energy in a form utilizable by the cell, and that it is, in fact, utilized for some purpose which gives the cell a biological advantage in the struggle for existance over those cells which do not have a comparable compound,...". However, the glycogen of Trit. augusta does appear to provide the organisms with a utilizable form of energy. This was observed when organisms with different levels of stored glycogen were subjected to starvation conditions (Figures 12 & 13). Organisms with high levels of stored glycogen remained motile and survived for a considerably longer period of time than did organisms containing lower levels of reserve glycogen. As previously pointed out, high glycogen reserves were steadily depleted during this period, indicating that organisms rich in glycogen reserves derive a distinct "biological advantage" in survival over organisms nearly devoid of stored glycogen. Ryley (1955) observed that Trit. foetus maintained motility and metabolism under non-nutrient conditions through fermentation of intracellular glycogen.

In view of the foregoing discussion, the glycogen stored by cells of Trit. augusta may be considered to serve as a functional carbon and energy reserve. However, the data obtained thus far does not rule out the possibility of another form of carbon and energy reserve in this organism (e.g. lipid or volutin). It was found that when aging cultures were examined, the organisms grown in excess glucose and possessing relatively high glycogen reserves died off rapidly after peak populations were attained. On the

other hand, organisms from cultures low in glucose, and thus containing minimal amounts of glycogen, remained viable for a considerably longer period of time (Figures 10 & 11). An attempt to explain these results necessitates a separate consideration of; 1) how organisms containing minimal glycogen reserves survived for considerable periods of time in the near absence of known energy sources, and 2) why organisms possessing relatively high glycogen reserves died off rapidly following the attainment of peak population.

Explanation of the first phenomenon may involve any one or a combination of three possible survival mechanisms: 1) the utilization of a major reserve energy source other than glycogen, 2) the limited utilization of some other component of the medium as an energy source besides glucose at a rate just sufficient for survival, and 3) a more economical utilization of minimal reserves or structural components at a reduced rate of metabolism just sufficient for survival. The first of these possibilities could easily involve stored lipid compounds. Halevy (1963) was unable to find evidence that any compounds of this nature were stored in quantities essential for energy reserves in the cytoplasm of the closely related trichomonad, Trit. foetus. The limited utilization of other compounds in the medium as exogenous carbon and energy sources remains to be demonstrated. The observations that these organisms were rather small and slender in form, as compared to the large spherical form of those with high

levels of reserve glycogen, does suggest that Trit. augusta is capable of utilizing structural cell components as sources of carbon and energy for survival and possibly very limited reproduction as has been proposed to occur in Paramecium aurelia and species of Glaucoma (Tetrahymena) (Harding, et al., 1937; and Kimball, et al., 1959). This phenomenon also was observed to occur in Amoeba proteus by Mast and Hahnert (1935) according to their description of morphological changes accompanying starvation of this organism. A more detailed analysis of cell components before and after periods of time in glucose free medium might clarify this observation.

The relatively rapid die off of organisms possessing high levels of glycogen reserves in cultures with excess glucose is probably the result of factors not related to glucose uptake or the glycogen reserves. They may die from accumulation of toxic wastes in the medium or from a critical deficiency of a nutrient or nutrients other than glucose. The decrease in the glycogen reserves of these organisms may have been the result of abnormal and unregulated catabolic reactions occurring in moribund cells.

The mean cell volumes of Trit. augusta were investigated at various cultural ages and with different levels of glycogen reserves in the hope of detecting the existence of more than one physiologically distinguishable cell type. The mean cell volume and frequency of larger cell volumes of Trit. augusta increased during the lag and logarithmic

phases of growth in the presence of both the 4 mM and the 20 mM glucose concentrations, and the large size was maintained during late phases of culture in the presence of the 20 mM concentrations of glucose (Figures 7b-g, 8 & 9). However, in medium with a 4 mM concentration of glucose the mean cell volume decreased and the frequency of small forms increased during the decelerating and maximal stationary growth phases (Figures 7h & 8). Therefore, the availability of excess glucose during the late phases of culture appears to enable Trit. augusta to maintain increased cell volumes. These observations are in agreement with those of Huntington and Winslow (1937), Ormsbee (1942), Loefer (1952), Scherbaum (1956), and Summers (1963) who used several species of bacteria as well as by Tetrahymena gelleii and T. pyriformis.

The glycogen concentration was calculated to be between 0.8-7.3% of the total protoplasmic weight. The specific gravity of glycogen is greater than that of water so the actual contribution to cytoplasmic volume would be less than the weight of the glycogen and certainly much less than the variation encountered in cell volume. This conclusion is based upon the assumption that glycogen is stored as a pool neither associated with additional cell structure nor combined with other compounds which would significantly increase the volume of the organism. These general considerations seem to rule out the volume of glycogen as the cause of increases in cell volume since the extremes of cell volumes represent a two-fold difference and glycogen

contributions to calculated cell weight was only 7.3%. These observations suggest that simultaneous increases and decreases in glycogen content and cell volume are the result of the influence of the particular environmental conditions upon two independent aspects of the physiology of this trichomonad.

Interest in the factors influencing the survival of Trit. augusta was stimulated by consideration of the necessity of its survival outside the host during the course of oral transmission in amphibia reported by Bishop (1930 & 1934). In order to infect a new host via the oral route, Trit. augusta must be able to endure unfavorable conditions outside its host, as well as the low pH of the stomach of a prospective host, without the protective advantage of a cyst-like stage (Kofoid and Swezy, 1915; Bishop, 1930 & 1934; Rosenberg, 1936; and Buttrey, 1954). Therefore, an attempt to determine if a physiologically more hardy stage exists in a population of basically delicate organisms seemed worth while.

Although at present the significance of the considerable range in cell sizes attainable by a given species of microorganism is poorly understood (Neidhardt, 1963), two features observed in this investigation seem worthy of comment. A population of predominantly small organisms containing minimal glycogen reserves was produced in an environment nearly devoid of known carbon and energy sources. These organisms were capable of surviving for a considerable

period of time in such an environment, however, they died rapidly upon suspension in an environment deficient in all organic nutrients. These observations suggest that this type of population is achieved automatically in the absence of glucose and may be capable of surviving during extended periods of hibernation of the host if carbohydrate were the limiting nutrient. On the other hand, the availability of excess glucose in the medium enabled this organism to accumulate high glycogen reserves and maintain large cell volume. Unlike the previous population these organisms died rapidly in culture following attainment of maximum populations, but were capable of surviving under non-nutrient conditions for a moderate period of time through the fermentation of glycogen reserves. These organisms would be particularly well adapted for surviving the adversities encountered during transmission to another amphibian via the oral route.

SUMMARY

1. Population, polysaccharide content, and cell volume were determined over the course of growth of Trit-richomonas augusta in a medium containing a limiting and a non-limiting concentration of glucose. Polysaccharide content, motility and survival time of organisms with radically different initial levels of stored polysaccharide were measured and compared during extended periods in aging cultures and under non-nutrient conditions.

2. In the presence of an excess concentration of glucose the level of total polysaccharide in culture continued to increase throughout all phases of growth. However, two peaks were observed in the polysaccharide concentration per organism; one during the lag and early logarithmic phases of growth, and a second during the decelerating and maximum stationary phases of growth. During the logarithmic growth phase, glycogen reserves decreased to a level intermediate between that in the organisms of the inoculum and those containing maximal reserves. Following an initial increase, Trit. augusta maintained large cell volumes through all phases of growth.

3. Organisms grown in medium with a limiting concentration of glucose continued to increase in numbers, but decreased in cell volume during the decelerating and maximum stationary phases of growth. They appeared to utilize their reserve polysaccharide as a source of energy

for survival and limited reproduction, as observed from a decrease in the total polysaccharide in culture during the above growth phases.

4. The maximal level of polysaccharide in Trit. augusta accounted for only 7.3% of the total cell weight of large organisms. Thus the volume of accumulated polysaccharide was not considered to be the cause of increased cell volumes in this organism.

5. When survival and the level of glycogen reserves were compared during phases of population decline, organisms possessing high polysaccharide reserves and large cell volumes from growth in excess glucose died off rapidly. This was believed to be the result of adverse environmental conditions. Organisms containing minimal polysaccharide reserves and small cell volumes as a result of growth in a medium nearly devoid of known energy sources remained viable in culture for a considerably extended period of time. This observation seemed to indicate that polysaccharide storage is not the only survival mechanism available to Trit. augusta.

6. When organisms possessing high levels of polysaccharide reserves were suspended in an inorganic buffered saline, they remained viable for moderate periods of time and polysaccharide reserves declined gradually. However, small organisms containing minimal polysaccharide reserves died off rapidly under these conditions.

7. The polysaccharide was accumulated by Trit. augusta

during periods when exogenous sources of energy were insufficient to meet the metabolic needs of the cell, and was broken down to yield energy in a form utilized by the cells, giving them a distinct "biological advantage" over cells without accumulated polysaccharide reserves. These observations indicated that the polysaccharide in Trit. augusta meets the requirements of a functional carbon and energy reserve.

8. The population of small organisms containing minimal glycogen reserves was achieved automatically in the near absence of glucose. These organisms may be expected to be capable of surviving similar environmental conditions during extended periods of hibernation of the host. The population of large organisms possessing high polysaccharide reserves was capable of surviving moderate periods of time in the absence of all organic nutrients. These organisms may be particularly well adapted to surviving the adversities encountered during oral transmission to another amphibian host.

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