THE RHIZOPLAST (FLAGELLAR ROOTLET) OF *NAEGLERIA*

THE RHIZOPLAST (FLAGELLAR ROOTLET) OF *NAEGLERIA*

By

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SCOPE AND CONTENTS:

The rhizoplast (flagellar rootlet) of the amebo-flagellate *Naegleria gruberi* has been studied in sectioned cells and in the isolated state. Since the organelle arises, as do the other components of the flagellar apparatus, through a *de novo* assembly, and possibly a *de novo* synthesis during the ameba-to-flagellate transformation, the characterization of the rhizoplast's nature may be of importance in the study of a differentiation process in a eukaryotic cell.

Structurally, the organelle is a periodically-banded, longitudinally fibrous structure arising in the basal body area of the cell and tapering towards its end in the cytoplasm

adjacent to the nucleus. The attachment of the organelle to the basal bodies is mediated through the interbasal body connector and the rhizoplast-associated microtubules. Attachment to the nucleus is unlikely as it has never been unequivocally demonstrated in electron-microscope studies.

Rhizoplasts exhibit a distinct periodicity composed of alternating electron-opaque and electron-transparent bands. Variations in the width of both bands has been observed and is discussed in terms of the possible role of the organelle as an anchor and stabilizing structure for the flagellar complex, with contractility and elasticity being discussed as possible mechanisms of this variation.

An isolation and partial purification of the rhizoplast has been achieved and is described with reference to its possible use as a tool in the biochemical analysis of the rhizoplast. An aggregation phenomenon of dissolved rhizoplast material by divalent cations has been observed and is discussed, keeping in mind the similar phenomena exhibited by the contractile proteins paramyosin and tropomyosin. Collagen, which exhibits a reaggregation from solution, is discussed and tentatively discarded as a possibility for rhizoplast material due to its tendency towards solubilization rather than reaggregation in solutions containing divalent cations.

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INTRODUCTION

Striated rootlets are found in almost all eukaryotic cells that bear cilia or flagella, with a few notable exceptions including spermatozoa of most animals (Pitelka, 1969). Information about these fibres has been confined mainly to the recognizance of their existence in cells and to descriptions of their structure (periodically-banded longitudinal fibres).

The present study was undertaken as an attempt to understand the nature of a particular type of rootlet, the rhizoplast (a striated rootlet of flagellates, connecting basal bodies and nucleus). The questions asked were in relation to structure, attachments to basal bodies and nucleus, and possible chemical nature with answers leading to possible insights regarding rhizoplast function and development.

The organisms used in the study are various strains of *Naegleria gruberi*, one of a small group of organisms known as amebo-flagellates, capable of existing either as amebae or as flagellates, depending upon the prevailing environmental conditions. Fulton and Dingle (1967) devised a method for obtaining, with a high degree of synchrony, a population of flagellates from a population of amebae by

removing them from a growth medium to a non-nutrient buffer. During the conversion process (transformation) amebae acquire flagella, basal bodies, and rhizoplasts through a *de novo* synthesis and assembly.

The structure of the rhizoplast of *Naegleria* has been described as a "transversely striated fibre which connects anteriorly with the kinetosomes and posteriorly may either join with the nucleus or end in the cytoplasm" (Schuster, 1963). This author reported a constant periodicity of 18 nm with a dark band 11 nm wide and a light band 7 nm in width. Dingle (1964) described the structure of the *Naegleria* rhizoplast as "a highly ordered, periodic structure", and further reported that the period is not constant as reported by Schuster, but varies between 6 and 19.5 nm for the dark band, and is constant at approximately 4 nm for the light. Outka and Kluss (1967), looking at the structure of a closely related amebo-flagellate, *Tetramitus rostratus*, reported that "the rhizoplast of *Tetramitus* has a non-uniform periodicity, as to both spacing and direction." They unfortunately did not give details but simply said that "the wide bands exhibited considerably greater variation in size than did the narrow bands." Aside from these reports on variability and on constancy in the *Naegleria* rhizoplast, descriptions in the literature of rhizoplast structure include no mention of constancy or variability of period, the former is assumed.

The complex nature of the mode of attachment of the rhizoplast to the basal bodies has been explored (Schuster, 1963; Dingle, 1964; and Dingle and Fulton, 1966) but has not been fully defined. Schuster proposed that attachment is through a complex arrangement of microtubules into a curved structure that he designates a "spur". Dingle referred to this same structure as a "palisade" of microtubules. Firm attachments were hypothesized by Schuster, but were not substantiated by electron micrographs and Dingle and Fulton reported"we cannot relate the chain of filaments (palisade) in any obvious way to the connection of basal bodies to the rhizoplast, since there is a gap at both ends of the chain." In no instance is mention made of the triangular banded structure seen between the basal bodies cut in cross section, even though it is quite obvious and has a constant arrangement with relation to the basal bodies. Attachment of the rhizoplast to the nucleus (implied in the definition) is uncertain. Schuster reported attachment at the surface of the nuclear membrane in *Naegleria*, and lack of attachment in *Tetramitus* whose rhizoplast ends free in the cytoplasm. Dingle and Fulton observed that the rhizoplast of *Naegleria* runs alongside the nuclear membrane, sometimes through a cytoplasm-filled groove formed by the membrane.

Rhizoplast function is unknown, but hypotheses concerning the question have been advanced, including:

1) a transmission function from nucleus to flagellum (impulse signalling the order to beat), 2) a contractile function mediating flagellar motion, and 3) an anchoring function providing mechanical stability to the flagellum-basal body complex during the flagellar beat cycle. The latter function seems most probable in light of past descriptions (Pitelka, 1969), but is as yet unproved.

Naegleria provides an ideal system in which to study rhizoplast development, since all parts of the flagellar apparatus are synthesized and assembled during the period of conversion from ameba-to-flagellate. Dingle and Fulton (1966) described the assembly and outgrowth of flagella during the transformation process. Outka and Kluss (1967) describeda similar phenomenon for the related amebo-flagellate, *Tetramitus rostratus*, reaching the same conclusions as the previous reference. Studies on basal body development in *Naegleria* have been initiated and are continuing (Dingle, 1967, 1970; and Fulton and Dingle, 1970). Developmental studies of the rhizoplast have not been previously attempted due to the limits of thin sectioning procedures of electron microscopy, which do not allow for the observation of whole organelles without resorting to the laborious task of preparing and observing serial sections.

Procedural approaches taken in this study include electron microscopy of sectioned material fixed in different

ways and treated so as to prevent flagellar beating activity. Careful analysis of electron micrographs, including detailed measurement of rhizoplast periodicity confirmed Dingle's original (1964) observation of variability and added fine-structural detail to the mechanism of attachment of the organelle at the basal body end. The characterization of the banding pattern of the *Naegleria* rhizoplast was enhanced by an isolation procedure releasing intact organelles from the cells. Isolation and attempted purification procedures led to the observation of some organelle properties not seen in sectioned material. For example, the fibrous nature of the rhizoplast not visible in sectioned material, was obvious in isolated organelles. Purification attempts led to studies involving dissolution and re-aggregation of rhizoplast material (rhizoplastin) into periodically-banded structures, a phenomenon which has been described for various proteins and protein systems including: collagen (Goldstein, Parlebas *et al.*, 1969), paramyosin (Hodges, 1952; Cohen, Szent-Györgyi, and Kendrick-Jones, 1970), and tropomyosin (Caspar, Cohen, and Longley, 1969).

This study defines the nature of the rhizoplast periodicity, the nature of the attachment at the basal body end, and provides an isolation procedure which may lead to insights concerning the molecular organization of the organelle. Arising from the findings of this study are

the distinct possibilities of defining the chemical nature and the development of the rhizoplast.

MATERIALS AND METHODS

Organisms

Cells used in this study are various strains of the amebo-flagellate *Naegleria gruberi*, as described most recently by Fulton (1970).

NB-1 was used for most of the initial rhizoplast work. Originally isolated in England by Pringsheim, NB-1 was cloned and designated as such by Fulton and Dingle (1967). NEG, used for much of the latter work was derived from a stock originally isolated by Schuster in California. Successive clonings by Fulton and Dingle led to the present NEG.

The bacterial associate used exclusively in this study for the *Naegleria* strains is *Aerobacter aerogenes*.

Media

The standard medium employed for plate growth and stock maintenance throughout this study was NM as described by Fulton and Dingle (1967). NM is made up in 800 ml batches in stoppered 1 litre Erlenmeyer flasks by mixing the components: Difco Bacto-peptone, 1.6 gm; dextrose, 1.6 gm; K_2HPO_4, 1.2 gm; KH_2PO_4, 0.8 gm; and Difco Bacto-agar, 16.0 gm into 800 ml glass distilled water. The medium is autoclaved

at 18 psi for 20 min and, when cool, poured to a depth of 7 to 8 mm in sterile plastic Petri dishes.

The *Aerobacter* medium used was Penassay Broth (PAB), which consists of 17.5 gm Difco Bacto Antibiotic Medium 3 per litre of glass distilled water. The material is thoroughly dissolved, distributed 6 ml to stoppered tubes, autoclaved at 18 psi for 20 min, and stored at 4°C.

Long term stocks of *Aerobacter* were maintained on slants of nutrient agar, prepared by adding 20.0 gm Difco Bacto-agar to 1 litre batch of PAB before autoclaving at 18 psi for 20 min. The medium was poured, 6 ml to each culture tube, stoppered and stored at 4°C until use.

Batch growth medium is described elsewhere in this section.

Stock Maintenance

Naegleria stocks are maintained on NM. Briefly, the method consists of inoculating cysts from a previous stock plate on NM with 0.2 ml *Aerobacter*. They are maintained at room temperature and transferred as needed. NB-1 was maintained on "spread cultures", in which a cyst-*Aerobacter* suspension is spread over the agar surface. NEG was maintained on "edge plates" (Fulton, 1970), since they do not readily encyst on the spread plates. *Aerobacter*, spread over the whole plate, is inoculated at one edge with a loop of cells taken from the origin of a previous stock plate.

Aerobacter was maintained by successive transfers to PAB from a long term stock which is maintained on nutrient agar slants.

Culture Methods

(a) Plate Growth

NM is not a nutrient medium for *Naegleria*, but is adequate for the growth of the bacterial associate, *Aerobacter*. When approximately 10^5 cysts and 0.2 ml bacteria are spread on an NM plate, the bacteria grow rapidly forming a bacterial lawn on the surface of the plate. *Naegleria* begin to excyst after 2 to 4 hours and engulf the already established bacterial culture. As amebae increase in number they become visible by virtue of their clearing of the bacterial lawn. After approximately 18 hours of culture at 34°C the amebae become confluent over the plate, giving rise to a "cleared" plate. In this culture situation, the doubling time for *Naegleria* is approximately 1.7 hours (Dingle, 1964).

Cells for transformation into flagellates are taken when the plates are 60+ percent cleared (estimated by visible observation), however once cells completely clear the plate they begin to encyst and thus plates completely cleared are observed for presence of cysts before being used.

(b) Liquid Batch Growth

When large quantities of cells are required, plate growth is inefficient, both in terms of expense and of time required for cell harvesting. Methods have been devised whereby up to 3 to 4 x 10^9 cells may be obtained overnight from 1 litre culture (Fulton and Guerrini, 1969; Dingle, unpublished).

Aerobacter, grown in 1 litre batches of PAB in 2.8 litre Fernbach flasks in a gyratory shaker-incubator (37°C at 200 rpm for approximately 9 hours - Gyrotory Shaker, Model G-25, New Brunswick Sci. Co., New Brunswick, N.J.) are harvested by centrifugation in 250 ml plastic bottles (Sorvall RC-2B Centrifuge with GSA rotor for 10 min at 5,000 rpm). Pellets are combined and suspended, either in Tris-Mg^{+2} buffer at pH 6.8 (10 m$\underline{M}$ Tris, 5 m$\underline{M}$ $MgSO_4$) or MOPS-Mg^{+2} buffer at pH 6.8 (5 m$\underline{M}$ MOPS, 5 m$\underline{M}$ $MgSO_4$), with one litre of the buffer containing the *Aerobacter* from 2 litres of PAB culture. Fulton and Guerrini (1969) report that $MgSO_4$ prevents bacterial clumping in the culture situation. Plate grown amebae are inoculated to an initial concentration of approximately 5 x 10^4 per ml into the buffer and the flask is placed onto an Eberbach flask shaker oscillating at 80 times per min (Eberbach Corp., Ann Arbor, Mich.) in a constant temperature room at 26 to 28°C. After 14 to 20 hours of growth the cell yield is routinely between 3 and 4 x 10^6 cells per ml.

Cell Counting

Cell concentrations were estimated using a Coulter Counter, Model "F" (Coulter Electronics Inc., Hialeah, Florida). Sensitivity settings providing suitable accuracy for the present work were as follows: for NB-1, threshold 20, attenuation 2 and aperture 64; for NEG, threshold 20, attenuation 2 and aperture 32.

The cell suspension to be counted is diluted in an electrolyte (0.4 percent NaCl in glass distilled water) to give between 1000 and 6000 counts for the 0.5 ml sample taken through the aperture, to avoid coincidence (Coulter Electronics Instruction Manual for the Model "F" Coulter Counter, 5th edition, 1967). Errors in counting due to the clumping of amebae are prevented by vigorous mixing of the diluted cell suspension on a vortexing mixer at top speed for 30 sec. Three counts per sample are taken and the average, corrected for the dilution factor, gives the cell concentration of the sample.

Flagellate Preparation

Fulton and Dingle (1967) describe a method for the synchronous transformation of *Naegleria* amebae to flagellates. Growing amebae from NM plates are suspended in a non-nutrient buffer, washed free of bacteria by differential centrifugation, and agitated in a shaking water bath at 25°C.

Amebae, grown on NM plates to late log phase or early

stationary phase, as evidenced by plate clearing, are suspended by pipetting 10 ml buffer (25 mM Tricine or 5 mM Imidazole at pH 7.0) onto the plate. A bacterial spreader sterilized in 70 percent ethanol and lightly flamed is used to remove amebae from the agar surface. The bacteria-cell suspension is poured into a 50 ml plastic centrifuge tube, and an additional washing is performed in the same manner. Amebae are washed free of bacteria by a differential centrifugation technique (Method C of Fulton, 1970).

The method is such that after 3 washings virtually all of the bacteria have been removed from the final suspension with minimal loss of amebae. After washing, the amebae are resuspended in sufficient buffer in a 125 ml Erlenmeyer flask to give a cell concentration of 2 to 7 x 10^6 cells per ml. The flask is immediately transferred to a reciprocating water bath oscillating at 100 to 125 1-inch strokes per minute at 25°C. The usual time period between initial suspension and placement of the flask into water bath is 6 or 7 min. Time zero (t = 0) in this procedure is at the point of introduction of buffer to the NM plates. At regular time points samples are removed from the flask, fixed in Lugol's Iodine (4 gm I_2 and 6 gm KI in 100 ml glass distilled water), and observed with phase contrast optics. Usually 100 cells per time point are scored for percent flagellates. A curve representative of many similar experiments is presented in Figure 1.

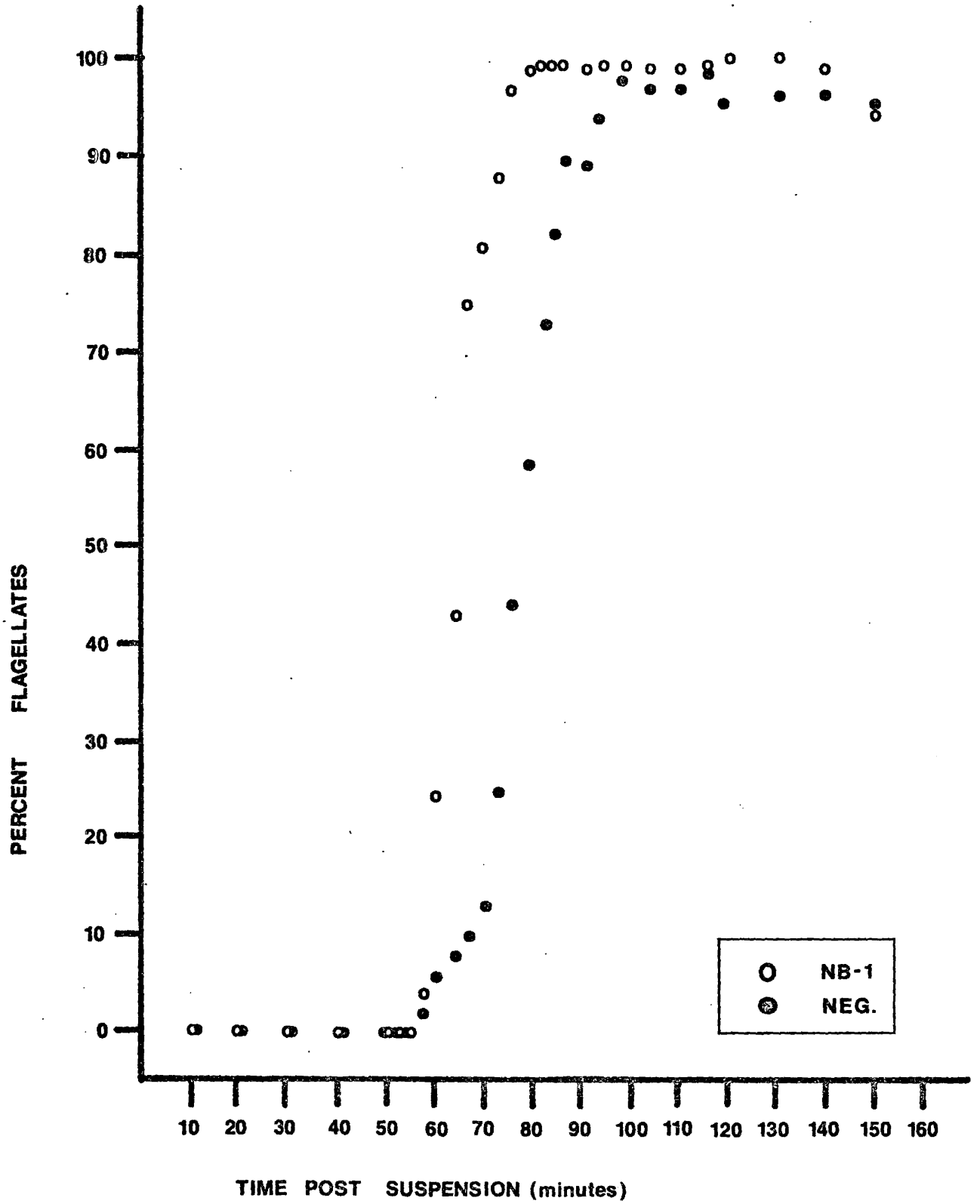
FIGURE 1

Transformation curve of amebae-to-flagellates

This figure shows the transformation of *Naegleria* amebae to flagellates under the conditions specified in the text (25 mM Tricine at pH 7.0, 25°C, 100 1 inch strokes per minute). Note that at the 120 minute point the cells are 90+ percent flagellates. It is at this time that the cells are taken for rhizoplast isolation.

NB-1 O

NEG ●


PERCENT FLAGELLATES
100
90
80
70
60
50
40
30
20
10
0
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
TIME POST SUSPENSION (minutes)
NB-1
NEG.

Rhizoplast Isolation

The techniques used in the isolation included lysis of cells in dilute NH_4OH, lysis of cells in dilute detergents and surfactants, and grinding of a cell suspension in a tissue grinder.

NH_4OH Lysis: Flagellates are suspended in a 0.7 percent solution of NH_4OH, either at room temperature, or on ice for various time periods. Observations of the suspension were made using phase contrast optics.

Detergent Lysis: Flagellates are resuspended in dilute solutions of various detergents at ice-bucket temperatures. Tween 80 and Tween 20 (polyoxyethylene sorbitan monooleate), Span 20 (sorbitan monolaureate), sodium deoxycholate, and sodium dodecylsulphate were used at concentrations of 1 percent or less. Observations were made using phase contrast optics.

Cell Grinding: This method involving cell grinding as a means of lysing flagellates is the one used throughout the study to obtain isolated rhizoplasts. Harvested flagellates are resuspended in approximately 2 ml extraction medium (the buffer in which the cells were transformed into flagellates plus sufficient $MgSO_4$ to give a final Mg^{+2} concentration of 2.5 mM) at 0 to 4°C and are poured into a previously chilled 7 ml capacity Dounce homogenizer (Kontes Glass Co. Ltd., Vineland, N.J.). Sixty to eighty strokes of the pestle are given and the homogenate is

poured into a conical centrifuge tube for preliminary purification by centrifugation. The homogenate is spun at rheostat setting 5 in the IEC for 1 min to pellet any remaining intact cells and large bits of cell debris. The supernatant is spun again at setting 7 for 2 min to pellet nuclei, bits of cell membrane and rhizoplasts. The resulting pellet is resuspended in 1 ml extraction medium and is spun at setting 5 for 1 min. The supernatant from this spin contains isolated rhizoplasts.

Rhizoplast Purification

Since the isolated rhizoplast supernatant contains not only rhizoplasts, but also mitochondria, bits of membrane, microtubules, and the like, purification procedures consisting of digitonin treatment, Sarkosyl treatment, urea treatment, treatment with the chelating agents EDTA and EGTA, pH adjustments, and sucrose-gradient centrifugation were undertaken in order to purify the preparation.

Digitonin Treatment: Isolated rhizoplasts were collected by centrifugation of supernatant at rheostat setting 7 of the IEC for 2 min. The pellet was resuspended in cold (0 to 4°C) digitonin medium described by Gibbons (1963) and used by him to remove membranes from isolated cilia and flagella. Gibbons' formula consisted of 30 m$\underline{M}$ Tris buffer at pH 6.8 containing 2.5 m$\underline{M}$ Mg^{+2} and 0.5 percent digitonin, for 1 to 4 hours at 0 to 4°C. Modifications of this

formula were used and included variations in buffers and buffer concentrations (Tris and Tricine, 30 mM, 25 mM and 5 mM), reduction in digitonin concentration (0.05, 0.10, 0.17, 0.25, 0.40 and 0.5 percent), and changes in the temperature of the treatment (room temperature or on ice).

Sarkosyl Treatment: The mild detergent Sarkosyl (sodium lauroyl sarcosine, obtained from Geigy Industrial Chemicals, Ardsley, N.Y.), has been reported to cause lysis of *Naegleria* amebae suspended in a 0.1 percent w/v solution (Fulton, 1970). Isolated rhizoplasts were treated with 0.2 percent w/v Sarkosyl in glass distilled water in the ratio of 1 part Sarkosyl to 1 part supernatant for 60 to 90 min at ice-bucket temperature. Since the presence of 2.5 mM $MgSO_4$ causes precipitation of Sarkosyl, the concentration was lowered to 1.25 mM simply by using Sarkosyl prepared in distilled water, thus resulting in the lower concentration when the detergent is mixed with the supernatant.

Urea Treatment: Isolated rhizoplasts were suspended in extraction medium containing various concentrations of urea (8 M, 6 M, 4 M, 2 M, 1 M, and 0.5 M) and was left for various time periods at ice-bucket temperature or at room temperature.

EDTA and EGTA Treatment: Isolated rhizoplasts were suspended in extraction medium containing either 5 mM EDTA (disodium ethylenediamine-tetraacetate), or 5 mM EGTA (ethyleneglycol-

bis (β-aminoethyl Ether) N, N'-tetraacetate) for various time periods on ice.

pH Adjustments: Routine transformation of cells into flagellates from amebae and cell grinding is done at pH 7.0 to pH 7.5. After isolation pH adjustments were attempted since it was thought that the rhizoplasts might be stable at pH values that were disruptive to other contaminants present in the supernatant. Isolated rhizoplasts were pelleted and resuspended in Tricine-Mg^{+2} (25 mM and 5 mM respectively) at pH 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 10.0, left standing for 2 hours in the cold and observed after uranyl acetate staining with the electron microscope.

Sucrose-gradient Centrifugation: It was thought that a gradient centrifugation procedure would separate rhizoplasts from the rest of the material present in the supernatant. After one or a combination of the above methods, the material remaining in the treatment tube is collected by a 10 min spin in the SS-34 rotor of the Sorvall RC-2 centrifuge at 5000 rpm and resuspended in about 1 ml extraction medium. The material is carefully added to a layered gradient of sucrose solutions: 3 ml each of 2.0 M, 1.75 M, and 1.5 M and 2 ml each of 1.25 M, 1.0 M, and 0.5 M sucrose in a 101 mm x 16 mm nitrocellulose tube (Beckman Instruments Inc., Spinco Division, Palo Alto, California). Sucrose solutions are prepared using extraction medium in order to

prevent the dissolution of rhizoplasts in the absence of sufficient Mg^{+2} during the centrifugation period. Gradients are spun for 60 min in the Sorvall RC-2 centrifuge equipped with HB-4 swinging-bucket rotor at 8000 rpm (10,444 x g) and are collected by dripping into fraction tubes. The fractions are observed with the electron microscope as above for presence of rhizoplasts and contaminating materials.

Methods for Electron Microscopy

(a) Fixation and Embedding

The fixatives used in this study consist of buffered agents (glutaraldehyde and osmium tetroxide) combined with the salts $CaCl_2$ and NaCl. Table 1 lists the concentrations of the components used in the various fixatives.

In brief, flagellates are prepared, harvested, fixed and pelleted. Pellets are rinsed with buffer, dehydrated and embedded in Araldite 502.

Different protocols were used in order to determine whether different fixations have any effect on observed rhizoplast periodicity: (1) FsGA followed by $FsOsO_4$; (2) $FsOsO_4$ alone; (3) TGA followed by $TOsO_4$; (4) $TOsO_4$ alone.

Harvested flagellates are resuspended in a small volume of the appropriate buffer in 12 ml conical glass centrifuge tubes. An equivalent volume of fixative is

TABLE 1

Solutions used during the fixation of *Naegleria* flagellates

The components of the various solutions used during fixation procedures in the preparation of sectioned material of *Naegleria* flagellates are given in the Table. The values in the Table correspond to the concentrations of the various components when they are in contact with the cells during fixation. The details of the fixation procedures are given in the text. All components except osmium tetroxide which is obtained from Ladd Research Industries were obtained from Fisher (Fisher Scientific Co., Ltd., Montreal, P.Q.).

TABLE 1

Solutions used during fixation of *Naegleria* flagellates

Component / Solution	Veronal Acetate	Tricine	Glutaral-dehyde	OsO_4	$CaCl_2$	NaCl	HCl
Fs-buffer	0.011 M				0.0016 M	0.01 M	0.008 N
Fs-GA	0.011 M		3%		0.0016 M	0.01 M	0.008 N
Fs-OsO_4	0.011 M			1%	0.0016 M	0.01 M	0.008 N
Tricine-buffer		0.0025 M			0.001 M	0.005 M	
Tricine-GA		0.0025 M	1.5%		0.001 M	0.005 M	
Tricine-OsO_4		0.0025 M		1%	0.001 M	0.005 M	

added to each tube with gentle swirling so as to thoroughly mix the two components. Cells are pelleted by hand centrifugation so they can be handled as if they were a piece of tissue. After 30 min at room temperature the fixative is removed by aspiration through a Pasteur pipette and is replaced by the appropriate buffer. If there is to be osmium post-fixation, it is carried out at this time. Also, if there is to be no glutaraldehyde fixation the first few steps are ignored. Osmium fixation is carried out on ice and is for 60 min. Dehydration is accomplished by a "half-step" method in which equal volumes of buffer and 30 percent ethanol are added to the pellet, left for 1 min, and replaced with 30 percent ethanol alone. Following steps include 50,75, 95 and 100 percent ethanols such that dehydration is complete at the end of the series. Three further changes of 100 percent ethanol are then carried out.

The resin used in the study, Araldite 502, is a mixture of 100 ml Araldite and 78 ml dodecenylsuccinic anhydride (Coulter, 1967). It is insoluble in ethanol, but soluble in propylene oxide, therefore substitution is necessary to ensure complete infiltration of the resin. Ethanol and propylene oxide are miscible thereby allowing for complete substitution after 3 changes of propylene oxide. The pellet is then soaked in Araldite 502 in propylene oxide (25 percent v/v) for 20 min, and then

50 percent v/v Araldite in propylene oxide for 60 min at room temperature. Three 1 hour changes in 100 percent Araldite at 60°C are followed by a transfer to Araldite plus accelerator (1.5 percent benzyldimethylamine, to accelerate polymerization) at room temperature for 6 to 12 hours. Finally, the pellets are transferred to BEEM capsules of preheated resin plus accelerator with sharpened applicator sticks. They are labelled and spun at top speed in the IEC to pellet the material within the capsules. The resin is cured for 60 hours at 60°C.

Non-swimming flagellates were also fixed with TGA followed by $TOsO_4$ to determine whether the observed variations in periodicity of the rhizoplasts were dependent upon motile, actively beating flagella.

DNP Treatment: Flagellates were treated with 5×10^{-3} M DNP (2:4-dinitrophenol, an inhibitor of oxidative phosphorylation, thus a poison affecting cellular processes dependent upon ATP). Five minutes on ice at pH 6.8 was sufficient time for loss of the swimming function. The cells were then pelleted, washed briefly with Tricine buffer and resuspended in TGA.

Vortex Treatment: The swimming function of flagellates was prevented by the removal of flagella with shearing force provided by a vortexing mixer. Samples were observed with phase optics until the population was at least 95 percent deflagellated. Pelleting was followed by fixation.

Chilling: Cells were chilled on ice until fewer than 5 percent of the cells had actively beating flagella. The time of cold treatment was usually less than 5 min, after which the cells were pelleted in the cold and resuspended in TGA. During fixation the mixture was allowed to slowly reach room temperature.

In all three cases fixation and embedding were as described earlier.

(b) Preparation of Sections and Grids

Grey to pale-gold (20.0 to 120.0 nm thick) sections were obtained from trimmed blocks using an LKB ultratome (LKB-Produkter, Sweden) equipped with glass knives freshly prepared on an LKB knifemaker. Sections were floated on water in a trough prepared on the knife with masking tape, and were picked up on 300 mesh copper grids, previously made "sticky" by dipping into a 0.15 percent formvar solution in ethylene dichloride, simply by touching the grid to the surface of the water. Grids were held in chloroform vapours for a short time to correct compression artifacts blotted from the underside, and air dried.

Sections were stained with uranyl acetate and lead citrate, either alone or in combination. Uranyl acetate staining was with a 2 percent solution in glass distilled water. Grids were floated, sections down, on drops of the

stain placed on small pieces of dental wax under small Petri dish lids for 15 to 20 min at room temperature. Grids were rinsed by dipping 10 times in a small volume of glass distilled water, blotted from the underside, and air-dried. Lead citrate staining was with the stain of Reynolds (1963). It was made up by dissolving 1.33 gm $Pb(NO_3)_2$ and 1.76 gm $Na_3C_6H_5O_7.2H_2O$ in 30 ml glass distilled water. The white precipitate that formed dissolved after the addition of 10 ml 1 N NaOH and a further 10 ml aliquot of glass distilled water. The pH was adjusted to 12 with 1 N NaOH. Due to the susceptibility of the stain to CO_2 and consequent contamination with lead carbonate, it is stored under a layer of mineral oil in the tubes.

The staining procedure is such that the stain is exposed as little as possible to the air. It is withdrawn from the storage tube with a previously scored Pasteur pipette. The end that penetrated the oil is gently removed and a drop of the stain is placed on a piece of dental wax under the lid of a small petri dish. Care is taken not to breathe, either on the stain or on the grid. After 10 to 20 sec the grid is removed from the stain, washed by dipping 10 to 15 times in glass distilled water, blotted from the underside and air-dried.

"In-block" staining with uranyl acetate was used in the latter stages of this work. Before the dehydration

sequence the pellets are subjected to a 2 percent solution of uranyl acetate in glass distilled water in the cold for 2 hours. It was found that this method was superior to the former method of uranyl acetate staining as the whole block and all sections produced from it were uniformly stained.

(c) Isolated Rhizoplasts

Two main methods of preparation were employed for the observation of isolated rhizoplasts; negative staining and shadow casting.

Negative Staining: Of the negative-staining agents available, 1 percent uranyl acetate in glass distilled water was used preferentially over 1 percent phosphotungstic acid and 2 percent ammonium molybdate since the latter two had a tendency to positively stain the organelles to such a degree as to obscure the cross striations. Uranyl acetate staining was carried out according to the method of Szent-Györgyi (personal communication). A drop of isolated rhizoplasts was placed onto a parlodion-carbon coated grid with a Pasteur pipette, allowed to sit 30 seconds, rinsed off with 5 drops of glass distilled water and then stained for 30 seconds with 3 to 10 drops of stain solution. Grids were blotted from the underside and air dried.

Shadow Casting: Shadow-cast specimens were prepared in the Edwards Vacuum Coating Unit (Model E12E4) using platinum-

carbon pellets from Ladd Research Industries. Isolated rhizoplasts were placed on grids, as for negative staining, rinsed with glass distilled water and air-dried. They were attached to a glass slide at one edge by a piece of Scotch tape. A thin coating of Pt-C was applied at high vacuum from an angle of 10° from the horizontal. In order to reduce contamination during the shadowing process the chamber of the vacuum unit was flushed with liquid nitrogen before use.

(d) Microscopy

Observations were made with either on RCA-EMU-3H operating at an accelerating voltage of 50 kV or a Zeiss EM-9 operating at an accelerating voltage of 60 kV. The RCA-EMU-3H was fitted with objective and condenser apertures of 50 and 250 microns respectively and the Zeiss with apertures of 25 and 80 microns. Micrographs were taken, in the respective machines, on 2 x 10 inch Kodak Electron Image Plates and 7 x 7 cm Scientia Film (Agfa Gevaert, Belgium), which were subsequently processed in Kodak H.R.P. Developer with Kodak Antifog I added. Fixation was for 15 min with frequent agitation in Kodak Rapid Fixer plus hardener. Negatives were printed on Ilford IB IP Photographic Paper of grades 3, 4 and 5. Development in Ilford Bromophen developer was followed by fixation for approximately 10 min in Kodak Acid Fixer and washing in

cool water for 60 min.

Measurements and Calibration

(a) Sectioned Material: Periodicities were measured directly from photographic prints using a Bausch and Lomb magnifying comparator with 7 x magnification of a 2 cm scale divided into units of tenths of mm. Both electron-opaque and electron-transparent bands were measured separately with the data being recorded as taken. Calculations of the width of the bands were made in nm. This method, which takes into account every band allows the determination of period variations within a single organelle as well as period variations between organelles.

(b) Negative Stained and Shadowed Material: Periodicities were measured directly from the photographic prints by a count-and-measure technique. Periods were counted over the length of the organelle on the print and the distance was measured. This method was used, instead of that used for the sectioned material, due to the difficulty in distinguishing between the boundaries of the electron-opaque and electron-transparent bands. The method was also found to be not as time-consuming as the one described previously, however variations within a rhizoplast could not be detected as readily.

(c) Magnification Calibration: In view of the quantitative nature of the measurements performed, it was found necessary to calibrate the magnification of the instruments used in the procedure.

Microscope magnification calibration was performed using a grating replica with 54,800 lines per inch (Ernest F. Fullam, Inc.). At least 100 lines were measured per calibration per magnification step. The Zeiss electron microscope was calibrated early on in the study and later when it was realized that a significant change may have taken place. However, the calibration revealed a change of less than 3 percent, a value which is within acceptable accuracy limits for the replica grating calibration procedure. The RCA microscope was calibrated only at one point during the study using the grating replica as the instrument was used only in the very early stages.

The enlarger used in the preparation of the photographic prints used for period measurements (Simmon Omega D-2 Enlarger, Simmon Omega Inc., Woodside, N.Y.) was calibrated before each use by the projection of the image of a measurement scale (a transparent plastic ruler) on the easel and the measurement of this scale at the level of the photographic paper.

RESULTS

Sectioned Material, Ultrastructure of the Flagellar Apparatus:

General Appearance of the Cells

Differences in the appearance of cells and cellular organelles fixed in the different ways were evident during electron microscope examination.

Apparent cytoplasmic density varied greatly with the fixative procedure. Doubly-fixed cells (both glutaraldehyde and osmium) exhibited cytoplasm much denser in appearance than those fixed in osmium alone. The latter cells appeared rather "empty" with fewer ribosomes and ribosome clusters. A tendency for these cells to lyse and lose cytoplasm was noticed and may be a possible explanation for the "empty" appearance.

In the osmium-fixed cells, cytoplasmic membranes - rough endoplasmic reticulum, mitochondrial membranes and cristae, membrane-bound vesicles - were seen much more often due to the partial loss of cytoplasm accompanying lysis. In doubly-fixed cells cytoplasmic density obscures these membranes and thus they are seen only infrequently. Golgi apparatus was not seen in any of the cells observed, confirming the mention of this fact by Schuster (1963).

Cytoplasmic microtubules were seen regularly only

in cells which had been doubly-fixed with the Tricine-buffered agents but were sometimes present in cells fixed differently.

In general, cells fixed in osmium alone presented more clearly the organization of the cytoplasm as seen unobscured by cytoplasmic density.

The Flagellar Apparatus of *N. gruberi*

The various parts of the flagellar apparatus described by Schuster (1963) and Dingle and Fulton (1966) include the nucleus, rhizoplast, rhizoplast-associated microtubules (Schuster's "spur" and Dingle and Fulton's "palisade"), basal bodies and flagella. These authors isolated, by lysis of flagellates, small numbers of flagellar apparatuses, which they observed by phase contrast and electron microscopy. The nucleus is considered a part of the apparatus since in these isolates they were often attached to the rest of the component parts. In sectioned material the rhizoplast is observed to run very close to the nuclear membrane, sometimes being seen in a membrane-bound groove near the nuclear surface. It has been very difficult to observe rhizoplasts actually penetrating the nuclear material, except in these aforementioned cytoplasm-filled grooves.

The following description of the parts of the flagellar apparatus will be generalized to the extent that

fixation procedures will not be mentioned.

Naegleria basal bodies and flagella have been described by Schuster (1963) and Dingle and Fulton (1966). Basal bodies are composed of 9 triplet microtubules arranged cylindrically giving a structure approximately 200 nm in diameter with a very distinctive cross-sectional appearance. There is a basal plate present in the organelle, below which a cartwheel arrangement of a central core and radiating spokes may be seen in cross section, as in Figure 14,2. Flagella are composed of an axoneme of nine doublet tubules and two central singlets surrounded by an extension of the cell membrane.

In thin section *Naegleria* rhizoplasts exhibit a distinct cross-striated pattern composed of alternating electron-opaque and electron-transparent bands. No further intraperiod bands were seen in either region throughout the study. The rhizoplast arises in the flagellate near the basal bodies and tapers towards its end in the cytoplasm, often alongside the nuclear membrane. Ignoring the obvious limitations of the technique of thin-sectioning, *Naegleria* rhizoplasts appear to vary in width between 50 and 200 nm, but the variation is much less than this in isolated organelles.

Only very rarely is a perfectly longitudinal section of the organelle obtained due to its sinuous path through the cytoplasm. For example, although the longest measured

rhizoplast observed in sectioned material was 7.8 microns the average observed length for 10 measurements was under 2 microns.

No membrane was seen surrounding the organelle, either in sectioned material or in isolated rhizoplasts. The organelle must then be a highly ordered structure capable of maintaining its integrity, both inside and outside the cell.

Distinct rhizoplast cross sections could not be unequivocally recognized due to the number of electron-opaque condensations of material present in the flagellate cytoplasm. Those condensations that were of adequate size, 100 to 200 nm, that were elliptical or oval could conceivably be rhizoplast cross sections, particularly as is the case in Figure 2, where such a condensation is observed in close association with a basal body.

In favourable sections the close relationship of the rhizoplast to the basal bodies may be observed, and appears to be maintained by two distinct morphological structures.

1. An inter-basal body connector appears to link two basal bodies, and runs alongside the rhizoplast. In cross section it appears to be triangular with the base of the triangle closely associated with the basal body proximal to the nucleus and the opposite apex closely associated with one or two of the triplets of the distal basal body. The

FIGURE 2

Basal body, longitudinal section

This figure shows a basal body of a TGATOsO_4-fixed cell cut in almost perfect long section. Note the outer tubules of the flagellum (ot), the cellular membrane (cm), the basal plate of the basal body (bp), the central core and radiating spokes comprising the cartwheel of the basal body (cw), the mitochondrion with dense matrix (m), and the electron-opaque condensation in the cytoplasm to the left of the basal body that is thought to be a rhizoplast in cross section (r). Magnification X 70,000.

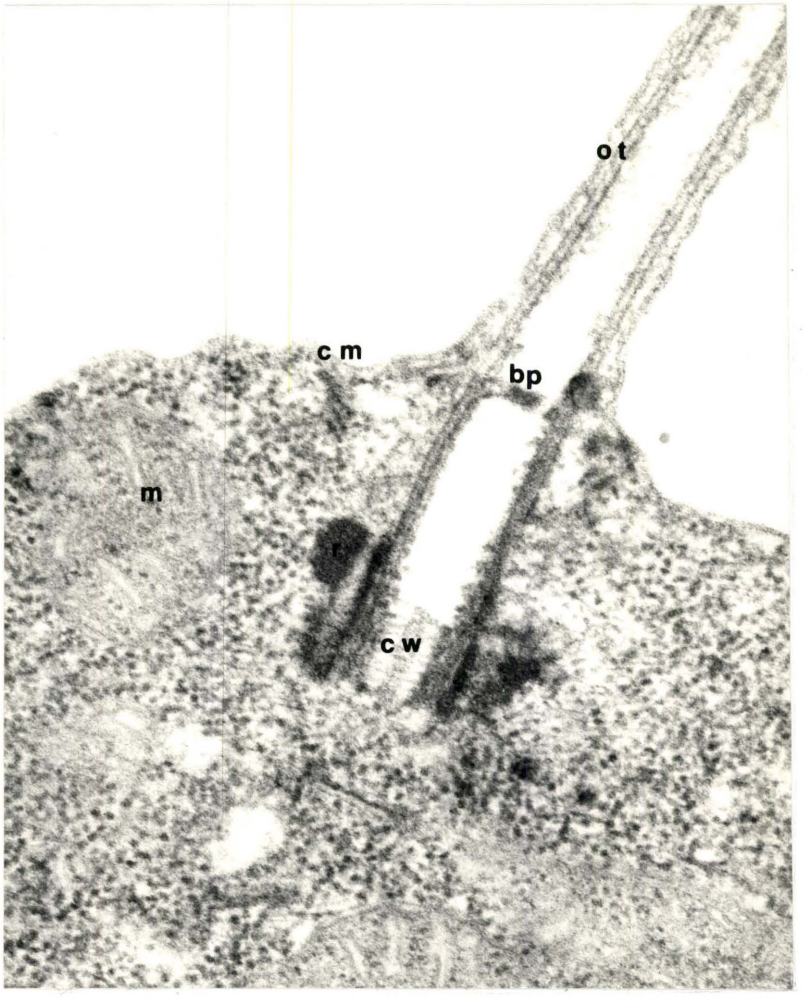


FIG. 2

structure itself shown in Figure 3 appear to be composed of bands of varying electron-opacity between the two basal bodies. In longitudinal section, the structure appears to be shorter than the basal body and exhibits the distinctive banding pattern seen in cross section Figure 4. A diagrammatic representation of the two photographs on which measurements are included (Figure 5) suggests that the structure seen in cross section in Figure 3 is indeed the structure seen in longitudinal section in Figure 4.

2. The second structure has previously been described as a "spur" (Schuster, 1963), and as a "palisade" (Dingle and Fulton, 1966). Due to the close association of the structure with the rhizoplast and its microtubular nature, it has been termed "rhizoplast-associated microtubules" or RAM in this thesis. In cross section, the structure appears as one, two, or three curving bands of microtubules - each composed of 8 to 14 joined tubules of center-to-center spacing 20 to 25 nm. Attachment to either the basal bodies or rhizoplast is uncertain in a visible sense but must presumably exist and be quite strong since isolated rhizoplasts invariably retain their basal bodies. In some sections the microtubular arrangement appears to be backed by a distinct electron-opaque band of material which may provide it with structural rigidity. In longitudinal section the RAM appears as a fan-like array of microtubules at one side of the basal body as is evident in Figure 6.

FIGURE 3

The rhizoplast and associated structures

This cell was fixed in TGATOsO_4, and the section shows a number of components of the flagellar apparatus - especially the basal bodies (Bb_1 and Bb_2), the interbasal body connector described in the text (ibc), the rhizoplast-associated microtubules (ram), and the cross-striated rhizoplast (r) with a total period of 17.2 nm comprised of 14.3 nm dark band and a 2.9 nm light band. Also noticeable is the dense cytoplasm (characteristic of cells fixed in this manner) and numerous mitochondria (m).
Magnification X 70,000.

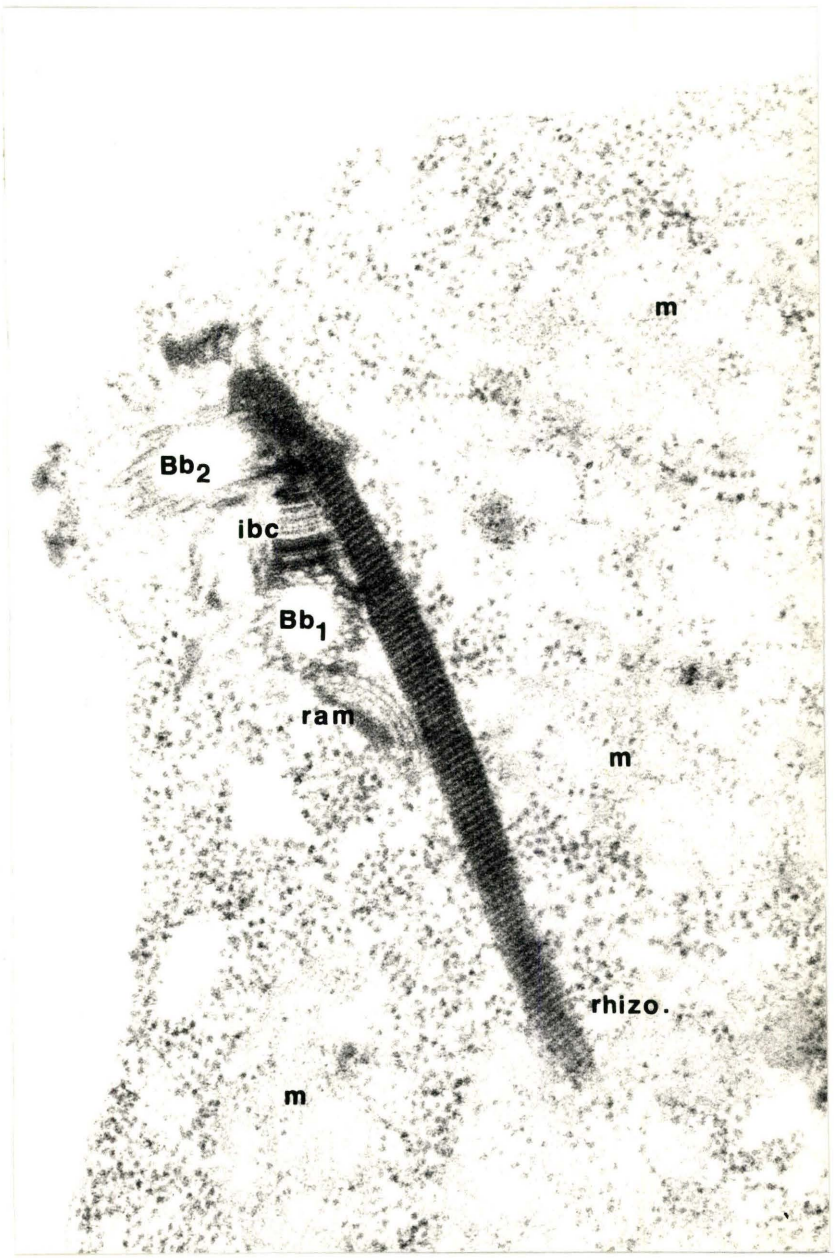


FIG. 3

FIGURE 4

The interbasal body connector, longitudinally sectioned

This figure shows a basal body in long section with an interbasal body connector to the left of Bb_1. Note the basal bodies (Bb_1 and Bb_2), the electron-opaque bands of the ibc and the flagellum (f). The cartwheel (cw) of the basal body is not clear but may be seen. Mitochondria (m) are also present in the section. Note also the dense cytoplasm of this TGATOsO_4-fixed cell.

Magnification X 70,000.

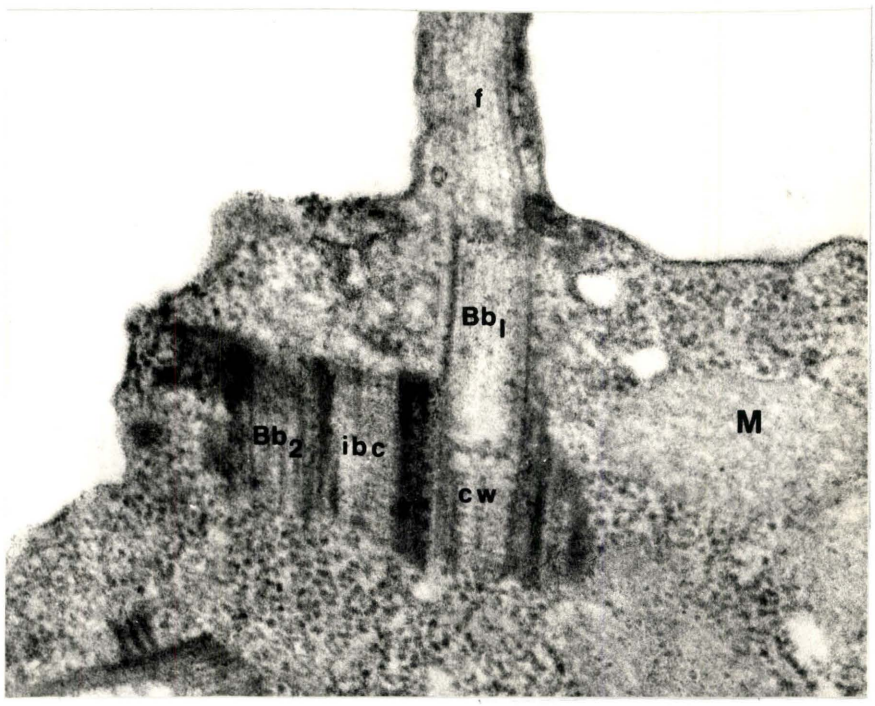


FIG. 4

In cases where more than two basal bodies are observed at the site of origin of a rhizoplast, additional RAMs and inter-basal body connectors, identical in appearance to those described earlier are usually found. In all cases observed the base of the inter-basal body connector is proximal to the nucleus. Figure 7 shows this multiple arrangement of basal bodies and connections.

Period Measurements on Sectioned *Naegleria* rhizoplasts:

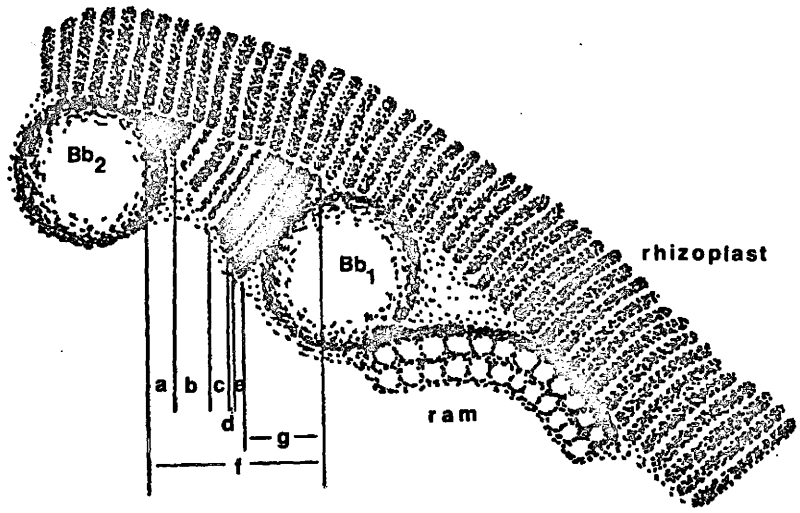
Various fixatives were used in an attempt to see if the rhizoplast periodicity differs when the organelle is fixed differently. Variations in period were first reported in *Naegleria* rhizoplasts by Dingle (1964) and by Dingle and Fulton (1966). Schuster (1963) reported a constant period of 18 nm for *Naegleria* rhizoplasts - the electron-opaque (dark) component being 11 nm wide and the electron-transparent component (light) being 7 nm. He used as his fixative, one related to that used by Dingle, the basis for both of them being a veronal acetate-buffered osmium tetroxide fixative with added salts. Since the one report indicates a constant period and the other a variable period, fixation methods may perhaps determine the appearance of the organelle.

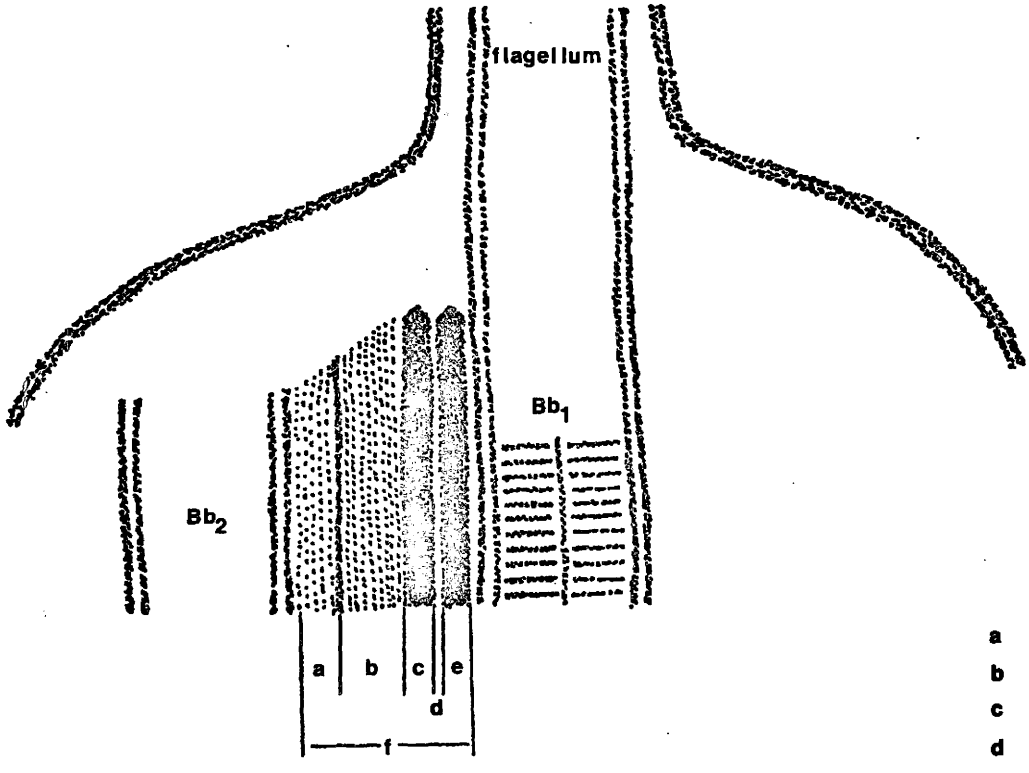
Generally, the only difference in the appearance of the rhizoplasts fixed in the different ways was in the sharpness or distinct nature of the periodic cross-bands. Those cells fixed in $FsOsO_4$ alone exhibited organelles that

FIGURE 5

The interbasal body connector, dimensions

Figure 5 shows in diagrammatic form the relationship between the interbasal body connector (ibc) seen in Figures 3 (cross section) and 4 (longitudinal section). Note the measurements of the bands of the ibc and the relationship between the measurements of the two figures, rhizoplast (r), basal bodies (Bb_1 and Bb_2), rhizoplast-associated microtubules (ram), and the cartwheel of the basal body (cw).





	xs (nm)	ls (nm)
a	73.7	76.7
b	66.3	66.3
c	35.4	39.8
d	2.9	2.9
e	31.0	35.4
f	229.3	221.1
g	145.0	

FIGURE 6

Rhizoplast-associated microtubules (ram), longitudinally sectioned

This figure shows two basal bodies (Bb_1 and Bb_2) in longitudinal section, with a longitudinally-sectioned rhizoplast-associated microtubular structure (ram) to the left of Bb_1. The electron-opaque condensation seen in the figure to the left of the ram is thought to be a very small section of a rhizoplast (r). The ram appears as a fan-like array of microtubules in the TGATOsO_4-fixed cell.

Magnification X 70,000.

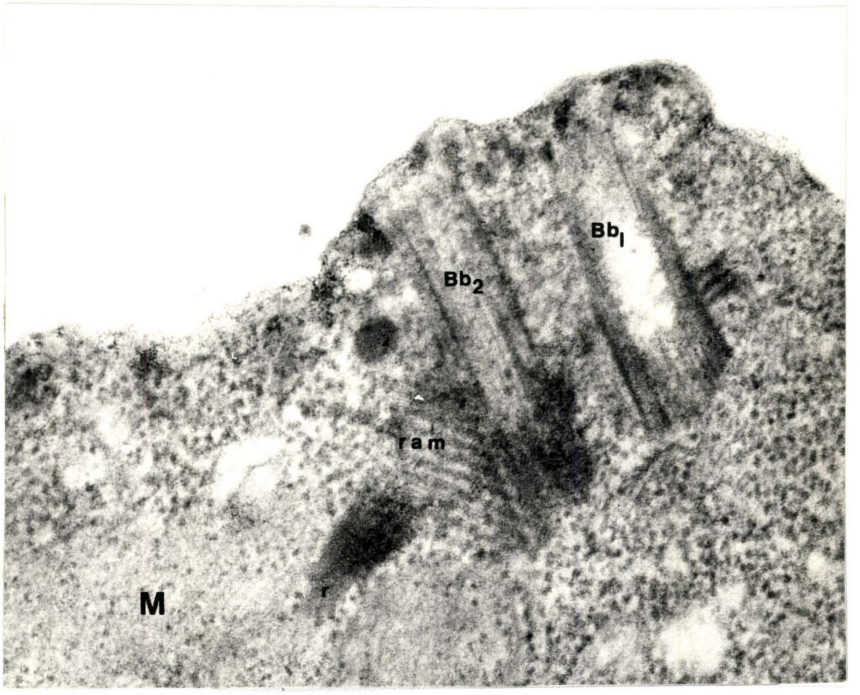


FIG. 6

FIGURE 7

Multiple basal bodies, ram's and interbasal body, cross-sectioned

This figure shows the case of multiple basal bodies (Bb_1, Bb_2, and Bb_3), multiple rhizoplast-associated microtubular structures (ram_1, ram_2, and ram_3), and multiple interbasal body connectors (ibc_1 and ibc_2). Note that the bases of the triangular ibc's are directed away from the periphery of the cell. The cartwheel arrangement in the basal bodies may be seen in Bb_1 and more clearly in Bb_2. The ram however, are not distinct as they were not sectioned in perfect cross-section. Numerous mitochondria (m) are visible, exhibiting dense matrix material seen in the TGATOsO_4-fixed cells.

Magnification X 70,000.

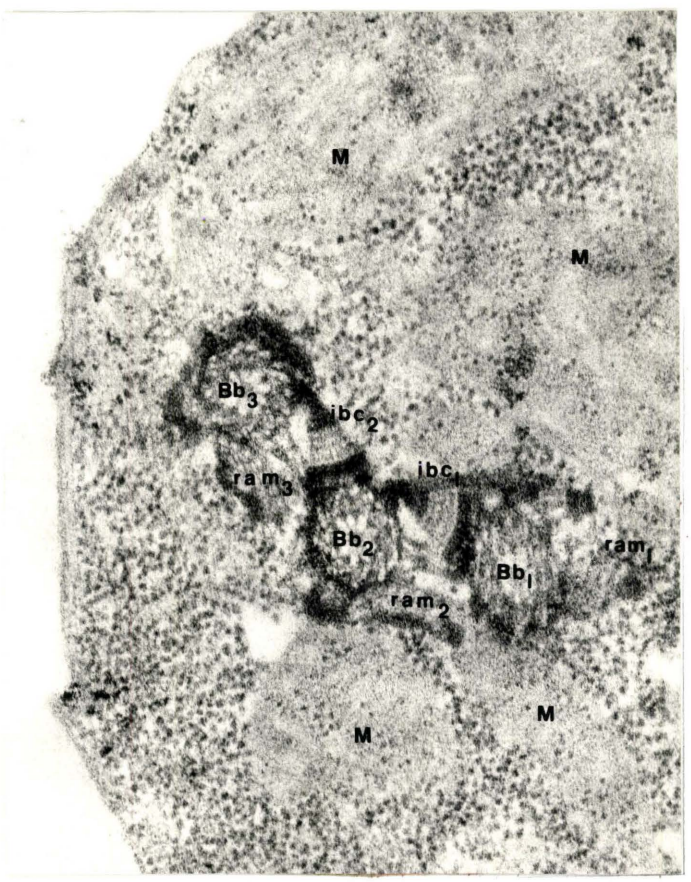


FIG. 7

were very sharp. A distinction between the dark and light bands of the pattern could be made much more easily than in the organelles fixed in the other ways.

The results of measurements of rhizoplast periodicity are presented in Tables 2 to 5 (Appendix I). In all cases reported, variations were observed between rhizoplasts and not within single rhizoplasts. For example, if a rhizoplast had a period of 18 nm (electron-opaque band plus electron-transparent band) it was relatively constant at 18 nm throughout its length. Yet, another rhizoplast may have a relatively constant period of 12 nm throughout its length. The total period is not regular between rhizoplasts, i.e., the dark component plus the light component does not give a constant value for all the organelles measured. Also, it was noticed that those rhizoplasts with dark bands having a width at or near the upper limit or the lower limit of the range of all the measured rhizoplasts do not necessarily have light bands whose widths are at or near the upper or lower limits of that range. Thus an organelle with a wide dark band may or may not have a wide light band.

On first glance, the Tables 2 to 5 seem to indicate that the rhizoplast period does vary from organelle to organelle. But, is the observed variability a physiological variability due to the nature of the proposed anchoring function of the rhizoplast - or is it an artifact? In an attempt to determine the answer to this question, cells

were treated before fixation to stop all flagellar motion with 2:4 DNP (an inhibitor of oxidative phosphorylation thus an inhibitor of ATP-dependent cellular processes, one of which is flagellar motion), chilling until no cells were seen swimming, or vortexing to physically remove flagellar function. If after treatment, the amount of period variation diminished or disappeared a more likely explanation of the variability other than artifact might be possible. The measurement data for treated organelles is presented in Tables 6 to 8 (Appendix I). The data of Tables 2 through 8 are represented diagrammatically in Figures 8, 9 and 10.

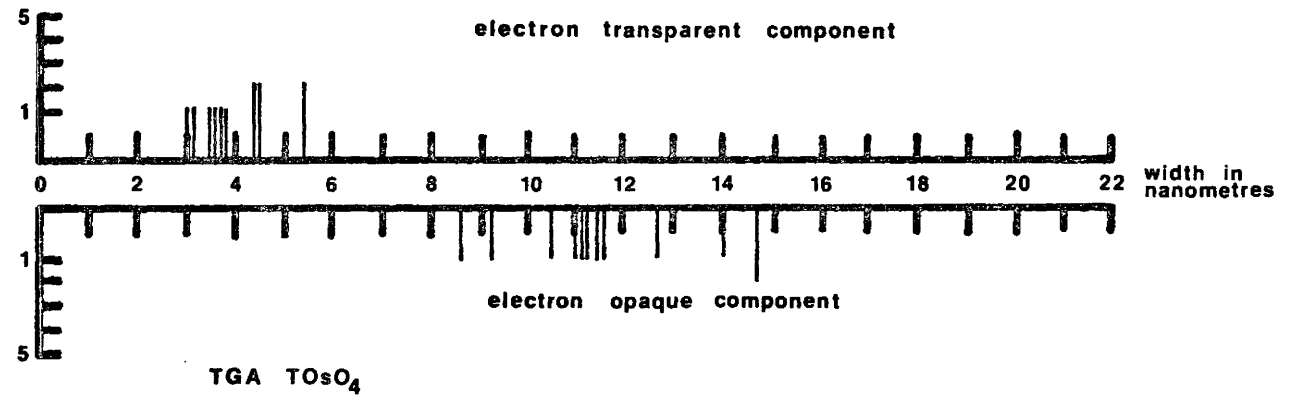
The photomicrographs included in this section are representative of a "wide-period" and a "narrow-period" organelle from each of the fixatives. Details of the effects of the fixation procedures on the other parts of the flagellar apparatus are given where appropriate, in the figure legends accompanying the figures.

Table 9 indicates various parameters calculated from the data of the measurements. Statistical procedures were applied to determine whether the observed variations are significant. In order to determine whether or not the variances derived from the data of treatments 2, 5, 6 and 7 are heterogeneous, a computation of Bartlett's test of homogeneity of variance was performed (Snedecor, 1956). In all three cases - dark band, light band, and the total

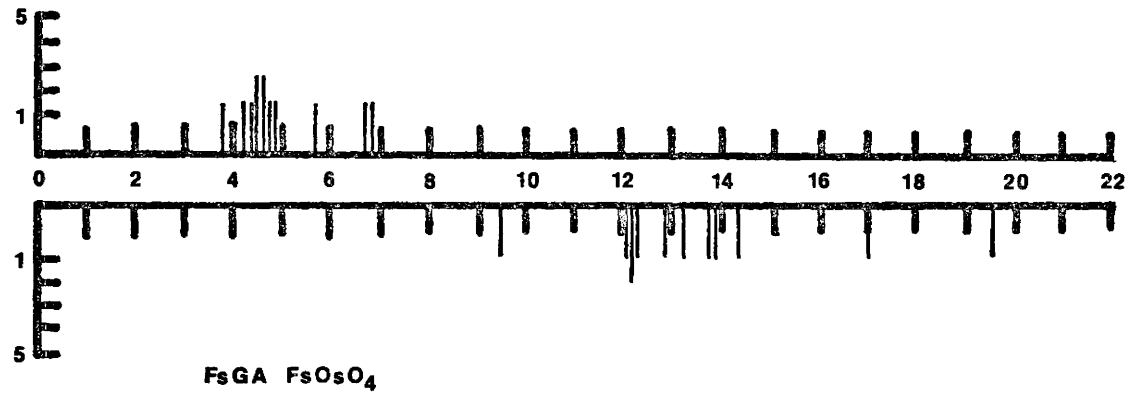
FIGURES 8, 9 and 10

Diagrammatic representations of the data of Tables 2 through 8

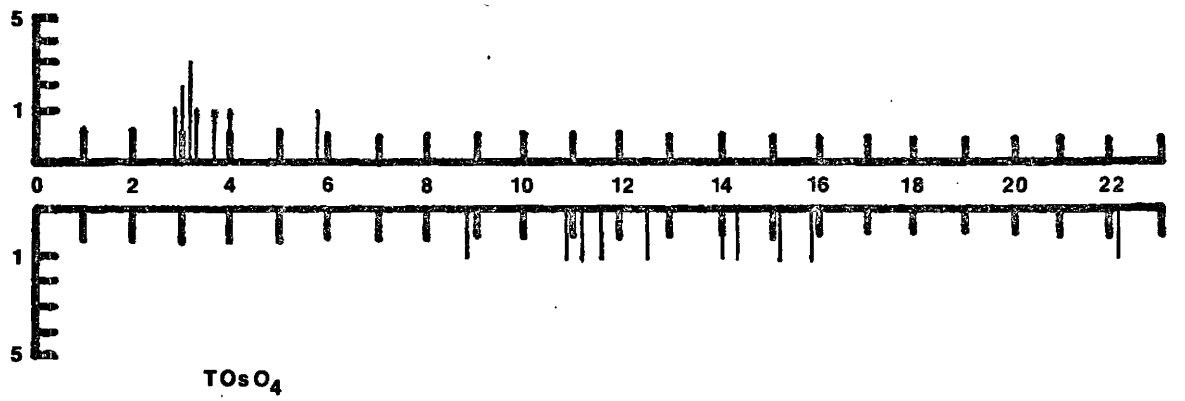
These figures are diagrammatic representations of the data presented in Tables 2 through 8. The abscissae represent width of light (top) and dark (bottom) bands in nanometers while the ordinates represent numbers of organelles with a particular band width. Figure 10 shows the data of Table 3 with specific band widths indicated. Note that the rhizoplasts with wide dark bands do not necessarily have wide light bands and vice versa.



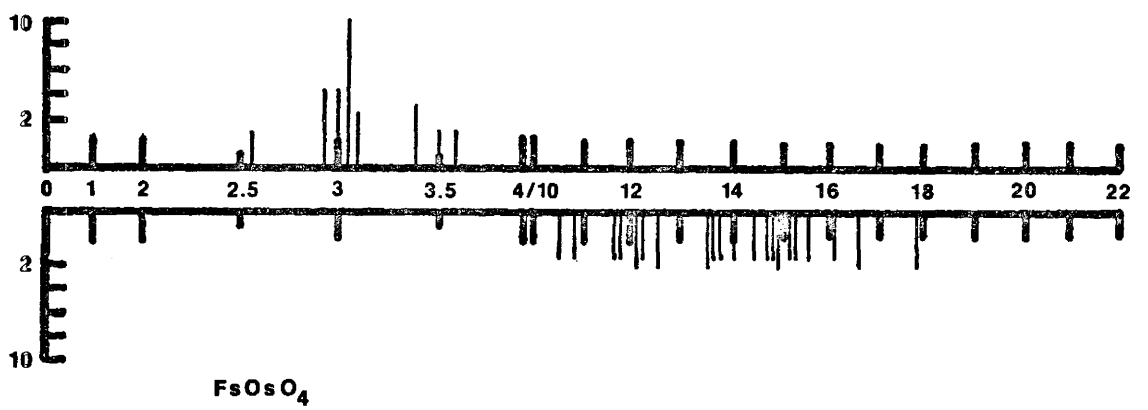
TGA $TOsO_4$



FsGA $FsOsO_4$



$TOsO_4$



$FsOsO_4$

FIG. 8

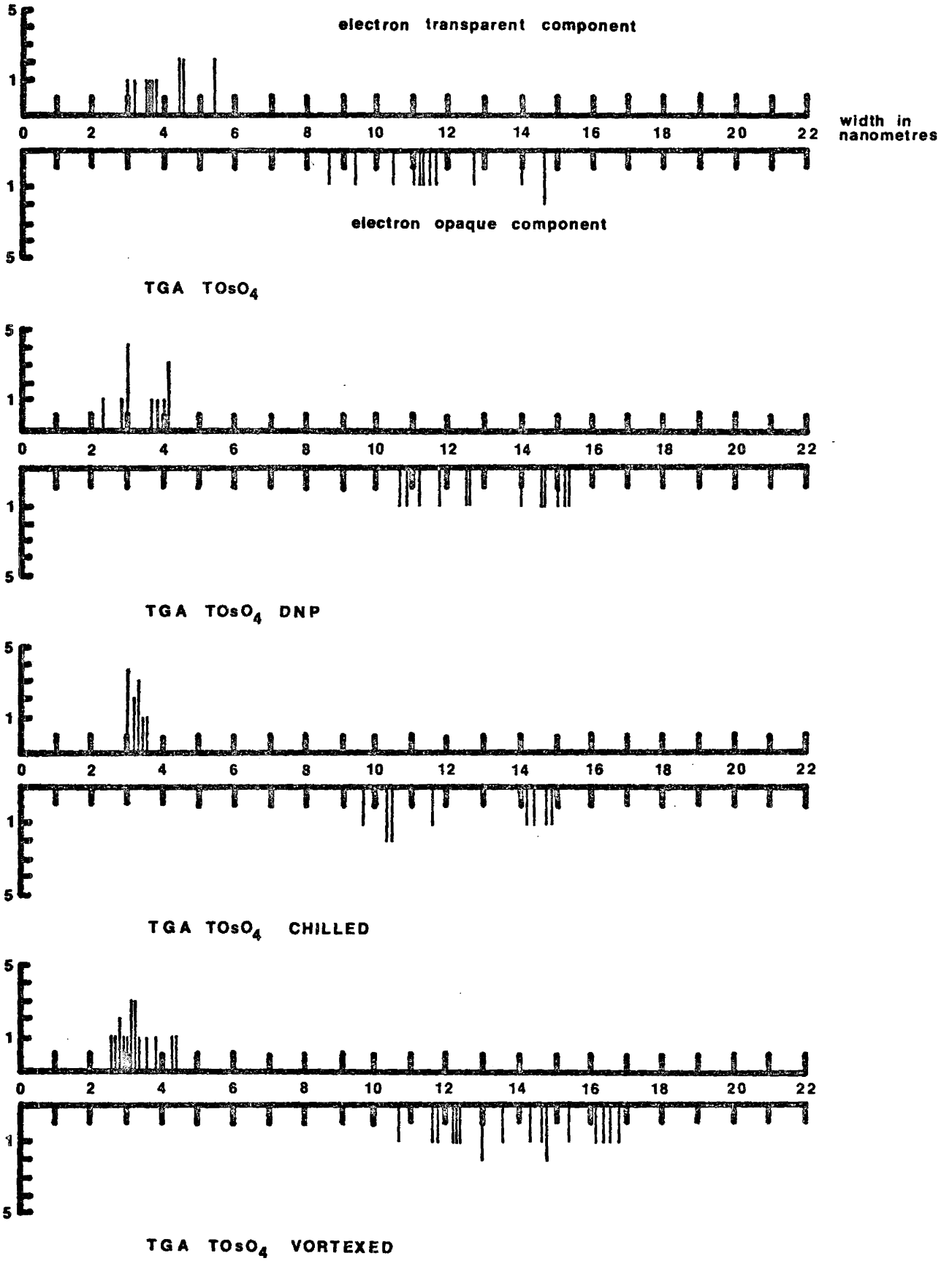
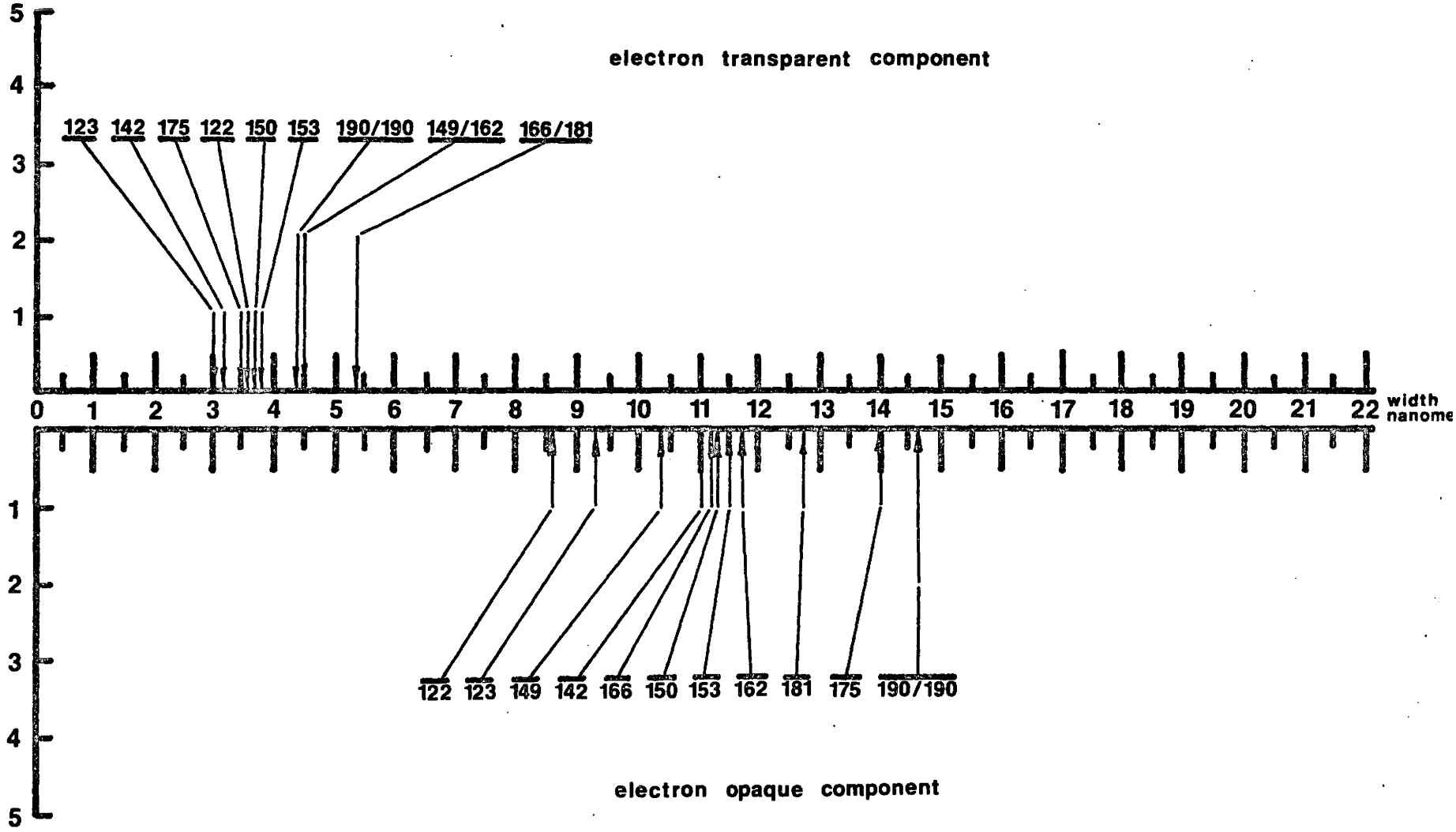

electron transparent component
width in nanometres
electron opaque component
TGA $TOsO_4$
TGA $TOsO_4$ DNP
TGA $TOsO_4$ CHILLED
TGA $TOsO_4$ VORTEXED

FIG.9



TGA $TOsO_4$

FIG. 10

TABLE 9

Statistical parameters calculated from rhizoplast periodicity measurement data

The table includes some of the parameters used in the statistical analysis of the measurement data for sectioned material. The values in the table were obtained in the standard ways (Spiegel, 1961);

$\overline{X}$ = mean = X/n,

σ = sample standard deviation = $\sqrt{\frac{\Sigma fX^2}{n} - (\frac{\Sigma fX}{n})^2}$,

σ^2 = sample variance,

S.E. = standard error = $\sigma / \sqrt{n}$, and

C.V. = coefficient of variation = $\sigma/\overline{X} \times 100$.

Please see the text for further details.

TABLE 9A

Statistical parameters calculated from rhizoplast periodicity measurement data

Fixative	No. of organelles	Dark Band					
		range nm	$\bar{X}$ nm	σ	σ^2	S.E.	C.V.%
FsGAFsOsO$_4$	12	9.4-19.5	13.59	±2.39	5.77	0.701	17.59
TGATOsO$_4$	12	8.6-14.6	11.74	±1.88	3.52	0.543	16.01
FsOsO$_4$	26	10.8-17.9	13.92	±1.84	3.4	0.363	13.22
TOsO$_4$	10	8.8-22.2	13.65	±3.54	12.50	1.116	25.93
TGATOsO$_4$ DNP	12	10.6-15.3	13.18	±1.72	2.97	0.651	13.05
TGATOsO$_4$ chilled	10	9.6-14.7	12.06	±2.06	4.26	0.497	17.08
TGATOsO$_4$ vortex	18	10.7-16.8	13.80	±1.91	3.66	0.450	13.74

TABLE 9B

Statistical parameters calculated from rhizoplast periodicity measurement data

Fixative	No. of organelles	Light Band					
		range nm	$\bar{X}$ nm	σ	σ^2	S.E.	C.V.%
$FsGAFsOsO_4$	12	3.8-6.9	4.99	±0.94	0.88	0.271	18.83
$TGATOsO_4$	12	3.0-5.4	4.12	±0.23	0.52	0.069	1.67
$FsOsO_4$	26	2.6-3.6	3.11	±0.28	0.08	0.055	8.99
$TOsO_4$	10	2.9-5.8	3.54	±0.84	0.71	0.266	7.51
$TGATOsO_4$ DNP	12	2.3-3.9	3.43	±1.33	1.76	0.384	38.83
$TGATOsO_4$ chilled	10	3.0-3.6	3.29	±0.20	0.04	0.063	6.08
$TGATOsO_4$ vortex	18	2.6-4.4	3.25	±1.39	1.94	0.328	42.76

TABLE 9C

Statistical parameters calculated from rhizoplast periodicity measurement data

Fixative	No. of organelles	Total Period					
		range nm	$\bar{X}$ nm	σ	σ^2	S.E.	C.V.%
$FsGAFsOsO_4$	12	13.8-25.2	18.56	±2.91	8.45	0.840	15.68
$TGATOsO_4$	12	12.2-19.0	15.86	±2.17	4.72	0.627	13.68
$FsOsO_4$	26	13.3-21.3	17.04	±1.86	3.45	0.365	10.91
$TOsO_4$	10	12.2-28.0	17.19	±4.18	17.49	1.322	24.32
$TGATOsO_4$ DNP	12	13.9-19.0	16.61	±1.94	3.79	0.560	11.67
$TGATOsO_4$ chilled	10	12.6-18.4	15.35	±2.17	4.69	0.686	14.14
$TGATOsO_4$ vortex	18	13.8-20.8	17.17	±1.94	3.72	0.457	11.30

period, the Chi-square value obtained from the computation revealed that the variances were not significantly different from each other at the 0.05 level of probability. Thus the test suggests that the amount of variation between the samples is not significant, and that the treatments are not effective in reducing the amount of variability as expected.

Within the treatments however, the coefficient of variation (CV) derived from the sample standard deviations shows that there is significant variability in rhizoplast period, not only in the total, but also in the dark and light bands. In a few cases, notably treatments 2, 3, 4 and 6 for the light band, the CV is less than 10 percent, suggesting that the higher CV for the total periods in these cases may be due largely to variation in the dark band. Unfortunately, no distinct trend was noticeable in the data, except that all the treatments give rise to significant variability in the organelle period, and that attempts at reducing or removing this variation were not successful.

Isolation and Purification of *Naegleria* Rhizoplasts:

(a) Isolation

Initially, the main problem of rhizoplast isolation was that of rupturing the cell without damaging the organelle. A number of attempts were made before the main

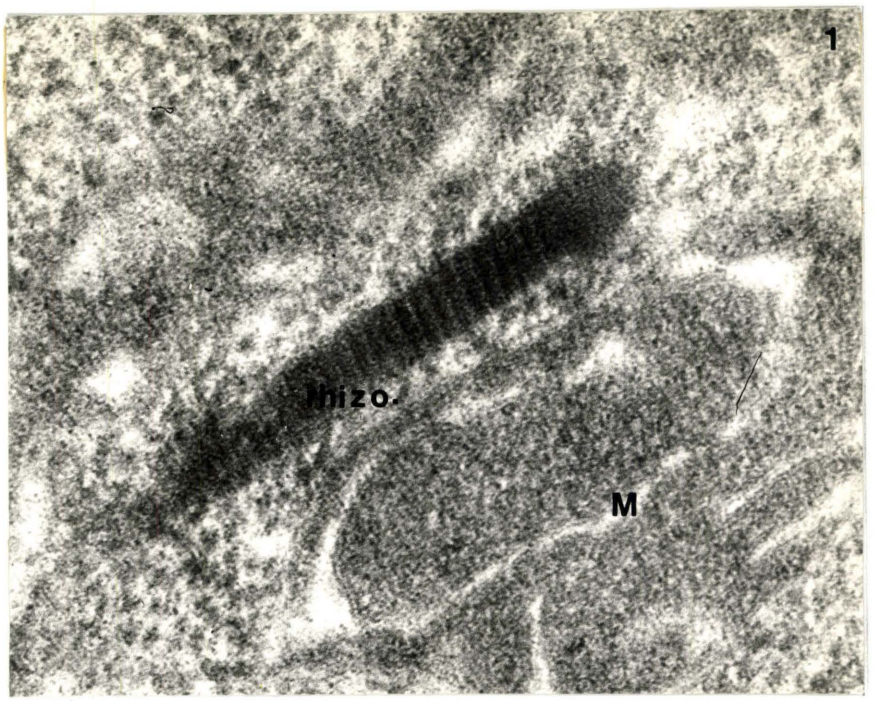
FIGURE 11

1. FsGAFsOsO$_4$-fixed rhizoplast, large period

The rhizoplast (r) in this FsGAFsOsO$_4$-fixed cell shows a large period of 25.2 nm - dark band of 19.5 nm and light band of 5.7 nm. A large mitochondrion (m) with a homogeneous matrix is present in the dense cytoplasm. Magnification X 70,000.

2. FsGAFsOsO$_4$-fixed rhizoplast, small period

This rhizoplast (r) shows a period of 17.0 nm composed of a dark band of 12.3 nm and a light band of 4.7 nm. Note the mitochondrion (m) with a distinct outer membrane and dense matrix material. The cytoplasm appears very dense with numerous ribosomes and ribosome clusters, as it does in all cells fixed with FsGAFsOsO$_4$.
Magnification X 70,000.



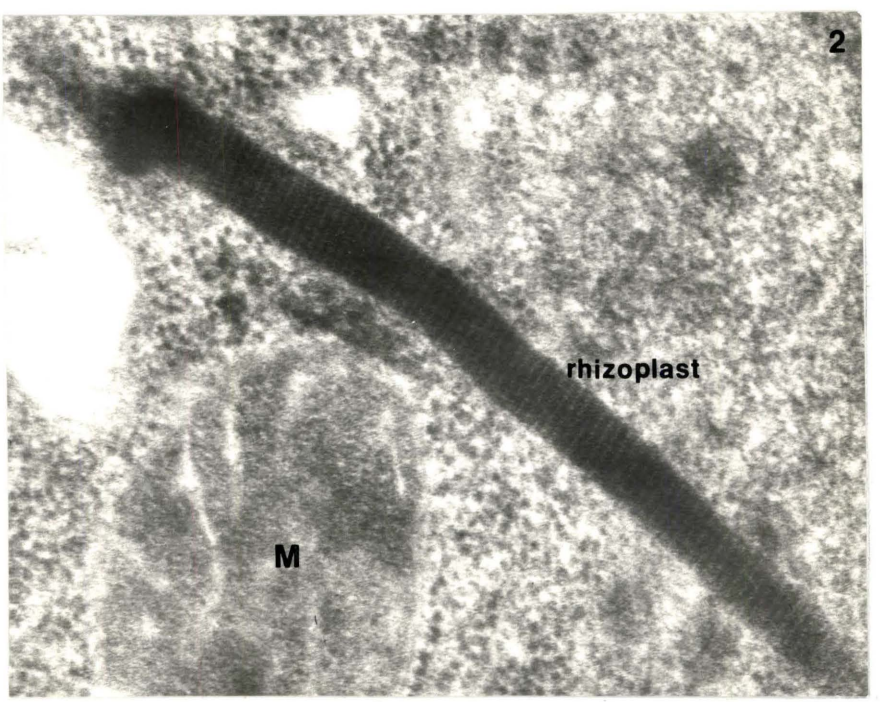


FIG.II

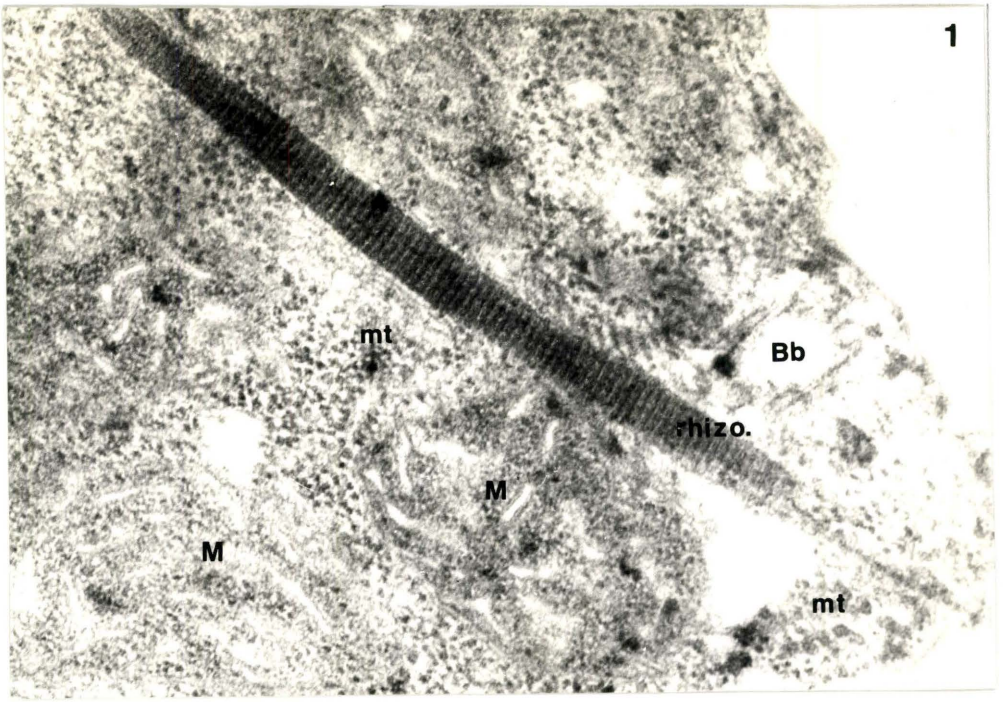
FIGURE 12

1. TGATOsO_4-fixed rhizoplast, large period

This cell was fixed with TGATOsO_4. The basal body (Bb) is tangentially sectioned and thus appears indistinct. The rhizoplast-associated microtubules (ram) are visible only as a number of vague microtubules alongside the rhizoplast (r). The rhizoplast itself exhibits a period of 19.0 nm composed of a dark band of 14.6 nm and a light band of 4.4 nm. Numerous mitochondria (m) are seen within the cell as are numerous structures that may be cytoplasmic microtubules (mt). Magnification X 70,000.

2. TGATOsO_4-fixed rhizoplast, small period

This section lacks contrast compared with Figure 12 1., perhaps due to the fact that the capsule from which this section was cut was not "in block" stained with uranyl acetate as was that from which Figure 12 1. was taken. The long rhizoplast (r) has a period of 12.3 nm composed of a dark band of 9.3 nm and a light band of 3.0 nm. The basal bodies (Bb_1 and Bb_2) are indistinct as are the rhizoplast-associated microtubular structures (ram_1 and ram_2) and the single interbasal body connector (ibc). Numerous mitochondria (m) appearing as grey masses in the cytoplasm are visible. Two membrane-bound vesicles (v) are also visible - one on each side of the rhizoplast. Magnification X 70,000.



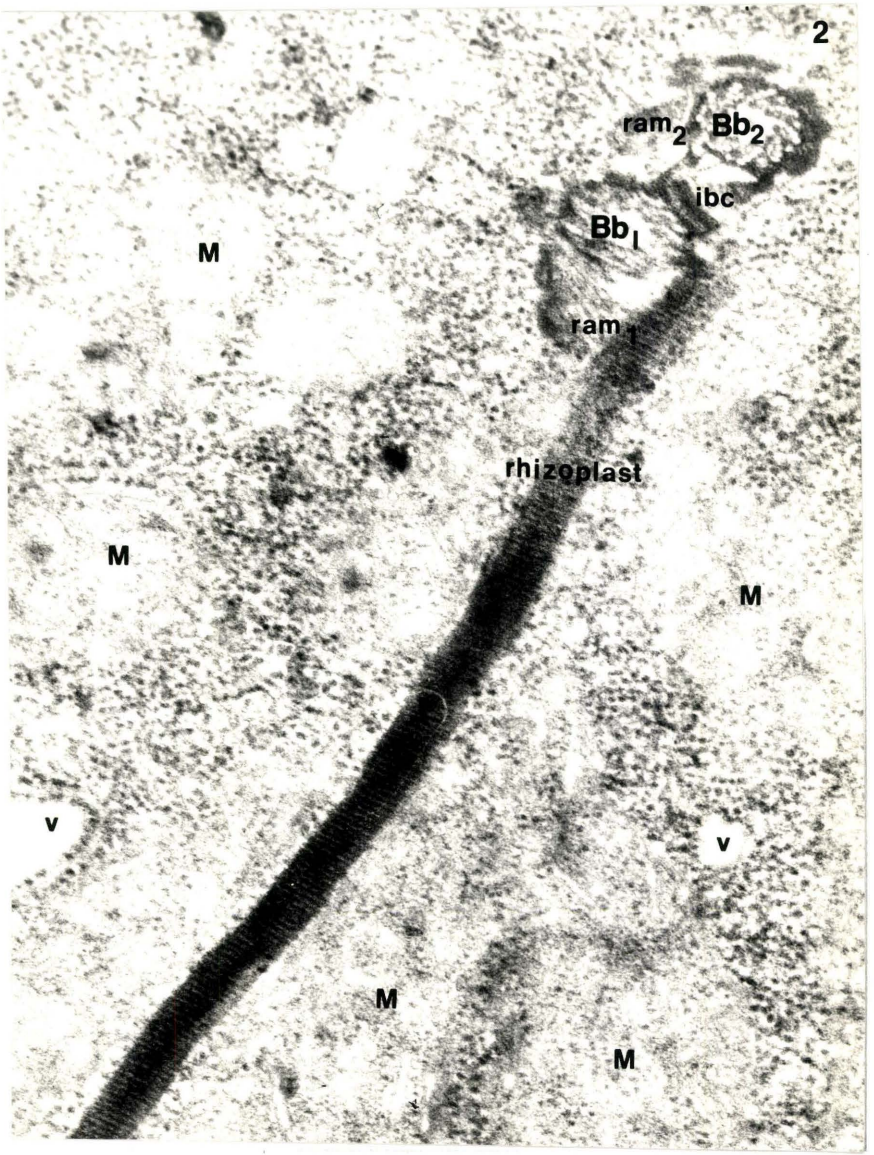


FIG. 12

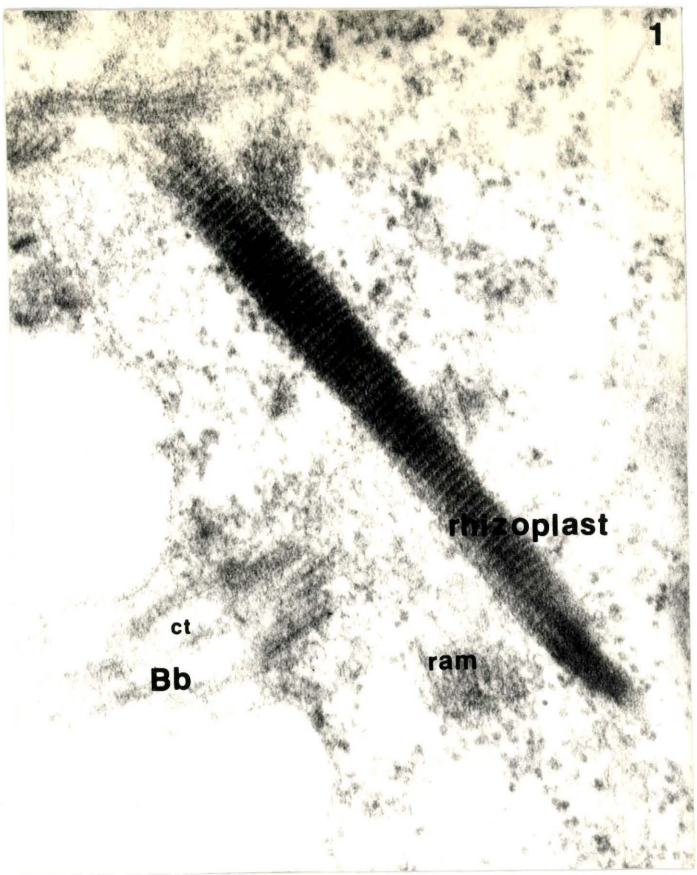
FIGURE 13

1. $FsOsO_4$-fixed rhizoplast, large period

This rhizoplast (r) exhibits a distinct period typical of $FsOsO_4$-fixed cells. The rest of the structures in the photomicrograph appear indistinct - the basal body (Bb) is sectioned almost at the junction of the basal body and the flagellum as evidenced by the central tubules seen in the central region of the organelle (ct). The rhizoplast-associated microtubules (ram) appear as a mass of electron-dense material only - the microtubular structure being indistinct. The cytoplasm is not very dense and suggests that perhaps the cellular membrane has been damaged during the fixation procedure. The period of the rhizoplast is composed of a 17.9 nm dark and a 3.5 nm light band for a total period of 21.4 nm. Magnification X 70,000.

2. $FsOsO_4$-fixed rhizoplast, small period

This rhizoplast exhibits a period of 13.3 nm composed of a dark band of 10.4 nm and a light band of 2.9 nm. Three basal bodies (Bb_1, Bb_2 and Bb_3) and two rhizoplast-associated microtubular structures (ram_2 and ram_3) are visible. The basal bodies have been sectioned distal to the basal plate region of the organelle. Mitochondria (m) and cytoplasm are distinctive of $FsOsO_4$-fixed cells. Magnification X 70,000.



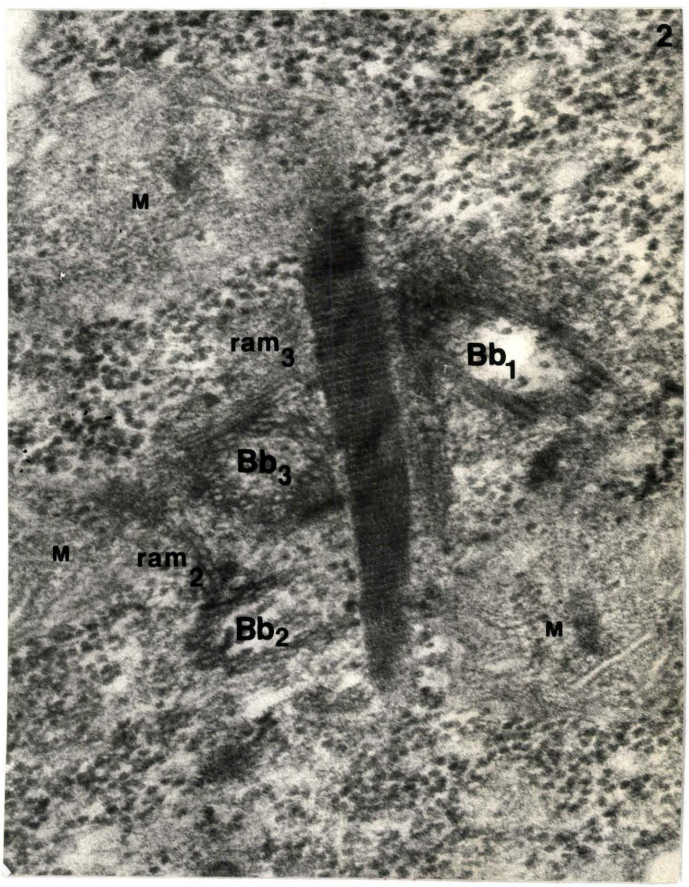


FIG. 13

FIGURE 14

1. $TOsO_4$-fixed rhizoplast, large period

This rhizoplast was fixed in $TOsO_4$ only. Note the "empty" cytoplasm with only a few ribosome clusters (rc) with a period of 14.3 nm for the dark band, 3.2 nm for the light band and a total period of 17.5 nm. The mitochondria (m) appear relatively empty with the membranes and cristae showing up very distinctly. A possible rhizoplast-associated microtubular structure (ram) lies just below Bb_1, however, its structure is not distinct in this photomicrograph. Magnification X 70,000.

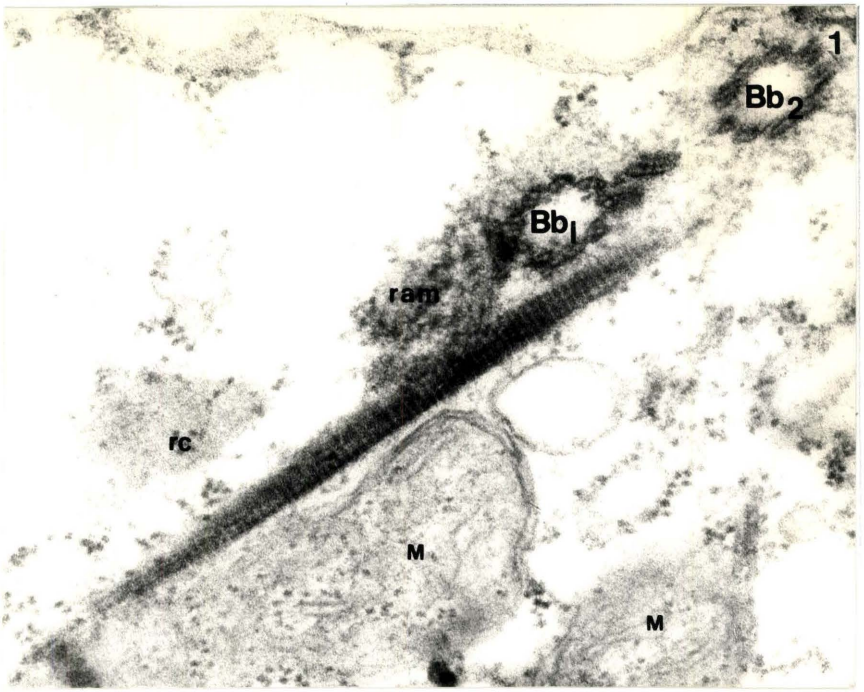
2. $TOsO_4$-fixed rhizoplast, small period

The organelle shown here is from a cell fixed only with $TOsO_4$. Note the dense cytoplasm and numerous ribosomes (this cell did not undergo lysis during the fixation procedure as did the one in Figure 11 left. The rhizoplast (r_1) exhibits a period of 11.2 nm composed of an 8.8 nm dark band and a 3.4 nm light band. Note the three basal bodies (Bb_1, Bb_2 and Bb_3) and the second rhizoplast (r_2) arising in the vicinity of Bb_3. Numerous mitochondria (m) are visible in the figure as are several microtubules (mt) above the basal bodies. Only one interbasal body connector (ibc) and two, or possibly three, rhizoplast-associated microtubular

FIGURE 14 (continued)

structures (ram_1, ram_2 and ram_3) are seen. The triplet structure of Bb_1 is quite distinct in this micrograph.

Magnification X 70,000.



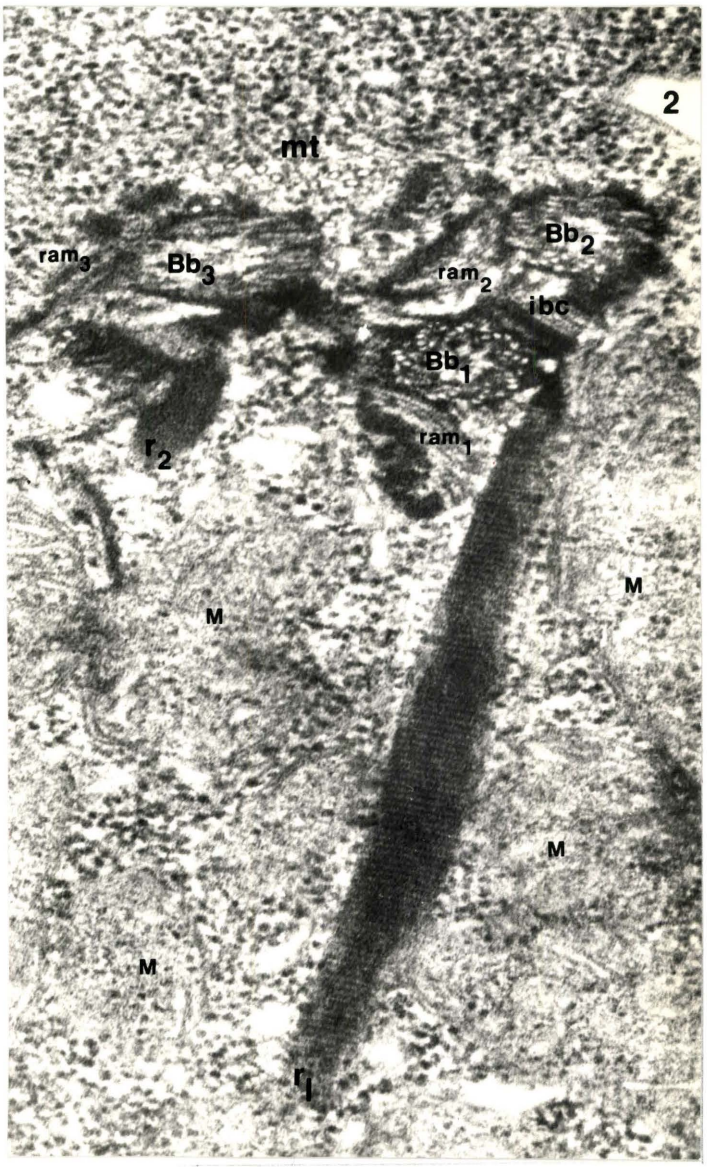


FIG. 14

FIGURE 15

1. DNP treated, TAGTOsO$_4$-fixed rhizoplast, large period

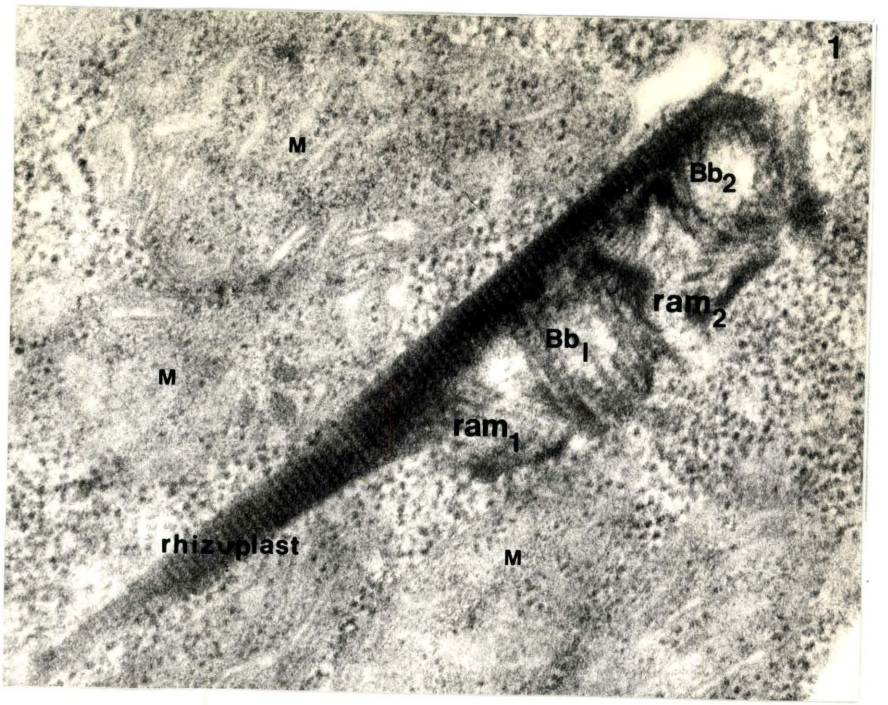
The rhizoplast (r) shown in this photomicrograph is from a TGATOsO$_4$-fixed cell treated with 5 x 10^{-3} M DNP before fixation. The period of 17.5 nm is composed of a dark band of 14.5 nm and a light band of 3.0 nm. The basal bodies (Bb_1 and Bb_2) are not distinct, however they are unmistakable. The two rhizoplast-associated microtubular structures (ram_1 and ram_2) have been sectioned tangentially and are indistinct, though a fan-like arrangement of the microtubules can be seen near Bb_1. Note that the rhizoplast is extended the full length of both basal bodies and that ram_1 seems to be closely associated with the organelle. Numerous mitochondria (m) are present in the section and show a relatively dense matrix. The "fading-out" of the rhizoplast distal to the basal bodies is commonly seen in sectioned material. Magnification X 70,000.

2. DNP treated, TGATOsO$_4$-fixed rhizoplast, small period

This section was taken from a cell pretreated with 5 x 10^{-3} M DNP before TGATOsO$_4$ fixation. Two very indistinct basal bodies (Bb_1 and Bb_2) and an interbasal body connector (ibc) may be seen in the figure just to the left of the rhizoplast (r) which exhibits a period of 15.6 nm (a dark band of 12.4 nm and a light band of

FIGURE 15 (continued)

3.2 nm). The cytoplasm is very dense with ribosomes and ribosome clusters and numerous mitochondria (m) may be seen. Magnification X 70,000.



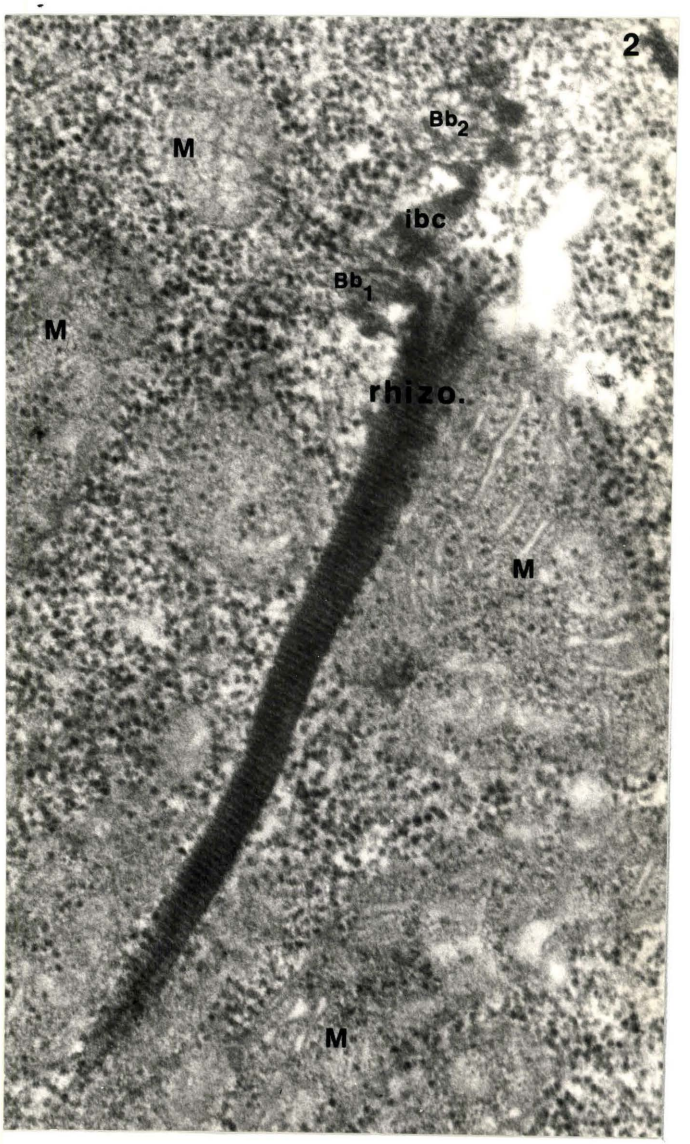


FIG.15

FIGURE 16

1. Vortexed, TGATOsO_4-fixed rhizoplast, large period

Cells were vortexed to remove flagella before being fixed in TGATOsO_4. The rhizoplast (r) in the figure is quite distinct and exhibits a period of 19.6 nm composed of a 16.8 nm dark band and a 2.8 nm light band. Two basal bodies (Bb_1 and Bb_2), two rhizoplast-associated microtubular structures (ram_1 and ram_2) and one interbasal body connector (ibc) with an associated band structure (abs) are present. The trifibred abs exhibits banding similar to the ibc and seems to join with three of the triplet microtubules of the basal body. This accessory to the ibc was seen only in this micrograph. The electron-opaque condensations of material (c) cannot be identified. Magnification X 70,000.

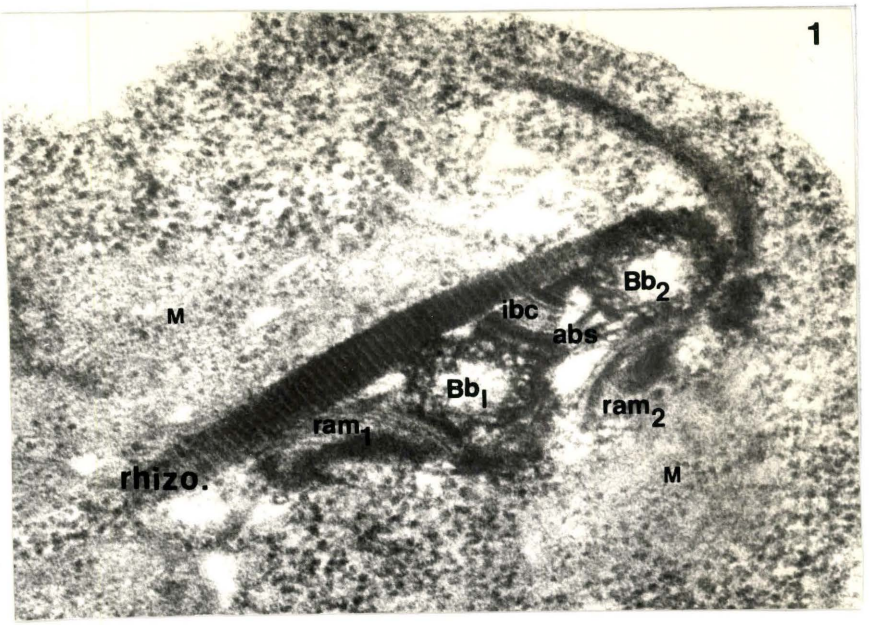
2. Vortexed, TGATOsO_4-fixed rhizoplast, small period

This figure shows a sectioned rhizoplast from a vortexed cell fixed in TGATOsO_4. The rhizoplast (r) exhibits a period of 14.6 nm with a dark band of 10.6 nm and a light band of 4.1 nm. The two basal bodies (Bb_1 and Bb_2) have been sectioned above the level of the basal plate and thus do not exhibit the cartwheel structure. The interbasal body connector (ibc) between the two basal bodies is distinct and evident attachments to both of the basal bodies is seen. The ram's (ram_1 and

FIGURE 16 (continued)

and ram_2) are poorly sectioned and are thus not clearly evident.

Magnification X 70,000.


1
M
ibc
abs
Bb2
Bb1
ram2
ram1
rhizo.
M

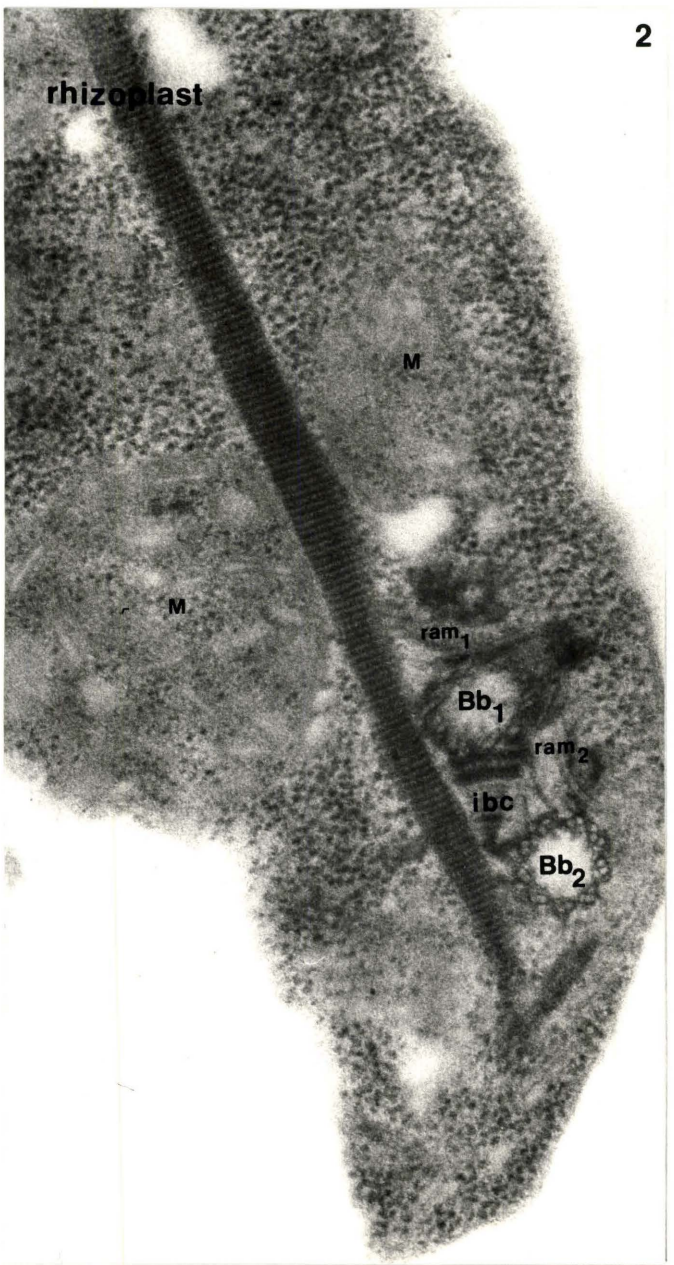

2
rhizoplast
M
M
ram1
Bb1
ram2
ibc
Bb2

FIG. 16

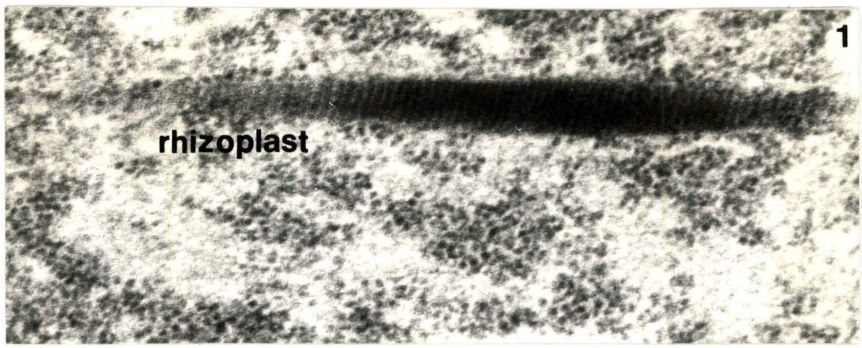
FIGURE 17

1. Chilled, TGATOsO_4-fixed rhizoplast, large period

The rhizoplast (r) in this photomicrograph exhibits a period of 18.4 nm composed of a dark band of 14.9 nm and a light band of 3.5 nm. The cytoplasm is not very dense and appears to be that of a lysed cell, perhaps as a result of the cold treatment before TGATOsO_4 fixation. Magnification X 70,000.

2. Chilled, TGATOsO_4-fixed rhizoplast, small period

This section shows four basal bodies (Bb_1, Bb_2, Bb_3 and Bb_4) and two rhizoplasts (r_1 and r_2) within a single cell. r_1 shows a period of 13.3 nm with a dark band of 10.3 nm and a light band of 3.0 nm. The second rhizoplast shows a period of 13.8 nm composed of a 10.4 nm dark band and a 3.4 nm light band. The basal bodies are typical of cold-fixed cells; no triplets could be seen and the cartwheels were indistinct. Five ram's (R) were seen in the section but were not clearly outlined. Note that R_3 seems to be associated with Bb_1 and r_2, indicating perhaps that multiple attachments of flagellar apparatuses is possible through the ram's and the ibc's. Cells with two or more rhizoplasts were seen rather frequently in the course of the study; they are not an unusual occurrence. The mitochondria (m) seem swollen and have a very dense matrix, perhaps a result of the cold treatment. Magnification X 70,000.


1
rhizoplast

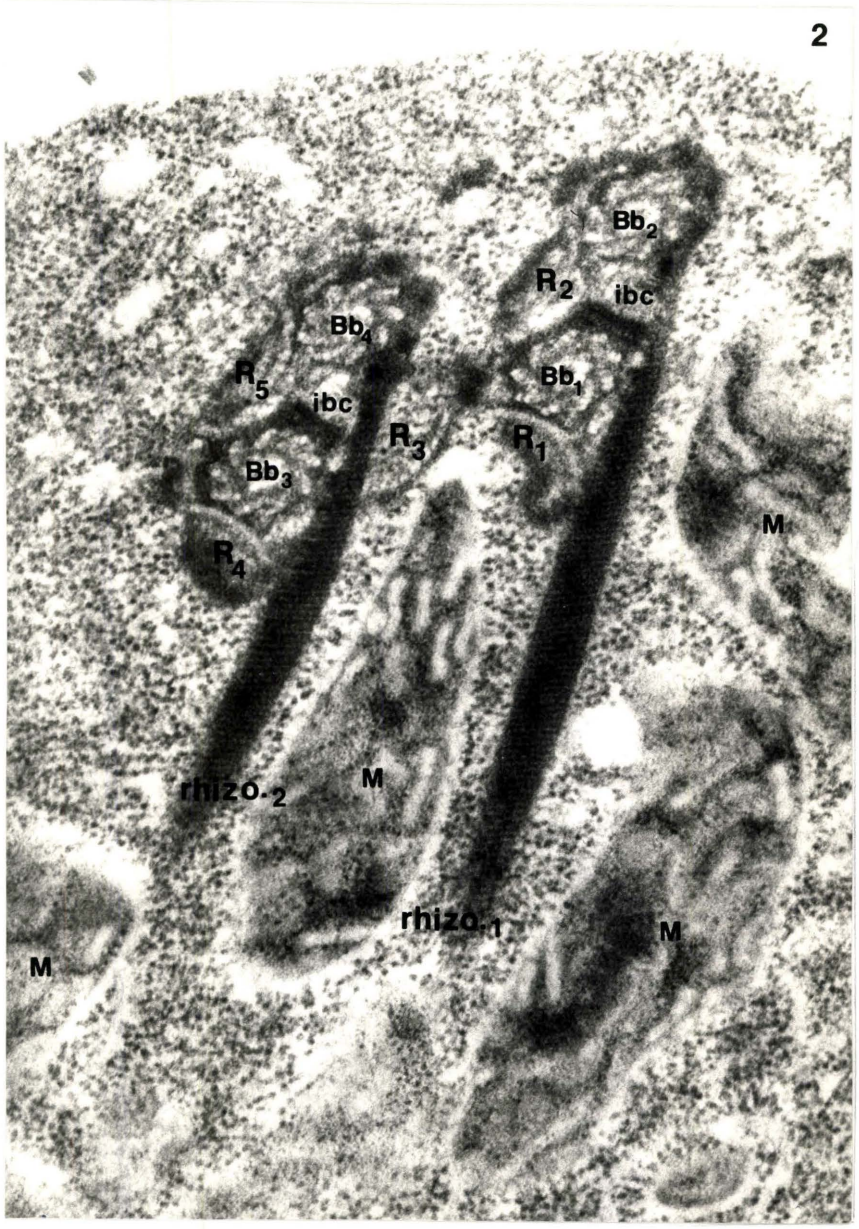

2
Bb2
R2
ibc
Bb4
R5
ibc
Bb1
R3
R1
Bb3
M
R4
rhizo.2
M
rhizo.1
M
M

FIG. 17

isolation technique used throughout this study was discovered to be the most effective.

Isolation of flagellar apparatuses by lysis of flagellates in 0.7 percent NH_4OH (Schuster, 1963) was unsuccessful although the cells lysed within 3 min at room temperature or on ice and some flagellar apparatuses were seen in the suspension (phase optics). The method was considered inadequate due to the small number of flagellar apparatuses isolated.

Tween 80, Tween 20, and Span 20 had no lytic effect on the flagellates, even after a 60 min treatment at a concentration of 1.0 percent. Sodium deoxycholate and sodium dodecylsulphate were too harsh, lysing cells after less than 5 min at a 1.0 percent concentration and damaging the rhizoplasts sufficiently that they were not visible under phase optics. Lower concentrations (0.5 and 0.1 percent) of the latter two detergents were used in another attempt to define the necessary conditions. The 0.5 percent concentration was found to be too harsh in both cases while the lower concentration was found to be ineffective in lysing the cells.

Grinding the flagellates in a tissue grinder of the Ten Broeck type was unsuccessful. A few strokes of the pestle were sufficient to deflagellate the cells, but even 50 percent lysis could not be attained after 100 or more strokes. The Dounce homogenizer was found to be effective

if the cell concentration was high - more than 10^7 cells per ml. More than 90 percent of the cells could be ruptured after 60 to 80 strokes of the pestle without damaging the rhizoplasts to a significant degree. The technique was modified by the addition of 2.5 mM $MgSO_4$, when it was realized that Mg^{+2} was likely a necessary requirement for the maintenance of rhizoplasts *in vitro*. The extraction media thus consisted of the buffers in which the cells were transformed into flagellates plus $MgSO_4$ to give a final Mg^{+2} concentration of 2.5 mM.

(b) Attempted purification

After the high-speed-low-speed centrifugation series designed to remove large and small contaminants from the suspension (1 min at rheostat setting 5 in the IEC followed by a 2 min spin at 7 of the supernatant followed by resuspension of the pellet and a 1 min spin at setting 5) the rhizoplasts were not the only entities present in the supernatant. Mitochondria, membrane fragments, microtubules, ribosomes, basal bodies and other unidentifiable materials were present on electron microscopic examination.

Since the majority of the material present as contaminant was membranous, Gibbons' digitonin treatment (1963), used for the removal of membranes from isolated flagella and cilia was attempted with the rhizoplast

suspension. Gibbons' formula dissolved rhizoplasts after less than 60 min. All variations mentioned in the MATERIALS AND METHODS section, including, buffer changes, temperature changes, exposure time and digitonin concentration did not result in the discovery of a suitable procedure capable of dissolving, or at least disrupting, the contaminants without damaging the rhizoplasts. The damaged organelles appeared fibrous in nature when negatively stained with uranyl acetate as seen in Figure 18.

Sarkosyl, a mild detergent, was used with more success. A 0.1 percent solution of Sarkosyl in buffer with 1.25 m$\underline{M}$ $MgSO_4$ (to maintain rhizoplast integrity) was found to be useful in the disruption of contaminants while leaving the rhizoplasts intact. As mentioned in the METHODS section, the Mg^{+2} concentration had to be lowered from 2.5 m$\underline{M}$ to 1.25 m$\underline{M}$ since the higher concentration caused precipitation of the detergent. It was found that the rhizoplasts would remain intact if the Mg^{+2} concentration was greater than 1 m$\underline{M}$ and that Sarkosyl precipitation was greatly reduced if the Mg^{+2} concentration was less than 1.5 m$\underline{M}$. The value of 1.25 m$\underline{M}$ was chosen because of its ease of preparation from the 2.5 m$\underline{M}$ extraction medium simply by adding an equivalent amount of 0.2 percent Sarkosyl in the appropriate buffer. Although the Sarkosyl treatment was useful in the disruption of the contaminants it did not dissolve them. A tendency for the disrupted material to stick to the basal body end

FIGURE 18

The fibrous nature of a damaged rhizoplast

This figure shows a portion of a damaged rhizoplast. This particular organelle shows the effect of a thirty minute treatment with Gibbons' digitonin formula (see the text for details of the treatment). The fibrous nature of the rhizoplast is evident in this micrograph. Note that even the small fibres (f) are cross-banded with the characteristic period of the organelle. The background material includes several unidentified structures that may be breakdown products from rhizoplasts or other cellular components (s).

Magnification X 70,000.

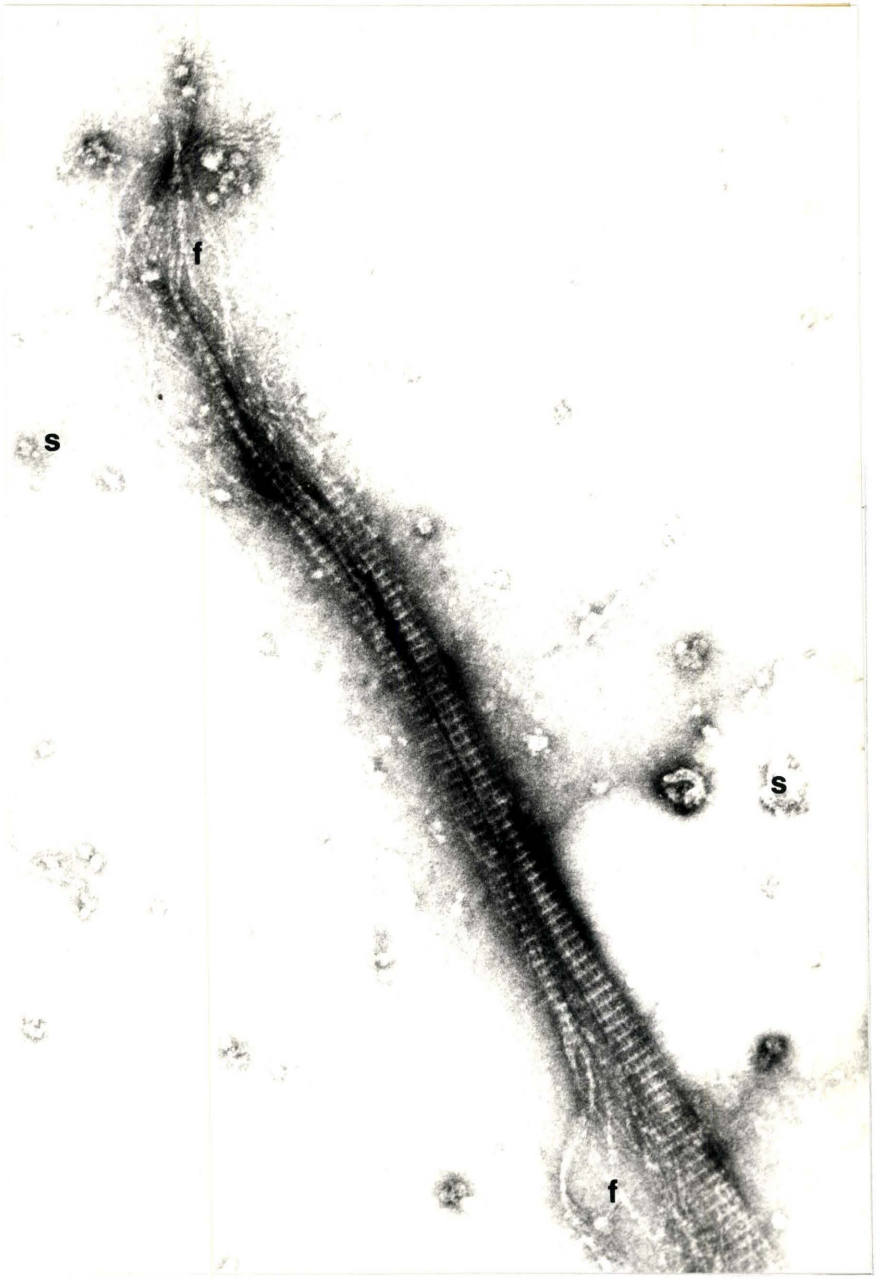


FIG. 18

of the rhizoplasts was evident even after vortex mixing and centrifugation of the suspension. The material could not be removed in this manner so urea treatments and treatment with chelating agents were initiated.

Urea treatments were used in an attempt to denature only this contaminating material, perhaps removing it from the rhizoplasts by disrupting hydrogen bonding, leaving the rhizoplasts intact. The higher concentrations - 8 M, 6 M, 4 M, and 2 M - disrupted everything present in the solution after 30 min at room temperature or after approximately 60 min on ice. The 1.0 M and 0.5 M were ineffective in removing contaminants even after 120 min, although some additional disruption of the material was noticed. After approximately 240 min in a solution of 1.0 M or 0.5 M urea the rhizoplasts began to appear fibrous and generally disrupted as is the organelle in Figure 18.

At this point in the study it was thought that the nature of the attachment of the contaminating material to the organelles was different from a simple hydrogen-bonding mechanism. Since $MgSO_4$ was present as a necessary part of the isolation medium, it was conceivable that the binding might be due to the Mg^{+2} ion. Removal of divalent cations by the addition of EDTA and EGTA to the isolated rhizoplast suspension was not useful. Rhizoplasts and contaminants suspended in 5 mM EDTA dissolved within 30 min in the cold. Electron microscope examination of negative-

stained preparations of the rhizoplast suspension revealed only very small unidentifiable bits of material. Nothing resembling a rhizoplast was seen, although a few small unidentifiable fibres of diameter 5 to 10 nm were seen. Rhizoplasts remained intact in cold 5 mM EGTA but the contaminants were still attached. Since EGTA has a much greater affinity for Ca^{+2} ion than Mg^{+2} ion it seems logical to assume that the presence of Mg^{+2} is a factor to be considered when trying to remove the contaminants. Longer exposure to the EGTA damaged the rhizoplasts before removing or disrupting the contaminants. After 120 min in the EGTA the rhizoplasts began to take on a fibrous appearance suggestive of breakdown.

The purification of rhizoplasts using detergents, centrifugation, urea, EDTA and EGTA was unsatisfactory. It was conceivable that the rhizoplasts would be stable at a pH at which the contaminants would be unstable. If this were the case, the purification procedures would be greatly simplified. Isolated rhizoplasts were pelleted and resuspended in cold 25 mM Tricine with 5 mM $MgSO_4$ added and after 2 hours at room temperature grids were made, negatively-stained, and observed, yielding the results presented, in a subjectively qualitative fashion in Table 10. As can be seen the rhizoplasts are stable at pH values ranging from 7.3 to 8.3 and the contaminants are stable in the overlapping range of 6.9 to 8.3. If the order of

TABLE 10

The pH stability of isolated rhizoplasts and contaminating materials

The Table shows in an arbitrary qualitative fashion the pH stability of rhizoplasts in 25 mM Tricine with 5 mM $MgSO_4$ at room temperature (22 to 23°C). The relative amounts of materials present after a two hour treatment are indicated by the number of "+" signs. Flagellates were prepared, and rhizoplasts were isolated in 25 mM Tricine at pH 6.8. The organelles were harvested by a 2 min spin at rheostat setting 7 in the IEC centrifuge and were resuspended in the various solutions. The pH's were re-adjusted and the two hour period was begun. After treatment, observations were made using uranyl acetate negative-stained grids.

TABLE 10

The pH stability of isolated rhizoplasts and contaminating materials

Test	1	2	3	4	5	6	7	8	9	10
pH before	4.1	5.0	6.0	6.5	7.0	7.5	8.0	8.5	9.0	10.1
pH after 2 hours	5.5	4.6	5.7	6.6	6.9	7.3	7.6	8.3	8.0	9.5
Rhizoplasts after 2 hours					+	+++	++++	++	+	
Contaminant after 2 hours			+	+	++	+++	+++	+++	+	

stability was reversed the method may have been of some use, however the approach is ineffective.

Sucrose gradient centrifugation was utilized along with a number of the above approaches. In general, the method was moderately successful as it was possible to concentrate the bulk of the rhizoplasts from a typical isolation (4 NM plates) into approximately 1 ml sucrose. It was thought that this would be a starting point for further purification as prior cleanup spins (1 min at rheostat setting 5 in the IEC followed by a 2 min spin of the supernatant at 7 followed by resuspension of the pellet and a 1 min spin at setting 5) and the gradient centrifugation greatly reduced the amount of contaminating material. The appearance of a gradient before and after spinning is shown in Figure 19. Electron microscopic examination of the fractions collected by dripping yielded the data that accompany the "after" tube.

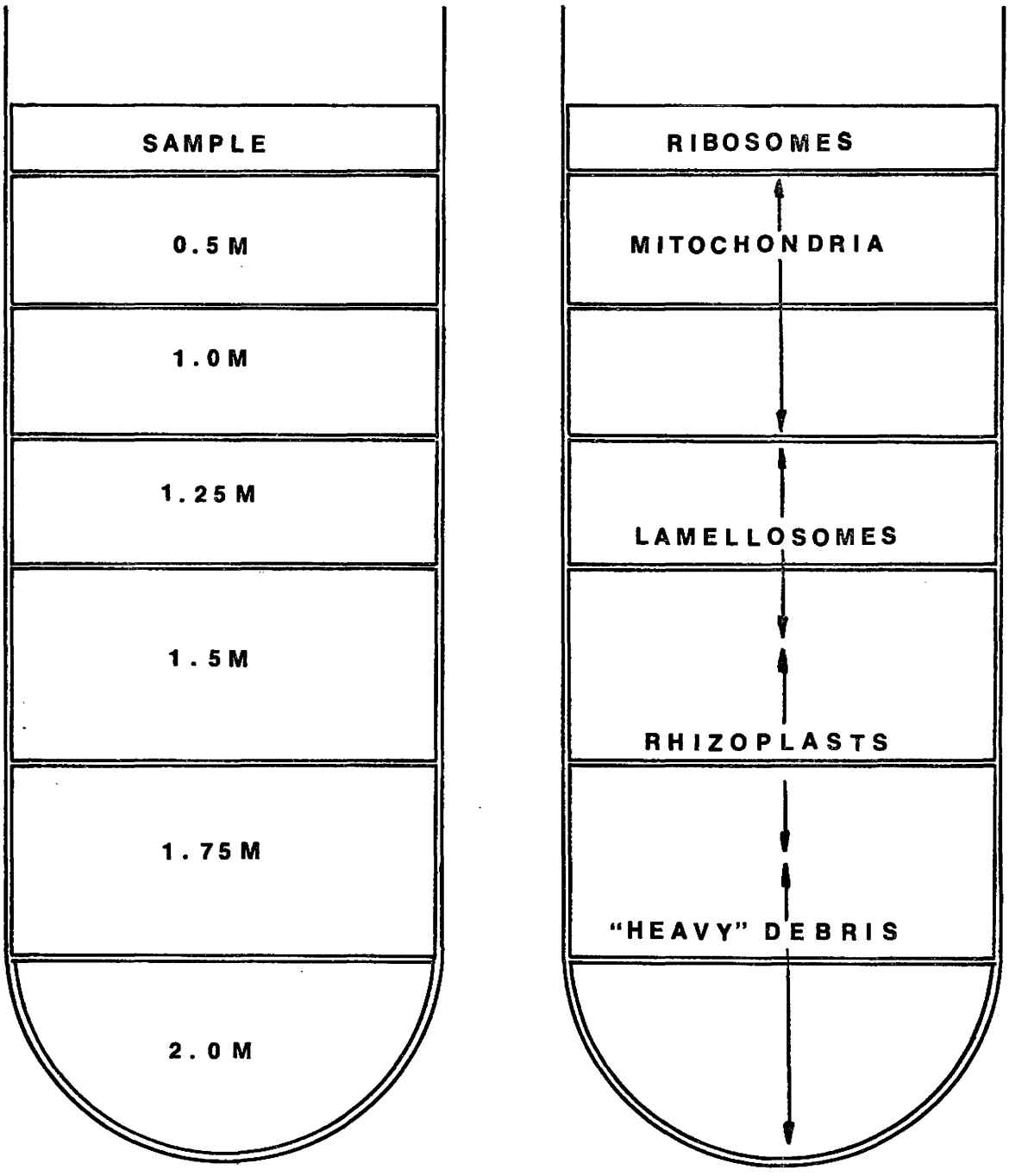
Total purification of the rhizoplast was not achieved during the course of the present work, however, the work has shown a number of properties of the nature of the purification problem. Presented here, as a summary to this section of the results, is a list of what the various attempts have shown about the system. This list may perhaps, in the future, show another way to approach and solve the purification problem.

1. Mg^{+2} ion is a possible requirement in the medium at a

FIGURE 19

The distribution of rhizoplasts and contaminants through a sucrose gradient

The figure shows the "before" and "after" appearance of sucrose gradient tubes run in an attempt to separate isolated rhizoplasts from the rest of the material in the solution. Details of gradient preparation may be found in the text as only a short description will be given here. The gradient was prepared as shown in the left tube, spun in the Sorvall RC-2 centrifuge for 60 min at 8,000 rpm in the HB-4 head (10,444 g). The tube on the right shows the approximate distribution of material through the tube as determined by examination of uranyl acetate negative-stained grids prepared from fractions collected from the gradient.


SAMPLE
0.5 M
1.0 M
1.25 M
1.5 M
1.75 M
2.0 M
RIBOSOMES
MITOCHONDRIA
LAMELLOSOMES
RHIZOPLASTS
"HEAVY" DEBRIS

concentration of 1.25 to 2.5 m$\underline{M}$ to maintain the structural integrity of the organelle.

2. Sarkosyl, at a concentration of 0.1 percent in suspension with 1.25 m$\underline{M}$ Mg^{+2} disrupts contaminants and leaves the rhizoplast intact.
3. EDTA and EGTA studies suggest that the observed "stickiness" of the contaminants is dependent upon the presence of Mg^{+2}.
4. The pH stability range of the rhizoplast is 7.3 to 8.3 units in 25 m$\underline{M}$ Tricine with 5 m$\underline{M}$ Mg^{+2} present at 22 to 24°C.
5. Under the conditions specified in the MATERIALS AND METHODS section of this thesis, rhizoplasts band at the 1.5/1.75 $\underline{M}$ interface of a sucrose gradient.

Isolated Rhizoplasts:

Isolated rhizoplasts were studied using two standard techniques of electron microscopy; negative-staining with uranyl acetate and shadow-casting with platinum-carbon.

(a) Negative-Staining

"Metal-embedding" is a term used to describe negative-staining since the material to be studied is embedded within a fine layer of electron-dense material. Uranyl acetate (1 percent aqueous solution) was used in preference to 1 percent phosphotungstic acid (PTA) and 2 percent ammonium molybdate since the latter two had a more positive-staining effect. With PTA the effect was so great that striations could not be seen on the organelles. Uranyl acetate staining was accomplished by applying stain for a short time to a grid on which a drop of suspension containing rhizoplasts had been placed, leaving it there for 30 sec and blotting it away with a small piece of filter paper. Not all of the rhizoplasts on the grid were negatively-stained, just those in areas where stain was deposited. As a result, both positively-stained and negatively-stained organelles are visible on a single grid.

Morphology of Isolated Rhizoplasts

Rhizoplasts appear as tapered fibres with one or more

basal bodies attached to the wider end. In some cases it was noticed that the rhizoplast splays out to a branched structure at the basal body end, having two or four branches. An average width at the point of basal body attachment was 228.7 nm for 10 measurements. The rhizoplast gradually tapers along its length to an approximate width of 20 nm. For 5 organelles measured the average length was 9.98 microns.

An obvious difference existed between the appearance of negatively-stained and positively-stained rhizoplasts. Figure 20 1. (positive) and 2. (negative) show this difference. Some loss of detail is evident but the most striking observation is the reversal of contrast. A diagrammatic representation of the reversal is presented in Figure 21.

Most of the rhizoplasts used for the period measurements described below were positively-stained, i.e., in an area of the grid where stain was not deposited. Since the technique by which the organelles were stained is designated "negative-staining", all rhizoplasts treated as such are referred to as being "negatively-stained".

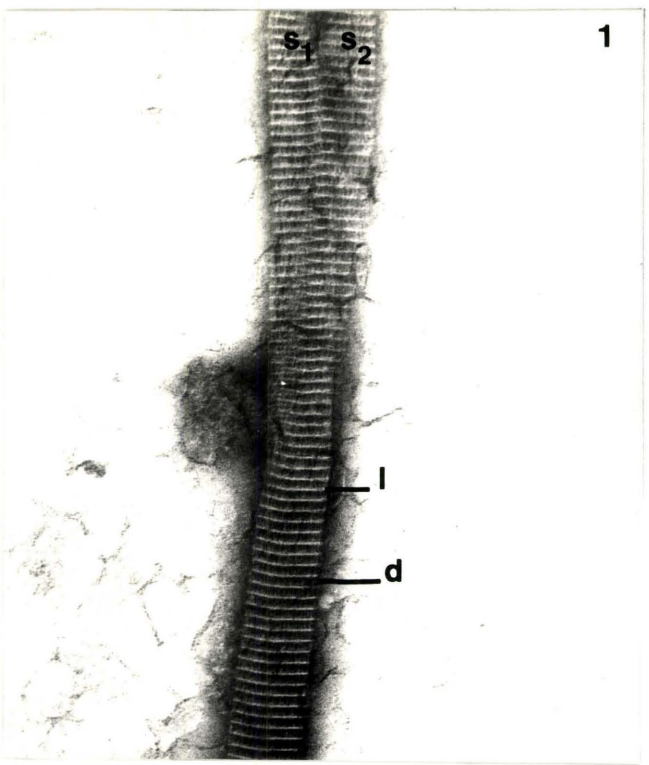
Isolated rhizoplasts were strikingly regular in appearance, however certain of those photographed were damaged and as such revealed fine structural features of the organelle not visible in intact rhizoplasts. The fibrous nature of the organelle is evident from Figure 22.

FIGURE 20

Comparison of a positively-stained and a negatively-stained rhizoplast

1. The figure shows a positively-stained isolated rhizoplast. Note the narrow light bands (l) and the relatively wider dark bands (d). The organelle is beginning to split into two segments (s_1 and s_2) which show identical periods. Magnification X 70,000.

2. This rhizoplast is negatively-stained with uranyl acetate (metal-embedded) and shows a reversal of contrast compared with Figure 20 1. The periods of both organelles are the same at 20.8 nm. Note that no "corrugation effect" can be seen in this micrograph as mentioned in the text. Magnification X 70,000.



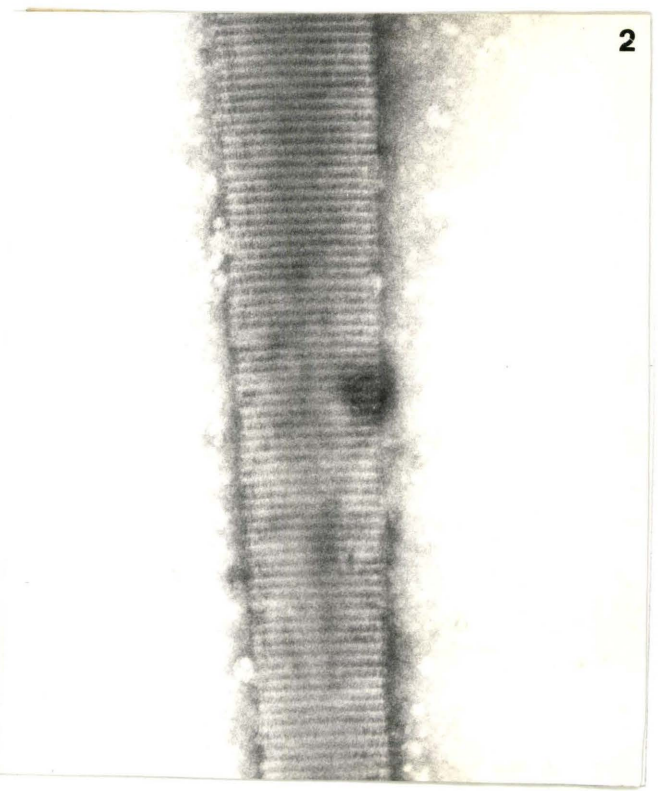


FIG. 20

FIGURE 21

Diagrammatic representation of the contrast-reversal phenomenon of Figure 20

The upper diagram depicts the positively-stained situation in which the indentations or gaps appear as more electron-transparent than the ridges. The lower diagram shows the negatively-stained situation in which the hatched area represents the deposition of stain on the organelle. Above each diagrammatic organelle is a representation of what is seen with the electron microscope.

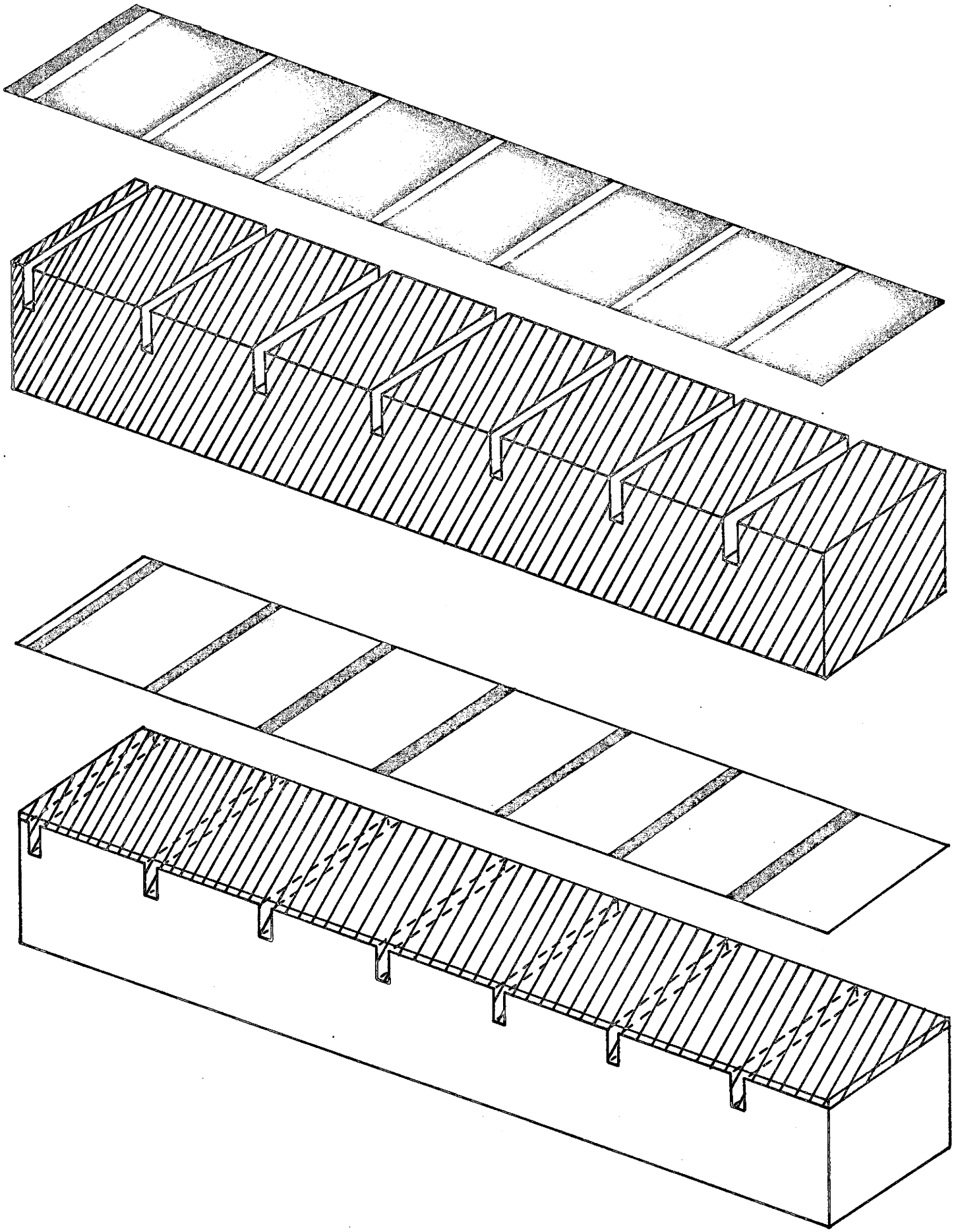


FIGURE 22

The fibrous nature of an isolated rhizoplast

The organelle in this figure shows the fibrous nature of the rhizoplast. It was not treated in any special way, but was left in the tube for some 60 hours before observation, and is thus in the process of disintegration. The small component fibre bundles are cross-banded and the splaying branches of the organelle seem to peel off of the main structure. Various unidentifiable structures, seen in most negative-stained preparations are present in the background. Magnification X 70,000.

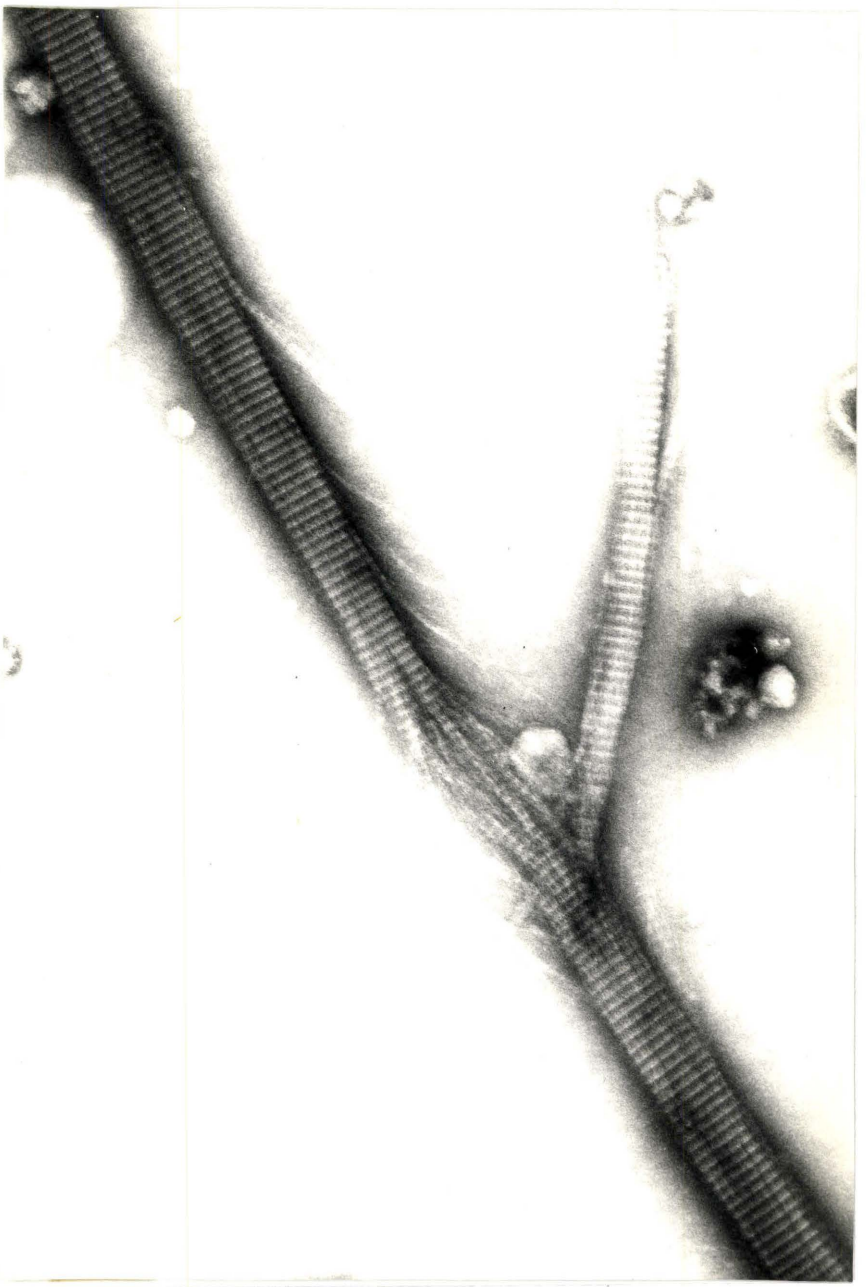


FIG.22

It may be noted that even the splayed fibrils exhibit the characteristic cross-banded pattern, and as such, may lead to the speculation that the organelle is composed of longitudinal fibre bundles with the final diameter of the organelle being dictated by the number of component bundles. In other instances, especially when rhizoplasts are greatly damaged during preparation, areas of the grid may be covered with non-striated fibres of width 14.6 nm (range of 10 measurements was 13.7 to 15.9 nm) and length 290 nm (range of 10 measurements was 220 to 380 nm) as seen in Figure 23. Whether or not these fibres are a result of the breakdown of rhizoplasts is uncertain, although they are not seen when there are intact organelles.

(b) Shadow-Casting

Negative-stained and sectioned material often gave the impression of the organelle being rippled on its surface or on the surface of the section. Since the process of shadow-casting reveals surface contours of the particles so treated, the question as to whether the rhizoplast is a rippled structure could be answered by observing shadowed material. The technique also gives a 3-dimensional appearance to objects on the grid and thus could be used to measure the diameters of the organelle by measuring the shadows and relating the shadow length to the object height.

FIGURE 23

Non-striated fibres, possibly breakdown products of rhizoplasts

This greatly damaged organelle (r) is surrounded by a number of non-striated fibres (f) approximately 15 nm in width. It is thought that these fibres may be breakdown products of rhizoplasts since they are not seen where intact organelles are seen. Various other structures are seen in the background material, including membrane fragments (mf) and clusters of unidentifiable material (c). The organelle was taken from solution after treatment with 0.1 percent Sarkosyl for 1 hour in the cold, placed on a grid and negatively-stained with 1 percent uranyl acetate.

Magnification X 70,000.

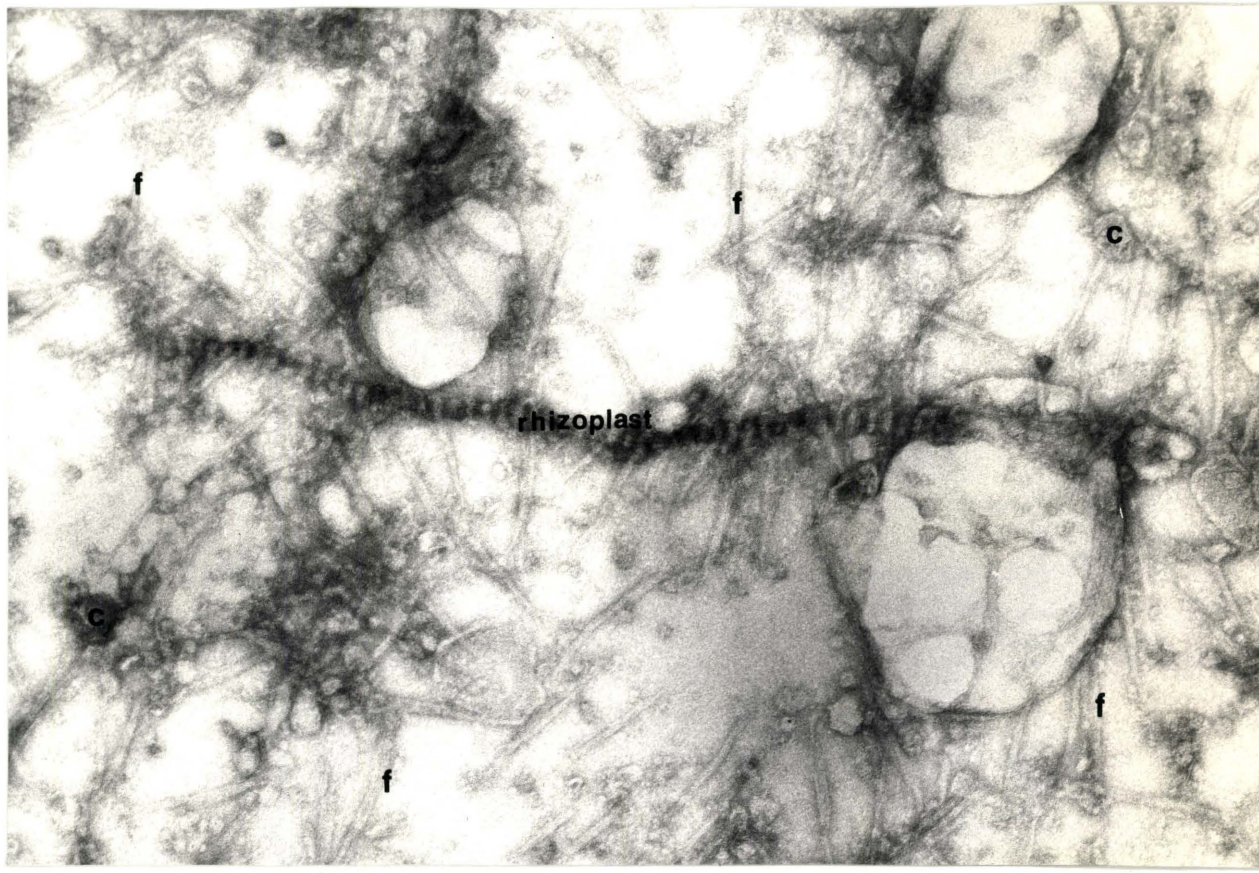


FIG. 23

Morphology of Isolated Shadow-Cast Rhizoplasts

The most striking observation in this section is that the rhizoplasts are rippled. Figure 24 shows the structure of a shadow cast organelle which displays a rippled surface. It is not possible to measure the depth of the ripples on the organelle but the extent of them is significant as they stand out sharply in the material. The rhizoplast in the figure tapers gradually from 306 nm at the basal body end to 110 nm at the point where the structure fades into the background. In this photograph much background material is present, as no attempted purification was done on the sample. Basal bodies are visible in Figure 24 though they are indistinct and appear only as raised structures from the surface of the organelle. In favourable preparations such as Figure 25 structural features resembling the tubules of basal bodies are seen but the clarity is not sufficient to warrant measurement.

The average width of 10 rhizoplasts at the basal body end was 308 nm with a range of 229 to 406 nm. The average length of 10 rhizoplasts was 11.2 microns with a range of 9.48 to 16.40 microns.

Measurements of height and width of rhizoplasts using shadows were performed on organelles that were not fixed before shadowing and on organelles prefixed in uranyl acetate before shadowing, with the hope of deducing

FIGURE 24

A shadow-cast rhizoplast

This full length rhizoplast displays the rippled nature of the organelle's surface as revealed by the procedure of shadow-casting. The grid from which this micrograph was taken was prepared using isolated organelles with no previous purification attempts. Thus, great numbers of ribosomes and ribosome clusters (rc) are evident in the background. The basal body (Bb) is indistinct in this photomicrograph.

Magnification X 25,000.

FIG. 24

FIGURE 25

A damaged shadow-cast rhizoplast

In this micrograph a rhizoplast that is damaged and branching as a result is shown. The background material has been reduced by a centrifugation sequence designed to remove small and large contaminants from the suspension of isolated organelles. Two basal bodies (Bb_1 and Bb_2) are present on this rhizoplast and suggestions of component microtubules are present. Note the rippled nature of the surface of the organelle. Magnification X 30,000.

FIG.25

the cross-sectional shape of the organelle. The results (Table 11) are consistent with an elliptical cross section having a long axis approximately 10 to 20 times the length of the short axis. Dehydration of the rhizoplast during the evaporation of the platinum-carbon which is done at high vacuum, may flatten the organelle exaggerating the elliptical shape. These results make it more reasonable to infer that the electron-opaque mass alongside the basal body in Figure 2 is a rhizoplast cross section. Although the ratio of long axis/short axis is only about 2.0 it must be considered that the organelle is still inside the cell, it is not isolated and it is subject to artifacts of sectioning. The organelle inside the cell is also not subject to the same degree of dehydration as the shadow-cast organelle.

As with the negative-stained material, the fibrous nature of the rhizoplast is apparent in Figure 25. As a consequence of damage during preparation, the organelle is beginning to break down into smaller fibres which display the periodic nature, comparable to the same region in the negatively-stained organelle of Figure 22.

Period Measurements of Isolated *Naegleria* Rhizoplasts

The periodic banding of the isolated organelle is not as variable as when the organelle is inside the cell. Measurements of 108 negative-stained (Table 12) and 50 shadow-cast (Table 13) rhizoplasts (Appendix I) indicate

TABLE 11

The cross-sectional shape of shadow-cast rhizoplasts

The cross-sectional shape of shadow-cast *Naegleria* rhizoplasts. The Table shows the results of measurements of rhizoplast cross-section dimensions using material, unfixed or pre-fixed with uranyl acetate before shadow-casting. The shadow:object ratio is 1:10 as indicated in the Table and the cross-section appears to be a flattened ellipse with a long axis 10 to 20 times the length of the short axis. No differences were observed between the prefixed and unfixed organelles except that the prefixed rhizoplasts tend to have a smaller long axis:short axis ratio.

TABLE 11

The cross-sectional shape of shadow-cast rhizoplasts

Treatment (fixed or unfixed)	Height of object/length of shadow	Shadow length (nm)	Rhizoplast height (nm)	Measured width of rhizoplast (nm)	long axis / short axis
fixed with UA	1/10	116	11.6	82	7.07
fixed with UA	1/10	139	13.9	246	17.70
unfixed	1/10	147	14.7	242	16.46
unfixed	1/10	150	15.0	242	16.13
unfixed	1/10	197	19.7	253	12.84

a variability that may be attributed solely to accumulation of errors in the enlargement of photographs, measurement procedures, and minor fluctuations in the instrument magnification.

Measurements could not be performed on the isolated organelles as they were on the sectioned material (a procedure that allowed for the detection of variability within a rhizoplast) since no regular electron-opaque-electron-transparent pattern could be discerned. Bands were counted and the distance spanned by them was measured to obtain an estimate of distance per band. In order to test this measurement procedure for accuracy of averaging, measurements were made in "units" of 10 bands along the length of an organelle. The averages were then compared to the average of all the measurements on the organelle combined. All measurements were within ±0.1 nm and thus it is felt that the method is as satisfactory as the direct method used in sectioned material.

Negative-stained organelles exhibited a period of 21.8 nm with a range of 20.0 to 23.6 nm and the shadow-cast organelles had a range of 19.3 to 22.6 nm with an average period of 21.0 nm.

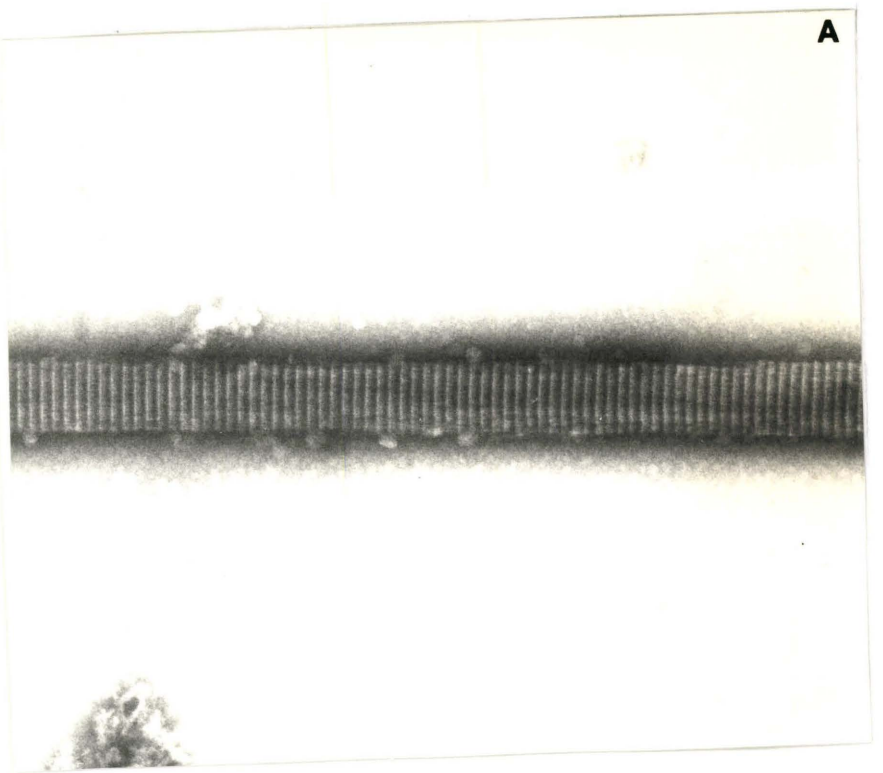
Visual observation of rhizoplasts fixed before the shadowing procedure with uranyl acetate gave the impression that the periodic pattern had been greatly disrupted. The comparison is shown in Figure 26A (negative-

FIGURE 26

Comparison of negative-stained and fixed-shadowed rhizoplasts

This negative-stained organelle (A) (period 22.1 nm) is presented as a comparison between A and B. The latter figure (B) shows two fixed-shadowed organelles. The upper one shows a period of 21.9 nm; the lower one a period of 21.7 nm, even though due to the apparent band separation in the upper organelle the difference seems to be greater.

Magnification X 70,000.



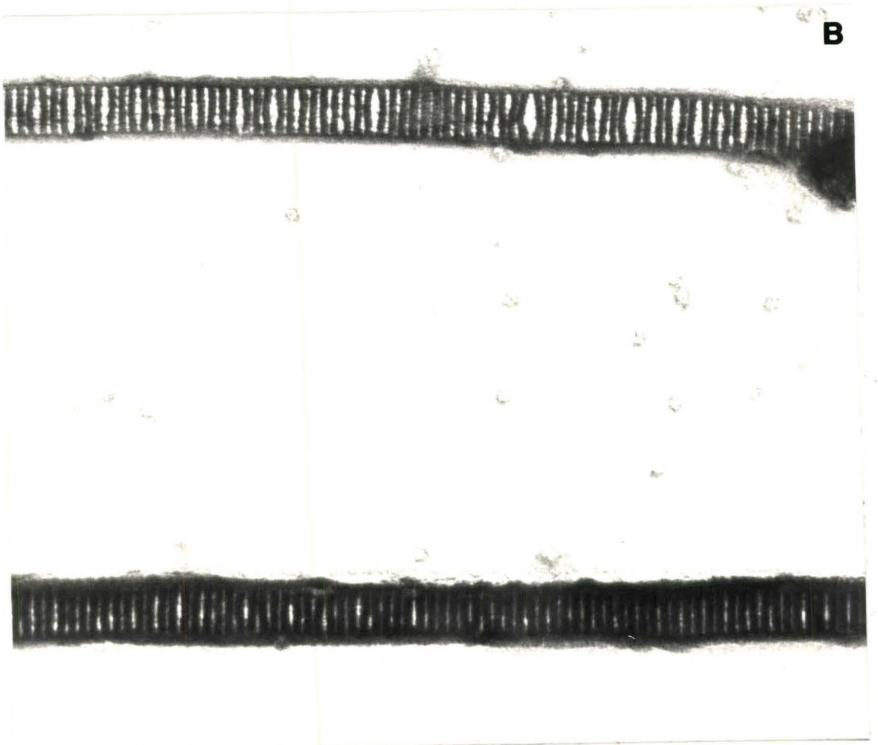


FIG. 26

stained, period 22.1 nm) and 26B (fixed in uranyl acetate then shadowed, period 21.7 nm for the upper organelle and 21.9 nm for the lower one. The difference in period between the upper and lower rhizoplasts in Figure 26B is about 0.2 nm but appears to be much more at first glance due to the apparent band separation in the upper one. The average of 6 period measurements on fixed-shadowed organelles gave a period of 21.1 nm with a range of 20.4 to 21.9 nm.

By the application of the appropriate statistical tests [the "F"-test of Fisher, (Snedecor, 1956)] to the data of this section, it was shown that the period of the negative-stained organelles is not significantly different from that of shadow-cast or fixed-shadowed organelles at both the 1 and 5 percent levels. A computation of Bartlett's test of homogeneity of variance also showed that the variances of data from shadow-cast and negatively-stained rhizoplasts were not significantly different at the 0.05 level of probability (Snedecor, 1956 and Appendix I). Thus it appears that isolated organelles are all similar in their periodic structure, leading to the speculation that isolated rhizoplasts tend to assume a common physiological state characterized by a periodic structure of 20.0 to 22.0 nm.

Dissolution and Reconstitution of Rhizoplasts

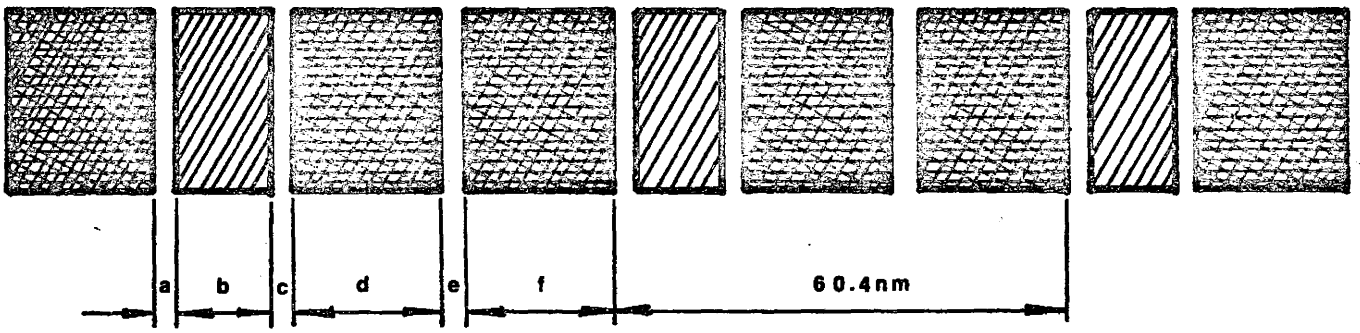
Observation of certain fractions collected from sucrose gradients revealed elongate cross-banded structures that appeared quite different from isolated rhizoplasts. Where these structures were seen, rhizoplasts themselves were absent or were in the final stages of breakdown and disintegration. Re-examination of the methods used in the experiments that gave rise to these structures showed, that in each case, Sarkosyl treatment was mistakenly performed when the Mg^{+2} concentration was less than the required 1.25 m$\underline{M}$ for the maintenance of the organelles. After Sarkosyl treatment however, and throughout the rest of each experiment, the $MgSO_4$ concentration was restored to, and maintained at, 2.5 m$\underline{M}$. The implication of these experiments is that the rhizoplasts had dissolved in the absence of sufficient $MgSO_4$ and had reaggregated upon the re-addition of sufficient $MgSO_4$.

The aggregates exhibit a morphology basically similar to that of rhizoplasts, that is, longitudinal structures with periodic cross-banding. The periodicity of the aggregates is distinctly different from that of native rhizoplasts - a more complex pattern with distinct subperiods. Figure 27 shows the differences between the two. The rhizoplast period is composed of alternating electron-opaque and electron-transparent bands with no subperiods evident in either band. The aggregate period is composed of 6 bands

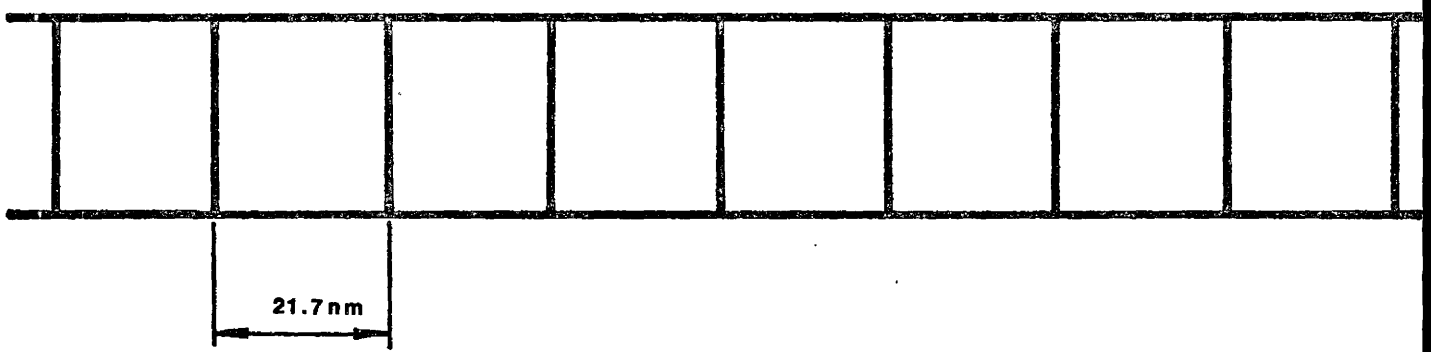
FIGURE 27

Aggregate period compared to rhizoplast period

This figure shows a comparison between the period of a rhizoplast and the period of one of the aggregates. The pattern of the aggregate period (top) is more complex than that of the rhizoplast (bottom), as it is composed of 6 separate bands. The numbers accompanying the figures indicate the width of the various bands with the total period in the case of the aggregate being 60.4 nm, and in the case of the rhizoplast, 21.7 nm.



a 2.95 nm
b 11.80 nm
c 2.95 nm
d 19.17 nm
e 4.40 nm
f 19.17 nm



comprising approximately 60.4 nm as indicated in Figure 27. The structural arrangement is seen in only a few of the photographed aggregates since in most cases the 4.40 nm band is not clear and the staining often obscures rather than reveals detail.

The periodicity of 15 aggregates was measured by the same technique used for the measurement of the isolated rhizoplast period. The data is presented in Table 14 and representative aggregates are shown in Figures 28 and 29. The average period for the 15 measured was 59.7 nm with a range of 54.5 to 64.3 nm. The standard deviation of ±6.5 nm compared to less than ±1.0 nm for isolated organelles indicates the wide variations in period.

Width measurements of 20 of the structures resulted in an average width of 132 nm with a range of 91.4 to 185.8 nm. Rhizoplasts, it will be recalled, taper gradually from 229 nm to approximately 20 nm. The length of the same 20 aggregates varied considerably from 1.2 to 13.1 microns with an average length of 3.2 microns. Rhizoplasts averaged about 10 microns in length with a range of about 7 to 16 microns.

Rhizoplasts showed evidence of a fibrous nature but the aggregates appeared "solid" in all cases with no apparent longitudinal substructure. The aggregates were not shadow-cast, thus it is not known whether they are rippled on their surfaces as are the rhizoplasts, however

FIGURE 28

Aggregate

The aggregate in this micrograph shows a period of 60.5 nm with all six bands visible in some areas along its length. Note the solid nature of the structure with no evidence of fibrous construction. Magnification X 70,000.

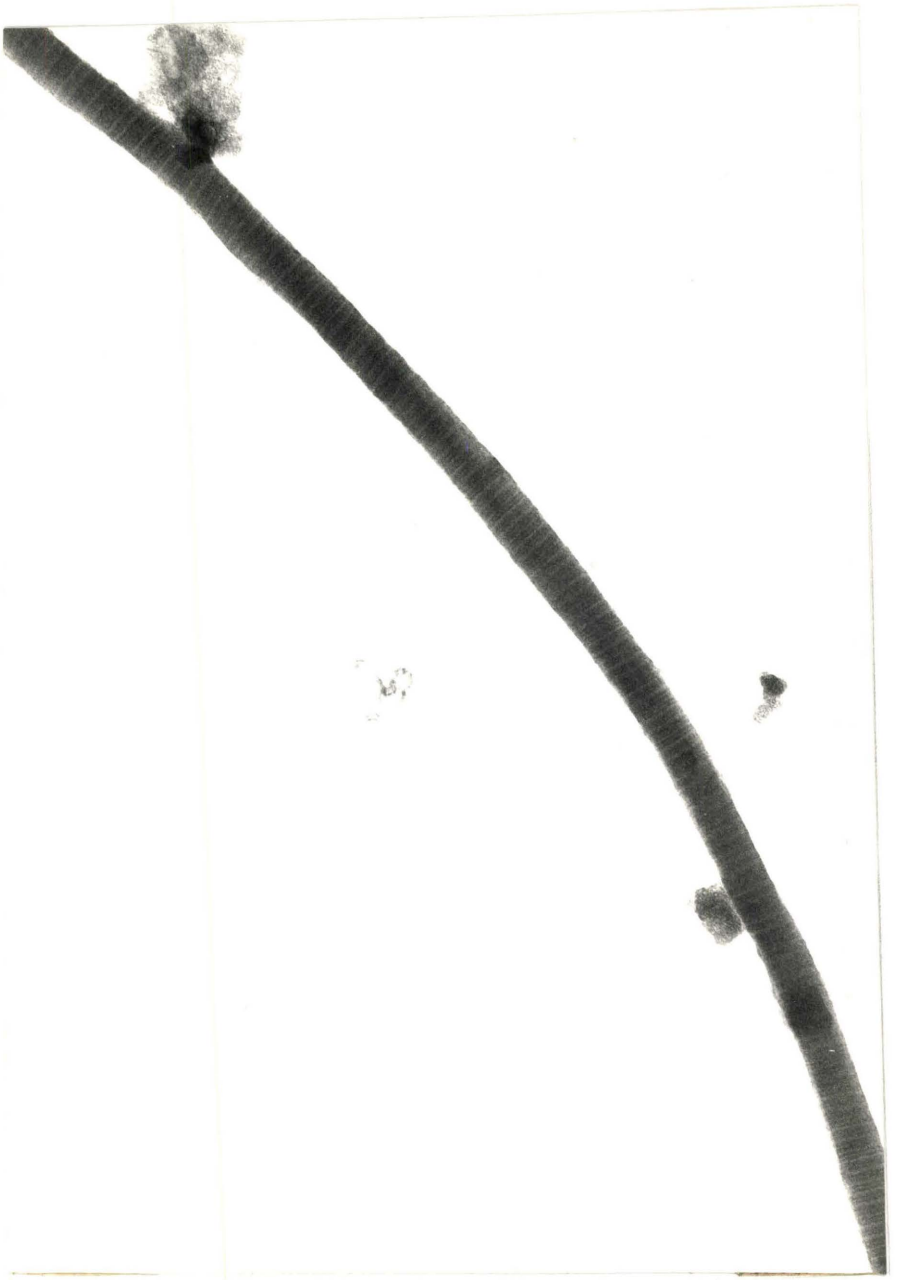


FIG. 28

FIGURE 29

Aggregate

This aggregate exhibits a period of 64.3 nm and shows a solid structural organization. The background material includes membrane fragments (mf), some small fibres (f) of similar dimensions to those in Figure 23, and other unidentifiable material. The aggregate itself has been negatively-stained with uranyl acetate.

Magnification X 30,000.

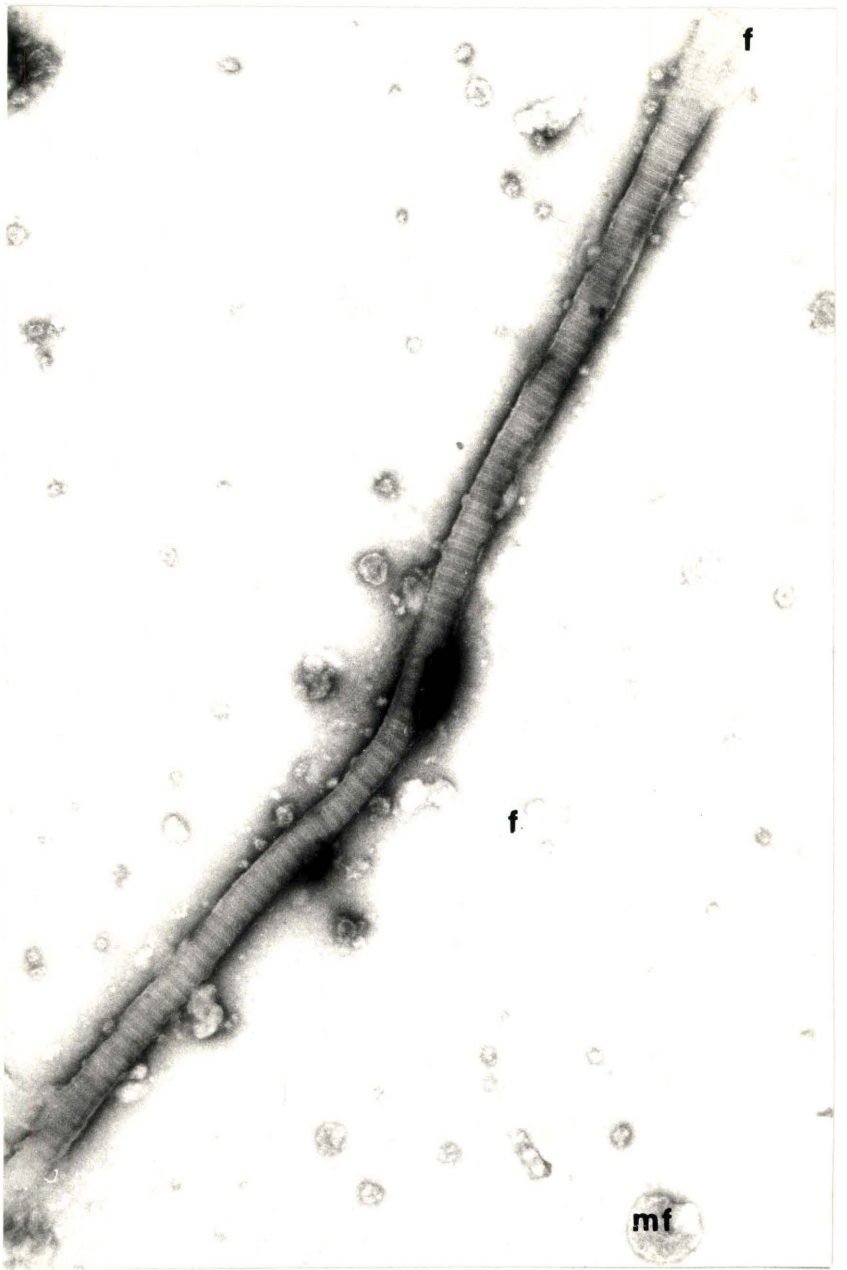


FIG. 29

TABLE 14

Aggregate Periodicity Measurements

The Table shows the results of period measurements of 15 aggregates. Statistical treatment of the data reveals that there is much more variation in these aggregates than in the isolated native rhizoplasts, the aggregates having a standard deviation of ±6.5 nm compared to approximately ±1.0 nm for the rhizoplasts. Details of the structure of the aggregates may be found in the text.

TABLE 14

Aggregate Periodicity Measurements

Photoseries	Number	Bands measured	Period nm
B	220	30	60.6
B	224	9	62.4
B	236	50	60.6
B	264	25	54.5
B	265	50	63.3
B	267	80	64.3
B	268	25	59.9
B	281	125	59.9
B	863	50	60.6
C	023	20	58.0
C	024	15	58.0
C	026	30	55.0
C	131	40	60.6
C	134	20	60.3
D	060	65	58.1
		mean	60.5

negative-stained preparations gave no evidence of a rippled nature, either on the top surface or on the sides. If the sides were rippled it would be seen in negative-stained preparations as a "corrugation effect" (Cohen, Szent-Györgyi et al., 1970).

Duplications of the original set of experiments resulted in the formation of aggregates but attempts to dissolve the rhizoplasts, centrifuge all particulate matter out of the solution and then reaggregate the organelles by the addition of divalent cations were not successful.

DISCUSSION

Descriptions of rhizoplasts and other ciliary-flagellar-apparatus-associated fibres have appeared regularly in the literature since Fawcett and Porter (1954) first described mollusc rootlets observed with the electron microscope. They described them as cross-striated fibres of diameter 60 to 100 nm with a major period of 55 to 70 nm, and noted the presence of subperiods within each major band.

Since that time, numerous reports have appeared concerning banding patterns of striated fibres, but the question of whether or not periodicity of a rhizoplast is constant has received little attention from most authors. Reported periods are given only as one figure (e.g., 30 nm) or at most, a small range (e.g., 30 to 35 nm). Original observations of periodicity variations in *Naegleria* rhizoplasts (Dingle, 1964; Dingle and Fulton, 1966) indicated that band period was not constant, but varied from approximately 10 to 24 nm. Measurements of rhizoplast periodicity presented in this thesis for sectioned material fixed in different ways, confirm the original observations and further weaken Schuster's (1963) contention that the *Naegleria* rhizoplast has a constant

period of 18 nm. Outka and Kluss (1967) reported variation in rhizoplast periodicity for another amebo-flagellate, *Tetramitus rostratus*, but unfortunately supplied no details, and simply gave confirmation to Dingle's (1964) observation that greater variation is found in the dark band of the organelle than in the light band.

Attempts to reduce or abolish variations in sectioned *Naegleria* rhizoplasts by poisoning cells with 2,4: dinitrophenol, removal of flagella by vortex mixing, and chilling the cells into a state of flagellar inactivity were statistically unsuccessful, suggesting that forces imposed on the rhizoplast by motile, actively beating flagella are not wholly responsible for the observed variations.

A constant periodicity was measured for isolated *Naegleria* rhizoplasts (negatively-stained, 21.7 nm and shadow-cast, 21.0 nm). Previous rhizoplast isolations have not been reported in the literature, however kinetodesmal fibres have been isolated (Sedar and Porter, 1955; Pitelka, 1965; Hufnagel, 1969). Sedar and Porter found periodicities of 30 to 50 nm for isolated kinetodesmal fibres of *Paramoecium multimicronucleatum*, while Pitelka reported 30 to 35 nm for the same organelles 10 years later. Hufnagel described a period of 35 to 40 nm for raffinose-isolated- and 29 to 34 nm for ethanol-isolated kinetodesmal fibres of *P. aurelia*. The values of the latter

two authors may be considered constant as there is less than a ±5 percent variation.

Findings of this thesis lead to the important question - what do variations mean? A number of answers may be considered: sectioning artifact, selection of samples from a random population of flagellates, and function of the organelle.

Sectioning artifacts may arise in a number of ways. The angle of sectioning determines the observed shape of a structure as seen in section. For example, a cube cut on the diagonal yields a rectangular shape, while a cube cut parallel to one side yields a square shape. A cylinder cut perpendicular to the long axis yields a circular shape and cut at any other angle, an oval or elliptical shape, the dimensions of which are determined by that angle. If this type of artifact was important in periodicity measurements of rhizoplasts, it would have shown up in the data as an inconsistency along the length of the organelle measured. This was not the case since periodicities were found to be constant within an organelle throughout its measured length. The rhizoplast twists and bends as it passes through the cytoplasm, although this does not seem to influence the periodicity. The only noticeable effect was the bending of the organelle out of the plane of the section, resulting in only short measurable structures in most of the sections. This occurred often, as judged by the

scarcity of very long measured rhizoplast sections. Compression artifacts during sectioning may play a role in influencing periodicity, but only a minor one since all sections were treated equally before observation. This seems to be the case, since single sections containing many cells yielded rhizoplasts with different periodicities.

Only a small number of rhizoplasts were measured for any one particular fixation procedure. Assuming that a random sample representative of the forms a rhizoplast may take in the cell, were taken from each population is logical. Obviously, all rhizoplasts in a flagellate population cannot be in an identical physiological state at the instant of fixation, just as all cells in a non-synchronous growing population cannot be in the same stage of the cell cycle at any one time. Inherent population variations may result in the observed period variations but this is highly unlikely since the population of cells used in the study has been cloned many times. A more valid question to ask would be - is rhizoplast fine structure regular, as is the "9 + 2" structure of *Naegleria* flagella?

If the rhizoplast is assumed to have an anchoring function (Pitelka, 1969), the observed variations may be related to this function, as no periodicity variation was observed in isolated, and supposedly non-functioning rhizoplasts. More will be said of this intriguing question later

in the discussion.

Purification of isolated rhizoplasts, attempted with the objective of obtaining material for biochemical analysis was unsuccessful, however numerous properties of the organelle were made apparent during these attempts. The fibrous nature of the *Naegleria* rhizoplast is not visible in sectioned material as is that of rootlets described by Gibbons (1961) and Hoffman and Manton (1962). Isolated *Naegleria* rhizoplasts, negatively-stained or shadow-cast, show longitudinally directed fibres only in cases where organelles have been damaged during isolation and preparation for electron microscopy, indicating that they may be somewhat more solid in structure than other rootlets.

There is a distinct $MgSO_4$ requirement for the maintenance of integrity of the isolated rhizoplast. Since EDTA will cause dissolution of isolated organelles even in the presence of 2.5 mM $MgSO_4$, it is logical to assume that the Mg^{+2} ion is the required component of the salt. Dissolving rhizoplasts in the absence of Mg^{+2} led to the finding that reaggregation of rhizoplast material was possible upon the addition of sufficient Mg^{+2} to a solution containing dissolved organelles. Aggregates exhibited a banding pattern more complex than that of native organelles, being composed of six subperiods spanning approximately 60 nm. Unfortunately, attempts to purify rhizoplasts by

dissolving the organelles in Mg^{+2}-free buffer, spinning down the particulate matter, and forming aggregates by the addition of Mg^{+2} to the supernatant were unsuccessful.

The data and descriptions presented in this thesis have raised a number of interesting questions, partially answering some of them to a degree of sophistication previously impossible. These ideas and concepts will be discussed separately below.

What is a rhizoplast? Structurally, the *Naegleria* rhizoplast is an elliptical longitudinally-fibrous structure in the cell which exhibits a variable banding pattern composed of alternating electron-opaque (9 to 20 nm) and electron-transparent (3 to 7 nm) bands. It arises in the basal body region as a fibre approximately 200 nm wide which tapers along its length as it passes through the cytoplasm close to, or running alongside the nucleus. Attachment to the basal bodies appears to be by two separate structures, the interbasal body connector and the rhizoplast-associated microtubules. Attachment to the nucleus is uncertain, but probably exists, however entry of the organelle into the nucleus is unlikely since it has never been observed, either by myself or by Dingle (1964).

The presence of variation in periodicity of sectioned rhizoplasts and the absence of it in isolated organelles suggests that variation may be a manifestation

of function. Current concepts of rhizoplast function include that of the organelle as an anchor for the basal bodies and flagella (Pitelka, 1969). The length of the organelle in *Naegleria* is such that it would provide ample leverage action, and perhaps the observed variation is a result of this function. Before firm hypotheses may be advanced, it must be determined whether the variation is active or passive. If active, one may envisage contractility as a means of providing anchorage. The rhizoplast would simply contract when necessary during the flagellar beating, with the amount of contraction being represented by the amount of variation. For example, the narrower the period, the more contracted the organelle and vice versa. If passive, one may envisage an elastic function for the organelle. When necessary the rhizoplast could be stretched to accommodate forces, thus providing the required stability to the basal body and flagellar apparatus. When not being stretched the organelle could conceivably remain in a "relaxed" state similar to an unstretched elastic band.

Speculations of this type generate interesting ideas worthy of inclusion in this discussion. If the organelle is contractile what is the nature of the contractile phenomenon? Is the organelle to be considered as a true intracellular muscle? It is now recalled that variations were not seen in single organelles but only between organelles.

Could the whole organelle contract equally in its function? Due to the limits of procedure, only a small portion of each organelle was observed in each section, generally less than 30 percent, if one assumes an average length of 10 microns. In one of the organelles measured, the length of the rhizoplast in section was almost 8 microns. The organelle exhibited a period of 20 nm composed of a 13 nm dark and a 7 nm light band. Variations were not observed within this organelle, but is it prudent to assume, on the basis of this one observation, that the rhizoplast period was constant throughout its length?

The possibility of elasticity of the organelle seems unlikely in light of the lack of a distinct connection of the organelle with the nucleus. In order for an elastic band to work, force must be applied at both ends, or at least force at one end and resistance at the other. Isolated rhizoplasts invariably have basal bodies, suggesting firm attachment, but rarely attached nuclei. Thus it appears that firm attachment exists at only one end, probably negating the elasticity hypothesis.

The aggregation phenomenon suggests a number of possibilities for the nature of rhizoplast material and perhaps some justification for presupposing a contractile function for the native organelle. A number of known proteins and protein systems may be induced to self-assemble *in vitro* into longitudinal structures with transverse

banding patterns. Included among these are paramyosin, tropomyosin, and collagen. The former two will be discussed in some detail since they are contractile proteins.

Paramyosin, a fibrous protein of molecular weight 220,000 is a large component of molluscan muscles specialized for prolonged tension maintenance. This protein may be solubilized in neutral salt solution at acid pH, and reaggregated into periodically-banded structures by lowering the ionic strength or raising the pH (Hodge, 1952). Divalent cations have recently been reported to induce the formation of periodic aggregates of paramyosin (Cohen, Szent-Györgyi, and Kendrick-Jones, 1970). The pattern of reconstitution may be influenced simply by using different concentrations of different cations, e.g., Ba^{+2}, Ca^{+2}, Pb^{+2}. Sr^{+2} as described in the reference. The various different, but basically similar, aggregate patterns obtained enabled the authors to deduce molecular length and hence hypothesize molecular packing within the reconstitutes.

Tropomyosin, a fibrous protein of molecular weight 70,000 is present in all muscles, and has aggregation properties somewhat similar to those of paramyosin. It is soluble at neutral pH and low ionic strength, and may be reconstituted into periodically-banded structures by the addition of divalent cations to the solution (Caspar, Cohen, and Longley, 1969). Examination of the aggregates using techniques of negative-staining, positive-staining and

X-ray diffraction led to the determination of molecular length and hypotheses concerning molecular packing in aggregates.

Collagen, a protein found in connective tissues exhibits reconstitution properties *in vitro* as well. Although the native rhizoplast is very much unlike any collagen structures previously described, rhizoplast material may be collagen-like in nature. A recent note (Goldstein, Parlebas et al., 1969) describes the periodically-banded structures obtainable from collagen solutions by precipitation with mono- and divalent cations. The authors report that divalent cations favour solubilization rather than precipitation of collagen, and that in cases where both types of ion are present in a collagen solution the aggregates exhibit structural characteristics produced by monovalent cations rather than divalent cations. The structures obtained by NaCl precipitation are difficult to distinguish from rhizoplast aggregates in appearance, except that more subperiods are visible and the period is regularly 64 nm. The authors stress that Mg^{+2} favoured the solubilization rather than the reaggregation of collagen, thus it is unlikely that the rhizoplast is of a collagenous nature. Making the idea even more untenable is the structure of the native organelle which is definitely not collagen-like.

It is evident from the last three paragraphs that

the approaches to be taken in further studies on rhizoplastin (rhizoplast material - designated as such due to the resemblance of the aggregation phenomenon to paramyosin and tropomyosin reaggregation) should be those presented in the above references. In the ideal situation, rhizoplastin would be composed of only one protein. Whether or not this is the case is uncertain, due to the difficulties encountered in the purification attempts. The aggregation properties of paramyosin, tropomyosin and collagen should surely be carefully considered in future thoughts on the rhizoplastin problem.

Answers to the questions raised in this report would aid considerably in the study of *Naegleria* rhizoplast development. At present isolation is possible, and may be used as a tool in future studies. Purification and identification of rhizoplast material would allow further studies including the origin and chemical nature of rhizoplastin, the time course and nature of rhizoplast assembly, and the nature of the changes undergone in the cell allowing rhizoplast assembly. Since the rhizoplast is an obviously large committment to the transforming *Naegleria*, understanding of the events leading up to its assembly within the cell would be a contribution to the understanding of cell differentiation in eukaryotic organisms.

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APPENDIX I

Periodicity Measurements on Rhizoplasts

Tables 2 through 8 and Tables 12 and 13.

Period Measurements on Sectioned *Naegleria* Rhizoplasts

Tables 2 though 8.

These tables present the data of period measurements on fixed sectioned rhizoplasts. Details of fixation and measurement will be found in the text.

TABLE 2

Rhizoplast Periodicity Measurements, FsGAFsOsO_4 Fixation

Photo Plate	number no.	Bands (d-1) measured	Electron opaque dark nm	Electron transparent light nm	Totals nm
2	2	50	12.2	4.7	16.9
6	1	20	13.9	4.5	18.4
11	3	65	13.8	4.2	18.0
14	3	50	17.0	4.5	21.5
18	3	200	12.9	6.9	19.8
20	4	80	12.2	4.9	17.1
22	3	120	12.1	3.8	15.9
23	2	110	9.4	4.4	13.8
27	5	10	19.5	5.7	25.2
29	3	25	13.5	4.8	18.0
33	2	30	14.3	6.8	21.1
37	3	75	12.3	4.7	17.0
		means	13.69	5.0[illegible]	18.6[illegible]

TABLE 3

Rhizoplast Periodicity Measurements, TGATOsO$_4$ Fixation

Photo number Series	No.	Bands (d-1) measured	Electron opaque dark nm	Electron transparent light nm	Totals nm
A	105	30	8.6	3.6	12.2
A	106	150	11.3	3.7	15.0
A	109	50	12.7	5.4	18.1
A	145	60	11.7	4.5	16.2
A	146	60	14.6	4.4	19.0
A	167	80	11.2	5.4	16.6
A	169	20	14.6	4.4	19.0
A	177	70	10.4	4.5	14.9
D	818	120	9.3	3.0	12.3
D	822	90	11.0	3.2	14.2
D	824	60	11.5	3.8	15.3
D	827	30	14.0	3.5	17.5
		means	11.7	4.1	15.9

TABLE 4

Rhizoplast Periodicity Measurements, $FsOsO_4$ Fixation

Photo number Series	No.	Bands (d-1) measured	Electron opaque dark nm	Electron transparent light nm	Total nm
E	129	45	13.6	3.0	16.6
E	132	40	14.8	3.4	18.2
E	134	100	15.2	3.2	18.4
E	136	30	15.1	2.9	18.0
E	137	35	16.6	2.9	19.5
E	140	60	16.6	3.1	19.7
E	143	60	10.4	2.9	13.3
E	148	30	13.8	3.0	16.8
E	149	120	14.9	2.9	17.8
E	151	40	15.5	3.1	18.6
E	187	35	14.0	3.1	17.1
E	188	40	12.6	3.1	15.7
E	190	70	14.7	3.1	17.8
E	191	50	11.8	3.1	14.9
E	192	30	16.1	3.5	19.6
E	193	35	11.9	3.2	15.2
E	195	60	17.9	3.4	21.3
E	196a	40	12.1	3.1	15.2
E	196b	20	12.3	3.1	15.4
E	198	70	12.6	3.1	15.7
E	199	20	12.1	3.0	15.1
E	201	50	14.9	3.0	17.9
E	202	110	14.4	3.1	17.5
E	203	70	13.6	2.6	16.2
E	204	20	10.8	3.6	14.4
E	205	140	13.7	3.4	17.1
		means	13.9	3.1	17.0

TABLE 5

Rhizoplast Periodicity Measurements, $TOsO_4$ Fixation

Photo number Series	No.	Bands (d-l) measured	Electron opaque dark nm	Electron transparent light nm	Totals nm
C	911	70	12.5	4.0	16.5
C	967	85	8.8	3.4	12.2
D	563	110	10.9	3.0	13.9
D	567	45	15.8	2.9	18.7
D	568	20	22.2	5.8	28.0
D	593	25	15.2	3.2	18.4
D	605	60	14.3	3.2	17.5
D	606	50	11.6	3.0	14.6
D	607	50	11.2	3.7	14.9
D	610	130	14.0	3.2	17.2
		means	13.7	3.5	17.2

TABLE 6

Rhizoplast Periodicity Measurements, DNP Treatment Before TGATOsO$_4$ Fixation

Photo number Series	No.	Bands (d-1) measured	Electron opaque dark nm	Electron transparent light nm	Total nm
A	181	30	12.6	4.1	16.7
D	534	85	10.9	3.0	13.9
D	538a	40	14.0	4.1	18.1
D	538b	110	14.5	4.0	18.5
D	591	80	11.8	2.3	14.1
D	595	20	12.5	3.0	15.5
D	599	25	15.3	3.7	19.0
D	602	70	15.2	3.0	18.2
D	616	120	11.2	3.9	15.1
D	618	130	10.6	4.1	14.7
D	620	80	14.6	3.0	17.6
D	636	55	15.0	2.9	17.9
		means	13.2	3.4	16.6

TABLE 7

Rhizoplast Periodicity Measurements, Vortexing to Remove Flagella Before TGATOsO$_4$ Fixation

Photo number Series	No.	Bands (d-1) measured	Electron opaque dark nm	Electron transparent light nm	Total nm
A	194	40	11.9	3.5	15.4
D	829	60	13.0	3.8	16.8
D	834	25	16.4	4.4	20.8
D	842	80	14.7	2.7	17.4
D	843	75	14.3	2.9	17.2
D	846	40	16.6	2.6	19.2
D	848	30	10.7	3.1	13.8
D	849	80	12.2	3.2	15.4
D	853	60	16.1	4.3	20.4
D	856	40	16.8	2.8	19.6
D	857	100	13.0	3.2	16.2
D	858	140	11.7	3.1	14.8
D	863	90	12.4	3.2	15.6
D	874	35	14.7	3.0	17.7
D	876	60	12.3	3.3	15.6
D	879	60	14.6	2.8	17.7
D	885	60	15.3	3.5	18.8
D	886	50	13.5	3.1	16.6
		Means	13.8	3.3	17.2

TABLE 8

Rhizoplast Periodicity Measurements, Chilled to Stop Flagellar Motion Before TGATOsO$_4$ Fixation

Photo number Series	No.	Bands (d-1) measured	Electron opaque dark nm	Electron transparent light nm	Total nm
E	030a	50	10.3	3.0	13.3
E	030b	75	10.4	3.4	13.8
E	034	70	9.6	3.0	12.6
E	052	60	14.9	3.5	18.4
E	055	60	10.4	3.4	13.8
E	056	60	10.3	3.0	13.3
E	057	60	14.1	3.3	17.4
E	058	110	11.6	3.6	15.2
E	059	160	14.3	3.3	17.6
E	060	60	14.7	3.4	18.1
		means	12.16	3.3[illegible]	15.4[illegible]

TABLES 12 and 13

Period Measurements on Isolated *Naegleria* Rhizoplasts

Table 12 and Table 13 present data of period measurements on 108 negative-stained isolated rhizoplasts and 50 shadow-cast isolated organelles respectively. The measurement procedure is described in detail in the METHODS and RESULTS section. The average period for negative-stained organelles was 21.8 nm while that of the shadow-cast organelles was 21.0 nm.

TABLE 12

Periodicity Measurements on Negative-Stained Rhizoplasts

Photo number Series	No.	Bands measured	Period nm	Photo number Series	No.	Bands measured	Period nm
A	236	100	20.9	D	163	150	22.0
A	238	70	23.6	D	191	95	20.9
A	239	300	21.6	D	209	130	21.7
A	240a	110	22.5	D	210	110	21.8
A	240b	120	22.4	D	211	130	21.7
A	241	80	23.6	D	220	130	22.0
A	242	100	22.4	D	229	330	20.0
A	244	190	22.1	D	230	70	21.8
A	245	170	22.4	D	231	220	20.0
A	246	200	22.3	E	290	130	22.6
A	247	100	21.6	E	291	290	20.3
A	248a	70	21.4	E	292	130	22.4
A	248b	70	21.7	E	293a	95	21.5
A	258	80	21.6	E	293b	125	21.6
A	285	110	21.7	E	294	130	22.4
A	286	110	21.8	E	295a	80	22.2
A	966	120	21.0	E	295b	95	22.4
A	967	55	20.5	E	296	125	22.3
A	968	110	21.7	E	297	130	21.9
A	969	130	21.7	E	298	130	22.2
A	973	110	21.0	E	299	130	22.2
A	974	120	21.0	E	300	130	22.2
A	976	120	21.2	E	301	135	21.9
B	120	130	21.3	E	302	130	22.1
B	143	120	21.5	E	303	130	22.2
B	144	130	21.3	E	304	130	22.3
B	232	100	21.5	E	305	135	21.9
B	864a	100	20.9	E	306	165	22.2
B	864b	70	21.5	E	307a	110	21.7
B	864c	100	20.9	E	307b	120	21.3
B	865	140	20.9	E	307c	80	21.6
B	866	140	21.2	E	308a	125	21.6
B	867	120	21.1	E	308b	75	21.8
D	121	160	21.7	E	309	130	21.9
D	122	90	21.7	E	310	130	21.9
D	124	130	21.1	E	311	135	21.8
D	128	70	21.9	E	312	130	22.0
D	131	260	21.1	E	313a	110	21.8
D	160	120	21.5	E	313b	130	22.1

(continued)

TABLE 12 (continued)

Photo number Series	No.	Bands measured	Period nm	Photo number Series	No.	Bands measured	Period nm
E	313c	85	22.3	E	328	130	22.1
E	316	130	21.9	E	329a	110	22.1
E	317	135	22.1	E	329b	65	22.6
E	318a	85	21.7	E	329c	120	22.0
E	318b	105	22.0	E	330	130	22.0
E	319	135	21.6	E	331	130	22.0
E	320	130	21.9	E	332	130	21.9
E	321	130	22.1	E	333	135	21.9
E	322	125	21.9	E	334a	120	22.2
E	323	130	22.1	E	334b	95	22.1
E	324a	130	21.8	E	335	130	21.8
E	324b	110	22.1	E	336	125	21.5
E	325	135	21.2	E	337	130	22.0
E	326	135	21.8	E	338	130	22.0
E	327	130	22.1	E	339	130	22.0

TABLE 13

Periodicity Measurements on Shadow-Cast Rhizoplasts

Photo number Series		Bands measured	Period nm	Photo number Series		Bands measured	Period nm
A	247	130	21.0	A	566	380	22.2
A	260	160	21.1	A	594	120	21.5
A	263	120	21.6	A	597	350	21.1
A	267a	130	20.9	A	598	190	21.6
A	267b	170	21.4	A	599	250	21.1
A	268	270	21.1	A	600	320	22.6
A	269	140	21.0	A	632	360	19.9
A	271	420	20.9	A	633	320	19.3
A	282	170	22.4	A	634	200	21.0
A	284a	90	21.5	A	636	350	20.4
A	284b	60	22.0	A	637a	230	21.2
A	285	150	21.8	A	637b	160	20.6
A	301	320	21.6	A	638	330	20.4
A	307a	290	22.8	A	641	340	20.5
A	307b	340	21.5	A	644	400	20.1
A	312	300	20.2	A	690	150	21.1
A	549	70	21.1	A	699	370	21.3
A	550	140	20.3	A	703	260	21.7
A	551	210	20.1	E	275	160	20.2
A	552	130	21.4	E	277	200	20.2
A	553	360	20.9	E	283	200	20.8
A	556	290	20.3	E	285	200	21.1
A	557	240	20.4	E	286	250	21.0
A	558	350	20.0	E	287	160	21.1
A	565	70	21.1	E	289	230	20.3

APPENDIX II

The Statistical Treatment of Rhizoplast Periodicity Measurements

Due to the nature of the data obtained in the measurements of rhizoplast periodicities (not definitive as to whether more variation exists in once case than another) various approaches to the analysis of the data were required.

In all cases standard parameters (Spiegel, 1961) were defined for the system. These included:

1. Mean = $\overline{X}$ = fX/n, where f = frequency, X = width of band, and n = sample size.
2. Standard deviation = $\sigma = \sqrt{\frac{\Sigma fX^2}{n} - \left(\frac{\Sigma fX}{n}\right)^2}$
3. Variance = σ^2 = (standard deviation)^2.
4. Standard error = S.E. = $\sigma/\sqrt{n}$
5. Coefficient of variation = $\sigma/\overline{X}$ x 100%.

These parameters were insufficient for the analysis of the sectioned material but were useful in the analysis of isolated-rhizoplast measurements. Application was made of the "F-test" of Fisher (Snedecor, 1956) in which variances are compared. The number of degrees of freedom are taken into account in the interpretation of the

significance of the ratio of F. It was shown by this method that there was no significant difference between the periods of negative-stained and shadow-cast organelles at the 1 percent level.

The coefficient of variation was used only as a guide in the interpretation of whether or not the differences of measurements in sectioned material were significant.

Bartlett's Test of homogeneity of variance (Snedecor, 1956), which relies on the use of the Chi-square table for significance-checking and on mean square deviations from the mean for arriving at the Chi-square test value was employed. This test was used since there was a difference in the sample sizes of the data, e.g., the control TGATOsO_4 data was composed of 12 measurements and the vortexed-TGATOsO_4 data was composed of 18 measurements. The method was used to test the hypothesis of whether or not the variances of the data of light band, dark band and total period measurements in the sectioned material were significantly different from each other to postulate a true variation. In each case of comparison (light bands, dark bands, and total periods) in the treated (DNP, vortexed and chilled) TGATOsO_4-fixed organelles and the untreated control TGATOsO_4-fixed organelles it was found that no significant differences were evident. Thus it was concluded that the treatments were not effective in reducing period variation as was expected. The same test was also used to check the

variances of shadow-cast and negative-stained organelles to see if they differed by a significant amount. The result obtained for the Chi-square value indicated that the variances were the same at the 1 percent level, confirming the conclusion reached using Fisher's "F-test".

Thus, with respect to statistical treatment of measurement data, the following conclusions may be drawn:

1. Variation does exist in all of the samples fixed in the different ways as evidenced by the range of measurements within a single set of data, and by the coefficient of variation exhibited by the measurements.
2. Bartlett's test was shown that the amount of variation in the TGATOsO_4-fixed cells' rhizoplasts is not reduced by pretreatment of the cells with DNP, or by removal of flagellar function, either by removal of flagella (vortexing) or by chilling.
3. Both the "F-test" of Fisher and Bartlett's test have shown that the measurements of rhizoplast periodicity in negatively-stained and shadow-cast organelles are similar (differences between the variances of the two populations were insignificant).