A HISTOCHEMICAL AND BIOCHEMICAL INVESTIGATION OF THE POLYSACCHARIDES, ESTERASES, AND DEHYDROGENASES OF *TETRAHYMENA PYRIFORMIS* (STRAIN W)

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
Lawrence Dale Koehler
1960

This is to certify that the

thesis entitled
A Histochemical and Biochemical Investigation
of the Polysaccharides, Esterases, and
Dehydrogenases of Tetrahymena Pyriformis W.

presented by

Lawrence Dale Koehler

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Zoology

R. A. Fennell
Major professor
R. A. Fennell

Date August 16, 1960

O-169

C-801

A HISTOCHEMICAL AND BIOCHEMICAL INVESTIGATION OF THE POLYSACCHARIDES, ESTERASES, AND DEHYDROGENASES OF *TETRAHYMENA PYRIFORMIS* (STRAIN W)

By

Lawrence Dale Koehler

AN ABSTRACT

Submitted to the School of Advanced Graduate Studies of
Michigan State University of Agriculture and
Applied Science in partial fulfillment
of the requirements for the
Degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1960

Approved Richard A. Fennell

ABSTRACT

Specimens of Tetrahymena pyriformis W were grown in bacteria-free tryptone media at 25°C . Polysaccharides were localized and stained in specimens of Tetrahymena with Best's carmine, the Feulgen-Bauer, and the periodic acid-Schiff reactions. Polysaccharides were localized throughout the cytoplasm as fine granules with all procedures. Formalin (10%) was superior to alcoholic fixatives for localization of polysaccharides.

Polysaccharides of T. pyriformis W consist primarily of glycogen. They were removed upon exposure to 1% diastase for 30 minutes at room temperature. No metachromasia was observed with toluidine blue. Methylene blue was not bound by the acid groups of the polysaccharides at pH 4.0 and below.

The concentration of polysaccharides was ascertained by the colorimetric procedure of Hooghwinkel and Smits (1957). Concentration of polysaccharides was low in 3 day cultures (0.31 mgm / 0.1 ml of organisms), increased to a maximum of 1.37 mgm per 0.1 ml of organisms by the 12th day, and declined to a minimum of 0.24 mgm per 0.1 ml of organisms on the 24th day.

Potassium was localized, by the procedure of Poppen et al. (1953), as a brownish precipitate uniformly distributed throughout the cytoplasm. The potassium of Tetrahymena was not localized in the same regions of the cytoplasm as glycogen.

The esterases were studied using the starch gel electrophoresis technique of Smithies (1955). Subsequent to electrophoresis the gel

strips were incubated in buffered solutions of various naphthyl esters. The free naphthol liberated was coupled with a diazonium salt to demonstrate 5 bands of esterase activity. With this procedure esterase activity, as demonstrated by intensity of bands, exhibited considerable differences at various culture ages. Band C exhibited high activity until the 12th day then it decreased to a minimum on the 16th day. On the other hand, band D increased in intensity between the 15th and 18th days. The intensity of enzyme activity in band E increased from a low in 3 to 6 day cultures to reach a maximum in 9 day cultures. It remained fairly constant until culture age reached 18 days and then decreased to zero in 24 day cultures. Bands A and B remained constant throughout the life of the culture.

Subsequent to electrophoretic separation of esterases, inhibitor specificity was noted for various bands of activity in the zymograms. Sodium taurocholate slightly stimulated enzyme activity in band D and moderately inhibited bands C and E. Phemerol chloride (2.4×10^{-3} M) heavily inhibited all bands of esterase activity in starch gel strips. Sodium arsanilate (3.2×10^{-1} M) completely inhibited esterase activity in bands A to E, **inclusive.** Sodium fluoride (7.1×10^{-1} M) inhibited esterase activity in bands A, B, C, and D, but stimulated esterase activity in band E. L-cysteine HCl (1×10^{-2} M), and dipterex (5.4×10^{-3} M) completely inhibited all bands of esterolytic activity in zymograms. Diazinon (1.3×10^{-5} M) slightly inhibited band B, and completely inhibited all other bands. Potassium iodide, eserine sulfate, RO 2-0683, RO 2-1250, and benzaldehyde were without effect on any of the

bands of esterase activity in concentrations used.

Certain of the dehydrogenases and DPN-linked diaphorases were identified in the cytoplasm of Tetrahymena. Succinate dehydrogenase, isocitrate dehydrogenase, and malate dehydrogenase, which are usually considered to be good indicators of Krebs' cycle activity, were present. Glycolytic pathways are also available to Tetrahymena since strong alcohol dehydrogenase, glycerophosphate dehydrogenase, and lactate dehydrogenase reactions were identified in the cytoplasm. β-hydroxybutyrate dehydrogenase activity, which was also localized in the cytoplasm, indicates that the fatty acids can be oxidized by this organism. Glutamate dehydrogenase activity, which may be concerned with either the synthesis of amino acids, the breakdown of glutamate, or the supply of succinate to the Krebs' cycle, was also identified.

Succinate dehydrogenase activity was ascertained, on a quantitative basis, by the procedure of Kun and Abood (1949). It was at a maximum in organisms from 3 day cultures, decreased to a minimum in 9 to 12 day cultures, then remained constant through the 24th day.

A HISTOCHEMICAL AND BIOCHEMICAL INVESTIGATION OF THE POLYSACCHARIDES, ESTERASES, AND DEHYDROGENASES OF *TETRAHYMENA PYRIFORMIS* (STRAIN W)

By

Lawrence Dale Koehler

A THESIS

Submitted to the School of Advanced Graduate Studies of
Michigan State University of Agriculture and
Applied Science in partial fulfillment
of the requirements for the
Degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1960

ACKNOWLEDGEMENTS

The author wishes to express his sincere thanks to Dr. R. A. Fennell for his suggestion of the research problem, and also for his guidance, interest, and ecnouragement during the course of this investigation. He also wishes to thank Mrs. Bernadette Henderson for her innumerable services.

The author is indebted to the National Institutes of Health for the Predoctoral Research Fellowship (CF-9369) which made this investigation possible.

CURRICULUM VITAE

Lawrence Dale Koehler

candidate for the degree of

Doctor of Philosophy

Final Examination: August 15, 1960

Dissertation: A Histochemical and Biochemical Investigation of the Polysaccharides, Esterases, and Dehydrogenases of *Tetrahymena pyriformis* (Strain W)

Outline of Studies:

Major subject:	Zoology
Minor subject:	Physiology

Biographical Items:

Born: February 19, 1932, Grand Rapids, Michigan.

Undergraduate Studies: Grand Rapids Junior College 1950-1952, Otterbein College 1952-1954.

Graduate Studies: Michigan State University 1956-1960.

Experience: Officer in the United States Marine Crops 1954-1956.

Professional Memberships: Society of Sigma Xi, Society of Protozoologists, and Michigan Academy of Science, Arts, and Letters.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

INTRODUCTION

The localization and demonstration of polysaccharides have been widely studied in vertebrate tissues. Dempsey et al. (1947), demonstrated the capacity of polysaccharides of tissues to bind methylene blue at various pH levels. This procedure was used for identification of the polysaccharides, e.g., acid polysaccharides were bound at hydrogen ion concentrations of pH 4.0 and below, while neutral polysaccharides were bound at higher pH ranges. Wislocki et al. (1947), observed metachromatic staining in various tissues. This reaction was attributed to glycogen, mucopolysaccharides, hyaluronidase, and nucleoproteins.

The periodic acid-Schiff (PAS) reaction, which has been widely used in histochemical studies, was first employed by McManus (1946), for demonstration of mucin. Both Lillie (1947), and Hotchkiss (1948), modified the procedure for the demonstration of a variety of polysaccharides in tissue sections. Hooghwinkel and Smits (1957), developed a colorimetric procedure for identification of saccharides using the (PAS) reaction. These workers found that the hexosamine groups of chondroitin-sulfuric acid and hyaluronic acid were still intact after oxidation with periodic acid. They also noted that although the hexuronic acids were oxidized, aldehydes were not demonstrated.

A review of the literature shows that a limited number of investigations have been undertaken for the identification and classification of polysaccharides in lower forms of animal life. Dawson and Happold (1943), reported that D-phenylalanine increased polysaccharide storage in specimens of Escherichia coli when cultured in a media made with glucose, but that it was without effect in a media made with mannose.

Gale (1947), maintained that formation of polysaccharides occurred in specimens of *E. coli* cultured in media with an optimum concentration of glucose. Following disappearance of glucose from the culture medium stored polysaccharides were metabolized. Dagley and Dawes (1949), showed that polysaccharides of *E. coli* increased in the logarithmic phase and then decreased in the stationary phase of growth. These workers maintained that cell division and synthesis of polysaccharides were not closely correlated. For a comprehensive review of the literature on classification, identification, and histochemistry of polysaccharides the reader is referred to Pearse (1960).

When 0.1 ml of organisms (*Tetrahymena geleii* W, now *T. pyriformis* W) was left in 1 ml of sterile tryptone solution the percent of organisms exhibiting glycogen decreased from 96 percent in 24 hours to 27 percent in 72 hours (Fennell, 1951). The activity factor for glycogen of *T. geleii* W (from vitamin-enriched tryptone solutions) reached a maximum in 216 hours, remained fairly constant until age of cultures reached 360 hours, and then rapidly decreased to almost zero in 504-hour cultures (Fennell and Marzke, 1954).

Fenn (1939), suggested that there was a correlation between the localization of potassium and glycogen in liver of rats. Poppen *et al.* (1953), noted a direct relationship between potassium and glycogen localization in sections of cardiac muscle from lambs.

The esterases that hydrolyze carboxylic acid esters have been studied intensively in recent years. Only a few detailed studies of this enzyme group have been reported for subhuman forms of animal life. Seaman and Houlihan (1951), carried out a general survey of the enzyme systems

in Tetrahymena geleii S and demonstrated an acetylcholinesterase which was correlated with ciliary activity. Fennell and Marzke (1954), employing the Tween technique, demonstrated esterase activity in T. geleii W, which was localized in the cytoplasm immediately adjacent to the nucleus. The Tween technique was carried out by incubating organisms in water-soluble esters of long-chain saturated fatty acids attached to polyglycols or polymannitols, in the presence of calcium ions. On hydrolysis, the liberated fatty acids form insoluble calcium soaps which are deposited at the site of enzyme activity. The calcium soaps are then converted to lead soaps and then to brown sulfied of lead with ammonium sulfide. Fennell and Pastor (1958), presented evidence for the existence of ali-esterases in T. pyriformis W. It was shown that esterases of Tetrahymena have a predilection for short-chain naphthyl esters. Pastor and Fennell (1959), studied the substrate specificity of esterases of Tetrahymena and found that they hydrolyzed saturated Tweens (polyoxyethylene sorbitan monolaurate, polyoxyethylene sorbitan monopalmitate, and polyoxyethylene sorbitan monosterate), alpha naphthyl acetate, and naphthyl AS acetate, but did not hydrolyze unsaturated Tweens (polyoxyethylene sorbitan monooleate). Esterases of Tetrahymena were also shown to be resistant to fixation in cold 10 percent formalin at pH 7.0, but not resistant to cold acetone. These workers also studied the effects of various inhibitors on activity of esterases.

With the introduction of zone electrophoresis in starch gel by Smithies (1955), it became possible to obtain a finer resolution of proteins in biological materials. Hunter and Markert (1957), using a modification of this technique, were able to demonstrate a number of

esterases in mouse liver.

During recent years many workers have studied the respiratory enzymes in microorganisms. Boell (1942, 1946), studied the effects of inhibitors on the oxygen consumption of *Paramecium calkinsi*. He noted that after treatment with sodium cyanide (0.01 M) or sodium azide (0.001 M) the oxygen consumption was reversibly depressed to 50 percent of the normal level. Humphrey and Humphrey (1947), demonstrated succinic dehydrogenase in homogenates of *Paramecium caudatum* with characteristics in common with those of the metazoan enzyme.

It was demonstrated by Pappenheimer and Hendee (1949), that extracts of diphtheria bacillus which actively oxidize succinate contain only traces of cytochrome c and cytochrome oxidase. Kun and Abood (1949a), described an enzyme preparation from the endotoxin of *Salmonella aertrycke* which oxidized succinate aerobically but did not contain a measurable amount of cytochrome oxidase. In a study of homogenates of *Tetrahymena pyriformis* GL, Ryley (1952), demonstrated that methylene blue was reduced anaerobically in the presence of succinate, and that there was no reduction of methylene blue under aerobic conditions. In contrast to the latter, Seaman (1950, 1952), has shown that homogenates of *T. pyriformis* S oxidize succinate aerobically and reduce cytochrome c in the presence of succinate and azide. Eichel (1956), assayed homogenates of *T. pyriformis* S for components of the DPNH (reduced diphosphopyridine nucleotide) oxidase system and concluded that they differ from those of the mammalian system. He maintained that cytochrome c oxidase does not function as a physiological electron carrier in *Tetrahymena*, and that cytochrome c is not the terminal oxidase.

Much of the recent work on the dehygrogenases includes studies of localization using the tetrazolium salts. The reduction of the latter to highly colored formazan dyes was first observed in plants by Kuhn and Jerchel (1941). Mattson _et al_. (1947), showed that the reduction of triphenyl tetrazolium chloride was not due to reducing sugars since reduction did not occur at pH 11.0 or below. When tissues were heated to 80°C for short periods they would no longer reduce triphenyl tetrazolium chloride to formazan. These workers concluded that some enzyme system in the tissues must be responsible for the reduction and they suggested that it was due to dehydrogenase systems which require coenzymes I and II. Kun and Abood (1949b), suggested reduction of triphenyl tetrazolium chloride was indicative of succinic dehydrogenase which does not require a coenzyme. A number of authors used triphenyl tetrazolium chloride in the study of animal tissues. Some of the objections to its use were the difficulty in penetration of cell membranes, false negative reactions, large crystal size of formazans, inadequate coloration, and the diffusibility of the formazan within tissues (Pearse, 1960). Others of the tetrazolium salts overcome these objections, e.g., 2,2'-(p-diphenylene)-bis-(3,5-diphenyl) tetrazolium chloride (Neotetrazolium chloride), 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride (Nitro-BT), and 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT). For a review of the literature on dehydrogenases and diaphorases the reader is referred to Scarpelli _et al_. (1958).

This investigation has as its objectives: (1) to localize and identify polysaccharides, and to ascertain the relationship between culture

age and polysaccharide content of organisms, (2) to ascertain whether or not a relationship exists between potassium and glycogen deposition in the cell, (3) to ascertain whether multiple esterase(s) exist in organisms, (4) to localize DPN-diaphorase and certain of the dehydrogenases, and (5) to clarify the role of the polysaccharides and enzymes in the aging processes of *Tetrahymena pyriformis* W.

MATERIALS AND METHODS

Specimens of Tetrahymena pyriformis W used for experimentation were cultured at 25° in a bacteria-free medium (10 gm Bacto-tryptone, 1 gm glucose, 1 gm potassium monobasic phosphate, 1 gm potassium dibasic phosphate, 1 gm sodium acetate, 0.1 gm yeast extract, and 0.0025 mgm thiamine hydrochloride in 1000 ml of glass-distilled water).

Ehrlenmeyer flasks (125 ml) containing 75 ml of culture medium were sterilized at 15 pounds pressure for 20 minutes. Bacteria-free cultures were established by inoculating each flask with 1 ml of stock solution in which cells were abundant. In most experiments tests were made with organisms 3 days subsequent to inoculation and at 3 day intervals thereafter throughout the life of the culture. In all experiments reported in succeeding pages age refers to days subsequent to inoculation of the culture.

Polysaccharides localization. Polysaccharides were localized in T. pyriformis W by means of three procedures: Best's carmine procedure (Bensley and Bensley, 1938), the Feulgen-Bauer method (Bensley and Bensley, 1938), and the periodic acid-Schiff (PAS) technique (Hotchkiss, 1948). All organisms used in localization studies were obtained by centrifuging 15 ml of culture solution for 3 to 4 minutes to concentrate organisms in the bottom of the centrifuge tube. Organisms were then affixed to albumin-coated glass slides and allowed to dry in air. Subsequent to drying, tests for localization of polysaccharides were performed. The effects of washing organisms in saline, and of resuspending them in the original culture medium for 30 minutes prior to affixing them to slides were noted. Some

organisms were also washed in warm 5 percent trichloroacetic acid for 30 minutes prior to fixation.

To determine the effects of various fixatives on polysaccharide localization, the organisms were fixed in absolute alcohol, Zenker's, Bouin's, alcoholic formalin, alcohol saturated with picric acid, and aqueous 10 percent formalin. Organisms were left in fixatives for 10 minutes to 24 hours prior to localization of the polysaccharides.

Colorimetric procedure for polysaccharides. Concentrations of polysaccharides present in the organisms from various ages of cultures were determined by the colorimetric procedure of Hooghwinkel and Smits (1957). In these experiments 0.1 ml of packed organisms was used in each test. Organisms were concentrated by centrifugation at 2400 ± 100 (386 x g.) rpm for 4 minutes. The organisms were then diluted to 1.0 ml with distilled water and homogenized by freeze-thawing 4 times in a solid CO_2-acetone mixture. The contents of each tube was made up to 5 ml with distilled water, and then 5 ml of 1½ percent periodic acid was added to each of the tubes. One hour was allowed for the periodic acid to react with the polysaccharides to form dialdehydes. The excess periodic acid was then neutralized by adding 0.1 ml of ethylene glycol. Subsequent to addition of the latter the mixture was dialyzed against running tap water for 24 hours. The contents of the dialysis tubes was transferred to 50 ml test tubes and each was made up to a volume of 25 ml . Three ml of potassium metabisulfite (2.66 gm of potassium metabisulfite and 53.3 ml of 1 N HCl made up to 200 ml with distilled water) was added to each tube, followed by the addition of 2 ml of Schiff's reagent (McManus, 1948). One hour subsequent to the addition of Schiff's reagent

the color intensity was read as percent absorption of light in a Bausch and Lomb Spectronic 20 colorimeter at a wavelength of 560 mμ.

Identification of polysaccharides. To ascertain the amount of color obtained due to glycogen with the PAS reaction, some of the organisms were treated with 1 percent malt diastase (Nutritional Biochemicals Corporation, Cleveland, Ohio) for 30 minutes prior to fixation. The PAS test was then run to demonstrate the polysaccharides remaining in the cell.

Toluidine blue staining was used to demonstrate metachromatic polysaccharides. Organisms were fixed in 10 percent formalin for 30 minutes followed by staining in 0.5 percent aqueous toluidine blue for 6 hours. Slides were then rinsed in distilled water and examined immediately for metachromasia.

Capacity to bind methylene blue was ascertained at various pH levels (e.g., pH of 3.0 to a pH of 9.0). All organisms were fixed in 10 percent formalin after affixing to glass slides. Slides were immersed in a 0.0005 M methylene blue solution, for 24 hours, which was maintained at the desired pH with Michaelis buffer. Following rinsing in distilled water they were examined immediately for binding of methylene blue.

Paper chromatography was used to identify the sugars in Tetrahymena homogenate. In these experiments 0.5 ml of T. pyriformis was collected by centrifugation and diluted to 1.0 ml with distilled water. Organisms were then homogenized for 2 minutes in a Vir-Tis homogenizer (23,000 rpm). The proteins were removed by adding 1 ml of 5 percent trichloroacetic acid and the precipitate was removed by centrifugation (386 x g.). The sugars of the supernatant fluid were precipiated by adding 5 ml of 95 percent ethanol. The granular precipitate

produced in this manner was washed in absolute alcohol. The precipitate was dissolved in 2 ml of 2 N HCl and hydrolyzed in a boiling water bath for 60 minutes. The hydrolyzate was neutralized with barium carbonate and 0.008 ml was applied to a spot on a sheet of Whatman No. 1 filter paper. Descending two dimensional chromatography was run using butanol: acetic acid: water (120:35:50, v/v) in the first direction and phenol: water (4:1, v/v) in the second direction. Chromatograms were air dried, sprayed with analine hydrogen phthalate and heated in an oven at 105°C for 10 minutes to demonstrate the presence of reducing sugars. Known sugars were chromatogramed separately and simultaneously with the sugars obtained from the homogenate and the resulting spots were compared.

Potassium and glycogen localization. Potassium was localized in the organisms using the cobaltinitrite technique of Poppen et al. (1953). Organisms were immersed in cobaltinitrite for 20 minutes, washed in distilled water at 0°C, and then immersed in ammonium sulfide for 5 minutes. Fixatives used in these tests were 10 percent aqueous formalin, alcoholic formalin, picrosulfuric acid, 10 percent acetic alcohol, and 95 percent ethanol. Potassium localization was followed by Best's carmine stain and the PAS test.

Electrophoretic separation of esterases. The esterases were studied using a modification of the starch gel electrophoresis technique of Smithies (1955), as developed by Hunter and Markert (1957). Specimens of T. pyriformis W were concentrated in a centrifuge tube and washed in a 0.0210 M borate buffer at pH 8.9. Two-tenths ml of packed organisms was diluted with buffer to 1 ml and homogenized in a Vir-Tis homogenizer (23,000 rpm). Starch gel strips were prepared according to the following

procedure: 12.5 gm of Starch-Hydrolyzed (Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada) was suspended in 100 ml of borate buffer (0.0210 M boric acid and 0.0084 M sodium hydroxide). The mixture was agitated in a flask while being heated over an open flame. The starch suspension became gelated and with further heating (about 3 minutes) it became a clear viscous liquid. Air bubbles were removed by applying a vacuum until the contents of the flask boiled vigorously. The liquid was then poured into plastic trays with internal dimension of 22.5 cm x 2.3 cm x 0.8 cm. The trays were covered with plastic covers and allowed to cool overnight. The homogenized sample was introduced into a slot cut transversely in the starch gel at 8 cm from one end. Three procedures were employed for introduction of homogenized organisms into the slots for separation of esterases. Homogenates were either added as a suspension in crude potato starch, included in pieces of Whatman No. 1 filter paper (with dimensions the same as the cross-sectional dimensions of the gel strip), or added as pure homogenates. The slots in gel strips used for inserting the sample of homogenized organisms were made using 2 razor blades welded together at a distance of about 2 mm from one another. Thus the quantity of sample used was 0.1 ml in all experiments. After addition of homogenates to the slot it was covered with a glass slide and then both slide and exposed surface of the gel were covered with mineral oil. Filter paper electrodes (2.4 cm x 15 cm) were placed on the ends of the gel strips and the strips were wrapped in Saran Wrap. The electrodes were then put into a 0.230 M borate buffer at pH 8.9.

Current was supplied with a variable voltage regulated power supply unit (Heath Company, Benton Harbor, Michigan). During electrophoresis a

current of 4 to 6 milliamperes, with a voltage drop of 6 volts per centimeter, was imposed along each gel strip. The voltage was measured with a high resistance vacuum tube voltmeter with electrodes about 23 cm apart, thus the total voltage across each strip was 138 volts. Current was applied for a duration of 6 hours. For details concerning trays, sample insertions, and power supply unit consult figs. 1 to 5, inclusive.

After application of the current to the starch gel strips for 6 hours they were removed from the trays, sectioned longitudinally (Figs. 6 and 7), and then transferred to an incubating medium consisting of 12 mgm of substrate in 3 ml of acetone, 60 ml of 0.2 M phosphate buffer at pH 6.5, and 40 mgm of a diazonium salt. Incubation time, in most cases, was for 3 hours after the addition of the substrate and coupler. Substrates used in this study were alpha naphthyl acetate; beta naphthyl acetate; 2-acetyl 3-napthoic acid anilide (Naphthyl AS acetate); 2-acetyl 3-(1,3 dimethyl analine) naphthoic acid (Naphthyl AS-MX acetate); and indoxyl acetate. Couplers used in this study were tetrazotized di-o-anisidine (Diazo blue B); 4 amino-3,1' dimethyl azo benzene (Fast garnet GBC); diazotized 2-amino-4-chloroanisole (Diazo red RC); and diazotized 5-benzamido-4-chloro-2-toluidine (Fast violet LB).

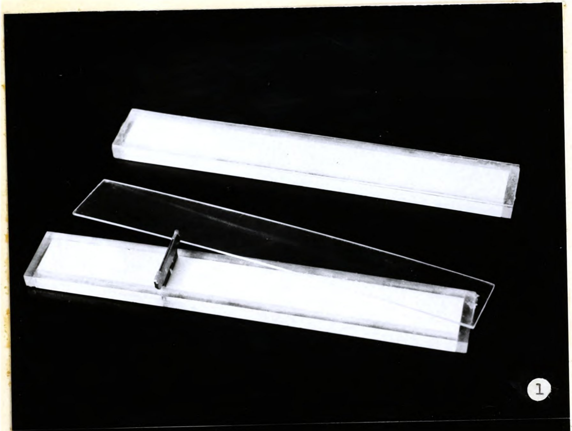
When inhibitors were employed one half of the gel strip was allowed to incubate in phosphate buffer containing the inhibitor for 30 minutes prior to addition of the substrate and coupler, while the remaining half (control) was incubated in phosphate buffer without the inhibitor for 30 minutes. Inhibitors used in this study were sodium taurocholate; phemerol chloride; potassium iodide; eserine sulfate; dimethylcarbamate of (2-hydroxy-5 phenyl benzyl) trimethylammonium bromide

FIGURE 1

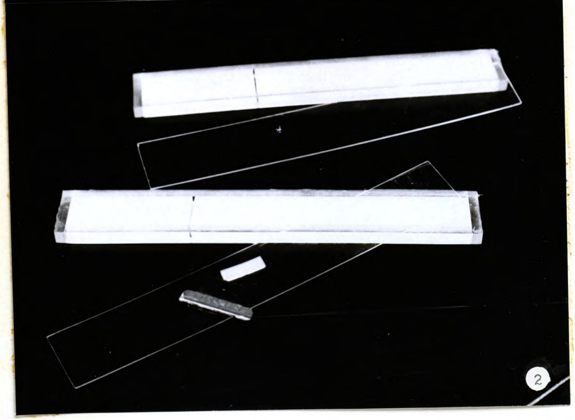
Trays containing hydrolyzed starch for electrophoresis. The lower tray has the plastic cover removed and the cutter (two razor blades welded together) inserted to remove a piece of gel, thus forming a slot for introduction of the sample.

FIGURE 2

Starch gel strips with plastic covers removed and slots cut into gels for introduction of the sample. The piece of gel removed from one of the slots is shown in the lower section of the photograph. The slot formed by this procedure has a volume of 0.1 ml.



1



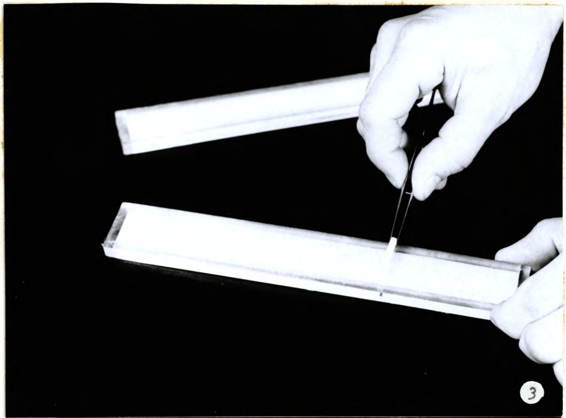
2

FIGURE 3

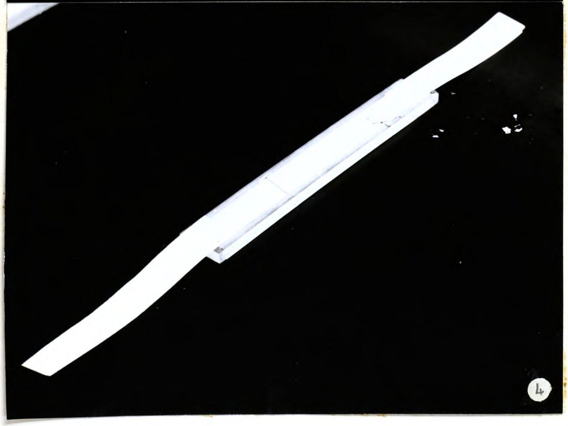
Starch gel strip showing sample being inserted into slot. The sample is being inserted on a piece of heavy filter paper cut with dimensions similar to those of the slot.

FIGURE 4

Starch gel strip, containing homogenized Tetrahymena, electrodes applied to ends of the gel strip.



③



④

FIGURE 5

Apparatus for starch gel electrophoresis.

A. Variable voltage regulated power supply unit. (Heath Company, Benton Harbor, Michigan).

B. Vacuum tube voltmeter (Heath Company).

C. Starch gel strips, containing sample, with filter paper electrodes immersed in bridge buffer solutions. Gel strips were wrapped in Saran Wrap.

D. Cathode bridge buffer.

E. Anode bridge buffer.

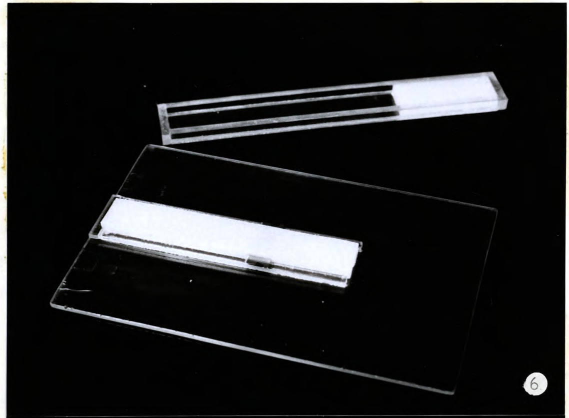

5
A
B
C
D
E
Heathkit VARIABLE VOLTAGE REGULATED POWER SUPPLY

FIGURE 6

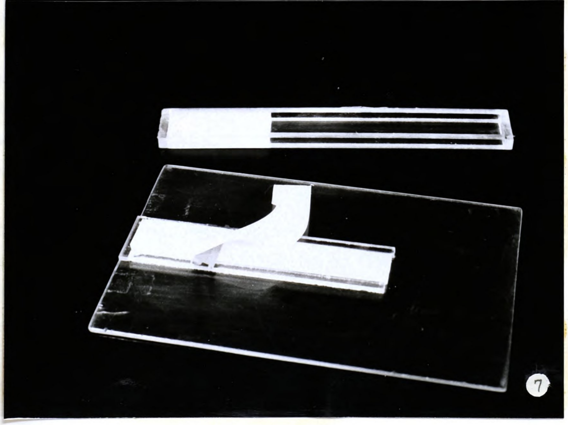
Sectioning of gel strips. Subsequent to application of current gel strips were removed from plastic trays and sectioned in a longitudinal plane.

FIGURE 7

Sectioning of gel strip showing the upper and lower sections. When inhibitor studies were conducted the lower section of the gel strip was placed in the inhibitor solution, while the upper section of the strip was used as control.



⑥



⑦

(RO 2-0683); p-chlorophenylmethyl carbamate of m-dimethylaminophenol methyl bromide (RO 2-1250); quinine hydrochloride; sodium arsanilate; sodium fluoride; L-cysteine hydrochloride; 0,0 dimethyl-1-hydroxy-2-trichloro methyl-phosphonate (Dipterex); 0,0 diethyl S-2 ethyl phosphorothioate (Systox); and 0,0 diethyl 0,2 isopropyl-4-methyl pyrimidyl thionophosphate (Diazinon). The intensity of bands of esterase activity in each was estimated by visual observation to determine whether there was a specific inhibitor for a particular band. Gel strips incubated with inhibitors were compared with control strips and numerical values were assigned to denote inhibitor effect as follows: +, acceleration; 0, no inhibition; 1, slight inhibition; 2, heavy inhibition; 3, complete inhibition.

Dehydrogenases and diaphorases. Succinate dehydrogenase was localized in specimens of T. pyriformis W by the method of Nachlas et al. (1957). Organisms were air dried on cover glasses, incubated in a mixture of sodium succinate (0.2 M), Tris buffer (0.2 M at pH 7.6), and Nitro-BT (1 mg/ml) for 10 minutes. Organisms were then fixed in formol-saline for 10 minutes and mounted in glycerine jelly. Organisms were then examined microscopically for the presence of blue diformazan deposits which are indicative of succinate dehydrogenase activity.

The method of Scarpelli et al. (1958), was used for localization of DPN diaphorase. Organisms were incubated in a mixture of MTT (1 mg/ml), cobaltous chloride (0.05 M), DPNH (0.01 M) which was adjusted to pH 7.0 with 0.2 M Tris buffer. Subsequent to incubation in the substrate solution organisms were fixed in formol-saline for 10 minutes and mounted in glycerine jelly. Black cobalt formazan deposits are indicative of diaphorase activity.

The method of Hess *et al.* (1958), was used for demonstration of DPN and TPN-linked dehydrogenases. Organisms were air dired on cover glasses and incubated in a medium consisting of the substrate, either DPN or TPN, sodium azide as respiratory inhibitor, and a tetrazolium salt. The hydrogen ion concentration of the incubation medium was adjusted to pH 7.0 with Tris buffer. β-hydroxybutyric dehydrogenase was localized using MTT as the tetrazolium salt and β-hydroxybutyric acid as the substrate. Others of the dehydrogenases were localized using Nitro-BT and the tetrazolium salt, DPN, sodium azide, Tris buffer, and the appropriate substrates, e.g., isocitrate dehydrogenase with DL-isocitric acid as substrate, malate dehydrogenase with sodium-L-malate as substrate, glutamate dehydrogenase with sodium-L-glutamate monohydrate as substrate, α-glycerophosphate dehydrogenase with DL-α-glycerophosphate as substrate, alcohol dehydrogenase with ethanol as substrate, and lactate dehydrogenase with DL-lactate as substrate.

Quantitative determination of succinate dehydrogenase. A quantitative estimate of succinate dehydrogenase activity was ascertained by a modification of the method of Kun and Abood (1949b), as modified by Jardetzky and Glick (1956). Organisms used in these experiments were obtained by centrifuging at 2400±100 rpm (386 x g.). One-tenth ml of organisms, diluted to 1 ml with 0.2 M phosphate buffer at pH 7.6, was used for each test. Two ml of 0.2 M sodium succinate was added to each tube, followed by the addition of 2 ml of tetrazolium salt solution (1 mg/ml). Both neotetrazolium chloride and triphenyl tetrazolium chloride were used as hydrogen acceptors. Tubes were incubated in the dark at room temperature for 2 hours subsequent to addition of the substrate.

One ml of 40 percent trichloracetic acid was then added to each tube to precipitate proteins. Following precipitation of proteins 8 ml of ethyl acetate was added to each tube and color intensity was read as percent absorption in a Bausch and Lomb spectronic 20 colorimeter. When triphenyl tetrazolium chloride was used as hydrogen acceptor color was read at a wavelength of 480mμ, and a wavelength of 520mμ was used with neotetrazolium chloride.

RESULTS

Localization of polysaccharides. Specimens of *T. pyriformis* W used for experimentation were obtained from cultures at various ages subsequent to inoculation. In most experiments culture age varied between 3 and 24 days. Polysaccharides were localized as discrete granules throughout the cytoplasm of the organism (Figs. 16-19). The granules were small when the PAS technique or the Feulgen-Bauer method was used, whereas Best's carmine stain produced fewer and larger particles. The PAS technique produced a reaction of greater intensity than the other methods.

Localization studies revelaed that the intensity of the reaction was very low in organisms from 3 day cultures, but the number and size of particles increased as age of the culture increased. The intensity of the reaction reached a maximum in cultures between 9 and 12 days subsequent to inoculation. A decline in the intensity of the reaction followed and polysaccharide content was nearly zero in organisms from cultures older than 21 days.

Observations revealed that alcoholic fixatives caused polarization of the granules while the mercuric chloride containing fixatives decreased the amount of polysaccharide demonstrable. On the other hand, uniform saccharide localization was observed when organisms were fixed in cold neutral aqueous 10 percent formalin. When specimens of *Tetrahymena* were fixed in the latter for varying lengths of time (15 minutes to 24 hours) the color intensity obtained with the PAS reaction was not affected.

Cells washed in normal saline for 30 minutes prior to fixation

showed a great reduction in polysaccharide content, whereas cells could be resuspended in the original culture medium without demonstrating any loss of the positive reaction.

Quantitative determination of polysaccharides. The quantity of polysaccharides present in *Tetrahymena* homogenates, from cultures of various ages, was ascertained by a modification of the method of Hooghwinkel and Smits (1957). A sensitivity curve was established using a known amount of glycogen (Nutritional Biochemicals Corporation, Cleveland, Ohio), plus reagents used in PAS reaction, and reading the percentage of light absorbed at various wavelengths (400 to 600mμ) by the resulting colored product (Fig. 8). A wavelength of 560mμ demonstrated greatest sensitivity.

A calibration curve was established using fixed increments (0.5 mgm to 3.0 mgm) of glycogen with reagents used for the PAS reaction. The color intensity was then determined by reading precent absorption of light at 560mμ (Fig. 9). The calibration curve was essentially linear in the range of concentrations used.

Quantitative tests were performed using fixed increments (0.1 ml) of *Tetrahymena* homogenates. It is evident that polysaccharide content was low in 3 day cultures, increased until the 9th day and declined from the 12th to the 21st day (Table 1, and Fig. 10).

Identification of polysaccharides. After exposure of organisms to a 1 percent malt diastase solution for 30 minutes at room temperature, the PAS test was negative for polysaccharides (no visible color could be observed). When organisms were incubated in 5 percent trichloroacetic acid prior to fixation, a weak PAS reaction was obtained.

FIGURE 8

Percentage absorption of light at various wavelengths (400 to 600 mμ) by the reaction product formed when the PAS reagents were combined with glycogen. Ordinates, percent absoprtion of light; abscissae, wavelength in millimicrons.

○, glycogen sample, 2.25 mgm.

△, glycogen sample, 1.15 mgm.

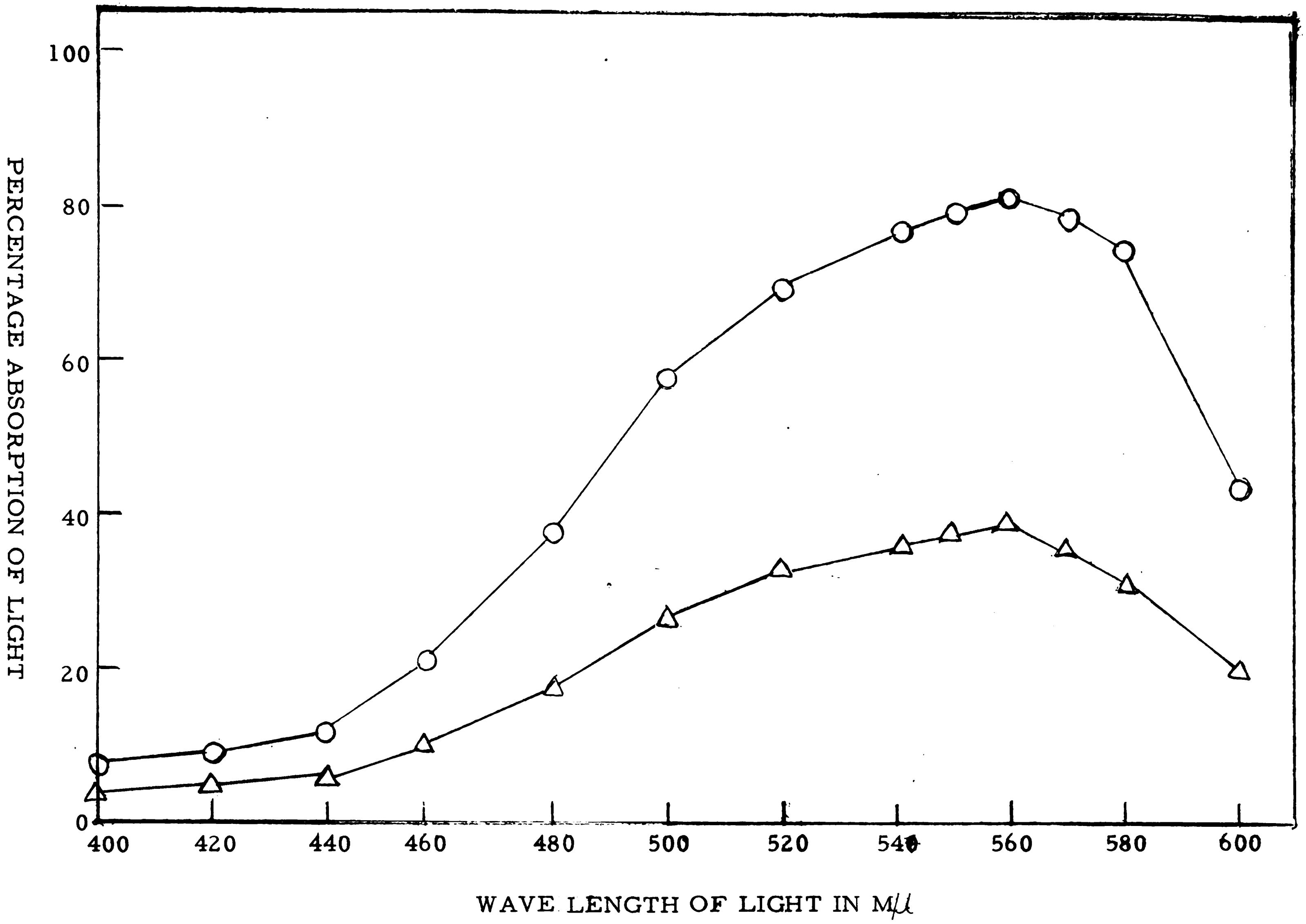
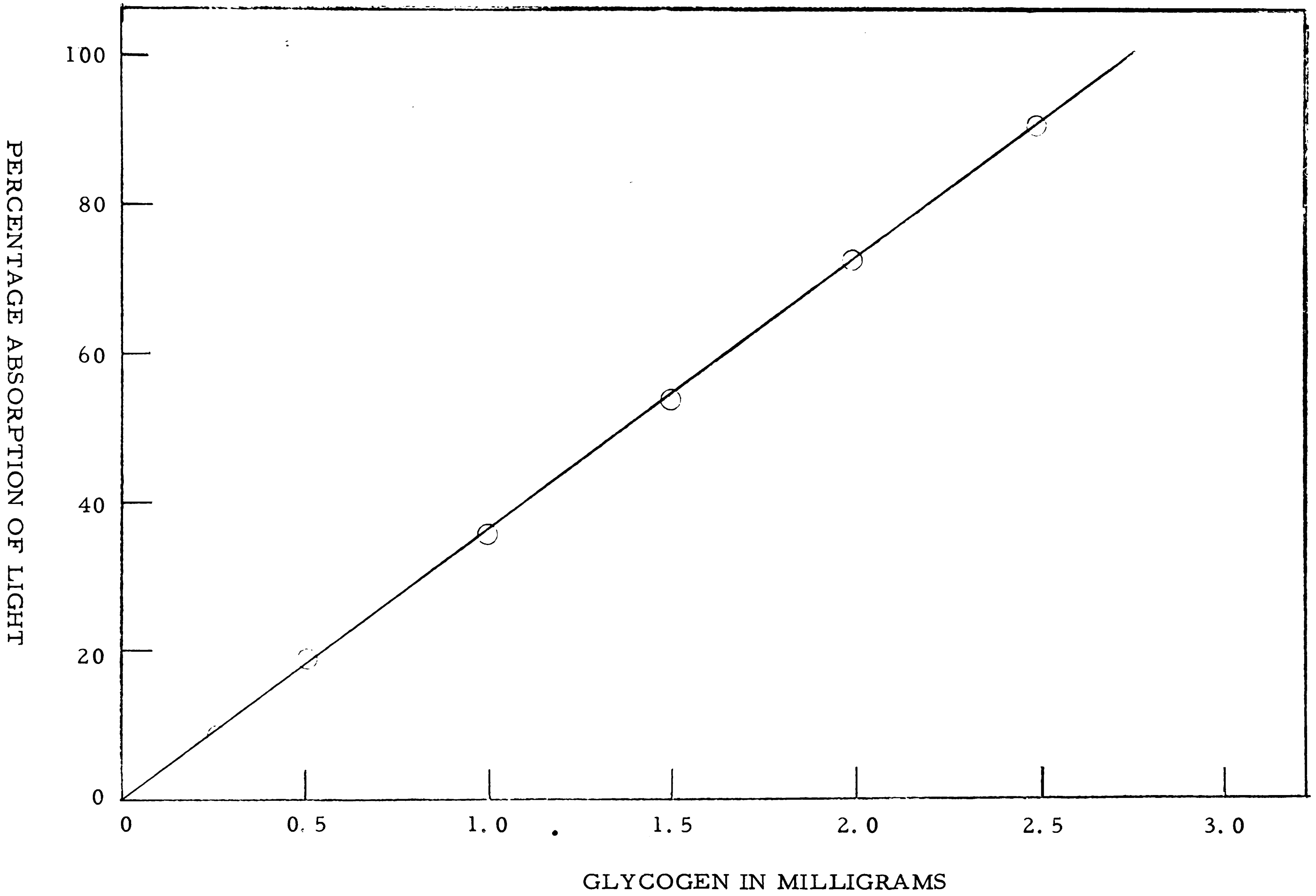

PERCENTAGE ABSORPTION OF LIGHT
100
80
60
40
20
0
400
420
440
460
480
500
520
540
560
580
600
WAVE LENGTH OF LIGHT IN Mμ

FIGURE 9

Calibration curve for glycogen. Relationship between percentage absorption of light and concentration of glycogen. The PAS reagents were used along with known increments of glycogen (0.5 to 2.5 mgm). Wavelength 560mμ.



100
80
60
40
20
0
PERCENTAGE ABSORPTION OF LIGHT
0
0.5
1.0
1.5
2.0
2.5
3.0
GLYCOGEN IN MILLIGRAMS

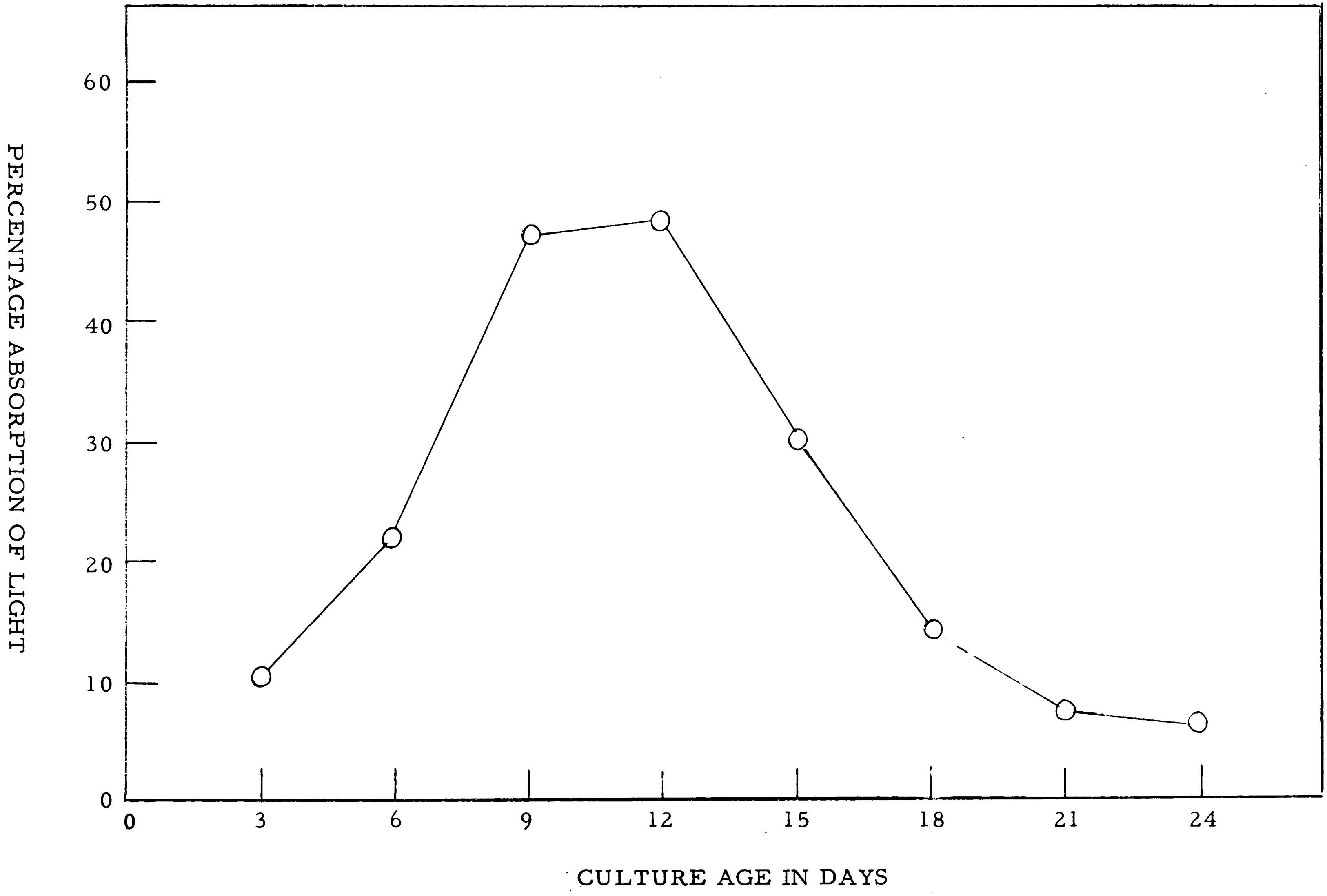
TABLE 1

Percentage absorption of light by the red-dye-product resulting from the PAS reaction using 0.1 ml homogenized organisms. All tests run in duplicate. Wavelength 560 mµ.

Age of Culture in Days	Series I	Series II	Series III	Series IV	Arithmetic mean
3	10.9	11.3	12.9	11.7	11.7
6	23.4	23.5	23.3	22.6	23.2
9	45.8	51.2	46.8	46.2	47.5
12	49.8	46.4	49.6	47.8	48.4
15	30.1	30.0	31.2	30.6	30.455
18	14.8	14.8	15.3	14.6	14.875
21	7.5	8.0	7.0	7.9	7.6
24	7.0	7.9	7.4	7.6	7.475

FIGURE 10

Relation between glycogen content and age of T. pyriformis W. The PAS reaction was conducted 3 days after inoculation of cultures and at 3 day intervals thereafter. One-tenth ml of homogenate of organisms was used for each test. Each point represents the average of 4 series of tests, and each test was duplicated. Ordinates, percentage absorption of light; abscissae, age of culture in days.


60
50
40
30
20
10
0
0
3
6
9
12
15
18
21
24
PERCENTAGE ABSORPTION OF LIGHT
CULTURE AGE IN DAYS

There was no metachromatic staining observed in specimens of Tetrahymena that were incubated in toluidine blue for 24 hours. Capacity to bind methylene blue was demonstrated over a wide range of pH values. Binding of the dye was very intense at pH levels of 9, 8, and 7; lower at pH 6 and 5 and absent at pH values of 4.0 and below.

Paper chromatography of homogenates of Tetrahymena revealed one sugar spot. This spot demonstrated a migration distance similar to that of a known glucose sample. When glucose and the sugar from the organisms were applied to the same sheet of chromatography paper only one sugar spot was identified.

Relation between potassium and glycogen localization. Potassium, when localized by the method of Poppen et al. (loc. cit.), appeared as dark brown granules throughout the cytoplams (Figs. 20 and 21). Potassium could not be demonstrated in organisms that were fixed in 10 percent aqueous formalin, or picrosulfuric acid. Following fixation in alcohol (15 to 30 minutes) potassium was localized as dark brown granules.

When the PAS or Best's carmine test was used for demonstration of saccharides, subsequent to potassium localization, reactions for glycogen were weak, or completely negative in some cases. Under these staining conditions the granules of potassium and glycogen were randomly distributed throughout the cytoplasm.

Electophoretic demonstration of esterases. Subsequent to separation of esterases, using electrophoretic procedures, 5 distinct transverse esterase bands were identified in the gel strips following incubation in either alpha naphthyl acetate, beta naphthyl acetate, naphthyl AS acetate, naphthyl AS-MX acetate, brom-2-naphthyl acetate, or indoxyl acetate as substrate. Diazo blue B or fast garnet GBC was used as coupler with the preceding substrate solutions, with the exception of indoxyl acetate which does not require a coupler. The strongest esterase bands were identified when alpha naphthyl acetate was used as substrate. When the sample was suspended on Whatman No. 1 filter paper instead of crude potato starch paste, the number of bands observed in gel strips was reduced from 5 to 4.

The effects of incubation of gel strips in inhibitors prior to incubation in alpha naphthyl acetate and fast garnet GBC are shown in table 2. Enzymatic hydrolysis of alpha naphthyl acetate showed slight inhibition of all bands with 8×10^{-2} M sodium arsanilate, while heavy inhibition was observed with a concentration of 1×10^{-1} M, and complete inhibition at a concentration of 1.6×10^{-1} M. Phemerol chloride (2.3×10^{-3} M) showed heavy inhibition of bands A, B, and D; and complete inhibition of band C. Sodium taurocholate (2×10^{-2} M) caused slight inhibition of bands C and E, and an acceleration of band D, while it had no affect on bands A and B. Sodium fluoride (1×10^{-1} M) slightly inhibited bands B, C, and D; and caused a marked acceleration of band E, while band A was unaffected. If the concentration of sodium fluoride was increased to 1 M there was complete inhibition of bands B, C, and D; band E was accelerated, and band A remained unaffected. L-cysteine hydrochloride at a concentration of 5×10^{-3} M accelerated band D while causing slight inhibition of bands B, C, and E; and did not affect band A. At a concentration of 1×10^{-2} M L-cysteine hydrochloride completely inhibited all bands. L-cysteine ethyl ester hydrochloride at a concentration of 3.3×10^{-3} M caused heavy inhibition

TABLE 2

Intensity of esterase activity in zymograms following incubation in various inhibitor solutions. Zymograms were developed in alpha naphthyl acetate as substrate, with fast garnet GBC as coupler. Degree of inhibition was evaluated on a subjective basis: 3, complete inhibition; 2, heavy inhibition; 1, slight inhibition; 0, no inhibition; and +, acceleration.

Inhibitor	Concentration	Age of Culture in Days	Extent Inhibition of Bands				
			A	B	C	D	E
Sodium Taurocholate	2×10^{-2} M	6	0	0	0	0	0
" "	"	11	0	0	0	0	0
" "	"	18	0	0	1	+	1
" "	"	21	0	0	1	+	1
Phemerol Chloride	1.2×10^{-3} M	9	0	0	0	0	0
" "	"	21	0	0	1	0	0
" "	2.4×10^{-3} M	21	2	2	3	2	0
Potassium Iodide	2×10^{-3} M	6	0	0	0	0	0
" "	4×10^{-3} M	11	0	0	0	0	0
" "	1×10^{-3} M	18	0	0	0	0	0
Eserine Sulfate	5×10^{-4} M	6	0	0	0	0	0
" "	8×10^{-3} M	6	0	0	0	0	0
" "	"	18	0	0	0	0	0
RO 2-0683	1×10^{-3} M	6	0	0	0	0	0
RO 2-1250	2.5×10^{-3} M	12	0	0	0	0	0
"	7.5×10^{-3} M	12	0	0	0	0	0
"	1.5×10^{-2} M	12	0	0	0	0	0
Sodium Arsanilate	4×10^{-2} M	12	0	0	0	0	0
" "	8×10^{-2} M	12	0	0	0	0	0
" "	1.6×10^{-1} M	12	3	3	3	3	3
" "	3.2×10^{-1} M	12	3	3	3	3	3
" "	8×10^{-2} M	18	1	1	1	1	1
" "	1×10^{-1} M	18	2	2	2	2	2
Sodium Fluoride	5×10^{-2} M	10	0	1	1	1	0
" "	1×10^{-1} M	12	0	1	1	1	+
" "	7.1×10^{-1} M	12	0	2	2	2	+
	1 M	12	0	3	3	3	+

TABLE 2 Continued

Inhibitor	Concentration	Age of Culture in Days	Extent Inhibition of Bands				
			A	B	C	D	E
L-Cysteine HCl	1×10^{-3} M	12	0	0	0	+	0
" "	5×10^{-3} M	18	0	1	1	+	1
" "	1×10^{-2} M	18	3	3	3	3	3
L-Cysteine ethyl ester HCl	7.5×10^{-4} M	12	0	0	0	0	0
"	3.3×10^{-3} M	12	2	2	2	2	2
Dipterex	2.3×10^{-4} M	6	3	3	2	3	2
"	1.0×10^{-3} M	6	3	3	2	3	2
"	5.4×10^{-3} M	6	3	3	3	3	3
Systox	2.7×10^{-4} M	12	0	0	0	0	0
"	1×10^{-2} M	12	1	1	0	0	1
Diazinon	1.3×10^{-5} M	12	3	1	3	3	3
Benzaldehyde	1.6×10^{-3} M	12	0	0	0	0	0
"	1×10^{-2} M	9	0	0	0	0	0
"	1×10^{-2} M	12	0	0	0	0	0

of all bands. Dipterex, 2.3×10^{-4} M caused heavy inhibition of bands C and E; and complete inhibition of bands A, B, and D; while at a concentration of 5.4×10^{-3} M it caused complete inhibition of all bands. Systox (1×10^{-2} M) caused slight inhibition of bands A, B, and E; and no inhibition of bands C and D. Bands A, C, D, and E were completely inhibited by 1.3×10^{-5} M diazinon while band B was only slightly inhibited. On the other hand, potassium iodide, eserine sulfate, RO 2-0683, RO 2-1250, and benzaldehyde showed no effects as inhibitors in the concentrations used.

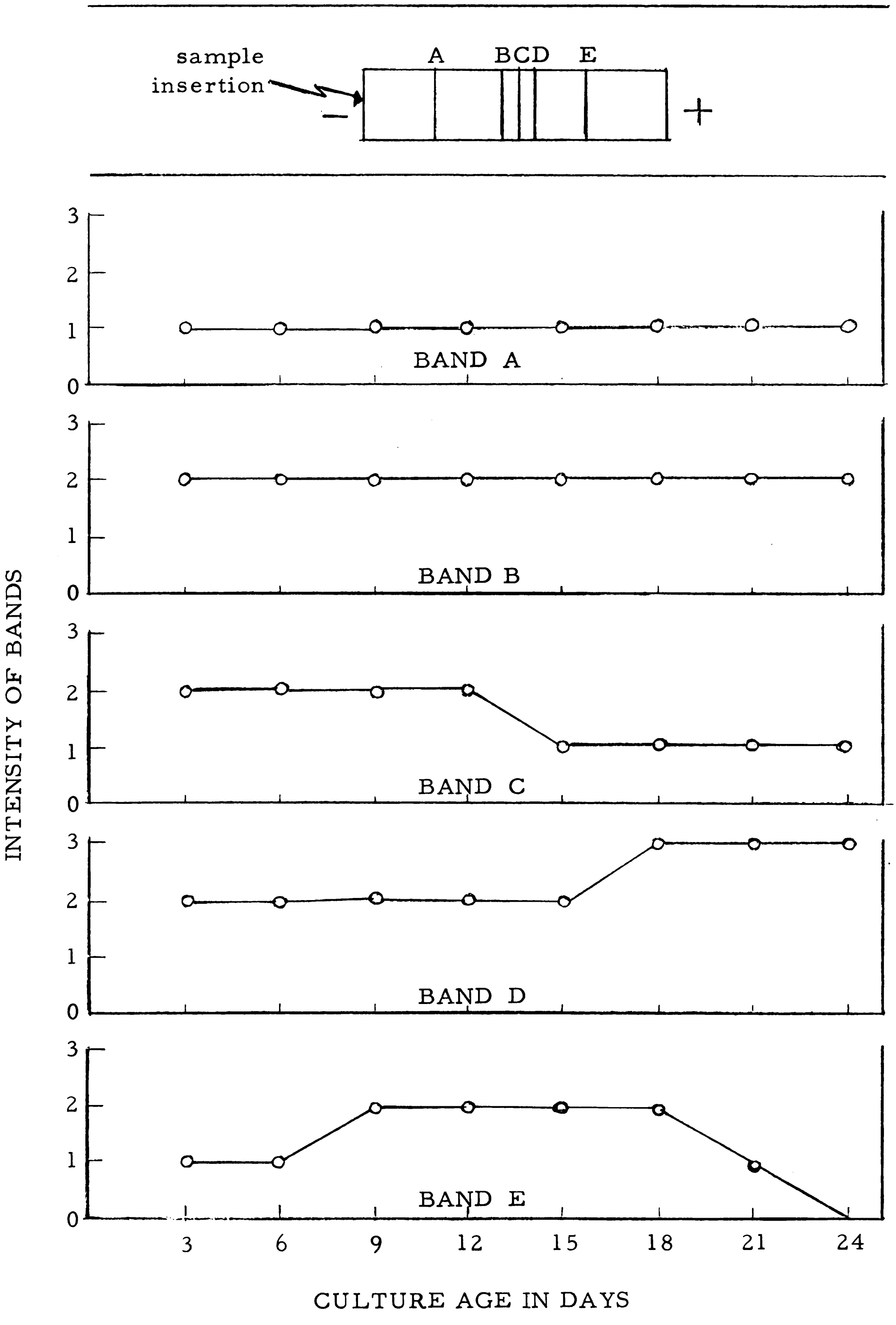
Separation of esterases with the starch gel procedure was made at 3 day intervals throughout the life of the culture. Considerable variation in enzyme activity was observed in homogenates from cultures of various ages (Fig. 11). The intensity of band E, exhibiting the greatest migration distance, increased as the culture aged from 6 to 9 days and decreased from the 18th to the 24th day, whereas the intensity of band D increased from the 15th day to the 18th day. Band C decreased in intensity from the 12th to the 15th day and remained constant from the 15th through the 24th day. The intensity of bands A and B were constant throughout the life of the culture.

Diaphorase and dehydrogenase localization. DPN diaphorase was localized in specimens of Tetrahymena pyriformis W using the method of Scarpelli et al. (1958). DPN diaphorase appeared as finely divided granules located throughout the cytoplasm, with an abundance of granules around and adjacent to the nucleus (Figs. 23-25).

When succinate dehydrogenase was localized in organisms reactive centers were more highly concentrated at the anterior end of the cell (Figs. 29 and 30). The distribution pattern was similar when either

FIGURE 11

Esterase activity in starch gel strips following incubation in alpha naphthyl acetate and coupling with fast garnet GBC to form an azo dye. Homogenates of T. pyriformis W (1 to 5 dilution) were used 3 days after seeding of cultures and at 3 day intervals until culture age reached 24 days. Intensity of esterase activity was evaluated on a subjective basis: 0, no band; 1, weak band; 2, moderate band; 3, strong band. Ordinates, intensity of esterase activity; and abscissae, age of organisms in days.


sample
insertion
A
BCD
E
−
+
INTENSITY OF BANDS
3
2
1
0
BAND A
BAND B
BAND C
BAND D
BAND E
3
6
9
12
15
18
21
24
CULTURE AGE IN DAYS

triphenyl tetrazolium chloride or nitro-BT was used as hydrogen acceptor.

When DPN and TPN-linked dehydrogenases were localized by the method of Hess _et_ _al_. (Pearse, 1960), enzyme-active areas were confined to small granules within the cytoplasm. Typical localization patterns for various dehydrogenases are shown in figs. 26 to 42 inclusive, e.g., β-hydroxybutyrate dehydrogenase (Figs. 26-28); isocitrate dehydrogenase (Figs. 31 and 32); glutamate dehydrogenase (Figs. 33 and 34); alcohol dehydrogenase (Figs. 35 and 36); lactate dehydrogenase (Figs 37 and 38); malate dehydrogenase (Figs. 39 and 40); and α-glycerophosphate dehydrogenase (Figs. 41 and 42).

Quantitative _determination_ _of_ _succinate_ _dehydrogenase_. Sensitivity curves were established by adding sodium succinate, sodium hydrosulfite, and a known amount of tetrazolium salt to a test tube. The intensity of the color, that developed due to reduction of the tetrazolium salt with hydrosulfite, was read as percentage absorption of light at various wavelengths (360 to 640 mμ). The sensitivity curve obtained when triphenyl tetrazolium chloride was used as hydrogen acceptor is shown in fig. 12. It is evident that a maximum absorption of light was attained at a wavelength of 480 mμ. When neotetrazolium chloride was used as hydrogen acceptor the maximum sensitivity was demonstrated at a wavelength of 520 mμ (Fig. 13).

Calibration curves were established using known increments of tetrazolium salts, which were reduced with sodium hydrosulfite. Subsequent to reduction of the tetrazolium salts, the intensity of the resulting color was ascertained as percentage absorption of light. The calibration curves for triphenyl tetrazolium chloride and neotetrazolium

FIGURE 12

Percentage absorption of light at various wavelengths (360 to 640 mμ following reduction of triphenyl tetrazolium chloride with sodium hydrosulfite to form a colored formazan. Ordinates, percentage absorption of light; abscissae, wavelength in millimicrons.

△ , 87 μgm of triphenyl tetrazolium chloride.

○ , 44 μgm of triphenyl tetrazolium chloride.

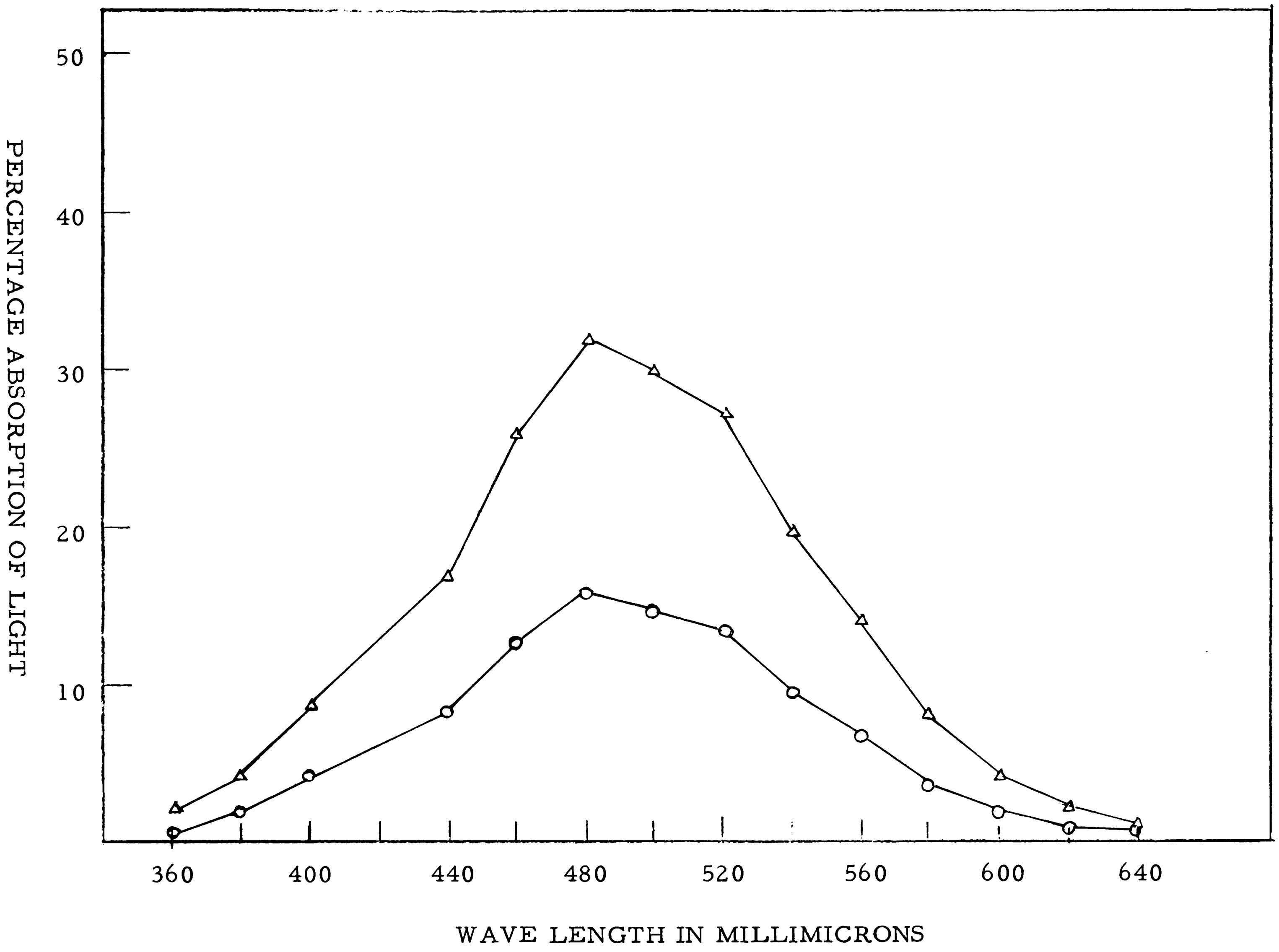
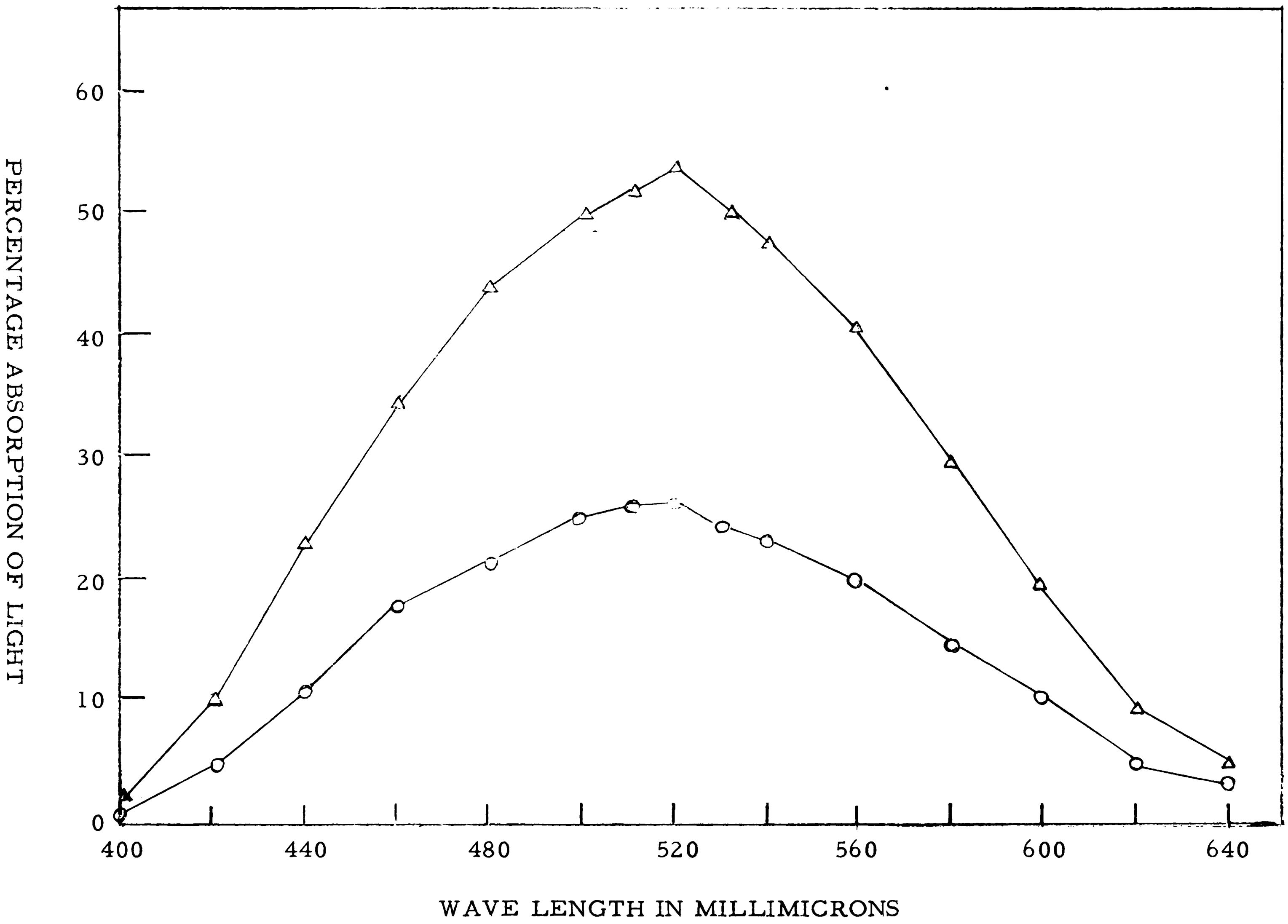

PERCENTAGE ABSORPTION OF LIGHT
50
40
30
20
10
360
400
440
480
520
560
600
640
WAVE LENGTH IN MILLIMICRONS

FIGURE 13

Percentage absoprtion of light at various wavelengths (400 to 640 mμ) following reduction of neotetrazolium chloride with sodium hydrosulfite to form a colored formazan. Ordinates, percentage absorption of light; abscissae, wavelength in millimicrons.

△ , 74 μgm of neotetrazolium chloride.

○ , 40 μgm of neotetrazolium chloride.


60
50
40
30
20
10
0
PERCENTAGE ABSORPTION OF LIGHT
400
440
480
520
560
600
640
WAVE LENGTH IN MILLIMICRONS

chloride are shown in fig. 14. It is evident that calibration curves obtained for both triphenyl tetrazolium chloride and neotetrazolium chloride are essentially linear.

Succinate dehydrogenase activity for organisms of various ages (3 to 24 days), is shown in tables 3 and 4 and fig. 15. It is evident that the pattern of succinate dehydrogenase activity is essentially similar with both neotetrazolium chloride and triphenyl tetrazolium chloride. However, with the former, dehydrogenase activity is highest in organisms from 3 day cultures and the minimum is reached in about 9 day organisms. Dehydrogenase activity then remains fairly uniform but higher than the minimum activity obtained with triphenyl tetrazolium chloride.

It is also evident in fig. 15, that when triphenyl tetrazolium chloride was used as an electron acceptor, dehydrogenase activity gradually decreased, reaching a minimum at 12 days, and then remained fairly uniform through 24 days.

FIGURE 14

Calibration curves for triphenyl tetrazolium chloride and neotetrazolium chloride. Known increments of tetrazolium salts were reduced with sodium hydrosulfite. Ordinates, percentage absorption of light; abscissae, weight of tetrazolium salt in micrograms.

○ , Calibration curve for triphenyl tetrazolium chloride. Wavelength 480mμ.

△ , Calibration curve for neotetrazolium chloride. Wavelength 520mμ.

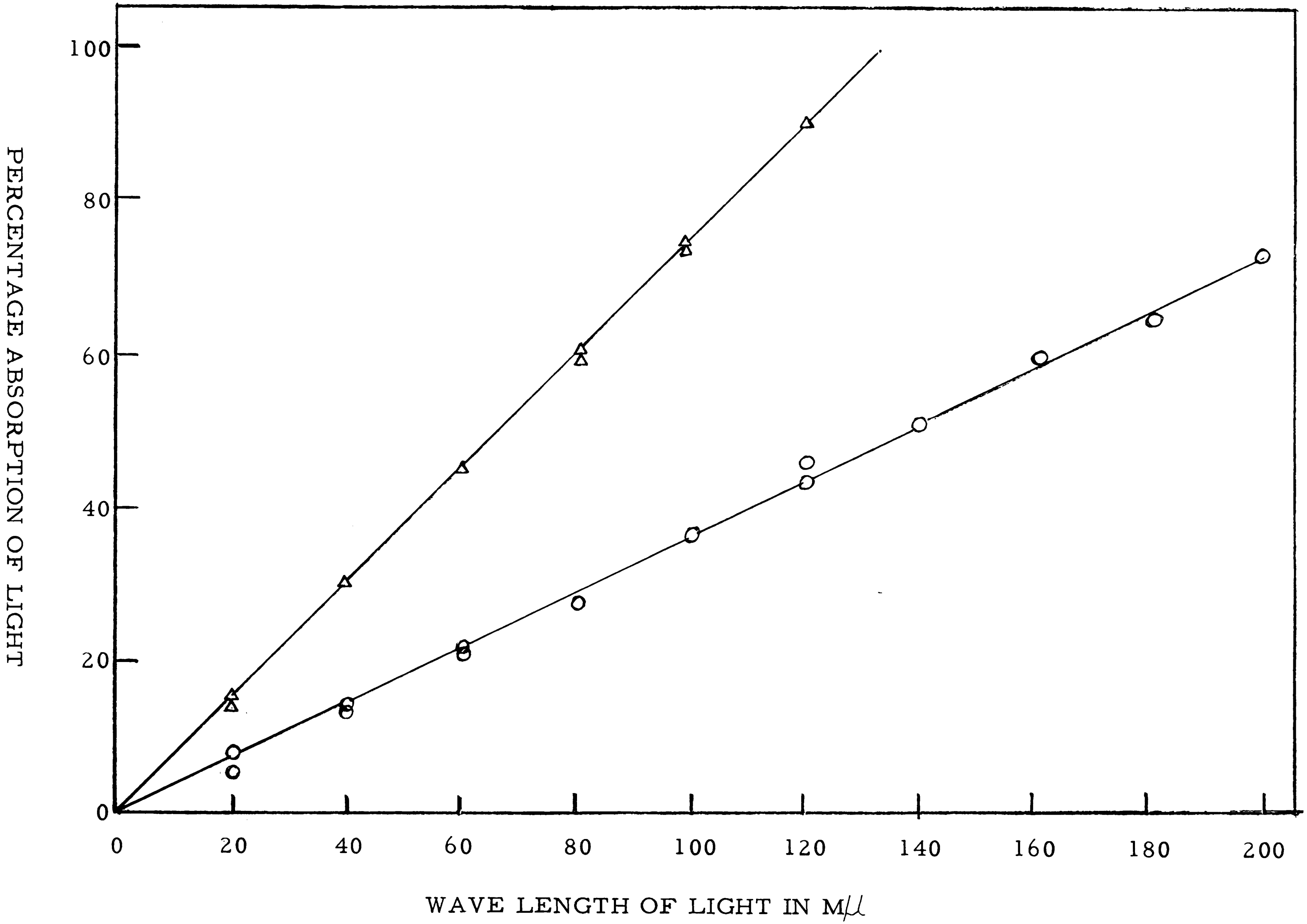

PERCENTAGE ABSORPTION OF LIGHT
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
200
WAVE LENGTH OF LIGHT IN Mμ

TABLE 3

Percentage absorption of light by formazan. Oxidation of sodium succinate by the dehydrogenases of Tetrahymena (0.1 ml) released hydrogen ions. The latter reduced tetrazolium chloride to a formazan. All tests were duplicated. Wavelength 480mμ.

Age of Culture in Days	Series I	Series II	Series III	Arithmetic Mean
3	33.0	31.9	34.6	33.17
6	25.0	24.3	22.8	24.03
9	18.6	19.3	18.9	18.93
12	16.0	17.0	16.8	16.60
15	15.3	14.9	15.9	15.37
18	16.0	15.7	15.2	15.63
21	13.9	14.8	14.6	14.43
24	14.6	14.1	15.3	14.67

TABLE 4

Percentage absorption of light by formazan. Oxidation of sodium succinate by the dehydrogenases of Tetrahymena (0.1 ml) released hydrogen ions. The latter reduced neotetrazolium chloride to a formazan. All tests were duplicated. Wavelength 520 mμ.

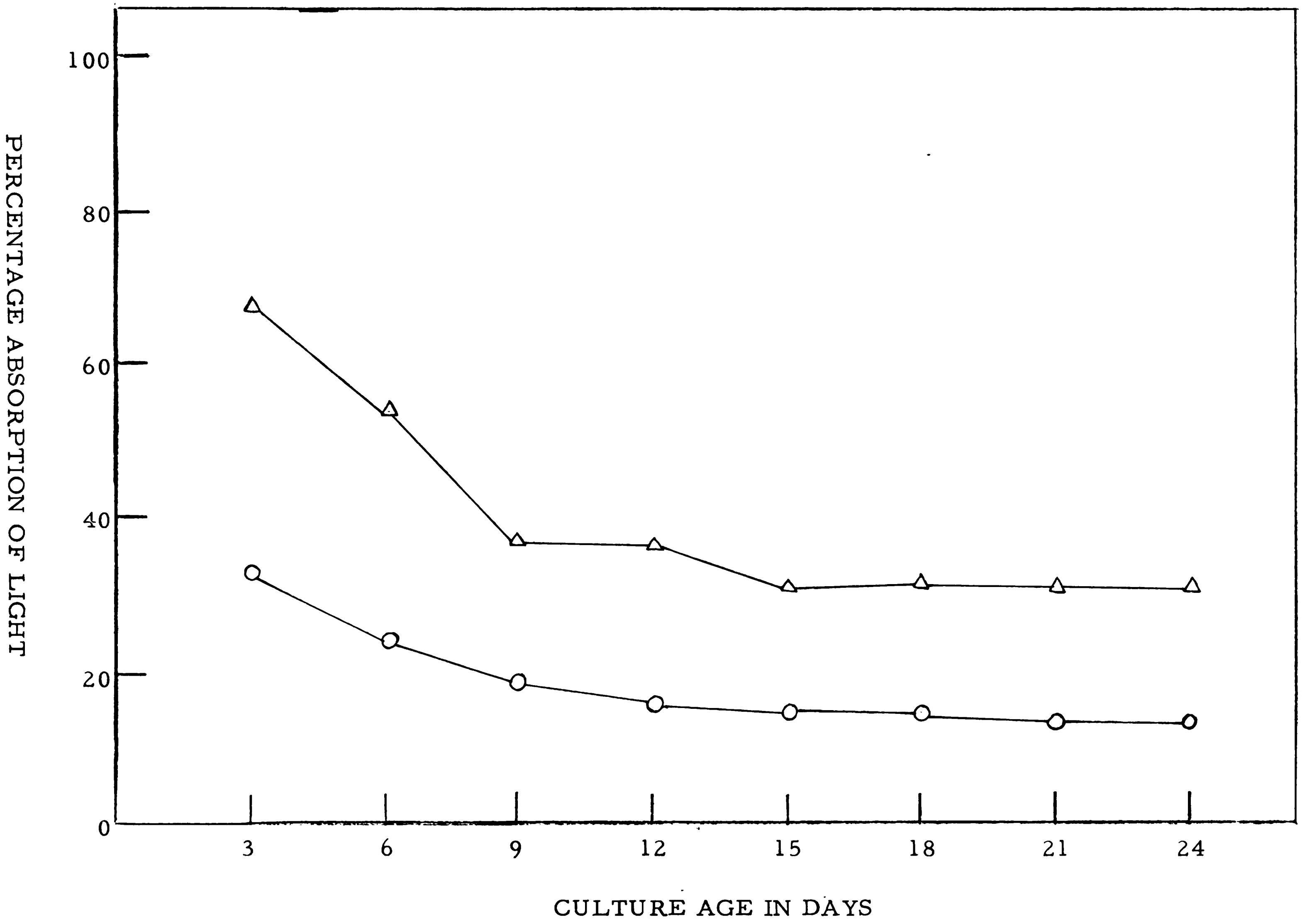
Age of Culture in Days	Series I	Series II	Series III	Arithmetic Mean
3	66.25	68.05	67.2	67.167
6	53.5	55.6	54.0	54.367
9	36.3	39.2	37.6	37.367
12	40.9	36.4	35.7	37.667
15	30.7	31.6	30.8	31.067
18	34.1	32.1	30.9	32.367
21	31.9	30.6	30.9	31.133
24	30.15	31.2	31.7	31.017

FIGURE 15

Relation between culture age in days and percentage absorption of light by formazan. Oxidation of succinate by dehydrogenases of *T. pyriformis* W (0.1 ml) released hydrogen. The latter reduced the tetrazolium salts to colored formazans. Each point represents the arithmetic mean of 3 series of tests, each test was duplicated. Ordinates, percent absorption of light; abscissae, age of cultures in days.

△ , Neotetrazolium chloride used as hydrogen acceptor. Wavelength 520mμ.

○ , Triphenyl tetrazolium chloride used as hydrogen acceptor. Wavelength 480mμ.


100
80
60
40
20
0
3
6
9
12
15
18
21
24
PERCENTAGE ABSORPTION OF LIGHT
CULTURE AGE IN DAYS

DISCUSSION

Polysaccharides. Many carbohydrates occur in nature either separately or in association with various amino, lipoid, or protein groups. Meyer (1938), classified carbohydrates as polysaccharides, mucopolysaccharides and glycoproteins. He revised this classification by dividing the carbohydrates into polysaccharides and protein-carbohydrate complexes (Meyer, 1945). The latter was divided into three groups, i.e., mucopolysaccharides, glycoproteins, and mucoproteins (mucoids).

Observations cited in the preceding pages indicate that polysaccharides of Tetrahymena pyriformis W consist primarily of glycogen. The PAS reaction was abolished upon exposure of the organisms to diastase. Diastase treatment at room temperature for 30 minutes is considered to be specific for removal of glycogen (Pearse, 1960). When the polysaccharides of Tetrahymena were isolated and hydrolyzed only glucose could be demonstrated by the paper chromatographic procedure. Since glycogen is the only commonly occurring animal polysaccharide which consists of glucose units, it would appear that Tetrahymena polysaccharides exist primarily as glycogen.

The concept that glycogen is bound to protein, and is therefore less easily extractable, led Willstatter and Rohdenwald (1934), to describe two types of glycogen, i.e., desmoglycogen, and lyoglycogen. The former is insoluble in warm water and weak trichloroacetic acid, and the latter is soluble in warm water and weak trichloroacetic acid. Meyer and Jeanloz (1943), maintained that glycogen is not chemically bound to protein, but that glycogen of high molecular weight is held by

mechanical inclusion in coagulated protein so that it cannot be extracted by washing.

The glycogen of Tetrahymena was completely removed following treatment of cells with warm water and weak trichloroacetic acid. These observations indicated that glycogen of these organism is similar to the lyoglycogen of Willstatter and Rohdenwald (loc. cit.).

Observations cited in this study revealed that glycogen of T. pyriformis W was localized in granules throughout the cytoplasm. The PAS reaction was more intense than the Feulgen-Bauer reaction. This is possibly due to the fact that chromic acid, used with the latter, is a stronger oxidizing agent than the periodic acid of the former. The chromic acid oxidizes the aldehydes that are formed while the periodic acid oxidizes the 1:2 glycol groups to dialdehydes and will not oxidize aldehydes. This is in agreement with Hotchkiss (1948), who maintained that periodic acid is unique as an oxidant in that it will not oxidize aldehyde groups. He thought that a greater number of aldehyde groups were present following periodic acid oxidation and as a consequence the intensity of the reaction was enhanced.

In order to demonstrate the histochemical localization of polysaccharides it is essential to choose an adequate fixative. In experiments reported for localization of polysaccharides a number of fixatives were employed. It was observed that alcoholic fixatives increased polarization of polysaccharides, while mercuric chloride fixatives reduced the amount of demonstrable polysaccharides. Cold neutral 10 percent formalin appeared to be the most adequate fixative for the polysaccharides of Tetrahymena. This is in general agreement with the work of Vallance-Owen (1948), in which he reported that

polysaccharides were fixed more adequately with formol-saline than with alcoholic fixatives. In contrast to the latter, Lillie (1947), reported that rat livers fixed in neutral formalin, with or without mercuric chloride, contained little or no glycogen. However, neutral 10 percent formalin did preserve glycogen in cat liver tissues. He maintained that neutral 10 percent formalin-alcohol was the best of all fixatives for glycogen.

Dempsey et al. (1947), classified the polysaccharides according to their capacity to bind the basic dyes, i.e., methylene blue at various pH values. They observed that with a basic dye maximum staining occurred at an alkaline pH and as the acidity is increased the dye uptake is decreased or absent. The polysaccharides of Tetrahymena demonstrated no capacity to bind methylene blue at a pH of 4.0 or below. It was assumed that relatively weak acid groups exist in this organism and that disassociation of acid groups is suppressed by acidities of about pH 4.0. Dempsey et al. (1947), maintained that acid mucopolysaccharides contain strong acid radicals and that disassociation is not suppressed at higher hydrogen ion concentrations.

Metachromatic dyes of the thiazine group have been used by many workers to demonstrate acid mucopolysaccharides. Michaelis and Granick (1945), suggested that the metachromatic color is due to formation of polymers (monomeric forms of the dye are blue while polymeric forms are red). The production of metachromasia was attributed to the presence of a certain minimum surface density of negative charges on the substrate (Pearse, 1960). Acid polysaccharides (containing SO_3H and COOH groups) would have adequate negative charges on their surface to induce formation of polymers. The observations presented in this study showed that

polysaccharides of *Tetrahymena* did not produce a metachromatic reaction with toluidine blue. On the basis of data resulting from this study, it was concluded that mucopolysaccharides are not present in sufficient quantities to give positive results with the tests used.

Quantitative studies of polysaccharide content of *Tetrahymena* revealed that there was an increase in stored polysaccharides in organisms from the 3rd to 9th day of culture age, and a decrease from the 12th to the 21st day of culture age. Gale (1947), worked on polysaccharide storage in *E. coli* and maintained that it occurred when there was glucose in the culture medium. Following utilization of the glucose from the culture medium, intracellular glycogen was then metabolized. The results reported in this paper indicate that the concentration of stored polysaccharides is reduced after the 12th day. Klamer (1960), has shown that most of the glucose is depleted from the culture medium prior to the 9th day when specimens of *Tetrahymena* are grown in a Bacto-Tryptone medium supplemented with glucose. This suggests that volume of glycogen stored could be closely correlated with glucose content of the medium in which specimens of *Tetrahymena* were cultured.

Poppen *et al.* (1947), maintained that there was a correlation between the localization of potassium and glycogen, and that possibly potassium was involved in the deposition of glycogen. In the present study potassium was localized as a brownish granular precipitate throughout the cytoplasm. The localization pattern was similar to that of glycogen, in so far as granules of both were randomly distributed throughout the cytoplasm.

Esterases. Esterases of vertebrate tissue are classified as non-specific esterases (ali-esterases and lipases), acetylcholinesterase, and pseudocholinesterase. The non-specific esterases hydrolyzed esters of naphthyl acetate. Cholinesterases, which hydrolyzed esters of choline, were classified by Augustinsson and Nachmansohn (1949), as true cholinesterase and pseudocholinesterase. The former cleaved its physiological substrate, i.e., low concentrations of acetylcholine, while higher concentrations of acetylcholine decreased its activity. On the other hand, pseudocholinesterase hydrolyzed acetylcholine more rapidly at higher concentrations. Further, the enzyme splits long-chain choline esters at a greater velocity than acetylcholine.

Pearse (1960), classified the non-specific esterases as: A esterase (arom-esterases) which is an organophosphate-resistant esterase and will hydrolyze di-isopropyl fluorophosphate (DFP) and diethyl p-nitrophenyl phosphate (E-600); B esterase (ali-esterase) or organophosphate-sensitive esterase; and C esterase which is organophosphate-resistant, but does not hydrolyze the organophosphate. Likewise, this author recognized acetylcholinesterase and pseudocholinesterase.

Markert and Hunter (1959), using starch gel electrophoresis, were able to demonstrate 10 distinct bands of esterase activity in zymograms of mouse tissues. These workers found that certain bands showed specificity of substrates and inhibitors. Observations cited in the present study showed a minimum of 5 esterase bands (bands were lettered from A to E in order of increasing electrophoretic mobility) in homogenates of T. pyriformis W. In these experiments some esterase activity could be observed at the site of sample insertion. The question arises as to why certain of the esterase bands did not exhibit electrophoretic mobility. This could be attributed to lower solubibility, variations in molecular size, or differences in electrical charges of the protein molecules. The evidence at hand does not demonstrate which of the preceding factors are involved in electrophoretic immobility of certain of the esterases.

Other observations cited in this study showed that the method of introducing the sample is an important factor in separation of the esterases. If the sample was introduced on Whatman No. 1 filter paper 4 bands of esterase activity could be demonstrated. Under these conditions the E band (the band showing the greatest electrophoretic mobility) was absent. It is interesting to speculate as to why this band was not observable. Since mobility of esterases is dependent on electrical charge it is possible that a firm union is made between the filter paper and the esterases. It was shown many years ago that congo red will not stain filter paper because the charge on the filter paper repels the dye, but on addition of Na^+ (cations) the filter paper is brilliantly stained (Gatenby and Beams, 1950).

Seligman _et al_. (1950) maintained that taurocholate accelerated lipase and inhibited non-specific esterase activity. Observations made in the present study showed that taurocholate accelerated esterolytic activity in band D. In the presence of taurocholate band D was observable within 5 minutes after addition of alpha naphthyl acetate and fast garnet GBC. On the other hand, band D appeared in controls (incubated in the absence of taurocholate) in about 20 minutes. Fennell and Pastor (1958), showed that taurocholate slightly increased esterolytic activity in homogenates of _Tetrahymena_. Thus the evidence at hand suggests that

lipase does exist in these organisms.

Inhibition of esterases by arsanilate has been demonstrated by a number of investigators. Nachlas and Seligman (1949), demonstrated that inhibition of liver and kidney esterases by 10^{-1} M arsanilate was more pronounced than inhibition of the pancreatic enzyme(s). Gomori (1948), observed that lipase and esterase were inhibited by arsanilate. In the present study 3.2×10^{-1} M arsanilate completely inhibited all esterase activity. These observations are in general agreement with the preceding observations.

Benzaldehyde has been cited as an inhibitor of lipase (West and Todd, 1957). In the present investigation the intensity of staining of bands A to E in the presence of benzaldehyde (1×10^{-2} M) was essentially the same as that of the same bands in control solutions. This confirms the work of Pastor and Fennell (1959), who found that benzaldehyde was without effect on esterases of _T. pyriformis_ W when alpha naphthyl acetate was used as substrate. Markert and Hunter (1959), reported preferential inhibition of bands of esterase activity in gels using RO 2-0683. In the present investigation no inhibition could be demonstrated using this compound.

Richter and Croft (1942), separated non-specific esterases and cholinesterases of tissues by treatment of sections with low concentrations of eserine sulfate. The latter completely inhibited acetylcholinesterase. Gomori and Chessick (1953), inhibited the hydrolysis of alpha naphthyl acetate with 10^{-5} M eserine sulfate. Pastor and Fennell (1959), found that esterase(s) of _Tetrahymena_ would hydrolyze myristoyl choline, lauryl choline, and benzoyl choline, but would not hydrolyze either acetyl thiocholine iodide or butyryl thiocholine iodide.

These workers also found that eserine (1×10^{-4} M) did not inhibit the hydrolysis of myristoyl choline but on increasing the concentration of the former to 8×10^{-3} M the hydrolysis was strongly inhibited. Observations cited in this study show that the esterases of Tetrahymena continue at high levels although the concentration of eserine is increased to 8×10^{-3} M. This suggests that the esterases of this organism have certain characteristics in common with that esterase of Planaria, which was also insensitive to low concentration of eserine (Hawkins and Mendel, 1946).

Nachlas and Seligman (1949) used naphthyl esters for study of esterase activity and maintained that sodium fluoride inhibited esterase activity. In the present study a concentration of 1×10^{-1} M caused a marked acceleration of band E, whereas bands A to D, inclusive, were strongly inhibited.

Dipterex, [dimethyl-(1-hydroxy-2, 2, 2-trichloroethyl) phosphate] in a concentration of 3.3×10^{-3} M, caused heavy inhibition of band C and completely inhibited all other bands. Diazinon, (0,0 diethyl 0,2 isopropyl-4-methyl pyrimidyl thionophosphate) in a concentration of 1.3×10^{-5} M, caused slight inhibition of band B and completely inhibited all other bands. Systox, which is a mixture of 2 active isomers, [0,0 diethyl 0-2-(ethylthiol) ethyl phosphorothioate, and 0,0 diethyl S-2-(ethylthiol) ethyl phosphorothioate] in a concentration of 1×10^{-2} M caused slight inhibition of bands A, B, and E, while it had no effect on bands C and D. Since all of these inhibitors are organic phosphate compounds, the variability of effects of different inhibitors on different bands cannot be explained at the present time.

Pearse (1960), who used others of the organic phosphate compounds found that the A esterase was inhibited by E-600 (10^{-2} M) and B esterase was inhibited by 10^{-5} M E-600. On the other hand, C esterase was organophosphate-resistant.

Observations cited in the present study showed that intensity of activity of certain of the bands was related to age of the organisms, e.g., band C exhibited high esterase activity until the 12th day and then it decreased to a minimum on the 16th day. On the other hand, band D showed an increase in intensity between the 15th and 18th days. Band E showed low activity in 3 to 6 day cultures, which was followed by increased activity until the 9th day. The intensity of band E then remained constant until the 18th day, and it gradually decreased to zero when the culture age reached 24 days. These results indicate that activity of individual esterases varies with age of organisms. Band D, which is believed to be a lipase, was very active in organisms from older cultures. This may indicate that the organisms metabolize short-chain esters during the logarithmic and early stationary phases of growth. In the late stationary phase long-chain esters may be metabolized.

Dehydrogenases and diaphorases. An enzyme which catalyzed the oxidation of reduced coenzyme I (DPNH) by methylene blue or cytochrome c was demonstrated by Green *et al.* (1937). This enzyme was characterized as a flavoprotein and referred to as DPN diaphorase (DPND). Adler *et al.* (1939), concluded that oxidation of coenzyme II (TPNH) was carried out by a separate enzyme (TPN diaphorase, TPND). The histochemical localization of DPND and TPND was first accomplished by Farber *et al.* (1956).

These authors used an exogenous dehydrogenase together with its substrate and oxidized coenzyme I in order to generate DPNH as substrate for the intrinsic dehydrogenase. Scarpelli _et_ _al_. (1958), described a simpler and more effective procedure for localization of diaphorases using DPNH and TPNH as substrates for the diaphorases. Pearse (1960), maintained that DPN and TPN diaphorase reactions obtained when DPNH and TPNH were used as substrates, demonstrated all sites of diaphorase activity. In both cases localization of the formazan is predominantly intramitochondrial.

Observations cited in the present investigation showed that DPND was localized in specimens of _T_. _pyriformis_ W as fine granules throughout the cytoplasm with an abundance of granules adjacent to the nucleus. Ryley (1952), maintained that homogenates of _T_. _geleii_ GL were unable to catalyze the oxidation of succinate in the presence of atmospheric oxygen, but succinate dehydrogenase activity was demonstrated by anaerobic reduction of methylene blue. On the other hand, Eichel (1954), demonstrated that homogenates of _T_. _pyriformis_ S oxidize succinate rapidly in the presence of atmospheric oxygen. He also maintained that antimycin, which is an extremely potent inhibitor of succinic-oxidase and diphosphopyridine nucleotide cytochrome c reductase systems in homogenates of vertebrate tissue had little effect on succinate oxidation in _T_. _pyriformis_ S unless there was a ten-fold increase of the inhibitor. He was unable to demonstrate cytochrome oxidase activity and he found that succinate dehydrogenase activity was present and insensitive to cyanide. Eichel (_ibid_.) concluded that hydrogen transport from succinate to molecular oxygen proceeded by a different pathway than that described for respiratory pathways of mammalian tissue. Eichel (1956), studied cell-free homogenates of T. pyriformis S for components of the DPNH oxidase system and concluded that they differ greatly from the mammalian system in that cytochrome c is not functional as an electron carrier.

In the present study, isocitrate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase were localized by the procedure of Hess et al. (1958). These reactions are usually considered to be good evidence of enzymes of the Krebs' cycle. Alcohol dehydrogenase, α-glycerophosphate dehydrogenase, and lactate dehydrogenase demonstrated that the glycolytic pathway is functional. Glutamate dehydrogenase was demonstrated in specimens of T. pyriformis W. This enzyme, when coupled with transaminases, can synthesize a variety of amino-acids from keto-acids and ammonia (Pearse, 1960). The glutamate in this case acts as an ammonia carrier. Glutamate dehydrogenase may also act in conjunction with ketoglutarate dehydrogenase and lipoate dehydrogenase resulting in additional succinate for oxidations in the citric acid cycle. According to Dixon and Webb (1958), there is also a possibility that glutamate may act like coenzyme I and II, as an intermediate hydrogen carrier, by undergoing alternate oxidation and reduction, catalyzed by glutamate dehydrogenase. Finally, glutamate dehydrogenase may take part in the synthesis of urea (Pearse, 1960). Others of our observations showed that β-hydroxybutyrate dehydrogenase was present in specimens of T. pyriformis W. This enzyme is important in the oxidation of fatty acids. Possibly the most important role of β-hydroxybutyrate dehydrogenase is to take part, with coenzyme A, in the oxidation of fatty acids coupled with the synthesis of active acetate (Pearse, 1960). On the basis of the evidence at hand, we can conclude that the metabolic pathways of Tetrahymena studied showed characteristics similar to those described for higher organisms.

The quantitative tetrazolium method for succinate dehydrogenase was based on the reduction of tetrazolium salts to insoluble, highly colored formazans. The tetrazolium salt acted as hydrogen acceptor during enzymatic oxidation of succinate to fumarate. The formazan was extracted with a fat solvent and the color intensity was ascertained by colorimetry.

Results obtained in experiments with the colorimetric procedure for succinic dehydrogenase indicate that enzyme activity is at a maximum in organisms from 3 day cultures, gradually decreased until the 9th to 12th day, and then it remained constant until the 24th day of culture age. In contrast to the latter, Thompson and Wingo (1959), who studied L-glutamic acid dehydrogenase in _T_. _pyriformis_ Y concluded that there was no significant difference in enzyme activity in the early logarithmic or late stationary phase of growth. Organisms during the late stationary phase of growth (cultures 18 to 24 days after seeding) exhibited a decrease in cell division, were less active, smaller, and more spherical in shape. Under these conditions the energy available to the organism due to the action of enzymes of the Krebs' cycle could be at a minimum, and as a consequence the succinate dehydrogenase activity would be reduced.

SUMMARY

1. Specimens of <u>Tetrahymena pyriformis</u> W were cultured in bacteria-free Bacto-Tryptone medium. Polysaccharides of the organisms were localized, in fine granules, throughout the cytoplasm.

2. Polysaccharides in the organisms were fixed by a variety of fixatives. Cold neutral 10 percent formalin was the most adequate fixative tried.

3. Carbohydrates of <u>T. pyriformis</u> W consist mainly of glycogen. Diastase treatment, for 30 minutes at room temperature, removed all polysaccharides from the cells.

4. Metachromasia could not be demonstrated in specimens of <u>Tetrahymena</u> with toluidine blue.

5. Acidic groups on the polysaccharides were not demonstrable. Binding of methylene blue was reduced at pH 5.0 and absent at pH 4.0 and below.

6. Glycogen was identified in homogenates of <u>T. pyriformis</u> W from all ages of cultures (3 to 24 days). It was low in 3 day cultures (0.31 mgm / 0.1 ml of organisms), increased to reach a maximum on the 12th day (1.37 mgm / 0.1 ml of organisms), then gradually declined to a minimum (0.24 mgm / 0.1 ml of organisms) on the 24th day.

7. Potassium was localized as a granular precipitate in specimens of <u>T. pyriformis</u> W from all ages of cultures. It was randomly distributed throughout the cytoplasm of organisms.

8. A minimum of 5 bands (A to E, inclusive) of esterase activity was demonstrated in homogenates of <u>Tetrahymena</u> by the starch gel electrophoresis procedure.

9. The intensity of esterase activity, as demonstrated by bands in starch gels, was related to age of cultures. Band C exhibited high esterase activity until the 12th day and decreased to a minimum on the 15th day. Band D increased in intensity between the 15th and 18th day. On the other hand, the intensity of band E was low in 3 day cultures, increased until the 9th day, then decreased from day 18 to 24.

10. Subsequent to electrophoretic separation of esterases of _Tetrahymena_, specificity of inhibitors was noted for various bands of activity in the zymograms. These studies indicated that band D could be due to lipase activity.

11. DPN diaphorase was localized throughout the cytoplasm of _T. pyriformis_ W. Enzyme activity was highest in the cytoplasm adjacent to the nucleus.

12. Enzymes of the Krebs' cycle were identified in specimens of _T. pyriformis_ W by localization of isocitrate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase.

13. It was demonstrated that specimens of _T. pyriformis_ W contain enzymes of the glycolytic pathway as shown by the presence of alcohol dehydrogenase, α-glycerophosphate dehydrogenase, and lactate dehydrogenase.

14. Glutamate dehydrogenase was localized in specimens of _T. pyriformis_ W. This enzyme functions in synthesis of amino acids from keto acids, breakdown of glutamate, and supplying succinate for the citric acid cycle.

15. β-hydroxybutyrate dehydrogenase was localized in specimens of _T. pyriformis_ W. This enzyme is involved in the oxidation of fatty acids.

16. Succinate dehydrogenase activity was demonstrated quantitatively in _Tetrahymena_. It was at a maximum in 3 day cultures, decreasing to a minimum in 9 to 12 days and then remaining constant through 24 days.

LITERATURE CITED

Adler, E., Euler, H.U., and Gunther, G. 1939 Diaphorase I and II. Nature, 143:641-642.

Augustinsson, K., and Nachmansohn, D. 1949 Distinction between acetylcholine-esterase and other ester-splitting enzymes. Science, 110:98-99.

Bensley, R. R., and Bensley, S. A. 1938 Handbook of histological and cytological technique. The University of Chicago Press, Chicago.

Boell, E. J. 1942 The effect of respiratory inhibitors on the oxygen consumption of *Paramecium calkinsi*. Anat. Record, 84:493-494.

Boell, E. J. 1946 The effect of sodium azide on *Paramecium calkinsi*. Biol. Bull., 91:238-239.

Dagley, S., and Dawes, E. A. 1949 Factors influencing the polysaccharide content of *Escherichia coli*. Biochem. J., 45:331-337.

Dawson, J., and Happold, F. C. 1943 The trypotophanase-tryptophan reaction. 6. Carbohydrate-amino acid relationships concerned in the inhibition of indole production by glucose in cultures of *Escherichia coli*. Biochem. J., 37:389.

Dempsey, E. W., Bunting, H., Singer, M., and Wislocki, G. B. 1947 The dye binding capacity and other chemohistological properties of mammalian mucopolysaccharides. Anat. Rec., 98:417-429.

Eichel, H. J. 1954 Studies on the oxidation of succinic acid by cell-free homogenates of *Tetrahymena pyriformis* S and W. J. Biol. Chem., 206:159-169.

Eichel, H. J. 1956a Respiratory enzyme studies in *Tetrahymena pyriformis*. II. Reduced diphosphopyridine nucleotide oxidase and reduced diphosphopyridine nuclootide cytochrome c reductase. J. Biol. Chem., 222:121-136.

Eichel, H. J. 1956b Respiratory enzyme studies in *Tetrahymena pyriformis*. III. Some properties of reduced diphosphopyridine nucleotide oxidase of cell-free homogenates. J. Biol. Chem., 222:137-144.

Faber, E., Sternbery, W. H., and Dunlap, C. E. 1956 Histochemical localization of specific oxidative enzymes. I. Tetrazolium stains for diphosphopyridine nucleotide diaphorase and triphosphopyridine diaphorase. J. Histochem. Cytochem., 4:254-265.

Fenn, W. D. 1939 The deposition of potassium and phosphate with glycogen in rat livers. J. Biol. Chem., 128:297-307.

Fennell, R. A. 1951 The relation between growth substances, cytochemical properties of Tetrahymena geleii, and lesion induction in the chorioallantois. J. Elisha Mitchell Sci. Soc., 67:219-233.

Fennell, R. A., and Marzke, F. O. 1954 The relation between vitamines, inorganic salts and histochemical characteristics of Tetrahymena geleii W. J. Morph., 94:587-615.

Fennell, R. A.,and Pastor, E. P. 1958 Some observations on the esterases of Tetrahymena pyriformis W. I. Evidence for the existence of aliesterases. J. Morph., 103:187-202.

Gale, E. F. 1947 The chemical activities of Bacteria. University Tutorial Press, Ltd., London.

Gomori, G. B. 1948 Histochemical domonstration of sites of cholinesterase activity. Proc. Soc. Exp. Biol. and Med., 68:354-358.

Gomori, G. B., and Chessick, R. D. 1953 Histochemical studies of the inhibition of esterases. J. Cell. and Comp. Physiol., 41:51-64.

Green, D. E., Dewan, J. G., and Leloir, L. F. 1937 CXXV. The β-hydroxybutyric dehydorgenase of animal tissues. Biochem. J., 31:934-494.

Hawkins, R. D., and Mendel, B. 1946 True cholinesterase with pronounced resistance to eserine. J. Cell.and Comp. Physiol., 27:69-85.

Hooghwinkel, C. J. M., and Smits, G. 1957 The specificity of the periodic acid-Schiff technique studied by a quantitative test-tube method. J. Histochem. Cytochem., 5:120-126.

Hotchkiss, R. D. 1948 A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. Archiv. Biochem., 16:131-141.

Humphrey, B. A., and Humphrey, G. F. 1947 Succinic dehydrogenase in protozoa. Nature, 159:374.

Humphrey, B. A., and Humphrey, G. F. 1948 Studies in the respiration of Paramecium caudatum. J. Exp. Biol., 25:123-134.

Hunter, R. L., and Markert, C. L. 1957 Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science, 125:1294-1295.

Jardetzky, C. D., and Glick, D. 1956 Studies in histochemistry. XXXVIII. Determination of succinic dehydrogenase in microgram amounts of tissue and its distribution in rat adrenal. J. Biol. Chem., 218:283-292.

Klamer, B., 1960 A chemical and cytochemical investigation of acid phosphatases of Tetrahymena pyriformis W. Ph.D. Thesis MSU.

Kun, E., and Abood, L. G. 1949a Biochemical properties of succinoxidase from Salmonella aertrycke. J. Biol. Chem., 180:813-823.

Kun, E., and Abood, L. G. 1949b Colorimetric estimation of succinic dehydrogenase by triphenyltetrazolium chloride. Science, 109:144-146.

Lillie, R. D. 1947 Studies on the preservation on histologic demonstration of glycogen Bull. Internat. Ass. Med. Museums, 27:23-61.

Markert, C. L., and Hunter, R. L. 1959 The distribution of esterases in mouse tissues. J. Histochem. Cytochem., 7:42-49.

McManus, J. F. A. 1946 Histological demonstration of mucin after periodic acid. Nature, 158:202.

Meyer, L. 1938 The chemistry and biology of mucopolysaccharides and glycoproteins. Cold Spring Harbor Symposia of Quantative Biology, 6:91-102.

Meyer, K. 1945 Classification of the mucopolysaccharide Adv. Prot. Chem., 2:249.

Meyer, K. H., and Jeanloz, R. 1943 Reserches sur l'amidon XXV Le glycogine du muscle matif. Helv. Chim. Acta, 26:1784-1798.

Michaelis, L., and Gronick, S. 1945 Metachromasia of basic dyestuffs. J. Amer. Chem. Soc., 67:1212-1219.

Nachlas, M. M., and Seligman, A. H. 1949 The histochemical demonstration of esterase. J. Natl. Cancer Inst., 9:415-425.

Nachlas, M. M., Tsuo, K., Souza, E., Cheng, C., and Seligman, A. M. 1957 Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. J. Histochem. Cytochem., 5:420-536.

Pastor, E. P., and Fennel, R. A. 1959 Some observations on the esterases of Tetrahymena pyriformis W. II. Some factors affecting aliesterase and cholinesterase activity. J. Morph., 104:143-158.

Pappenhermer, A. M., and Hendee, E. D. 1949 Diphtheria toxin. V. A comparison between the diphtherial succinoxidase system and that of beef heart muscle. J. Biol. Chem., 180:597-614.

Pearse, A. G. E. 1957 Intracellular localization of dehydrogenase systems using monotetrazolium salts and metal chelation of their formazans. J. Histochem. Cytochem., 5:515-527.

Pearse, A. G. E. 1960 Histochemistry, theoretical and applied. 2nd edition, J. A. Churchill Ltd., London.

Poppen, K. J., Green, D. M., and Wrenn, H. T. 1953 The histochemical localization of potassium and glycogen. J. Histochem. Cytochem., 1:160-173.

Richter, D., and Croft, F. G. 1942 Blood esterases. Biochem. J., 36:746-757.

Ryley, J. F. 1952 Studies on the metabolism of the protozoa. 3. Metabolism of the ciliate Tetrahymena pyriformis (Glaucoma piriformis) Biochem. J., 52:483-492.

Seaman, G. R. 1951a Enzyme systems in Tetrahymena geleii S. I. Anaerobic dehydrogenases concerned with carbohydrate oxidation. J. Gen. Physiol., 34:775-783.

Seaman, G. R. 1951b Localization of acetylcholinesterase activity in the protozoan, Tetrahymena geleii S. Proc. Soc. Exp. Biol. and Med., 76:169.

Seaman, G. R. 1952 Enzyme systems in Tetrahymena geleii S. IV. Combination of arsonoacetate with carboxyl affinity points on the succinic dehydrogenase. Arch. Biochem. Biophys., 35:132.

Seaman, G. R., and Houlihan, R. K. 1951 Enzyme systems in Tetrahymena geleii. S. II. Acetylcholinesterase activity. Its relation to motility and to coordinated ciliary action in general. J. Cell. and Comp. Physiol., 37:309-321.

Seligman, A. M., Mallomo, M. C., and Nachlas, M. M. 1950 The colorimetric determination of lipase and esterase in human serum. Amer. J. Physiol., 159:337-341.

Smithies, O. 1955 Zone electrophoresis in starch gels. Group variations in the serum proteins of normal human adults. Biochem. J., 61:629-641.

Thompson, W. D., and Wingo, W. J. 1959 Comparatine enzymatic studies on cells of Tetrahymena pyriformis Y. from cultures of two stages of growth: L-Glutomic Acid dehydrogenase. J. Cell. Comp. Physiol, 53:413-420.

Vallance-Owen, J. 1948 The histological demonstration of glycogen in necropsy material. J. Path. Bact., 60:325.

West, E. S., and Todd, W. R. 1957 Textbook of Biochem., p. 441. Macmillan Co., New York.

Willstatter, R., and Rohdenwald, M. 1934 Über den Zustand des Glykogens in der Leber im Muskel in Leukocyten (Zur Kenntnis der Protein bindung physiologisch wichtiger Stoffe.) Hoppe Seyl. Z., <u>225</u>:103-124.

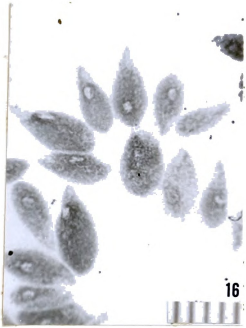
Wislocki, G. B., Bunting, H., and Dempsey, E. W. 1947 The metachromasia in mammalian tissue and its relationship to the mucopolysaccharides. Amer. J. Anat., <u>81</u>:1-37.

PLATE 1

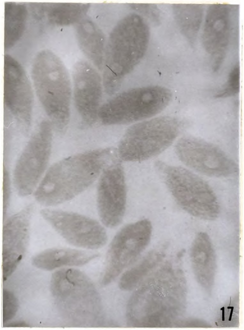
EXPLANATION OF FIGURES

Specimens of Tetrahymena pyriformis W fixed in 10% formalin and stained for polysaccharides with the periodic acid-Schiff technique (Hotchkiss, 1948). Micrometer scale insert graduated in 0.01 mm.

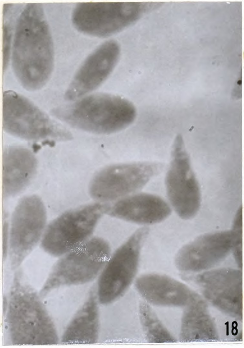
Fig. 16. Organisms from 6 day cultures;
Fig. 17. 9 day cultures;
Fig. 18. 12 day cultures; and
Fig. 19. 18 day cultures.



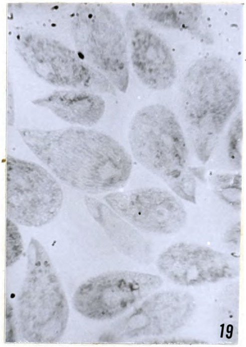
16



17



18



19

PLATE 2

EXPLANATION OF FIGURES

Specimens of Tetrahymena pyriformis W fixed in 95% ethanol and stained for potassium. Micrometer scale insert graduated in 0.01 mm.

Fig. 20. Organisms from 9 day cultures; and

Fig. 21. 15 day cultures.

Fig. 22. Zymograms showing bands of esterase activity when homogenates of Tetrahymena pyriformis W were separated by electrophoresis. Bands of esterase activity were developed by incubation of starch gel strips in alpha naphthyl acetate and fast garnet GBC. The point of sample insertion was at the bottom of the picture. All bands migrated toward the anode.

a. Homogenates of 12 day organisms; esterase activity inhibited with sodium arsanilate (8×10^{-2} M).

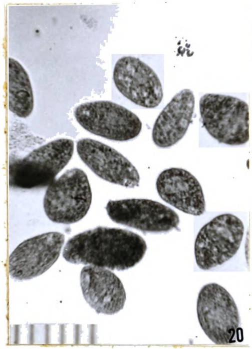
b. Homogenates of 12 day organisms; esterase activity inhibited with L-cysteine HCl (1×10^{-3} M).

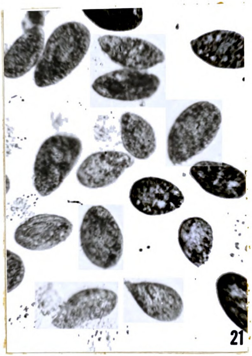
c. Homogenates of 12 day organisms; esterase activity inhibited with sodium fluoride (1×10^{-1} M).

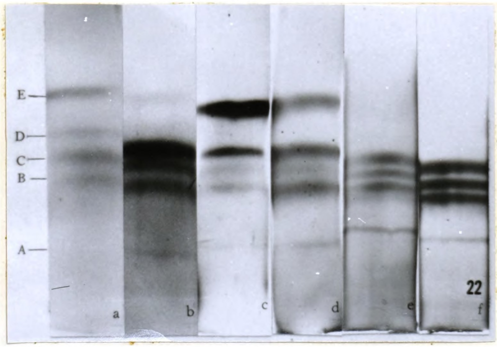
d. Homogenates of 12 day organisms; control.

e. Homogenates of 24 day organisms.

f. Homogenates of 12 day organisms, sample inserted on filter paper.


20


21



22

PLATE 3

EXPLANATION OF FIGURES

Specimens of <u>Tetrahymena pyriformis</u> W air dried on slides and stained for diaphorase and dehydrogenase activity. Micrometer scale insert graduated in 0.01 mm.

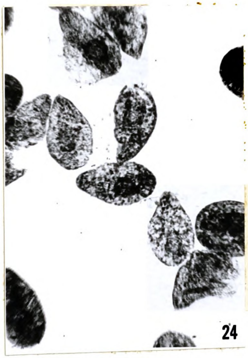
Fig. 23. Organisms from 6 day cultures showing DPN diaphorase activity;

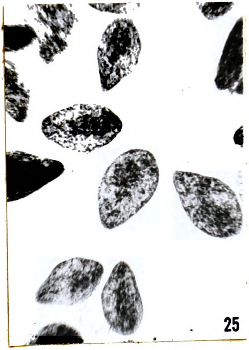
Fig. 24. Organisms from 12 day cultures showing DPN diaphorase activity;

Fig. 25. Organisms from 18 day cultures showing DPN diaphorase activity; and

Fig. 26. Organisms from 6 day cultures showing β- hydroxybutyrate dehydrogenase activity.


23


24


25


26

PLATE 4

EXPLANATION OF FIGURES

Specimens of Tetrahymena pyriformis W stained to show dehydrogenase activity. Micrometer scale insert graduated in 0.01 mm.

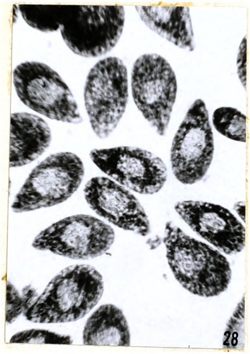
Fig. 27. Organisms from 12 day cultures showing β-hydroxybutyrate dehydrogenase activity;

Fig. 28. Organisms from 18 day cultures showing β-hydroxybutyrate dehydrogenase activity;

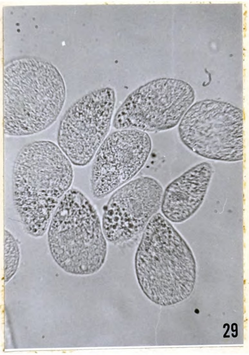
Fig. 29. Organisms from 9 day cultures showing succinate dehydrogenase activity; and

Fig. 30. Organisms from 15 day cultures showing succinate dehydrogenase activity.

27



28



29

30

PLATE 5

EXPLANATION OF FIGURES

Specimens of Tetrahymena pyriformis W stained to show dehydrogenase activity. Micrometer scale insert graduated in 0.01 mm.

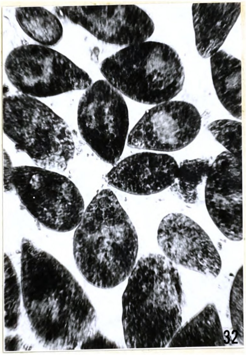
Fig. 31. Organisms from 6 day cultures showing isocitrate dehydrogenase activity;

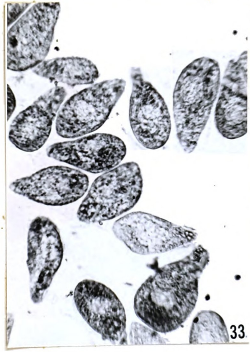
Fig. 32. Organisms from 15 day cultures showing isocitrate dehydrogenase activity;

Fig. 33. Organisms from 9 day cultures showing glutamate dehydrogenase activity; and

Fig. 34. Organisms from 15 day cultures showing glutamate dehydrogenase activity.


31


32


33

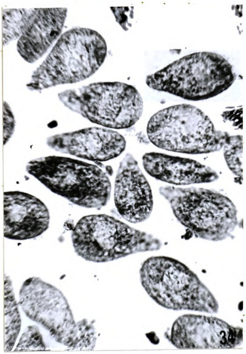

34

PLATE 6

EXPLANATION OF FIGURES

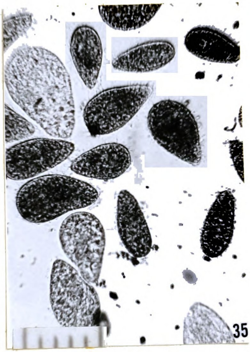
Specimens of Tetrahymena pyriformis W stained to show dehydrogenase activity. Micrometer scale insert graduated in 0.01 mm.

Fig. 35. Organisms from 12 day cultures showing alcohol dehydrogenase activity;

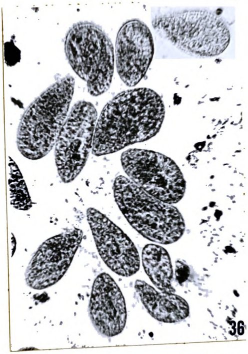
Fig. 36. Organisms from 18 day cultures showing alcohol dehydrogenase activity;

Fig. 37. Organisms from 15 day cultures showing lactate dehydrogenase activity; and

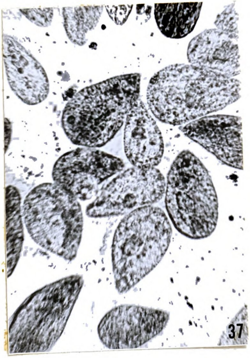
Fig. 38. Organisms from 24 day cultures showing lactate dehydrogenase activity.



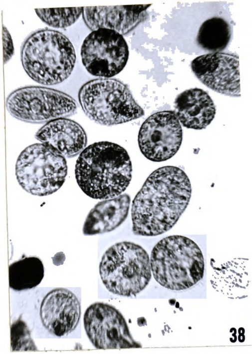
35



36



37



38

PLATE 7

EXPLANATION OF FIGURES

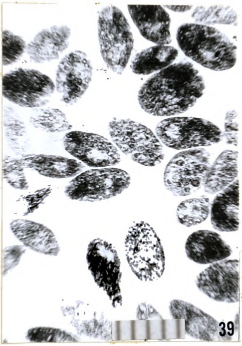
Specimens of *Tetrahymena pyriformis* W stained to show dehydrogenase activity. Micrometer scale insert graduated in 0.01 mm.

Fig. 39. Organisms from 9 day cultures showing malate dehydrogenase activity;

Fig. 40. Organisms from 15 day cultures showing malate dehydrogenase activity;

Fig. 41. Organisms from 12 day cultures showing α-glycerophosphate dehydrogenase activity; and

Fig. 42. Organisms from 18 day cultures showing α-glycerophosphate dehydrogenase activity.


39


40


41


42