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Autonomous features of kinetosomes and mitochondria in Tetrahymena pyriformis GL: an ultrastructural, nutritional, and biochemical study

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Autonomous features of kinetosomes and mitochondria in

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An ultrastructural, nutritional, and biochemical study

by

Frank David Seydel

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I. INTRODUCTION

The mechanisms governing morphogenesis, function, and interaction of cellular components and organelles have occupied the interest of biologists since the mid-1800's when advances in microscopy and staining first made organelles and manifestations of cellular processes visible. Elucidation of the basic principles of heredity around the turn of the century led microscopists to consider a number of organelles as repositories of genetic information. Based on morphological evidence of continuity, kinetosomes, mitochondria, chloroplasts, nuclei, and sometimes other organelles were considered to be autonomous or partly autonomous. During the ensuing decades, most of the interest was focused on the nucleus because of the ease of observing it and because of its apparent preponderant role in determining hereditary. However, with the advent of molecular biology in the last two decades, attention has returned to the possibility of autonomy in kinetosomes, mitochondria, and chloroplasts. The possibility of kinetosomal autonomy is an issue which is currently vigorously debated. In contrast, at least a degree of autonomy for mitochondria and chloroplasts has been established, but knowledge of which processes are directly under mitochondrial control or chloroplast control is still fragmentary.

The research described in this dissertation was originally undertaken to study the nature of kinetosomal autonomy. The possibility of autonomy in kinetosomes would be strengthened by demonstration of nucleic acids (NA) within the organelles. Enzyme extractions of fixed cells and of thin-sectioned material and regulation of the cellular NA
pools were the principal investigative methods. *T. pyriformis* was selected for the research organism for several reasons. First, it had been intensively studied and much was known concerning its growth characteristics, physiology, ultrastructure, and genetics. Second, the organism could be grown rapidly and axenically in a defined medium. Thus, large quantities of *T. pyriformis* uncontaminated by other organisms could be obtained and growth could be regulated by the nutritional constraints of the medium. This was especially valuable for biochemical studies.

The evidence presented in this dissertation suggests that no NA is present in kinetosomes, and further, that an understanding of the function of kinetosomes can only be achieved by more complete knowledge of kinetosomal composition. Therefore, differential solubilization and separation of kinetosomal components, the prerequisites for biochemical analyses, were investigated. During this research information concerning the involvement of folic acid in the protein synthetic process of mitochondria was obtained. Such evidence strengthens the possibility of mitochondrial autonomy.
II. HISTORICAL PERSPECTIVE

A. Function and Ultrastructure of Kinetosomes

1. Function of kinetosomes

The kinetosome occurs in organisms of all eukaryotic taxa except conifers and angiosperms (S. Wolfe, 1972; Fulton, 1971; Gibbons, 1967). It is almost invariably present at the base of a cilium, including the modified stereo-cilium present in a sensory receptor cell, flagellum, or sperm tail (Fulton, 1971; Dowling, 1967; Gibbons, 1967; Pitelka and Child, 1964); and it is ultrastructurally identical with the centriole (Gall, 1961). The development of the classical terms "basal body," "kinetosome," and "centriole" has a long and complicated evolution.

Briefly, "basal body" referred to the dark staining item present at the base of a cilium or flagellum in a number of types of cells, especially ciliated epithelia and spermatozoa of vertebrates. In their work with ciliates, Chatton and Lwoff termed this dark staining item a "cinetosome" in order to emphasize its apparent involvement in motility. (See Chatton and Séquela (1940) for a discussion of the evolution of the kinetosome concept.) "Centriole" was a similarly dark staining structure at the center of the mitotic spindle. Since electron microscopic (EM) studies have revealed that all three terms refer to a single morphological entity, "kinetosome" will be used in this dissertation except where the use of the classical term provides historical accuracy or where an apparent functional difference is significant.

Studies of the kinetosome in a wide variety of organisms have prompted diverse suggestions of function, which can be assembled into
four categories. First, a number of hypotheses have suggested that the
kinetosome initiates, controls, or is otherwise involved in the motility
of cilia and flagella (Stubblefield and Brinkley, 1967). Because of its
complex association with other cellular microtubules and fibrils, the
kinetosome has been associated with coordination of ciliary or flagellar
beat (e.g., Pitelka, 1969). Most of the support for these hypotheses
was based on classical studies of the "neuromotor apparatus," a
concept which no longer seems tenable (Wise, 1965). This concept appears
to have influenced the motility model designed by Stubblefield and
Brinkley (1967), but there is no definitive evidence for such a model.

Second, the importance of the centriole in mitosis as the focus of
the spindle and aster has long been known. (See Fulton (1971) and
Pickett-Heaps (1969) for reviews.) Third, the kinetosome is known to
participate in the formation and maintenance of cilia and flagella.
These latter organelles have rarely been demonstrated in the absence of
kinetosomes. During morphogenesis, a cilium appears only after the
kinetosome has migrated to its proper place in association with the
pellicle (e.g., Allen, 1969; Randall, et al., 1963; Sorokin, 1962;
Sotello and Trujillo-Cenoz, 1958); if a cilium or flagellum is removed,
the organism will reform the organelle provided that the kinetosome has
been left intact (e.g., Rosenbaum and Child, 1967; Dembowska, 1926).
However, if the kinetosome is removed along with the cilium, the kineto-
some must first be reformed before the cilium is reestablished (Dembowska,
1926).
Fourth, and of immediate interest to this thesis, is the role of the kinetosome in the production of daughter organelles. On a functional basis, an existing kinetosome is involved in formation of a new kinetosome (e.g., Allen, 1969; Dippel, 1968; Lwoff, 1950; Chatton and Séquela, 1940); the centriole is involved in the formation of daughter centrioles (e.g., Anderson and Brenner, 1971; Sorokin, 1968; Gall, 1961); and the centriole is involved in the formation of kinetosomes (e.g., Fulton, 1971; Sorokin, 1968; Dirksen and Crocker, 1966; Renaud and Swift, 1964; Gall, 1961).

2. Ultrastructure and composition

Because the kinetosome is a cylindrical structure measuring 0.2 μm in diameter and in most organisms 0.5 μm in length, it was at the limit of resolution of the light microscope. Nothing could be seen of its internal structure; it was thus considered to be a "basal body."

Numerous EM observations have revealed the ultrastructure of kinetosomes and centrioles, and the details have been summarized and discussed in several recent articles and reviews (Anderson, 1972; J. Wolfe, 1972; Fulton, 1971). Only the major features are presented here.

The walls of the cylinder are composed of nine sets of triplet microtubules. Even when the microtubular arrangement of the cilium is abnormal, the nine-part triplet ultrastructure of the kinetosome remains constant (Baccetti et al., 1970), with two possible exceptions (Van Deurs, 1973; Phillips, 1967). The triplets are set at an angle to the tangent of the cylinder, 60-70° at the proximal end and 20-30° at the distal end, such that the triplet microtubules twist from the proximal
to the distal end. The innermost tubule of the triplet, the A tubule, is continuous with the A tubule of the ciliary axoneme. The middle tubule of the triplet; the B tubule, is continuous with the B tubule of the axoneme; and the outermost tubule, the C tubule, does not extend into the cilium. A cartwheel structure consisting of nine spokes each radiating from an axis to the A tubule of each triplet occurs at the proximal end of the kinetosome and centriole. At the distal end of kinetosomes only a terminal elaboration occurs which has been called variously the basal plate (Wolfe, 1970; Munn, 1970), terminal plate (Allen, 1969; Pitelka and Child, 1964), and transverse plate (Hufnagel, 1969a). "Terminal plate" seems preferable to the other terms for three reasons. First, "terminal plate" appears to have historical precedence. Second, "basal plate" is a potential source of confusion since it already refers to two other classically described structures: the collection of kinetosomes forming a cirrus base in hypotrichs (Pitelka, 1969), and the dense structure at the juncture of the base of the sperm tail with the nucleus (S. Wolfe, 1972). Third, the term "basal" presents some potential confusion itself for while it marks the basal end of the cilium it marks the distal end of the kinetosome. On the other hand, the plate does mark the terminus of both the kinetosome and the ciliary axoneme, and, therefore, "terminal plate" seems most descriptive.

In some organisms, notably T. pyriformis, an electron-dense, amorphous core occupies the lumen of the cylinder and an electron-dense sheath encloses the proximal half or third of the cylinder (Allen, 1967). In some other organisms electron-dense satellites or feet occur adjacent
to kinetosomes (J. Wolfe, 1972). A variety of fibers and microtubules appear in conjunction with kinetosomes of various species. Those appearing in *T. pyriformis* will be described in Sec.IV.A.2.

Little is known of the molecular composition of kinetosomes. Schafer and Chandler (1970) reported, using the technique of electron microprobe x-ray analysis on thin sections, the presence of chlorine, potassium, silicon, and sulfur in guinea pig kidney centrioles but not in the adjacent cytoplasm. J. Wolfe (1972) noted that isolated kinetosomes subjected to acrylamide gel electrophoresis produced three bands. Two of the three bands matched the microtubule bands of isolated cilia (Sec.II.D.2.) and the third was unique. Only three cytochemical studies have been performed. Cholinesterase has been localized in the kinetosomes and associated fibers of *T. pyriformis* (Schuster and Hershenov, 1969), and adenosine triphosphatase (ATPase) has been identified in the kinetosomes of *Helix aspera* spermatozoa (Anderson and Personne, 1969) and human retinal cells (Matsusaka, 1967). The possibility of nucleic acids being present in kinetosomes will be discussed below.

B. Morphogenesis of Kinetosomes

1. Morphological relationships

Centriolar duplication was first described by Van Beneden and by Boveri in 1887. In 1898 Lenhossek and Henneguy described the conversion of centrioles to kinetosomes (Fulton, 1971). Numerous studies since then have examined the origin and fate of the kinetosome, but all of the light microscopic (LM) work was hindered by the small size of the organelle. Essentially, three major schools of thought persisted. One school
believed that centrioles and kinetosomes were autonomous organelles, centrioles giving rise by division to daughter centrioles and to kinetosomes, and kinetosomes giving rise to kinetosomes. These ideas were discussed in Lwoff (1950). A second school felt that centrioles and/or kinetosomes could arise de novo and then disappear when no longer needed. A third school felt that centrioles and kinetosomes were merely artifacts and, therefore, discussions of their origin were fruitless. Studies with electron microscopy have confirmed that kinetosomes are not artifacts and that replication does not occur by fission or budding from the parent. Rather, several modes of formation have been reported.

De novo formation, or assembly of the intact organelle from ultrastructurally unrecognizable precursors, appears to occur in ameboflagellates during the tranformation from ameba to flagellate (Fulton and Dingle, 1971; Outka and Kluss, 1967; Schuster, 1963), in the hypotrich Oxytrichia during excystment (Grimes, 1973), and in a number of protists and plants during mitosis and meiosis (Pickett-Heaps, 1971).

Alternatively, the existing centriole or kinetosome may participate in the formation of daughter organelles (procentriole or prokinetosome) either directly or indirectly. In the directed situation, the daughter forms perpendicularly to the proximal end of the parent at a distance of 100 nm (Allen, 1969; Dippel, 1968). When several daughters form, they tend to maintain this perpendicular orientation and an equidistant spacing from each other such that a cross section through the cartwheel region of the parent shows the daughters arranged as spokes in a wheel around the parent (Sorokin 1968; Gall, 1961). In the
indirect situation, first described in rat tracheal epithelium (Dirksen and Crocker, 1966) masses of fibrogranular material appear in association with pre-existing centrioles. These masses form "condensation forms" around which several daughter kinetosomes form, each oriented with its axis towards the center of the condensation form and separated from it by an approximately 100 nm space containing fine fibrous material.

Similar observations, varying only in minor detail, have been made on developing ciliated epithelia of chick trachea (Kalnins et al., 1972), rhesus monkey oviduct (Anderson and Brenner, 1971), mouse oviduct (Dirksen, 1971), mouse nasal passages (Frisch, 1967), rat lung (Sorokin, 1968), and Xenopus trachea (Steinman, 1968).

Blepharoplasts in ferns appear to be similar to condensation forms (Mizukami and Gall, 1966). The new kinetosome elongates and turns perpendicularly to the cell surface. Eventually it contacts the surface and initiates cilium formation.

2. Possibility of autonomy

The involvement of the parent organelle in the formation of the daughter, at least in some systems, suggests that the organelle is to some degree autonomous. Two major lines of evidence have been advanced.

a. Morphological and morphogenetic complexity

First, the kinetosome is an organelle with a high degree of morphological detail and symmetry. Second, the formation and maturation of the daughter kinetosome involves a precise sequence of events. At least in ciliates, the daughter forms anteriorly and at a 90° angle to the parent, and it then elongates as it turns parallel to the parent and becomes established
on the pellicle. Third, the kinetosome, especially in ciliates, is often integrated into an elaborate pellicular network of organelles including microtubules, fibers, striated fibers, vacuoles, and membranes. Such organization at least suggests the need for genetic regulation. Indeed Nanney (1968) suggested that the number of ciliary rows, or kineties, is a hereditable characteristic which is independent from nuclear regulation, as determined by conjugation studies. Sonneborn (1970) reviewed this subject, citing especially his classic experiment in which a section of pellicle was removed from a Paramecium and reoriented backwards; organization of the reversed kineties was maintained, and new kinetosomes retained the reversed position through all succeeding cell generations. These kinds of studies, along with all of the other information, provide a substantial basis for some degree of cortical, and perhaps kinetosomal, autonomy.

b. Presence of nucleic acids in kinetosomes

The identification of DNA as the hereditary substance and RNA as the intermediate in the expression of the hereditary message, and the insight into the replication process provided by the Watson-Crick model for the structure of DNA meant that the demonstration of DNA in an organelle would strongly suggest a hereditary role for that organelle. (See Pollock (1970) for a review of the significant events and discoveries leading to the current understanding of molecular genetics.) Thus those organelles which classically had been considered to possess hereditary potential and autonomy, kinetosomes, mitochondria, and chloroplasts, were examined for the presence of nucleic acids. As regards kinetosomes, both positive and
negative evidence has been reported.

(1) **Light microscopic cytochemistry**  
Randall and Fitton-Jackson (1958) and Randall (1959) reported that kinetosomes of *Stentor* and *T. pyriformis* stained with the Feulgen reagent, which is specific for DNA. However, Randall and Disbrey (1965) noted that "these earlier cytochemical studies...proved difficult to corroborate." McDonald and Weijer (1966) reported finding DNA in *Neurospora* centrioles. Randall and Disbrey (1965) and Smith-Sonnebom and Plaut (1967) reported DNase-extractable acridine orange fluorescence in the kinetosomes of *T. pyriformis* and *Paramecium*, respectively.

(2) **Autoradiography with $^3$H-thymidine**  
Randall and Disbrey (1965) also performed LM autoradiography and found labelling associated with the kineties of *T. pyriformis*. However, Rampton (1962) and Stone and Miller (1965) using light microscopy, and Pyne (1968) using electron microscopy, found no labelling associated with kinetosomes of *T. pyriformis*. Smith-Sonneborn and Plaut (1969, 1967) reported LM evidence of $^3$H-thymidine incorporation into *Paramecium* kinetosomes. However, Sonnèuborn (1970) could find no association between pellicular silver grains and kinetosomes, using electron microscopy. Sukhanova and Nilova (1965) indicated labelling in *Opalina* kinetosomes. Dirksen and Crocker (1966) reported no labelling in centrioles of embryonic rat trachea using electron microscopy. With LM observations of membranellar regeneration under various conditions, Younger et al. (1972) could not associate the labelling in the membranellar bands of *Stentor* with kinetosomes.
(3) Electron microscopic observations

Only a few efforts to localize nucleic acids by EM cytochemistry have been observed. Swift et al. (1964) indicated no staining in *T. pyriformis* kinetosomes by uranyl acetate, which preferentially stains for DNA. Brinkley and Stubblefield (1970) and Stubblefield and Brinkley (1967) suggested that a helix was present in the lumen of centrioles of Chinese hamster tissue culture cells and that it might be DNA; they indicated that the kinetosomal triplet feet were removable with RNase.

(4) Biochemistry

Seaman (1960) first reported the presence of DNA (6% as much DNA as protein) in isolated kinetosomes of *T. pyriformis*. Subsequent studies have reported decreasing amounts of DNA with increasing purity of the kinetosome fraction. Argetsinger (1965) and Hoffman (1965) both reported less than 1% as much DNA as protein. Flavell and Jones (1971) could find no DNA in the isolated pellicles of *T. pyriformis* and Hufnagel (1969b) found only variable amounts of DNA, identical to nuclear DNA for *P. aurelia*.

C. Electron Microscopic Cytochemistry of Nucleic Acids

The controversy concerning the presence of nucleic acids in kinetosomes suggested alternative methods of experimentation might be useful. Conflicting results could be due to small amounts of NA, amounts at the limits of detection by the above mentioned methods. Electron microscopy should permit observation of those hypothetical small amounts of NA, if it were combined with a cytochemical procedure to make the NA visible. Therefore, a number of cytochemical procedures were reviewed.
1. Stains

   a. Stains of fixed tissue prior to embedment  
   A number of workers have developed procedures permitting localization of DNA in thick sections due to silver grains deposited after fixation of the tissue (Peters and Giese, 1970; Bryan and Brinkley, 1963; Jurand et al., 1958; Bradfield, 1954; Bretschneider, 1949). Gautier and Schreyer (1970) have reported a "Feulgen-like" stain for electron microscopy. However, none of the procedures appeared sensitive and specific enough for the amount of DNA likely to be present in kinetosomes.

   b. Stains of thin sections after embedment  
   A number of stains have been developed, most of them producing metal deposition on thin sections: bismuth, indium, iron, mercury, ruthenium, silver, tin, uranium, and vanadium (Hayat, 1970). With the exception of the indium (Outka, 1971), the specificity was inadequate. All of the metal stains were low in contrast.

2. Extraction of nucleic acids

   An alternate approach to staining NA is the extraction of the NA from tissues. The NA can then be localized by comparison of extracted and non-extracted specimens. Three general methods are possible.

   a. Extraction of nucleic acids from tissues prior to fixation and embedment  
   The work of Mentre (1969; 1968) is typical of this method. Mentre incubated isolated rat liver nuclei in DNase and RNase, then fixed them in OsO₄ and embedded them in Epon. The level of extraction was good, but the ultrastructure was unsatisfactory.
b. Extraction of nucleic acids from fixed tissues prior to embedment

Aldridge and Watson (1963) were able to verify by quantitative methods the removal of RNA from acrolein-fixed rat liver using cold perchloric acid; they found that RNase was ineffective. Molenaar et al. (1970) reported that removal of 94% of the DNA from isolated, glutaraldehyde-fixed yeast nuclei by DNase resulted in only minor change in the ultrastructural appearance of the chromatin strands; and removal of 84% of the RNA by RNase caused only minor loss in density of the 12 nm granules of the nucleus, and of the nuclear membrane-associated ribosomes. Koshiba et al. (1970) examined the nuclear pore regions of Novikoff hepatoma cells fixed in glutaraldehyde and then treated with DNase, RNase, and various proteases. Ribosomes were affected only by the RNase.

Most other reports in the literature concerning the use of nucleases for extractions in fixed tissue prior to embedment, (e.g., Herrmann and Kowallik, 1970) appear to be based on the procedure established by Kislev et al. (1965) for formaldehyde-fixed tissues. No quantitative estimates have been performed with formaldehyde fixation.

c. Extraction of nucleic acids from thin sections after embedment

Leduc and Bernhard (1962) fixed rat pancreas in formaldehyde and embedded it in three different water-soluble plastics—Durcupan, Aquon, or glycol methacrylate (GMA). Nucleases and proteases were effective only on GMA sections, but "almost catastrophic extraction and distortion of tissues" resulted from the formaldehyde-GMA procedures. Leduc et al. (1963) experimented with three additional fixatives, acrolein,
acrolein-formaldehyde, and glutaraldehyde. Again, the tissue (rat pancreas) was embedded in GMA. Glutaraldehyde produced the best fixation, but the tissue was resistant to DNase. RNase and proteases were effective on all the samples. Lord and Lafontaine (1969) obtained results similar to Leduc et al. (1963) using plant meristem tissue fixed in formaldehyde and embedded in GMA.

Monneron and Bernhard (1966) obtained good ultrastructural preservation and good extraction with both nucleases and proteases for rat pancreas, initially fixed in glutaraldehyde, post-fixed in OsO$_4$, and embedded in Epon. In order to obtain extraction, the osmium had to first be removed by treating the thin sections with a hydrogen peroxide hydrolysis.

D. Analysis of Proteins in Kinetosomes

Very little attention has been paid to components of the kinetosome other than nucleic acids, yet knowledge of the molecular composition of the kinetosome would greatly facilitate understanding of function. Two basic approaches were considered for analyzing the protein components of the kinetosome.

1. Extraction of proteins from thin sections

Several workers have studied the extraction of cell components by various proteolytic enzymes (e.g., Lord and Lafontaine, 1969; Monneron, 1966; Monneron and Bernhard, 1966) but only Anderson and André (1968) appear to have examined kinetosomes specifically. They fixed tissues from three animals in glutaraldehyde, post-fixed them in OsO$_4$, and embedded them in Epon-Araldite. They treated the thin sections with hydrogen
peroxide and then pronase. The core and microtubules of the kinetosome were digested, but adjacent cytoplasmic microtubules remained.

2. Analysis of components from isolated kinetosomes

Ciliates are advantageous organisms for isolation of kinetosomes because large numbers of cells can be easily grown and each one has a thousand or more kinetosomes. The available procedures can be organized into two groups. In one group belong the methods for isolation of the ciliate pellicle. The majority of these procedures were based on the work of Child and Mazia (1956) in which the cells were fixed in -20°C ethanol, warmed to 4°C, and disrupted. Addition of digitonin solubilized all cellular components except the pellicles, which were collected by centrifugation. Rubin and Cunningham (1973), Flavell and Jones, (1971), Satir and Rosenbaum, (1965), Hoffman, (1965), Argetsinger, (1965), and Seaman, (1960) have all utilized this approach on T. pyriformis, as has Hufnagel (1969a) on P. aurelia. Rubin and Cunningham (1973) then disrupted the pellicles in an attempt to isolate the kinetosomes and selectively solubilize kinetosome components with phosphotungstic acid and separate them with sucrose gradient centrifugation; but the purity of the isolate was low and the identification of the centrifugation bands was uncertain. An importantly different method for isolating pellicles was that of Nozawa and Thompson (1971) in which ethanol fixation was avoided by isolating the pellicles in a relatively high ionic strength buffer.

The second group of procedures for obtaining large quantities of kinetosomes is based on the tendency of the oral apparatus to remain
intact during disruption of the cell in hypertonic solution (Wolfe, 1970), in a saturated indole solution (Whitson et al., 1966), or in a solution of tertiary butanol (Williams and Zeuthen, 1966). The stability is due to the numerous microtubules and fibrils interconnecting the oral apparatus. Unfortunately, it has not been possible to differentially solubilize the microtubules, fibrils, and kinetosomes for chemical analysis.

Since ciliary microtubules are extensions of kinetosomal microtubules, they are presumably similar to or identical with kinetosomal microtubules. Although questions of purity have to date hindered chemical analysis of kinetosomes, some information can be inferred from studies involving the chemical analyses of ciliary microtubules. The major methods of isolating cilia and their components have been reviewed by Warner (1972). Mohri (1968) isolated the primary microtubular protein from sea urchin spermatozoa flagella and called it "tubulin." Tubulin is now known to consist of two similar proteins of 55,000-60,000 molecular weight (Witman et al., 1972a; Everhart, 1971; Fine, 1971; Jacobs and McVittie, 1970; Stephens, 1970; Renaud et al., 1968). The amino acid composition of the two tubulins has been determined (Witman et al., 1972a, b; Stephens, 1970) and Witman et al. (1972b) have made a tentative correlation of these two tubulins with the subunit positions in the doublet microtubules. Approximately 20-25 secondary proteins are associated with the axoneme (Warner, 1972), including dynein, a protein with ATPase activity which is present in the arms of the A microtubule (Gibbons, 1967).
E. Function and Ultrastructure of Mitochondria

Mitochondria are essential cytoplasmic organelles in nearly all eukaryotic organisms, except for those few which are anaerobic (Cornford, 1971). Mitochondria perform a number of essential metabolic reactions, particularly those involved with the utilization of energy by the cell (for a review, see Borst (1969)). The ultrastructure of mitochondria has been intensively studied (e.g., Munn, 1969; Fawcett, 1966); it is a pleomorphic organelle bound by two membranes. The composition and properties of the two membranes differ (Ernster and Kuylenstierna, 1970). The inner membrane is invaginated to form plates or tubules called cristae. The space within the plates or tubules (intra-cristal space) is continuous with the region between the inner and outer membranes. The region bound by the inner membrane is called the matrix. The locations (matrix, intermembrane space, inner or outer membrane) of many of the enzymes known to be present in mitochondria have been established, primarily by biochemical means (for a review, see Sager (1972). Under certain conditions, negatively stained inner membranes appear to have numerous globules attached to them by means of stalks (Fernandez-Morán et al., 1964). Racker and Horstman (1967) have reported that the globules appear to be composed of molecules of ATPase (see Munn (1969) for further discussion).

A number of different granules and densely staining inclusions have been recorded in mitochondria of cells from various growth conditions and sources (Kimura, 1972; Thomas and Greenawalt, 1968; Kurosumi et al., 1966; Elliott and Bak, 1964; Greenawalt et al., 1964; Roth and Minick,
1961), including pathologic conditions (Byrnes et al., 1972; Ishihara et al., 1972; Watari et al., 1972; Anderson, 1967; Frei and Sheldon, 1961; Sheldon and Zetterqvist, 1956). Except for the demonstration of phosphate ions in certain granules (Thomas and Greenawalt, 1968), the compositions and functions of these granules are unknown.

F. Morphogenesis and Autonomy of Mitochondria

Lehninger (1964) suggested three major categories for proposals concerning the reproduction and genesis of mitochondria (see Baxter (1971) for recent review.)

1. De novo synthesis from submicroscopic precursors

The possibility of de novo synthesis was suggested by LM studies (Harvey, 1946; Beckwith, 1914) where sea urchin larvae which had apparently been freed of mitochondria by centrifugation still developed mitochondria. Electron microscopy has revealed that the centrifuged larvae still possessed mitochondria (Baxter, 1971).

2. Formation from other membranous structures

This idea is based primarily on occasional electron micrographs which seem to suggest mitochondria "budding off" from the nuclear membrane, the Golgi apparatus, endoplasmic reticulum (ER), or outer cell membrane. There are also biochemical similarities between mitochondrial membranes and other cellular membranes.

3. Growth and division of pre-existing mitochondria

A preponderance of evidence has accumulated in favor of the third suggestion, growth and division of pre-existing mitochondria. Numerous micrographs suggest a prokaryote-like division either by constriction into
smaller organelles or by the formation of internal partitions (Tandler, 1973). Further, biochemical data collected from numerous sources, especially Neurospora, T. pyriformis, and rat liver, indicate that mitochondria possess the hereditary and synthetic machinery necessary for growth (Linnane et al., 1972; Sager, 1972; Baxter, 1971). Of particular interest to this study has been the work on the mitochondria of T. pyriformis in which DNA has been isolated and its molecular weight characterized (Flavell and Jones, 1970b; Suyama and Miura, 1968), DNA polymerase has been identified (Keiding and Westergaard, 1971; Westergaard, et al., 1970), and ribosomes have been isolated (e.g., Chi and Suyama, 1970).

G. Nature of the Mitochondrial Genetic and Protein Synthetic System

The replication, transcription, and translation processes appear to be distinct from the nuclear and cytoplasmic processes of eukaryotes and similar to those of prokaryotes in a number of ways.

1. Properties of the DNA

The buoyant density of the mitochondrial DNA is distinct from that of the nuclear DNA (Hufnagel, 1969b; Suyama and Miura, 1968; Flavell and Jones 1970b). Further, it appears to be circular in many cases, as is the case for bacterial genomes (Sager 1972).

2. Responses to antibiotics

Mitochondria are sensitive to a number of antibiotics. Ethidium bromide inhibits DNA synthesis (Charret, 1972; Meyer et al., 1972; Horwitz and Holt, 1971) and RNA synthesis (Mahler and Dawidowicz, 1973). Chloramphenicol reduces the number of cristae present and inhibits
protein synthesis (Kislev et al., 1973; de Pijper and Hülsmann, 1973; Storrie and Attardi, 1973; Adoutte et al., 1972; Smith-Johannsen and Gibbs, 1972; Lenk and Penman, 1971; Mason et al., 1970; Mager, 1960, Turner and Lloyd, 1970). Chlortetracycline (Mager, 1960), oxytetracycline (de Pijper and Hülsmann, 1973), and rifampicin (Sager, 1972) also have been cited as inhibitors of mitochondrial growth and protein synthesis. All of these antibiotics also inhibit similar processes of bacteria (Linnane et al., 1972; Sager, 1972, S. Wolfe, 1972).

On the other hand, cycloheximide and α-amanitin, inhibitors of eukaryotic cytoplasmic protein synthesis, have no effect on mitochondria (Sager, 1972; Conklin and Chou, 1971; Mager, 1960).

3. Characteristics of the ribosomes

Mitochondrial ribosomes in general are smaller, have a greater sensitivity to magnesium-ion concentration, and have lower sedimentation coefficients than cytoplasmic ribosomes (Chi and Suyama, 1970). In these ways they appear to be similar to bacterial ribosomes (e.g., Sager, 1972). In addition mitochondrial ribosomes are distinct from cytoplasmic ribosomes in the electrophoretic patterns of constituent ribosomal proteins, in the base compositions of the ribosomal RNA's (rRNA's), and in the hybridizability of the rRNA's with nuclear and mitochondrial DNA (Chi and Suyama, 1970).

4. Nature of the transfer RNA's (tRNA's)

At least 15 mitochondrial tRNA's which are distinct from cytoplasmic tRNA's have been identified (Linnane et al., 1972). Of particular interest is the occurrence in the mitochondria of rat liver,
yeast, and Neurospora of a methionyl tRNA (met-tRNA) specifically capable of being enzymatically formylated, whereas the corresponding cytoplasmic met-tRNA is inactive in this respect (Sager, 1972). In bacteria but not in eukaryotic cytoplasm, formylmethionyl-tRNA (f-met-tRNA) is required for the initiation of protein synthesis in ribosomes (Blakely, 1969).

H. Evolutionary Origin of Mitochondria

1. Endosymbiont hypothesis

Altmann (1890) proposed that mitochondria had originally been bacteria which had become established as permanent endosymbionts in eukaryotic cells. The current renewed interest in this hypothesis has been based on a number of observations. First, mitochondrial growth and division appear to have elements of autonomy with a genetic and protein synthetic system distinct from the system present in the nucleus and cytoplasm. Indeed mitochondrial heredity has been reported and a number of specific mitochondrial mutants have been identified (Sager, 1972; Adoutte and Beisson, 1972).

Second, a number of similarities appear to exist between mitochondria and bacteria. They are similar in size and shape. Mitochondria are mostly prolate spheroids about .3 μm-5 μm long and .3 μm-2 μm wide; this compares well with many coccus and bacillus bacteria. Both mitochondria and bacteria are often somewhat pleomorphic (Baxter, 1971) and apparently they both divide by division. The composition of the outer mitochondrial membrane is similar to other cellular membranes and contains cholesterol (Ernster and Kuylenstierna, 1970), while the inner mitochondrial membrane is more like that of bacteria and lacks cholesterol.
Also, the genetic and protein synthetic systems of mitochondria and bacteria show many similarities.

Third, examples exist for such an endosymbiotic process. A number of cases have been reported where prokaryotes or where eukaryotic algae have established either temporary or permanent endosymbiotic relationships in ciliate and invertebrate hosts (Gibson et al., 1971; Mackinnon and Hawes, 1961).

2. Alternative hypotheses

The endosymbiont hypothesis is by no means universally accepted. Several problems are evident. The mitochondrial genome is small and its capacity has been considered inadequate to code for all mitochondrial proteins (e.g., Linnane et al., 1972; Lehninger, 1964). In addition, certain components of the mitochondrial protein synthetic system, such as ribosomal proteins and peptide chain elongation factors, appear to be synthesized in cytoplasmic ribosomes (Linnane et al., 1972; Baxter, 1971).

Because of the small size of the mitochondrial genome, Meyer (1973) has argued that the bacterial episomes, and not a complete bacterial genome itself, were the source of the DNA. Raff and Mahler (1972), on the other hand, have suggested that as the prokaryotic cell evolved into a eukaryotic cell, its mesosomes transformed into mitochondria and that certain portions of the nuclear genome were committed to the mitochondrion for convenience.

I. Regulation of Nucleic Acid Pools by Nutritional Control

T. pyriformis was the first ciliate to be grown in axenic culture and the first for which the complete nutritional requirements were
established (Elliott and Hayes, 1953; Kidder et al., 1950). Knowledge that *T. pyriformis* required a preformed pyrimidine ring, plus recent knowledge of many of the pathways involved in nucleic acid metabolism (e.g., Plunkett and Moner 1971; Zeuthen and Villadsen, 1970) suggested that nucleic acid pools could be regulated by nutritional control and that processes or organelles dependent on nucleic acid could thereby also be regulated.

Heinrich et al. (1957) demonstrated that deletion of folic acid prevented the synthesis of thymidine monophosphate from deoxyuridine monophosphate. However, they concluded that folic acid was also involved in another pathway or pathways, since addition of thymidine did not eliminate the folic acid requirement. Wykes and Prescott (1968) concurred with this conclusion, noting that the addition of thymidine to folic acid-starved *T. pyriformis* caused only a minimal growth response. They suggested that folate derivatives might be required for the synthesis of f-met-tRNA, which could be involved in the initiation of protein synthesis. In such a case, the results might be detectable by both ultrastructural and biochemical means. The procedures for obtaining cell-free preparations of *T. pyriformis* have been reviewed by Conklin and Chou (1971) and the procedures for isolating *T. pyriformis* mitochondria have been discussed by Schwab-Stey et al. (1971).

Zeuthen and Villadsen (1970) reported another method for controlling the thymidine monophosphate pool. They demonstrated that folic acid analogues allowed partial division synchrony in *T. pyriformis*, which they
attributed to the inhibitory effect of the analogues on the thymidine synthetase reaction.
III. MATERIALS AND METHODS

A. General Procedures

1. Culture conditions and procedures

   a. Proteose peptone medium

   Tetrahymena pyriformis GL was routinely grown axenically in a proteose peptone medium because of its simplicity of assembly and ability to promote rapid growth. The medium consisted of 2% proteose peptone (Difco, Detroit, Mich.), 0.1% liver extract (Nutritional Biochemical Co., Cleveland, O.), and 0.1% K$_2$HPO$_4$, pH 7.1. Cells were routinely grown in 5 ml of medium in 16 x 125 mm screw-cap culture tubes slanted to provide maximum aeration. When large quantities of cells were required, 1000 ml Erlenmeyer flasks containing 300 ml medium were used. These flasks were maintained on a Con-Torque Rotary Shaker (Eberbach Corp., Ann Arbor, Mich.) at 128 rpm. All cultures were grown at ambient temperature, approximately 23°C.

   Aliquots were aseptically withdrawn from cultures for cell counts and optical density (OD) measurements. Cells were prepared for counting by mixing a volume of cells with an equal volume of fixative. Cell counts were made in quadruplicate on either a Bright-Line Hemocytometer (A. O. Spencer, Buffalo, N.Y.) or a Speirs-Levy Eosinophile Counting Slide (C. A. Hausser and Son, Philadelphia, Pa.). Optical density was measured at 535 nm on a Spectronic 20 Spectophotometer (Bausch and Lomb, Rochester, N.Y.) using an adaptor for 16 x 125 mm culture tubes.

   Cultures were routinely monitored by OD measurements. Cells were inoculated from logarithmic phase cultures into new medium at an initial
OD of 0.01; cultures were harvested or subcultured at an OD of 0.25, which corresponds to a concentration of $10^5$ cells/ml.

b. Defined medium For experiments involving nutritional deletions, T. pyriformis was grown in a defined medium. The components and instructions for assembly of the medium are given in Sec. VII. For most experiments cells were grown in 15 ml of medium in 50 x 150 mm culture tubes using Dispo Plugs (Scientific Products, Chicago, Ill.). Tubes were slanted to provide maximum aeration. Growth was routinely monitored by OD which was measured at 560 nm in a Spectronic 20, using an adaptor for the 50 x 150 mm tubes. Cells were inoculated to produce an initial OD of 0.01. They were subcultured at the end of the logarithmic phase of growth; this point varied depending on the nutritional parameters. Cell counts were made as in the above section. When large quantities of cells were required, the cells were grown in 500 ml prescription bottles (see Sec.III.D.1. for details). All cultures were grown axenically at ambient temperature, approximately 23°C.

2. Microscopy

Cells and cell fractions were observed on a Universal Microscope (Carl Zeiss, Inc., Oberkochen, West Germany) equipped with a 0.9 numerical aperture (na) condenser and a x40 (0.65 na) phase objective. Fluorescence was observed on a Zeiss Photomicroscope equipped with HBO-200 high-pressure mercury arc illumination, exciter filters UG1 and UG5, barrier filters BG44 and BG53, a 1.4 na achromatic aplanatic condenser, and a x40 (0.65 na) phase objective or a x100 (1.3 na) oil-immersion phase objective.
Cells were fixed in $\text{OsO}_4$ and $\text{HgCl}_2$ and prepared for scanning electron microscopy according to the procedure of Marszalek and Small (1969); micrographs were taken by Dr. M. E. Cornford on a JSM-S1 microscope (Japanese Electron Optics Co., Tokyo, Japan). Preparative procedures for transmission electron microscopy varied and will be described in connection with individual experiments; micrographs were taken by Dr. D. E. Outka on an HU-11E microscope (Hitachi, Ltd., Tokyo, Japan) at 50 kV.

B. Cytochemistry

1. Identification of nucleic acids by acridine orange fluorescence

Cells were obtained from one-day old proteose peptone cultures. Both intact cells and isolated pellicles were fixed, treated with nucleases, and stained with acridine orange (Fisher Scientific Co., Fair Lawn, N.J.) according to the procedures of Kasten (1967), Smith-Sonneborn and Plaut (1967), and Randall and Disbrey (1965).

2. Extraction of nucleic acids from fixed cells

   a. Fixation procedures

Both RNA and DNA extraction from fixed cells was measured in an initial experiment. Twelve hundred ml of cells were harvested and concentrated by centrifugation in 250 ml plastic centrifuge bottles in an RC2B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) at 1500 g for 6 minutes at $4\,^\circ\text{C}$. The cells were resuspended in fresh, cold proteose peptone medium. An aliquot was pelleted and resuspended in pH 7.4 buffer containing 0.1 M potassium phosphate + 0.5 M glycerol (glycerol-phosphate buffer) and then homogenized in an ice bath with a Biosonik Ultrasonic probe.
(Bronwill-Blackstone Co., Rochester, N.Y.) until cell breakage was complete. Perchloric acid (PCA) in glycerol-phosphate buffer was then added to yield a final concentration of 5% PCA. The remaining cells were fixed by mixing a volume of cells with an equal volume of double-strength Karnovsky's fixative; after 20 minutes the cells were pelleted and resuspended for an additional 10 minutes in fresh, single-strength Karnovsky's fixative, consisting of 0.2 M glutaraldehyde, 0.3 M paraformaldehyde, 0.1 M sodium cacodylate, and 0.01 M CaCl₂, pH 7.4 (Karnovsky, 1965).

In two additional experiments, only RNA extraction was analyzed. In these experiments 300 ml of cells were harvested in 50 ml round-bottom centrifuge tubes in a clinical centrifuge (International Equipment Co., Needham Heights, Mass.) at 850 g for 6 minutes. In one experiment, efforts were made to improve RNA extraction from Karnovsky-fixed cells. Washing the cells in 0.2 M glycerol prior to fixation to remove the medium, short fixation (10 minutes), and agitation during the extraction were each tested for their efforts on nucleic acid extraction. In the other experiment, the effect of other fixatives on RNA extraction was investigated. Cells were prewashed three times in 0.2 M glycerol and fixed for 30 minutes in one of four fixatives: single-strength Karnovsky's; 0.5 M formaldehyde; 0.5 M glutaraldehyde; and 0.5 M acrolein. Each fixative was made up in 0.1 M sodium cacodylate + 0.01 M CaCl₂ and adjusted to pH 7.4.

After fixation, the samples of each experiment were washed three times in 0.5 M glycerol + 0.1 M sodium cacodylate (glycerol-cacodylate
buffer) and three times in glycerol-phosphate buffer. The fixation and subsequent washes were all performed at 4°C.

b. Extraction procedures. Four procedures were used to extract the NA from the fixed cells. Hot PCA hydrolysis, in which the sample was incubated in 5% PCA in glycerol-phosphate buffer for one-half hour at 90°C, served as the basis of comparison for the other procedures—cold PCA, RNase, and DNase. The cold PCA hydrolysis solution contained 10% PCA in glycerol-phosphate buffer. Samples were incubated for 18 hours at 4°C. The RNase hydrolysis solution contained 0.2 mg/ml RNase (Beef pancreas, Worthington Code RAF, Worthington Biochemical Corp, Freehold, N.J.) in 0.5 M glycerol, pH 6.5. The RNase contained 2700 units/mg of activity. The solution was boiled for 20 minutes to free it from possible contamination by DNase. The DNase hydrolysis solution contained 0.1 mg/ml DNase (beef pancreas, Worthington Code DPFF, Worthington Biochemical Corp.) in 0.003 M MgSO₄ + 0.5 M glycerol, pH 6.5. DNase was supplied electrophoretically separated from RNase. The enzyme was verified to possess 2300 units/mg of activity by measuring the increase in ultraviolet (uv) absorption at 260 nm observed during the course of depolymerization of DNA by DNase (Worthington Enzyme Manual, 1969). The enzyme hydrolyses were performed at 37°C.

After hydrolysis, the supernatant was saved and the sample of cells was either placed into an additional hydrolysis solution or placed into glycerol-cacodylate buffer and stored at 4°C until the hydrolyses had been completed on all the samples. Controls, which were performed
on all samples, were treated identically except that PCA or nuclease was omitted from the hydrolysis solution.

c. Assay procedures for nucleic extraction

(1) Biochemistry  Optical density of the supernatant from each extraction was measured at 260 nm and 280 nm with a DU Quartz Spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.) modified with a Gilford OD converter and a Gilford digital readout (Gilford Instrument Laboratories, Inc., Oberlin, O.)

Both DNA and RNA were measured colorimetrically. In order to eliminate contamination from organic residues, reactions were carried out in 16 x 125 mm screw-cap glass tubes which had been soaked 24 hours in sulfuric acid-dichromate solution, rinsed five times in tap water, and finally rinsed three times in distilled water. DNA was measured by means of the indole test (Ceriotti, 1952) as modified by Keck (1956): tubes containing 2 ml of test substance, 1 ml of 3 N HCL, and 1 ml of 0.06% indole were placed in a boiling water bath for 10 minutes; the tubes were cooled, nonspecific coloring material was twice extracted with four ml of amyl acetate; and the OD of the extracted solutions was read at 490 nm. RNA was measured colorimetrically by means of an orcinol test (Shatkin, 1969). Standard curves were obtained for yeast DNA and RNA (Nutritional Biochemical Co., Cleveland, O.) in 0%, 5%, and 10% PCA in glycerol-phosphate buffer. The DNA and RNA were stored desiccated in a vacuum chamber. Optical densities for these reactions were recorded on a Spectronic 20. All samples were measured in duplicate for both reactions.
(2) Electron microscopy  Samples which had been stored at 4°C in glycerol-cacodylate buffer until all hydrolyses were completed were washed in this buffer twice more, post-fixed in 2% OsO₄ in glycerol-cacodylate, pH 7.4, for one hour in the cold, washed twice in glycerol-cacodylate buffer, twice in distilled water, and stained and post-fixed for 20 minutes in 0.5% aqueous uranyl acetate (Kellenberger et al., 1958). The samples were then washed twice in distilled water, dehydrated rapidly in a graded acetone series, infiltrated in a graded series of Epon-Araldite in acetone (Anderson and Ellis, 1965), and polymerized in Epon-Araldite in capsules at 60°C for 72 hours. The blocks were sectioned on an Om U-2 Ultramicrotome (Reichert, Vienna, Austria) with a diamond knife. Sections on grids were stained in 1% uranyl acetate in 50% ethanol and then in 0.1% or 0.3% lead citrate (Venable and Coggeshall, 1965; Reynolds, 1963). Stain solutions were filtered prior to use (Rowden, 1969).

3. Enzyme hydrolysis on sectioned material

For this experiment cells were grown in the defined medium. Cells near the end of logarithmic growth were harvested by centrifugation and fixed in a solution of 0.5 M glutaraldehyde, 0.1 M sodium cacodylate, and 0.08 M sucrose, pH 6.9, for 20 minutes. The fixed cells were washed twice in 0.1 M cacodylate + 0.08 M sucrose, post-fixed in 2% OsO₄ in 0.1 M cacodylate, pH 6.0, for 30 minutes, washed three times in distilled water, and stained and post-fixed for 20 minutes in 0.5% aqueous uranyl acetate (Kellenberger et al., 1958). The cells were washed in water, dehydrated rapidly in a graded acetone
series, and infiltrated in a graded series of ERL in acetone. ERL is a low viscosity embedding medium (Spurr, 1969) obtained from Polysciences Inc., Warrington, Pa. The cells were placed in capsules and polymerized at 60°C for 55 hours. The blocks were sectioned on an Om U-2 Ultramicrotome with a diamond knife. Thin sections were picked up on grids which had been prepared with parlodion films and then carbon coated.

These grids were subjected to enzyme hydrolyses modified from the procedures of Anderson and André (1968) as follows: the grids with sections were floated in spot plate depressions on a 3% aqueous solution of hydrogen peroxide for one hour at 37°C, rinsed in distilled water and immersed in an enzyme solution at 37°C for 10 hours. Pronase (Calbiochem Corp., La Jolla, Calif.) at a concentration of 0.5 mg/ml was adjusted to pH 7.4; 0.2 mg/ml RNase was adjusted to pH 6.5. Control grids were treated in solutions which contained no enzyme.

After the hydrolysis, the grids were rinsed with distilled water and then stained five minutes in 1% uranyl acetate in 50% ethanol, followed by a 0.1% lead citrate stain (Venable and Coggeshall, 1965). Stain solutions were filtered prior to use (Rowden, 1969).

C. Nutritional Deficiencies

1. Culture conditions and procedures

*Trichomonas pyriformis* GL was grown in defined medium in 50 x 150 mm tubes as described in Section III.A.1. Uridine, thymidine, and/or folic acid were omitted, or 0.05 mM amethopterin or 0.05 mM amethopterin + 200x uridine (Zeuthen and Villadsen, 1970) was added to the various experimental media (Table 1).
<table>
<thead>
<tr>
<th>Sample</th>
<th>Uridine</th>
<th>Thymidine</th>
<th>Folic Acid</th>
<th>Amethopterin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. U+T+F+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>2. U+T+F-</td>
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<tr>
<td>3. U+T-F+</td>
<td>+</td>
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<tr>
<td>4. U+T-F-</td>
<td>+</td>
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<td>-</td>
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<td>5. U-T+F+</td>
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<tr>
<td>6. U+T+F+A+</td>
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<tr>
<td>7. U+T+F+A+</td>
<td>x200</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

Cells were either inoculated directly into the experimental media or they were washed three times in medium lacking uridine, thymidine and folic acid (U-T-F-) and then inoculated. Each variant was tested in duplicate, and each growth experiment was repeated three or more times.

Cell counts and OD determinations (Section III.A.1.) were used to measure cell growth. Tubes were inoculated at an initial OD of 0.01. When an individual tube approached stationary phase, one portion of the culture was inoculated into a tube containing fresh experimental medium. Another portion was inoculated into control medium (U+T+F+) in order to determine recovery from the deletion. Cells were fixed and prepared for electron microscopy as in Section III.B.3.

Cell volume was calculated from measurements of length and width. Dimensions of fixed cells were determined with a Zeiss light microscope by means of an ocular micrometer which had been calibrated against a stage micrometer. Each set of dimensions reported represents an
average of 25 cells. Assuming that *T. pyriformis* approximates a prolate ellipsoid (Corbett, 1958), the volume \( V \) can be calculated according to the following equation: 
\[
V = \frac{4}{3} \pi abc
\]
where \( a \) is the major semiaxis and \( b \) and \( c \) are the minor semiaxes. Measurements of low-power electron microscopic cross sections indicate that one minor semiaxis is approximately \( \frac{2}{3} \) of the other. Since the cells would most probably orient themselves so that the longer minor axis would be horizontal on the microscope slide, the longer one was assumed to be the one measured. Hence, \( a = \frac{\text{length}}{2} \), \( b = \frac{\text{width}}{2} \), \( c = \left(\frac{2}{3}\right) \frac{\text{width}}{2} \), and \( V = \frac{1}{9} \pi ab^2 \).

2. Analysis of micrographs

Micrographs were taken on 3½ x 4 inch glass electron image plates (Eastman Kodak Co., Rochester, N.Y.). The electron density of mid-longitudinal sections of kinetosomes was determined by scanning selected portions of the glass plate negatives with an RB Analytrol Densitometer (Beckman Instruments, Inc., Palo Alto, Calif.) equipped with a film densitometer attachment. Two scans were required per kinetosome to include most of each kinetosome's axial core. The densitometry curves were compared to the micrographs and the areas under the curves which corresponded to the regions of the kinetosomal axial core and the microtubular triplet wall were measured with a planimeter (Frederick Post Co., Chicago, Ill.) to obtain a core/microtubule ratio (C/M).

For computations of mitochondrial area and number per cell, electron micrographs were selected which transected the cell in the region of the macronucleus. At least 6 cells were selected from each nutritional variant; the cytoplasmic area was measured with a planimeter. The
length (l) and width (w) of at least 20 mitochondria in each cross section were measured, and the mean l and w were used in computing the average mitochondrial cross sectional area (A): \( A = \frac{1}{4} \pi lw \).

D. Reduction of Protein Synthesis in Mitochondria

1. Culture conditions and procedures

Several antibiotics were tested for their ability to inhibit growth. Chloramphenicol (Parke, Davis and Co., Detroit, Mich.), chlortetracycline HCl (Lederle Laboratories Division, American Cyanimid Co., Pearl River, N.Y.), streptomycin sulfate (Parke, Davis and Co.), neomycin sulfate (Nutritional Biochemical Co., Cleveland, 0.), and erythromycin stearate (Abbott Laboratories, North Chicago, Ill.) were tested at concentrations of 50, 150, and 450 mg/l. Cycloheximide (Nutritional Biochemical Co.) was tested at concentrations of 2, 6, and 18 mg/l. The agents were added from stock solutions to U+T+F+ defined medium to produce the desired concentrations. Cells were grown in 15 x 125 mm tubes and growth was measured by OD (see Sec. III.A.1. for details).

The minimum concentration of the above drugs effective in inhibiting growth were used for the amino-acid incorporation experiment, which consisted of four samples: U+T+F+ cells, control, U+T+F- cells, U+T+F+ + 50 mg/l chloramphenicol, and U+T+F+ + 6 mg/l cycloheximide. The chloramphenicol was added at the time of inoculation, but the cycloheximide was added 10 hours prior to harvest. Cells were grown in 125 ml medium in 500 ml screw-cap prescription bottles, which were placed on their sides to provide maximum aeration. Cell growth was monitored by aseptically removing 10 ml aliquots for OD determinations and cell counts.
Cells from one-day old prescription bottle cultures in defined medium were prewashed in buffer consisting of 2 g KH$_2$PO$_4$, 2 g K$_2$HPO$_4$, and 2 g sodium acetate per liter, pH 6.9, and were inoculated to produce an initial OD of 0.01.

2. Isolation and assay procedures

Mitochondria were isolated by a procedure adapted from Flavell and Jones (1970b). When the control was in mid-logarithmic growth, the cultures were harvested by centrifugation at 800 g for 5 minutes in a floor model centrifuge (International Equipment Co., Needham Heights, Mass.). The cells were resuspended in ten volumes of cold 0.3 M sucrose, 2 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM 2-amino-2-hydroxymethyl-1,3-propanediol HCl (Tris), pH 7.2; and homogenized in a sonicator (L and R Manufacturing Co., Kearney, N.J.). Cell disruption was monitored by phase microscopy.

The cell suspensions were spun at 750 g for 8 minutes in an RC2B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) and the pellet containing whole cells and nuclei was discarded. The supernatant was then centrifuged at 5500 g for 8 minutes to sediment the mitochondria. The pellet was resuspended in the same volume of homogenization medium and again centrifuged at 5500 g for 8 minutes; this step was repeated twice.

Lowry determinations for protein (Shatkin, 1969) were performed on aliquots of the mitochondrial fractions. Bovine serum albumin (Armour and Co., Chicago, Ill.) was used to prepare a standard curve. Optical density of the samples was measured at 650 nm on a Lumetron Colorimeter (Photovolt Corp., New York, N.Y.).
The procedure for assaying amino acid incorporation was modified from Rosenbaum and Holz (1966) and Mager and Lipmann (1958): 0.5 ml samples of mitochondrial fractions were added to 0.5 ml of incubation medium consisting of 100 mM Tris, pH 7.6, 6 mM MgCl$_2$, 10 mM adenosine triphosphate, and 0.4 mM D,L-lysine HCl-2-C $^14$ (New England Nuclear Corp., Boston, Mass.) The lysine had a specific activity of 3.8 Curies/M. In addition, when chloramphenicol or cycloheximide had been present in the culture medium, it was added at the same concentration to the incubation medium. Samples were incubated for five hours at 26.5$^\circ$C, the temperature optimum for T. pyriformis, and the reactions were stopped by the addition of an equal volume of 10% trichloracetic acid.

The samples were centrifuged in a clinical centrifuge (International Equipment Co.) and the mitochondrial pellets were dissolved in 0.5 ml of 1 N NaOH and transferred to Tri-Carb liquid scintillation vials (Packard Co., La Grange, Ill.). Fifteen ml of scintillation gel (Heintz et al., 1968) was added. The vial was shaken several times and the radioactivity was measured in a Tri-Carb liquid scintillation spectrometer.

E. Isolation of Pellicles and Pellicular Components

1. Isolation of pellicles

Figure 1 illustrates the steps involved in obtaining, isolating, and disrupting the pellicles. To obtain the pellicles 300 ml proteose peptone cultures were chilled at 4$^\circ$C in a dry ice-acetone bath and centrifuged at 160 g for 5 minutes in a floor model centrifuge (International Equipment Co., Needham Heights, Mass.). The cells were
Culture
Chill to 4°C in dry ice-acetone bath
160 g, 5 min.

Supernatant (medium)  Pellet (cells)
Resuspend in high ionic strength (N-T) buffer
100 g, 5 min.

Supernatant  Pellet (washed cells)
Resuspend in N-T buffer, homogenize gently
1020 g, 5 min.

Pellet (deciliated cells)  Supernatant (cilia)
Resuspend in N-T buffer, homogenize

Homogenate  Homogenate
High ionic strength procedure (N-T) for isolation of pellicles and pellicular components; place on sucrose gradient 4000 g, 5 min.

Pellet (nuclei and unbroken cells)  Supernatant (Pellicle and Particulate Fraction)
Resuspend in N-T buffer 1250 g, 5 min.
Figure 1. Scheme for the isolation of pellicles and pellicular components from T. pyriformis.
resuspended in cold, high ionic strength buffer (N-T buffer, Nozawa and Thompson, 1971) consisting of 0.2 M potassium phosphate, pH 7.2, 0.1 M NaCl, and 3 mM Na₂EDTA and washed by another 5 minutes centrifugation at 100 g.

The washed cells were resuspended in 12 ml of N-T buffer and gently homogenized by hand in a loose-fitting, ground-glass homogenizer until most of the cilia had been removed from the cells, as determined by phase microscopy. The suspension was then centrifuged at 1000 g for five minutes to sediment the deciliated cells. The pellet, about 2 ml of deciliated cells, was resuspended in 10 ml of N-T buffer and homogenized vigorously by hand in a tight-fitting, ground-glass homogenizer. To isolate the pellicles, the cell homogenate was layered on a discontinuous N-T buffered sucrose gradient (0.3 M, 10 ml; 1.0 M, 15 ml; 1.5 M, 15 ml) and centrifuged in 50 ml conical-bottom tubes in a swinging bucket rotor at 4000 g for 5 minutes. Three major zones were separated: Zone A, a top band down through the 0.3 M layer; Zone B, a discrete band of pellicles and pellicular components at the interface between the 1.0 M and the 1.5 M layers; and Zone C, a pellet of unbroken cells and nuclei at the bottom. All materials were maintained in an ice bath during the entire process.

Several modifications of this N-T procedure were employed. First, a Teflon-glass homogenizer (A. H. Thomas Co., Philadelphia, Pa.) was substituted for the ground-glass one used above in obtaining the pellicles. The Teflon plunger was connected to a small motor (Talboys Engineering Corp., Emerson, N.J.) and was rotated at 150 rpm to detach
the cilia from the cells and at 350 rpm to disrupt the cells. Second, a different method was employed to isolate the pellicles from other cellular components. The homogenate was centrifuged at 450 g for 5 minutes to sediment the nuclei and unbroken cells. The supernatant was then centrifuged at 1250 g for 5 minutes to pellet the pellicles. The pellet, about 2 ml, from the 1250 g centrifugation was resuspended in 10 ml of N-T buffer and recentrifuged at 1250 g; this step was repeated three times.

2. Disruption of pellicles

The pellicular pellet was disrupted in one of two ways (Figure 1). In one method, the pellet was resuspended in 10 ml of cold solution consisting of 0.15 M sucrose, 15 mM Tris, 2.5 mM Na₂EDTA, 11% ethanol, 30 mM KCl and 0.6% Triton X-100 (octyl phenoxy polyethoxyethanol, Sigma Chemical Co., St. Louis, Mo.); this solution will be called "STEEP + Triton X" (Witman, et al., 1972a). The suspension was incubated for 14 hours in the cold and then the pellicles were gently disrupted, as monitored by phase microscopy, by three strokes with a B-32 small glass hand homogenizer (Kontes Glass Co., Vineland, N.J.). In the second method, the pellet was resuspended in 10 ml of cold, low ionic strength buffer consisting of 2 mM EDTA, 10 mM Tris, and 300 mM sucrose, pH 7.2; the pellicles dissolved in several minutes (Flavell and Jones, 1970b).

3. Preparation of samples for electron microscopy

The pellicular preparations were prepared for examination by electron microscopy by first placing a drop of sample on a glow-
discharged, carbon-coated grid. The grid was then rinsed with distilled water and negatively stained, or it was fixed by adding a drop of 10% formalin in 0.1 M sucrose, pH 8.5, to the grid, rinsed with 0.3% Photo Flo (Kodak Co., Rochester, N.Y.) and then negatively stained (Miller and Bakken, 1972). Several stains were used: 1% phosphotungstic acid (PTA), pH 7.2; 4% uranyl acetate, pH 7.3; 4% uranyl formate, pH 7.3; and 1% uranyl acetate + 1% PTA in 70% ethanol. Excess solution was removed from the grid with filter paper after each step.
IV. RESULTS

A. General Observations on *Tetrahymena pyriformis* GL

1. Growth

*Tetrahymena pyriformis* GL was routinely grown in proteose peptone medium (Figure 2) because growth was rapid and the medium was easy to prepare. Generation time was 3.2 hours. Although Scherbaum and Zeuthen (1955) were able to achieve a generation time of 2.3 hours in proteose peptone medium by growing the cells at the optimum temperature, 28.5°C, other aspects of their growth curves are similar to the ones presented here. Their reported cell density at maximum stationary phase of $8 \times 10^5$ cells/ml compares closely with the cell density of $7 \times 10^5$ cells/ml in Figure 2. The curves for cell number and OD are similar, indicating that OD is reliable for routine monitoring of the culture. Cells were harvested for experiments in mid-logarithmic growth at an OD of 0.23, i.e., $10^5$ cells/ml.

Normal generation time for *T. pyriformis* in defined medium was seven hours. Details concerning the growth, including growth curves, are presented in Sec.IV.C.1.

2. Ultrastructure

The major features of the ultrastructure of the holotrichous ciliate, *T. pyriformis*, have been described previously by a number of workers. *T. pyriformis* has a precise pattern of ciliation which can conveniently be described as oral and somatic ciliature (Figure 3). There are four sets of oral cilia. Though distinct, the cilia of each set are closely spaced and cohere together; hence, they function as a
Figure 2. Growth curves for *T. pyriformis* in proteose peptone medium. Cells were grown at ambient temperature, about $23^\circ C$, in 300 ml of medium in 1000 ml Erlenmeyer flasks on a rotary shaker (see Sec.III.A.1. for details).

Figure 3. Scanning micrograph of OsO$_4$- and HgCl$_2$-fixed *T. pyriformis*. The organism's mouth can be clearly seen in the upper right side of the micrograph. The three membranelles (Mb$_1$, Mb$_2$, Mb$_3$) are on the organism's left and the undulating membrane (UM) is on the organism's right. The cilia in each of the four mouth structures cohere together. The somatic cilia are arranged in rows, or kineties. One such kinety (Ki) is indicated by the double-headed arrow. Somatic cilia (C) are up to 6 $\mu$m long. X 3,500.
"membrane." On the organism's right is a single row of cilia called the undulating membrane; on its left are three membranellae, each of which has three rows of cilia. It is this quadripartite, or tetrahymenal, nature of the oral ciliature which provides the organism with its name (Mackinnon and Hawes, 1961).

The somatic cilia are arranged in meridians. Each cilium is attached at the level of the pellicle to a kinetosome. The kinetosome is the key structure in an integrated morphological unit called a kinetid (Allen, 1969, 1967). The kinetid (Figure 4; see Table 2 for symbol explanations in micrographs.) includes, in addition to the cilium and the kinetosome, the kinetodesmal fiber, the terminal plate, the circumciliary ring, and the several microtubular systems originating at the kinetosome and linking it to other cortical elements. The kinetodesmal fiber always originates at the anterior right corner of a kinetosome and extends anteriorly, connecting the kinetosome with other kinetosomes in the meridian. The longitudinal and basal microtubules also interconnect the kinetosomes of a meridian (Franke, 1971). Together, all the kinetids in a meridian form a complex morphological structure called a kinety. In addition, transverse microtubules cross-link kineties. Except in the oral region each new kinetosome, or prokinetosome, always occurs anteriorly to the parent kinetosome and to the left of the kinetodesmal fiber.

Several additional organelles are also prominent within the cytoplasm. Ribosomes, endoplasmic reticulum, and peroxisomes are distributed throughout the cytoplasm. Mitochondria also occur throughout the
Table 2. Abbreviations used in micrographs

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>cilium</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>K</td>
<td>kinetosome</td>
</tr>
<tr>
<td>M</td>
<td>mucocyst</td>
</tr>
<tr>
<td>Mi</td>
<td>mitochondrion</td>
</tr>
<tr>
<td>N</td>
<td>nucleus</td>
</tr>
<tr>
<td>Nc</td>
<td>nucleolar cleft or cavity</td>
</tr>
<tr>
<td>Nm</td>
<td>nuclear membrane</td>
</tr>
<tr>
<td>Np</td>
<td>nuclear pore</td>
</tr>
<tr>
<td>Nu</td>
<td>nucleolus</td>
</tr>
<tr>
<td>P</td>
<td>peroxisome</td>
</tr>
<tr>
<td>ac</td>
<td>axial core</td>
</tr>
<tr>
<td>am</td>
<td>alveolar membrane</td>
</tr>
<tr>
<td>ap</td>
<td>annulus of nuclear pore</td>
</tr>
<tr>
<td>at</td>
<td>attachment site of kinetodesmal fiber to kinetosome</td>
</tr>
<tr>
<td>ax</td>
<td>axosome</td>
</tr>
<tr>
<td>bp</td>
<td>breaking point of cilium</td>
</tr>
<tr>
<td>bt</td>
<td>basal microtubule</td>
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<tr>
<td>cc</td>
<td>circumciliary ring</td>
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<td>cr</td>
<td>crista</td>
</tr>
<tr>
<td>cs</td>
<td>central sheath of cilium</td>
</tr>
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<td>cv</td>
<td>coated vesicle</td>
</tr>
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<td>cartwheel</td>
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<td>chr</td>
<td>condensed chromatin</td>
</tr>
<tr>
<td>e</td>
<td>epiplasmic layer</td>
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<tr>
<td>h</td>
<td>hole in pronase-treated section</td>
</tr>
<tr>
<td>im</td>
<td>intranuclear microtubules</td>
</tr>
<tr>
<td>kd</td>
<td>kinetodesmal fiber</td>
</tr>
<tr>
<td>kf</td>
<td>kinetosomal triplet foot</td>
</tr>
<tr>
<td>lb</td>
<td>lipid body</td>
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<tr>
<td>lt</td>
<td>longitudinal microtubules</td>
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<tr>
<td>mr</td>
<td>mitochondrial ribosome</td>
</tr>
<tr>
<td>md</td>
<td>doublet microtubules of cilium</td>
</tr>
<tr>
<td>mt</td>
<td>triplet microtubules of kinetosome</td>
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<tr>
<td>ocm</td>
<td>oral connective microtubules</td>
</tr>
<tr>
<td>ocf</td>
<td>oral connective filaments</td>
</tr>
<tr>
<td>pdr</td>
<td>partially digested ribosome</td>
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<tr>
<td>pm</td>
<td>pellicular membrane</td>
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<tr>
<td>pr</td>
<td>parasomal ring</td>
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<tr>
<td>ps</td>
<td>parasomal sac</td>
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<tr>
<td>r</td>
<td>ribosome</td>
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<td>rp</td>
<td>central rod of nuclear pore</td>
</tr>
<tr>
<td>rs</td>
<td>radial spoke</td>
</tr>
<tr>
<td>s</td>
<td>kinetosomal sheath</td>
</tr>
<tr>
<td>tp</td>
<td>terminal plate</td>
</tr>
<tr>
<td>tt</td>
<td>transverse microtubules</td>
</tr>
</tbody>
</table>
Figure 4. Section of *T. pyriformis* tangential to cell surface. The cell was grown in defined medium, fixed in glutaraldehyde, post fixed in OsO₄, and embedded in ERL. Portions of two somatic kineties are visible (compare with Figure 21). A kinetodesmal fiber leads anteriorly from each kinetosome. At the upper right of the micrograph is a kinetid sectioned at the upper level of the terminal plate, the zone of transition between the kinetosome and the cilium. The third microtubule of each triplet has terminated, but the central pair of ciliary microtubules has not yet begun. A parasomal sac is anterior to each kinetid at the level of the pellicle. Mucocysts and peroxisomes are randomly oriented on the cell surface; mitochondria, on the other hand, are in rows which alternate with the kineties. Numerous tubular cristae, typical of protozoa, and ribosomes occupy the mitochondrial matrix. Numerous ribosomes are also present in the cytoplasm both free and along the endoplasmic reticulum (ER). Uranyl acetate-lead citrate. X 36,000
cytoplasm. Just below the pellicle, the mitochondria are arranged in rows which alternate with the kineties. The mitochondria contain numerous tubular shaped cristae. The matrix, which occupies the space between the cristae, contains ribosomes with properties distinct from those of the cytoplasm (Chi and Suyama, 1970). Mucocysts, striated, membrane-bound structures of unknown function, are randomly arranged along the pellicle.

A double membrane separates the nucleus from the cytoplasm. The outer membrane and the inner membrane are 9 ± 1 nm; they are separated by a space of 15 ± 1 nm (Figure 6). Both membranes have the typical unit membrane appearance. Nucleoli are distributed predominantly in a layer on the periphery of the nucleus, and condensed chromatin predominantly inside that layer, towards the center of the nucleus. The nucleoli are electron-dense amorphous bodies consisting primarily of fibers and granules; the proportion of these components to each other varies with changes in cultural conditions (Satir and Dirksen, 1971; Nilsson and Leick, 1970). Not infrequently, cavities or clefts in the nucleoli are visible. Charret (1969) has demonstrated by tritiated thymidine uptake that nucleolar organizer activity occurs in these locations when a deoxyribonucleoprotein granule is present. This granule (see Sec.IV.B.2.) has a different electron density than the nucleolus, but one similar to the condensed chromatin. Condensed chromatin appears as amorphous electron-dense masses in electron micrographs; little can be seen of any substructure. Numerous small granules and fibers ranging in size down to 10 nm in diameter appear in the nucleoplasm.
B. Cytochemistry

1. Failure to localize nucleic acids in kinetosomes by acridine orange fluorescence

The presence of DNA in ciliate kinetosomes, as indicated by acridine orange fluorescence, was claimed by Smith-Sonneborn and Plaut (1967) and by Randall and Disbrey (1965). Such presence would provide the basis of a genetic mechanism for kinetosomal replication and would account for the fact that the daughter kinetosome always occurs next to the parent. Despite considerable effort, no staining which could be correlated with the kineties was demonstrated. Rather, there was often general staining in the pellicle which could not be removed by nucleases. Further, when the rigorous procedures of Kasten (1967) were employed, there was no staining in the pellicles.

2. Enzymatic and chemical extraction from intact fixed cells

a. Biochemistry One explanation for the failure of light microscopic cytochemistry to demonstrate NA is that the NA might be present in amounts too small to be detected; in this case, the resolution afforded by electron microscopy would be required. Fixation prior to nuclease hydrolyses was employed to prevent the loss or deterioration of cellular components. Thus comparison at high resolution of structures before and after nuclease hydrolysis might reveal selective removal of NA from components and organelles, even if the NA were a minor component. The following three experiments were performed in order to obtain a quantitative estimate of the level of enzymatic extraction of NA from fixed T. pyriformis.
Table 3. Extraction of NA from Karnovsky-fixed *T. pyriformis*—ultraviolet and orcinol test data

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ultraviolet</th>
<th>Orcinol</th>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control^a</td>
<td>Exp. a</td>
<td>% of</td>
<td>260/280</td>
<td>Control^b</td>
<td>Exp. b</td>
<td>% of</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sample 1</td>
<td></td>
<td></td>
<td></td>
<td>Sample 1</td>
</tr>
<tr>
<td>1. unfixed cells hot PGA</td>
<td>---</td>
<td>390</td>
<td>100</td>
<td>---</td>
<td>1.8</td>
<td>---</td>
<td>430</td>
</tr>
<tr>
<td>2. hot PGA</td>
<td>15</td>
<td>380</td>
<td>100</td>
<td>1.5</td>
<td>1.6</td>
<td>18</td>
<td>440</td>
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<tr>
<td>3. cold PGA</td>
<td>5</td>
<td>50</td>
<td>15</td>
<td>1.8</td>
<td>1.8</td>
<td>8</td>
<td>70</td>
</tr>
<tr>
<td>4. a. 14 hour RNase</td>
<td>5</td>
<td>190</td>
<td>50</td>
<td>1.1</td>
<td>2.4</td>
<td>0</td>
<td>190</td>
</tr>
<tr>
<td>b. 26 hour RNase</td>
<td>6</td>
<td>100</td>
<td>25</td>
<td>1.3</td>
<td>2.0</td>
<td>0</td>
<td>110</td>
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<tr>
<td>c. hot PGA</td>
<td>0</td>
<td>120</td>
<td>30</td>
<td>---</td>
<td>1.9</td>
<td>0</td>
<td>160</td>
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<td>105</td>
<td></td>
<td></td>
<td></td>
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<td>105</td>
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<tr>
<td>5. a. 14 hour DNase</td>
<td>9</td>
<td>60</td>
<td>15</td>
<td>1.1</td>
<td>2.0</td>
<td>7</td>
<td>70</td>
</tr>
<tr>
<td>b. 26 hour DNase</td>
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<td>20</td>
<td>1.5</td>
<td>2.4</td>
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<td></td>
<td>95</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>90</td>
</tr>
</tbody>
</table>

^a Figures are given in picograms of NA/cell

^b Figures are given in picograms of RNA/cell
(1) **Extraction of RNA and DNA from Karnovsky-fixed cells**

Nucleic acid was completely removed from fixed cells by hot PCA hydrolysis (compare Sample 2 with Sample 1, Table 3); hence the hot PCA hydrolysis was used as a quantitative control for the enzyme hydrolyses. The 14-hour and 26-hour RNase hydrolyses combined extracted 75% of the NA; on the other hand, cold PCA hydrolysis (Sample 3) removed 15%, and 14-hour and 26-hour DNase hydrolyses removed 35%. Both the sequence of RNase hydrolyses followed by PCA (Sample 4), and the sequence of DNase hydrolyses followed by PCA (Sample 5) extracted amounts of NA approximately equal to that extracted by hot PCA alone (Sample 2).

Purified yeast NA solutions (Table 4) were used as standards for the experimental hydrolyses. Not-highly-polymerized RNA and DNA were used in order to minimize the effects of hyperchromicity in the standards.

### Table 4. 260/280 ratios for yeast nucleic acids used as standards in glycerol-phosphate (G-P) buffer

<table>
<thead>
<tr>
<th></th>
<th>G-P Buffer + 0% PCA (nuclease extraction)</th>
<th>G-P Buffer + 5% PCA (hot extraction)</th>
<th>G-P Buffer + 10% PCA (cold extraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>2.2 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>DNA</td>
<td>1.9 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
</table>

The similarity of the 260/280 ratios for the various samples to those of the standards indicated that little or no protein dissolved during
the hydrolysis treatments. Minimal dissolution, less than 3% of the NA, occurred in the controls. The actual orcinol values were slightly higher than the corresponding uv values; however, the figures for percentage of extraction of NA for the uv and the orcinol measurements were similar, ± 5% (Table 3). For example, for unfixed, homogenized cells, the orcinol value was 110% of the uv value and for the hot PCA hydrolysis of fixed cells, the orcinol value was 115%.

According to the indole test values for the hot PCA hydrolysis in homogenized cells, *T. pyriformis* had 18 pg of DNA/cell, or 4.5% of the uv value for the amount of NA per cell. By comparison, Scherbaum (1957) reported that *T. pyriformis* grown in proteose peptone medium at 28.5°C possessed 13.6 pg/cell of DNA or 5.5%; Scherbaum et al. (1959) reported, for the same conditions, a value of 17.5 pg/cell DNA. The hot PCA hydrolysis removed 70-80% of the indole-positive substance from fixed cells. For reasons not understood at this time, the remainder of the indole test data was not consistent, and further conclusions were not possible.

The amount of NA removed by DNase was equal to 30% of the cell's total NA whereas, both the indole test and the work of others indicate that the amount of DNA removed could only have been in the order of 3-5%. Results of the orcinol test and the similarity of the 260/280 ratio with that of RNA (Table 4) indicate the remainder of the NA must have been RNA. Thus, the DNase may have been contaminated with RNase even though the DNase was stated to be electrophoretically pure. In any event, the removal of a major amount of RNA during the DNase hydrolyses
made it impossible to correlate ultrastructural changes with the loss of a specific nucleic acid. Hence, the DNase extractions were not continued in later experiments.

In summary, the results of the extraction experiment on Karnovsky-fixed cells indicate that *T. pyriformis* has approximately 400 pg of NA per cell. According to this research and that of Scherbaum (1957), less than 6% of the NA was probably DNA and hence, both the uv values and the orcinol test values could be considered to be equivalent to the total NA in *T. pyriformis*, within the limits of error of the testing procedure. Hot PCA hydrolysis removed essentially all the NA, and so in later experiments the hot PCA value will be considered to be 100% of the cellular NA. The 14-hour and 26-hours RNase hydrolyses combined extracted 75% of the NA, whereas less than 3% was extracted in the controls.

(2) Efforts to improve RNA extraction from Karnovsky-fixed cells In the second experiment, several parameters were tested in an effort to improve RNA extraction (Table 5). The experimental procedure in Part A of this experiment was the same as that in the first experiment above, and the results were in good agreement. The uv data for the sequence of hydrolyses (Sample 2), added up to 115% of the hot PCA treatment (Sample 1), and the 14-hour and 26-hour RNase hydrolyses combined removed an amount of NA equal to 75% of the hot PCA extraction and 65% of the complete hydrolysis sequence (Sample 2). For the orcinol test, the hydrolysis sequence adds up to 109% of the hot PCA treatment, and the two RNase hydrolyses remove 70%
Table 5. Efforts to improve RNA extraction from Karnovsky-fixed *T. pyriformis* cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ultraviolet</th>
<th>Orcinol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control a</td>
<td>Exp a</td>
</tr>
<tr>
<td>Part A: standard procedure—not washed prior to 30 min. fixation, no agitation during extraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. hot PCA</td>
<td>18</td>
<td>410</td>
</tr>
<tr>
<td>2. a. 14 hour RNase</td>
<td>9</td>
<td>180</td>
</tr>
<tr>
<td>b. 26 hour RNase</td>
<td>9</td>
<td>140</td>
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<tr>
<td>c. hot PCA</td>
<td>0</td>
<td>160</td>
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<tr>
<td>Total of sample 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Part B: washed in 0.2 M glycerol (G) prior to fixation; no agitation during extraction

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ultraviolet</th>
<th>Orcinol</th>
</tr>
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### Part C: washed in G prior to fixation, plus agitation during extraction

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### Part D: washed in G prior to fixation, 10 min. fixation, plus agitation during extraction

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*Figures are given in picograms of NA/cell*

*Figures are given in picograms of RNA/cell*

*The fixation and extraction procedure is identical to that of previous experiment (Table 3)*
of the hot PCA treatment and 65% of the hydrolysis sequence. In general, orcinol values compare well with uv values. Dissolution of NA or protein in the controls was minimal.

Because proteose peptone medium has a high content of proteins and polypeptides, fixation of *T. pyriformis* in the medium by admixture with double-strength Karnovsky's fixative might cause denaturation and precipitation of the proteins and polypeptides onto the pellicular surface of the organisms. These proteins could conceivably dissolve during the hydrolyses and elevate the uv values, or they might physically interfere with diffusion of nucleases across the pellicle. To avoid these possibilities, the cells were washed in 0.2 M glycerol prior to fixation in single-strength Karnovsky's fixative. Glycerol was selected because it would be used later during the hydrolysis procedure, because it was not toxic to *T. pyriformis* as cacodylate buffer might be, and because it would not form a precipitate upon contact with the fixative as phosphate buffer would. Transfer of organisms into various dilutions of glycerol indicated that 0.2 M was optimal for survival.

Within the limits of the method used, there was no detectable effect on enzymatic hydrolysis due to prewashing (Part B, Table 5). Agitation (Part C, Table 5) facilitated the speed of enzymatic extraction, presumably by increasing the outward rate of diffusion of the hydrolysis products. The uv data and orcinol data suggest that 65% and 60%, respectively, of the NA was removed in the first (14-hour) RNase hydrolysis when agitation was employed, compared to values of 40-45%
removed without agitation (Part B, Table 5). Also, the control values, especially in the second (26-hour) RNase treatment, were higher with agitation; the low 260/280 ratio for this treatment suggests that some of this material may have been protein. The primary effect of the shorter fixation (Part D, Table 5) was that the second RNase hydrolysis control solution removed nearly as much NA as the enzyme and probably also more protein.

In summary, the orcinol values are generally somewhat higher than the corresponding uv values, but percentages of extraction for the orcinol and uv data show good correlation for this experiment. The data in Table 5 is in good agreement with that in Table 3 except that the Table 5 values for PCA extractions following the RNase extractions are about 30% higher. Prewashing had no effect on enzymatic hydrolysis; agitation speeded the process of enzymatic hydrolysis but did not increase the total amount of NA extracted.

(3) Effect of three other fixatives on RNA extraction

This experiment was performed to determine if other electron microscopic fixatives might permit more complete enzymatic hydrolysis. Formaldehyde, glutaraldehyde, and acrolein were tried; Karnovsky's fixative was used as a control (Table 6). As in the previous experiments for Karnovsky-fixed cells (Part A), the amount of NA extracted during the hydrolysis sequence generally exceeded the amount extracted during hot PCA hydrolysis alone by as much as 20%; and orcinol values again generally exceeded the uv values by 5-10%. Agitation facilitated more rapid extraction of NA (compare the 14-hour hydrolysis with that in
Table 6. Effect of other fixatives on RNA extraction from T. pyriformis

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### Part C: Glutaraldehyde fixation

1. Hot PCA 12 390 100 1.8 1.7 33 410 100

2. a. 14 hour RNase 13 300 80 2.7 2.1 29 270 65
   b. 26 hour RNase 35 80 20 1.3 1.1 33 40 10
   c. Hot PCA 21 100 **25** 2.4 1.4 25 70 **15**

Total for sample 2 120 95

### Part D: Acrolein fixation

1. Hot PCA 16 360 100 1.2 1.6 37 350 100

2. a. 14 hour RNase 14 280 80 1.9 2.1 33 290 80
   b. 26 hour RNase 34 30 10 1.4 1.4 33 20 5
   c. Hot PCA 6 80 **20** 2.1 1.6 20 60 **15**

Total for sample 2 110 105

---

*a* In all cases, cells were prewashed in 0.2 M glycerol prior to 30 min. fixation. Agitation was employed during extractions.

*b* Figures are given in picograms/cell of NA

*c* Figures are given in picograms/cell of RNA

*d* Procedure is identical to that of Part C, Table 5.
the non-agitated system seen in Table 5, 80% vs. 50%). Note that the values for hot PCA extraction following the enzyme hydrolyses were lower than those in Table 5 and correspond more nearly with those of the original experiment (Table 3).

All the fixatives in Table 6 permitted at least 65% NA extraction during the first enzyme hydrolysis. With the exception of formaldehyde fixation, the dissolution in the controls was low. The controls for the second (26-hour) RNase extraction showed somewhat higher values than the first (14-hour) extraction and the low 260/280 values indicated that some protein was extracted.

Formaldehyde was inferior to the Karnovsky, glutaraldehyde, and acrolein fixatives in terms of NA preservation in *T. pyriformis*. Since both the uv and orcinol measurements gave high control values, and since the 260/280 ratios were normal, the data indicated that 60% of the NA was lost from the cell without any enzymatic treatment in the first 14 hours of hydrolysis. In the next 26 hours of hydrolysis some protein was lost, according to the 260/280 ratio.

**b. Electron microscopy** Once information on the degree of extraction of NA from fixed *T. pyriformis* had been obtained (see Sec.IV. 8.2.a.(l).), cells which had undergone substantial extraction could be examined for any ultrastructural change; fixation of these cells prior to the hydrolyses should have reduced the chances of nonspecific ultrastructural damage. Since there were few biochemical or ultrastructural differences in terms of total NA extraction among the several extraction experiments (Tables 3, 5, and 6), the cells from the
initial RNA and DNA extraction experiment are presented as being representative of the series.

(1) RNase control There are no identifiable ultra-structural differences between control Karnovsky-fixed cells held in buffer for 40 hours at 37°C (Figures 5, 9, and 13) and those held in buffer for only 30 minutes at 4°C. The cytoplasm possesses endoplasmic reticulum (ER) and numerous mitochondria and ribosomes, a situation characteristic of rapidly growing cells. Due to the conditions of growth used in the experiments (agitation of cells in proteose peptone medium with no added glucose), no glycogen is observable; ribosomes appear to occupy all of the cytoplasmic space not occupied by other organelles. The ribosomes measure $21.5 \pm 0.5$ nm in diameter, and they stain intensely with uranyl acetate-lead citrate. The kinetosomes (Figures 9 and 13) appear identical to those described by other workers (e.g., Allen, 1967). All nuclear components are normal.

(2) RNase hydrolysis Biochemical data from the preceding section (Table 3) indicates that 75% of the RNA has been extracted by RNase hydrolysis on Karnovsky-fixed *T. pyriformis* (Figures 6, 10, and 14-16). After hydrolysis, the ribosomes stain less intensely with uranyl acetate-lead citrate and the average diameter is $14 \pm 1$ nm, a 30% reduction in size. Mitochondria and ER appear similar to controls. No change is apparent in the kinetosomes; the microtubular triplets, the axosome, the terminal plate, the axial core, and the cartwheel are consistently present. The "feet" of the triplets, structures present in the cartwheel region (Figure 16), are not removed by RNase,
in contrast to the results reported by Stubblefield and Brinkley (1967) for Chinese hamster kinetosomes.

Subtle change is apparent in the nucleus. There is some loss of staining intensity and granularity, but no decrease in size. Nucleolar organizer regions and their associated granules are still visible. Condensed chromatin is unchanged; fibers 10 nm or larger in diameter and small granules are still present in the nucleoplasm.

(3) Hot PCA control The PCA control (Figures 7 and 11) has suffered considerable general ultrastructural damage. The unit membrane of the ER, the mitochondria, and the nucleus appear less defined. Ribosomes stain less intensely relative to the nucleoli than in the RNase control and appear less distinct, but they have a similar diameter. No change is apparent in the kinetosomes. Nucleoli appear less granular. Nucleolar clefts and their associated granules are still visible. The condensed chromatin also appears less granular. Much of the nucleoplasm is gone but what remains appears similar to that present in the RNase control, i.e., small granules and fibers 10 nm or larger.

(4) Hot PCA hydrolysis Hot PCA treatment (Figures 8 and 12) increases the ultrastructural damage produced by the 90°C incubation. The cytoplasm has a reticulated appearance. Ribosomes are less visible in such cytoplasm, but they are still present, still stain intensely, and are still 21 nm in diameter as in the RNase control, despite the fact that all of the cellular RNA has been extracted (Table 3). This contrasts with the RNase hydrolysis where a removal of 75% of the RNA causes a 30% reduction in ribosomal size. As in the
other conditions reported here, no major alterations are visible in
the kinetosomes; all the major structural components are still present.

The nuclear membrane can still be resolved as two unit membranes,
and the dimensions approximate those of normal cells; 9 nm for the
outer membrane, 7 nm for the inner membrane with a total thickness
for the entire structure of 33 nm. However, the nuclear membranes
are usually obscured by aggregations of dense material along them. The
nucleoli appear contracted or condensed, and little internal structure
is visible except for nuclear clefts and cavities. Condensed chromatin
also has lost its finely granular and fibrillar appearance. Much of
the nucleoplasm is now absent.

Neither prewashing nor agitation during hydrolysis had any
discernable effects on ultrastructure. Because the plastic blocks
from the final experiment failed to polymerize properly, the effects
that different fixatives might have on ultrastructure after NA extraction
could not be determined.

3. Enzyme hydrolysis on sectioned material

Several workers (Anderson and Andre, 1968; Monneron, 1966;
Monneron and Bernhard, 1966) have reported the successful enzymatic
extraction of protein but inconsistent results for the extraction of RNA
from material embedded in Epon-Araldite. Compared to Epon-Araldite,
ERL has considerably lower viscosity, less stability in the electron
beam, and more intense staining of embedded material; these characteristics
suggested that ERL might have less cross-linking than Epon-Araldite, and
hence might afford enzymes greater accessibility to embedded components.
Figures 5-6. Effect of RNase hydrolysis on Karnovsky-fixed *T. pyriformis*. Cells are from the initial RNA and DNA extraction experiment seen in Table 3. Uranyl acetate-lead citrate. X 43,000

Figure 5. Control for RNase hydrolysis; cells were incubated at 37°C for 40 hours in glycerol-phosphate buffer. The ribosomes stain intensely. Note the nucleolar cavity containing the chromatin-like granule. Arrows indicate 10 nm diameter fibers in the nucleus.

Figure 6. RNase hydrolysis at 37°C for 40 hours. Ribosomes are 30% smaller and less intensely stained. No differences are apparent in the nucleus. The unit membrane structure of the two nuclear membranes can be resolved at several locations. Again, note the nucleolar cavities. A bundle of intranuclear microtubules is present in this section. Arrows indicate 10 nm diameter fibers in the nucleus.
Figures 7-8. Effect of PCA hydrolysis on Karnovsky-fixed cells. Cells are from the initial RNA and DNA extraction experiment seen in Table 3. Uranyl acetate-lead citrate. X 43,000

Figure 7. Control for PCA hydrolysis; cells were incubated at 90°C for one-half hour in glycerol-phosphate buffer. Ribosomes are less distinct than in RNase control cells. Arrows indicate 10 nm fibers in the nucleus.

Figure 8. PCA hydrolysis at 90°C for one-half hour. Because the cytoplasm has a coagulated or reticulated appearance, ribosomes, although of normal size, are less apparent. The unit membrane structure of the two nuclear membranes can be resolved at one location, but the structure is generally obscured by aggregations of material along the membrane. Note that the nucleoli seem more compact and more granular than in the control. Much nucleoplasm appears to be absent.
Figures 9-12. Longitudinal sections of kinetosomes from different treatments of the initial RNA and DNA extraction experiment seen in Table 3. All of the procedures cause varying degrees of general ultrastructural damage, but no specific damage is discernable in the kinetosomes. The microtubular triplets, the axosome, the terminal plate, the axial core, and the cartwheel are consistently present. Endoplasmic reticulum is often seen in close association with kinetosomes. Uranyl acetate-lead citrate. X 102,000

Figure 9. Control for RNase hydrolysis; cells were incubated at 37°C for 40 hours in glycerol-phosphate buffer

Figure 10. RNase hydrolysis at 37°C for 40 hours. Note that the ribosomes are 30% smaller than in the control.

Figure 11. Control for PGA hydrolysis; cells were incubated at 90°C for one-half hour in glycerol-phosphate buffer

Figure 12. PGA hydrolysis at 90°C for one-half hour
Figure 13. Cross section of kinetosome from RNase control of initial RNA and DNA experiment seen in Table 2. Cells were incubated at 37°C for 40 hours in glycerol-phosphate butter. Strands of material (arrows) connect the axial core to the triplets. ER is in close proximity to the kinetosome. Uranyl acetate-lead citrate. X 180,000

Figures 14-15. Cross sections of kinetosomes from RNase hydrolysis of initial RNA and DNA extraction experiment. Cells were incubated in RNase at 37°C for 40 hours. Sections are approximately through the midregion of the kinetosomes. No loss of structure is apparent in the axial core or in the triplet wall. Strands of material (arrows) connect the axial core to the triplets. Uranyl acetate-lead citrate. X 180,000

Figure 16. Cross sections of kinetosome from RNase hydrolysis of initial RNA and DNA extraction experiment. Cell was incubated in RNase at 37°C for 40 hours. Section is through the proximal, or cartwheel, region of a kinetosome. The appearance is identical to that of control kinetosomes. There is no loss of visible structure. Note the presence of electron-dense appendages or "feet" extending centripetally from microtubule A of each triplet. Uranyl acetate-lead citrate. X 180,000
a. Untreated control  Thin sections which had not been subjected to the hydrolysis procedure were used in assessing any potential effects of the various hydrolysis steps. It is easier to observe the ultrastructure of the various nuclear and cytoplasmic organelles of cells grown in defined medium (Figures 17, 21, 25, 27, and 29) compared to those of cells grown in proteose peptone medium (Figure 5) because there are fewer interchromatin granules occupying the nucleus, and fewer ribosomes occupying the cytoplasm. Otherwise, the ultrastructure is similar. In the nucleus (Figure 17) nucleoli are found along the nuclear membrane. Fibers, the smallest of which are 10 nm in diameter, and small granules and condensed chromatin are found in the nucleoplasm. Nuclear pores (Figure 21) occur frequently but at random intervals within the nuclear membrane. Ribosomes are plentiful along the nuclear membrane and in the cytoplasm.

The mitochondria, kinetosomes, mucocysts, peroxisomes, and ER all appear normal (Figures 25, 27, and 29). Figure 27 shows a cross-section through the three rows of cilia comprising one of the oral membranellae. Since the section cuts at an angle, structures present at various levels of the kinetid can be seen, including the axial core, the terminal plate, the axosome, and the central pair of microtubules. In Figure 27 the radial spokes and central sheath give the ciliary axoneme a striated appearance (Birge and Doolin, 1969).

b. Hydrogen peroxide control  Treatment of sections with hydrogen peroxide and subsequent incubation in buffer produced only minimal effects (Figures 18 and 23). The sections have a mottled
appearance, with particular regions staining less intensely than the background. However, there is no alteration in the appearance of, or loss of, any component in the nucleus or cytoplasm.

c. RNase hydrolysis  Only a few differences from the controls are apparent in the RNase-treated sections. There is some loss of stain intensity immediately around the nucleolus (Figure 19). The effect is especially noticeable in the clefts or cavities. Also, there is some reduction in the number of granules and fibers in the nucleoplasm. The condensed chromatin is similar to that of untreated sections (Figure 17). In the cytoplasm, the mottle noticed in the hydrogen peroxide controls is present (Figure 24). There is no loss of components or visible stain intensity in the mitochondria or kinetosomes. Ribosomes are numerous and of normal size (21 nm).

d. Pronase hydrolysis  Ten-hour pronase treatment caused considerable extraction. In the nucleus (Figure 20), both the condensed chromatin and the nucleoli have been largely digested, leaving behind faintly visible reticular or fibrillar components. In the ground substance of the nucleus are fibers which measure as little as 5 nm in diameter compared with a minimum of 10 nm in the control (Figures 17 and 21).

The nuclear pores show selective extraction (Figure 22); note that the dense annulus is digested but the dense central rod is resistant. Except for the pores, the two nuclear membranes, as well as the pellicular and alveolar membranes (Figures 26, 28, and 30), endoplasmic reticulum (Figures 20 and 30), mucocyst membrane (Figure 30), and the
two mitochondrial membranes (Figures 20, 28, and 30) are resistant to pronase. The lumina of the mitochondrial tubular cristae are extracted (Figures 20, 28, and 30) but the matrix appears resistant. Mucocysts are not affected by the extraction; even the striations are still visible (Figure 30).

Most components of the cilium and kinetid are affected by the ten-hour pronase treatment (Figures 26, 28, and 30). In the cilium, the doublet microtubules, the central pair of microtubules, and the axosomal granule are removed. Only the radial spokes, the central sheath, and possibly the matrix remain unaffected. In the kinetid, the kinetosomal triplets, the axial core, the sheath, the circumciliary ring and adjacent epiplasm, the cartwheel spokes, the kinetodesmal fiber, the longitudinal and transverse microtubules, and the proximal cap of oral kinetosomes are susceptible to digestion. Only the terminal plate, the cartwheel axis, and possibly the matrix are resistant.

If the protein comprised a major portion of a particular region of the ERL section, it appears that enzymatic removal of the protein reduced the structural support so that that region of the section "dropped out"; in other cases resistant material (protein or other components) remained and substructure can still be seen. For example, the cilium in Figure 28 appears to undulate gently in and out of the section. In some areas the central pair is completely gone; in adjacent areas of the cilium which skim the pair, the striations of the central sheath still remain (Birge and Doolin, 1969).
Ribosomes are another example where the ultrastructural appearance of an organelle after extraction depends upon the amount and thickness of the organelle contained in the section. The number of intact ribosomes has been greatly reduced (Figures 20 and 30), and they now measure 17 nm compared with 21 nm in the control (Figures 17 and 29). However, numerous lightly staining spots and fibers occur in the cytoplasm which appear to be the undigested remains of ribosomes. Occasionally, holes in the section the diameter of ribosomes occur. These may be locations where several ribosomes, aligned vertically in the section, "dropped out." Apparently the 17 nm diameter ribosomal "cores" were either more stably cross-linked or were situated in the middle of the section such that the surrounding plastic provided steric hindrance to the pronase.

C. Nutritional Deficiencies

1. Growth data

The complex nutritional requirements of *T. pyriformis* and the ability to grow the organism in a completely defined medium (Kidder et al., 1950; Elliott and Hayes, 1953) permit physiological control through selection of nutritional conditions. The requirement for a preformed pyrimidine ring (Kidder et al., 1950) suggested manipulation of processes affecting organelles dependent on nucleic acid. In the case of kinetosomes, specifically, a detectable structural change in the organelle or the prevention of formation of genetically dependent daughter organelles could indicate a requirement by the kinetosome for the missing pyrimidine. Lack of thymidine should
Figure 17. Untreated control section for enzyme extraction on sectioned material. Cells were grown in defined medium, fixed in glutaraldehyde, post-fixed in OsO₄, and embedded in ERL. The ultrastructure is similar to that in Figure 5, but both the nucleus and cytoplasm appear less compact when cells are grown in defined medium. Condensed chromatin and fibers (arrows) are present in the interior of the nucleus. The smallest fibers appear to be 10 nm diameter. Nucleoli are aligned along the nuclear membrane; frequently they possess cavities and clefts. Many ribosomes of 21 nm diameter are present on the outer nuclear membrane and in the cytoplasm. Uranyl acetate-lead citrate. X 48,500
Figure 18. Control section treated in hydrogen peroxide and subsequently in water for ten hours. The treatment has produced some subtle effects. The micrograph has a mottled appearance, particularly in the cytoplasm. However, there is no loss of any cytoplasmic component. Mitochondria and peroxisomes compare well with untreated ones. Ribosomes are abundant and of normal size (21 nm). In the nucleus, fibers are present in the nucleoplasm; nucleoli and condensed chromatin are perhaps more granular. Uranyl acetate-lead citrate. X 48,500
Figure 19. Section treated in RNase for ten hours. The micrograph has a mottled appearance, especially in the cytoplasm. However, there is no loss of any cytoplasmic component. Ribosomes are abundant and of normal size. In the nucleus the condensed chromatin and the fibers appear normal. However, there is reduction of staining intensity in the nucleoplasm in the immediate vicinity of the nucleolus. The effect "outlines" the nucleolus, and it is especially noticeable in the nucleolar clefts. Uranyl acetate-lead citrate. X 48,500
Figure 20. Section treated for ten hours with pronase. The micrograph has been purposely overdeveloped in order to emphasize the details of the remaining ultrastructure. Very little structure is left in the condensed chromatin. Only slightly more structure remains in the nucleoli. Many fibers (white arrows) still are present in the nucleoplasm, but now they measure as small as 5 nm in diameter. Many of the lumina of the mitochondrial cristae are now empty (black arrows). The number of intact ribosomes is reduced from that present in the control; they now measure 17 nm compared with 21 in the control. Often, smaller and less dense structures suggestive of partially digested or unraveled ribosomes are present. Occasionally, holes in the section the size of ribosomes can be seen, where polysomes may have "dropped out" during the digestion. Uranyl acetate-lead citrate. X 48,500
Figure 21. Untreated control cell; high magnification of the nuclear membrane region. Note that the annulus and central rod of the nuclear pore are composed of electron-dense material. Ribosomes are present along the outer nuclear membrane and in the cytoplasm. Uranyl acetate-lead citrate. X 110,000.

Figure 22. Ten-hour pronase treated section; high magnification of the nuclear membrane region of Figure 20. The micrograph has been purposely overdeveloped in order to emphasize the remaining ultrastructure. The annular material of the nuclear pores has been completely digested, but a portion of the central rod remains. Fibers as small as 5 nm in diameter can be seen in the nucleus (arrows). Most of the nucleolus has been digested, leaving only a faint reticulum. Uranyl acetate-lead citrate. X 110,000.
Figure 23. Control section treated in hydrogen peroxide and subsequently in water for ten hours. The cytoplasm shows a mottled effect, but all components, e.g., peroxisomes, mitochondria, kinetosomes, and ribosomes, are normal. Uranyl acetate-lead citrate. X 43,000

Figure 24. Section treated in RNase for ten hours. Mottling of the cytoplasm is apparent. Mitochondria are normal. Ribosomes are plentiful and of normal size (21 nm). All components of the kinetosome are present. Uranyl acetate-lead citrate. X 43,000
Figure 25. Untreated control for enzyme extraction on sectioned material; cross section through oral membranelle. The section cuts more deeply from top to bottom and from left to right. Note that one ciliary central tubule originates at the axosome and the second originates distally to the axosome. Uranyl acetate-lead citrate. X 43,000

Figure 26. Section treated for ten hours with pronase; cross section through oral membranelle. The micrograph has been purposely overdeveloped in order to emphasize the remaining ultrastructure. The section cuts more deeply into the cell from top to bottom and from left to right. The three ciliary rows of the membranelle are numbered. Only one kinetosome of the third row is visible; its terminal plate and digested tubules are visible at the upper left. The central pair of ciliary microtubules, the doublets, the axosome, the triplets, the axial core, and the cartwheel spokes have all been digested; only the ciliary membrane, the ciliary and kinetosomal matrices, the terminal plate and the center of the cartwheel remain. Uranyl acetate-lead citrate. X 43,000

Figure 27. Untreated control for enzyme extraction on sectioned material; longitudinal section of kinetosome and associated structures. Note that one ciliary tubule originates at the axosome and the second originates distally to the axosome. The central sheath gives the central pair of microtubules a striated appearance. Dense material of the epiplasmic layer underlies the inner alveolar membrane. In the region of the kinetosome, the epiplasm (arrows) extends along the fold of the inner and outer alveolar membranes almost to the pellicle, forming a collar around the kinetosome. Uranyl acetate-lead citrate. X 43,000

Figure 28. Section treated for ten hours with pronase; longitudinal section of kinetosome and associated structure. The micrograph has been purposely overdeveloped in order to emphasize the remaining ultrastructure. In the cilium the microtubular doublets and the central pair of microtubules have been extracted. However, the radial spokes and the central sheath appear resistant. In the kinetosome the axosome and the axial core have been digested. Only the cartwheel axis, the terminal plate, and the kinetosomal matrix remain. The epiplasmic layer has been digested except where it adjoins the terminal plate (arrows). The lumina of the mitochondrial cristae (arrowheads) were completely digested. Membranes and mucocysts appear intact. Uranyl acetate-lead citrate. X 43,000
Figure 29. Untreated control for enzyme extraction on sectioned material; section tangential to cell surface. Compare with Figure 4. The section cuts more deeply into the cell from right to left. Portions of two kineties are visible. The kinetodesmal fibers "point to" the anterior end of the cell, which is in the upper left. Each kinety is composed of units called kinetids, each consisting of a kinetosome and its associated cilium, kinetodesmal fiber, and longitudinal and transverse sets of microtubules. The mitochondria are arranged in rows which alternate with the kinetids; mucocysts, on the other hand, appear to be randomly oriented on the cell surface. Peroxisomes are occasionally present. Numerous cytoplasmic ribosomes are present; mitochondria possess their own ribosomes. Uranyl acetate-lead citrate. X 43,000

Figure 30. Section treated for ten hours with pronase; section tangential to the cell surface. The micrograph has been purposely overdeveloped in order to emphasize the remaining ultrastructure. The section cuts more deeply into the cell from left to right. The kinetosomal triplets, axial core, longitudinal microtubules, sheath material, and kinetodesmal fiber have all been digested by the pronase. One small portion of the fiber remains undigested (white arrow). At the left is a cilium which has been sectioned just above its junction with the cell surface. The doublets and central pair have digested. In this particular section some mitochondrial cristae (arrowheads) have been digested while others are still intact. Remaining ribosomes measure 17 nm compared with 21 nm in the control. Holes the size of ribosomes are occasionally present in the section. Mucocysts appear to be resistant to the enzymes; note that the striated pattern is still visible. Membranes also appear to be resistant. Uranyl acetate-lead citrate. X 43,000
prevent DNA synthesis, and lack of uridine should prevent RNA synthesis. Although *T. pyriformis* cannot convert thymidine to uridine, it can methylate deoxyuridine monophosphate to thymidine monophosphate in the presence of folic acid (Wykes and Prescott, 1968). The synthesis of thymidine should thus be prevented by addition of a folic acid analog (Zeuthen and Villadsen, 1970) or by withholding of folic acid.

Folic acid participates in four other metabolic pathways (Blakely, 1969): the synthesis of the amino acids serine and methionine, the synthesis of purines, and the formylation of methionyl transfer RNA in prokaryotes. *T. pyriformis* is a favorable organism for folic acid manipulation because serine, methionine, and a preformed purine ring, the products of folic acid reactions, can all be supplied in the defined medium, thus preventing the problems produced by their absence from obscuring the ultrastructural picture. Only the formylation of methionyl transfer RNA to formyl methionyl transfer RNA (f-met-tRNA) used in initiation of a prokaryotic ribosomal protein synthesis (Linnane et al., 1972) and the methylation of uridine should be affected by the manipulations.

The results of the nutritional deficiencies have been previously published in preliminary form (Seydel and Outka, 1972).

---

**a. U+T+F+ medium**

Complete defined medium U+T+F+ served as the control (Figures 31 and 32) for the several nutritional deletions. The centrifugation required in the prewashing of cells had no effect on the shape of the curves for control cultures (Figure 31). In each subculture OD increase exceeded cell number increase by
nearly one generation (Figure 31 and Table 7); but the difference
cannot be explained by an increase in cell volume since the cells did
not double in volume with each subculture (Table 8). The volume did
increase somewhat (60%) during the first two growth curves indicating
that the cells achieved an optimum size during extended logarithmic
growth. The curves from the prewashed cultures are closer together,
because the corresponding experimental cultures required more frequent
subculturing. Growth in U+T-F+, U+T+F+A+, and U(x200)+T+F+A+
media was similar to the control.

b. U+T+F- medium _T. pyriformis_ ultimately ceased
growing in medium lacking folic acid, U+T+F-. Evidently the concen-
trations of folic acid required by the organisms was low enough that an
amount sufficient to support several generations could be transferred
in the initial inoculum, since growth in terms of length of time
(Figure 34 versus 33) and as measured by OD (Table 7) declined twice as
quickly when the inocula were prewashed in U-T-F- medium. The cell
number values are in general agreement with the OD values but the first
and second subcultures each showed one-half generation less growth in
cell number than in OD. Cell size was similar to that of the control
(Table 8). Cells from all stages, including the final subculture resumed
normal growth within 24 hours after transfer to control medium. The
growth curves for amethopterin inhibition were identical to the folic
acid deficiency curves.

c. U+T-F- medium When a thymidine deficiency was superimposed
upon the folic acid deficiency, U+T-F-, both the prewashed and unwashed
Table 7. Cell generations in defined media

<table>
<thead>
<tr>
<th>Subculture</th>
<th>Prewashed</th>
<th>Not washed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Medium</td>
</tr>
<tr>
<td></td>
<td>U+T+F+</td>
<td>U+T+F-</td>
</tr>
<tr>
<td></td>
<td>OD</td>
<td>#</td>
</tr>
<tr>
<td>First</td>
<td>5.1</td>
<td>4.3</td>
</tr>
<tr>
<td>Second</td>
<td>5.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Third</td>
<td>4.8</td>
<td>4.0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>14.9</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} A generation is defined as a doubling of the OD value or of the cell number (\#)

\textsuperscript{b} The third subculture is not included in the total because it extends beyond the termination of the other experimental cultures
Table 8. Cell dimensions<sup>a</sup> and volumes in defined media—prewashed

<table>
<thead>
<tr>
<th>Days</th>
<th>U+T+F+ Dimensions µm</th>
<th>U+T+F+ Volume µm³ x 10³</th>
<th>U+T+F- Dimensions µm</th>
<th>U+T+F- Volume µm³ x 10³</th>
<th>U+T-F- Dimensions µm</th>
<th>U+T-F- Volume µm³ x 10³</th>
<th>U-T+F+ Dimensions µm</th>
<th>U-T+F+ Volume µm³ x 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40±4 x 20±3</td>
<td>5.4</td>
<td>40±4 x 20±3</td>
<td>5.4</td>
<td>40±4 x 20±3</td>
<td>5.4</td>
<td>40±4 x 20±3</td>
<td>5.4</td>
</tr>
<tr>
<td>1</td>
<td>49±2 x 19±2</td>
<td>6.2</td>
<td>45±5 x 20±3</td>
<td>6.4</td>
<td>60±3 x 27±3</td>
<td>14.5</td>
<td>43±3 x 21±2</td>
<td>6.4</td>
</tr>
<tr>
<td>1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47±4 x 22±3</td>
<td>7.9</td>
<td>46±3 x 21±3</td>
<td>7.0</td>
<td>57±7 x 27±5</td>
<td>14.7</td>
<td>45±3 x 22±2</td>
<td>7.2</td>
</tr>
<tr>
<td>2.5</td>
<td>50±5 x 21±2</td>
<td>7.9</td>
<td>48±7 x 21±3</td>
<td>7.3</td>
<td>63±8 x 22±3</td>
<td>23.8</td>
<td>45±3 x 25±1</td>
<td>9.8</td>
</tr>
<tr>
<td>3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50±4 x 22±2</td>
<td>8.4</td>
<td>50±3 x 22±2</td>
<td>8.2</td>
<td>79±6 x 35±4</td>
<td>33.3</td>
<td>47±3 x 26±1</td>
<td>11.0</td>
</tr>
<tr>
<td>5.4</td>
<td>50±4 x 22±2</td>
<td>8.7</td>
<td>45±4 x 22±2</td>
<td>7.8</td>
<td>66±11 x 39±7</td>
<td>35.9</td>
<td>47±4 x 21±1</td>
<td>7.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Dimensions are reported as length and width, respectively.

<sup>b</sup>Cultures were subcultured at these points, except for U-T+F+ which was subcultured only at 3.4 days.
cultures declined more quickly (Figures 35 and 36) than the corresponding cultures with folic acid deficiency alone, U+T+F-. Again, the prewashed cultures declined twice as quickly as the unwashed ones in terms of days (Figure 35 versus 36) and cell generations as determined by OD measurements (Table 7). In the first two subcultures the OD continued to increase after the cell number stopped increasing; volume determinations (Table 8) suggest the OD increase was due to enlargement of the cells. In the prewashed situation only cells from the first subculture, and in the unwashed situation, only cells from the first and second subcultures, grew when transferred to control medium.

d. U-T+F+ medium In the case of a uridine deficiency, U-T+F+, prewashing had little effect (Figures 37 and 38). In both the prewashed and unwashed situations the growth rate was slower than in the other experimental conditions, but the growth appeared to continue indefinitely. Difference in the first subculture in the number of generations as determined by OD versus cell number (Table 7) can be attributed to an increase in volume. At the start of the second subculture there is a burst of division and a decrease in cell size. Cells from any stage of uridine deficiency resumed normal growth within 24 hours after transfer to control medium.

2. Electron microscopy

a. U+T+F+ cells The ultrastructure of these cells served as a control for the changes produced by growth in media lacking one or more specific nutritional requirements. The ultrastructural features of the nucleus and cytoplasm (Figures 39, 43, 47, and 59) are summarized
Figures 31-32. Growth of *T. pyriformis* in U+T+F+ defined medium. This medium served as a control for several experimental deletions. Generation time was 7.5 hours. Growth in U+T+F- medium was identical to the control. The arrows indicate the points of subculture into fresh medium.

**Figure 31.** Cells were washed in U-T-F- medium prior to the experiment.

**Figure 32.** Cells were not washed in U-T-F- medium prior to the experiment. Cells were prepared for electron microscopy at the end of the third subculture.
Figures 33-34. Growth of T. pyriformis in U+T+F- defined medium. The arrows indicate the points of subculture into fresh medium.

Figure 33. Cells were washed in U-T-F- medium prior to the experiment.

Figure 34. Cells were not washed in U-T-F- medium prior to the experiment. Cells were prepared for electron microscopy at the end of the third subculture.
Figures 35-36. Growth of *T. pyriformis* in U+T-F- defined medium. The arrows indicate the points of subculture into fresh medium.

Figure 35. Cells were washed in U-T-F- medium prior to the experiment.

Figure 36. Cells were not washed in U-T-F- medium prior to the experiment. Cells were prepared for electron microscopy at the end of the third subculture.
Figures 37-38. Growth of *T. pyriformis* in U-T+F+ defined medium. The arrows indicate the points of subculture into fresh medium.

Figure 37. Cells were washed in U-T-F- medium prior to the experiment.

Figure 38. Cells were not washed in U-T-F- medium prior to the experiment. Cells were prepared for electron microscopy at the end of the second subculture.
in Tables 9 and 10. All organelles are normal and have been discussed previously (Sec. IV. A. 2. and IV. B. 3. a.). The densitometry scans of kinetosomes produced an average C/M of .32.

b. U+T+F- cells The nuclei of T. pyriformis grown in the absence of folic acid are normal (Table 10). Chromatin and nucleoli are present; nuclear pores contain their customary electron dense annuli and central rods (Figures 40 and 43). In the cytoplasm, ribosomes are abundant. However, two classes of unidentified cytoplasmic bodies occur more frequently in these cells than in controls: very electron-dense bodies, and light grey bodies similar to lipid bodies in tritrichomonads (Cornford, 1971). There are no changes in the structure of kinetosomes (Figure 60), and prokinetosomes are observed.

The folic acid deficiency has a pronounced effect on the mitochondria (Table 9). The mitochondria are half as large as normal, U+T+F+ mitochondria, however, they are also twice as numerous so that the \( \frac{\text{number of mitochondria}}{\text{um}^2} \) of mitochondrion/\( \text{um}^2 \) of cytoplasm is approximately normal. The tubular cristae, although 15% larger than normal, are poorly defined and much less numerous than normal. In addition, electron-dense inclusions occur in 17% of folic acid deficient mitochondria. The inclusions are generally elongated and associated with, but apparently not necessarily enclosed by, cristae (Figures 48-53). In favorable longitudinal and transverse sections, striations may be observed (Figure 53; compare with U+T-F- cell, Figure 57). Such inclusions are only very infrequently observed in normal cells; there is only one published report of dense cristal inclusions (Williams and
Table 9. Condition of mitochondria from cells in various nutritional conditions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Length $^a$ (µm)</th>
<th>Width $^a$ (µm)</th>
<th>Area $^a$ (µm)$^2$</th>
<th>No. Mito./ (µm)$^2$ cytopl.$^b,c$</th>
<th>(µm)$^2$ Mito./ cytopl.</th>
<th>Diameter of Cristae $^a$ (nm)</th>
<th>No. of Cristae</th>
<th>Appearance of Matrix</th>
<th>Appearance of Inclusions$^b$</th>
<th>% Mito. with Inclusions$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>U+T+F+</td>
<td>.97±13</td>
<td>.77± 7</td>
<td>.59</td>
<td>.20; 15%</td>
<td>.12</td>
<td>47±4</td>
<td>normal</td>
<td>normal</td>
<td>≤1</td>
<td></td>
</tr>
<tr>
<td>U+T+F-</td>
<td>.68±6</td>
<td>.50± 3</td>
<td>.27</td>
<td>.50; 15%</td>
<td>.13</td>
<td>54±6</td>
<td>reduced</td>
<td>normal</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>U+T-F-</td>
<td>.96±17</td>
<td>.82±14</td>
<td>.62</td>
<td>.25; 38%</td>
<td>.16</td>
<td>53±5</td>
<td>reduced</td>
<td>sparse</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>U-T+F+</td>
<td>1.01± 7</td>
<td>.64± 3</td>
<td>.51</td>
<td>.14; 21%</td>
<td>.07</td>
<td>46±2</td>
<td>normal</td>
<td>normal</td>
<td>≤1</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ A minimum of 20 mitochondria, approximately one-half quadrant, from each of 6 complete cross sections was measured.

$^b$ The mitochondria from 6 complete cellular cross sections (about 900) were counted for each condition.

$^c$ Relative standard deviation is included with each figure.
Table 10. Ultrastructural characteristics of cells in various nutritional conditions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Condensed Chromatin</th>
<th>Nucleoli</th>
<th>Nuclear Pores</th>
<th>Ribosomes</th>
<th>Cytoplasmic Vacuoles</th>
<th>Kinetosome C/M Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Prokinetosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>U+T+F+ normal appearance, abundant</td>
<td>normal No. and size</td>
<td>normal</td>
<td>numerous</td>
<td>normal</td>
<td>present</td>
<td>.46 ± .10 ( .32-.62 )</td>
<td>present</td>
</tr>
<tr>
<td>U+T+F- normal appearance, abundant</td>
<td>normal No. and size</td>
<td>normal</td>
<td>numerous</td>
<td>normal</td>
<td>present</td>
<td>.48 ± .10 ( .37-.64 )</td>
<td>present</td>
</tr>
<tr>
<td>U+T-F- absent</td>
<td>few present, very large and dark</td>
<td>much dense material associated with pore region</td>
<td>few</td>
<td>normal</td>
<td>present</td>
<td>.51 ± .09 ( .34-.67 )</td>
<td>present</td>
</tr>
<tr>
<td>U=T+F+ normal appearance, abundant</td>
<td>normal No. and size</td>
<td>normal</td>
<td>few</td>
<td>excessive number</td>
<td>present</td>
<td>.30 ± .14 ( .21-.60 )</td>
<td>present</td>
</tr>
</tbody>
</table>

<sup>a</sup>A minimum of 13 kinetosomes was scanned for each condition; the range is given in parentheses.
Luft, 1968; Figures 2 and 13). Less frequently, striated regions that
are not filled with densely staining material occur within or between
cristae. They may (Figures 48 and 49) or may not (Figures 50 and 52)
appear in association with the dense striated cristal inclusions. The
mitochondrial effect is severe enough to halt growth (Figure 34).

c. U+T-F- cells A thymidine deficiency superimposed upon a
folic acid deficiency is fatal. Observation of cells sampled just
prior to cessation of growth (Figure 36) indicates the reason. The
nuclei are extremely abnormal (Table 10). No condensed chromatin is
apparent and the nuclear ground substance is reduced from normal
density (Figures 41 and 45). The few nucleoli which exist are greatly
enlarged and very dark and they have lost their granular appearance;
they are reminiscent of nucleoli during cellular starvation (Satir
to be normal but annuli and central rods are nearly obscured by an
accumulation of electron-dense material.

Few ribosomes are present in the cytoplasm or on the nuclear
membrane. The dark bodies and light, lipid-like bodies noted in the
U+T+F- cells are also present in this condition (Figure 41). The
mitochondrial defects are identical to those for a folic acid deficiency,
but often more pronounced (Table 9). The few cristae present are very
poorly formed, and the matrix is often lacking in substance (Figure 54,
especially, and Figures 55 and 56). Those cristae present are 15%
larger than in control cells. Approximately 18% of these mitochondria
have dense striated cristal inclusions (Figures 54-57), a figure
similar to that for U+T+F- mitochondria. Again, in favorable sections, striations are visible (Figure 57). There are no changes in the structure of kinetosomes (Figure 61); prokinetosomes are observed and appear to be normal.

d. U+T+F+ cells The nuclei and nuclear pores of T. pyriformis grown in uridine deficient medium are normal; the cytoplasmic bodies associated with U+T+F- and U+T-F- cells do not occur (Figures 42, 46, and 58 and Table 10). Mitochondria are normal (Figures 46 and 58 and Table 9). However, these cells do show several abnormalities. The number of ribosomes is reduced (Figure 46) and the cytoplasm has many large, empty vacuoles (Figures 42 and 58). All components of kinetosomes are present, but in 40% of the kinetosomes the axial core appears greatly reduced in density (Figure 62). This appearance is confirmed by the densitometric scans of kinetosomes (Figure 62 and Table 10). The number of kinetosomes with reduced cores lowers the M/C ratio from normal (.30 versus .45) and the increased variation in core density is reflected in the greater standard deviation for this condition (+ .14 versus ± .10).

D. Reduction of Protein Synthesis in Mitochondria

Mager (1960) demonstrated that chloramphenicol and chlortetracycline inhibit the in vitro incorporation of amino acids in isolated mitochondria from T. pyriformis W, but not in isolated microsomes. Further, Turner and Lloyd (1970) reported a reduction of mitochondrial cristae in chloramphenicol-treated T. pyriformis ST, an effect similar to that produced by a folic acid deprivation for T. pyriformis GL
Figures 39-42. Sections of cells grown in the absence of specific nutritional requirements. Cells were not washed prior to inoculation into various defined media. Cells were sampled and fixed at the end of seven days. Uranyl acetate-lead citrate. X 6,000

Figure 39. Control, U+T+F+ cell. Cytoplasm contains numerous ribosomes and mitochondria with many microtubular cristae, typical of protozoa. In the nucleus, nucleoli can be seen adjacent to the nuclear membrane, and condensed chromatin is present. Compare with Figures 4, 17, and 30

Figure 40. U+T+F- cell just prior to cessation of growth. The mitochondria are smaller and more numerous than in normal cells, and the cristae are poorly formed and less numerous. Often a densely staining inclusion is present in the mitochondrion; such an inclusion is rarely seen in normal mitochondria. A variety of vacuoles, such as the very dark ones, are abundant in a cell suffering from folic acid deficiency. The nucleus appears normal

Figure 41. U+T-F- cell just prior to cessation of growth. The mitochondrial defects are similar but more pronounced than those in a U+T+F- situation. The cristae are even less numerous and the matrix is sparse. Dense-staining inclusions are often present. A variety of vacuoles, such as the lightly staining one, can be seen. The nucleus is unusual; it is larger than normal, no condensed chromatin is visible, and the few nucleoli present are enlarged and very dark

Figure 42. U-T+F+ cell. The mitochondria and nucleus are normal. However, the cytoplasm is highly vacuolated and the cytoplasm that is present has less background material
Figures 43-46. Sections from the region of the nuclear membrane of cells grown in the absence of specific nutritional requirements. Cells were not washed prior to inoculation into various deficient defined media. Cells were sampled and fixed at the end of seven days. Uranyl acetate-lead citrate. X 48,500

Figure 43. Control, U+T+F+ cell. The nuclear appearance is typical for T. pyriformis (compare with Figures 17 and 21). Condensed chromatin and numerous fibers are present. The nuclear pores are normal; both the annulus and the central rod consist of dense material. Numerous ribosomes are present in the cytoplasm and on the nuclear membrane.

Figure 44. U+T+F- Cell just prior to cessation of growth. The nucleus and nuclear pores appear normal in this condition. Numerous ribosomes are present in the cytoplasm and on the nuclear membrane, as in the control.

Figure 45. U+T-F- cell just prior to cessation of growth. No condensed chromatin occurs in this nucleus and nuclear and cytoplasmic ground substance appears sparse. The annulus and central rod of the nuclear pores are almost obscured by electron-dense material. Few ribosomes are present in the cytoplasm or on the nuclear membrane.

Figure 46. U-T+F+ cell. The nucleus and nuclear pores appear normal. However, few ribosomes are present in the cytoplasm or along the nuclear membrane. Little background is present in the cytoplasm.
Figures 47-58. Mitochondria from cells grown in the absence of specific nutritional requirements. Uranyl acetate-lead citrate

Figure 47. Mitochondrion from control U+T+F+ cell. Numerous tubular cristae approximately 47 nm in diameter occur within the matrix. X 36,000

Figures 48-51. Mitochondria from U+T+F- cells just prior to cessation of cell growth. The cristae are larger than in the control, approximately 54 nm in diameter, and they are fewer in number and more poorly formed. Electron-dense, generally elongated inclusions are found in 17% of these mitochondria. They are found between two or more cristae (Figure 48), adjacent to cristae (Figure 48) or within cristae (Figures 48, 49, and 51). In favorable sections striations within the cristal inclusions are visible (Figure 51), and less frequently striated regions within or between cristae, either associated with or lacking dense-staining material, also occur (Figures 48, 49, and 50). X 36,000

Figure 52. Enlargement of a portion of Figure 50. Striations are associated with several cristae; no dense cristal inclusion is present. X 110,000

Figure 53. Enlargement of a portion of Figure 51. The dense cristal inclusion is apparently cut in cross section; striations are visible. X 110,000

Figures 54-56. Mitochondria from U+T-F- cells just prior to cessation of cell growth. The mitochondria appear similar to the U+T+F- mitochondria. However, the matrix is more lacking in substance (e.g., Figure 54). The cristae, though larger (53 nm) than in the control, are poorly formed and few in number. Striated inclusions are present in 18% of these mitochondria. X 36,000

Figure 57. Enlargement of portion of Figure 55. The inclusion is apparently cut in longitudinal section. X 110,000

Figure 58. The mitochondrion from a U-T+F+ cell is similar to the control in size and frequency of cristae. Note, however, the vacuolated appearance of the cytoplasm. X 36,000
Figures 59-62. Comparison of densitometric scans of mid-longitudinal sections of kinetosomes from cells grown in the absence of specific nutritional requirements. Two scans of each kinetosome were required in order to include most of each axial core; the arrows indicate the region included in each scan. The scan was divided into portions equivalent to the axial core and to the microtubular triplet walls, and the area of each portion was measured with a planimeter to obtain a core/microtubule ratio (C/M). The kinetosome with the median C/M for each nutritional situation is portrayed with its corresponding scans (compare with Table 10), and the C/M derived from each pair of scans is indicated below the scans. Uranyl acetate-lead citrate. X 93,000

Figure 59. Control, U+T+F+ cell. The actual densities of the core and the microtubules are reduced for this kinetosome due to the thinness of the section. However, the C/M is typical for the control

Figure 60. U+T+F- cell immediately prior to cessation of growth. The C/M is normal

Figure 61. U+T-F- cell immediately prior to cessation of growth. The C/M is normal

Figure 62. U-T+F+ cell. The kinetosome core is reduced in density compared with the cores in the other three conditions; hence the C/M is lower
(U+T+F- and U+T-F- cells, Sec.IV.C.2.). Since eukaryotic mitochondria share some similarities with prokaryotes, particularly in the nature of the protein synthetic process, and since folic acid is required for initiation of prokaryotic protein synthesis (Sager, 1972), it seemed reasonable to investigate the effect of a folic acid deprivation on amino acid incorporation ability of mitochondrial ribosomes.

Chloramphenicol, chlortetracycline HCl, and cycloheximide were used as inhibitory controls in the experiment; in addition, several other antibiotics, streptomycin, erythromycin, and neomycin, known to arrest bacterial growth, were tested.

Before performing an experiment on incorporation of amino acids in isolated mitochondria, studies were performed to determine the minimum concentration of each agent effective in inhibiting growth. Chloramphenicol and chlortetracycline HCl were equally effective in arresting growth; 150 mg/l arrested growth after three mass doublings. Hager (1960) also reported that the two antibiotics had similar effects. However, since the chlortetracycline produced a precipitate upon standing, which made OD measurements difficult, it was not used for the amino acid incorporation experiment. Neither streptomycin sulfate, neomycin sulfate, or erythromycin stearate had any effect on the growth of T. pyriformis, even after three subcultures, in concentrations as high as 450 mg/l. The antibiotics may have had difficulty in crossing the cell membrane. Cycloheximide at 6 mg/l produced immediate cessation of growth. Although Frankel (1970) reported recovery of T. pyriformis from
0.2 mg/l cycloheximide, while still in the presence of the drug, no recovery was observed at the concentrations used in this experiment.

Mitochondria from cells grown for four generations (cell number and OD) in defined medium lacking folic acid showed 30% of the amino acid incorporation ability of control mitochondria but a normal amount of mitochondrial protein per cell (Table 11). Thus, the net incorporation ability per cell was reduced (31.4 x 10^3 decays per minute [dpm] versus 57.5 x 10^3 dpm). In vitro incubation of mitochondria in the presence of 150 mg/l chloramphenicol produced 80% inhibition of amino acid incorporation ability. In vivo incubation in the presence of chloramphenicol produced no inhibition; however, these cells showed a great reduction in quantity of mitochondrial protein. Thus, the net incorporation ability per cell was reduced (31.4 x 10^3 dpm/10^6 cells versus 57.5 x 10^3 dpm/10^6 cells). Cycloheximide had no effect, either in vivo or in vitro, on uptake of lysine into the mitochondrial suspension; and it had no effect on the mg mitochondrial protein/10^6 cells.

E. Electron Microscopy of Pellicular Components

Extensive effort has been devoted to determining the molecular composition of cilia (e.g., Witman et al., 1972a, b; Gibbons, 1967). However, although the cilium is structurally continuous with and morphogenetically dependent upon the kinetosome, relatively little attention has been given to determining its molecular composition. In part this has been due to lack of a procedure for obtaining
Table 11. Effect of folic acid deprivation and antibiotics on C-14-lysine incorporation into the proteins of the mitochondrial system of *T. pyriformis*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Decays/min./µg Mitochondrial Protein</th>
<th>µg Mitochondrial Protein/ 10^6 Cells</th>
<th>Net Incorporation Ability/ 10^6 Cells (decays/min, x 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (U+T+F+)</td>
<td>2,180</td>
<td>26.4</td>
<td>57.5</td>
</tr>
<tr>
<td>150 mg/1 chloramphenicol, <em>in vivo</em>^a^</td>
<td>2,730</td>
<td>11.5</td>
<td>31.4</td>
</tr>
<tr>
<td>6 mg/1 cycloheximide, <em>in vivo</em>^b^</td>
<td>2,000</td>
<td>25.8</td>
<td>51.7</td>
</tr>
<tr>
<td>U+T+F-</td>
<td>710</td>
<td>25.0</td>
<td>17.7</td>
</tr>
<tr>
<td>Control + 150 mg/1 chloramphenicol, <em>in vitro</em></td>
<td>340</td>
<td>26.4</td>
<td>9.0</td>
</tr>
<tr>
<td>Control + 6 mg/1 cycloheximide, <em>in vitro</em></td>
<td>2,300</td>
<td>26.4</td>
<td>60.7</td>
</tr>
</tbody>
</table>

^a^ 150 mg/1 chloramphenicol was also present during the *in vitro* incubation of the mitochondria

^b^ 6 mg/1 cycloheximide was also present during the *in vitro* incubation of the mitochondria
sufficient quantities of kinetosomes reasonably pure from other components. The following experiments were performed in an effort to develop procedures for isolating kinetosomes and selectively solubilizing some of the numerous components of the kinetosome.

1. Isolated pellicular fraction

The high ionic strength buffer of Nozawa and Thompson (1971) stabilized the disrupted pellicle so that large fragments could be observed (Figure 63). Kineties were preserved, but the relationships between and within kinetids have been disturbed. Some kinetodesmal fibers remain attached to their kinetosomes. The stubs of ciliary axomemes can be seen extending from their kinetosomes through the pellicle on the distal side of the terminal plate and circumciliary ring. Detachment of cilia from the pellicle is nearly complete; only rarely are ciliary microtubules found still attached (Figure 68) or in the pellicular fraction (Figure 83).

Many free kinetodesmal fibers and kinetosomes are found in the pellicular fraction. The kinetodesmal fibers appear intact (Figures 64 and 65). Rectangular components of the attachment site and the banding pattern are clearly evident. The major repeating unit of 33 nm is composed of a dark 14 nm portion and a lighter 19 nm portion. The brief alcohol fixation on the grid stabilized the fibers against the disruptive effect of the PTA (compare Figures 64 and 65).

Free kinetosomes usually have their terminal plates and axial cores still intact and the parallelism of the microtubular triplets is maintained (Figures 66 and 68). Dense material is associated with the
proximal third of the kinetosome (Figures 63 and 68); comparison of this micrograph with thin-sectioned material (Figures 4, 9, 25, 69, and 70), indicates that this material is sheath. The slight bulge of the microtubular triplets in this region suggests that this material provides structural support for the microtubules. A small remnant of material, perhaps cartwheel, is attached to the proximal end of some kinetosomes (Figure 68). Note that the cilia generally detach 120 nm above the terminal plate and axosome (Figures 63 and 68); even when a fragment of the axoneme remains attached, the position of the breaking point is obvious. Lack of a proximal cap of dense material found in oral kinetosomes (see below) and the small distance between the two kinetosomes in Figure 67 indicates that they comprise a parent-daughter pair. Because the kinetodesmal fibers and the accessory microtubules have been removed, orientation is lost, and it is impossible to determine which is the parent and which the daughter.

The pair of kinetosomes in Figure 67 requires careful interpretation. The close distance between them (compare with Figure 27), the proximal caps of the dense material, and the fibrils radiating from them and interconnecting them at their proximal ends all indicate they are oral kinetosomes (Allen, 1969; Nilsson and Williams, 1966). Ciliated kinetosomes are usually disrupted at the breaking point, 120 nm above the terminal plate, but these kinetosomes are only 500 nm long, the distance from proximal cap to terminal plate. No terminal plates are present. Wolfe (1970) discusses kinetosomes of the undulating membrane of the oral apparatus, where one row of kinetosomes is ciliated and
possesses terminal plates and the other row lacks these features. The pair in this micrograph is apparently from the non-ciliated row of the undulating membrane.

The terminal plates in longitudinal kinetosomal view (Figure 68) present the same two-layered or two-banded appearance that they do in thin-sectioned material (Figures 9, 25, and 59), and the same intricate pattern of rings and spokes in face or tangential view (Figure 66, see also Figure 75) as they do in thin-sectioned material (Figures 69 and 70). Spokes radiate from a central ring of nine apposed pentagons. The spokes intersect a middle ring of 150 nm diameter and join a scallop-shaped outer rim of 320 nm outer diameter. The microtubule doublets pass through the terminal plate centripetally to the middle ring, at a distance of 60 nm from the outer edge of the plate.

2. Disrupted pellicles

a. STEEP + Triton X procedure Phase microscopy examination of the pellicular fraction incubated 14 hours in STEEP + Triton X (Witman et al., 1972a) revealed that the pellicle fragments were still present. However, three strokes with a hand homogenizer were sufficient to disrupt the fragments, due to the solubilization of their membranes by Triton X. The kinetodesmal fibers show varying degrees of degradation. In some cases the pattern of banding is still visible (Figure 76); but in others the fiber has dissociated longitudinally into long fibrils with no striations apparent, and only the site of attachment to the kinetosome remains intact (Figure 71). As in the high ionic strength procedure of Nozawa-
Thompson, some kinetodesmal fibers remain connected to their kinetosomes; but dissociation of the fiber can be so complete that only the attachment site is intact (Figures 71 and 76).

The cilia in general have detached at a breaking point 120 nm above the terminal plate (Figures 71, 74, and 77), although some microtubular doublets have broken off closer to the terminal plate. Axial cores and sheaths are also lost during this procedure (Figures 74 and 77). A small remnant of material is attached to the proximal end of the kinetosomes even after the Triton X treatment (Figures 74 and 77); the nature of this material is not known, but it is probably not membraneous. It could be cartwheel material.

Terminal plates are still attached to kinetosomes (Figures 71, 74, and 77) but they are also seen free (Figure 75). The pattern of rings and spokes compares well with the thin-sectioned appearance (Figures 69 and 70) and also with isolated plates of other workers (Cunningham and Rubin 1973; Munn, 1970; Wolfe, 1970); the dense material connecting the microtubules with the outer ring is removed during the pellicle isolation. It is not equivalent to the spokes since the microtubules pass between the spokes (Figure 70).

Circumciliary ring complexes are frequently encountered in this procedure (Figures 72 and 73). The complex consists of a small oval ring in the region of the parasomal sac and a 320 nm inner diameter round ring, into which the 320 nm diameter terminal plate fits. Since the diameter of a kinetosome microtubular cylinder is 210 nm in diameter (Figures 71, 74, and 77), the microtubules pass through the plate at a
distance of 60 nm from the outside margin. The two rings are asymmetrically surrounded by a whorl consisting of a dense-staining epiplasm. That the surrounding material is not membranous is suggested by its stability in Triton X and the lack of positive staining by uranyl formate. A spike occurs on the organism's right or left. The spikes' relationship to other kinetid components and to the typical thin-sectioned appearance is also unknown. The small distance between the two complexes in Figure 72 suggests that the anterior complex is associated with a daughter kinetosome.

b. Low ionic strength buffer procedure Observations obtained while isolating mitochondria from *T. pyriformis* (Sec. IV.D.) indicated that the pellicular fragments disintegrated spontaneously after being placed for a few minutes in the low ionic strength buffer of Flavell and Jones (1970b). Fragments of membrane can be seen which possess a typical hexagonal, or "honeycomb," pattern of subunits (Figure 78). Kinetodesmal fibers appear in various stages of dissociation; the attachment site is heavily negatively stained (Figures 80, 81, and 82).

No intact kinetosomes were found in this preparation; the coherence of the microtubular triplets was destroyed, and no axial cores or sheaths remained (Figure 79). The microtubular bundles still pass through the terminal plate, however, and exhibit a breaking point 120 nm above the plate. The microtubules can be seen to penetrate the plate between the outer and middle rings.
Figures 63-70. Components of pellicular fraction isolated in high ionic strength buffer and collected by discontinuous sucrose gradient centrifugation.

Figure 63. Positively stained whole mount of a portion of a T. pyriformis pellicle. Oral apparatus (OA) is at top. Portions of several somatic kineties can be seen. The shearing forces of glass-to-glass homogenizations removed cilia and disrupted the arrangement of kinetosomes, kinetodesmal fibers, and accessory microtubules within the kineties. Kinetosomes still have the sheath. Uranyl acetate-PTA in 70% ethanol. X 10,000.

Figures 64-65. Negatively stained kinetodesmal fibers. Lateral striations with a periodicity of 33 nm are clearly visible. Each band is composed of a 14 nm wide dark band and a 19 nm wide light band. The fiber in Figure 65 has undergone lateral swelling. Figure 64, uranyl acetate-PTA in 70% alcohol; Figure 65, PTA. X 39,000.

Figure 66. Free kinetosome appearing in pellicular fraction. The terminal plate, still attached to kinetosome, has been slightly deformed by its drying upon the grid. Uranyl acetate-PTA in 70% ethanol. X 39,000.

Figure 67. Two kinetosomes appearing in pellicular fraction. The close proximity of the kinetosomes, the fibers connecting them, and the dense material serving as proximal caps (pc) indicate that these are oral kinetosomes. Uranyl acetate-PTA in 70% ethanol. X 39,000.

Figure 68. Two kinetosomes showing intact terminal plates, axial core, and axosome. A breaking point is apparent between the left kinetosome and its cilium. The triplets appear somewhat collapsed in the midregion. Note the dense sheath material in the proximal region. A fragment of material is attached to the proximal end of the lower kinetosome (arrow). Uranyl acetate-PTA in 70% ethanol. X 39,000.

Figures 69-70. Thin sections showing terminal plate. Cells were grown in defined medium, fixed in glutaraldehyde, post fixed in OsO₄, and embedded in ERL. The plates have an intricate nine-fold symmetry. See text for details. Figure 69, U+T+F- cell; Figure 70, U-T+F+ cell. Uranyl acetate-lead citrate. X 39,000.
Figures 71-77. Components of pellicular fraction isolated with the Teflon-glass homogenization procedure. The pellicular fraction was incubated for 14 hours in STEEP + 0.7% Triton X-100 and then gently disrupted with a hand glass homogenizer.

Figure 71. Two kinetosomes and several kinetodesmal fibers. A portion of one kinetosomal fiber is still attached to its kinetosome, but the others are unattached and have been disrupted into arrays of long fibrils joined together only at the attachment site. Uranyl formate. X 39,000.

Figures 72-73. Ciliary pore complexes. The circumciliary ring and parasomal ring are placed slightly asymmetrically in a surrounding whorl of epiplasm. A spoke or fibril (arrows) protrudes from one side of the whorl except in the case of the right or anterior, prokinetosome-associated complex in Figure 72. Uranyl formate. Figure 72, X 22,000; Figure 73, X 39,000.

Figure 74. Three isolated kinetosomes with terminal plates. Most ciliary microtubules have detached at the breaking point. One kinetosome still has the axosome attached. The central kinetosome has one attached longitudinal tubule and opposite a fragment of a transverse tubule; the attachment site of the kinetodesmal fiber is also present. None of the kinetosomes have proximal dense sheaths. Uranyl formate. X 60,000.

Figure 75. A number of free terminal plates. The diameter of the plates is 320 nm, exactly equal to the inside diameter of the circumciliary ring (Figures 60 and 61). The microtubular doublets pass through the clear region (arrow). PTA. X 39,000.

Figure 76. Kinetodesmal fiber in early stage of disruption. The striations along its length are still clearly visible. The attachment site is heavily outlined by the negative stain. Uranyl formate. X 39,000.

Figure 77. Isolated kinetosome with terminal plate. No sheath is present. An unidentified component is present at the base of the kinetosome (arrow). Uranyl formate. X 39,000.
Figures 78-83. Components of pellicular fraction isolated with Teflon-glass homogenization procedure and resuspended in low ionic strength buffer. Uranyl acetate. X 60,000

Figure 78. Pellicular membrane. When glow-discharged grids are used, this sort of pellicular image is common for non-detergent preparations. Note the hexagonal pattern with spacings of 125-150 nm

Figure 79. Isolated terminal plate with portions of microtubular bundles passing through it. The microtubular doublet is 120 nm long, the usual length for the distance between the breaking point and the plate. Several doublets (arrows) can be clearly seen to pass through the plate between the outer and middle rings

Figure 80. Kinetodesmal fiber in early stage of disruption. The dark band is 14 nm wide and the light band is 19 nm wide; the total period is thus 33 nm (compare with Figures 52, 53, and 64)

Figure 81. Kinetodesmal fiber in an intermediate stage of disruption. The longitudinal fibrils are still connected to each other near the attachment site, and a few bands can still be seen (arrows)

Figure 82. Kinetodesmal fiber completely disrupted. The fibrils are connected only at the attachment site

Figure 83. One of the central pair of microtubules. A row of projections (arrows) occur on either side, about 15 nm apart (compare with thin section, Figure 23). Protofilament substructure of the microtubules is evident. The occurrence of a central microtubule in the pellicular fraction indicates either that deciliation was not complete or that the pellicular fraction was not entirely pure
V. DISCUSSION

Suggestions of autonomy of cellular organelles have been made since the mid-1800's, yet only recently have there been sufficient advances in knowledge and methodology to permit serious investigation of the mechanisms governing growth, development, and continuity of organelles. The often inadequate experimental procedures used and the conflicting results obtained concerning kinetosomal autonomy prompted the investigation described in this dissertation.

The ultrastructural results obtained from cytochemical, nutritional, and pellicular isolation studies of Tetrahymena pyriformis GL will be discussed, particularly as they relate to the issue of kinetosome autonomy. In addition, the nutritional studies provided information about certain features of mitochondrial autonomy, and this situation was further explored by an amino acid incorporation study on isolated mitochondria. The present status concerning mitochondrial autonomy and its evolutionary implications will be considered.

Finally, the research of others on kinetosome autonomy, especially as it relates to the possibility of kinetosomal DNA, will be re-evaluated and consideration will be given to the role of the kinetosome in cellular morphogenesis.

A. Cytochemistry

1. Failure to localize nucleic acids in kinetosomes by acridine orange fluorescence

The studies of Randall and Disbrey (1965) and Smith-Sonneborn and Plaut (1967) provided the most substantial evidence for the presence of
DNA in kinetosomes. These studies were based in large part on acridine orange fluorescence. Numerous difficulties existed in interpreting these fluorescence results (compare with Fulton, 1971). First, because of the limit of resolution imposed by the light microscope, it was difficult to determine accurately exactly which organelles were fluorescing. Mitochondria, in particular, are aligned in rows just beneath the pellicle (Figures 3 and 20), and these rows alternate with the kineties. *T. pyriformis* mitochondria are known to possess DNA (Suyama and Miura, 1968) and, in an unclean preparation, they could conceivably have accounted for the fluorescence reported. Second, acridine orange was known to stain a number of other compounds besides nucleic acids (Kasten, 1967). Third, nuclear or mitochondrial DNA, released by disruption of those organelles could have adsorbed to the pellicle (Hufnagel, 1969b). Fourth, the green fluorescence in the pellicles could be detected during only 60% of the life cycle, and it was gone not only after treatment with DNase, but also after treatment with RNase, protamine, and histone. The difficulties suggested that re-examination of these key studies was necessary.

The fluorescence work of Randall and Disbrey and of Smith-Sonneborn and Plaut could not be verified in the present study. No fluorescence could be correlated specifically with the kineties. Further, when the pellicles were carefully and vigorously washed by repeated centrifugations, in an effort to remove mitochondria and/or adhered DNA, and when the careful procedures of Kasten (1967) were followed in order to eliminate nonspecific staining, no acridine orange fluorescence could be observed.
D. Prescott (personal communication, 1972) also was unable to verify
kinetosomal DNA by acridine orange fluorescence.

2. Enzymatic and chemical extraction of nucleic acids from intact
fixed cells

a. Biochemistry

(1) Extraction of RNA and DNA from Karnovsky-fixed cells

(a) Concentrations of nucleic acids in T. pyriformis
The values of 18 pg/cell DNA and approximately 400 pg/cell RNA here
reported are in reasonable agreement with those in the literature. Of
the few studies which have attempted to quantify the concentrations of
DNA and RNA present in T. pyriformis, only two report values for strain
GL. Scherbaum (1957) used colorimetric procedures to obtain values of
250 pg/cell RNA and 13.6 pg/cell DNA for cells grown in proteose peptone
medium at the optimum temperature of 28.5°C. Later, Scherbaum et al.
(1959) using the same methodology and growth conditions, obtained a
value of 17.5 pg/cell DNA; no attempt was made to determine RNA. The
35% upward revision of values suggests that a refinement in technique
led to reduced loss of DNA. Hence, loss of RNA in the earlier experiment
of Scherbaum (1957) is also probable and accounts for his RNA values
being lower than in the present experiment; this is especially likely
as an explanation since the values obtained in the present experiment
are consistent with those of the subsequent two extraction experiments.

(b) Degree of extraction of nucleic acids from fixed
cells

Although extraction of nucleic acids from fixed cells and
tissues is a common cytochemical procedure (Pearse, 1968), little
information is available concerning the degree of extraction which takes place. The results described in this dissertation (Sec.IV.B.2.a.) supply some necessary information concerning the degree of extraction. First, it is significant that all of the NA present in Karnovsky-fixed cells could be extracted by PCA in this experiment, while maintaining a reasonable approximation of the original ultrastructure. Second, RNase was able to extract 75% of the RNA from Karnovsky-fixed cells with little ultrastructural change. By way of comparison, Molenaar et al. (1970) obtained similar results with RNase, achieving 85% extraction of RNA from glutaraldehyde-fixed, isolated yeast nuclei with minimal effects on ultrastructure. Third, cold PCA, although reported by Aldridge and Watson (1963) to be able to completely extract RNA from acrolein-fixed rat liver within 18 hours, was ineffective on the Karnovsky-fixed T. pyriformis in this experiment, extracting only 15% of the RNA and producing no visible ultrastructural effect (D. Outka and S. Jenkins, personal communication, 1971). The most probable explanation for the discrepancy relates to the difference in fixatives.

Fourth, although the activity of the DNase could be verified by its ability to produce a hyperchromicity effect on standard yeast DNA and by its ability to remove fluorescence from nuclei of acridine orange-stained cells, its ability to extract measurable amounts of DNA could not be determined. The indole test for DNA (Ceriotti, 1952) modified by Keck (1956) was selected over the diphenylamine test (Burton, 1956) in part because the 5% PCA hydrolysis at 90°, which was used as a control for the enzyme hydrolyses, destroys the chromogen for the diphenylamine
reaction (Webb and Lindstorm, 1965). While it is possible that the use of amyl acetate for the removal of nonspecific color (Keck, 1956), rather than chloroform, may have contributed to the variability of the results (Hubbard et al., 1970), a more likely explanation is that the DNase may not have penetrated the Karnovsky-fixed membrane as easily as RNase or may not have had sufficient accessibility to the DNA in the nucleoprotein complex.

Fifth, the ability of the DNase to extract, during the 40 hours of incubation, 35% of the cellular orcin-positive substance suggests either that conditions of extraction were effective in removing RNA from Karnovsky-fixed cells or that the DNase was contaminated with RNase. In view of the fact that 80% of the cellular RNA is present in the ribosomes of *T. pyriformis* (Nilsson and Leick, 1970), and only 2% of the cellular RNA occurs in the nucleus (Leick, 1970), the RNA detected could only in very small part have been nascent RNA liberated by the hydrolysis of the DNA template. Thus, the more likely explanation for the occurrence of RNA in the hydrolystate was the presence of RNase in the DNase; such contamination of commercial DNase of the kind used has been demonstrated (Zimmerman and Sandeen, 1966).

(2) Efforts to improve extraction from Karnovsky-fixed cells

None of the three variations in fixation and extraction procedure had any effect on the amount of RNA extracted. The original fixation procedure, in which a volume of cells was mixed with an equal volume of fixative, was adopted from Williams and Luft (1968). This procedure was designed to produce instantaneous fixation and avoid any stress
created by centrifugation and concentration of the cells. However, since proteose peptone medium consisted of a proteolytic digest of beef, it was felt that the admixture of the medium and the fixative could possibly have bound polypeptides to the cell surface. In such a case a network of polypeptides might have physically restricted the flow of enzyme across the cell membrane, which still retained some osmotic properties (Hayat, 1970). In fact, however, prewashing the cells in glycerol-phosphate buffer to remove the proteose peptone had no effect on the extraction level.

Agitation during hydrolysis increased the degree of extraction from about 75% to 85% of the cells' nucleic acids but the major effect was the increase in rate of extraction of RNA from approximately 50 to 75% during the first hydrolysis. The short (10 minute) fixation did not stabilize the NA as well against prolonged incubation, since the last control incubation (14 hours) removed a considerable amount of RNA.

(3) Effects of three other fixatives on RNA extraction

Formaledhyde, glutaraldehyde, and acrolein were similar to Karnovsky's fixative in terms of allowing RNA extraction. Approximately 80 to 85% of the cellular NA was extracted. This compares well with the work of Molenaar et al. (1970) in which 84% of the RNA could be extracted from isolated, glutaraldehyde-fixed yeast nuclei. Formaldehyde, however, appears to be an extremely poor fixative for NA preservation, at least if the cells are maintained in a buffer solution for any length of time, since as much RNA was extracted in the buffer as in RNase.
In none of the extraction experiments could more than approximately 85% of the cell's RNA be extracted by RNase. Even with formaldehyde fixation, which permitted extraction without enzyme, 15% of the RNA remained. These results suggest that a certain amount of RNA is cross-linked by the fixative and is, thereby, made inaccessible to the enzyme.

b. Electron microscopy

(1) RNase control The ultrastructure of cells incubated in buffer for 40 hours compared well with cells treated in buffer for only 30 minutes. No alternation of any cellular component was visible, and the biochemical data indicated no significant loss of nucleic acid or protein.

(2) RNase hydrolysis The ultrastructural effect produced by RNase appears to be dependent on the proportion of RNA contained in a structure. Nucleoli, which contain 10-15% RNA (Busch and Smetana, 1970) showed only slight loss of granularity. However, ribosomes which contain approximately 50% RNA (Spirin and Gavrilova, 1969) showed both a reduction in stain intensity and a decrease in size, from 21 nm to 14 nm. These results compare well with those of Molenaar et al. (1970) for isolated yeast nuclei (with ribosomes attached to the membrane) fixed in glutaraldehyde. It is probable that all of the messenger RNA and tRNA molecules, which are relatively small and not tightly bound to proteins, have been removed by the enzyme hydrolysis. Even so, since 80% of T. pyriformis RNA is rRNA (Nilsson and Leick, 1970), a minimum of at least 70% of the rRNA must have been removed to account for 75%
total RNA extraction by RNase. It is likely that 70-75% of the rRNA extracted was distributed towards the exterior of the ribosome, and included especially the loops of RNA which cover the surface of the ribosome (Vasquez and Kleineschmidt, 1973), whereas the 25-30% of the rRNA which was not RNase-extractable occurred in the interior of the ribosomes, where its tight binding to protein made it sterically inaccessible to the RNase. Removal of the RNA in the ribosome "cortex" and the concomitant collapse of the protein with which it had been associated would account for the decrease in size. The remaining rRNA and the ribosomal protein would account for the remaining stain intensity.

Randall and Disbrey (1965) visually estimated that the DNA present in a kinetosome of *T. pyriformis* produced an intensity of acridine orange fluorescence equal to that of a T-2 bacteriophage, which has $10^{-16}$ g DNA. Since a *T. pyriformis* ribosome has $3 \times 10^{-18}$ g RNA (Chi and Suyama, 1970) the amount of DNA present in the T-2 phage and hence presumably in the kinetosome should be equivalent to the amount of RNA present in 33-100 ribosomes. A longitudinal section might then possess an amount of DNA equivalent to the RNA of 8-25 ribosomes. Not only should such an amount be stainable (Sec.V.E.3.), but it should require the presence of significant amounts of RNA to mediate its protein syntheses. However, no extraction effect, such as reduction in the size or staining intensity of the axial core, a likely location for transcription and translation, or in other structures of the kinetosome, could be seen.
Although Stubblefield and Brinkley (1967) reported that kinetosome triplet feet were removed by RNase hydrolysis in glutaraldehyde-fixed Chinese hamster kidney cells, no removal of the triplet feet could be detected in *T. pyriformis* kinetosomes. The feet may not be completely homologous structures, since in hamster cell kinetosomes they extend the length of the triplets, and in *T. pyriformis* they occur only in the cartwheel region. However, the single set of serial sections presented by Stubblefield and Brinkley (1967) was low in magnification and not convincing because one third of the feet still appeared to be present after treatment.

(3) **Hot PCA control**  
The general loss of crispness in the cytoplasm, the contraction of the condensed chromatin, and the apparent loss of some nucleoplasm can probably be attributed to the heat denaturation of proteins produced by incubation in glycerol-phosphate buffer for one-half hour. Nevertheless, the ultrastructural framework of all organelles, including the kinetosome, was intact and recognizable. Since neither nucleic acids nor proteins were detected in the supernatant, nucleoplasm could not actually have been lost, but instead the fibrils and granules probably aggregated and coalesced into larger structures, thereby leaving vacant regions.

(4) **Hot PCA hydrolysis**  
The denaturation produced by the 90°C temperature was considerably exacerbated by the presence of PCA (see Sec.IV.B.2.(b).(3)). The cytoplasm has coalesced into a reticulum. This gave the appearance of structures being missing but no protein could be detected in the supernatant.
Less ultrastructural loss of NA could be detected with this procedure than with RNase hydrolysis despite biochemical evidence that all of the NA had been removed. Whereas the gentler extraction of RNA by RNase may have allowed the proteins in the ribosomal exterior "to collapse," the harsh PCA treatment may have essentially "fixed" the ribosomal protein and thus there was no change in size. Again, the apparent loss of nucleoplasm may have been due to coalescence of the fibers and granules with each other or with nucleoli or the condensed chromatin. No loss of kinetosomal structures was observed; the obscure ultrastructural status of NA extraction in the nucleus precludes conclusions concerning loss of NA from the kinetosome.

c. Value of extraction from fixed cells for localization of nucleic acids

Nucleic acid cytochemistry is a difficult subject. Nucleic acid stains bind to specific functional groups which may also be present in other cellular components. In addition the staining reactions are modified by the proteins with which the nucleic acids are associated. Hence, stains for nucleic acids are only relatively specific, with the possible exception of indium (Outka, 1971). This difficulty in assessing chemical composition of cellular structures is emphasized by results on extraction in fixed cells, where most of the NA has been removed by nuclease or PCA, yet, even in organelles containing major amounts of NA, only subtle differences in ultrastructure may be apparent. The situation is undoubtedly compounded by the effects of the fixative on the protein and NA since some fixatives, notably formaldehyde, appear to allow visibly more intense staining or more extraction (e.g.,
3. Enzyme hydrolysis on sectioned material

   a. Hydrogen peroxide control     No significant extraction of any cellular components was obtained in ERL-embedded *T. pyriformis* by hydrogen peroxide incubation or subsequent incubation in water for ten hours. This is similar to the results reported by Anderson and André (1968) for Epon-Araldite, by Monneron and Bernhard (1966) for Epon, and by Monneron (1966) for GMA. An advantage of pronase over some other proteolytic enzymes such as pepsin and trypsin is that a neutral pH is optimal. Hence nonspecific extraction in acid buffer is not a problem (Monneron, 1966). RNase was used at pH 6.5 and presents no problem in this regard.

   b. RNase hydrolysis     The results reported here and those by Lord and Lafontaine (1969) Monneron and Bernhard (1966), and Leduc and Bernhard (1962) suggest that the type of plastic and the amount of protein present in a ribonucleoprotein structure combine to produce an extraction gradient. GMA is a water-soluble embedment. It is, therefore, more hydrophilic than epoxy resins and it may have fewer cross-links; thus it is presumably more easily penetrated by enzymes and should allow greater extraction. Hence, ribosomes (50% RNA) were greatly reduced in contrast and nucleoli (15% RNA) were somewhat reduced in contrast (Lord and Lafontaine, 1969; Leduc and Bernhard, 1962). Epon on the other hand, is an epoxy resin which is not water soluble, but some extraction is still possible. No pictures were shown by Monneron and
Bernhard (1966), but they reported that RNase had no effect on the nucleolus but did "attack ribosomes."

ERL is also an epoxy resin; the results reported here indicate less effect by RNase than in the other three reports discussed. No effect was observed in the ribosomes and no effect was observed in the granular or fibrillar areas of the nucleoli. However, material in the nucleolar clefts and cavities and circumnucleolar material was removed. It is known that the clefts and cavities are sites of nucleolar organizer activity (Charret, 1969). The availability of these regions to enzyme probably related to changes in conformational state of the RNA as it was synthesized in the organizer region and then moved away from the nucleolus. Interchromatin granules, which are also ribonucleoprotein structures, shared the same digestibility characteristics. They may have been more susceptible to RNase than the ribosomes themselves because their conformation exposed more key binding sites, or because they were less tightly associated with protein.

Based on all of this information, a gradient of ease of extractability can be proposed: nucleolar clefts, circumnucleolar region, and interchromatin granules > ribosomes > nucleoli.

c. Pronase hydrolysis Pronase is a powerful proteolytic enzyme isolated from Streptomyces griseus, and it produced radical changes in the ultrastructure of ERL-embedded T. pyriformis.

(1) Nucleoproteins In those areas of a section where there had existed a high protein concentration little plastic remained after the digestion, perhaps because the plastic could not infiltrate
those regions easily. Thus, large assemblies of nucleoprotein, i.e. condensed chromatin, which is 70% protein (Stein, 1972), and nucleoli, which are 85% protein (Busch and Smetana, 1970), were nearly completely digested; only a few fibrils, possibly consisting of NA or of plastic, remained. In contrast, ribosomes and fibers in the nucleoplasm were affected but generally not completely digested. One might expect that because of their small size, these structures would have been readily attacked. However, it appears that their small size in fact protected them.

A gradient of digestion was evident in the ribosomes. Occasionally very light spots the size of ribosomes were found in the sections; they were often associated with endoplasmic reticulum or nuclear membrane. This association suggests that where polysomes were aligned vertically in the section, the enzyme could remove a column of nucleoprotein and leave an empty region. Other ribosomes could be seen which had the appearance of the spread or unfolded chymotrypsin-treated ribosomes prepared by Vasquez and Kleinschmidt (1973). These evidently were close to or on the surface of the ERL section. Removal of the protein permitted them to "spread" along the plastic surface. And finally, some ribosomes were reduced in size from 21 to 16 nm but otherwise appeared intact. In this case the ribosomes were probably completely embedded in the 50-60 nm thick section. Steric hindrance perhaps produced by cross-linking formed between the ribosomal interior protein and other cytoplasmic structures because of the glutaraldehyde fixation may have prevented the pronase from hydrolysing the majority of the ribosomal
protein. Only the exterior portion of the ribosome, that is, the portion loosely associated with loops of RNA, was available to the pronase. The ten nm diameter nuclear fibers may also have been saved from complete digestion by pronase because they were surrounded by ERL.

(2) Nuclear pores A number of workers have attempted to assess the composition of nuclear pores by cytochemical means. (Little good evidence has been obtained, however.) Koshiba et al. (1970) fixed Novikoff hepatoma cells in glutaraldehyde and treated them with pepsin, pronase, subtilisin, DNase, and RNase prior to embedment; they reported that pepsin digestion removed the pores, pronase reduced the density of the pores, and subtilisin had little effect. RNase and DNase had no effect on the pores, although RNase digested the nuclear membrane-associated ribosomes. Their results are subject to question because of poor ultrastructural preservation of the nuclear region in the pictures presented. Beaulaton (1968) treated glutaraldehyde-fixed lepidopteran secretory cells with pepsin and reported that the pores were digested. Mentre (1969) treated isolated rat nuclei with RNase and DNase before fixation in OsO₄ and embedment. Mentre claimed that the nuclear pores were susceptible to RNase, but not to DNase; however, his micrographs were not convincing. Cole (1969) fixed frog oocytes in glutaraldehyde, embedded them in GMA, and incubated them in enzymes. He claimed in his abstract that RNase or pronase decreased the affinity of the central rod for uranyl acetate stain, but DNase did not. Finally, Franke and Falk, (1970) exposed Epon-embedded thin sections of onion root tip
to a regressive stain procedure and concluded that the pore granule could be either ribonucleoprotein or protein.

The image of the nuclear pore obtained in Sec.IV.B.3.d. is in good agreement with that reported for *T. pyriformis* by Wunderlich and Speth (1972). Dense material fills the periphery of the pore, and a dense rod occupies the axis of the pore. Contrary to some reports for vertebrate cells (Stevens and Andre', 1969), the presence of a central granule seems to be a constant feature for *T. pyriformis*. It is perhaps significant that the central rod was resistant to the action of pronase whereas the annulus was susceptible. Similar results were reported by Abelson and Smith (1970) for green monkey kidney cells. This difference in the proteins or nucleoproteins of the rod and annulus may somehow be related to the regulatory function proposed for the pores (Wunderlich, 1972).

(3) Mitochondria Pronase appears to have had a more variable effect on the mitochondria than on many other organelles, since the cristae were in various states of digestion. The pronase appears to have specifically extracted the interior regions, or lumina, of the cristae; it does not appear to have extracted the matrix. This is similar to the result demonstrated by Monneron and Bernhard (1966) for mitochondria in various rat tissues embedded in Epon, although they described the effect as occurring in the matrix. Curiously, the effect of pronase in ERL and in Epon appears to be opposite to its effect in GMA, in which it did extract the matrix but not the intracrystal lumina (Monneron, 1966).
Kinetid

Most components of the kinetid were digested. Only the cartwheel axis, the luminal matrix, and the terminal plate of the kinetosome and the matrix, radial spokes, and central sheath of the cilium remained undigested. Anderson and André (1968) reported a differential extractability of microtubules from tissues of several invertebrate species embedded in Epon. The microtubules of the kinetosome were extracted by pronase and pepsin, but adjacent cytoplasmic tubules were not. However, in this present study all microtubules, including longitudinal and transverse microtubules as well as kinetosomal triplet and ciliary doublet microtubules, were extracted. This could have been due to a longer digestion time in this study, or perhaps ERL infiltrates microtubules less adequately than does Epon. It is notable that the epiplasmic layer was extracted except where the circumciliary ring inserted into it. Both the ring and the terminal plate were resistant to the pronase. This could indicate a modification of the protein necessary for anchoring the kinetid in the pellicle. The extraction of the kinetodesmal fiber is consistent with the proteinaceous nature ascribed to it from pellicular isolation studies (Rubin and Cunningham, 1973).

d. Value of cytochemical extractions employing ERL-embedded material

ERL permits visualization of extraction only of RNA which is not tightly bound to protein. Thus it suffers, to a somewhat greater extent, from the same difficulties reported by others (Lord and Lafontaine, 1969; Anderson and André, 1968; Monneron and Bernhard, 1966; Leduc et al., 1963; Leduc and Bernhard, 1962). Considering the difficulty in observing any
ultrastructural difference even when considerable RNA extraction is known to occur (i.e., extraction from fixed cells prior to embedment, Sec.V.A.2.b.(2).) great care must be employed in deciding if the RNA has been removed from the thin sections. Regardless, the method appears to be of limited value for cytochemical localization.

In contrast to the situation with RNase, ERL does permit considerable extraction of proteinaceous components, including nucleoproteins, microtubular protein, mitochondrial intracristal material, epiplasm, and kinetodesmal fiber. However, absence of extraction should not, without other data, be taken to imply that a structure is not proteinaceous, since membranes, mucocysts, and peroxisomes are resistant.

B. Nutritional Deficiencies

1. U+T+F+ cells (control)

The generation time in defined medium was longer than in proteose peptone medium, 7 hours versus 3.2 hours. The slower growth, incidentally, produced an ultrastructural image in which organelles were more easily observed because of the reduced numbers of ribosomes. Otherwise the cells were normal. U+T+F+ cells were also normal, indicating that T. pyriformis was able to methylate the pyrimidine ring in the presence of folic acid, in agreement with the results of Wykes and Prescott (1968), Elliot and Hayes (1953), and Kidder et al. (1950).

2. U+T+F+A+ cells and U(x200)+T+F+A+ cells

Amethopterin had no effect on the rate of growth. Zeuthen and Villadsen (1970) experimented with several concentrations of amethopterin. Only the highest concentration, equivalent to the one in the present
study, produced immediate and full inhibition of DNA synthesis; and after three hours new synthesis began at 35% of the control rate. *T. pyriformis* exhibits rapid recovery from or insensitivity to nearly all drugs tested against it (Conklin and Chou, 1971; Frankel, 1970). It apparently manifests complete recovery from amethopterin as well, since the long-term growth curve showed no effect. Heyer and Frankel (1971) propose that the resistance to many drugs results from the ability of *T. pyriformis* to form a component which brings about extrusion of the drug. Regardless, amethopterin could not be used as a means of seriously depleting the DNA pool in *T. pyriformis* in order to regulate DNA-containing organelles.

3. **U+T+F- cells**

Deletion of folic acid from defined medium caused reduction and finally cessation of growth. Examination of the metabolic pathways in which folic acid is known to participate (Blakely, 1969) indicates that this effect could not have resulted from reduced synthesis of methionine, serine, purines, or thymidine, since all of those compounds were exogenously supplied. However, folic acid is also required for the synthesis of f-met-tRNA in prokaryotes, and possibly in the apparently prokaryotic-like mitochondria of eukaryotes.

EM observations indicate that the defect produced by folic acid deletion was indeed specifically localized in the mitochondria. The decrease in number of cristae, the swelling of the cristae, and the poor appearance of the cristal membranes were similar to the effects described for the mitochondria of *T. pyriformis* grown in the
presence of chloramphenicol (Turner and Lloyd, 1970) or ethidium bromide (Charret, 1972; Meyer et al., 1972). Ethidium bromide has also been shown to inhibit DNA replication in *T. pyriformis* (Charret, 1972), and chloramphenicol to inhibit translation (Mason et al., 1970; Mager, 1960); thus the ultrastructural effects produced by these two agents appear to be directly related to a protein deficiency.

F-met-tRNA has been isolated from the mitochondria of several eukaryotes. F-met-tRNA no doubt serves the same function in eukaryotic mitochondria as it does in prokaryotes where it is required for this initiation of protein synthesis in prokaryotes (e.g. Sager, 1972). Taken together, the evidence indicates that the changes in the ultrastructure were caused by an inability of the mitochondria to initiate protein synthesis, in turn caused by an absence of folic acid necessary to formylate met-tRNA to f-met-tRNA. With the loss of mitochondrial activity, the cell was deprived of its source of energy and thus growth ceased. If folic acid was again supplied, growth resumed.

The effect on mitochondrial protein synthetic apparatus was more gradual than that produced by addition of chloramphenicol or ethidium bromide. *T. pyriformis* is evidently able to use and conserve folic acid efficiently, since it was able to sustain six cell divisions in the absence of exogenous folic acid and since the small amount of folic acid carried over in the inoculum if the cells were not prewashed extended the amount of growth possible.

The nature and significance of the striated inclusions associated with the mitochondrial cristae are unknown. These inclusions averaged
300 x 60 nm in the micrographs. This is considerably larger than the spherical polyphosphate granules which measure about 30 nm in diameter (Thomas and Greenawalt, 1968; Fawcett, 1966, Greenawalt et al., 1964). Mitochondria from a number of sources produce granules under various conditions of stress: prolonged vitamin A overdose in mouse pancreas (Watari et al., 1972); vitamin A deficiency in mouse cornea (Sheldon and Zetterqvist, 1956), Cushing's syndrome in human adrenal cortex (Ishihara et al., 1972), croton oil-induced hyperplastic mouse epidermis (Frei and Sheldon, 1961), regenerating kidney (Anderson, 1967), folic acid overdose (Byrnes et al., 1972), neonatal rat tongue (Kimura, 1972), hibernating snake renal tubules (Kurosumi et al., 1966), and *T. pyriformis in stationary phase cultures* (Elliott and Bak, 1964: Roth and Minick, 1961). However, there appears to be no similarity in the morphology of the granules and no underlying physiological mechanism. The inclusions observed in the present study appear to be unique because of their elongate shape and striated structure, and they are, to date, unique to *T. pyriformis*. They have been seen in this laboratory only very rarely in normal cells, and Williams and Luft (1968) have indicated they were occasionally seen in their cells. Striated regions not filled with dense-staining material were sometimes seen in the folic acid deficient mitochondria. They could have represented an early stage in the formation of dense inclusions since the striations had a similar interperiod spacing.

4. WT-F- cells

When cells were grown without an exogenous supply of thymidine and
folic acid, an additional effect was superimposed on the mitochondrial effect since the cells ceased growth more quickly. Furthermore, the defect, if allowed to persist until growth ceased, was irreversible. Studies from other organisms reveal that without an exogenous supply of thymidine and without the ability to synthesize thymidine monophosphate from uridine monophosphate via the thymidine synthetase pathway (which requires a folic acid derivative), cells cannot synthesize DNA. Such a situation generally produces an unbalanced growth that results in "thymine-less death" (Cohen, 1971). Examination of the ultrastructure corroborates the idea that T. pyriformis may have died a "thymine-less death."

T. pyriformis has a polyploid macronucleus; about 80-90 copies of each chromosome are present (Flavell and Jones, 1970a; Raikov, 1969). Apparently each cell apportioned its existing chromosomes between its daughters, until after four successive divisions, only a few copies of each chromosome were left, thus accounting for and correlating with the empty appearance of the macronucleus. Remarkably there was no condensed chromatin visible, implying that all of the remaining deoxyribonucleoprotein was in the dispersed state. This level of DNA may have been too low to provide an adequate rate of transcription necessary for an organism as large as T. pyriformis, an explanation which would account for the reduced numbers of ribosomes present in the cytoplasm. The nucleoli were very condensed and large, reminiscent of those reported during general cell starvation (Satir and Dirksen, 1971).
The mitochondria in these cells presented an ultrastructural appearance similar to, but more exaggerated than, those of the U+T+F-decient cells. This situation probably was due to two factors. First, the thymidine deficiency affected the ability of the mitochondria as well as the nucleus to synthesize DNA and, therefore, the low level of transcription magnified the reduced level of translation. Second, the mitochondria were deprived of gene products normally supplied by the nucleus.

In contrast to the severe effects observed in the nucleus and mitochondrion, the two organelles known to possess DNA in *T. pyriformis*, no effect was observed in kinetosomes due to thymidine deficiency. This strongly suggests that DNA is not involved in kinetosomes.

5. U-T+F+ cells

Wykes and Prescott (1968) reported that *T. pyriformis* was unable to demethylate thymidine or one of its phosphorylated forms in order to form deoxyuridine. Hence cells grown in the absence of uridine apparently were unable to synthesize RNA. This difficulty was reflected in the reduced numbers of ribosomes present in the cytoplasm. The highly vacuolated appearance may also have been a reflection of reduced amounts of proteins present. Comparing the severe effect produced in the nucleus by the absence of thymine and its derivatives, with the normal appearance of the nucleus in the U-T+F+ deficiency, one would conclude that all NA bases must have been present; in other words, *T. pyriformis* must be able to synthesize deoxycytidine from thymidine. Most of the pathways for interconverions of NA derivatives in *T. pyriformis* have not been
worked out, but apparently the organism can demethylate 5-methyl deoxycytidine but not thymidine.

Also, in contrast to the obviously affected cytoplasm and to the effect on mitochondrial protein synthesis caused by the folic acid deficiency, the mitochondria appeared normal. The conclusion is that the mitochondria were not affected by the uridine deficiency, either because they were able to selectively concentrate uridine, or because they possess a pathway for demethylation of thymidine.

In contrast with the other nutritional conditions, in the uridine deficiency approximately 40% of the kinetosomes from cells demonstrated axial cores of reduced density. The density loss could have been due to either a reduction in RNA or protein content. The reduction in the cores was as much as 50%; if that portion of the core were RNA, it should have been detected by the cytochemical extractions in fixed cells (Sec.V.A.1.b.). However, no losses were detected by cytochemistry, and it thus seems reasonable to assume that the axial core is primarily protein. The fact that only some cores exhibited a reduction might be explained by assuming that the cores constituted microtubule subunits (especially tubulin) in a highly concentrated form. Normally, when cilia are broken off, regeneration proceeds immediately (Rosenbaum and Carlson, 1969). Rannestad and Williams (1971) and Williams et al. (1969) have demonstrated the existence of a large tubulin pool in T. pyriformis. It would be advantageous to have the pool located at least in part in highly concentrated form in the kinetosome such that when a cilium becomes broken off, as is fairly frequent in nature (Blum, 1971),
regeneration could proceed as soon as possible. However, in the absence of new protein synthesis, the core tubulin material once used up, could not be fully replaced; hence 40% of the kinetosomes with reduced cores.

C. Reduction of Protein Synthesis in Mitochondria

Nutritional and ultrastructural evidence previously obtained indicates that a folic acid deficiency causes reduced protein synthesis in mitochondria of \textit{T. pyriformis}. This possibility was further examined by studying the ability of folic acid deficient mitochondria to incorporate C-14-labelled lysine into protein. Chloramphenicol was used as the control for the experiment because it has been reported to produce similar effects on growth (Mager, 1960) and ultrastructure (Turner and Lloyd, 1970).

U+T+F-cells were capable of six cell divisions before the folic acid deficiency halted growth. Cells which had undergone four divisions still showed a normal amount of mitochondrial protein, but by this time exhibited only 30% of the amino acid incorporating ability of normal cells, which suggests that the ultrastructural degeneration occurred after this time. This reduction in protein synthetic capacity, together with the ultrastructural picture, provides strong evidence for the role of a folic acid derivative in mitochondrial protein synthesis in \textit{T. pyriformis}. Indeed, f-met-tRNA has been demonstrated in mitochondria of \textit{Neurospora}, yeast, and rat liver (Sager, 1972).

The inhibitory effect of chloramphenicol on growth of \textit{T. pyriformis} and on the \textit{in vitro} incorporation of a labelled amino acid into
mitochondrial protein was similar to that reported by Mager (1960). Remarkably, mitochondria from cells which had been grown in the presence of chloramphenicol showed no inhibition of amino acid incorporation, even when incubated in vitro in chloramphenicol. However, the amount of mitochondrial protein was approximately 50% that of normal cells. This suggests that the chloramphenicol had indeed inhibited mitochondrial protein synthesis and growth but that the remaining mitochondria may have developed resistance to the drug. Chlortetracycline HCl produced a growth response similar to chloramphenicol, and Mager (1960) reported that it also produced inhibition of mitochondrial protein synthesis.

In contrast, cycloheximide, a powerful cytoplasmic protein synthesis inhibitor, (Linnane et al., 1972), although it immediately halted growth when added to the medium, had no effect either in vivo or in vitro on mitochondrial amino acid incorporation or the amount of mitochondrial protein. Frankel (1970) reported spontaneous recovery of T. pyriformis from 0.2 mg/l cycloheximide; however, no recovery was observed at the 6 mg/l concentration which was used in this experiment. Streptomycin, erythromycin, and neomycin demonstrated no inhibitory effect on cell growth in this study even though Conklin and Chou (1971) reported that similar concentrations of streptomycin and erythromycin produced 34% and 10% inhibition, respectively, of amino acid incorporation into protein in a cell-free cytoplasmic fraction of T. pyriformis. This suggests that the intact organism may be able to actively exclude these antibiotics (Heyer and Frankel, 1971).
Mitochondria of protozoa are ultrastructurally different from many organisms in that they possess tubular cristae, and *Trypanosoma pyriformis* mitochondria have a larger DNA chromosome than the mitochondria of many organisms. Nevertheless, the results of these inhibition and deficiency studies, taken together, leave little doubt that in terms of fundamental biosynthetic pathways, *Trypanosoma pyriformis* mitochondria are closely related to mitochondria of other eukaryotic organisms, and also to prokaryotes.

Mitochondria undoubtedly do not have the genetic capacity to synthesize all of their own proteins (e.g., Storrie and Attardi, 1973; Sager, 1972; Baxter, 1971), but the EM picture implies that the proteins synthesized by the mitochondrion perform key structural roles (as well as possible enzymatic roles). Comparison of the chemical analyses of isolated mitochondria from normal and folic acid deficient cells may permit identification of some of the mitochondrial gene products. The contrast between *Trypanosoma pyriformis* cytoplasm and mitochondria in terms of the role of folic acid and the contrast in inhibitory effects produced by chloramphenicol and cycloheximide provide further substantiation for distinct protein synthetic mechanisms in the two systems. This distinction plus the similarity of the mitochondrial mechanism with that of prokaryotes, provide additional support for the endosymbiont hypothesis of eukaryotic cell evolution.

D. Isolation of Pellicles and Pellicular Components

1. Comparison of procedures for isolation of pellicular components

The procedures developed in this study for isolation of pellicular components, particularly kinetosomes, are significant because they provide
considerably purer preparations than are possible with either of the other
two methods reported in the literature: isolation of the oral apparatus,
or alcohol fixation of the cells followed by digitonin solubilization.
The oral apparatus is easily isolated from other cellular components
(Wolfe, 1970; Whitson et al., 1966; Williams and Zeuthen, 1966), and it
provides an abundant source of kinetosomes (approximately 160); however,
all efforts to date to separate the kinetosomes from the numerous
fibrils and microtubules interconnecting the oral apparatus have failed
(J. Wolfe, 1972).

Attempts to isolate the kinetosomes from pellicles using the
procedure of Child and Mazia (1956) have also failed to provide
sufficient purity for chemical analysis. The procedure is based upon
fixation of *T. pyriformis* in a 10-30% ethanol solution at -20°C in order
to confer stability to the pellicle during disruption of the cells.
The fixed, disrupted cells were returned to room temperature, and a
solution of 1% digitonin in a relatively low ionic strength buffer
(150 mM sucrose, 15 mM Tris, 2.5 mM EDTA, and 30 mM KCl) was added to
solubilize cellular components except the pellicles. The pellicles were
collected by centrifugation and disrupted by grinding or by sonication
to free the kinetosomes and other components.

Rubin and Cunningham (1973) attempted to improve the purity of the
kinetosomal preparation by careful control of the fixation conditions,
and by extremely vigorous sonication of the pellicles in order to more
completely free the kinetosomes from other components. They stated
that "solubility of the membranous organelles in digitonin after this
mild fixation is rapid and complete" and "basal body structure is maintained if the cells are disrupted without ethanol fixation, but separation of the basal bodies from DNA, membranes, and cell debris is very difficult." Apparently separation was difficult even with fixation, and solubility of the membranes in digitonin was incomplete since the micrographs of the kinetosomal preparation showed considerable contamination from a variety of membranous structures. It must be concluded that ethanol fixation only partially counteracted the tendency for cellular structures to form a "sticky web" (Rubin and Cunningham, 1973) in the presence of low ionic strength buffer, and in fact, it may have stabilized other cellular components as well as the pellicle against the action of digitonin.

In contrast, in the present study deciliated cells were homogenized in the presence of a relatively high ionic strength buffer (0.2 M potassium phosphate, 0.1 M NaCl, and 3 mM EDTA). Nozawa and Thompson (1971) noted that the high ionic strength buffer was critical for maintaining intact pellicles. It should be emphasized that the high ionic strength buffer avoided the "sticky web" problem and permitted improved isolation of the pellicles from other cellular components, while better preserving the integrity of pellocular components such as kinetosomes. The clean pellicles could then be disrupted to obtain the pellocular components by either the STEEP + Triton X procedure of Witman et al. (1972a) or the low ionic strength procedure of Flavell and Jones (1970b).
2. Features and relationships of pellicles and pellicular components revealed by the isolation procedures

Large sections of *T. pyriformis* pellicle as well as individual pellicular components could be obtained by homogenization of the deciliated cells in the relatively high ionic strength buffer. The major features of the kinety were easily seen in the isolated pellicles. Many of the kinetodesmal fibers were still attached to the kinetosome, although a number were also found free in the preparation. The 33 nm spacing of the repeating unit was in close agreement with the 32 nm spacing for the negatively stained fibers reported by Munn (1970). This is in contrast to the 23 nm periodicity reported for thin-sectioned kinetodesmal fibers (Allen, 1967). Not enough is known to decide which is the correct view. The sheath material and axial core were still associated with the kinetosome at this stage of isolation. The cilia generally did not break off from the kinetosome at the terminal plate, but rather 120 nm above it. This agrees with the micrographs of Nilsson and Williams (1966) for isolated oral apparati of *T. pyriformis*. *T. pyriformis* cilia are known to be easily sheared from the pellicle, and Blum (1971) discusses the theoretical value to cells of a breaking point between the cilium and flagellum; but the reason the cilia break at the precise point where they do is unknown.

Comparison of the two methods for dissociating the pellicle indicates variations in solubility and stability among the pellicular components. The STEEP + Triton X procedure of Witman et al. (1972a), which employed low ionic strength buffer (15 mM Tris, 2.5 mM EDTA, 30 mM KCl, 11%
ethanol, and Triton X) solubilized membrane components and also possibly transverse and longitudinal microtubules (since they could not be observed in the grid preparations). Thus gentle hand homogenization was sufficient to disperse the pellicles.

Components resistant to the solubilization could be readily observed in the preparation. The kinetodesmal fibers were evidently slowly soluble in the STEEP + Triton X solution since various stages of dissociation into longitudinal fibers could be observed. The site of attachment of the fiber to the kinetosome was the most resistant region. The kinetosome, except for the axial core and sheath, was resistant. Indeed the cylindrical geometry of the kinetosome was well maintained. The loss of the axial core and sheath from the kinetosome is consistent with the proposal that the material may be a tubulin pool concentrated in the kinetosome for quick use and, therefore, not stabilized by bonding, as tubulin assembled in the microtubules would be. Some kinetosomes may have been solubilized, however, since a number of free terminal plates were found. It is not clear how the tangential or face view corresponds with the double-banded appearance of the plate in longitudinal section of the kinetosome. The terminal plates were displaced by the procedure from the circumciliary rings into which they fitted.

Whereas most of the epiplasm was dissociated by the STEEP + Triton X procedure, the circumciliary ring and its associated whorl of epiplasm evidently possessed stability which paralleled their resistance to pronase (Sec.V.A.3.c.).
The close proximity occasionally observed between two epiplasmic whorls suggests a parent-daughter relationship for the kinetosomes with which the whorls had been associated. Nothing is known about the sequence of events involved in modifying the pellicle for the insertion of a newly matured kinetosome, or about the coordination of those events with kinetosome morphogenesis.

The second procedure for dissociating the pellicles also used a low ionic strength buffer, but no ethanol. The instant dissociation of the pellicles and the generally greater ultrastructural damage observed in pellicular components suggests that the ethanol fixation provided significant preservation. Kinetosomes presented a splayed appearance instead of one of a coherent cylinder. The stability conferred by the alcohol on kinetosomes probably was temporary, since pictures of splayed kinetosomes are common in the work of others who used ethanol fixation followed by digitonin solubilization in low ionic strength buffer. The low ionic strength of the buffer appeared to be the major factor in dissociation of the kinetodesmal fiber, since there was little difference in appearance between this procedure and the STEEP + Triton X procedure. Portions of membrane with no structures attached could be seen in this procedure. The honeycomb appearance is similar to that described for other membranes (DePierre and Karnovsky, 1973).

The purity of the pellicular preparation obtained by homogenization of cells in high ionic strength buffer plus the observations concerning the differential solubilities of various pellicular and kinetosomal
components to STEEP + Triton X or the low ionic strength buffer provide a substantial basis for exploring the molecular composition of kinetosomes (see Sec.V.E.6.).

E. Re-examination of Kinetosomal Autonomy

1. Presence of nucleic acids in kinetosomes

Classically the major argument for kinetosomal autonomy related to the morphogenetic continuity between parent and daughter kinetosomes. In terms of the recent understanding of molecular genetics this continuity suggested the need for NA, and nearly all recent studies of the subject have concentrated on this facet. However, there have been no positive reports of the presence of NA in kinetosomes in the last four years, while the number of negative reports, including this dissertation, has steadily increased. It appears that the major reports concerning the presence of DNA may have been due in part to wishful thinking (see Fulton (1971) for a discussion of this possibility), and thus the evidence needs careful re-examination.

a. Light microscopic cytochemistry Knowledge concerning the presence and amount of NA in cell organelles under various conditions has long been an important concern of biologists. The direct visualization afforded by microscopy has been a popular means of localization of NA; consequently several light microscopic stains for demonstrating the presence of DNA and/or RNA have been developed. Two which appear to be most specific for DNA are the Feulgen stain and acridine orange stain (Pearse, 1968), and these stains have consequently been applied to kinetosomes in order to determine if DNA is present in these organelles.
Randall (1959) and Randall and Fitton-Jackson (1958) reported that the kinetosomes of *T. pyriformis* and *Stentor* respectively stained distinctly with the Feulgen reagent. However, Randall and Disbrey (1965) commented that the earlier results could not be corroborated. McDonald and Weijer (1966) reported that *Neurospora* kinetosomes showed a high density of Feulgen staining. The size of the Feulgen-positive bodies varied greatly, ranging up to 1.2 x 2.4 μm in diameter whereas kinetosomes are only 0.2 x 0.5 μm in size. Further, the identity of these bodies as kinetosomes was not confirmed by electron microscopy.

By far the most convincing reports to date concerning the existence of kinetosomal DNA have been those of Smith-Sonneborn and Plaut (1967) and Randall and Disbrey (1965). The evidence in those two papers was based in large part on acridine orange staining. The difficulties involved in interpreting their results and the negative results obtained in the research for this thesis and elsewhere have already been discussed (Sec.V.A.1.).

b. Autoradiography with ³H-thymidine  

The fluorescence results of Smith-Sonneborn and Plaut (1967) and Randall and Disbrey (1965) were corroborated, according to them, by the autoradiography results on ciliate pellicles. However, there are also difficulties in interpreting these results as evidence for kinetosomal DNA. First, their LM pictures do not show highly ordered rows of silver grains which can be specifically correlated with kineties. Second, Randall and Disbrey performed no enzyme extraction controls. Third, the best evidence for an association of label with kineties occurred after 4-5 fissions in
the presence of $^3$H-thymidine, when less than half of the label was DNase-
extractable (Smith-Sonneborn and Plaut, 1967). Paramecium is capable of
demethylating thymidine (Berech and Van Wagtendonk, 1962), and thus
a number of other compounds present in the pellicle could have been
labelled. Fourth, a curious selectivity to the labelling occurred
(Smith-Sonneborn and Plaut, 1969). Feeding the Paramecia exogenous
thymidine produced labelling in both the nucleus and the pellicles, but
feeding the Paramecia with bacteria prelabelled with thymidine produced
only nuclear labelling. Fulton (1971) suggests this phenomenon also may
have been due to exogenous thymidine labelling of compounds other than
or in addition to DNA.

Sukhanova and Nilova (1965) reported incorporation of thymidine
into kinetosomes of Opalina, but their autoradiographs are not convincing.
All other LM attempts to demonstrate the presence of kinetosomal DNA
(Younger et al., 1972; Dirksen and Crocker, 1966; Stone and Miller,
1965; Rampton, 1962) have been negative.Careful EM studies (Sonneborn,
1970; Pyne 1968) in which the resolution was adequate to visualize
kinetosomes, have been negative.

c. Electron microscopic observations Only Brinkley and
Stubblefield (1970) and Stubblefield and Brinkley (1967) have claimed
to have demonstrated the presence of NA in kinetosomes with electron
microscopy. Numerous faults exist with their interpretation. No
evidence was presented to indicate that the helix identified in the
lumen of the hampster kinetosomes was DNA. Indeed, the micrographs of
the helix are not convincing, the diameter of the helical filament varies
by a factor of three in the various micrographs and the pitch of the helix is different in each micrograph. Although the kinetosomal triplet feet were reported to be RNase-extractable, the single series of serial sections do not demonstrate the removal of all feet. Finally, the triplet feet were not RNase-extractable in *T. pyriformis* in the work performed in this dissertation (Sec.IV.B.2.b.(2)).

Outka (1971) was unable to detect any DNA in kinetosomes by means of indium staining of thin-sectioned *T. pyriformis*. Other EM stains which are generally used for demonstration of DNA were rejected because of lack of sensitivity, contrast, or specificity (see Sec.II.C.1. and V.A.). In large part these difficulties prompted the EM cytochemical procedures described in this dissertation. To recapitulate briefly, no change was visible in kinetosomes as a result of NA extraction from fixed cells; further, enzymatic extractions on thin-sectioned material showed that the major components of kinetosomes were proteins, although nucleo-proteins could not be excluded (Sec.V.A.3.b.).

**d. Biochemistry** The only positive report of kinetosomal NA obtained by chemical analysis of isolated kinetosomes was that of Seaman (1960). His work is very suspect because only LM examination of the isolated fraction was employed. All other reports have been negative (Sec.II.B.2.b.(4)). It can be argued that since the kinetosome is not membrane-bound, the NA could have been lost during the isolation procedures. However, no pellicular DNA with a buoyant density distinct from nuclear or mitochondrial DNA could be found (Hufnagel, 1969b).
e. Control of nucleic acid pools

Inhibition by actinomycin D of DNA replication did not prevent *Stentor* from regenerating membranellar bands (Younger et al., 1972). The work in this dissertation (Sec.V.B.) indicates that depletion of the thymidine monophosphate pool in *T. pyriformis* drastically affected the ultrastructure of known repositories of DNA, the nuclei and mitochondria, but it had no effect on the ultrastructure of kinetosomes. The reduction in the density of the kinetosomal axial core during uridine deficiency was probably related to a reduced level of protein synthesis rather than to a reduced amount of RNA.

2. Morphological and morphogenetic relationships

As indicated above, the classical argument for hereditary autonomy in kinetosomes is based on morphological relationships between parent and daughter kinetosomes. However, *de novo* formation of kinetosomes presents a problem to the proposal of autonomy. The fact that kinetosomes seem remarkably similar in function and structure for all eukaryotic organisms (Sec.II.A.1.) strongly suggests a common evolutionary and hereditary basis. Thus, the absence of kinetosomal continuity in numerous organisms (Sec.II.B.2.b. and Pickett-Heaps, 1971; 1969) argues against such continuity in other organisms necessarily being due to a hereditary mechanism located in the kinetosome itself.

Rather, the morphological relationship between parent and daughter organelles is probably an evolutionary refinement developed in organisms with a highly specialized ciliature. For organisms with a highly plastic cell membrane, such as ameboflagellates during the ameboid-to-flagellate transformation, the precise location of the newly formed kinetosome
with respect to the cell surface is probably less important. Likewise, for cells such as those in ciliated vertebrate epithelia, which are said to use an indirect intermediate like a deuterosome (Sorokin, 1968) or condensation form (Dirksen and Crocker, 1966) the initial formation of kinetosomes is quite random with respect to the cell surface, although eventually the arrangement of the kinetosomes does become well ordered.

In ciliates, however, utilization of cilia as locomotory and food-gathering organelles reaches its highest development. For these organisms, the placement of kinetosomes is undoubtedly more critical. Since the present status of the evidence indicates that no NA is present in kinetosomes, the geometry of the daughter kinetosome and the placement of the daughter relative to the parent cannot explain kinetosomal genetic autonomy or partial autonomy, but rather must be accounted for by other mechanisms. In T. pyriformis, at least, there are strands of material which maintain a 1-to-1 correspondence between similar parts of parent and daughter kinetosomes (Outka and Seydel, 1972). Thus the parent may contribute accumulated tubulin and other proteins to the daughter for use in a directed, self-assembly process.

It is evident from the ultrastructural studies of the isolated pellicular fraction that there is a significant portion of the morphogenetic story which has not even been described, i.e. the pellicular elaborations and modifications which coincide with the maturation of the prokinetosome and its positioning in the pellicle. A better understanding of kinetosomal function and development will be obtained only by a more complete knowledge of the composition of and interrelations between
the numerous components which are present in or associated with the kinetosome. The isolation procedures described in this dissertation, with the improved purity and the selective solubilization of certain components, may open the way to such a chemical analysis of pellicular components.
VI. SUMMARY

T. pyriformis provided a good model system in which to study the issue of autonomy in two different cytoplasmic organelles, kinetosomes, and mitochondria. Because of the numerous problems associated with other studies reporting the presence of kinetosomal nucleic acids (NA), including the limits of resolution imposed by the light microscope, and the inability to verify acridine orange fluorescence, an electron microscopic examination was initiated. Cytochemical extractions on fixed cells and on thin-sectioned material embedded in ERL and regulation of nucleic acid pools were the principal methods employed.

Colorimetric and ultraviolet spectrophotometric analyses of supernatants verified that significant amounts of NA could be removed by nucleases and by perchloric acid from Karnovsky-fixed cells. However, the ultrastructural changes were subtle, and no changes could be detected in kinetosomes. ERL-embedded materials were only minimally susceptible to the action of ribonuclease but highly susceptible to pronase. Most components of the kinetosome were digested, including the axial core, a possible repository of kinetosome or ciliary precursor protein. Reduction of the DNA pool had no ultrastructural effect on kinetosomes although it strongly affected nuclei and mitochondria. Reduction of RNA caused a concomitant reduction in protein synthesis, reflected in a reduced number of ribosomes, an altered cytoplasmic appearance, and a reduced axial core in some kinetosomes.

Lack of evidence for kinetosomal NA and indications that the parent kinetosome acted to direct the formation and self-assembly of the
daughter suggested that deeper understanding of kinetosome function could be achieved by further knowledge of the composition of kinetosome components. High ionic strength buffer, isolation of pellicles, and subsequent dissolution of the pellicles in two low ionic strength buffers provided considerable improvement in purity over previous isolation methods (necessary for chemical analyses) and revealed additional ultrastructural details and relationships.

Mitochondria, in contrast to kinetosomes, appear to possess a considerable degree of autonomy, including their own DNA, ribosomes, and transfer RNA's. Absence of folic acid produced severe ultrastructural defects in the mitochondria, which correlated with the reduced ability of these mitochondria to incorporate labelled lysine into mitochondrial protein. These facts suggest an additional similarity between mitochondria and prokaryotes, the requirement for folic acid in the initiation of protein synthesis, and further substantiate at least a degree of mitochondrial autonomy.
VII. APPENDIX: DEFINED MEDIUM

The defined medium described by Kidder et al. (1950) has been modified for the experiments described in this thesis. The medium was assembled in three parts. To avoid precipitation of components, each part was autoclaved separately and the parts were then combined aseptically to produce 1 liter of medium. Each part was adjusted to pH 6.8-6.9 prior to autoclaving, and the pH of the combined medium was always checked before use. Lipoic acid was obtained from Calbiochem Corporation, La Jolla, California. All other organic compounds were obtained from Nutritional Biochemical Company, Cleveland, Ohio.

<table>
<thead>
<tr>
<th>Part A - 500 ml</th>
<th>mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_2HPO_4$</td>
<td>1100</td>
</tr>
<tr>
<td>$KH_2PO_4$</td>
<td>900</td>
</tr>
<tr>
<td>$NaO_2CCH_3$</td>
<td>1000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Part B - 250 ml</th>
<th>mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Na_2EDTA^1$</td>
<td>150</td>
</tr>
<tr>
<td>$MgSO_4 \cdot 7H_2O$</td>
<td>100</td>
</tr>
<tr>
<td>$CaCl_2$</td>
<td>50</td>
</tr>
<tr>
<td>$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$</td>
<td>25</td>
</tr>
<tr>
<td>$CuCl_2$</td>
<td>5</td>
</tr>
<tr>
<td>$FeCl_3 \cdot 6H_2O$</td>
<td>1.25</td>
</tr>
<tr>
<td>$MnCl_2 \cdot 4H_2O$</td>
<td>0.50</td>
</tr>
<tr>
<td>$ZnCl_2$</td>
<td>0.50</td>
</tr>
</tbody>
</table>

^1 Ethylenediaminetetraäcetic acid
<table>
<thead>
<tr>
<th>Part C - 250 ml</th>
<th>mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>D,L-Alanine</td>
<td>110</td>
</tr>
<tr>
<td>L-Arginine HCl</td>
<td>206</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>122</td>
</tr>
<tr>
<td>Glycine</td>
<td>10</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>233</td>
</tr>
<tr>
<td>L-Histidine HCl</td>
<td>87</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>138</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>344</td>
</tr>
<tr>
<td>L-Lysine HCl</td>
<td>272</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>124</td>
</tr>
<tr>
<td>D,L-Phenylalanine</td>
<td>320</td>
</tr>
<tr>
<td>L-Proline</td>
<td>250</td>
</tr>
<tr>
<td>D,L-Serine</td>
<td>394</td>
</tr>
<tr>
<td>D,L-Threonine</td>
<td>376</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>72</td>
</tr>
<tr>
<td>D,L-Valine</td>
<td>162</td>
</tr>
<tr>
<td>Adenylate</td>
<td>25</td>
</tr>
<tr>
<td>Guanylate</td>
<td>25</td>
</tr>
<tr>
<td>Thymidine&lt;sup&gt;1&lt;/sup&gt;</td>
<td>25</td>
</tr>
<tr>
<td>Uridine&lt;sup&gt;1&lt;/sup&gt;</td>
<td>25</td>
</tr>
</tbody>
</table>

<sup>1</sup>These components were prepared as stocks at 100x the concentration of the complete medium. The stocks were autoclaved separately, and were added to the complete medium according to the requirements of the specific experiment.
Part C cont.

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine HCl</td>
<td>1.0</td>
</tr>
<tr>
<td>Pyridoxal HCl</td>
<td>1.0</td>
</tr>
<tr>
<td>Ca pantothenate</td>
<td>0.1</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>0.1</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.1</td>
</tr>
<tr>
<td>Lipoic acid</td>
<td>0.001</td>
</tr>
<tr>
<td>Folic acid(^1)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

All vitamins except folic acid were prepared as a stock solution which was 250\(\times\) the concentration of the complete medium. The vitamin stock was stored frozen. Lipoic acid is nearly insoluble in water; hence it was dissolved in 95\% ethanol at a concentration of 40 mg/l and 1 ml of this solution was added per 160 ml of vitamin stock. The alcoholic lipoate solution was stored at -20\(^{\circ}\)C.

\(^{1}\)These components were prepared as stocks at 100\(\times\) the concentration of the complete medium. The stocks were autoclaved separately, and were added to the complete medium according to the requirements of the specific experiment.


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