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Osmium tetroxide vapor fixation of dividing and regenerating Blepharisma

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OSMIUM TETROXIDE VAPOR FIXATION
OF DIVIDING AND REGENERATING
BLEPHARISMA.

Iowa State University of Science and
Technology, Ph.D., 1964
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OSMIUM TETROXIDE VAPOR FIXATION OF DIVIDING
AND REGENERATING BLEPHARISMA
by
Robert Allan Jenkins
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Dean of Graduate College
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INTRODUCTION

A principal organizational activity of the cell is the formation of organelles. This process, for which the term "organellogenesis" might be invoked, whether duplicative or de novo in nature, represents the means by which products of cellular syntheses are ordered and assembled to form precise macromolecular complexes that integrate to complete the intricate, physical chemical interactions demanded of a living system. These developmental processes at the macromolecular level are fundamental to differentiation and morphogenesis. Cellular processes preliminary to the genesis of organelles, energy utilization, nucleic acid function, and biosynthesis, are reasonably well defined biochemically; however, almost no information is available which relates to the assembly of organelles and organelle complexes. At the present time, in vitro systems sufficient to simulate conditions existing in the living cell at the time of organellogenesis are not possible, so that the cell represents the only satisfactory model system. It is at this level of investigation that the electron microscope can be effectively utilized.

The choice of an appropriate system for an electron microscopic study of this dynamic process (organellogenesis) will be influenced by at least five considerations: (1) the organelle should have a characteristic and easily discernable microstructure in order to facilitate the recognition of developmental stages; (2) the organelle will be more easily located in thin sections if a particular intracellular location can be expected; (3) ideally, the system (cell) should be independent of alien or homologous contact with other cells so that modification by association does not
occur and so that environmental stimuli may be imposed with greater exactness; (4) the system becomes especially useful if means are available for evoking cellular responses which lead to the formation of a particular class of organelles; (5) because of the inherent difficulty encountered in the complete survey of even a single cell, the organelle would preferably be present in considerable numbers.

The tubular, filamentous complexes found in ciliate protozoa closely approach the ideal suggested by these considerations with the kinetosome and associated cilium representing optimum subjects. During division and regeneration in ciliates, there occurs a very intense and precise ordering of materials into complex, filament systems such as the mitotic apparatus, kinetosomes, cilia, kinetodesmal fibrils and rootlet fibrils. The brief time period through which these systems are formed can be approximated either by the selection of early division stages or even more efficiently if the oral apparatus is removed by merotomy and the regenerative process is closely followed.

The research reported in this paper was undertaken in an effort to acquire new information concerning phenomena essential to the formation and function of certain filamentous structures of a ciliate protozoan. Of particular salience are the filaments of the micronuclear and macronuclear division and the stomatogenic processes. The nature of these continuous filament systems will be described and aspects of their formation, function, and preservation for electron microscopy will be discussed.

The heterotrich ciliate Blepharisma was selected for this investigation because of its optimum size, well-developed oral ciliature, amenability to merotomy, pronounced regenerative capacity, and nuclear
morphology and because many physiological aspects of the regenerative process are known.

Fixation techniques featuring minor variations in the nature of the vehicle have been employed in almost every study of protozoa with the electron microscope; they usually represent, however, only token modifications of the basic Palade fixative (Palade, 1952). Since major difficulties were encountered in achieving satisfactory fine structure preservation by usual methods in this investigation, an unconventional method, osmium tetroxide vapor fixation, has been employed. Osmium tetroxide vapor was first used by Schultz (Baker, 1950) in the year 1865 for the fixation of Noctiluca, a marine protozoan, and has been utilized since that time primarily for the study of protozoa and the Golgi complex by light microscopy. Osmium tetroxide vapor has been used exclusively in this investigation for the fixation of dividing Blepharisma, giant amebae and paramecia, and for regenerating Blepharisma. Electron micrographs evaluating this technique will be presented and conditions essential to the tissue preservation effected by this method are discussed.
MATERIALS AND METHODS

Experimental Organisms and Culture

*Blepharisma undulans americanus* Suzuki was obtained from Dr. William Balamuth, Department of Zoology, University of California, Berkeley. A second strain, similar to *B. undulans americanus*, was obtained from General Biological Supply, Chicago, Illinois and will be referred to as *Blepharisma* sp. Turtox strain. Stock cultures were maintained in 0.1% hay-lettuce infusion buffered with 5 mM phosphate at pH 6.8-7.0 (Giese, 1945) and inoculated with *Bacillus subtilis* at least 24 hours before the introduction of *Blepharisma*. Mass cultivation was in 10-cm culture dishes containing approximately 30 ml of fluid. All regeneration experiments utilized animals from single clones grown in 6-8 ml of infusion in square watch glasses. Both types of culture were maintained at 22-23°C and transfers were made weekly. Careful selection was made against cultures displaying a lack of dividing forms, decreased viability, or the slender morphology typical of starvation.

Organisms entering division show increased angularity just posterior to the hypostome due to the formation of the new contractile vacuole of the proter or presumptive anterior daughter; in addition the posterior half of the cell begins to show a pronounced elongation. At this time the macronucleus is approaching the compact stage, and the micronuclei are undergoing mitosis (Suzuki, 1954). Cultures examined one or two days after transfer contain many such dividing organisms, and reliable selection is possible under intermediate magnification (30-50X) of a dissecting microscope. In order to follow changes in macronuclear morphology,
dividing cells were fixed with osmium tetroxide vapor for 15-20 seconds, stained by a modification of the methyl green-pyronin procedure of Jordan and Baker (1955), and observed with the phase-contrast microscope. Early dividers of both strains were selected and prepared for electron microscopy.

The original culture of *Paramecium multimicronucleatum* Powers and Mitchell was obtained three years ago from Carolina Biological Supply Company, Elon College, North Carolina. Organisms showing the earliest indication of the division furrow were selected from newly established hay-infusion cultures and prepared for electron microscopy.

The giant ameba, *Pelomyxa carolinensis* Wilson, used in this study was obtained from Dr. E. W. Daniels, Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois. Cultures were maintained in distilled water in 10-cm finger bowls at 22-23°C. The organisms were fed with *Paramecium multimicronucleatum* and *Chilomonas paramecium* Ehrenberg; increased division was induced by heavy feeding 8-12 hours before organisms were to be selected. Organisms with the characteristic mulberry appearance indicative of nuclear division (Kudo, 1947) were washed once in demineralized distilled water and fixed by exposure to osmium tetroxide vapor.

**Transection and Regeneration of Blepharisma**

Single animals not showing division morphology were selected from one, week-old clone culture. Such cultures usually contained more than 300 organisms and, therefore, ample numbers for all experiments used. For each sample, 30 organisms were selected and transferred to square watch glasses containing sterile culture medium diluted with balanced salt
solution (Giese, 1945). Organisms were transected at a site just posterior to the hypostome; anterior fragments were discarded and the desired posterior fragments without mouth parts were collected with a fine micro-pipette and transferred to dilute culture medium. Transection was completed under a dissecting microscope (60-90X) by using a fine glass needle or eyelash attached to a glass rod (Moore, 1924); no quieting methods were employed. The cutting of a group was accomplished in the shortest time possible, usually less than eight minutes.

In order to establish the period of time required for the regeneration of the adoral zone of membranelles, posterior fragments of known age were placed on microscope slides in a small drop of methyl cellulose (Marsland, 1943) or prepared as hanging-drops and observed at frequent intervals under the phase-contrast microscope.

The work of Giese and McCaw (1963a) suggested that posterior fragments might be held at 10°C without significant regeneration so that larger numbers of regenerators of essentially the same age would be accumulated. A water bath at 10°C was utilized, and organisms were introduced immediately after cutting to preconditioned solutions. Organisms were examined under the phase-contrast microscope at frequent intervals in order to ascertain their rate of regeneration as measured by the appearance of the oral primordia. Since the origin of new basal bodies might occur at any stage of regeneration previous to their production of oral cilia, organisms were observed over the entire regeneration interval.

From a single clone, 12 groups of approximately 30 organisms each were placed in dilute culture fluid in square watch glasses. Transection was completed as previously described with 15-minute intervals separating
the beginning of each operation. The posterior portions from each group, usually about 20, were placed in one depression of a spot plate containing dilute culture fluid. The spot plate was then placed in a moist chamber or covered with a glass plate to minimize evaporation through the post-transection period of regeneration. At post-transection times from 20 minutes through 3 hours and 40 minutes, at 20 minute intervals, all organisms of eleven groups were fixed for electron microscopy; regenerators showing the loss of general body form were excluded. The remaining group was allowed to complete regeneration and served as the cut-control for the experiment. Another group of organisms from the same clone, serving as the uncut-control, was passed through the same fluids and subsequently fixed for electron microscopy.

In a second experiment only three groups were transected, and handled as described. Intervals of 15 minutes separated the cutting of the last organism in each operation. Each group was prepared in a hanging-drop over the depression of a microconcavity microscope slide. The first group served as the "decoy" or "leader" and was observed at frequent intervals under the phase contrast microscope; such exposures to strong illumination were brief since intense visible light is known to affect morphogenetic events (Hirshfield and Giese, 1953). When early primordia of the adoral zone of membranelles were visible in 30-50% of the regenerators of this preparation, the two remaining experimental groups were immediately fixed for electron microscopy.
Specimen Preparation and Electron Microscopy

In the preliminary stages of this study it became apparent that *Blepharisma* would be difficult to prepare for electron microscopy. Fixation with standard procedures (Palade, 1952; Caulfield, 1957; Millonig, 1962 and Roth et al., 1963) gave unsatisfactory results. The inclusion of CaCl₂ (0.02 to 0.002 M), varied amounts of sucrose (0.04 g/ml to 0.16 g/ml), and adjustment of buffer concentration over a wide range resulted in alteration in the quality of fixation, but in no case was the preservation optimal. Perhaps some proper combination of conditions exists which would give good fixation but was not found. Osmium tetroxide vapor was useful in the light microscope observations of this study and has been used especially by protozoologists for nearly a century to fix cells for the light microscope (Pease, 1961). Therefore, its application to electron microscopy seemed appropriate even though previous studies of sectioned cells had not utilized such a method. Form, dimension, and fine structure were so well preserved that osmium tetroxide vapor was utilized to fix all organisms examined in this study with the electron microscope.

Protozoa were micropipetted in approximately 0.025 ml of culture fluid and placed at the center of a clean No. 2, 22mm coverslip. The coverslip was then quickly inverted in order to prevent spreading of the drop and placed over the mouth of an 9-ml specimen bottle containing 3-4 ml of 2% osmium tetroxide. In this position, the coverslip acted to close the container, and the hanging-drop was about 35 mm from the solution surface. Fixation times were one to four minutes for *Blepharisma* (three minutes
became standard), three minutes for *Paramecium*, one and one-half to five minutes for amebae, always at 25°C; much longer or shorter times have not been investigated.

The coverslip was removed at the end of exposure and eased gently into 50% ethanol in a square watch glass to begin dehydration of the specimen. Dehydration was accomplished by passing the organisms through a second change of 50% ethanol (a total of 10 minutes, in 50%), one change of 75% ethanol for 15 minutes, one change of 95% ethanol for 10 minutes, and two changes of absolute ethanol for 10 and 20 minutes each.

Infiltration with methacrylate was then performed by passing the organisms through two monomer changes of 10 and 30 minutes. The methacrylate embedding mixture consisted of 57% n-butyl methacrylate, 43% ethyl methacrylate, 1.5% v/v divinyl benzene (Watson, 1961) and 1% w/v benzoyl peroxide; the mixture was dried before use by filtering through anhydrous powdered sodium sulphate. Individual organisms were placed in separate, size #2, gelatin capsules; filled capsules were agitated to help locate the single organism at the bottom and after one hour were placed in an oven for polymerization at 60°C for 12 hours.

Methacrylate blocks were examined under the dissecting microscope to select organisms in the preferred orientation or division stage and trimmed with a razor blade to give a block face 0.25 to 0.5 mm². Free hand sections of ameba were cut, mounted in immersion oil, and examined by phase microscopy to allow selection of particular division stages. Because of the small size of the regenerating *Blepharisma* and of the
organelles being investigated and because any or all parts of the cell cortex might be the site of significant development, no part of the regenerating organism was expended. The use of thick sections including the whole organism showed good detail under the phase microscope, but no efficient method was found to mount the specimen for thin sectioning.

Thin sections were cut on an LKB Ultrotome equipped with freshly broken glass knives or a Dupont diamond knife. Section thicknesses were in the grey-silver range, 35-60 μ, with slightly thicker sections occasionally cut for survey purposes. In the case of regenerators, 2-μ sections were cut at levels through the organism, placed on glass slides, and examined by phase microscopy. Adjacent sections were used where possible; however, the major effort was in the maintenance of serial order through the organism. Sections were stretched by brief exposure to chloroform vapor and mounted on 200-mesh parlodion-filmed copper grids.

Sections were stained at room temperature by one of the following procedures: 1% potassium permanganate for 20-40 minutes (Lawn, 1960), 0.75% calcium permanganate for 10-20 minutes, 2% uranyl acetate for 40 minutes (Watson, 1958), or lead citrate for 12 minutes (Reynolds, 1963).

All sections were examined with an RCA EMU-3F electron microscope operated at 100 KV; a 150-μ condenser aperture and a 35-μ objective aperture were employed. Objective-lens astigmatism was compensated at the beginning of each period of use. Micrographs were taken on medium contrast Kodak Projector Slide plates at magnifications ranging from 2,400 to 20,000 and printed on Kodak Kodabromide F2 through F4 papers.
Determination of Osmium Tetroxide Concentration in Hanging-drop Preparations

The method of Frigerio and Nebel (1962) was adapted for the determination of osmium tetroxide concentrations in the droplets at the end of fixation exposures. Known concentrations of osmium tetroxide were prepared, and 1 ml of each was diluted to 100 ml with distilled water. To 1 ml of this solution, the following reagents were added, in order: 2 ml distilled water, 1 ml of 1 M HCl, and 1 ml of 1 M KI. The optical density of each was determined at 649 μm within one hour of preparation. The data obtained were plotted against concentration and gave a straight line indicating adherence to the Beer-Lambert Law; this plot represented the calibration curve for the determination of unknown concentrations.

Hanging-drop samples were prepared by placing 0.025 ml distilled water on a microscope coverslip and immediately inverting it over 2% osmium tetroxide; all conditions approximated as closely as possible those used in fixation. Each droplet was exposed for a definite period ranging from one to six minutes, after which the preparation was dropped immediately into a small glass-stoppered weighing bottle. Four samples were collected for each determination, and the total (0.1 ml) was diluted to 10 ml with distilled water; a 1-ml aliquot of this solution was handled as described for known solution concentrations. Hanging-drop concentrations were determined by plotting the measured optical density for each exposure on the calibration curve. The concentrations of the samples determined in this way were then plotted against time. All measurements of optical density were made with a Beckman DU Quartz Spectrophotometer.
at a wavelength of 649 μm with a slit width of 0.05 mm and 1 cm cuvettes.
LITERATURE REVIEW

Osmium Tetroxide Fixation

The present study is concerned, in part, with the utilization of osmium tetroxide fixation by a procedure not usually employed; the significance is better analyzed if pertinent information relating to osmium tetroxide fixation is reviewed.

The higher resolution of the electron microscope impressed early workers with the importance of delicate tissue preservation. It was recognized that fixed material must be devoid of extreme changes in volume and texture. Because of the harsh conditions imposed on the sample during examination in the electron microscope (desiccation and electron bombardment), protoplasmic structures must be rendered as stable as possible. Aside from these considerations, the criteria used in selection of fixative agent were primarily empirical.

Work of Porter, Claude, and Fullam (1945), Claude and Fullam (1946), and Gessler and Fullam (1946) demonstrated that osmium tetroxide gave the most adequate preservation of tissue form and fine structure. Cytologists had long used the vapors of osmium tetroxide to fix delicate protozoa and tissue culture cells (Pease, 1961), and since the tissue culture cell was one of the first objects of study, the use of osmium tetroxide fixation followed quite logically; in this application Porter, Claude, and Fullam (1945) and Porter and Kallman (1953) observed that there was no appreciable shrinkage or change in cell outline and that the electron microscopic image was consistent with light microscopic observations of the living cells. Gessler and Fullam (1946) and Claude and
Fullam (1946) used solutions of osmium tetroxide and found them superior to other fixatives for tissues prepared for thin sectioning; less distortion in the tissue fine structure was observed after dehydration, embedding, and sectioning techniques.

Dalton, Kahler, Striebich, and Lloyd (1950) examined the effects of various fixative solutions (Zenker-acetic, 10% formalin, Zenker Formol, Champy, Flemming, Carnoy, and 2% osmium tetroxide) on the hepatic cell of the mouse. These workers concluded that osmium tetroxide gave the best preservation of the solutions tested and that further improvement might be made by the addition of bichromate and lanthanum sulfate to the osmium tetroxide solution; the use of bichromate is still practiced.

The first and one of the most significant experiments dealing with varied conditions of osmium tetroxide fixation was conducted by Palade (1952). By using tissues which had taken up neutral red *in vivo*, he was able to demonstrate that during fixation a decrease in pH preceded the advancing fixation front. Fine structure damage during fixation was attributed to this wave of acidification that moved ahead of the osmium tetroxide penetration. To secure a constant pH, a 1 or 2% solution of osmium tetroxide buffered with the acetate-Veronal buffer of Michaelis at a pH of 7.4 was utilized for fixation. The results obtained were judged by comparison with tissue culture monolayers fixed in osmium tetroxide vapor, which Palade considered to be the best cytological fixation obtainable at that time, and found to be of equal quality. However, many of the differences noted by Palade in this study now have been attributed to various degrees of post-mortem change, and the significance of buffering is questioned (Sjöstrand, 1956). Whatever the cause for
improvement, the Palade formula gave reasonably consistent fixation of most tissues and, with concomitant improvements in embedding and thin sectioning, marked the beginning of significant contributions by electron microscopists to the study of the cell.

In an early effort to better define the action of osmium tetroxide, Porter and Kallman (1953) subjected films of globulin and albumin to osmium tetroxide vapor and a number of other cytological fixatives. The samples were examined under the electron microscope, and it was demonstrated that only osmium tetroxide gave fine homogeneous films. In a second experiment, the reaction of osmium tetroxide with solutions of albumin and fibrinogen was investigated; an initial gelation was followed after long exposure by decomposition to a liquid state.

Osmium tetroxide is a powerful oxidizing agent and is extremely volatile; it melts at 41°C to give a pale yellow liquid which boils at 131°C. It is a nonelectrolyte, is neutral to litmus, and is highly soluble in saturated fats and carbon tetrachloride. The chemical reactions of osmium tetroxide with more than 280 biologically significant substances were investigated by Bahr (1954). In vitro reactions with the sulfhydryls of proteins, unsaturated lipids and sterols, amino groups in terminal positions and not salt linked, sulphide sulfur, and certain hydroxyls and carbonyls were demonstrated. The more important materials showing no reaction were carboxyls, paraffin chains, peptide bonds, salt linkages, most carbohydrates, and nucleic acids. Wolman (1955, 1957) applied techniques of cytochemistry to an analysis of osmium tetroxide fixation and demonstrated its blocking or destruction of basic amino groups; his conclusions were in general agreement with those of Bahr. A recent study
(Wigglesworth, 1964) reaffirms the reduction of osmium tetroxide by unsaturated lipids, demonstrates a 30% reduction in osmium uptake if free amino groups are blocked, and shows a 40% decrease in osmium tetroxide staining following deamination with nitrous acid.

These experiments base evidence for reaction on visible darkening of the reaction mixture either in the test tube or as observed by light microscopy. Such criteria may be misleading, since there is evidence that a visible color-change is not a conclusive method of judging osmium tetroxide reactions (Porter and Kallman, 1953 and Wolman, 1957); reactions between osmium tetroxide and the chemical components of the cell will also depend on the constituents of the vehicle (buffer, neutral salts) and on the physical-chemical state of the reaction sites during fixation (Afzelius, 1962). It is therefore somewhat dangerous to extrapolate in vitro evidence to varied conditions or to in situ reactions and mechanics of fixation. For example, the non-reactivity of DNA with osmium tetroxide has recently been questioned; Schreil (1964) has demonstrated a gelation of DNA (in vitro) when osmium tetroxide is used at pH 6.0 and in the presence of 0.1% tryptone and 0.1% CaCl₂.

The fixation of unsaturated lipids in cell membranes may result from the formation of mono- or diesters by oxidation of double bonds between chains in close apposition (Wigglesworth, 1957 and Pearce, 1960). The lower oxides of osmium would act to unite chains and so render lipid components less soluble. Cell components known to contain much lipid, such as membranes, are usually well preserved by osmium tetroxide, and the contrast of membranes in electron micrographs is indicative of a concentration of heavy metal. To account for the fixation of complex cellular
proteins and carbohydrates becomes more difficult. It is almost certain that ethylenic double bonds are not the only points where links are formed; in addition alcoholic groups and especially 1,2-glycols, sulfhydryls, and amino groups are probably involved (Wolman, 1955). The failure to elucidate the mechanisms of osmium tetroxide fixation stands as an impressive commentary on the paucity of knowledge concerning chemical fixation and the nature of in situ cell substance (Gersh, 1959). We continue to rely, therefore, primarily on empirical methods for improvement in the quality of fixation.

It is generally accepted that buffered osmium tetroxide fails to adequately fix some types of cells or cellular components (Afzelius, 1962). As a result, modifications in the standard Palade formula have been made, the most significant by Caulfield (1957), Kellenberger et al. (1958), and by workers in Sjöstrand's laboratory (Sjöstrand, 1956). Other workers have questioned the use of a buffered vehicle for osmium tetroxide and propose the use of molten osmium tetroxide, 40% osmium tetroxide in carbon tetrachloride (Afzelius, 1962), or slightly acid 1% osmium tetroxide in distilled water (Malhotra, 1962a, 1962b; Claude, 1961). These authors suggest that there is no advantage in using buffered solutions. In fact Claude states that osmium tetroxide fixation carried out in buffered alkaline media favors the progressive removal of tissue components by extraction, slowly in fixation but more rapidly and to a greater extent in distilled water during the subsequent washing of the specimen. Sjöstrand (1956) argues that the pH is not of prime importance and that the buffer only acts to adjust the tonicity of the fixative. With Rangan (1960) and Tahmisian (1964), he believes the tonicity of the fixative to
be a most important factor.

Many workers have stressed the importance of short exposures of tissue to osmium tetroxide; the criterion is the suspected and sometimes demonstrated extraction of components after the necessary initial fixation has been completed (Porter and Kallman, 1953; Bahr, 1954; Wolman, 1955; Dallam, 1957; and Pease, 1961). More recently, Huxley and Zubay (1961) and Griffin (1963) have advocated brief osmium tetroxide fixation, the former in relation to selective staining of nucleic acids, the latter on the basis of gross changes observed throughout fixation.

The excellence of fixation achieved with osmium tetroxide vapor was attested to by Porter and Kallman (1953) and used by Palade (1952) as the standard of comparison. With the advent of sectioning, larger specimen samples were more often utilized and a buffered osmium tetroxide solution became the fixative of choice. However, osmium tetroxide vapor fixation may offer certain advantages. Its knowledgeable application certainly involves the considerations included in this review.

Biology of *Blepharisma undulans*

**General**

*Blepharisma undulans* has been the subject of numerous and varied investigations since the first description by Stein in 1867. *B. undulans americanus* (Suzuki, 1954) will be examined in detail where the literature allows; however, since some workers have not recognized differences within the species (Suzuki, 1954; Hirshfield, Chunosoff, and Bhandary, 1963), it is not always possible to identify the particular organism.

*B. undulans americanus* (Suzuki, 1954) has a body length of 80-300 μ;
bacteria-fed forms are consistently 150-225 μ in length. The body has a faint rose color due to the presence of pigment granules which lie just below the pellicle in 7-10 longitudinal rows; the rows are equally spaced and run postero-anterior between the kineties or rows of cilia which usually number about 30 and are located within grooves of the pellicular membrane. Well-nourished animals are pyriform or spindle-shaped while undernourished ones assume an elongated cylindrical appearance. The anterior half of the body is somewhat flattened laterally whereas the posterior half is more rounded. The peristome is a narrow groove usually limited to the anterior third or half of the body; an undulating membrane borders the posterior half of the right peristomal edge. The membranelles, composed of from one to three transverse rows of cilia fused into an elongate triangular structure, are about 80 in number and are arranged in an antero-posterior row along the left peristomal margin to form the adoral band which extends continuously to the cytostome. A single, contractile vacuole is located at the posterior tip and empties through the oblong cytopyge.

The vegetative macronucleus consists of 3-8 ovoid or spherical nodes of varied size which are interconnected by slender strands. The micronuclei, usually 2-20 in number, are small, spherical bodies measuring about 1 μ in diameter and lying close to the macronuclear membrane but never enclosed within it. Both types of nuclei are stained intensely by the Feulgen reaction.

Stolte (1924) described the general morphology of *B. undulans* Stein and recognized the importance of culture conditions in the physiological variability of the organism. He was able to predict the occurrence of
conjugation and encystment from observations of cytoplasmic vacuolization and reduced division frequency. Additional investigations of morphological features have been conducted by Moore (1934) with special emphasis on the formation and transient structure of the contractile vacuole and by Chambers and Dawson (1925) on the undulating membrane. The latter authors were able to tease the membrane into short lengths only to observe an almost immediate reformation to the membrane-like unit.

The pigment granules of Blepharisma have attracted the attention of several investigators. Weisz (1950) utilized cytochemical techniques in an effort to elucidate their chemical composition and physiological significance. The granules demonstrated a strong basophilia that was not lost when the pigment was removed by alcohol or ether, also treatment with RNase or HCl did not destroy the basophilia. Sudan black B and Janus green B stain the granules intensely. He concluded that phospholipid and mitochondrion-like substances were present.

Aware of the ability of Blepharisma to exist for 1-3 days in complete anaerobiosis, Beadle and Nilsson (1959) examined pigmented and nonpigmented organisms in the absence of oxygen, found no difference in vitality, and concluded that the pigment was not required in anaerobic metabolism. The pigment can be extruded by the organism without injury; up to 30% of the total will be lost by organisms placed in 0.9% NaCl (Prabhakara Rao, 1963). Calcium chloride inhibits extrusion induced by other salts (magnesium chloride shows no inhibition) while the addition of EDTA results in a marked increase in extrusion.
Division and stomatogenesis

The behavior of the macronucleus is the most readily observed feature of division and has been described for several strains by a number of workers (Young, 1939; Suzuki, 1954, 1957; McLoughlin, 1957; Bhandary, 1959; Helson, Pecora and Hirshfield, 1959; Seshachar and Bhandary, 1962). The essential features are very similar although variations occur from strain to strain; the most significant variation is whether dissolution of central lobes occurs during the condensation of the macronucleus (Young, 1939; McLoughlin, 1957).

The onset of division in *B. undulans americanus* as described by Suzuki (1954) first is recognized by a posterior expansion and by the appearance of a new adoral zone for the presumptive posterior daughter. Only then do the macronuclear nodes begin to condense and eventually fuse into a compact central mass. As the division progresses, the condensed macronucleus elongates to assume the form of a rod that may be as long as 150 μ. The macronucleus begins to show a constriction at the mid-level exactly corresponding with the site of the division furrow, and the terminal ends become somewhat enlarged until only a slender strand connects the two presumptive daughter macronuclei; cytotomy is completed as the two daughter macronuclei are separated. Nodulation then occurs in each which results in two new vegetative moniliform macronuclei. The entire process is completed in 2-3 hours.

Little is known about the division of the micronuclei in this organism except that the mitosis is correlated with macronuclear changes. The nonsynchronous mitosis apparently begins as the macronucleus reaches the compact stage and is usually completed at the time of greatest macronu-
clear elongation (Bhandary, 1959; Suzuki, 1957). The micronuclear figures are concentrated but not uniformly aligned in the central area near the compact macronucleus. A telophase separation of daughter micronuclei up to 30 μ is not uncommon.

Stomatogenesis during cell division has been fully described by Suzuki (1957) and Eberhardt (1962). Just posterior to the peristome of the proter lies a ramifying zone (V-shaped area) where the kineties show bifurcation especially during early division. An increased number of kinetosomes gives rise to an anarchic field in this area, and each kinetosome sprouts a short cilium. (As these events are completed, the macronucleus begins condensation.) Just prior to the appearance of the new cilia, the kinetosomes migrate and give rise to transverse rows, with the result that the floor of the peristome is clearly established. The kinetosomes bearing short cilia which at first beat without coordination now become ordered as rudimentary membranelles, and the cilia increase in length and become morphologically complete. Following these events (the macronucleus has reached the compact stage), the old anterior peristome undergoes partial dedifferentiation; that is, the undulating membrane separates into its component cilia, and the adoral spiral shows a subtle detorsion. The new peristome of the opisthe begins to show a depression and spiralization, and the oral area of the proter is redifferentiated. Oral parts are presented diagramatically in Figure 3. (The latter events correspond to the elongation of the macronucleus). Finally, the division furrow forms, the oral area of each daughter is completed, and cell division occurs.
Oral regeneration

Regeneration of excised parts is a noted capacity of certain ciliate protozoa, particularly heterotrichs. Oral regeneration is induced by removal of that body portion bearing the feeding organelles. The resulting development of a new oral apparatus is nearly identical to the stomatogenesis in the presumptive posterior daughter during cell division, except that it occurs in the absence of a formed cytostome.

Moore (1924), Suzuki (1957), and Eberhardt (1962) have carried out extensive investigations of the morphogenetic phenomena associated with regenerating Blepharisma and are in general agreement on a number of associated conditions and processes. The most significant are as follows: only the oblation of a portion of the peristome induces oral regeneration, dorsal fragments lacking stomatogenic kineties regenerate normally (all kineties are equipotential), any significant portion of the macronucleus is capable of supporting regeneration and some macronuclear material is absolutely essential, the complete absence of micronuclei appears to be of no immediate consequence, and both nuclear types undergo changes typical of cell division (macronuclei reorganize but do not divide) in the same time relationship as during the stomatogenesis in division.

Suzuki (1957), in the most comprehensive study to date, has also demonstrated an induction by the formed peristome during division and by the anal region during development of a new oral anlage in regeneration.

A number of investigations have been performed in an effort to discover basic physiological control processes which are active during regeneration. Hirshfield and Pecora (1955) treated posterior fragments
with colchicine and found that $10^{-4}$ M solutions greatly impeded the restoration of the peristome. They proposed that a disruption of the forming fibrillar system was caused by the colchicine; however, they did not eliminate the possibility of a membrane, macronuclear, or general cortical involvement.

Ultraviolet irradiation (ca. 260 mJ) retards regeneration and causes an apparent delay in macronuclear reorganization (Giese and Lusignan, 1961a). Irradiation is most effective if used immediately after cutting, whereas later exposures result in little retardation. In contrast, X-irradiation of regenerating organisms shows the most pronounced delay at about three hours after the cutting operation when the macronucleus is reaching the compact stage and the micronuclei are approaching division (Giese and Lusignan, 1961b).

The effect of temperature extremes on the regeneration rate has been studied by Giese and McCaw (1963a). Almost complete blockage of regeneration is reported at $10^\circ$C; the macronuclear cycle during regeneration is lengthened to 25 hours at $13^\circ$C as compared to 5 hours at $25^\circ$C, and if posterior fragments are allowed to regenerate for 2-3 hours and then are placed at $10^\circ$C, regeneration does not occur. The regenerative process is also delayed by high temperatures.

Giese and McCaw (1963b) treated regenerating blepharismas with a number of substances designed to inhibit various cell processes. They report that blockage of oxidative pathways by azide or cyanide had little effect on regeneration rate while inhibition of anaerobic metabolism by iodoacetic acid or urethane served as a pronounced impediment. The involvement of nucleic acid in the immediate processes of regeneration was
tested by treating regenerators with chloramphenicol and RNase; early
treatments (coincident with cutting) are reported to show very effective
inhibition the degree of which decreased when the substances were applied
after short regeneration times. A very pronounced inhibition was found
for $7 \times 10^{-2} \text{M}$ mercaptoethanol; treatment for 1-2 hours resulted in a
doubling of regeneration time.

The series of experiments just alluded to implicates three, funda-
mental cellular functions as being integral to regeneration: assembly of
organelles (filament formation), nucleic acid function, and anaerobic
metabolism. Colchicine, mercaptoethanol and cold treatment (see: Inoue',
1964) most probably act to disrupt or retard filament assembly essential
to regeneration (ionizing radiations might also act in this way). Nucleic
acid function, particularly the formation of messenger RNA for synthetic
direction and control, is undoubtedly affected by ionizing radiation, RNase,
chloramphenicol, and reduced temperature; these same factors may also act
to prevent processes essential to macronuclear reorganization. The
anaerobic metabolic capacity of Blepharisma has been described (Beadle
and Nilsson, 1959); therefore, it is not particularly surprising that
anaerobic poisons (urethane and iodoacetate) show pronounced deleterious
effects. The use of these chemical and physical inhibitors in restrictive
combinations or perhaps in carefully designed experiments involving isotopic
labeling will result in a more complete understanding of regeneration.

**Fine structure**

Blepharisma has not been extensively studied by electron microscopists.
Utsumi and Yoshizawa (1957) report 0.2 to 0.4 $\mu$-granules in the macro-
nucleus and describe the interphase micronucleus as having an obscure inner structure and morphological connection to the macronucleus. Food vacuoles were found to have many small, finger-like projections extending into their interior. Numerous oval, filamentous or spherical granules were observed in the endoplasm; the inner surface of the granule membrane showed very small microvilli-like projections. The pigment granules of colorless and pigmented strains were examined by Inaba, Nakamura, and Yamaguchi (1958). Colorless organisms possessed granules 0.2-0.4 μ in diameter while the granules of the pigmented strain were usually 0.5 μ in diameter, and both types of granules are said to be bounded by a distinct, dense membrane 40-60 μ in thickness.

The most recently reported electron microscope study is that of Seshachar (1964) on the vegetative nuclear apparatus of *B. intermedium*. Bodies with a diameter of 0.05 to 0.2 μ and larger bodies 0.4-0.6 μ in diameter are observed in the macronucleus. Certain of the large bodies contain very dense particles most of which are 10-80 μ in diameter and sometimes appear doughnut-shaped. The micronucleus is surrounded by a double membrane; the central contents are basket-like and very dense but contain many interspaces.

Comprehensive studies of the infraciliature are not presently available.

The fine structure, particularly of the infraciliature, of other heterotrichs has been studied in more detail; similarity is apparent in those examined (Finley et al., 1964). In *Stentor polymorphus*, Randall and Jackson (1958) have described dense macronuclear inclusions 60 μ in diameter and larger bodies with a diameter of from 1-5 μ; no bodies
identifiable as micronuclei were found. The cytoplasm is described as being highly vacuolated and generally hyaline. Distinctive heavy-walled vacuoles with closely associated 125-A fibrils are found at the posterior terminus of the buccal cavity. These workers identify three different fibrillar systems. The first consists of fibrillar bundles (km fibers) running parallel to the body length at about 0.3 μ below the cell surface; the component fibrils are about 20 μ in diameter and about 30 comprise a bundle. These authors propose that these bundles which show connection to kinetosomes are homologous to kinetodesma and may function in contraction. Lying deeper in the cytoplasm are the so-called M bands which are fibrous networks extending in an antero-posterior direction and are also implicated in contractile function. A fibrillar root system composed of 22-μ fibrils is connected with the kinetosomes of the membranelles and extends into the endoplasm for about 20 μ; the complex from each membranelle is fan-shaped and bifurcates to connect neighboring root complexes into a cohesive unit.

Yagi and Shigenaka (1963) and Finley, Brown, and Daniel (1964) have reported almost parallel observations on the ectoplasm of Spirostomum ambiguum. Structures apparently identical to the km fibers observed by Randall and Jackson are observed in Spirostomum; in addition, a fibrous structure resembling the M bands of Stentor is found at the transition region between the ectoplasm and endoplasm. A third fibrillar complex is composed of fibrils 20-26 μ in diameter and aligned directly beneath the pellicle in an anterior-posterior direction.

The observations of Roth (1957), Roth and Shigenaka (1964), Beams and Anderson (1961), and Pitelka (1961), dealing with protozoan fibrillar
systems are also of value in interpreting the present work. Reviews by Fawcett (1961), Sleigh (1962), Pitelka (1963), and Grimstone (1961) provide excellent sources for comparison of ciliate fibrillar systems and have been used extensively.

Ciliate Morphogenesis

The state of knowledge concerning ciliate morphogenesis has been given the following evaluation by A. V. Grimstone (1961).

"Even more daunting than the functional problems, however, are those of morphogenesis. What processes bring into being these organelles, with all their multiplicity of parts, their asymmetry, their linking together of specific components in constant ways? It is not unduly pessimistic to say that at present we are scarcely in a position even to formulate hypotheses on the nature of the processes involved."

The multiplicity of problems and implications in the study of morphogenesis in ciliate protozoa have been reviewed by Balamuth (1940), Summers (1941), Lwoff (1949, 1950), Weisz (1951), Trager (1963) and Sonneborn (1963). The comprehensive monograph of Tartar (1961) covers the information accumulated by himself and other workers on morphogenesis of the genus Stentor and serves in many ways as an excellent review of regenerative and morphogenetic processes. The phenomena associated with morphogenesis have been studied by a number of different approaches, some of which are briefly reviewed.

Regeneration

Much of the research utilizing merotomy, the cutting away of cell parts, has been carried out on the heterotrich protozoa. These organisms possess great regenerative ability, well defined and elaborate oral
ciliature, a macronucleus extending over much of the body length, and a size convenient for handling. The magnitude of the regeneration process is better appreciated by considering the nature of the events which take place in the restoration of the oral apparatus. For example in *Stentor*, Tartar (1961) has estimated that the formation of the membranelllar band requires the multiplication of approximately 15,000 kinetosomes and their precise alignment into rows, then the development of cilia and interconnecting rootlets and associated fibers. Finally this entire structure must coil and shift into position at the anterior end of the organism; the whole process is usually completed in four hours.

*Stentor* has been used extensively by Tartar, (the magnitude of this work is reviewed, Tartar, 1961) and by Weisz (1948a, 1948b, 1949, 1951, 1955) in a concerted effort to determine the controlling and modifying mechanisms of the morphogenetic processes active during oral regeneration. These authors, by means of a great variety of cutting experiments, grafts and other cortical rearrangements, and the application of inhibitory substances, have tested such a variety of conditions that the following conclusions can be drawn from their numerous observations: (1) the ectoplasm is shown to be virtually totipotent throughout in regard to oral differentiation; (2) while nucleated ectoplasm shows almost unimpeded regeneration, nucleated endoplasm alone is totally incapable of restorative processes; (3) a cortical pattern of polarized, anisotropic, lateral striping is always present no matter how the ectoplasm is fragmented; (4) the new oral anlage always arises at the junction of the wide and narrow stripes (always within the fine stripe zone); (5) excision of the head or any appreciable portion of the feeding organelles leads to
regeneration; (6) the nuclear involvement is identical to that already described for Blepharisma (during reorganization the macronucleus adjusts in size to the mass of the regenerating fragment); (7) regeneration imposes no need for exogenous nutrient; finally, (8) the process can be blocked by certain chemical agents or by imposed cortical rearrangements.

These findings have contributed much to the understanding of morphogenetic processes, but their consequence is primarily a delineation of the problem; explanations of the phenomena are not yet possible.

Morphogenesis has also been studied in Condylostoma spatiosum by Suhama (1957, 1961). In experiments involving regeneration of the oral area, he has demonstrated that incomplete removal of the oral ciliature or treatment with acriflavin or lithium chloride shows very marked inhibition of the developmental processes. Observations of kinetosomal production and cortical position effects are reported. The macronucleus is essential to the process and undergoes a reorganization; the micronuclei divide mitotically when the macronucleus reaches the compact stage. Yagiu and Nakata (1956) report that in the vegetative division of Condylostoma nearly all micronuclei undergo mitosis while only about 50% divide in regeneration.

Hanson (1962) has used ultraviolet microbeam irradiation to damage selected gullet areas of homopolar doublet Paramecia aurelia.¹ The use of doublets for the study of regeneration in paramecia is necessary because no regeneration takes place in this species in the absence of a

¹Homopolar doublets are animals possessing two gullets and other associated parts of the oral area, one of which can be destroyed without observable effect on either the organisms vitality or subsequent division frequency.
mouth (Tartar, 1961); in addition to the visible damage, ultraviolet irradiation affects the capacity of the organism to produce an oral apparatus on that meridian during division. In terms of inheritance over a number of fissions, complete loss of the one oral apparatus and the ability to produce one on that oral meridian as well as the return to a complete doublet morphology are found to occur.

**Morphogenesis associated with cystment processes**

The phenomena associated with excystment and encystment provide still another avenue for the study of ciliate morphogenesis. Hashimoto (1963) has observed a thickening of certain fibrillar structures which are present in the resting cystic stage and which are involved in the origin of primordia. Encystment-regeneration interaction has also been observed (Hashimoto, 1962); the posterior fragment always encysts more rapidly than does the anterior fragment derived from the same precystic animal. Formed adoral membranelles apparently tend to inhibit encystment processes in *Condylostoma* as they do in the initiation of oral primordia in regenerating *Stentor*.

**Morphogenesis as a division phenomenon**

The work of Frankel (1960a, 1960b, 1964) illustrates another approach to the study of ciliate morphogenesis. Frankel places the period of kinetosomal replication in synchronously dividing *Tetrahymena* and *Glaucome* just after the completion of a division and well before the beginning of stomatogenesis for the following division. The newly appearing kinetosomes are always close to the subequatorial portion of kinety one; their rowing and subsequent arrangement into membranelles follow, and the entire
process is completed in approximately 80 minutes (Frankel, 1960b). In conjunction with temperature synchrony, the use of high temperatures (33.5-35°C) is shown to block the formation of primordia completely while a somewhat lower temperature (33°C) results in the development of branching or extra membranelles. Cells with abnormal oral organelles divide and yield a posterior product with similar abnormal oral structures which are rejected and resorbed (Frankel, 1964).

Suzuki (1957), Tartar (1961), Eberhardt (1962) and others have studied stomatogenesis in division and have compared it with the process occurring during regeneration.

**Physiological regeneration**

At seemingly irregular intervals, certain heterotrich ciliates are found to form an oral primordium which serves neither for division nor regeneration but merely replaces oral structures already present. The process has been called reorganization or physiological regeneration; Weisz (1951) feels the latter term is more appropriate since the process differs little from regeneration. The factors inducing physiological regeneration are still unknown and, generally its occurrence is unpredictable. In *Blepharisma* (Suzuki, 1957) and *Stentor* (Tartar, 1961) a slightly smaller than normal, oral primordium forms; the original mouth parts de-differentiate and near-buccal portions are fully resorbed. Next, the anterior original area and new primordium unite to form a continuous oral area. Concomitant with these changes the macronucleus undergoes reorganization without division, and some micronuclei divide mitotically. Tartar (1961) regards physiological regeneration (reorganization) as a wholly
spontaneous and intrinsic response to certain disproportionalities or
disarrangements of parts of the cell which restores a more normal
relationship. The study of physiological regeneration is especially
interesting because of the subtle ways in which it differs from and yet
resembles both division and regeneration.

Ciliate morphogenesis, whether observed in regeneration, division,
cystment processes or reorganization and/or in a number of different
organisms, is apparently reliant upon the same basic mechanisms of
initiation, development, and maintenance. The cell cortex and associated
structures are essential in establishing and supporting the highly
differentiated oral ciliature; a capacity so pronounced that the role of
such preformed structures in ciliate heredity is now a problem of intense
investigation (Sonneborn, 1963). The cortex is not completely autonomous
or totipotent since in all developmental processes a stringent macronuclear
involvement is evident. The way in which nucleus and cortex interact to
form and maintain the precise surface pattern of ciliates is an intriguing
and cogent biological problem.

The Duplication of Kinetosomes: Electron Microscopy

A focal point in light microscope investigations of ciliate cortical
differentiation is the structure at the base of all cilia, the basal body
or kinetosome. At the present time both of these terms are in popular
usage and reasons for preference of one or the other are discussed by
Fawcett (1961) and Picken (1960). The present author has elected to use
the term "kinetosome" for the following reasons. First, no demonstrations
of absolute obligate structural association are known; the structure may
exist free of the cilium in vivo and in biochemical isolation procedures. Second, the term is more appropriately used especially in protozoology because of the wide usage of the associated terms "kinety" and "kinetodesma".

Lwoff (1950) has defined the kinetosome as a visible, cytoplasmic particle endowed with genetic continuity which always arises from the division of a pre-existing kinetosome. There are, however, some observations indicating that kinetosomes arise de novo from sub-organellar-level pools (Ehret and Powers, 1959). In this regard, the presence of nucleic acids in kinetosomes has been tested in a variety of ways: biochemical isolation (Seaman, 1960 and Hoffman, 1964); cytochemically (Randall and Jackson, 1958); electron microscopic techniques (Rampton, 1963; Swift, Adams, and Larsen, 1964). The general conclusion is that RNA is probably present while DNA is either absent or present in insignificant amounts. Even if evidence for the presence or absence of DNA in kinetosomes was conclusive, hypotheses for self-duplication are still possible (Hoffman, 1964), and the issue of genetic continuity would not be resolved.

Since the kinetosome is near the limit of resolution of the light microscope, it has always been impossible to identify its origin, movement and functional role. Precise descriptions of kinetosome fine structure were not possible until the development of the electron microscope; recent observations show that this organelle is a cylinder composed of nine triplet-filaments equally spaced from each other and from a central axis. The cylinder is usually 150-225 μm in diameter while the length is more variable, averaging 500 μm. The fine structure of kinetosomes is reviewed by Gibbons and Grimstone (1960), Fawcett (1961), and Sleigh
(1962). At the present time we do not know how this fibrous structure is reproduced or how it is involved in the formation of a cilium (Pitelka, 1963). In an effort to answer these questions, a number of workers have expended considerable effort.

Randall and Hopkins (1962) examined regenerating and dividing *Stentor* and embryonic chick trachea, Ehret and DeHaller (1963) extensively studied dividing *Paramecium*, and Sedar and Porter (1955), Roth (1960), Elliot (1962), and Roth and Shigenaka (1964) have examined different dividing ciliates; in no case was evidence found for the division of kinetosomes. Schuster (1963), in a study of the ameba-flagellate transformation of *Naegleria*, has observed structures that he suggests may be prekinetosomes developing in the apparent absence of formed kinetosomes.

The structural resemblance of kinetosomes and centrioles has prompted many workers to suggest that they may be interchangeable (Gatenby, 1961). The work of Sotelo and Trujillo-Cenoz' (1958), Tokuyasu and Yamada (1959), Sorokin (1962), Schuster (1963), and Berlin (1964) as well as earlier light microscope studies leaves little doubt that centrioles do give rise to ciliary structures. Such structural and functional similarity may imply a similar means of duplication.

Gall's careful study (1961) resulted in the identification of short centrioles which he termed "procentrioles" near to and at right angles to the mature centriole. In a later study, Gall (1963) observed clusters of small centrioles during the early stages of sperm development in the water fern *Marsilea*; however, no mature centriole was found to be associated with the cluster. Berlin (1964) reports an end-to-end association of short centrioles in *Albugo* and suggests that the centrioles of this organism
reproduce themselves by an end-to-end process.

Observations similar to those just alluded to have not been made for protozoa.

The Formation of Cilia: Electron Microscopy

The role of kinetosomes in the formation of cilia has not been clearly established. Rouiller and Faure'-Fremiet (1958), King, Beams and Tahmisian (1961), Randall and Hopkins (1962), and Roth and Shigenaka (1964) have reported the presence of dense granules within kinetosomes without shafts and also within those bearing newly formed shafts.

Limited observations of ciliary formation have been made by Sotelo and Trujillo-Genoz' (1958) for the neural epithelium of the chick embryo and by Tokuyasu and Yamada (1959) for developing retinal rods. These authors agree that no development occurs until the centrioles approach the plasma membrane where a protrusion containing numerous small vesicles is formed; details of fibrogenesis were not observed. Sorokin (1962) has made observations on the development of rudimentary cilia by fibroblasts and smooth muscle cells. The sprouting of the axoneme appears to take place before the centriole has migrated to the cell surface; however, a membranous vesicle is closely appressed to one end of the centriole during early development. The shaft seems to develop from a dense mass at the distal end of the centriole and appears in early stages as an assortment of small vesicles and short filaments.

In the dividing ciliate, Diplodinium, a particular tuft of cilia apparently lags in development during cell division and, therefore, these cilia are in the process of development at the time when most new cilia
are structurally complete. Roth and Shigenaka (1964) report that the cilia of this tuft are composed of about nine filaments with normal, nine-doublet morphology near the kinetosome but, at a slight distance beyond, the doublet morphology is not present, and the filaments are randomly arranged within a now somewhat flattened ciliary membrane. It is suggested that the tips of the peripheral filaments are formed first and in association with the kinetosomes; later development gives rise to the doublet nature and spatial arrangement.

If, as has been suggested by Faure-Fremiet (1961) and Hobbs (1964), the development of a cilium is completed within 10-20 minutes, it is not surprising that all the details of ciliary formation have not yielded to electron microscopic examination.
OBSERVATIONS AND RESULTS

The observations and results of this investigation are reported in four sections: "Fixation", "General Fine Structure", "Regeneration", and "Division". The degree of success achieved in this study is due, at least in part, to the utilization of osmium tetroxide vapor fixation; therefore, pertinent findings regarding evaluation of this technique are presented first. Since only cursory electron microscopic observations of Blepharisma have been published, many of the morphological details observed through the course of this study are described. Previous investigations directed specifically toward the elucidation of the fine structure of regenerating and dividing ciliates have been, in most respects, generally disappointing. These processes were studied in the most detail, and observations which have not been made by other investigators are reported.

Fixation

Blepharisma possesses a highly vacuolated cytoplasm (Fig. 4) and is extremely sensitive to osmotic change (Giese and McCaw, 1963b); both conditions are characteristic of tissues which are difficult to fix for electron microscopy. In this study, early attempts at fixation utilized Veronal acetate-buffered, 1% OsO₄ at pH 7.6 and containing 3mM CaCl₂; however, fixation was not satisfactory, and this basic formula was adjusted by the addition of varied amounts of sucrose, the deletion of CaCl₂, and/or variation in buffer strength. The addition of sucrose (8 mg/ml) improved the general appearance of fixed organisms; however, coagulation of the cytoplasmic ground substance was marked, and many
large, empty, angular vacuoles were present (Figs. 5 and 6). Glutaraldehyde fixation in conjunction with a number of vehicles consistently resulted in a severe "crinkling" or crenation of the cell and fine structure preservation was not acceptable.

Through preliminary phases of this study, osmium tetroxide vapor had been used to lightly fix organisms before they were stained with methyl green-pyronin; good preservation of form and dimension was observed even in exposures as short as 20 seconds. The use of osmium tetroxide vapor fixation for electron microscopy was then attempted, and preservation by this method is considered to be the best so far obtained by the author.

The ciliates, Blepharisma and P. multimicronucleatum, cease activity (locomotion) 10 to 15 seconds after initial exposure to osmium tetroxide vapors, show an almost immediate darkening in color, and retain a very life-like form. Giant amebae cease protoplasmic streaming within 15 to 20 seconds after initial exposure, and the pseudopodia are preserved with little alteration of form. In dividing amebae (often nearly 1 mm in diameter), the darkening of the cytoplasm is complete to the organism's center in one to one and one-half minutes. For Blepharisma, the quality of fixation is dependent on the size of the fixation droplet and the physiological condition of the organisms.

Fine structure preservation

One of the most striking features observed in osmium tetroxide vapor-fixed Blepharisma is the delicate appearance of the cytoplasmic ground substance (Figs. 4, 16 and 42) which lacks the coarse coagulation typical of standard fixation. True vacuoles show rounded forms rather than the
Fig. 1. Calibration curve for the determination of the osmium tetroxide in hanging-drop preparations. Points of the calibration curve are indicated by circles; the absorbancies of the unknowns are indicated by crosses. Osmium tetroxide concentrations employed in fixation were determined from this curve.

Fig. 2. The concentration of each unknown, as determined in Fig. 1, is plotted against the time for which the droplet was exposed to osmium tetroxide vapor. The relationship is apparently linear for the exposure times employed.
Fig. 1 Determination of OsO₄ concentration in hanging-drop preparation

Fig. 2 Rate of OsO₄ concentration increase
Fig. 3. *Blepharisma undulans americanus* Suzuki. Drawing is altered slightly from Suzuki (1954). A short section of a ridge shows rowed pigment granules (PG); adoral membranelles (M), the undulating membrane (UM) and gullet (GU) are peristomal features of most prominence. The moniliform macronucleus (MA) and the micronuclei (MI) are shown in close contact, a condition usually observed in electron micrographs (Fig. 4). The kinetosomal rows (KS), food vacuoles (FV) and the contractile vacuole (CV) are also shown. The heavy line (X) marks the site at which organisms were transected for regeneration experiments.
Fig. 4. Survey micrograph of a *Blepharisma* sectioned longitudinally. A single lobe of the macronucleus appears near the center of the micrograph and is surrounded by several micronuclei. Mitochondria, pigment granules, and a variety of vacuoles and vesicles are present throughout the cytoplasm. Golgi bodies are numerous and intensely stained. The gullet is cut in approximate cross-section (lower right) and the distinctive vacuoles associated with the gullet terminus are obvious. Portions of rootlet fibers are visible (upper right) adjacent to an area of the pellicle from which pigment granules have been voided. Vapor-fixed and stained with lead citrate. Magnification: X 6,300
Fig. 5. The dense coagulation and high degree of vacuolization of the cytoplasm which results from the fixation of *Blepharisma* in a 1% osmium tetroxide solution buffered with Veronal-acetate at pH 7.6 and containing 8 mg/ml sucrose. Stained with potassium permanganate. Magnification: X 12,000

Fig. 6. The macronucleus (MA) is uniformly dense, limited by a double membrane, and surrounded by a thin layer of cytoplasm; dense bodies usually present in ciliate macronuclei are absent. Fixation as in Fig. 5 and stained with potassium permanganate. Magnification: X 19,000
angular or spherical outlines expected from tissue shrinkage or swelling, and the cell membrane shows close cytoplasmic apposition without discontinuities or blebs (Figs. 16 and 18).

The cilia and infraciliature are well preserved (Figs. 15, 22 and 41), mitochondria are without recognized swelling or shrinkage (Figs. 19 and 46), and the Golgi apparatus shows a typical structure (Fig. 33, G). The envelopes of the nuclei are clearly defined (Figs. 47, NE; also 42 and 47), and the nucleoplasm does not give the impression of marked extraction (Figs. 10, 42 and 46); moreover, its appearance is comparable to that previously described for Blepharisma (Utsumi and Yoshizawa, 1957; Seschachar, 1964) and for Paramecium (Jurand, Beale, and Young, 1962).

A sloughing of pigment material occurs more frequently than in standard fixation; either the entire granule is voided with the granule membrane and cell membrane becoming continuous (Fig. 28, PG) or the contents of the granule are partially expelled or extracted leaving the membrane with a highly crenated appearance (Fig. 16, PG). Organisms fixed in vapor for less than two minutes contain many prominent, oval granules that are presumably stored food material of a glycogenoid nature (Weisz, 1950) and that stain intensely with lead citrate (Fig. 29, OF); longer fixations result in a decreased stain-ability (Fig. 32, OF).

The dividing micronucleus contains a very well organized mitotic apparatus consisting of tubular filaments that are 15 μm in diameter and appear circular in cross-section with a dense cortex and less dense center (Fig. 50, SF). Filaments identical to these surround the macronucleus during the latter stages of its amitotic division (Fig. 25, EF).

In order to evaluate the vapor fixation method, different cells of
known and similar structure were utilized. The general features and specifically the filament morphology of the rather labile mitotic apparatus of the giant ameba are well known (Roth and Daniels, 1962), and specific fixation conditions for its preservation have been demonstrated (Roth and Jenkins, 1962; Roth et al., 1963). The mitotic apparatus of dividing ameba fixed by osmium tetroxide vapor contains 15-μm filaments with adhering fine material, all morphologically identical to those observed after osmium tetroxide fixation in the presence of stabilizing solutions (Figs. 7 and 8, SF). Furthermore, some filaments seem to show periodicity along their lengths (Fig. 8, SF). The Golgi apparatus, mitochondria, chromosomes, and fragments of the nuclear envelope of ameba have the same appearance as when fixed by orthodox procedures (Fig. 7).

Preliminary observations of dividing P. multimicronucleatum fixed by osmium tetroxide vapor have also been made as a further test of the general applicability of the method. Longitudinal sections of an organism which showed only the slightest indication of the developing division furrow show 16-μm filaments lying within the micronucleus near the envelope and apparently lacking connection to or continuity with other structures (Fig. 9, F). The micronucleus at this stage is approximately 2-2.5 μ in diameter and is bounded by an envelope consisting of two membranes separated by a less dense space 15-20μm in width and having numerous pores about 35 μm in diameter, some of which show a single, dense 2 to 3 μm granule at their center (Fig. 12, NP). The central portion of the micronucleus contains a mass of dense granular and/or course fibrillar material; the balance of the nucleoplasm consists mainly of fine, particulate material and long tortuous fibers (Fig. 12, CR). In some sections, loose
Fig. 7. Early metaphase mitotic apparatus of the giant ameba Pelomyxa carolinensis. The nuclear envelope (NE) shows many discontinuities but a double nature is maintained. The mitochondria (M), Golgi bodies (G), and chromosomes (CH) are well preserved. The spindle filaments (SF) are surrounded by fine material and show excellent preservation. Osmium tetroxide vapor-fixed for three minutes and stained with potassium permanganate. Magnification: X 26,000

Fig. 8. Higher magnification of the filaments from the mitotic apparatus of the giant ameba. The spindle filaments (SF) show indication of a periodic structure along their lengths and are surrounded by fine material (FM). Osmium tetroxide vapor-fixed for three minutes and stained with potassium permanganate. Magnification: X 65,000
bundles of fibers approximately 4 m in diameter are seen in the central portions of the micronucleus (Fig. 13, NF); a faint periodicity is evident within the bundle. Vivier and André' (1961) have described similar fibrillar structures within the macronucleus of *P. caudatum*. Enclosed within bulbous extensions of the outer membrane of the envelope are numerous bacterium-like bodies (Figs. 9 and 14, BLO) whose diameter, about 0.4 μ, cell wall structure, and mesosome-like membranous whorls are typical of bacterial cells. These bodies resemble in most respects the bacteria found in the buccal cavity (Fig. 15, B) and the μ particles described by Beale and Jurand (1960).

The macronucleus has an irregular outline and is enclosed by an envelope similar to that of the micronucleus except that pores are more numerous and slightly larger in size (Fig. 11, NP). The macronucleus contains large numbers of electron dense bodies of two distinct sizes; the smaller and most numerous are about 100 m in diameter while larger and more granular appearing bodies 0.5 μ in diameter are present in most sections (Fig. 10, SB and LB). The macronuclear structure is nearly identical to that described for *P. aurelia* (Ehret and DeHaller, 1963) and for *P. caudatum* (Jurand, Beale, and Young, 1962). Bacterium-like bodies are found near to but apparently not in contact with the macronucleus (Fig. 11).

Unstained sections of material fixed in osmium tetroxide vapor show about the same amount of contrast as do unstained sections of material fixed by routine osmium tetroxide procedures. However, the use of heavy metal stains may be more effective following osmium tetroxide vapor fixation; lead citrate (Reynolds, 1963) has been found to give particularly
Fig. 9. Portion of the micronucleus (MI) from *Paramecium multimicronucleatum*. The nuclear envelope is clearly double, shows numerous pores, and has bulbous extensions of the outer membrane which contain bacterium-like bodies (BLO). Filaments (F) are present and may be remnants of the micronuclear, mitotic spindle. Fixed for three minutes by osmium tetroxide vapor and stained with potassium permanganate. Magnification: X 62,000

Fig. 10. The macronucleus contains large bodies (LB) which are granular in appearance and many dense small bodies (SB). Vapor-fixed and stained with potassium permanganate. Magnification: X 18,000

Fig. 11. Higher magnification of the macronucleus of *Paramecium*. The nuclear pores (NP) are prominent. A bacterium-like organism is shown near the nuclear envelope, but connection to the envelope is not clear. Osmium tetroxide vapor-fixed and stained with potassium permanganate. Magnification: X 37,000
Fig. 12. Survey of a portion of the micronucleus of Paramecium. The dense chromatin (CR) is present at the center of the nucleus. The nuclear envelope shows pores and associated annuli in both tangential and cross-section (NP). Numerous bacterium-like bodies surrounded the micronucleus. Osmium tetroxide vapor-fixed and stained with lead citrate. Magnification: X 27,000

Fig. 13. Nuclear fibers (NF) associated in bundles are present in the micronucleus; a periodic structure is apparent and suggests a helical form for the individual fibers. The double nuclear envelope (NE) is clearly resolved. Osmium tetroxide vapor-fixed and stained with lead citrate. Magnification: X 43,000

Fig. 14. Bacterium-like bodies (BLO) are clearly shown to be included within bulbous extensions of the outer membrane of the nuclear envelope. Cell wall structure and internal organization are visible within the bacterium-like bodies. Osmium tetroxide vapor-fixed and stained with potassium permanganate. Magnification: X 37,000

Fig. 15. A section through the buccal cavity of Paramecium includes bacteria (B) and cross-sections of many cilia (C). Note similarity of bacteria and the bacterium-like organisms in Fig. 14. Osmium tetroxide vapor-fixed and stained with potassium permanganate. Magnification: X 45,000
intense staining of some constituents (Figs. 29 and 34).

In order to better evaluate osmium tetroxide vapor fixation, especially since extremely short fixation times are employed, the concentration of osmium tetroxide in the hanging drop was determined by spectrophotometric measurements of the colored OsI₆⁻ complex. The results of the spectrophotometric determinations of osmium tetroxide concentrations are presented graphically in Fig. 1. The points on the Beers' Law plot (solid line) are represented by circles; the optical density of each unknown is plotted on this calibration curve, and each point is marked by a cross. In Fig. 2 the concentrations at 1-minute intervals up to 6 minutes are plotted against time; the relationship is linear for the times tested, and the rate of increase is approximately 0.10% per minute. Exposure to vapor for 3 minutes has been used routinely; and, therefore, at the end of the fixation period the osmium tetroxide concentration has only reached a level near 0.30%. The concentration of osmium tetroxide active as the fixative agent is very low as compared to routine methods and also acts for a very short period of time.

General Fine Structure

Light microscopy

During periods of rapid proliferation, *B. undulans americanus* Suzuki and *Blepharisma* sp. Turtox strain are found to attain a nearly consistent length of 200 μ and under the conditions of culture are only faintly pigmented. Under the conditions of culture used in this investigation, *B. undulans americanus* and *Blepharisma* sp. Turtox strain were found to be so similar that further distinction between the strains will not be
made in the course of the reported observations. The prominent morphological features are shown diagramatically in Fig. 3. Under the phase-contrast microscope, the cytoplasm of well-fed organisms contains a large number of vacuoles which are usually hyaline in appearance and greatly variable in size. A single, large, contractile vacuole is found at the posterior tip; this structure appears to lack the radial canal system recognized in paramecia but rather seems to form by the coalescence of smaller vesicles. The contractile vacuole serves as an efficient meter of culture conditions; for example, an early indication of starvation is its elongated and pointed appearance. Introduction of organisms to unfavorable culture conditions (extremes in tonicity or pH) has been observed to result in distortion (bowing or flexing) of the contractile vacuole area and/or the voiding of comparatively large amounts of material through the cytopyge.

Few details of peristomal structure are revealed by phase observation of living or osmium tetroxide vapor-fixed cells. The undulating membrane is distinct, and its distal margin is usually frayed so that the component cilia can be distinguished. The membranelles composing the adoral band consist of three rows of closely aligned cilia except at the anterior tip and posterior terminus (at the gullet) where it appears that double and single rows are present. The membranellar band spirals in a clockwise direction and extends into the gullet region.

The macronucleus is easily observed by the use of phase microscopy, is usually located along the dorsal side of the organism, and extends, as a moniliform chain of four to five nodes, in an antero-posterior direction (Fig. 3, MA). The nodes present a dense, homogeneous appearance and often
contain small, highly refractile spherules. The use of methyl green-pyronine or acid methyl-green as vital stains has invariably resulted in cytolysis of the organisms. Fixation for 20 to 30 seconds by osmium tetroxide vapor averts this cytolysis and does not seem to interfere with the staining reaction. Dilute methyl green-pyronin stains the cytoplasm pink and the macronucleus blue-green and was used to facilitate the study of macronuclear morphology. Micronuclei have not been observed under the phase microscope in either stained or unstained cells.

The longitudinal ridges and furrows of the cell surface are well defined; the ridges are approximately 4 μ wide at mid-body while the furrows along which the cilia are arranged are very narrow. Pigment granules occupy only the region under the ridges, so that the furrows appear as clear zones separating the rows of granules. In living organisms, pigment granules are observed to move away suddenly from the cortex into the flowing endoplasm.

Old and declining cultures often contain great numbers of conjugating organisms. Exconjugants transferred to fresh culture fluid are viable and undergo division after what appears to be a growth period of usually 72 hours or more (conjugants are almost invariably smaller than vegetative forms); the progeny continue to divide at normal intervals. Giant forms are also present in old cultures, particularly those to which *Chilomonas* or rice grains have been added.

**Electron microscopy**

The surface of *Blepharisma* is bounded by a pellicle which consists of at least two membranes, each approximately 7 μ in width, which are
separated by a less dense space of 7 μm (Fig. 16, P). This structure is most evident in regions between the membranelles and in sections cut transverse to the long axis of the cell (Fig. 18); in some cases a third membrane is suggested (Fig. 57) while at other times only a single limiting membrane is clearly resolved (Fig. 24). Closely applied sub-pellicular filaments (Fig. 18, SPF), flattened vesicles (Fig. 57), and variable planes of section all result in difficulty in precisely delimiting a consistent structure for the pellicle. The dimensions and general appearance of the pellicular membranes are also affected by the type of stain employed.

A well organized filament system is present directly beneath the inner membrane of the pellicle. In transverse section the system is seen to be composed of 15 to 16-μm filaments which appear tubular in cross-section, are arranged parallel to one another, and are spaced at regular intervals, usually about 21 μm (Fig. 18, SPF). Examination of transverse (Fig. 18) and longitudinal sections (Fig. 19, lower right) reveals that the filaments are always oriented parallel with the longitudinal axis of the organism and follow the surface contours but are absent directly beneath the lowest depression of the furrows (Fig. 18). The complex will be referred to as the sub-pellicular filament system. A similar organization of filaments has been reported for Spirostomum (Yagi and Shigenaka, 1963; Finley et al., 1964).

Longitudinal fibers situated to the right of each somatic kinetosomal row and just below the cell surface have been described from observations made with the light microscope (Suzuki, 1957); filament bundles are found at this site in both transverse (Fig. 18, KS) and longitudinal (Fig. 19,
thin sections. In cross-sections the fibrils appear as distinct rows of tubular filaments with a diameter of 15-16 μm (Fig. 18) and follow the rule of desmodexy (Chatton and Lwoff, 1935). The component filaments of each fibril arise at each kinetosome and run in a right-posterior direction to join with the kinetodesmal bundle (longitudinal view: Fig. 19). No connection is observed between the filaments nearest to the right side of the ectoplasmic ridge and the inner membrane of the pellicle complex as has been reported for Spirostomum (Yagiu and Shigenaka, 1963; Finley et al., 1964); there is, however, less space separating the sub-pellicular filaments in this region (Fig. 18). The length of the filaments composing a bundle has not been established although in several instances individual filaments are continuous past five pairs of kinetosomes. Consistent with terminology used throughout this report, this structure will be called the kinetodesmal fibril, and the component elements will be referred to as kinetodesmal filaments.

Survey electron micrographs reveal the presence of a poorly-defined fibrous band located at the ecto-endoplasmic boundary (Figs. 18 and 58, TB); examination of transverse sections indicates that it is probably present at this site for the length of the organism. The components of this band are not tubular in appearance and seem to lack precise alignment. At times, high magnification micrographs of longitudinal sections show a network of fine elements approximately 4 μm in diameter (Fig. 21, FR). A trabecular band of fibers similar in both morphology and location to that just described has been observed in Spirostomum (Yagiu and Shigenaka, 1963; Finley et al., 1964).

The cilia of Blepharisma present structure typical of that described
Fig. 16. Longitudinal section along the newly formed band of adoral membranelles in a dividing Blepharisma. Each membranelle consists of three ciliary rows; the cilia bear membranous extensions which have enlarged spherical tips (CP) and kinetosomes show connection to the rootlet fibers. The pellicle (P) is composed of two membranes between membranelles; a single membrane is found between the cilia of a membranelle. The cytoplasm of this area contains many ribosome-like particles, pigment granules (PG) and a single fibrous shaft (FS). Many filaments are present scattered in the ectoplasm (upper left). Osmium tetroxide vapor-fixed and stained with lead citrate. Magnification: X 38,000

Fig. 17. The rootlet fibers extend deep into the endoplasm. The fibers are cut in cross-section and show component 20 μm-tubular filaments. Definite order of filaments in the bundle is noted, but interconnection between filaments is not apparent. Osmium tetroxide vapor-fixed and stained with calcium permanganate. Magnification: X 72,000
Fig. 18. A section cut directly across the longitudinal axis of *Blepharisma*. Subpellicular filaments (SPF) are present just beneath the pellicular membrane. Numerous pigment granules are located under the ridges (center) but absent from the furrows (near KS). Kinetodesma (KS) appear in cross-section and the fibrous band (TB) is present a short distance from the cell surface. Osmium tetroxide vapor-fixed and stained with potassium permanganate. Magnification: X 25,000

Fig. 19. Kinetodesmal filaments arise at each kinetosome and run in a right-posterior direction to join with the kinetodesmal fibril (KS). Subpellicular filaments appear near the pellicle (lower right). Pigment granules show well-defined membranes, and mitochondria are uniformly dense and contain many tubular cristae. Osmium tetroxide vapor-fixed and stained with calcium permanganate. Magnification: X 43,000
for most ciliates. The ciliary shaft consists of the classical filament pattern, nine doublets arranged around two single central filaments; the entire structure is bounded by a unit membrane which is continuous with the pellicular membrane (Fig. 24, C). The filaments of the ciliary shaft are surrounded by a fine matrix which becomes particularly prominent near the base (Fig. 22, FM). The central filaments terminate at a dense granule located near the distal end of the kinetosome, just above the transverse septum at about the level of the cell surface (Fig. 24, K). As the distal end of the cilium is approached, the peripheral filaments first appear single, then fewer in number, and finally terminate while the central pair extends to very near the tip (Fig. 24, A). The body cilia occur in the ectoplasmic furrows apparently only in pairs (Figs. 19 and 21).

The membranelles of the adoral zone are composed of closely aligned cilia which have a morphology slightly different from somatic cilia. Aligned in rows of two or more often in rows of three (Figs. 22, MR; also 20 and 41), the cilia show the normal, filament morphology, but the limiting membrane bears numerous long filamentous projections, each of which ends in a spherical enlargement (Fig. 16, CP). These projections are usually between 0.3 and 0.6 μ in length and have a diameter near 16 μ except at the bulbous end which is about 50 μ in diameter. Similar structures are reported for the membranellar cilia of Stentor (Randall and Jackson, 1958) and Nyctotherus (King et al., 1961) and for the cirri of Euplotes (Roth, 1956).

The kinetosomes are about 160 μ in diameter and 450 μ in length. Near the level of the plasma membrane, just beneath the slightly concave
septum and the dense granule at which the central filaments terminate (Fig. 24, K), the kinetosome appears as a cylinder of nine, triplet filaments (Fig. 20, K). Deeper cross-sections nearer the proximal end show the triplet filaments to be oriented so that each makes an angle of 30 to 40 degrees with the tangent to the cylinder (Fig. 22, right center). A cartwheel-like structure with a single tubular filament at the center is present at the proximal end of the kinetosome (Fig. 36, arrow); this morphology is similar to that described for the kinetosomes of other ciliates (Gibbons and Grimstone, 1960). The proximal end of the kinetosome appears, at least in the case of membranelles, to be closed by a dense bar which is continuous with the rootlet filaments (Figs. 24, K). The kinetosomes of membranellar cilia are interconnected by fibrous strands (Fig. 20, I) seen only in cross-sections at precise levels, probably near the mid-level of the kinetosome. Found at one side of the outer kinetosomal row of a membranelle is a closely appressed row of six or seven tubular, 19-μm filaments that extends away from the kinetosome at an oblique angle; the component filaments are parallel with the long axis of the kinetosome (Fig. 22, arrow) and extend toward the surface.

Attached to the proximal end of each kinetosome of the adoral membranelles is a number of tubular filaments (Figs. 24 and 34, RF); these 20-μm filaments converge to form the rootlet fibers and extend slightly posterior and toward the endoplasm where they join with the rootlet bundles of other membranelles to form a rather dense fiber (Figs. 26, 34 and 41, RF). In transverse sections within the endoplasm, the fibers show filaments with a circular cross-section and a uniform separation from each other (Fig. 17, RF); interconnection between the filaments and...
Fig. 20. Cross-sections of kinetosomes (K) in *Blepharisma* show peripheral filaments that are triplets and fibrous interconnections (I) between adjacent rows. Kinetosomes are sectioned near the distal end in the upper row and progressively deeper so that the lower row is sectioned near the proximal end. Osmium tetroxide vapor-fixed and stained with calcium permanganate. Magnification: X 71,000

Fig. 21. The ectoplasmic-endoplasmic boundary in *Blepharisma* is marked by a fibrous band. Fibers are non-filamentous and lack precise alignment although some organization is present. Kinetodesmal filaments (KF) are shown as are paired kinetosomes (below FR). Osmium tetroxide vapor-fixed and stained with calcium permanganate. Magnification: X 54,000
precise packing of the filaments may occur.

The pigment granules are very electron dense bodies about 0.3 μ in diameter, are bounded by a single membrane (Fig. 16, PG), and are either concentrated under the ridges of the pellicle in longitudinal rows (Fig. 18) or are scattered throughout the endoplasm (Fig. 4). Considerable variation in the appearance of the limiting membrane and general structure of the granule is found. When stained with lead, the membrane usually appears dense with diffuse limits (Fig. 16, PG), while permanganate staining results in a better definition of the membrane (Fig. 19, center). The internal content of a granule is usually extremely dense and homogeneous (Fig. 19); however, some show dark centers with a lighter cortex (Fig. 16) while others have light centers with a darker cortex (Fig. 57). High magnification micrographs of extremely thin sections show the granule matrix to have a well defined crystalline array (Fig. 30, PG). The granules closely resemble those described for a colorless strain of Blepharisma (Inaba et al., 1958). On occasion the usual content of a granule appears to be partially removed (Fig. 16, upper); in other instances the granule membrane appears fused with the cell membrane, and the granule has been voided to the outside (Fig. 4).

In addition to the components just described, the ectoplasm contains mitochondria, a few small vesicles and ribosome-like particles (Figs. 16 and 18) and appears to extend not much deeper than the proximal end of the kinetosomes (Fig. 41); the ectoplasm-endoplasm transition is, at least under the conditions of fixation employed, difficult to identify.

The endoplasm contains many membrane-limited vacuoles of varied size and shape (Figs. 4, 41 and 42) the majority of which lack contents of high
electron density. The membranes present the usual unit form and show a random accumulation of dense clumps when lead staining is employed (Fig. 34). In addition to the large vacuoles, a great number of small, membrane-bounded vesicles are found that contain small granules, vesicles, or fine material (Figs. 16 and 18). The inner surface of the membrane of a certain ubiquitous, oval or elongate body bears tiny microvillus-like projections which extend about 16 μm into their interior (Figs. 46 and 53). These vesicles often appear to contain a granular material (Fig. 46) and are apparently identical to bodies described for Blepharisma by Utsumi and Yoshizawa (1957).

Food vacuoles contain bacteria and are found to have either a smooth membrane (Fig. 4) or numerous finger-like projections extending into the vacuole content (Fig. 23, FV). The projections are about 0.6 μm in length and consist of a finger of cytoplasm 30 μm in diameter that is surrounded by the vacuole membrane (Fig. 23, VP).

Immediately posterior to the buccal cavity is a zone of closely packed vacuoles, each limited by a dense membrane (Fig. 4, lower right). The vacuoles are found to take a peculiar configuration in their close association with each other (Fig. 42, arrow). Randall and Jackson (1958) have described an essentially identical zone in Stentor.

Lead stained sections show a great number of oval granules (Figs. 29, OF; also 4 and 42), while after permanganate staining only empty vacuoles of corresponding form are present (Figs. 18 and 48).

The endoplasm contains numerous typical Golgi bodies (Figs. 4 and 34, G) that are composed of a few flattened vesicles about 70 μm in diameter and are intensely stained by lead citrate. Small spherical
Fig. 22. Membranelles (MR) consist of three closely aligned ciliary rows; kinetosomes of these rows show varied structure according to the level of sectioning. The proximal ends of cilia show a very high concentration of fine material (FM) surrounding the filaments. Numerous filaments are found randomly oriented in this region. A row of six or seven tubular filaments (unlabeled arrow) extends away from the kinetosome and is directed along the longitudinal axis of the kinetosome (in this micrograph, filament rows are associated only with the right-most kinetosomal row of each membranelle). Blepharisma fixed with osmium tetroxide vapor, and section stained with potassium permanganate. Magnification: X 53,000

Fig. 23. In Blepharisma, food vacuoles (FV) often bear tiny villi-like projections (VP) which extend into the vacuole content. A bacterium (B) has been partially digested. Osmium tetroxide vapor fixed and section stained with calcium permanganate. Magnification: X 41,500
Fig. 24. Longitudinal section through cilia and kinetosomes of *Blepharisma*. The ciliary filaments are clearly resolved (C), the distal end of the kinetosome (K) is closed by a concave septum at about the cell surface, and the central filaments of the cilium terminate in a dense granule at this level. The rootlet filaments are shown in connection with a dense bar which closes the proximal end of the kinetosome. The membrane of the pigment granule (PG) is delineated. Osmium tetroxide vapor fixed and section stained with calcium permanganate. Magnification: X 65,000

Fig. 24A. Cross-sections through the tips of cilia of *Blepharisma*. The cilium at the left shows nine single filaments surrounding the two central filaments; the cilium at the right is sectioned very near the tip and shows only the two central filaments. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 45,000

Fig. 25. Extranuclear, 15-μm filaments (EF) are located about the periphery of the dividing macronucleus, always just outside the nuclear envelope. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 60,000

Fig. 26. A dense band of filaments, presumed to be the rootlet fiber (RF) is present deep in the endoplasm at a site just posterior to the gullet. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 20,000
vesicles and various amounts of dense material are found within the central portion of the Golgi (Fig. 33, G). Mitochondria vary somewhat in size and shape but usually appear as dense, oval or rod-shaped bodies 0.5 to 0.75 μ in diameter. The tubular cristae are 26 μ in diameter and are involutions of the inner of the two limiting membranes (Fig. 46, M). Pigment granules and ribosome-like particles are also present in the endoplasm (Figs. 4, 16 and 26).

Nuclei will be described in the section dealing with division.

Regeneration

**Light microscopy**

The transection of *Blepharisma* occurs without apparent loss of cytoplasm; the two resulting fragments are capable of active and directed movement. The anterior fragment, bearing the oral apparatus, swims in a more exaggerated spiral than does the complete organism, but ciliary coordination is apparently maintained. The progress of regeneration in anterior fragments has not been followed; it has been noted, however, that the general body form is restored in less than 90 minutes. The site of transection is indicated in Fig. 3.

The posterior fragment, lacking mouth parts, maintains its general form; the anterior end usually becomes rounded initially but in latter stages it becomes obviously pointed. It is capable of active swimming and, in nearly all cases, is more active immediately following transection than was the organism from which it was derived. The posterior fragments were studied in the greatest detail since their regeneration requires reformation of the extensive oral ciliature; the following
observations are concerned only with these fragments.

Phase microscope observation of regenerating fragments 30 to 90 minutes after cutting reveals a very active endoplasmic streaming, more active than that in uncut animals. The only other obvious change is a decrease in active movement occurring with some variability of onset and duration in the latter part of this time interval. At about 100 minutes, the fragments are somewhat longer and more slender than initially, and a ridge or groove becomes evident along one side near the anterior end. In some cases a concentration of pigment granules was indicated nearby.

The first indication of the new oral apparatus is the appearance of adoral membranelles. The row of membranelles extends along the longitudinal axis of the fragment; each membranelle is composed of cilia which are clearly shorter than those of the mature unit. The time required for the appearance (regeneration) of the oral primordium is reasonably constant for fragments from a single clone; however, an exact and predictable time requirement for all organisms of any experiment has not been established. At 23°C the earliest observation of primordia is from 150 to 210 minutes after transection. Restoration of body form and morphological completion of the peristome requires approximately 5 hours. A small percentage, less than 10%, of the posterior fragments lose the elongated form typical of normal regeneration, become markedly rounded, and do not regenerate.

Fragments placed in hanging drops containing methyl cellulose to retard movement regenerated normally in most cases. In one experiment, however, organisms treated in this way showed no signs of regeneration after five hours and had cytolyzed at the end of 12 hours. Therefore,
regenerating organisms to be utilized for electron microscopy were not held in fluid containing methyl cellulose.

The use of low temperature treatment, 10°C for 3 to 4 hours immediately following transection, was investigated as an aid to the manipulation of large numbers of cut organisms and is effective in blocking morphological regeneration, but samples returned to 23°C regenerate with greater variation in time. Transected organisms were also held at 5°C for 2 to 3 hours with apparent blockage of regeneration and few visible deleterious effects. Organisms treated in this way have been observed to divide within 24 hours after return to 23°C. Low temperature blockage of regeneration may prove to be a valuable experimental method in an electron microscope approach to regeneration problems but requires further investigation.

Electron microscopy

Low power survey micrographs show that the ridges of the pellicle at the point of transection are ragged in appearance (Fig. 28). The pellicle is interrupted by numerous, globular depressions resulting from the extrusion of entire pigment granules some of which are present between the ridges and near the pellicle (Fig. 28, PG). The posterior half of the same fragment shows pigment granules neatly layered beneath the pellicle; sloughing of granules is not apparent (Fig. 27, PG). The voiding of pigment granules is frequently observed but never to the extent shown near the site of transection. A slightly higher than normal vacuolization of the wound-area cytoplasm is often found (Fig. 28), but significant cortical or cytoplasmic changes have not been recognized at
Fig. 27. Posterior end of a regenerating fragment fixed two hours after transection. Pigment granules (PG) are found neatly layered beneath the pellicle, and numerous vacuoles (V) are present. The cytoplasm lacks significant modification from the interphase condition. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. *Blepharisma*. Magnification: X 7,900

Fig. 28. Anterior end of the same regenerating fragment shown in Fig. 27. Many pigment granules (PG) have been voided by the organism, possibly in response to the initial contact with osmium tetroxide in fixation, and numerous cup-like depressions (unlettered arrows) are present in the cell surface. The ragged appearance of this area results from the cutting operation. Section stained with potassium permanganate. Magnification: X 8,900

Fig. 29. Short vapor fixation (1.5 minutes) of *Blepharisma* is satisfactory in most respects. A pronounced effect of short fixation is the intense staining of the oval, stored-food granules (OF) with lead citrate. Magnification: X 27,000

Fig. 30. High magnification of a thin section through a single pigment granule (PG). The unlettered arrow indicates the direction of lines which indicate a crystalline structure; lines are separated by a less dense space of approximately 2.5 μm. Osmium tetroxide vapor-fixed and section stained with calcium permanganate. Magnification: X 150,000
early stages.

During regeneration, the macronucleus undergoes reorganization without division (Suzuki, 1957). A single, macronuclear mass becomes evident at about three hours post-operation. Numerous, small vesicles with attached ribosome-like particles are closely associated but not continuous with the macronuclear envelope (Fig. 32, RLP). Dense round bodies, 0.45 to 0.80 µ in diameter (Fig. 40, DB), are found within the macronucleus in about the same frequency as in vegetative cells; pores are neither more nor less obvious, and no nuclear blebs are found.

The few non-dividing micronuclei observed in regenerating organisms do not always display the typical interphase morphology. The electron-dense center is not observed (Fig. 46, MI); rather the entire nucleoplasm appears to be filled with a fine, fibrous matrix (Fig. 47). These micronuclei are about 2 µ in diameter, have more obvious pores in the nuclear envelope, and following lead staining, have dense particles randomly distributed throughout the nucleoplasm (Fig. 47, PB).

Dividing micronuclei are frequently present in fragments fixed three to three and one-half hours after cutting. The division is mitotic, intranuclear, and acentric, and appears identical to that found during cell division (Fig. 31, MI).

The cytoplasm of the regenerating fragment contains a great number of ribosome-like particles, particularly during the two to three hour period (Fig. 32). Numerous randomly distributed 250-µ vesicles containing 25-50 A granules of moderate density are found (Fig. 33); other vesicles of approximately the same size but having heavier walls and containing a 150-µ spherical core are also present (Fig. 47, HV). An increased number
Fig. 31. The dividing micronucleus in a regenerating fragment of *Blepharisma* fixed three hours after merotomy. The chromosomes (CH) are present at the equatorial plate (metaphase), many tubular spindle filaments (SF) are present, and the nuclear envelope is intact and clearly double. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 37,500

Fig. 32. At approximately two hours after transection, regenerating fragments show the presence of many ribosome-like particles, both free and attached to membranous vesicles (RLP), in close association with the macronucleus (MA). The staining of stored food material (OF) by calcium permanganate is less intense than that resulting from lead citrate. Osmium tetroxide vapor-fixed. *Blepharisma*. Magnification: X 32,000

Fig. 33. During early stages of regeneration, the occurrence of paired Golgi bodies (G) has been found in a number of fragments. Numerous vesicles which contain assorted smaller vesicles and/or granules are also present. Regenerating fragment was fixed one hour and 45 minutes after merotomy. Osmium tetroxide vapor-fixed and section stained with lead citrate. Magnification: X 21,000
of paired Golgi bodies is found in some cases (Fig. 33, G).

Significant morphological changes in mitochondria, glycogenoid granules, pigment granules or changes in the distribution of these components are not observed.

The anterior one-half of regenerating fragments particularly the cortical area has been studied to identify elements of fine structure related to the formation of kinetosomes and/or cilia. At the present time only limited observations can be reported even though the period of development of these organelles has been closely bracketed.

The kinetosomes of the primordia are easily recognized by their precise alignment, usually in rows of three (Fig. 22, MR). In one organism, transverse sections of this region show three distinct rows of kinetosomes, but only two, well-defined rows of cilia (Fig. 35). The kinetosomal rows not showing expected continuity with a ciliary row do have, however, one imperfect cilium at the predicted position (Fig. 35, IC). In addition, kinetosomes are apparently lacking in these rows and in or near their place very heavy-walled vesicles are found (Fig. 35, HV). Also, the triplet-filament structure of kinetosomes in these rows is difficult to define. The distal end of the kinetosome and the basal region of the ciliary shafts have much fine material surrounding the filaments, and numerous heavy-walled 100 to 150-μ vesicles and an assortment of flattened membranous structures are found within this zone (Fig. 35).

Sections cut parallel with the band of new membranelles so that the kinetosomes of a number of membranelles are then cross-sectioned show numerous pairs of kinetosomes (Fig. 36, K), some of which are in discrete
Fig. 34. Regenerating *Blepharisma* fixed with osmium tetroxide vapor three hours and 20 minutes after transection. A portion of a single kinetosomal row of one new membranelle is sectioned longitudinally. Small dense granules (PB) are found under the ciliary membrane; such granules are more prominent following uranyl acetate staining. Flattened vesicles and vesicles containing granules of medium density (unlettered arrows) are present near the kinetosomes. A well-formed Golgi body (G) and tubular filaments connected to the proximal end of the kinetosomes rootlet filaments (RF), are visible. Section stained with lead citrate. Magnification: X 52,000

Fig. 35. Distinctive heavy-walled vesicles (HV) are located within the area of new membranelle formation; these vesicles appear at sites where kinetosomes are expected to be. The left-most row of each membranelle shows irregular kinetosomes (unlettered arrow), irregular spacing of kinetosomes, and spaces where kinetosomes are expected. Note that the cilia associated with the two well-formed kinetosomal rows are normal while only a single imperfect cilium appears in conjunction with the left-most row. It is characteristic that newly formed cilia show much fine material (FM) near their proximal end. *Blepharisma* allowed to regenerate for three hours before fixation with osmium tetroxide vapor. Section stained with potassium permanganate. Magnification: X 40,000
Fig. 36. Section cut along the longitudinal axis of the adoral band of membranelles so that the kinetosomes of many membranelles are found in cross-section. Most kinetosomes (K) are arranged in precise rows of two, however, some lack apparent alignment into well defined rows. Numerous heavy-walled vesicles (HV) are present within the area. Regenerating Blepharisma fixed with osmium tetroxide vapor three hours and ten minutes after transection. Section stained with potassium permanganate. Magnification: X 30,000
rows while others apparently lack orientation. Note again the presence of numerous spherical, heavy-walled, flattened vesicles (Fig. 36, HV). That the area beneath and near to the kinetosomes of new cilia often contains numerous flattened vesicles resembling the cisternae of Golgi bodies is even more evident when the kinetosomal rows are sectioned longitudinally along a single row of one membranelle (Fig. 34, arrows). Vesicles bounded by a single membrane and containing granules of medium density are also prominent (Fig. 34). Termination of rootlet filaments at the dense bar closing the proximal end of the kinetosome and an apparent interconnection of the kinetosomes at their proximal end is indicated.

At no time has anything resembling the division or formation of a kinetosome been observed.

Division

Light microscopy

For the most part, observations of division parallel those made by Suzuki (1954). Since a description of his work appeared in an earlier section, only supplementary observations will be reported.

Newly established, mass cultures of Blepharisma contain numerous dividing forms during the first two or three days of growth. The frequency of division is variable, but an average time of 18 hours has been determined.

Organisms entering division are found to be slightly less active than non-dividers and are recognized by a disproportionately lengthened posterior half. Phase microscope examination of such organisms discloses
a well-formed oral primordium which consists of a membranellar band
aligned parallel to the kineties; this region of the organisms ventral
surface is straight in outline during the early stages.

The condensed macronucleus is present at about one hour following
the recognition of an early divider. The loss of central nodes during
the condensation of the macronucleus has not been observed. As division
proceeds, the macronucleus begins to elongate and forms a distinctly rod-
shaped structure extending for more than 85 percent of the animal's
length; it then begins to show constriction at the division line which
initially is largely free of pigment granules. Constriction and separa­
tion of daughters is effected approximately two hours after the first
recognition of division. Micronuclei were not observed in light microscope
studies.

Electron microscopy

The vegetative macronucleus consists of 4-5 nodes interconnected by
strands (Fig. 42, MA). Dense bodies of two general sizes are present
within the macronucleus. The larger bodies, about 0.5 μ in diameter, have
a diffuse appearance (Fig. 38, LB) are few in number, and, after lead
staining, show numerous 12 to 20 μ electron opaque granules (Fig. 42,
PB). Smaller bodies, 0.15 μ in diameter, are more numerous and present
a more compact appearance (Fig. 38, SB). The balance of the nucleoplasm
consists of a moderately dense matrix (Fig. 38). Infrequently, dense
spherical bodies, 0.45 to 0.80 μ in diameter, composed of a poorly defined
and very compact reticulum of 4 μ fibers are present (Figs. 39 and 40,
DB). The macronucleus is bounded by an envelope consisting of two unit
membranes separated by a less dense space 7-8 μ in width. Numerous pores 50 μ in diameter with associated annuli are clearly evident in sections cut transverse (Fig. 42, NP) or at a tangent to the envelope (Fig. 37, NP).

Approximately midway through the division process, the macronucleus has completed condensation, and the nucleoplasm has become more homogeneous (Fig. 49, MA). Being partially obscured by an apparent agglomeration of fine material, the envelope is less distinct (Fig. 49). Tubular filaments 15 μ in diameter are found closely applied to the cytoplasmic side of the nuclear envelope (Figs. 25 and 49 EF). The dense, round bodies found in interphase and reorganizing macronuclei (Fig. 40, DB), as well as round bodies of similar size but of lower density, are observed (Fig. 52, RB) within the nucleoplasm.

The elongated, dividing macronucleus is very dense; the large and small, dense bodies typical of the vegetative form are once again obvious (Fig. 45, MA). Sections cut at a right angle to the long axis of the elongate macronucleus reveal many 15-μ filaments closely appressed to the outside of the nuclear envelope (Fig. 25, EF); at this stage and in this plane of section, only circular cross-sections were found. Corresponding filament profiles are not seen within the macronucleus; however, in one instance membrane-limited bundles of 15-μ filaments were observed in the nucleoplasm (Fig. 44, FB). Larger filament bundles, circular in cross-section and enclosed by a single membrane, are found outside the macronucleus (Figs. 44 and 52, FB). These filaments are tubular, 15 μ in diameter, and in some cases appear to be associated in pairs (Fig. 44, FB). In any one section, the bundles are quite consistent in the number of filaments present; 45-50 are shown in each bundle in Figure 44.
Fig. 37. Tangential section of the interphase macronucleus of *Blepharisma*. The nuclear envelope shows numerous pores, some of which show a dense central granule (NP); nucleoplasm is to the left in the micrograph. Vesicles with attached ribosome-like particles (RLP) are present near the macronucleus. Osmium tetroxide vapor-fixed and section stained with lead citrate. Magnification: X 80,000

Fig. 38. Interphase macronucleus of *Blepharisma*. The nucleoplasm consists of small dense bodies (SB) and large bodies (LB) which are granular in appearance and may be nucleoli, surrounded by a matrix of fine material. Osmium tetroxide vapor-fixed and section stained with calcium permanganate. Magnification: X 32,000

Fig. 39. High magnification of the spherical dense bodies found in the macronucleus of *Blepharisma*. The very dense reticulum consists of fibers approximately 4 μ in diameter. Osmium tetroxide vapor-fixed and section stained with calcium permanganate. Magnification: X 130,000

Fig. 40. Lower magnification of a section through the macronucleus of *Blepharisma*. The dense appearance of the large, spherical bodies (DB) is in pronounced contrast to the nucleoplasm. The double nuclear envelope is clearly evident. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 40,000
Fig. 41. Survey micrograph of the area near the anterior end of 
*Blepharisma*. Component rootlet filaments (RF) arise at 
the proximal end of the kinetosomes of each membranelle 
and unite to form the large rootlet fiber. Many mitochondria 
are present throughout the cytoplasm. Note the comparatively 
large vacuolar areas which lack electron dense material. 
Osmium tetroxide vapor-fixed and section stained with lead citrate. Magnification: X 7,300

Fig. 42. The moniliform interphase macronucleus (MA) of *Blepharisma* 
is connected by short extensions. The nuclear envelope is 
clearly double and has numerous pores (NP). When stained 
with lead citrate, the larger bodies (Fig. 38) show many 
small dense granules (PB). Closely appressed vacuoles 
associated with the terminus of the buccal funnel are in­
cluded (unlettered arrow). Numerous, faintly-stained 
stored food granules and dense mitochondria are randomly 
distributed. Osmium tetroxide vapor-fixed and section 
stained with lead citrate. Magnification: X 16,500
In this same figure the membrane enclosing a bundle appears to be continuous with a large dense mass; filament cross-sections are also observed outside the limiting membrane and at the periphery of the dense mass. Longitudinal sections of filament bundles have not been obtained so the filament length is not known.

If the plane of sectioning parallels the long axis of the fully extended macronucleus, electron micrographs reveal a great number of 15-μm filaments all of which are oriented parallel to long axis of the nucleus (Fig. 43, EF); that the filaments are outside the envelope is demonstrated in cross-sections (Fig. 25, EF). It is established, therefore, that the elongate macronucleus has a great number of filaments located just external to its periphery and directed only along the longitudinal axis. Annuli are clearly visible in the envelope at this stage (Fig. 43, arrow). Evidence for any method for precise segregation of chromatin material during the amitotic macronuclear division has not been found.

The interphase micronucleus is a nearly spherical body, 1.5 to 2 μ in diameter, and is limited by a two-membrane envelope in which pores are present but not prominent (Fig. 46, NP). The nucleoplasm has a cortex consisting of a fine matrix surrounding an electron-dense reticular, central region which lacks a well defined granular or fibrillar structure (Fig. 46, CR). The structure of the micronucleus is essentially identical to that described for B. intermedium (Seshachar, 1964). Micronuclei may be located throughout the cytoplasm but most are localized near the macronucleus (Fig. 4).

In early metaphase when the chromosomes are aligned at the equatorial plate, well-defined 15-μm filaments are present in large numbers (Fig. 48);
Fig. 43. A section cut along the longitudinal axis of the dividing macronucleus of *Blepharisma*; the plane of section is immediately adjacent to the cytoplasmic side of the nuclear envelope, a portion of which is included (unlettered arrow). The macronucleus returned to the plane of section at the left but is not included in the micrograph. Numerous tubular filaments (EF) which compose the extranuclear division apparatus are directed along the long axis of the nucleus. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 31,000

Fig. 44. The elongated, dividing macronucleus in *Blepharisma* sectioned at an exact right angle to Fig. 43. The macronucleus (upper left) contains membrane-limited filament bundles (FB); few extranuclear filaments are present in this particular section. The larger filament bundles (FB) to the right, also membrane limited, are each composed of 45-50 tubular filaments, sometimes associated in pairs. The lower bundle is near to a dense mass; the membrane of the filament bundle extends into the structure. These filament bundles are probably cross-sections through late anaphase micronuclear mitotic figures. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 44,000

Fig. 45. Longitudinal section of the dividing macronucleus in *Blepharisma*. The enlarged end of the nucleus is to the left; approximately one-fourth of the elongated macronucleus is included in the micrograph. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 6,500
some filaments appear to terminate at chromosomes while others pass between chromosomes, apparently extending from pole to pole. The nuclear envelope remains intact, but its component membranes are not always clearly observed. At this stage, the micronucleus is barrel-shaped with each pole slightly depressed; no structures resembling centrioles or astral filaments are found at the poles (Fig. 48, MI). In other nuclei, the mitotic figure appears more elongated, and very early separation of chromosomes is indicated (Fig. 49, CH). Near cross-sections (Fig. 50, SF) of the micronucleus at this stage reveal great numbers of spindle filaments evenly distributed throughout the nucleus and completely contained within the bounds of the envelope; filaments cut in exact cross-section present a circular profile with a dense cortex and less dense center. The several metaphase figures of one organism do not display a uniform axial orientation, but the alignment of closely associated figures is usually similar (Fig. 48, MI).

The chromosomes appear as 250-mµ masses with little internal structure aside from a generally fibrous appearance (Figs. 50 and 54, CH). Well defined structures (kinetochores) marking connections with spindle filaments were not observed (Fig. 54, CH). The matrix surrounding the filaments (Fig. 49, FM) closely resembles the nucleoplasmic substance of the condensed macronucleus (Fig. 49, MA) and demonstrates no differential concentration during division. Neither material adhering to the mitotic filaments as described for amebae (Roth and Daniels, 1962) nor material layered just inside the micronuclear envelope (Roth and Shigenaka, 1964) is observed in Blepharisma.

The very late anaphase micronucleus shows the chromatin to be
Fig. 46. The interphase micronucleus in *Blepharisma* contains a dense central mass (CR), presumably chromatin, surrounded by a less dense nucleoplasm. The nuclear envelope is composed of two unit membranes separated by a uniform space and contains a comparatively low number of nuclear pores (NP). Mitochondria (M) with well-defined tubular cristae and an overall dense appearance are typical of *Blepharisma* fixed by osmium tetroxide vapor. The cytoplasm (to the left of the nucleus) contains many ribosome-like particles and small vesicles with many small villi-like projections extending into the granule. Section stained with potassium permanganate. Magnification: X 56,000

Fig. 47. Micronuclei in regenerating *Blepharisma* and occasionally in organisms thought to be in interphase (Fig. 4), sometimes lack the dense central mass typical of non-dividing micronuclei (Fig. 46). A moderately dense material fills the nucleus and after lead staining numerous extremely dense granules (PB) are present. The nuclear envelope is very distinct and contains numerous pores. Nuclei of this morphology are consistently larger than those known to be in interphase; this morphology may represent the change described for micronuclei about to enter division. This nucleus is from a regenerating fragment; note the great number of ribosome-like particles, also the heavy-wall vesicles (HV). Osmium tetroxide vapor fixation. Magnification: X 40,000
Fig. 48. Survey micrograph of a dividing Blepharisma. A portion of the macronucleus (MA) is included; two metaphase micronuclei (MI) show the nuclear envelope to be intact, fibrous chromosomes aligned at the equatorial plate, well-defined 15 μm filaments, and much fine material within the mitotic apparatus. Osmium tetroxide vapor-fixed and section stained with calcium permanganate. Magnification: X 26,000
Fig. 49. Higher magnification of a dividing micronucleus in *Blepharisma*. Spindle filaments (SF) are surrounded by fine material (FM) which is much like the nucleoplasm of the macronucleus (MA). Filaments extend continuously from pole to pole or show apparent termination at the fibrous chromosomes (CH). The nuclear envelope is intact but the double nature is less obvious and fine material appears to be attached. Ribosomes are absent from the mitotic apparatus; filaments appear to terminate at the nuclear envelope, no centrioles are present. The macronucleus is at the condensed stage and filaments (EF) are found closely appressed to the cytoplasmic side of the nuclear envelope. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 37,000
accumulated in a large mass at one end of the new daughter, and well
defined spindle filaments are present (Fig. 53, SF). By early telophase,
the chromatin mass has regained its spherical form, and the dense inter-
phase reticulum is obvious (Fig. 51, CR). Daughter micronuclei are
separated by distances greater than 20 μ.

The micronuclear division is apparently asynchronous (all micronuclei
do not enter division at the same time), since interphase micronuclei and
metaphase figures are regularly found in the same section.

By the time nuclear changes are observable in division, the stomato-
genic process is well underway. Just posterior to the new contractile
vacuole of the proter, the new adoral zone of membranelles (Fig. 55, AM)
is well developed, coincident with micronuclear metaphase. The cilia
appear to be morphologically complete, kinetosomes are well aligned, and
rootlet fibrils are well formed; the vesicles and abundant ribosome-like
particles observed in association with the newly forming primordia of
regenerators are present.

Near the posterior terminus of the peristome of the proter and less
often in other cortical regions of nondividing organisms, elongated
fibrous structures are occasionally found. These trichite-like shafts
appear in cross-section as circular bundles approximately 90-100 μ in
diameter and are composed of closely packed, 8 to 10-μ filaments (Figs.
57 and 58, FS). The shafts are hollow when appropriately sectioned (Fig.
57, left) and are directed toward the cell surface. Their tapered end
is sometimes found to distend the pellicle which appears in all cases to
be continuous over their tips (Figs. 57 and 58).

In this same region numerous vesicles are present, the walls of
which consist of at least two appressed membranes plus fine adhering material; there is a strong morphological resemblance between these vesicles (Fig. 58, HV) and the pellicular membrane complex. Anomalous ciliary shafts with unusual filament patterns (Fig. 58, IC) are present in the same area. Ciliary shafts that lack surrounding membranes have been found lying free in the cytoplasm (Fig. 58, CS); the filaments are well defined, are properly oriented, and are followed in the section plane for approximately 2.5 μ.

Organisms in the latter stages of division, when the macronucleus has reached its greatest elongation and the division furrow is prominent, usually contain numerous mitochondria showing greater length than usual (2.5 μ as in Fig. 56) and well defined constrictions (Fig. 56). These forms are also found in other stages but much more infrequently.

Observations of cytokinesis and the developing division furrow, cytokinesis, have not been included in this study and will perhaps be the subject of a future investigation.
Fig. 50. Near cross-section through the micronucleus in metaphase. The micronucleus is sectioned through the metaphase plate at a slight angle so that the chromosomes (CH) appear to be displaced to one side. The spindle filaments (SF) are tubular in cross-section and appear to be present evenly through the micronucleus. The nuclear envelope (NE) is intact except near SF where a break attributed to polymerization damage is present. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 54,000

Fig. 51. Late telophase micronucleus shows the chromatin mass (CR) typical of interphase reformed, and only a few spindle filaments (SF) remain intact. The macronucleus (MA) is beginning to lengthen at this time; however, distinct morphological changes are not obvious. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 47,000

Fig. 52. Macronucleus at the condensed stage often has membrane-limited filament bundles arranged near its envelope; these bundles are thought to represent cross-sections through micronuclear, late anaphase mitotic figures. A moderately dense, round body (RB) is found within the macronucleoplasm. Blepharisma fixed with osmium tetroxide vapor and section stained with potassium permanganate. Magnification: X 16,000
Fig. 53. A telophase, daughter micronucleus in *Blepharisma*. The chromatin mass (CR) has reformed and appears as if extending chromosome-chromosome filaments (SF) had forced it to one side. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 39,000

Fig. 54. High magnification of the metaphase plate and immediate area. The fibrous chromosomes lack structures identifiable as kinetochores; component fibers are not clearly resolved. Continuous and chromosomal spindle filaments (SF) can be distinguished; a faint periodicity is sometimes apparent along the filaments. Osmium tetroxide vapor-fixed and section stained with calcium permanganate. Magnification: X 90,000
Fig. 55. The new adoral zone of membranelles (AM) in dividing Blepharisma. By the time the micronuclei have entered division, the stomatogenic process is well underway. The cilia are morphologically complete, and the rootlet fibers are well formed. A small portion of the condensed macronucleus (MA) is visible in the lower left corner of the micrograph. The new contractile vacuole (CV) of the proter is essentially completed at this time. Osmium tetroxide vapor-fixed and section stained with potassium permanganate. Magnification: X 8,100

Fig. 56. Dividing and regenerating Blepharisma, particularly late stages, often show long mitochondria which are sharply constricted at the mid-line. Under the phase microscope, dumbbell-shaped bodies are observed to be present in the moving endoplasm during the latter stages of division and regeneration and may represent constricted mitochondria. This mitochondrion is approximately 2.5 μ in length and could quite logically be interpreted as a "dividing" form. Osmium tetroxide vapor fixed and section stained with potassium permanganate. Magnification: X 59,000
Fig. 57. Section just posterior to the peristome of the proter in a dividing *Blepharisma*. The cortex in this area shows numerous fibrous shafts (FS), many protrude the pellicle which remains continuous over their tips. Cross-sections of cilia display the typical filament pattern. Osmium tetroxide vapor fixed and section stained with calcium permanganate. Magnification: X 50,000

Fig. 58. The same area as in Fig. 57 but from a different dividing organism. Fibrous shafts (FS) are present near the surface and some distend the pellicle (lower right). Imperfect cilia (IC) which have a poorly organized filament pattern are also found. The unlettered arrow marks the site of the ectoplasmic-endoplasmic fibrous band. Heavy-walled vesicles (HV) are present near the surface and lack internal structure; their walls resemble the pellicle complex. A well formed ciliary shaft (CS) is found within the cytoplasm; the filaments are in the appropriate orientation, however, a membrane does not limit the structure. Osmium tetroxide vapor fixation and stained with calcium permanganate. Magnification: X 35,000
The last ten years have seen the development of procedures for the isolation and analysis of cell fractions, even to the level of the nucleotide and amino acid sequences of their respective polymers. The structure in terms of molecular configuration is known for a number of isolated, biologically important substances; moreover, factors such as ionic strength, pH, temperature, and chemical reactivity are used to manipulate the macro-molecular form, and in a great number of cases only slight variations in the immediate environment induce pronounced configurational changes. There exists, however, a considerable disparity between this level of investigation and our knowledge of the nature of macromolecular form and interaction in the protoplasmic milieu. Even more obvious is the paucity of information concerning the specific changes induced in cytoplasmic constituents in toto by the action of physical and chemical agents used to establish a "life-like" chemical and structural preservation. As a matter of fact, the biochemistry of dissolution and precipitation of chemical moieties during preservation, the "fixation" of cells, is so poorly understood that only broad generalities are advanced as explanation.

Until the development of the electron microscope, the finer elements of cellular structure could not be resolved; therefore, the degree of preservation, particularly in terms of specific molecular interaction was not of practical importance. At the present time, resolution at the macromolecular level is consistently obtained, and considerations of
Fixative action become increasingly important especially as macromolecular function is elucidated. Osmium tetroxide has been the fixative agent of choice and will remain an essential and basic tool in electron microscopy for many years to come. All contributions toward understanding more clearly the conditions and mechanisms of its fixative action will aid in realizing its technical possibilities and in interpreting the results obtained.

Fixation invariably involves formation of new crosslinks between tissue molecules; if such links cause the clumping together of particles, the result will be bad fixation. Bonding more extensive than is required may result in the formation of irregular coacervates with empty areas between them. In this regard, Wolman (1955) proposes that good fixation would be typified by many links between many molecules with attractive forces of intermediate strength. Herein lies the problem which confronts the electron microscopist; what agent and combination of conditions are requisite to attain accurate preservation of cellular fine structure?

The reactivity of osmium tetroxide with a great number of biologically significant materials (Bahr, 1954) and tissue components (Wolman, 1957) has established a basis from which possible explanations of fixative action can be developed, but little work has been reported which elucidates the specific, chemical reactions involved. Wolman (1955) and Wigglesworth (1957) propose that osmium tetroxide-cross-linking at ethylenic double bonds, particularly in oriented lipids, is an important mechanism in fixation. The chemical reaction of osmium tetroxide with sulfhydryls, terminal amino groups, and possibly alcoholic groups may result in the formation of crosslinks or in a denaturation of proteins effecting
insolubility. The delineation of such reactions has not been attained and awaits careful chemical analysis. Wolman (1957) suggests that osmium tetroxide reacts with tissue components in at least three different ways as judged by the reaction product; no interpretation is offered for this observation. A lucid explanation for osmium tetroxide fixation is not currently available.

It is the ancillary conditions of osmium tetroxide fixation with which this research bears directly. Conditions of fixation, concentration of osmium tetroxide, buffer importance and pH, and fixation time will be considered in reference to osmium tetroxide vapor fixation. The preservation of the labile spindle filament will also be discussed.

Concentration

The preponderance of research reporting the use of osmium tetroxide as the fixative agent reveals a nearly consistent utilization of 1% concentrations. Lower concentrations are seldom reported while higher concentrations (2%) are occasionally encountered. It is difficult to establish a rationale for such concentrations, at least, in instances where the ratio of fixative to tissue volume is great and little extraneous matter is added with the tissue. Palade (1952) suggested originally that a 2% solution might be used to advantage, and later Palay and Palade (1955) reported superior results when 3% or 4% solutions were employed for tissues particularly rich in reducing substances. Frigerio and Nebel (1962) contend that a slight decrease below 1% osmium tetroxide levels results in serious variability in micrographic product, particularly in quantitative work. Observations made in the current work are in apparent
contradiction to the blanket use of the generally recommended concentra-

tions.

The concentration of osmium tetroxide in hanging-drop preparations, as similar as possible to those utilized in the fixation of protozoa, indicates that the preservation of tissue fine structure attained in this study has been achieved with much lower levels than are customarily employed. Spectrophotometric measurements reveal that the hanging-drop increases in osmium tetroxide concentration at approximately 0.10% per minute so that at the end of three minutes the concentration has reached only 0.3%.¹ When compared to the concentrations and times generally employed, these values appear to be extremely low; it is clear, nevertheless, that these concentrations are sufficient to produce a satisfactory and probably superior preservation of protozoan fine structure. However, it is not known at the present time how significant the method of fixation itself is in achieving these results. That the concentrations of osmium tetroxide currently employed may be unnecessarily high for many applications is apparent.

Buffering and pH

The utilization of buffered osmium tetroxide stems from Palade's original experiments (1952) which demonstrated that a wave of acid pH, to which Palade ascribed tissue damage, preceded the fixation front that is

¹These values are thought to be accurate to the limit imposed by the difficulties encountered in the collection and handling of the osmium tetroxide vapor-exposed droplets. The fact that the extinction values obtained for the $\text{OsI}_6^{2-}$ complex at known osmium tetroxide concentrations are essentially identical to values obtained by Frigerio and Nebel (1962) is indication that the technique was correctly applied.
defined by the darkening of the tissue. It may be more correct to recognize the zone of pH change as the actual fixation front, for if the reactive groups with acidic and basic behavior present in cytoplasm are examined in terms of their in vitro reactions with osmium tetroxide as determined by Bahr (1954) and in terms of cytochemical detection (Wolman, 1957; Wigglesworth, 1964), it is apparent that amino and sulfhydryl groups are reactive while carboxyls are not; therefore, an acid pH change is expected at the site of reaction. The zone of darkening represents the region in which further reduction of osmium occurs with the resulting formation of darker, lower oxides that are not contributory to actual tissue preservation.

That the shift to an acid pH actually distorts fine structure preservation is not definitely established. Furthermore, it may be difficult to establish that if the acid pH shift does damage spatial orientation or chemical integrity, that such damage is more serious than that resulting from exogenous buffer salts flooding the tissue in advance of the penetrating osmium tetroxide.

Robbins (1961) suggests that osmium tetroxide kills the cell but does not instantly fix it; if this concept is factual, there is an appreciable period of time between the exposure of the intracellular to the extracellular environment and the total macromolecular immobilization by osmium tetroxide. The ultimate quality of fixation is probably determined during this brief prefixation period of interaction between the intracellular and the extracellular environments. With buffered fixatives, it is almost certain that buffer salts reach most regions of tissue pieces before the osmium tetroxide. The buffer-mediated extraction
becomes important, therefore, not only during fixation (Palade, 1956; Claude, 1961) and the following steps (Claude, 1961) but also during any interval preceding the arrival of the fixative agent.

The inconsequence of omitting the buffer in osmium tetroxide has led Claude (1961) and Malhotra (1962a, 1962b) to question the significance of buffering fixatives and, further, to advocate its deletion in many applications. Sjöstrand (1956) maintains that pH is of little consequence during fixation and that the buffer salts are effective only as they adjust the tonicity of the fixative; this concept was reemphasized by Tahmisian (1964). However, the importance of osmotic phenomena affecting fixation may also be challenged since there is evidence that the physico-chemical barriers so important in living systems are profoundly disturbed or are no longer effective once the membranous structures have been modified by fixation (Claude, 1961).

Osmium tetroxide vapor fixation as employed in this investigation represents osmium fixation without controlling pH or tonicity. It may be (1) that control of these conditions is generally unimportant as suggested by others, (2) that the rapidly increasing concentration of osmium tetroxide when applied as vapor to the fixation droplet allows changes of pH and tonicity to be minimized, or (3) short fixation of this type is optimal because it is carried out only with endogenous conditions of pH and salt concentrations. It is certain that a labile cell structure has been preserved with fewer disrupting influences than other methods imposed, so a stronger inference than before can be made that the filament is the in vivo structure of the mitotic apparatus. The obvious conclusion is that the necessity of adding buffers to osmium fixative solutions is
once again questioned and, furthermore, that the deltion of buffer is advisable under certain conditions.

**Fixation time**

Palade (1956), in an analysis of osmium tetroxide fixation, clearly demonstrated the extraction of proteinaceous materials from tissues exposed to buffered fixatives for extended periods; he attributed this extraction to the acetate-Veronal buffer. Claude (1961) and Pease (1961) have emphasized the importance of short fixation times. The latter author reports that fixation times as short as three minutes are satisfactory in most respects and suggests that complete fixation occurs very quickly, within minutes or even seconds of the time when the fixative reaches the tissue.

The fixation times employed in this study reaffirm the adequacy and possibly, the desirability of extremely short fixation. Fixation times were not in excess of three minutes, and the degree of preservation was excellent in terms of the criteria suggested by Pease (1961). In light of the low osmium tetroxide concentrations employed, such brief fixation is decidedly unorthodox as compared to usually recommended procedures. On the basis of the fixation achieved in this investigation, it is recommended that much shorter fixation times than those routinely employed should often be used and should be seriously investigated.

**Osmium tetroxide vapor fixation and the preservation of spindle filaments**

The fibers of the mitotic spindle are described by Inoue (1964) as labile structures existing in a dynamic state of flux which are capable of being readily built up, broken down, or reorganized depending on the
activity of centers and the physiological state of the cell. This concept is extended by the proposal of Roth (1964) who suggests a functional metastability in which elongation of the filaments is accomplished by intussusception of materials along their lengths, and shortening is accomplished by the removal of materials. The instability of the mitotic apparatus is further emphasized by Mazia (1961) and specific conditions necessary for the preservation of the isolated mitotic apparatus are recognized (Harris and Mazia, 1962).

With these considerations in mind, it is not at all surprising to find that the fine structure of the mitotic apparatus has been poorly preserved by the usual techniques of fixation for electron microscopy. In some cases modifications have been made in fixation (Sato, 1958; Lehmann, Henzen, and Geiger, 1962) that have so coarsened the fibrous structures that little or nothing was added to the light-microscope image. Only when divalent cations were added to osmium tetroxide fixatives (Roth and Daniels, 1962; Harris, 1962) were the tubular filaments of the mitotic apparatus preserved with clarity and consistency. The demonstrated stability of the isolated mitotic apparatus at pH 6.2 or lower (Mazia, et al., 1961; Kane, 1962) has been extended to fixation of dividing cells for electron microscopy (Roth and Jenkins, 1962; Harris, 1962) and results in filament preservation in the absence of divalent cations. Thus, two conditions for the preservation of spindle filaments with osmium tetroxide are recognized; fixation in the presence of divalent cations and fixation at an acid pH in the absence of divalent cations. In addition, fixation with glutaraldehyde followed by post-fixation with alkaline osmium tetroxide in the absence of divalent cations has resulted

Osmium tetroxide vapor fixation results in good preservation of the spindle filaments of the micronuclear mitotic apparatus of Blepharisma and the mitotic apparatus of giant amebae as well as tubular filaments within the micronucleus of P. multimicronucleatum. If we accept the conclusion of Roth and Jenkins (1962) and Harris and Mazia (1962) that exact osmium tetroxide fixation conditions are essential in preserving the labile spindle filament structure, then some concordant explanation for stabilization by osmium tetroxide vapor is required.

The conditions of vapor fixation in the hanging-drop are defined first by a slightly acid pH. Although the pH was not determined for the fixation droplet, the culture fluid and distilled water were both known to be near pH 6.8. The preservation of the spindle filament structure might, therefore, be attributed to the acid pH. However, it is also evident that the pH was not controlled by the addition of exogenous buffer and so could vary considerably (become more acid?) during fixation. The importance of an acid pH in filament structure preservation by vapor fixation might be investigated by adjusting the droplet to a slightly alkaline pH with a dilute buffer.

Roth et al. (1963) suggest that divalent cations participate in stabilization of spindle filaments by cross-linking component elements probably at adjacent sites. It is not known whether divalent cations are essential in in vivo formation and/or function of filaments or if the cation is effective only during the fixation process. If the first
alternative is correct, a well preserved filament, corresponding closely to the in vivo structure would result from a fixation procedure not extracting or displacing divalent cations. The second choice introduces the possibility that divalent cations act to stabilize filaments in a non-physiological manner.

Alkali salt solutions (NaCl, KCl) are known to have a dispersing and eventually liquifying effect upon organic hydrophilic gels; the effect can be balanced by adding small amounts of alkaline earth salt (Ca,Mg) in the case of physiological materials as well as colloidal models (Höber, 1945). Harris and Mazia (1962) suggest that osmic fixation as used for other cell structures would be adequate if the osmium tetroxide were given a chance to fix the structure before it disorganized; therefore, the instability of the spindle filament in the presence of the considerable Na⁺ contained in the Palade fixative may result from the more rapid penetration by the sodium and its dispersing effect upon the spindle protein before the osmium tetroxide has effected fixation. The stabilizing effect exhibited by the addition of divalent cations might be explained in terms of the hypothesis reiterated by Höber (1945); that a normal function may require the existence of a certain colloidal state, and this necessitates a certain balance of mono- and polyvalent cations. If a preponderance of monovalent cations is present, dispersity of a structural colloid is abnormally great; the proper amount of divalent cation increases or insures the necessary rigidity. The hypothesis considered earlier, that divalent cations (Ca++) are essential in spindle filament formation and function, would suggest that an "antagonism" results from the introduction of large amounts of monovalent cation
(Na\(^+\)), that the divalent cation (Ca\(^{++}\)) is replaced, and that the filaments disperse, all before the osmium tetroxide reaches the necessary sites. In fixation by osmium tetroxide vapor, large amounts of Na\(^+\) are absent and therefore, no flood of exogenous salts precedes the penetrating osmium tetroxide. Thus, it may be that there is no induced dispersion of the highly labile spindle filament protein and preservation results.

Two possible explanations remain for the ability of osmium tetroxide vapor to preserve spindle filament structure; both are concerned, as were the above considerations, with the highly labile nature of the filaments. The first considers the importance of rapid penetration in fixation. If the spindle filament is maintained in function and structure by a dynamic process (Roth, 1964), then cell death would allow disassembly of the filament integrity unless fixative reaches the necessary sites very rapidly. Osmium tetroxide is notoriously slow in penetration; however, the penetrating power of osmium tetroxide vapor is reported to be much greater than that of solutions, at least as applied to objects such as nerve tissue suspended over a 2% solution (Breusch, 1942). Whether or not the same effect is achieved by placing cells in a small droplet of fluid is not known; however, it may be that the very low initial concentration coupled with the rapid gradient increase in some way allows a more rapid penetration into the cell, and the spindle filament is fixed before dispersion can take place. The second is in reference to the demonstrated extraction of material from cells during fixation (Palade, 1956; Claude, 1961). It follows that components of marginal stability would be most effected, and thus the length of time for which the tissue is in contact with the fixative becomes important. Preservation of the
filament morphology by osmium tetroxide vapor may result only from fixation for the extremely short times used so that fixative mediated extraction does not occur.

The stabilizing action of osmium tetroxide vapor fixation cannot be explained resolutely at this time; the preceding considerations are, at best, a heuristic approach and demand further experimentation before definitive conclusions can be made. However, this study has established the fact that osmium tetroxide vapor fixation is valuable for certain studies of sectioned cells and that a further understanding of osmium tetroxide fixation will result when the parameters of vapor fixation are determined.

Filament Systems

Ciliate protozoa are distinguished primarily by two striking features, their highly differentiated cortex and their nuclear contingent which consists of the distinctly different micro- and macronuclei. The cortex is predominantly the expression of a precise disposition of filamentous components, and thus the morphogenetic processes of ciliate stomatogenesis are the result of filament formation and organization into functional systems. In mitosis the involvement of fibrous elements is substantiated by the excellent, polarization microscope studies of Inoue' (1953, 1964) on living cells and by the recent electron microscopic studies of mitosis (Roth and Daniels, 1962; Harris, 1961, 1962; Roth and Shigenaka, 1964; Schuster, 1964). Only the recent observations of Roth and Shigenaka (1964) have clearly demonstrated the presence of tubular filaments within the dividing micronucleus and indicated the existence of an extranuclear
filament system possibly concerned with macronuclear division. It is apparent that the filaments of the mitotic apparatus as well as those of protozoan, filament systems have the same basic, tubular morphology and size.

The present investigation has permitted a more complete description of the micronuclear division apparatus, the extranuclear filament system of the dividing macronucleus, and the cortical filament system of dividing and regenerating Blepharisma; these findings will be discussed relative to the structure, function, and formation of the nearly ubiquitous component, the tubular filament.

The formed filaments of the ectoplasm and infraciliature

The subpellicular filament system is composed of tubular filaments approximately 16 μm in diameter that lie just under the pellicle in an orientation parallel with the long axis of the cell. A similar system is described for the flagellate Peranema (Roth, 1959) and for Spirostomum (Yagi and Shigenaka, 1963; Finley, et al., 1964) but has not been found in the heterotrichs Condylostoma or Stentor. Roth identifies the complex as the "subpellicular filament system" and suggests that it may be involved in the slow, contractile movements of Peranema. Yagi and Shigenaka also suggest a contractile function; they propose that the system serves in the spiral contraction of Spirostomum and identify the components as "subpellicular fibrils". Finley et al. (1964) use the term "peripheral ectomyoneme" for the same structures but do not speculate on a possible function. The subpellicular filament system is well developed in Blepharisma; however, this organism is only slightly contractile at best
(Pitelka, 1963), and observation of organisms in culture indicate that blepharismas are able to produce only a flexing or bending of the anterior one-third of their body. Perhaps the system serves primarily in mechanical support and secondarily, in limited contraction.

The birefringent fibers observed in living Stentor were demonstrated by Randall and Jackson (1958) to consist of stacks of filaments, "Km fibers", and were identified as homologues of kinetodesma. The system has also been described in Condylostoma (Yagiu and Shigenaka, 1960) and Spirostomum (Yagiu and Shigenaka, 1963) where it was called the "longitudinal fibrillar bundle" and again in Spirostomum (Finley et al., 1964) where it was called the "lateral ectomyoneme".

Since, in each case, these structures were said to be identical to the traditional, kinetodesmal fibrils and since unnecessary new terms should be avoided, the use of "kinetodesmal fibril" for the compound structure and "kinetodesmal filament" for component 15 to 16-mu tubular elements is suggested.

Hypotheses of kinetodesmal filament function are made largely on evidence from their association with other organelles. In Blepharisma as in the other heterotrichs studied, the association with the somatic kinetosomes and the overlapping pattern displayed by the compound fibrils suggests a system designed for conduction or coordination. This hypothesis agrees with that advanced by Yagiu and Shigenaka (1963) to explain the metachronal wave of cilia.

The rootlet fibrils of the adoral membranelles in Blepharisma are composed of 20-mu, tubular filaments that are joined together to form the heavy bundles extending deeper than other filament systems so far con-
sidered. That the synchronous and intense stroke of the cilia of a membranelle requires a good anchor is almost a certainty. Since other connections exist between the kinetosomes of membranellar cilia, coordination need not be dependent solely on the rootlet filaments, and a supporting or anchoring role for this filament system may be suggested. Sleigh (1962) states that all information available in regard to rootlet function in Stentor points to the fact that they serve to anchor the bases of the membranelles.

Morphologically the kinetosomes and cilia of Blepharisma are typical. However, membranellar cilia do bear membranous projections which terminate in bulbous swellings and undoubtedly serve to unite by entanglement the component cilia of each membranelle (Roth, 1956; King et al., 1961).

The last complex to be considered differs considerably from the 16 and 20-µm filaments described earlier; it consists of very fine fibers approximately 4 µm in diameter that form a rather loose bundle at the ecto-endoplasmic boundary. This system is found in Spirostomum and has been called both a "contractile fibrillar system" (Yagiu and Shigenaka, 1963) and an "endomyoneme" (Finley et al., 1964). Neither Blepharisma nor Spirostomum show the structure to be as well-developed as the so-called M-bands of Stentor (Randall and Jackson, 1958) or a system found in Isotricha (Roth, 1964) for which these authors suggest a motile function. Both Randall and Jackson (1958) and Yagiu and Shigenaka (1963) call attention to the morphological similarity between these fibers and the myofilaments of smooth muscle in higher animals; the latter authors speculatively designate this structure as "...an exactly true contractile element". Examination of the micrographs presented by these authors and
Finley et al. (1964) would indicate that the same system is at least as well developed in *Blepharisma* as in *Spirostomum*. In light of the limited contraction exhibited by *Blepharisma*, it is premature to designate a contractile function.

Controversies over terminology seldom result in useful contributions. As Finley et al. (1964) have observed, this matter is secondary and will be resolved with sufficient observations. A functionally neutral and consistent terminology which will simplify reference to heterotrich fine structure can now be derived and should be followed. The terminology suggested here closely follows the early literature and excludes inferences to function.

It is a paradox that the beautiful fibrillar systems of ciliates cannot be assigned specific functions. As Roth (1958) has suggested, there is need for questioning the validity of morphological comparison of filamentous components in different organisms without knowledge of chemical composition, origin, or function. Even with morphological and chemical characterization now available, knowledge of filament function has progressed little beyond the speculations reviewed by Taylor (1941) who suggested four elementary functions: (1) elasticity, (2) mechanical support, (3) contractility, and (4) conductivity. That filaments participate in movements or structural rigidity is almost certain, but the precise identification of function for a particular filament system or of theories explaining means by which the functions are completed rest primarily on teleological bases.

Until experimental approaches employing *in vivo* alteration of these filament systems are devised such as those used by Inoue (1964) and
Roth (1964) for study of the mitotic apparatus, electron microscopic investigation can contribute little to an elucidation of filament function.

Filament formation in stomatogenesis

The formation of kinetosomes is generally assumed to be an essential process of stomatogenesis; it should be remembered, however, that the kinetosome represents an instrument in morphogenesis and cortical heredity but is not the cause (Sonneborn, 1963). The important role played by the kinetosome is emphasized by the fact that a large proportion of the cortical filaments are known to be, or suspected of being, physically connected with kinetosomes. It has also become generally accepted that tubular filaments are formed under the influence of particular cell structures (Gall, 1961; Mazia, 1961; Pitelka, 1963; Roth and Shigenaka, 1964; Inoue', 1964); therefore, the kinetosome becomes a focal point of any investigation directed toward the elucidation of ciliate morphogenesis.

At the present time the hypotheses for the formation of new kinetosomes can be considered under two broad categories: (1) formation dependent upon a preformed unit which would include bipartition, budding, and induction; (2) formation independent of preformed kinetosomes, that is, a de novo origin.

The views of Lwoff (1949, 1950) represent the kinetosome as a genetically autonomous structure that always arrives from the division of a pre-existing kinetosome. Porter (1960), Grimstone (1961), and Ehret and DeHaller (1963) all express reasons for the doubtful validity of this concept. Mazia (1961) summarizes the currently held objections to kinetosomal replication by division when he suggests that the division
of the kinetosome is as difficult to envision as the formation of a bacteriophage by direct division. In light of the paucity of evidence so far accumulated for division it seems most probable that kinetosomes do not originate from pre-existing ones by a fission process.

On the basis of evidence from studies of centriole replication, Mazia (1961) argues that kinetosomes reproduce themselves from a germinal part and suggests a three-step process by which replication is accomplished. Dirksen (1961) explains the occurrence of centrioles in artificially activated sea urchin eggs by proposing that a precursor (germinal part) exists in the egg and activation induces aggregation of essential materials with subsequent formation of centrioles.

In the snail *Viviparous*, Gall (1961) has found centrioles which appear to develop from smaller centriole-like forms that he terms "procentrioles". The origin of the procentriole is still unknown but its close association with a mature centriole might implicate an inductive process more than a generative one. In this connection, Grasse' (1961) has concluded from examining many flagellates with the electron microscope and without observing evidence for direct genesis of a new blepharoplast that this organelle is formed de novo by induction. After producing evidence for the presence of RNA and the absence of DNA in kinetosomes, Hoffman (1964) considers the possibility that enzymes bound to the kinetosome synthesize some linking agent which is spatially oriented in the vicinity of the kinetosome and gives rise to a replica by this inductive process. Also, Randall and Hopkins (1962) believe on the basis of electron microscopic observations, that existing basal bodies may supply "unorganized components" towards the genesis of a new neighbor and have tried diligently,
therefore, but without success to demonstrate the presence of DNA. Like theories involving a generative mechanism formation by induction is appealing but difficult to verify.

The de novo origin of kinetosomes has been supported by Ehret and Powers (1959) and Ehret and DeHaller (1963). In the latter study, small circular vesicles were found at sites where new kinetosomes were expected to appear. This possible kinetosomal precursor resembles a rather thick-walled body which Randall and Hopkins (1962) believe gives rise to the filamentous kinetosome. In transforming amebae, Schuster (1963) has observed a 22-mµ structure composed of ordered granules and bounded by portions of the endoplasmic reticulum which he proposes are centers of kinetosomal production.

The present study similarly has not resulted in the observation of direct kinetosomal replication by division, budding, or intimate inductive association. However, a 100 to 150-mµ, heavy-walled vesicle found at sites where kinetosomes are subsequently expected is implicated and closely resembles the suspected kinetosomal precursor described by Ehret and DeHaller (1963) and by Randall and Hopkins (1962). Since intermediate stages have not been observed, the evidence rests only on the coincident occurrence of the visicle at strategic locations.

An electron microscopic study designed to investigate basic processes associated with the replication of kinetosomes must allow for at least three major difficulties (Pitelka, 1963): (1) in protozoa the replication of kinetosomes commonly precedes all other events and is generally thought to be completed by the time visual signs of morphogenetic activity are apparent, (2) if new kinetosomes are organized independently of old ones,
nothing may be recognized until the new kinetosome is present and, (3) the sample available to electron microscopic examination is so minute in both time and space that a rapid process occurring in a very small area is very elusive. Concerted efforts in defining the critical stage of regeneration during which replication of kinetosomes occurs will allow the first and last consideration to be minimized, and the replicative process will undoubtedly yield to such an approach. The regenerative inhibition shown by colchicine (Hirshfield and Pecora, 1955; Giese and McCaw, 1963b), mercaptoethanol, and cold treatment (Giese and McCaw, 1963a, 1963b) when applied at early stages and possibly in combinations might serve as methods for synchronizing large numbers of regenerating organisms. It is probable that these treatments serve to interfere with the assembly of filaments and/or kinetosomes. In fact, the forming filaments may have a lability similar to the spindle filaments. Therefore, regenerating forms in which stomatogenesis is delayed by chemical or physical treatments should be examined under the electron microscope after fixation under conditions that are known to preserve the labile spindle filament.

In regard to the subject of shaft formation in cilia, the extent of knowledge is limited to preliminary observations comparable in precision to those made for kinetosomal replication. The observations of Sotelo and Trujillo-Cenoz' (1958), Tokuyasu and Yamada (1959), Roth and Shigenaka (1964) suggest that shaft formation occurs only at or near the cell surface. This study of Blepharisma has shown a concentration of material at this location in forming cilia and thus lends some support to the concept of intussusception of molecules at about the distal end
of the kinetosome.

Sorokin (1962), Manton (1957), and Schuster (1963) have observed formed ciliary shafts at a considerable distance interior from the cell surface, and Sorokin and Schuster attribute the presence of these axonemes to formation at this site while Manton interprets their presence as related to resorption.

The present investigation has revealed somewhat aberrant cilia, ciliary shafts within the endoplasm, and trichite-like fibrous shafts in the so-called growing zone posterior to the mouth of the prospective proter (Suzuki, 1957). Present observations do not allow interpretation of the significance of these structures, but continued study of this zone at earlier division stages will be valuable.

The formation mechanisms of kinetosome and cilia are not currently known. However, the information summarized above when correlated with biochemical and physiological findings will aid in exposing the nature of these essential morphogenetic events.

Filaments of the nuclear apparatus

The electron microscope has clearly revealed the filamentous components within the mitotic apparatus of numerous animal cells (Harris, 1961, 1962; Roth and Daniels, 1962; Kane, 1962; Dales, 1963; Thomas, 1964; Robbins and Gonatas, 1964; Schuster, 1964; Roth and Shigenaka, 1964; Szollosi, 1964) and also within a growing number of plant cells (Roth, Wilson, and Bowen, 1963; Ledbetter and Porter, 1963, Berlin, 1964; Allen, 1964). The spindle filament is shown to possess a consistent morphology. In cross sections a dense cortex surrounds a less dense center; the
diameter is somewhat variable extending from 15 to 30 μm although the range is more consistent for animal cells where the diameters range between 15 and 20 μm. This tubular filament is now known to be a component of the mitotic apparatus of four types of nuclear division; anastral, astral, acentric without breakdown of the nuclear envelope, and centric without apparent breakdown of the nuclear envelope. The acentric division in which the nuclear envelope remains intact is typical of ciliate, micronuclear mitosis and introduces several interesting problems relating to the currently held concepts of the mitotic process.

For many years, the study of dividing ciliates did not disclose the presence of discrete filaments in either the macro- or micronucleus (Roth, 1959; Roth and Minick, 1961; Elliot, et al., 1962; Elliot, 1963); only the observations of Roth, and Roth and Minick indicated the possible presence of filaments associated with the amitotic macronuclear division. Recently, Roth and Shigenaka (1964) have demonstrated filaments within dividing macro- and micronuclei as well as in the area surrounding the nuclei. Several reasons are suggested for the inability to elucidate the fine structure of dividing ciliate nuclei: (1) nuclear division is difficult to predict since it is completed before gross morphological signs are visible, (2) ciliate fixation is often less than optimal, and (3) specific conditions necessary for spindle filament preservation have not been employed. These restrictions are overcome in this study, and the fine structure of ciliate nuclear division is more clearly demonstrated than in any previous report.

The micronuclear divisions are mitotic, acentric, intranuclear, and asynchronous. The distinctive division spindle forms within the confines
of the nuclear envelope, and the mitotic process is apparently completed without disruption of the membranes.

The formation of filaments within an intact nuclear envelope presents a question of considerable importance; what is the source of the protein which is aggregated to form the spindle filaments? The fully-formed, mitotic apparatus represents a large amount of specific protein which must be derived without free mixing of nucleo- and cytoplasmic materials; thus the filament precursors either are synthesized in the micronucleus or in cytoplasm and carried into the nucleus across the nuclear envelope. The possibility that the protein is of nuclear origin must be considered remote since the ciliate micronucleus apparently lacks both nucleoli and ribosomes and is thought to be metabolically inert. Cytochemical evidence for the presence or absence of RNA in micronuclei is not conclusive. As much as 14% has been reported (Moses, 1950) while other studies have not demonstrated the presence of RNA (Raikov, 1959, 1962). The micronucleus of Blepharisma does not show particles which could be interpreted as ribosomes; Roth and Shigenaka (1964) report a similar finding in Diplodinium. The presence of RNA in the micronucleus will perhaps be resolved by the use of RNase digestion at the electron microscope level or by biochemical studies on isolated micronuclei. All indications are that the micronucleus engages only in replicative synthesis (DNA) and is not a site of protein synthesis.

The absence of recognizable micronuclear RNA supports the concept that the spindle protein exists in the cell before the inception of mitosis (Went, 1960) and that the process of spindle filament formation or aggregation is independent of precursor synthesis in both time and
location (Roth, 1964). Mazia (1961) maintains that nuclear protein is not sufficient for the formation of the mitotic apparatus, and Mazia and Harris (1962) suggest that the mitotic apparatus in sea urchin eggs forms in the cytoplasm and engages the chromosomes at the time of nuclear envelope breakdown. These considerations lead to the assumption that the filament precursor must be transferred through the intact nuclear envelope.

The swelling of premitotic micronuclei (Suzuki, 1957) may represent, if not the actual up-take of precursor, an indication of permeability changes in the nuclear envelope. When precursors are inside the confines of the envelope, conditions of pH, ion concentration, hydration or the presence of a linking agent (RNA?) may effect the formation of spindle filaments.

In Diplodinium, Roth and Shigenaka (1964) found a layer of fine material just inside the nuclear envelope of the anaphase micronucleus; and suggested that this material may represent an intranuclear store of precursor protein for filaments. No such layer exists in the mitotic apparatus of Blepharisma; however, the entire nucleus is filled with a fine homogeneous material which might serve as the protein reserve for filament formation and extension.

Turning to the initiation of filament formation, Inoue' (1964) has shown and emphasized that the fibers of the mitotic apparatus are oriented and organized by "centers" such as centrioles and kinetochores. Examination of many mitotic figures in Blepharisma has not revealed the presence of kinetochores within chromosomes or of centrioles either within or outside the confines of the nuclear envelope. Schuster's study (1964)
of intranuclear meiosis in *Didymium* and the work of Roth and Shigenaka (1964) also indicate an acentric, intranuclear figure while Berlin (1964) found well defined centrioles associated with the intranuclear mitosis of *Albugo*. If the presence of a "center of aggregation" (Inoue', 1964) is essential, the most likely center for acentric and intranuclear filament formation is at the kinetochore, for which precise structure has not been defined.

However, the role of centers of formation must be questioned. First, there is little evidence from electron microscopic studies that kinetochores exist as discrete entities; however, they may be so highly labile that fixation designed to preserve the formed or forming filament is not sufficient to preserve the fine detail of a structure representing a site of aggregation. Secondly, within the mitotic apparatus filaments are present which are continuous from pole-to-pole and lack connection to chromosomes or centrioles and direct contact with the cytoplasm. In micronuclear division, the lengthening of the continuous filaments is considerable and is probably responsible for the great separation of telophase daughter nuclei. Since these filaments are essentially independent of direct association with known "centers", it is appropriate to implement a hypothesis which considers their formation as resulting from changes in the microenvironment such as pH, ionic strength, or the presence of a specific protein or linking agent; such a concept entails growth of the filament by insertion of material along its length (Roth, 1964).

It is known that the division of micronuclei in *Blepharisma* is correlated with the mid-division stage (condensation) of the macronucleus.
The prerequisites for cell division are (Scherbaum, 1963) thought to consist primarily of a multitude of synthetic reactions aimed at the duplication and separation of highly complex structures and, since the macronucleus is known to be metabolically active and is essential to and reorganizes during regeneration, it is reasonable to suggest that some macronuclear product is essential to the initiation of mitosis. All micronuclei do not divide in synchrony, and it appears that some may forego division completely in both cell division and regeneration. The factors which initiate division are of considerable interest generally and further studies of macronuclear-micronuclear interdependence may yield valuable information.

Turning to the macronucleus, all evidence indicates that its division is by an amitotic process. Tubular filaments have been observed within the macronucleus (Roth, 1959; Roth and Minick, 1960; Roth and Shigenaka, 1964), but a well organized functional apparatus of significance in macronuclear division is not recognized. The latter authors report an extranuclear filament system which encloses both the macronucleus and micronucleus in Diplodinium. They also state that the system becomes more highly developed during division and, therefore, suggest some involvement in the amitotic process. In Blepharisma the dividing macronucleus undergoes a very great increase in length within a short period of time. Just prior to this extension, filaments are observed for the first time in close apposition to the cytoplasmic side of the nuclear envelope. Later, when the macronucleus is undergoing elongation, these filaments are found in great numbers and are consistently directed along the longitudinal axis of the macronucleus; cross-sections indicate their
uniform distribution circumferentially about the macronucleus.

If these filaments show the same lability as do filaments of the mitotic spindle, then colchicine or cold treatment of cells at particular stages should result in filament disruption and function could be deduced from division delay or the absence of the system in electron micrographs of appropriately handled material. The occurrence of membrane bounded filament bundles within the macronucleus may also be significant in division, but the number is apparently so small that such a role is not likely. The filament bundles found enclosed in membranes outside the macronucleus are not thought to be involved in macronuclear division but are, instead, cross-sections through late anaphase, micronuclear division figures. As Roth, (1964) has pointed out, no adequate mechanism of macronuclear division is yet suggested. Therefore, a filament system showing preferential alignment, a high degree of development, and occurrence in the proper time sequence is justifiably considered to be involved in a prospective mechanism.

The tubular filament is clearly a component of the micronuclear mitotic apparatus; its morphology is consistent with that found for other organisms. Problems inherent in intranuclear mitosis and the inter-dependence of micronuclear and macronuclear division will require intense effort and the application of varied approaches for their resolution. The importance of the extranuclear filament system in macronuclear division is hypothesized and must be examined in greater detail.
SUMMARY

1. Protozoan cells were fixed for electron microscopy by three minute exposures of small hanging-drop preparations to the vapors of 2% osmium tetroxide. *Blepharisma*, *paramecia*, and giant amebae were prepared by this method, and fine structure preservation is considered superior to that resulting from the use of buffered osmium tetroxide solutions.

2. Hanging-drops exposed to osmium tetroxide vapors within a closed container show an increase in osmium tetroxide concentration at the rate of approximately 0.1% per minute. Fixation is effected by a rapidly increasing osmium tetroxide gradient and at concentrations less than 0.3%. Short fixation times and/or low concentrations are recommended and the importance of buffers, salts, or other vehicles is questioned.

3. Filaments of the mitotic apparatus are preserved by osmium tetroxide vapor fixation. The stabilization of these labile structures is attributed to (a) short fixation, (b) the absence of a buffer effected dispersion, (c) rapid penetration of osmium tetroxide when applied as a vapor, (d) the slightly acid pH existing in the fixation droplet and intracellularly, or (e) to some combination of these factors.

4. A comprehensive study of the fine structure of *Blepharisma* has been made. Four distinct filament systems are described; a bimodal distribution of tubular filaments is evident, and a non-tubular fibrous element is also recognized. Possible functional roles are discussed.
and a consistent terminology is suggested.

5. **Blepharisma** were transected to yield astomate fragments which were studied at progressive stages of regeneration. Examination of the cortex and nuclei reveals: (a) micronuclei divide mitotically, (b) organelle concentration is not obvious during any stage, except for an apparent increase in both the Golgi apparatus and ribosome-like particles at very early stages, (c) morphological indications of increased macronuclear activity are not apparent, (d) replication of kinetosomes is not observed; a heavy-walled vesicle present in the proper time and place is implicated as a possible precursor, and (e) a heavy concentration of fine material near the proximal terminus of newly formed or forming cilia is thought to be involved in the development of the ciliary filaments.

6. The dividing macronucleus is surrounded by many 15 μm-filaments which are oriented parallel with its long axis and arranged circumferentially at a site just outside the nuclear envelope. This system is identified as the extranuclear mitotic apparatus and is thought to be essential in macronuclear division.

7. The micronuclear division division is mitotic, acentric, intranuclear, and asynchronous. The distinctive division spindle composed of many 15 μm-filaments, forms within the confines of the nuclear envelope, and the mitotic process is apparently completed without disruption of the membranes. Well defined fibrous chromosomes are present; kinetochores have not been observed.
LITERATURE CITED


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