Neutral red granules and food vacuoles in Tetrahymena pyriformis

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GRASSMICK, Robert Alan, 1936-  
NEUTRAL RED GRANULES AND FOOD VACUOLES IN  
TETRAHYMENA PYRIFORMIS.  

Iowa State University, Ph.D., 1971  
Zoology  

University Microfilms, A XEROX Company, Ann Arbor, Michigan
Neutral red granules and food vacuoles

in Tetrahymena pyriformis

by

Robert Alan Grassmick

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of

The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Zoology (Protozoology)

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1971
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>Neutral Red Granules</td>
<td>4</td>
</tr>
<tr>
<td>Cytochemistry of Cytoplasmic Granules</td>
<td>6</td>
</tr>
<tr>
<td>Vacuole Formation and Digestion</td>
<td>8</td>
</tr>
<tr>
<td>Synchronous Division</td>
<td>9</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>13</td>
</tr>
<tr>
<td>Growth and Starvation Conditions</td>
<td>13</td>
</tr>
<tr>
<td>Neutral Red Staining and Acid Phosphatase Localization</td>
<td>14</td>
</tr>
<tr>
<td>Food Vacuole Formation</td>
<td>17</td>
</tr>
<tr>
<td>Electron Microscope Observations</td>
<td>19</td>
</tr>
<tr>
<td>RESULTS</td>
<td>20</td>
</tr>
<tr>
<td>Food Vacuole Formation</td>
<td>20</td>
</tr>
<tr>
<td>Penetration of the Food Vacuole by Neutral Red Granules</td>
<td>29</td>
</tr>
<tr>
<td>Fine Structure of Neutral Red Granules</td>
<td>30</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>34</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>51</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>53</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>59</td>
</tr>
<tr>
<td>ABBREVIATIONS AND LINE SCALES</td>
<td>60</td>
</tr>
<tr>
<td>PLATES</td>
<td>61</td>
</tr>
</tbody>
</table>
INTRODUCTION

Corliss (1954b) suggests that Antony Van Leeuwenhoek may have observed a species of *Tetrahymena* in 1676; at least one of his earliest descriptions seems to fit some small ciliate in what is known as the *Colpidium-Glaucoma-Leucophrys-Tetrahymena* group. It is impossible to make any definite statements about the early reports referring to *Tetrahymena* since Corliss (1954a) states that it has been allocated in its past history to more than five genera under some 15 specific names. The full name for the organism used in this study, according to Corliss (1953), should be *Tetrahymena pyriformis* (Ehrenberg, 1830) Lwoff, 1947 following petition for approval from the International Commission on Zoological Nomenclature.

Corliss (1954b) recognized over 500 papers dealing with the genus *Tetrahymena*, written in eight different languages and distributed in more than 100 journals. Many more papers have been added to that list in the past 17 years by the cell biologists, geneticists, physiologists, biochemists, morphologists, and general biologists who have studied, more specifically, *T. pyriformis*.

The popularity of *T. pyriformis* as a research organism is in part due to its adaptability to numerous laboratory experiments. Lwoff (1923) made a major contribution to the domestication of *T. pyriformis* when he isolated a strain of "*Glaucoma pyriformis*" in a non-particulate, axenic medium. This historic feat has made possible a seemingly endless series of investigations in cell physiology and biochemistry.

Lwoff's isolation of *T. pyriformis* in axenic conditions was
surpassed only by the efforts of the many individuals who strived to develop a chemically defined synthetic medium in which to grow *T. pyriformis*. Kidder and his associates are responsible, more than anyone else, for working out the amino acid and vitamin requirements for the organism. It was Seaman (1952) and Slater (1952), however, who independently achieved success in growing *T. pyriformis* in a totally defined chemical synthetic medium. The organism was then available for refined biochemical studies. Regarding the development of a totally defined chemical synthetic medium Elliott (1959) stated, "This was a landmark in nutrition because no 'animal' had been grown on a diet in which every molecule was known." There can be little doubt that the growth of *Tetrahymena* in defined medium is the primary reason for the popularity of the organism in many research laboratories today.

Elliott and Gruchy (1952) and Elliott and Nanney (1952) reported conjugation in *Tetrahymena*, and their findings led to increased research in the genetics and cytogenetics of this ciliate. Study of *Tetrahymena* in so many areas has made the advantages of using the organism apparent. *Tetrahymena* is rapidly becoming as well-known to protozoologists throughout the world as *Paramecium* and *Amoeba*. The Biology of *Tetrahymena* (in press by Appleton-Century-Crofts, New York), edited by A. M. Elliott will soon take its rightful place with such publications as The *Genus Euglena* by Gojdics (1953), The Biology of *Paramecium* by Wichterman (1953), and The Biology of *Stentor* by Tartar (1961).

It is ironic that an organism so widely studied by protozoologists, cellular biologists, cell physiologists and electron microscopists
continues to reveal new information when used for experimental purposes. Prior to this work, an area of study that has been completely neglected in *T. pyriformis* is the relationship of the cytoplasmic granules that stain vitally with neutral red to the intracellular digestion of the organism. Studies of this type on *T. pyriformis* are conspicuous by their complete absence when a comparison is made to similar studies conducted on *Amoeba proteus* and *Paramecium caudatum*.

Lack of information on neutral red granules in *Tetrahymena* led to the initiation of this study. At various times in the past, investigators have suggested that the neutral red granules in protozoa actually represent cytoplasmic bodies containing hydrolytic enzymes and this study is an attempt to relate neutral red stained granules in the cytoplasm of *Tetrahymena* to the mechanisms of intracellular digestion. Intracellular metabolism is universal throughout all levels of the plant and animal kingdoms, and results obtained in this study may be significant in many organisms other than the protozoa. This study was primarily concerned with the old question of whether neutral red granules penetrate the food vacuoles in protozoa, and with the morphological nature of the neutral red granule at the fine structural level. No other publications on the relationship of neutral red granules to digestion in *Tetrahymena* were found at the initiation of this work in 1966 and this remains true at the completion of this study.
LITERATURE REVIEW

Neutral Red Granules

Neutral red stainable inclusions in the cytoplasm and near the food vacuoles of Paramecium caudatum were first reported by Prowazek (1897). Numerous studies since that time have related intracellular enzyme activity in protozoa to the process of digestion within the food vacuoles. Kitching (1956) described many of these studies in a review of food vacuoles and devoted considerable discussion to the numerous studies on neutral red granules.

The granules stainable with neutral red have long been associated with digestive enzymes. Prowazek (1897) termed these neutral red stainable inclusions "Fermentträger," but he did not attempt to explain how the enzymes gain access to the food vacuoles. Nirenstein (1905) stated that in P. caudatum the neutral red inclusions entered the food vacuoles during the acid phase of digestion. His work was supported by that of Rees (1922). Fortner (1926) and Müller (1932) reported the penetration of food vacuoles by neutral red granules in P. caudatum. MacLennon (1941), who used the vital staining technique on numerous species of protozoa, demonstrated that vitally stained granules actually pass into the food vacuoles of many protozoa.

Several investigators have failed to observe the penetration of food vacuoles by neutral red granules. Mast (1947) specifically stated that one vacuole he observed in P. caudatum "throughout practically its entire existence" was never penetrated by a neutral red granule. Hall and Dunihue (1931) reported no relationship between neutral red granules and
food vacuoles in *Vorticella sp.* comparable to that reported in *Paramecium caudatum*. Hall and Alvey (1933) made no mention of neutral red granules penetrating the food vacuoles in *Colpidium colpoda*. Adherence of neutral red granules to vacuoles was not observed by Hall and Nigrelli (1937) in *Paramaecium bursaria*. One of the most recent reports dealing with neutral red granules and food vacuoles in protozoa is that of Rosenbaum and Wittner (1962). In their statements concerning several hundred organisms, Rosenbaum and Wittner report that "at no time were neutral red granules ever seen to actually enter the food vacuole or to subsequently appear within the vacuoles of healthy animals."

Koehring (1930) suggested that neutral red reached the inside of the vacuoles through the "canalicular system" of Cosmovici (1933). Since the entire "canalicular" theory of Cosmovici has been refuted by many workers, it follows that one must accept the theory suggested by Koehring with some reservation. The reports by Hall (1929) on *Feranema trichophorum* refutes the "canalicular" theory in that neutral red granules are present in the cytoplasm even though *F. trichophorum* is primarily autotrophic. Hall (1931) reported that neutral red granules do not collect on the surface of the food vacuoles and apparently do not penetrate the food vacuoles in *Stylonychia sp.* He again refuted the suggestion of Koehring that neutral red enters the cells only along the route normally taken by food particles since he observed neutral red granules in dividing forms of *Stylonychia sp.*, in which feeding activities had apparently stopped. Mast and Doyle (1935) categorized neutral red granules in *Amoeoba proteus* into two groups, one type in the cytoplasm...
and another type within the food vacuole, but they make no mention of passage from one form to another.

**Cytochemistry of Cytoplasmic Granules**

Until the last decade there were very few cytochemical studies on the composition of the cytoplasmic granules in protozoa. One cytochemical procedure that was commonly employed did provide consistent results and was commonly accepted by early workers. Granules that stain with neutral red are known to blacken if exposed to osmium tetroxide (OsO₄) vapors. Once they turn black, the granules cannot be destained with hydrogen peroxide. The latter characteristic separates them from the mitochondria which also blacken upon exposure to OsO₄ vapors but can be reversibly bleached by exposure to hydrogen peroxide. This staining phenomenon is apparently universal in that it has been observed not only in protozoa but in plant cells by Dangeard (1918) and metazoan cells by Parat (1928). Early workers used a variety of methods in their OsO₄ impregnation. Bowen (1928) discussed in detail more than five accepted methods of OsO₄ impregnation in a review article.

More recently, localization of digestive enzymes by cytochemical technique has become a common practice. Gomori (1952) added impetus to this field when he authored a book dealing with principles and practice of microscopic histochemistry. Acid phosphomonoesterase activity has been studied in *Trichonympha turkestancia* by El Mofty (1957), and Rosenbaum and Wittner (1962) studied this group of enzymes in *P. caudatum*. In *T. pyriformis* acid phosphatase localization has been studied by Elliott and Hunter (1951), Seaman (1961a), Elliott and Bak...
(1964), Klamer and Fennell (1963), Elliott (1965), Elliott and Clemons (1966), Elliott and Zieg (1968), and Levy and Elliott (1968). Other workers have studied many other enzymes and the ultrastructure of *T. pyriformis* but their results are beyond the scope of this paper. For further information on the enzyme activity and ultrastructure in *T. pyriformis*, a review is forthcoming in *The Biology of Tetrahymena* (in press by Appleton-Century-Crofts, New York), edited by A. M. Elliott. For a good review of acid phosphatase activity, not only in protozoa but in several plant and animal representatives, there is a comprehensive article by deDuve and Wattiaux (1966).

In many of the studies of enzyme activity, an attempt has been made to identify the activity with feeding or the mechanism of digestion in protozoa. The relation of neutral red to this phenomenon has been almost entirely disregarded in recent years. Rosenbaum and Wittner (1962) attempted to delineate the cytochemical characterization of neutral red granules in *P. caudatum* by indirect procedures. They succeeded in showing a positive correlation, under various experimental conditions, between the neutral red granules and granules which were positive for the acid phosphatase reaction when treated according to the method of Gomori (1952). This same procedure produced a negative correlation when the workers compared neutral red granule distribution to enzymes such as aminopeptidase, *β*-glucuronidase, and non-specific esterases. The only other literature that reports an attempt to correlate neutral red granules with specific organelles is that of Koenig (1965) on rat renal nephrons and neurons. Other than the attempts by Rosenbaum and Wittner (1962) and Koenig (1965),
investigators make only suggestions that the neutral red granules actually represent cytoplasmic bodies containing hydrolytic enzymes. The close association between the two organelles, as revealed from these varied studies, suggests that the function of the neutral red granules is in some way related to digestion within the food vacuoles.

Vacuole Formation and Digestion

Numerous descriptions of food vacuole formation in protozoa have been written by Nirenstein (1905), Rees (1922), Hall (1929), Koehring (1930), Cosmovici (1933), Mast and Doyle (1935), MacLennon (1941) and Mast (1947). A good review of the early work on food vacuoles and nutrition in protozoa was written by Hall (1967).

In the past two decades, protozoologists have attempted to describe the biochemical processes involved in addition to the mechanism of food vacuole formation. Müller and Röhlich (1961), Müller and Törö (1962), and Müller, Röhlich, and Törö (1965) have made major contributions to the study of food vacuoles and protozoan nutrition in Paramecium and Tetrahymena. By numerous short-term observations they have been able to categorize food vacuole formation, digestion, and egestion into four separate intervals. They have elucidated the sequence of events through which a food vacuole passes during its existence and described the time intervals of the events in terms of stages. During Stage I, which lasts about five minutes, a young food vacuole is characterized by the absence of acid phosphatase activity. During Stage II enzyme activity appears at the periphery in a granular form for about 1 1/2 minutes. A high level
of enzyme activity is maintained throughout Stage III. Since the total
time from vacuole formation to egestion is approximately 60 to 90
minutes, by far the largest part of the vacuoles time is spent in Stage
III. Stage IV is referred to as the egestion stage.

Levy (1967), Levy and Hunt (1967), Levy and Elliott (1968), and
Levy, Gollon, and Elliott (1969) have attempted to clarify the pathways
involved in the conversion of cytoplasmic molecules and organelles from
one form to another. They have demonstrated that starving cells of
*T. pyriformis* reach a low level of lipid reserve at 9 hours of star-
vation and that carbohydrate reserves are low by 8 hours of starvation.
Acid phosphatase showed a net decrease in quantity, but specific activity
remained relatively constant during these studies.

**Synchronous Division**

The rhythmic formation of food vacuoles observed in this study is
related to synchronous cell division in the cultures. Synchronized
division in cultures of *T. pyriformis* has been accomplished by numerous
experimental methods. The heat-shock synchronization method was first
described by Scherbaum and Zeuthen (1954). Although Scherbaum and
Zeuthen are given credit by most authors as the first workers to induce
synchronized division in *T. pyriformis*, synchronization was reported
earlier when Browning, Brittain, and Bergendahl (1952) reported a
synchronized "burst" in *Tetrahymena geleii* immediately subsequent to
inoculation in fresh medium.

James and Read (1957) described the effects of incubation temperature
on cell size in cultures of *T. pyriformis*. This work also indicates a
Corbett (1964) demonstrated that synchronously dividing cultures of T. pyriformis are obtainable from the supernatant of cultures centrifuged at 550 g for 6 minutes. This work indicated the presence of a synchronized portion of the population even though the population was not entirely synchronized. Hamburger and Zeuthen (1957) had previously shown that cells synchronized by heat-shock would remain in synchrony for at least three cycles following their resuspension in a saline solution. The work of Corbett takes on more meaning when considered in the light of the statement made by Campbell (1957) in a review of synchronization of cell division in which he stated, "The growth of a population which is the aggregate of all these clones is balanced over arbitrary time intervals, because events in different clones are not in phase with each other." Campbell stressed that there may be synchrony within a clone without it appearing in a total population.

The cultures originally synchronized by the heat-shock method of Scherbaum and Zeuthen remained in synchrony for only three major cycles after which they began to return to the asynchronous condition. Padilla and Cameron (1964) increased the length of time a culture of T. pyriformis could be held in synchronous division. They demonstrated that repetitive synchrony can be induced in Tetrahymena by the use of temperature cyclings within its physiological range coupled with conditions of continuous culture.
A procedure for the synchronization of *Tetrahymena* cell division based upon control of the partial pressure of oxygen in the culture flask was reported by Rooney and Eiler (1967). By alternate exposure to normal growth conditions and hypoxic shock, these investigators closely reproduced the results obtained by Scherbaum and Zeuthen in heat-shock treated cultures. Cells synchronized by thermal shock increased in size while those treated with hypoxic shock were not greatly altered.

Wille and Ehret (1968) produced synchronized division in *T. pyriformis* by a sudden increase in irradiance or by a sudden decrease in irradiance. In their work they obtained positive results only if light transition occurred after some critical time in the late exponential growth phase.

Stone (1968) accomplished synchronization of *Tetrahymena* cultures by first inhibiting their normal mitosis with Vinblastine sulfate (Eli Lilly and Company, Inc.) followed by removal of the inhibitor by washing. Wunderlich and Peyk (1969) induced synchronous division in *T. pyriformis* by inhibiting division with colchicine and Colcemid. The cells entered a synchronous division burst by a phenomenon referred to by the author as "endogenous recovery." This experimental procedure differs from the work of Stone in which the inhibitor, Vinblastine, had to be removed from the culture medium to induce division.

The most recent report of synchronous cell division in *T. pyriformis* is that of Cameron and Jeter (1970). They were able to induce synchrony in dividing cells by subjecting the cultures to 24 hours of starvation.
Following starvation and the return to a normal growth medium, the cultures divided synchronously after 4 hours of feeding.
MATERIALS AND METHODS

Growth and Starvation Conditions

The sexually active strain of *Tetrahymena pyriformis*, variety 1, mating type I, was used throughout this investigation. The original culture was provided in 1966 through the courtesy of Dr. Alfred M. Elliott, Department of Zoology, University of Michigan, Ann Arbor, Michigan. Unless otherwise noted, the cells were grown axenically for 72 hours at 25 C in 500 ml Erlenmeyer flasks containing 100 ml of proteose peptone medium as described by Elliott and Hayes (1953). The original medium, which contained no glucose, was modified by the addition of 1 gm of glucose per liter and the usual 0.1 gm of yeast extract was increased to 1 gm per liter of medium. In the modified medium the culture reached the late log growth phase at approximately 72 hours at 25 C.

When conditions related to starvation were under investigation, 72-hour cultures were axenically washed from the culture medium and resuspended in a sterile inorganic buffer solution first described by Dryl (1959) and referred to throughout this dissertation as Dryl's solution. Washing was accomplished by dispensing the cultures in the peptone medium into 50 ml centrifuge tubes and concentrating the cells into a soft pellet in a clinical-type centrifuge operated for 3 minutes at a speed sufficient to produce 160 g. The supernatant of proteose peptone was decanted and the cells resuspended in the centrifuge tubes by the addition of Dryl's solution. If the cells in the pellet were not immediately resuspended by the addition of Dryl's solution the centrifuge tubes were gently agitated with a vortex mixer. The cultures were centrifuged and
resuspended in Dryl's solution three times to insure complete removal of all nutrient growth medium. The cells were then resuspended in 200 ml of Dryl's solution and returned to specially designed vessels in the 25 C incubator.

Special containers (Fig. 1) and procedures were necessary for starvation studies to make certain that the ciliates obtained after washing were kept in a medium completely free of available nutrient materials. Since the active cells are negatively geotropic they remained near the surface of the inorganic solution above the standpipe where no food material was available but where they could be drawn from the vessel for further experimentation as needed. Even dying cells or cells injured in centrifugation, which would normally be used as food by active ciliates, settled to the bottom of the containers and were unavailable as food for the active cells. Starved cells were removed by means of the pinchcock and tube fitted to the bottom of the vessels; this caused very little agitation within the containers and prevented resuspension of the dead cells and particulate matter which settled on the bottom.

Neutral Red Staining and Acid Phosphatase Localization

The neutral red stain used in this study was Toluylene Red, C. I. number 825. It was obtained as a dark green powder but produced a red color when dissolved in either alcohol or water. The technical name for neutral red is aminodimethylaminotoluaminozine hydrochloride. It has a molecular weight of 288.78, chemical formula of $C_{15}H_{17}ClN_4$, and a molecular structure as follows:
Figure 1. Special vessels for the starvation of T. pyriformis. Dead cells and cellular debris settled to the bottom of the containers. Active ciliates that remained near the surface above the standpipe were drawn out of the containers by releasing the pinchcocks.
Several methods of staining with neutral red were employed by previous workers. All of the cells treated with neutral red in this work were stained in a centrifuge tube. A 6 ml sample of cells was drawn from the culture into a 15 ml centrifuge tube. A Pasteur pipette was used to add 2 drops (0.074 ml) of 0.5% (w/v) aqueous neutral red to the tubes. The contents of the tubes were gently mixed with a vortex mixer and then set aside. After 3 minutes the medium and stain in which the ciliates were suspended was removed. The remaining cells were rinsed three times in Dryl's solution according to the procedure described earlier for removing proteose peptone medium prior to starvation. The end result of this staining process was a large number of cells with well-stained neutral red inclusions suspended in a medium completely free of any neutral red stain. When large numbers of cells were required for investigation, 50 ml of culture was placed in a 50 ml centrifuge tube and 12 to 16 drops (0.444 to 0.592 ml) of 0.5% (w/v) aqueous neutral red added. Healthy stained cultures were maintained for periods up to 7 days and all patterns of feeding, reproduction, and locomotion appeared normal.

For acid phosphatase localization the ciliates were treated according to the method originally described by Gomori (1952) as modified
by Elliott and Zieg (1968). Controls for this procedure were maintained by eliminating the glycerophosphate substrate.

Food Vacuole Formation

The starved cells used in feeding experiments were obtained in the same manner as those used for the starvation studies. To study food vacuole formation, the starved ciliates were removed from Dryl's solution and mixed with equal quantities of "food." For most experiments the "food" was the same proteose peptone medium used for growth of the cultures; however, in the experiments dealing with the rate of food vacuole formation the "food" was a 1.25% (v/v) aqueous India ink solution.

To determine the rate of food vacuole formation, cells were washed and placed in vessels for starvation. Samples were drawn from the container at 1-hour intervals for 28 hours and were then mixed in a ratio of 1:1 with India ink in a Corning deep depression slide which had been thermally equilibrated in a 25 C incubator prior to use. After 15 minutes the cells were removed from the depression slide and immediately fixed with 2% gluteraldehyde solution at 4 C. The fixed cells were then placed on a slide which had quadrants etched upon the surface. A microscope with a 40x bright field objective was used to observe the cells in ten of the randomly selected quadrants. The total number of cells and vacuoles in each quadrant was counted and the mean number of vacuoles for each of the ten quadrants was recorded. Cells were then photographed using a 35 mm camera mounted by a bellows attachment to the camera tube of the microscope. In addition to fixed specimens, living cells were also studied thoroughly and photographed.
To study the fusion of neutral red granules with newly formed food vacuoles, the ciliates were first starved until devoid of all food vacuoles formed prior to starvation. Throughout this experiment care was taken to prevent the contamination of the cultures or depression slides with any extraneous matter that might be stained by neutral red. After 10 to 12 hours of starvation the cells were stained with neutral red. By starving the cells prior to staining, cells were obtained containing neutral red granules but completely lacking food vacuoles. These starved cells containing neutral red granules, but lacking food vacuoles, were mixed with proteose peptone medium which was then incorporated into the cells in newly formed vacuoles. The relationship between food vacuoles and neutral red granules was then studied and photographed. During this experiment, samples of the feeding cells were placed on slides and covered with cover slips which were completely sealed to the slides with melted beeswax. Wet mounts prepared in the described manner allowed for continued observation for 2 to 4 hours.

Statistical analysis of the data concerning food vacuole formation was carried out according to methods of Arkin and Colton (1939) and Snedecor (1956). Three trials of the experiment to determine time of vacuole formation and the number of vacuoles formed were compiled for analysis. The means, modes, and standard deviations were all computed from this compiled data according to the methods of Snedecor (1956). The Student's t test was employed to determine whether a significant difference existed between the means of the 28 samples. Analysis was made between means of consecutive samples and between means of samples.
located at peaks and depressions of food vacuole formation. A model suggested by Arkin and Colton (1939) for linear regression and coefficient of correlation was used to determine whether a significant correlation existed between all of the means and the respective modes derived from the experimental data.

**Electron Microscope Observations**

Specimens were prepared for observation with the electron microscope according to accepted standard procedure. Cells starved for 10 to 12 hours were concentrated into soft pellets with a centrifuge and immediately fixed with 3% gluteraldehyde for 20 minutes or 1% OsO₄ for 30 minutes. Control cells were occasionally fixed in a combination of both gluteraldehyde and OsO₄ as a safeguard against artifacts induced by fixation. The cells were rinsed three times for 30 minutes each in a phosphate buffer at pH 7.3. An alcohol series of 25, 50, 70, 95, and 100% ethyl alcohol, for 5 minutes each, was used to dehydrate the cells. The method described by Luft (1961) was used to embed the cells in Epon 812.

Following polymerization of the Epon 812, the embedded specimens were sectioned with glass knives made on a LKB 7801A Knifemaker and mounted on a LKB 8800A Ultrotome III. Only sections within the range of 60 to 100 nanometers (nm) were picked up on 150 mesh Formvar coated copper grids. Sections were stained with methanolic uranyl acetate or uranyl acetate followed by lead citrate according to Reynolds (1963). Prepared specimens were examined with an RCA Model EMU-3F electron microscope at 50Kv.
RESULTS

Food Vacuole Formation

Some initial studies were made to determine the times at which *T. pyriformis* most actively form food vacuoles. It was easily demonstrated that cells removed from proteose peptone culture medium and placed in sterile Dryl's solution would void themselves of all proteose peptone food vacuoles in less than 4 hours. Cells returned to proteose peptone medium or India ink at random times following starvation did not always readily form new vacuoles. To determine the time at which food vacuoles were most actively formed, a series of experiments was designed to observe food vacuole formation in organisms of a starving culture at 1-hour intervals for 28 hours. The time at which the cells were removed from the proteose peptone medium by washing was designated 0-hour of starvation. After the cultures had been washed free of the proteose peptone medium and resuspended in Dryl's solution, samples were removed from the starvation vessels at 1-hour intervals and mixed with India ink for 15 minutes to allow time for vacuole formation. The cells were then fixed with 2% gluteraldehyde and scored for the number of black food vacuoles present. The mean number of vacuoles formed the first time the experiment was attempted provided some very interesting data (Fig. 2, Trial 1). The number of vacuoles formed from the India ink particles remained very low for the first few hours following 0-hour of starvation and then reached a peak at 7 hours of starvation. This peak was followed by a decline in vacuole formation at 8 hours of starvation. The increased
Figure 2. The results of three independent trials illustrating the mean number of India ink vacuoles formed by ciliates starved for periods up to 28 hours. Note the number of feeding peaks that fall near a 4-hour rhythm beginning with 7 hours of starvation.
and decreased feeding activity then followed a rhythmic pattern of 4-hour intervals with an approximate 2-hour period separating the high and low periods of activity.

To lend support to the initial data the experiment was repeated two more times, under the same conditions, with basically the same results (Fig. 2, Trials 2 and 3). During the three trials it was noted that a considerable number of cells in the culture during the low periods of feeding activity were in a state of fission (Fig. 14). Although the cells in fission were not counted, they appeared often enough to indicate that more dividing cells were present during low periods of feeding activity than during high periods of feeding activity.

The results obtained from the three trials were compiled for statistical analysis of dependability. The compiled data were used to determine the range, mode, mean, and standard deviation of the mean for each period of starvation (Table 1). It is significant to note that none of the ranges for standard deviations of the means overlapped when adjacent periods of high and low feeding activity, separated by 2-hour intervals, were compared (Fig. 3, bottom graph). The difference between the standard deviations of the means indicated a significant difference in the number of vacuoles formed even though the means for the number of vacuoles formed in the periods of low feeding activity were arithmetically very near to the means for the number of vacuoles formed during any given period of high feeding activity.

Statistical analysis supported the relationships suggested by the bottom graph on Fig. 3 that the feeding activity of starved cells was
Table 1. The standard deviations of the means, ranges, modes, and means of the numbers of vacuoles formed by starving *T. pyriformis*.

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\(^a\) Data from 20 observations, two trials. All other data from 30 observations, three trials.
Figure 3. The relationship between the modes and means of vacuoles formed during 15 minutes of feeding following starvation for periods up to 28 hours. Lower graph illustrates statistical significance of samples showing no overlap between the standard deviations of the means at peak periods and those at low periods of vacuole formation.
cyclic. When analyses between means of consecutive periods of starvation were conducted, most of the intervals showed no statistical difference (Table 2). When comparisons were made between means from samples observed at the high and low levels of feeding activity, most comparisons demonstrated a significant difference (Table 3).

The tabulated information in Table 1 illustrated a statistical relationship between the mode and mean of the number of vacuoles observed during any given period of starvation. When the mean for the number of vacuoles formed was high at certain hours of starvation (i.e., 7, 15, and 23 hours), the mode for the vacuoles formed was also quite high. There was also a correlation between low modes and means for the vacuoles formed (i.e., 9, 20, and 28 hours of starvation). The correlation between the mode and mean for any given period of starvation was more apparent when observed on one graphic illustration (Fig. 3, top and bottom).

The modes and means for the vacuoles formed were analyzed statistically. Linear regression and the coefficient of correlation were determined to compare the mean number of vacuoles formed at each sample period during starvation with the mode for the vacuoles formed during that same period. The line of linear regression, along with a 95% confidence interval for the line, is shown in Fig. 4. All of the sample comparisons except one fell within the 95% confidence interval. The one point not falling within the above range was the sample of cells taken at 1 hour of starvation at which time India ink vacuoles were never formed. This was, nevertheless, a valid sample as it occurred in each trial and
Table 2. A comparison between the mean numbers of food vacuoles formed at successive 1-hour intervals of starvation

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*No significant statistical difference between means.
Table 3. A comparison between the mean numbers of food vacuoles formed at high and low periods of feeding activity

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^aNo significant statistical difference between means.

^bSignificant statistical difference between means at 95% confidence level. All other figures shown are significant at, or above, 95% confidence level.
Figure 4. Graph of linear regression illustrating significant correlation between the means and modes from Table 1. Shaded area represents 95% confidence interval. Coefficient of correlation equals 0.729.
could not be discarded. The coefficient of correlation between the modes and means was 0.729, a value well above the point of question for biological materials.

Penetration of the Food Vacuole by Neutral Red Granules

Following the observation that the highest level of food vacuole formation occurred at 11 hours of starvation, an experiment was designed to study the relationship between neutral red granules and food vacuoles. Cultures starved in Dryl's solution for 10 hours and completely void of any food vacuoles were stained with neutral red. The granules which stained red were normally located in the posterior portion of the cells (Figs. 6, 10, and 12). Two types of granules stained with neutral red, those which stained around the periphery and those which stained as solid red spheres (Figs. 6, 15, and 17). When the stained cells lacking food vacuoles were then reintroduced into proteose peptone medium 1 hour after staining they immediately formed numerous clear proteose peptone vacuoles. The vacuoles were quickly surrounded and, in less than 25 minutes, penetrated by most of the neutral red granules (Figs. 7, 13, and 15). Both types of stained granules entered the food vacuoles.

Cells starved for 18 hours did not have neutral red granules dispersed in the posterior portion of the cytoplasm but rather had them evenly distributed throughout the cytoplasm in the peripheral area just under the cell membrane (Figs. 17 and 18).

Since most of the cells used for this work were starved for 10 1/2 to 11 hours, cells treated for this period of time were selected for the cytochemical localization of acid phosphatase activity. Cells starved
for 10 1/2 to 11 hours showed a definite positive reaction for acid phosphatase in the posterior region of the cells (Fig. 11). No acid phosphatase activity was observed anterior to the area of the micronucleus and macronucleus. The positive reaction for acid phosphatase occurred in the same region in which neutral red granules appeared in the control cells (Figs. 10 and 12).

Fine Structure of Neutral Red Granules

Cells of *T. pyriformis* starved 10 1/2 hours were selected for detailed study of fine structure for a number of reasons. The initial work of this paper demonstrated that in less than 4 hours starving cells void themselves of the food vacuoles formed prior to the onset of starvation. Biochemical analyses of starving *T. pyriformis* by Levy and Elliott (1968) demonstrated that most of the stored lipids, carbohydrates, and proteins were depleted by 8 to 9 hours of starvation. The portion of this study devoted to food vacuole formation indicated that starving cells most actively form food vacuoles at 11 hours of starvation. It was apparent that, if the cells were starved for 10 to 10 1/2 hours before observation with the electron microscope, they would probably be in the process of switching from the use of normal stored products for self-maintenance to some other intracellular mechanism necessary for survival under conditions of starvation.

Very interesting fine structural detail was observed when cells of *T. pyriformis* were starved for 10 to 10 1/2 hours. Large fields of rough endoplasmic reticulum were commonly observed in the perinuclear region (Figs. 19, 20, 21, and 22). These fields were commonly seen enclosing,
or adjacent to, mitochondria. Vesicles commonly gave the appearance of coming from the rough endoplasmic reticulum (Figs. 21 and 22).

Other organelles appeared with nearly the same frequency as the fields of rough endoplasmic reticulum. These were dense membrane bound bodies containing material similar to, but often more concentrated than, the ground cytoplasm. The bodies containing concentrated cytoplasm were often seen near the fields of rough endoplasmic reticulum, but more commonly in a close spatial relationship to a mitochondrion (Figs. 23, 24, 25, and 30).

Mitochondria in 10 1/2 hour starved cells were primarily located in two areas of the cytoplasm; posterior to the macronucleus (Fig. 42), or underlying the region of the cell membrane (Fig. 26). Mitochondria with a normal appearance were quite common in the cells, but various stages of degenerating mitochondria were also apparent. A series of stages of mitochondrial degradation was observed. The mitochondria engulfed ground cytoplasm containing small pieces of rough endoplasmic reticulum (Figs. 27 and 28) or engulfed the vesicles, described earlier, which had previously engulfed material similar to the ground cytoplasm (Figs. 29, 30, 31, 32, and 33). Occasionally, mitochondria were observed that contained not only ground cytoplasm and membrane bound structures containing cytoplasm (Fig. 35), but pieces of material that were obviously canalicular-type rough endoplasmic reticulum (Fig. 36).

Mitochondria in extreme stages of degradation were observed (Figs. 37, 38, 39, and 40) and some of them were enclosed in membranes with obvious cytoplasmic material (Fig. 40). Occasionally a mitochondrion
was seen containing another mitochondrion, the latter being in a later stage of degradation than the mitochondrion in which it was enclosed (Fig. 41).

When *T. pyriformis* were starved for 10 1/2 hours, fixed with *OsO₄* and observed with the electron microscope, only one group of organelles had the same size range, posterior location, and appeared with the same frequency as the solid neutral red granules in living *T. pyriformis*. The organelles that correlated with the solid neutral red granules had the appearance of degenerating mitochondria. At low magnification the structures were very dense since they were very osmophilic (Fig. 42). At high magnification it was possible to resolve some structural detail within the organelles (Fig. 45), but the true nature of the organelles was not revealed until the negative images obtained with the electron microscope were printed on positive photographic paper. The true identity of the structures was not detected during the early stages of this investigation since normal exposure and development of the positive photographic paper produced dense black bodies which appeared to be solid amorphous masses (Fig. 49). When the technique of overexposing and under-developing the positive photographic paper was applied, the exact nature of the organelles became apparent (Figs. 37, 38, 39, 40, and 46). The degenerating mitochondria appeared dense, round, and slightly smaller in size than the normal mitochondria. How the method of printing altered the appearance of the organelles is apparent on Figs. 46, 47, 48, and 49. All of the prints were made from the same negative on the same positive photographic paper; the only variation was in the time of exposure and
length of development of the paper.

The concentration of neutral red used in all of the experiments had no visible detrimental effect on the protozoa when they were observed alive or when they were fixed and observed with the electron microscope. Cultures stained with the vital stain remained in good condition up to 7 days and all patterns of feeding, reproduction, and locomotion appeared normal. The cells stained with neutral red prior to fixation for electron microscope observations (Figs. 20, 22, 23, 24, 30, 33, 38, 40, and 41) showed no apparent difference, at the fine structure level, from the unstained controls (Figs. 19, 21, 25, 26, 29, 30, 31, 32, 34, 35, 36, 37, and 39).

Two organelles were observed at the electron microscope level that had a strong affinity for OsO₄. The bodies containing concentrated cytoplasm (Figs. 23, 24, 25, and 30) were enclosed by what appeared to be a membrane which was strongly osmophilic. The other organelles, demonstrating a strong affinity for OsO₄, were the degenerating mitochondria (Figs. 37, 38, 39, 40, 41, 42, 43, 44, and 45). Both of these organelles correlated with the size and distribution of the neutral red granules observed in living cells.
This study was made to relate the neutral red granules observed in the cytoplasm of *T. pyriformis* to the mechanisms of intracellular digestion. The fine structure of the neutral red granules was observed with the electron microscope. To study the relationship of the neutral red granules to intracellular digestion however, it first became necessary to study the feeding patterns of *T. pyriformis*. The initial attempt to study feeding patterns was to observe numerous cells taken, during their late log growth phase, from proteose peptone medium. This procedure proved unsuitable for the study of neutral red granules and their relationship to food vacuoles since all of the cells were filled with food vacuoles upon removal from the culture medium. When these cells were stained with neutral red it was impossible to determine whether the neutral red granules were surrounding freshly formed vacuoles or vacuoles nearly ready for egestion. A procedure was developed in which the protozoa were placed in sterile Dryl's solution and allowed to digest and egest all of the vacuoles within their cytoplasm. This method of obtaining cells free of vacuoles was used throughout the remainder of the study. The geotropic behavior of *Tetrahymena* was utilized in the experimental plans to obtain completely starved animals, since the active cells could be easily removed from the vessels at desired intervals without mixing them with injured cells or cell particulate matter from the bottom of the containers. The physical separation between the active ciliates and the injured and dead cells insured that the active cells remained in a true state of starvation. Without this procedure, true starvation was more
difficult to obtain than originally anticipated because active *T. pyriformis* became cannibalistic and formed food vacuoles from cytoplasm or particulate matter of injured cells.

After a procedure to obtain large numbers of active starved ciliates was developed, the starved cells were stained with neutral red and the method by which neutral red enters a cell was investigated. The method by which neutral red enters a cell is still open to question but this study illustrates conclusively that it does not enter the cells through the oral apparatus. The large number of dividing cells observed during periods of low feeding activity served as an aid in studying the method of entry of neutral red into the cells. Samples stained with neutral red, observed, and photographed during periods of low feeding activity contained numerous cells in fission and they stained as readily with neutral red as non-dividing stages. Since the oral parts of *T. pyriformis* degenerate during fission and do not regenerate until late in the process, it is apparent that dividing cells stained during the early stages of division must incorporate the neutral red by some method other than through the oral apparatus. The entrance of neutral red into the cells by some mechanisms other than the oral apparatus is in direct disagreement with the suggestion of Koehring (1930). However, because the entire "canalicular" theory upon which Koehring based her work has been refuted, the dividing forms that stained with neutral red in this work should be accepted as evidence that neutral red does indeed enter the cells along some pathway other than the oral apparatus. This would also agree with the work of Hall (1929) who found that neutral red entered *P. trichophorum,*
an autotroph with no oral apparatus.

The presence of neutral red granules in both well fed cells and cells starved for 12 to 18 hours indicates that neutral red does not stain food storage compounds such as carbohydrates or lipids. This conclusion agrees with the biochemical analyses of Levy and Elliott (1968) in which food storage compounds were depleted by approximately 9 hours of starvation.

The fact that the distribution of neutral red granules in feeding cells correlates with the localization of acid phosphatase activity also indicates that the granules stained by neutral red are not stored reserves but, rather, are particles related in some way to enzyme activity. This suggestion is not new since numerous investigators have suggested that neutral red granules actually represent bodies of hydrolytic enzymes, and the close association of neutral red granules to the food vacuole logically suggests their function to be that of producing digestion within the vacuole. It is highly possible that some of the neutral red granules observed in protozoa are actually the "lysosomes" of deDuve and Wattiaux (1966).

To study the relationships of neutral red granules to food vacuoles, the cells were starved to void them of any old food vacuoles; they were then stained with neutral red followed by feeding with proteose peptone medium. The expected, immediate formation of a large number of new vacuoles, did not always occur and in fact was non-predictable in the pilot studies. True knowledge of the feeding patterns involved in starving T. pyriformis was not gained until an experiment was designed to
determine when this organism actually forms food vacuoles. When samples of the starving cells were fed every hour with India ink, the \( \frac{1}{4} \)-hour cyclic feeding pattern became apparent. The feeding pattern is obviously related to the patterns of cell fission within the starving population. As stated previously, *Tetrahymena* has no oral apparatus during fission since the apparatus degenerates early in the process and does not appear as a functional structure until near the end of fission. Since the temperature and medium used in this work was conducive to cell division every \( \frac{1}{4} \) hours, it is not surprising that the feeding pattern followed a \( \frac{1}{4} \)-hour cycle. When the cells of the population are actively forming food vacuoles they are not in a state of fission and, conversely, when they are in fission they are not able to form food vacuoles. Synchronized cell division in *T. pyriformis* is not new since it has been produced by various methods employed by Browning, Brittain, and Bergendahl (1952), Scherbaum and Zeuthen (1954), Corbett (1964), Wille and Ehret (1968), and Cameron and Jeter (1970). However, synchronized vacuole formation of a population has not previously been demonstrated in *Tetrahymena*. The synchronous vacuole formation is probably an indirect result of the centrifugation process used to remove the cells from the proteose peptone medium just prior to starvation. Corbett (1964) demonstrated that cultures could be partially synchronized in their division by the process of centrifugation. Cells in fission are retained in the pellet formed in the conical tip of the centrifuge tube while non-dividing stages can be decanted and lost with the supernatant. The removal of specific stages of division from the culture leaves a partially
synchronized culture entering the starvation process. The cells were full of proteose peptone vacuoles and stored chemical reserves and, for the first 5 to 6 hours following removal from the growth medium, they did not feed actively when offered India ink as "food." As old food vacuoles were gradually utilized, which took slightly over 4 hours, the India ink was more actively incorporated into new food vacuoles. The synchronized feeding behavior of the cells became more apparent after food vacuole formation reached a peak at 7 hours of starvation.

When the last set of experiments on the relationship between neutral red granules and food vacuoles was designed, all of the information known about the nutrition and feeding behavior of T. pyriformis was incorporated into one experiment. Pilot studies in this work had previously shown that the old vacuoles in starving cells were eliminated by approximately 4 hours of starvation. Levy and Elliott (1968) had shown that most of the stored chemical reserves are utilized by 9 hours of starvation, and detailed sampling and feeding in the present study indicated a high level of feeding activity at 11 hours of starvation. It was apparent that cells starved 10 1/2 to 11 hours would have no food vacuoles remaining from the original proteose peptone culture medium, would be completely lacking in stored chemical reserves, and would rapidly form food vacuoles. It was desirable to have large numbers of cells in this condition so the cells were cultured, starved, and stained with neutral red in quantity. The centrifuge method of staining employed in this study is completely different than the methods used by previous workers. Hopkins (1938) stained cells for his work by adding neutral red directly
to the growth medium at concentrations of one part or less of neutral red to 60,000 parts of medium. Hall (1931) added a drop of culture medium containing cells to a slide which had been previously coated with a neutral red solution and allowed to air dry. He also used the method of Hopkins and added neutral red directly to the culture medium. Procedures similar to those used by Hall were employed by Koehring (1930) and Dunihue (1931). Rosenbaum and Wittner (1962) used a modification of Hall's slide technique by applying the cells to a slide along with a drop of 0.925% neutral red dissolved in absolute ethyl alcohol. They also obtained results by adding one drop of 0.025% aqueous solution to 10 ml of medium containing living organisms. The vital staining methods employed by previous workers had one of two apparent disadvantages. Using slides coated with a dry film of neutral red provides a very small sample of stained cells. The other frequently used method, that of staining cells directly in the culture medium without removing the excess stain, makes it very difficult to ascertain whether new food vacuoles obtain their red color indirectly by coalescing with neutral red granules or directly from the neutral red stain in the surrounding medium. Staining ciliates for this study by employing the centrifuge method not only provided very consistent and dependable results but also had the advantage of providing a large number of stained cells. The centrifuge method of staining cells is quite simple and the stained cells exhibit no apparent impairment from the process.

Large numbers of cells uniformly containing neutral red granules and completely lacking food vacuoles were a major tool in this study. Cells
in this condition removed from the vessels in which they were starved were observed and photographed, and the distribution of neutral red granules was determined. The cells were then mixed with fresh proteose peptone and constantly observed with the light microscope. Neutral red granules were observed to surround the new proteose peptone vacuoles and actually penetrate them within a very short time. This observation clearly re-enforces the suggestion stated previously that neutral red granules represent dynamic organelles related to intracellular digestion.

The penetration of food vacuoles by neutral red granules observed in this work agrees with similar observations by Nirenstein (1905), Rees (1922), Fortner (1926), Müller (1932), and MacLennon (1941). Some of the past disagreement over whether or not the neutral red granules penetrate the food vacuole no doubt arose because of the type of food used in the formation of food vacuoles. Prior to the period of axenic cultures and chemically defined media, it was standard procedure to feed protozoa other protozoa, yeast, or bacteria. The organic food sources used by early investigators studying neutral red probably led to much confusion because some of the organisms used for food themselves stained with neutral red. Apparently some early workers suggested the food vacuoles were red due to the penetration of neutral red granules; other workers attributed the red color of the vacuoles to the direct staining of the organism used for food and concluded that vacuole color was in no way related to the penetration of the vacuoles by the neutral red granules from the cytoplasm.

Another problem in interpretation was probably encountered by some
of the early workers who used inert materials for "food." Rosenbaum and Wittner (1962) suggest that *Paramecium* will form food vacuoles upon ingesting digestible as well as non-digestible particles but that some degree of selectivity was observed in the intracellular response to the two types of vacuoles. In their work the organisms forming food vacuoles on China black retained the same distribution of neutral red granules as did the starved cells. Thus, no neutral red granules were observed to penetrate the vacuoles of China black. The variation in types of "food" used probably accounts for some of the early reports of penetration and non-penetration of food vacuoles by neutral red granules.

The use of *Tetrahymena* and proteose peptone in the present study helped surmount the obstacles which confronted all of the previous workers attempting to determine whether neutral red granules actually penetrate food vacuoles. *Tetrahymena* cultured, starved, and stained with neutral red under axenic conditions could be observed forming vacuoles when mixed with proteose peptone. The freshly formed proteose peptone vacuoles were not only composed of digestible material, but they were large and clear since they had not previously been exposed to neutral red stain. As the neutral red granules migrated to the periphery of the newly formed food vacuoles they could be easily observed with the light microscope, and within less than 10 minutes some of the neutral red granules appeared within the food vacuoles. The position of the neutral red granules could be accurately determined by carefully focusing the high-power objective. The neutral red granules were demonstrably within the limits of a food vacuole when the periphery of the larger food vacuole
and the periphery of the smaller neutral red granule located within the food vacuole were both clearly seen in the same plane of focus (Fig. 7).

Some observations were made in this work to characterize the nature of the neutral red granules. It was impossible to demonstrate conclusively that neutral red granules possess enzyme activity, because with the Gomori technique, it was not possible to preserve neutral red while at the same time retaining acid phosphatase activity. The reverse was also true; since neutral red is a vital dye it could not be applied to dead organisms stained first for acid phosphatase activity. The localization of the acid phosphatase activity did, however, parallel the distribution of neutral red granules. The bodies containing concentrated cytoplasm and the degenerating mitochondria observed in this study correlate with structures observed by numerous previous workers such as Elliott (1965), Elliott and Bak (1964), Elliott and Clemons (1966), and Levy and Elliott (1968) which are positive for acid phosphatase activity.

Another cytochemical procedure employed in this study suggests that the bodies containing concentrated cytoplasm and degenerating mitochondria observed with the electron microscope are the equivalent of the neutral red granules observed at the light microscope level. The history of the second procedure employed is not only of value, but also of historical interest. The major conflict over neutral red granules in protozoa was debated in the literature throughout most of the 1920's and 1930's. The major interest in the question was then dropped in the late 1930's apparently for lack of a suitable method to obtain a definite answer to the problem. One significant paper by Mast appeared in 1947, and the
more recent work of Rosenbaum and Wittner (1962) attempted to correlate the neutral red granules with a definite enzyme group. However, no investigators have attempted to describe the morphology of the neutral red granules.

A fixation technique employed by many early workers was put to significant use in this work. In the past, workers studying neutral red commonly fixed cells with OsO$_4$ vapors while observing the cells through a light microscope. All of the early reports agree that the neutral red granules in protozoa are osmophilic and turn deep black when exposed to OsO$_4$ vapors. This concept was well established prior to the advent of the electron microscope. Since OsO$_4$ is one of the major fixatives used in preparing material for the electron microscope, it follows that some old staining techniques applied to a new research instrument could help solve an old problem. Cells stained with neutral red and fixed with nothing other than OsO$_4$ consistently showed only two organelles that were highly osmophilic, the bodies containing concentrated cytoplasm and the degenerating mitochondria. These organelles correspond in size and distribution to the neutral red granules observed in living cells with the light microscope. The fact that two types of organelles falling in the same size range were highly osmophilic suggests that these organelles correspond to the two types of neutral red granules observed in living cells. The solid dense neutral red granules in living cells correlate in size and distribution with the degenerating mitochondria; the neutral red granules that appear the most red around the periphery of the granule correlate with the bodies containing concentrated cytoplasm that have a
highly osmophilic limiting membrane. The results obtained in this study indicated that staining with neutral red prior to fixing the cells with OsO₄ did not make the organelles more osmophilic. Controls not stained with neutral red contained highly osmophilic organelles as did the cells stained first with neutral red. It is evident that the characteristic of the granules that makes them highly osmophilic is probably the same characteristic that binds neutral red stain in the living cells. The neutral red granules that blackened when exposed to OsO₄ vapors by early workers would have darkened without prior staining with neutral red.

The electron microscope observations in this study indicate that normal mitochondria actively engulf pieces of rough endoplasmic reticulum directly from the ground cytoplasm or indirectly by engulfing bodies containing concentrated cytoplasm, the lysosomes of Elliott (1965), which already have pieces of rough endoplasmic reticulum within their limiting membranes. Either of these two processes provide a method by which normal mitochondria could obtain the hydrolytic enzymes necessary for the formation of initial autophagic vacuoles. Elliott and Bak (1964) observed mitochondria similar to two of the stages observed in this study and shown in Plates VII and VIII. They suggested that some of their electronmicrographs illustrated "donut shaped" mitochondria with ground cytoplasm both inside and outside the circular mitochondria. Since Elliott and Bak observed only a small number of these unusual organelles in aging cultures, they did not conclude that the mitochondria were actively engulfing cytoplasm as demonstrated in this study (Figs. 29, 30, 31, and 32). Although the culture conditions are quite different, it is
interesting that not only circular mitochondria but also the degenerating mitochondria were observed in both studies when the cells were under conditions of cellular stress. The individual degenerating mitochondria probably coalesce to form the membrane bound aggregates of several mitochondria observed by Levy and Elliott (1968). Their work demonstrated acid phosphatase activity within the membrane bound aggregates, and their results essentially agree with the observations of this study. The hydrolytic enzymes are retained within the starving cells by coalescence of degenerating organelles with normal organelles (Fig. 41). The continued coalescences and digestion of these organelles could lead to the accumulation of residual bodies observed by several workers. Residual bodies are highly osmophilic membrane packed structures observed in both metazoan and protozoan cells. In their recent review article deDuve and Wattiaux (1966) suggest that residual bodies are depositories for undigestible membranes.

Some of the degenerating mitochondria in Tetrahymena were located in the periphery of the cell following starvation, and their location correlates with the peripheral distribution of neutral red granules in Tetrahymena starved for 18 hours. These results in Tetrahymena are similar to those obtained by Rosenbaum and Wittner (1962) and possibly account for the peripheral distribution of the neutral red granules they observed in Paramecium starved for 24 hours.

The electron microscope observations in this study provide some clues to the answer of another controversial question appearing in some of the early literature on neutral red granules. Dunihue (1931) and
Hall (1931) agreed that neutral red and Janus green B were specific for two different organelles in the cytoplasm of protozoa. Hopkins (1938) observed bodies, each of which seemed to be divided into two areas, with one area positive for neutral red stain and the other for Janus green B. The early stages of mitochondrial degeneration observed in the present investigation would account for the earlier reports by investigators. At a very critical time the normal portion of the degenerating mitochondrion would probably stain with Janus green B while the membrane of the body containing concentrated cytoplasm would stain with neutral red. The identity of a structure staining in this manner could not be resolved at the level of the light microscope.

The true nature of the degenerating mitochondria remained unclear during the early stages of this investigation. When positive electronmicrographs are printed the mitochondria and ground cytoplasm are commonly used as criteria for determining the quality of fixation of the biological material and contrast or tone of the prints. The true nature of the highly osmophilic degenerating mitochondria was not detected until the criteria normally used for printing positive electronmicrographs were disregarded. The method employed in this study was to overexpose and underdevelop the positive paper used for making electronmicrographs. This technique, although causing the mitochondria and ground cytoplasm to appear very unclear, made the true nature of the highly osmophilic granules more apparent (Figs. 46, 47, 48, and 49).

The schematic drawing (Fig. 5) is a composite of several investigations illustrating the stages of mitochondrial degeneration and possible
Figure 5. Schematic illustration of the relationship between mitochondria and cytoplasm containing bodies. This is a composite from the works of several authors. Stages C, E, and F are reported in detail for the first time in this study. Stages C, E, F, and H correspond to figures on Plates VI, X, IX, and III, respectively.
site of production for the bodies containing concentrated cytoplasm. Stages designated as A and B on the schematic drawing have been suggested by the work of Levy and Elliott (1968) and Elliott and Clemons (1966). Stage A represents a lysosome fusing with a forming food vacuole, and stage B illustrates a lysosome fusing with pinocytic vesicles. A stage not illustrated on the drawing was observed and misinterpreted by Elliott and Bak (1964), but it was clearly demonstrated in this study to be a mitochondrion engulfing cytoplasm (Figs. 27, 28, 29, 30, 31, and 32). The stage designated as G on the illustration has been demonstrated by Elliott, Travis, and Work (1966). Stage G represents several degenerating mitochondria inside one autophagic vacuole. The site designated as H has been observed by Levy, Gollon, and Elliott (1969) and in this study (Fig. 22); it is the area where bodies containing concentrated cytoplasm are commonly seen pinching off from fields of rough endoplasmic reticulum. Stages C, E, and F have not been described in detail prior to this study and correspond to the figures on Plates V, VI, X, and IX. Stage D was observed by Elliott and Bak (1964) and was commonly seen in material used for this investigation. Stage D represents the dense degenerating mitochondria. The entire schematic composite presents a modification of the proposed mechanism of intracellular digestion in animal cells suggested by deDuve and Wattiaux (1966).

Elliott and Clemons (1966) suggested a mechanism of intracellular digestion for T. pyriformis, but they presented very little evidence demonstrating a mechanism for the acquisition of hydrolytic enzymes
by mitochondria destined to become autophagic vacuoles. Levy and Elliott (1968) presented more electronmicrographs depicting autophagic vacuoles containing degenerating mitochondria but stated "how the enzyme enters the vacuole or how the vacuole is formed remain unknown." The electronmicrographs obtained in this study definitely suggest a route by which hydrolytic enzymes could enter the mitochondria.

The stages of mitochondrial degradation observed in this study were probably overlooked by other workers due to the length of time the cells were starved prior to fixation for observation with the electron microscope. All electronmicrographs in publications prior to this study depict organelles from cells starved much less than 10 hours, or longer than 12 hours. Failure of most research workers to observe *Tetrahymena* at 10 to 12 hours of starvation is the probable reason that only stages A, B, D, G, and site H of Fig. 5 have been observed in detail prior to this study; they all occur before 10 hours or after 12 hours of starvation. As mentioned earlier, the period of 10 to 11 hours of starvation is very significant in cultures of *T. pyriformis* because at this time they no longer contain any stored reserves and must change to another form of intracellular digestion. The alteration in feeding behavior observed originally at the light microscope level thus suggested a change in intracellular digestion and indications of the change were actually visible at the fine structural level in *T. pyriformis*.

Neutral red granules have been described in several different protozoa by Kitching (1956), but it is of interest to consider some of the other situations where enzyme activity has been correlated with
neutral red granules in other kinds of cells. Ogawa, Mizuro, and
Okamoto (1961) demonstrated the correlation of acid phosphatase activity
with neutral red granules in neural cells grown in culture. Lacy and
Chalice (1957) related what they called neutral red bodies to the
topographical location of the Golgi apparatus in vertebrate cells.
Although the mechanisms involved in the staining of neutral red granules
have never been definitely established, information is beginning to
accumulate from several areas of research on neutral red granules which
seems to support the term "Fermentträger" suggested by Prowazek over
70 years ago.
SUMMARY

1. *T. pyriformis* has an inherent feeding rhythm which is directly related to a division rhythm.

2. *T. pyriformis* changes from the mechanism used for the digestion of stored foods to a mechanism for digesting cytoplasmic organelles at approximately 10 hours of starvation at 25°C. A correlation between the change in feeding patterns and a change in morphology of several cell organelles is evident at the same period of starvation.

3. The morphology of the degenerating organelles in starving *T. pyriformis* is most apparent when the electron microscope negatives are used to produce underdeveloped positive prints.

4. The location of neutral red granules in *T. pyriformis* is variable and depends upon the nutritive state of the organism. In cells actively forming food vacuoles or starved a short time, most of the granules are located in the posterior portion of the cells. In cells starved for over 12 hours, vitally stained granules are distributed throughout the periphery of the cell.

5. Actively feeding organisms show an accumulation of neutral red granules around the forming food vacuoles. The neutral red granules enter the food vacuoles.

6. Two types of organelles apparent with the electron microscope are highly osmophilic and agree in size and distribution with neutral red granules. The bodies containing concentrated cytoplasm and degenerating mitochondria are both highly osmophilic, a characteristic of neutral red granules long recognized by individuals experimenting with
neutral red.

7. Neutral red does not enter the organisms through the oral apparatus. Cells having no oral apparatus during fission stain as well with neutral red as do actively feeding stages.

8. Neutral red does not stain food reserves such as carbohydrates or lipids in *T. pyriformis* because most of the organisms observed in this study had depleted their stored food reserves prior to staining.

9. The distribution of acid phosphatase activity within the organisms correlates with the distribution of neutral red granules. This agrees with numerous other reports that neutral red stains organelles containing hydrolytic enzymes.

10. The distribution, behavior, and morphology of the neutral red granules in *T. pyriformis* suggests that these cytoplasmic components play a significant role in the digestion of food vacuoles as well as digestion in autophagic vacuoles.
LITERATURE CITED


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ACKNOWLEDGMENTS

I sincerely thank Dr. Benton W. Buttrey for making his research facilities available to me throughout most of this study and his editing of this dissertation, and other publications, has been most helpful. His concern for my personal welfare the past four years has been deeply appreciated.

Drs. David R. Griffith, Nels R. Lersten, John A. Mutchmor, and Martin J. Ulmer will always be remembered by me as receptive, courteous, and willing to give advice whenever I solicited their aid. Their criticism and suggestions on my research and dissertation were of great value to me.

I would be remiss if I should not remember Dr. Alfred M. Elliott, now retired from the Department of Zoology, University of Michigan. It was he who originally stimulated and encouraged my interest in protozoology.

My wife, Mary Alice, has been a great help in typing and organizing this dissertation and actually made it a reality with her encouragement. She unselfishly sacrificed her own professional and personal interests over the past four years to maintain a home that has been my constant "port during the storm" of graduate work.

I extend my appreciation to my children, Teddy John and Laura Lee. They, and the other members of their generation, provided much of my incentive during the past years and motivated me to establish some lifelong goals.

This work was supported in part by Grant No. 68084, a National Science Faculty Fellowship.
ABBREVIATIONS AND LINE SCALES

RER - Rough endoplasmic reticulum
M - Mitochondrion
MN - Macronucleus
CB - Body containing concentrated cytoplasm
GC - Ground cytoplasm
CM - Cell membrane
SE - Site of entry of CB or GC into M
EC - Engulfed cytoplasm
PV - Proteose peptone vacuole

Figs. 6-9 Line scale equals 20 micrometers.
Figs. 10-18 Line scale equals 30 micrometers.
Figs. 19-22 Line scale equals 1.0 micrometer.
Figs. 23-29 Line scale equals 0.5 micrometer.
PLATES
Fig. 6. Living *T. pyriformis* 11 hours after it had been removed from proteose peptone culture medium and starved in saline solution. Stained with neutral red 1 hour prior to the time the photograph was taken. Absence of food vacuoles is quite apparent. Distribution of neutral red granules in the posterior portion of the cell is typical of cells in this condition.

Fig. 7. Living *T. pyriformis* treated like that in Fig. 6 but followed by feeding proteose peptone for 25 minutes. Clear vacuoles of proteose peptone are numerous and some quite clearly demonstrate that neutral red granules are incorporated into the vacuoles following vacuole formation.

Fig. 8. *T. pyriformis* starved 11 hours in saline solution followed by a 15-minute exposure to an India ink suspension. Cell was fixed in 2% glutaraldehyde.

Fig. 9. Cell treated like that in Fig. 8. Five large black "food" vacuoles of India ink are quite apparent and easily distinguished from the number of vacuoles formed in the cell in Fig. 8.
Fig. 10. Living *T. pyriformis* starved 10 hours in saline solution followed by staining with neutral red. Neutral red granules appear very black in color since a green filter was used to enhance contrast. Note the posterior location of most of the granules.

Fig. 11. Living *T. pyriformis* starved 10 hours in saline solution followed by treatment for the localization of acid phosphatase activity. Note the location of the reaction product in the posterior portion of the cell which corresponds to the distribution of neutral red granules in Figs. 10 and 12.

Fig. 12. Living *T. pyriformis* treated like that in Fig. 10 but showing a large contractile vacuole in the posterior portion of the cell surrounded by neutral red granules. There are no apparent neutral red granules inside the contractile vacuole.

Fig. 13. Living *T. pyriformis* starved 10 1/2 hours in saline solution followed by staining with neutral red. After the cells were stained they were allowed to feed on proteose peptone for 25 minutes. Neutral red granules can be seen distributed evenly around the periphery of the macronucleus and the edges of the two food vacuoles which also show some color from granules which have penetrated the vacuoles. Note the absence of neutral red granules around the periphery of the contractile vacuole.

Fig. 14. Living *T. pyriformis* treated like that in Fig. 13. This dividing stage indicates that neutral red stain is not taken into the cell through the oral apparatus since the oral structures degenerate in the cells during the early stages of fission.

Figs. 15-16. Living *T. pyriformis* treated like that in Fig. 13 but showing a more posterior location of the neutral red granules and food vacuoles.

Fig. 17. Living *T. pyriformis* starved 18 hours in saline solution followed by staining with neutral red. Note the even distribution of neutral red granules within the cell indicating the peripheral distribution of most of the granules. Two types of stainable structures are present, those which are completely dense and those showing red stain only around the periphery.

Fig. 18. Living *T. pyriformis* treated like that in Fig. 17. The even distribution of neutral red granules is apparent in this cell with the exception of a group of granules just anterior to the contractile vacuole.
Figs. 19-49. Electronmicrographs from *T. pyriformis* starved 10 1/2 hours and fixed with 1% OsO₄.

Fig. 19. Control cell not stained with neutral red. A mitochondrion surrounded by rough endoplasmic reticulum.

Fig. 20. Cell stained with neutral red. A mitochondrion and closely related rough endoplasmic reticulum. No apparent difference in ultrastructure can be noted due to the use of neutral red stain.

Fig. 21. Control cell not stained with neutral red. The areas of rough endoplasmic reticulum are most commonly seen in the cytoplasm immediately adjacent to the macronucleus.

Fig. 22. Cell stained with neutral red. A large field of rough endoplasmic reticulum showing a close relationship to three mitochondria each of which has incorporated a body containing concentrated cytoplasm. Small pieces of rough endoplasmic reticulum appear to be pinching off and becoming part of the ground cytoplasm.
Fig. 23. Cell stained with neutral red. A body containing concentrated cytoplasm with an osmophilic membrane (arrow) shown very close to a mitochondrion in the upper part of the micrograph. The texture of the material inside the body containing concentrated cytoplasm corresponds to that located in the ground cytoplasm. Note the concentration of endoplasmic reticulum inside the body containing concentrated cytoplasm as compared to the ground cytoplasm.

Fig. 24. Similar to Fig. 23 but a more definite concentration of endoplasmic reticulum is apparent within the body containing concentrated cytoplasm when it is compared to the ground cytoplasm of the cell.

Fig. 25. Control cell not stained with neutral red. There is no apparent difference induced in the cells due to the use of neutral red stain. The membrane around the body containing concentrated cytoplasm (arrow) is osmophilic like those in Figs. 23 and 24.

Fig. 26. Control cell not stained with neutral red. Normal mitochondria are commonly located against the cell membrane in well fed cells or cells in early stages of starvation.
Fig. 27. A mitochondrion, from a series of serial sections, which appears as a "U" shaped structure and leaves some question as to whether the area designated SE is the site of entry for the cytoplasm into the mitochondrion or an area located between the two ends of the mitochondrion.

Fig. 28. A section from a series following that in Fig. 27 illustrating that the cytoplasm actually has a site of entry as indicated by the grazing section of the mitochondrion through the area that appears completely open on Fig. 27.
Fig. 29. Control cell not stained with neutral red. Mitochondrion with a body containing concentrated cytoplasm within the limits of the outside mitochondrial membrane (long arrow) which is evident as a continuous membrane around both organelles. The inside mitochondrial membrane gives rise to some of the tubules (short arrow) within the mitochondrion. The body containing concentrated cytoplasm has a limiting membrane entirely separate from either of the mitochondrial membranes. Note the concentration of rough endoplasmic reticulum within the body containing concentrated cytoplasm as compared to the ground cytoplasm outside the mitochondrion.

Fig. 30. Cell stained with neutral red. A mitochondrion and three bodies containing concentrated cytoplasm. Two of the bodies lay just outside while one is partially enclosed within the mitochondrion.

Fig. 31. Control cell not stained with neutral red. A mitochondrion with some rough endoplasmic reticulum just outside the organelle and a body containing concentrated cytoplasm enclosed within. The area showing an unclear delineation of membranes is probably the site of entry of the body containing concentrated cytoplasm. Concentration of endoplasmic reticulum within the body is apparent when compared to the ground cytoplasm just outside the mitochondrion.

Fig. 32. Control cell not stained with neutral red. Mitochondrion underlying the cell membrane. The usual rough endoplasmic reticulum is apparent just outside the mitochondrion and a body containing concentrated cytoplasm is almost entirely enclosed within. A vesicle (arrow) similar to those commonly seen in the ground cytoplasm is incorporated within the body containing concentrated cytoplasm.
PLATE VII

Fig. 33. Cell stained with neutral red. A mitochondrion with a large amount of engulfed cytoplasm completely enclosed. The endoplasmic reticulum within the mitochondrion is more concentrated than outside the ground cytoplasm. The organelle is just underlying the cell membrane.

Fig. 34. Control cell not stained with neutral red. A large field of view illustrating the common occurrence of cytoplasm within the mitochondria. The membrane at the end of the arrow appears to be engulfing rough endoplasmic reticulum to possibly form a body containing concentrated cytoplasm.

Fig. 35. Control cell not stained with neutral red demonstrating the possibility that a mitochondrion can actively incorporate rough endoplasmic reticulum by two methods, engulfing rough endoplasmic reticulum from the ground cytoplasm or by incorporating bodies which already contain the rough endoplasmic reticulum.

Fig. 36. Control cell not stained with neutral red. Rough endoplasmic reticulum is engulfed in several forms as indicated by this micrograph. The engulfed cytoplasm in this case contains small pieces of rough endoplasmic reticulum as well as canalicular forms (arrow inside organelle). The canalicular form inside the mitochondrion is similar to the form indicated by the arrow on the outside of the mitochondrion.
Figs. 37-40. Control cells not stained with neutral red are shown in Figs. 37 and 39. Cells stained with neutral red are shown in Figs. 38 and 40. Degenerating mitochondria become more dense, round, and slightly smaller in size as autodigestion progresses. The outer membranes (arrow) fit loosely around the degenerating organelle leaving a halo between the outer membranes and the more dense contents. Organelles in this stage of degeneration are very osmophilic compared to the less osmophilic cytoplasm surrounding them. Material having the appearance of cytoplasm can be seen in the area at the end of the short arrow in Fig. 40. This corresponds to the location of similar material in the earlier stage shown in Fig. 29.
Fig. 4. Cell stained with neutral red. Three different stages of autodigestion within mitochondria are apparent. The mitochondria at the upper left and lower right of the figure appear to be normal. The degenerating form indicated by the short arrow is similar to those in Figs. 37-40. The remaining mitochondrion has not only engulfed some cytoplasm but contains a degenerating mitochondrion (long arrow). Close observation reveals tubular remnants within both degenerating organelles marked by arrows. The late stage of degeneration is also apparent from the large halo between the degenerating material and the outside membranes.
Figs. 42-45. Control cell not stained with neutral red. Shows increasing magnification of some of the organelles posterior to the macronucleus. The same degenerating mitochondrion is indicated by an arrow in all of the figures. Fig. 42 illustrates the tendency of the degenerating organelles to be very osmophilic when compared to the ground cytoplasm and other organelles. A proteose peptone vacuole can be seen very near to the degenerating structure and appears to contain some endoplasmic reticulum. Fig. 45 illustrates that high magnification and close observation reveals membranes within the degenerating mitochondrion. The compressed membranes become more obvious when printed like the micrographs in Figs. 37-40.
Figs. 46–49. Control cell not stained with neutral red. A series of prints made from one electron microscope negative. The apparent variation is from the difference in exposure and development of the positive photographic paper. Fig. 46 illustrates the texture of the degenerating organelle (arrow) is most apparent when the print is underdeveloped. The ground cytoplasm and mitochondrion are not clear in either Figs. 46 or 47. The normal method of developing prints to show the detail of the mitochondria and ground cytoplasm as shown in Figs. 48 and 49 makes the detail in the degenerating structure much less obvious.