DEVELOPMENT OF THE FLAGELLAR ROOTLET OF *NAEGLERIA*

By

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Abstract

Naegleria gruberi amoebae differentiate rapidly and synchronously to flagellates, assembling a complete flagellar apparatus de novo within two hours. The flagellar apparatus consists of two flagella, their associated basal bodies and a flagellar rootlet. This study is directed towards an understanding of the development of the flagellar rootlet. The rootlet has been isolated by differential centrifugation and the major rootlet protein has been identified by solubilization of the organelle in urea, high salt, detergents or extremes in pH.

During the amoeba to flagellate differentiation at least 62% of the major rootlet protein assembled is synthesized de novo as measured by means of an isotope dilution experiment. Most likely the major rootlet protein is completely synthesized de novo since it is absent in amoebae but present in developing flagellates prior to the appearance of rootlets. This protein, whose subunit molecular weight is 170,000 daltons, has been purified by elution from polyacrylamide preparative gels. The sequence of rootlet development has been elucidated by indirect immunofluorescence using a specific antibody directed against the purified major rootlet protein. Rootlets, which are first detected in developing flagellates 70 min after the initiation of differentiation, reach their maximum length of 13 μm within 40 min. During the differentiation, the appearance of rootlets in the population follows that of flagella, but is delayed by about

5 min. When flagellates revert to amoebae, the rootlets disappear from cells shortly after the flagella are lost, and they reappear just minutes after the flagella become visible in redifferentiating flagellates.

The controls which govern the number of organelles assembled during differentiation are not absolute and cells may assemble three, four or occasionally more flagella. Furthermore, the controls of organelle number may be deranged by high temperature shocks applied at precisely defined intervals during the differentiation such that the resulting flagellated populations may develop nearly three times the normal number of basal bodies and flagella. In these multiflagellated populations the increase in the mean number of rootlets per cell is proportional to the increase in the mean number of flagella such that the two flagella to one rootlet relationship of a typical (biflagellate) flagellar apparatus is maintained. These results suggest that the temperature effect which leads to uncontrolled basal body and flagellum assembly simultaneously deranges whatever controls the number of rootlets assembled.

As the number of flagella and rootlets per cell increases, the mean length of the organelles decreases. These do not counterbalance, however, and there is a net increase in the total length of organelle assembled. Measurements of the total amount of the major rootlet protein in control and temperature-shocked populations show no increase in the multiflagellated cells. Rather, there appears to be an increase in utilization of the available major rootlet protein to provide for the increased rootlet assembled.

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This thesis is dedicated to my husband Douglas.

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Chapter 1

Introduction

"We have seen that all organisms are composed of essentially like parts, namely of cells; that these cells are formed and grow in accordance with essentially the same laws; hence, that these processes must everywhere result from the operation of the same forces."

Schwann, 1839 (cited in Wilson, 1925)

Both Schleiden and Schwann in 1839 suggested that the basic unit of all biological systems was the cell and that all cells function using similar physiological and morphological principles. Since that time, many studies have been devoted to the understanding of how cellular structural components interact in a coordinated fashion.

Of the organelles first observed and studied, the nucleus was investigated intensively because of its ubiquity, its pronounced morphological changes during cell division and the possibility, following the rediscovery of Mendel's work, that it might possess a component involved in the transmittance of heritable characteristics. Other organelles, the mitochondria, chloroplasts, membranes and cilia came to be studied carefully because of their key roles in cell functions, e.g., energy generation and cell movement. Structures such as rootlets or cytoplasmic microtubules and microfilaments received less attention until the last ten years because of technical difficulties with fixation, isolation or characterization and the less obvious nature of their functions.

By the early 1920's, rootlets had been described in a variety of ciliated and flagellated cells, e.g., the mussel Anodanta, the earthworm Lumbricus, the snail Aplysia and in several protozoans (Wilson, 1925). Since that time, rootlets have been found in most ciliated and flagellated cells from both protozoans and metazoans, with the notable exception of sperm. Cells containing rootlets are involved in such diverse functions as respiration, feeding, locomotion, reproduction, excretion and sensory perception. Little is known, however, about the molecular composition or organization of rootlets or about their development or function. Although no specific function has been conclusively demonstrated, rootlets may serve as a non-extensible anchor providing positional stability to the basal body-flagellum complex; alternatively, a tensile or contractile function modifying flagellum motion has been proposed (Fawcett, 1958a; Pitelka, 1969).

Rootlets are tightly attached to the basal bodies and, as they extend through the cytoplasm, are often intimately associated with the nucleus as well (Pitelka, 1969). Although there are considerable size variations among rootlets from different cell types, the organelle consistently displays a characteristic banding pattern of alternating electron opaque and electron lucent regions. Subperiods are often found within each major band. Rootlets of metazoans usually display major repeat periods of about 65 nm, e.g., 56 to 66 nm for ciliated fetal thymic cell rootlets (Sebuwufu, 1968), 65 nm for Aequipecten irradians ciliated gill epithelium rootlets (Stephens, 1975a),

50 nm for the flame cell rootlet from rotifers (Mattern and Daniel, 1966) and 70 nm for Polychaerus caudata ciliated integument rootlets (Olson and Rattner, 1975). The periodicities of rootlets from protozoans, in contrast, are generally 35 nm or less, e.g., 32 nm for Tetrahymena pyriformis rootlets (Munn, 1970), 30 to 35 nm for Paramecium multimicronucleatus rootlets (Pitelka, 1969) and 12 nm for the flagellar rootlet of Tetramitus rostratus (Outka and Klus, 1967).

The exact values cited for rootlet periodicities was questioned by Simpson and Dingle (1971) following their detailed electron microscopic study of the flagellar rootlet of Naegleria gruberi. They demonstrated that the Naegleria flagellar rootlet exhibited variable periodicity in situ, with an average repeat period of 18.5 nm, but a range of 13.8 to 25.2 nm. In contrast, the isolated organelle, perhaps reflecting a "relaxed or extended" state, had a relatively constant periodicity of 21.7 nm and ranged only from 20.0 to 23.6 nm. The variable periodicity exhibited in rootlets from different cells led Simpson and Dingle (1971) to suggest that the periodicity may reflect a functional state. Two other lines of evidence support this idea. Treatment of the alga Platymonas with exogenous adenosine triphosphate resulted in a drastic change in the periodicity of the rootlet suggesting that the alteration in banding pattern may be energy-dependent (Salisbury and Floyd, 1978). In addition, phosphatase activity has been found in the dark bands of the isolated striated rootlet of the alga Polytomella (White, et al., 1978).

The characteristic periodicities seen with the electron microscope in both isolated and intact rootlets may result from the specific organization of constituent filaments, 4 to 5 nm in diameter. Thin filaments have been observed in rootlets from the ciliated gill epithelium of Anodanta cataracta (Gibbons, 1961) and Aequipecten irradians (Stephens, 1975a), Tetrahymena (Munn, 1970) and ciliated tail cells of larval amphioxus (Flood, 1975). The molecular organization leading to the striated appearance of the rootlet is unknown but it may be similar to that which results in cross-banding of comparable supramolecular structures like collagen (Hodge and Schmitt, 1960) and paramyosin (Cohen et al., 1971) or to tropomyosin (Cohen, 1975) and fibrinogen (Tooney and Cohen, 1977).

Only two papers in the literature report serious efforts to characterize the molecular components of isolated rootlets. Solubilization of the rootlets from Tetrahymena pyriformis with phosphotungstate led Rubin and Cunningham (1973) to tentatively identify a 21,000 dalton peptide as a rootlet component. From a detailed solubility study of basal rootlets from the ciliated gill epithelium of Aequipecten irradians, Stephens (1975a) identified two proteins with subunit molecular weights of 230,000 and 250,000 daltons as the major rootlet protein components. Rootlets have been isolated from a variety of other systems, e.g., Paramecium (Metz et al., 1953; Hufnagel, 1969), Oedogonium zoospores (Hoffman and Manton, 1962), Tetrahymena (Satir and Rosenbaum, 1965; Munn, 1970; Wolfe, 1970), Naegleria flagellates (Simpson, 1970) and Polytomella agilis (Brown et al., 1976), but these

studies were primarily concerned with detailed descriptions of the arrangement of basal bodies, striated fibres and accessory structures.

The flagellar rootlet of the amoebo-flagellate Naegleria gruberi lends itself to a detailed biochemical and ultrastructural investigation because of its large size and unusual stability. For example, the isolated organelle is 13 μm in length. It is stable for at least 24 h at 2°C, despite the fact that amoebae actively phagocytoze bacteria and cells must contain large amounts of proteases (Green, 1974). Since the rootlet is present only in the flagellate form of Naegleria, another advantage of this system is the potential for studying rootlet development as part of an overall programme of differentiation involving the synthesis and assembly of basal bodies, flagella, cytoplasmic microtubules and the rootlet into an integrated unit, the flagellar apparatus (Fulton and Dingle, 1967).

The Naegleria Cell Differentiating System

The differentiation of amoebae to flagellates is so synchronous and reproducible that it has proven useful as a model cell differentiation system, permitting the construction of a timetable of differentiation events (Dingle, 1977; Fulton; 1977). Initiation of differentiation, T_0, can be triggered by a decrease in the nutrient supply, a decrease in temperature, an increase in mechanical agitation or a decrease in electrolyte concentration (Fulton, 1974). All events are expressed on a time scale relative to the appearance of flagella, an obvious and easily measurable parameter for monitoring the differentiation. Events

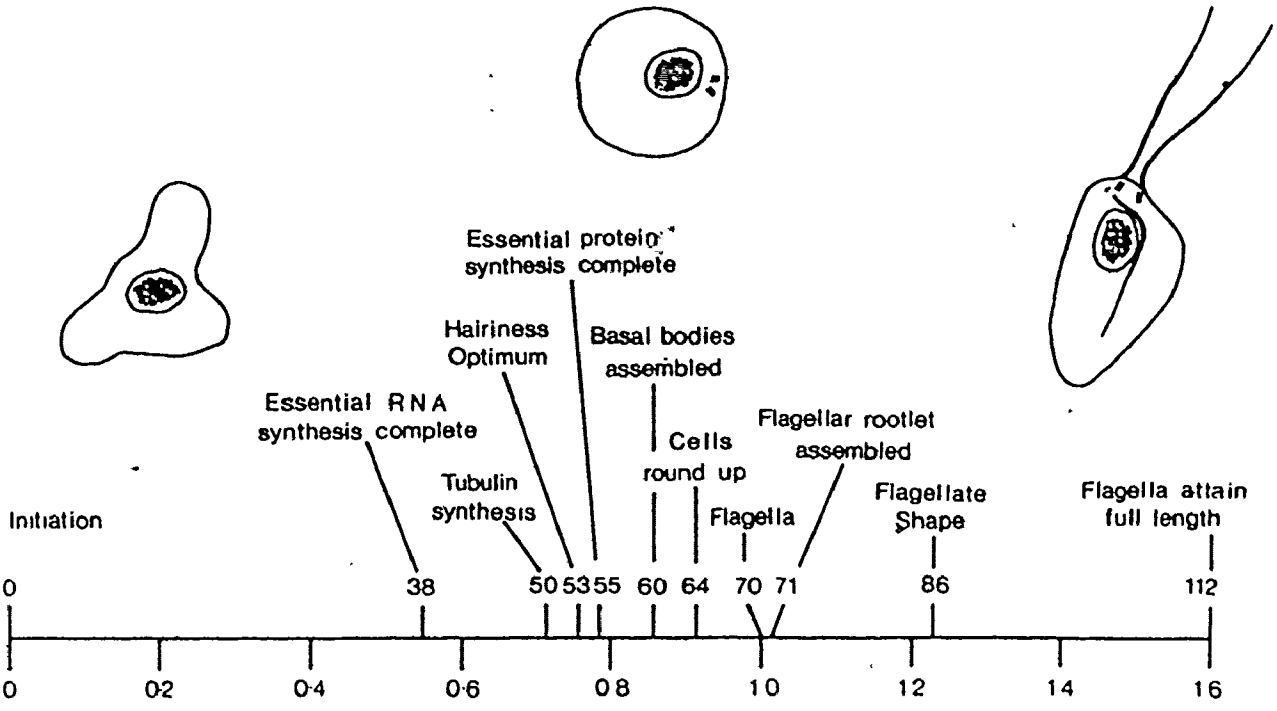
are expressed in terms of T_{50}, i.e., the time after T_0 when 50% of the cells have attained a specific state. For example, using strain NB-1, the T_{50} for appearance of flagella at 25°C is 70 min. A timetable of the major events during the amoeba to flagellate differentiation is summarized in Fig. 1 (from Dingle, 1977).

The sequence of flagellum development in synchronously differentiating Naegleria flagellates has been described in detail (Dingle and Fulton, 1966; Fulton and Dingle, 1967). The basal body assumes a position near the plasma membrane with its microtubules perpendicular to the cell surface. Flagellum development probably involves elongation of the developing axoneme by distal addition of flagellar components in a manner similar to that described for regenerating flagella of Chlamydomonas (Whitman, 1975). The flagellar membrane elongates to accommodate the developing axoneme. By the time flagella attain a length of 1.5 to 2 μm, they become visible in the light microscope and undergo their first beats. Within 20 min of the appearance of flagella, cells are free-swimming flagellates.

The sequence of basal body development in Naegleria has been elucidated and is similar to that of other systems. In a detailed ultrastructural study, Fulton and Dingle (1971) constructed a time course of appearance of basal bodies during the amoeba to flagellate differentiation. Basal bodies were first seen 10 min before the appearance of flagella. T_{50} for basal body appearance was 0.86 relative to the appearance of flagella (Fig. 1). Basal bodies probably

Figure 1

An outline of the major events occurring during the amoeba to flagellate differentiation in Naegleria. Since the data have been collected from several laboratories using different strains and culture conditions, they have been presented on a time scale relative to the appearance of flagella (below line). T_{50} for the appearance of flagella has been arbitrarily set at 1.0. To illustrate the absolute times of these differentiation events, T_{50} for the appearance of flagella for strain NB-1 at a differentiation temperature of 25°C has been set at 70 min and all events expressed in minutes after the initiation of differentiation (above line) (from Dingle, 1977).


Essential protein synthesis complete
Hairiness Optimum
Basal bodies assembled
Essential RNA synthesis complete
Cells round up
Flagellar rootlet assembled
Tubulin synthesis
Flagella
Flagellate Shape
Flagella attain full length
Initiation
0
38
50
53
55
60
64
70
71
86
112
0
02
04
06
08
10
12
14
16

arise de novo during the differentiation since no centrioles are seen at the spindle poles during mitosis nor has any basal body precursor been identified (Fulton and Dingle, 1971). Dingle (1977) has subsequently found various differentiating stages of the characteristic "9 triplets" microtubule cartwheel arrangement in developing flagellates.

An obvious omission from this account of assembly of the three major organelles into a functioning flagellar apparatus is a description of flagellar rootlet development. A quantitative electron microscopic study of rootlet appearance is precluded because of its long and narrow morphology; the probability of seeing a rootlet at an early stage of development would be extremely low. Rootlets with the characteristic banding pattern have been seen in thin sections as soon as 65 min after the initiation of differentiation (Dingle and Fulton, 1966). Green (1974) measured the appearance of rootlets in developing flagellates by lightly fixing cells and scoring for the presence of a rootlet by gentle lysis of each cell with detergent. Although in this method rootlets were only briefly visible before they too were solubilized, he was able to construct a time course of the appearance of rootlets which paralleled flagellum appearance (Fig. 1).

Accompanying the assembly of basal bodies, flagella and rootlet into a functioning flagellar apparatus are distinct cell shape changes: from amoeboid to spherical to the fusiform flagellate shape. Relative to the appearance of flagella, the times of appearance of spheres and the flagellate shape are 0.96 and 1.26, respectively (Fig. 1).

RNA synthesis is required for flagellate differentiation (Yuyama, 1971; Walsh and Fulton, 1973; Dingle, 1977). By adding actinomycin D, an inhibitor of RNA synthesis, at various times during the differentiation period, a T_{50} of 0.55 relative to the appearance of flagella was measured for the onset of insensitivity to the inhibitor (Fig. 1). This has been interpreted to show that approximately halfway through the differentiation period, the cells have synthesized the required messenger RNAs to permit their normal differentiation. Actinomycin D has been shown to inhibit the de novo synthesis of flagellar tubulin messenger RNA (Lai, 1978). In addition, there is a shift in total RNA synthesis 5 min after the initiation of differentiation to a pattern of preferential messenger RNA synthesis (Walsh and Fulton, 1973). During the differentiation there are changes in poly A-containing RNA (Walsh et al., 1976) but not in the RNA polymerases (Soll and Fulton, 1974).

Flagellate differentiation is also dependent on protein synthesis. Using cycloheximide, an inhibitor of protein synthesis, Dingle (1977) measured a T_{50} of 0.78 for the onset of insensitivity to the inhibitor (Fig. 1). Although the flagellar apparatus is undoubtedly composed of many new proteins not present in amoebae, the only one which has been shown to be synthesized de novo to date is flagellar tubulin. Using a radioimmunoassay which employed an antibody specific for flagellar tubulin, Kowit and Fulton (1974a) demonstrated that flagellar tubulin is synthesized de novo with a

T_{50} of 0.71 (Fig. 1). Flagellar tubulin has also been synthesized in vitro using messenger RNA which exists in developing flagellates, but not in amoebae (Walsh and Fulton, 1975).

Regulation of Cell Differentiation

In multicellular systems, cell differentiation appears to be an ordered sequence of events at both the molecular and cellular levels of organization. For example, during pancreas differentiation, a mass of "undifferentiated" cells develop into a number of distinct cell types, each exhibiting specific biochemical functions (Rutter et al., 1968). A multicellular system well suited for investigating the control of specific events during cell differentiation is the slime mold Dictyostelium discoideum. During a 24 h period Dictyostelium amoebae aggregate and form a fruiting body containing three cell types each with specific functions (Loomis, 1976). During this differentiation there is an ordered sequence of biochemical and morphological events occurring in a cascade fashion such that inhibition of one step prevents continued development. Temporal mutants of Dictyostelium exist in which the sequence of events during fruiting body formation occurs in less than the normal 24 h period. Monitoring the synthesis of specific enzymes during the differentiation demonstrated that on a relative time scale neither the time of appearance of the enzymes nor the duration of synthesis had been altered (Loomis, 1970). These results suggest that in such mutants the precise, ordered sequence of biochemical and morphological

events had not been altered.

The question of regulation during cell differentiation can be posed at the organelle level. In many protozoa the control over flagellum number and distribution is so precise that these two parameters have been used as taxonomic criteria, e.g., chryosomonad, phytomonad and trichomonad flagellates (Hall, 1953; Grell, 1973). In contrast, the swarm cells of Didymium nigripis initially develop one flagellum but may develop a second after several hours (Kerr, 1960). Occasionally mammalian sperm are found which are biflagellated (Fawcett, 1961) and multiflagellated sperm are often found in the snail Viviparus (Gall, 1961). The expression of flagellum number and length may not be constant throughout the organism's development, despite a constant genotype. An obvious example is in mammals in which spermatozoa possess one flagellum (Fawcett, 1958), the adenohypophysis has two (Wheatley, 1967) and hundreds of cilia are present on ciliated epithelium of trachea (Dirksen and Crocker, 1966) and lung (Sorokin, 1968).

The "9+2" microtubular arrangement is typical of most cilia and flagella but there are many exceptions in birds, insects, annelids and flatworms (Bachetti and Afzelius, 1976). Usually a species' sperm displays one microtubular organization; however, exceptions are found in the order Eoacanthocephala in which a few sperm tails from a given individual are structurally distinct from the prevalent type (Marchand and Mattei, 1977). Mammalian fetal lung cells develop primary cilia with a "9+0" microtubular arrangement

and only later do those cells which differentiate into ciliated epithelium develop a "9+2" arrangement (Sorokin, 1968).

There are two reasons why Naegleria lends itself to a study of regulation of organelle number and size. Firstly, the controls which govern the number of organelles assembled during the differentiation are not absolute. Naegleria flagellates normally develop two flagella but one, three, four or occasionally five may be present. Secondly, the controls of organelle number may be deranged by high temperature shocks applied at precisely defined intervals during the differentiation such that the resulting flagellate population may develop nearly three times the normal number of basal bodies and flagella (Dingle, 1970). Furthermore, by varying the time and duration of the temperature shock, populations may be experimentally manipulated to give any intermediate value. The question arises whether in these multiflagellated populations the number and size of flagella and rootlets are affected to the same extent.

Statement of Problem

This thesis is concerned in a broad sense with events occurring during cell differentiation. Specifically it describes the development of a major cell organelle, the flagellar rootlet, during the differentiation of amoebae to flagellates in the protozoan Naegleria gruberi. It also describes the isolation and biochemical characterization of the flagellar rootlet, necessary prerequisites to the subsequent localization of developing rootlets by indirect

immunofluorescence. In addition, some evidence is presented to support the idea that the assembly of basal bodies, flagella and rootlets is under some form of coordinate control.

Chapter 2

Materials and Methods

2.1 Organism

The strain of the amoebo-flagellate Naegleria gruberi used in this study was originally isolated by Pringsheim in England and after successive subcloning of this isolate by Fulton and Dingle (1967) was designated strain NB-1. This strain was always grown in association with the bacterium Klebsiella pneumoniae.

2.2 Culture Media

Naegleria amoebae were grown and stock cultures maintained on agar medium designated NM by Fulton and Dingle (1967). NM consisted of 1.6 g Difco bacto peptone, 1.6 g dextrose, 1.2 g K_2HPO_4, 0.8 g KH_2PH_4 and 16 g bacto agar in 800 ml DW (demineralized and then glass distilled water). The medium was autoclaved and poured into plastic petri plates. Large batches of amoebae were grown on PM (similar to NM except that the bacto peptone concentration was doubled) in three quart pyrex trays each containing 500 ml PM.

Stock cultures of Klebsiella were maintained on NM and working cultures were grown in Penassay Broth (PAB) which consisted of 1.75% Difco bacto medium 3, autoclaved in 6 ml aliquots.

2.3 Stock Maintenance

Naegleria NB-1 stocks were maintained on spread cultures on NM. Briefly, this method involved removing approximately 2×10^5 cysts from a previous stock plate, suspending the cysts in 0.5 ml DW

with vigorous agitation and adding 0.1 ml Klebsiella (working culture). The cyst-suspension was then spread over the agar surface. Stocks were maintained at room temperature and transferred when needed.

Klebsiella stocks were maintained on NM as streak cultures. Successive transfers involved removing a loopful of bacteria and making two streaks across the agar surface. Working cultures were prepared by inoculating PAB with a small loopful of bacteria. This culture was then incubated at 34°C for approximately 20 h, until stationary phase growth was achieved.

All manipulations of Naegleria and Klebsiella stocks were done using aseptic techniques.

2.4 Culture Methods

Approximately 18 h before Naegleria amoebae were needed 2×10^5 cysts and 0.1 ml Klebsiella (working culture) were spread on a NM agar plate and grown at 34°C. Under these conditions the bacteria grow rapidly forming a bacterial lawn on the surface of the plate. Naegleria excyst in 2 to 4 h and engulf the already established bacterial culture. As the amoebae reach stationary phase and deplete the bacterial food source, they become visible since a portion of the bacterial lawn has been cleared, thus giving rise to a "cleared" plate. Amoebae for differentiation were taken when plates were 20 to 90% cleared. Yield under these conditions was $2\text{-}4 \times 10^7$ cells/plate.

Large quantities of amoebae were obtained by inoculating each pyrex tray containing PM with approximately 2×10^6 cysts plus

1 ml, Klebsiella and incubating at 34°C. Approximately 24 h later, trays were 50 to 80% cleared and each contained 3-5 x 10^8 amoebae.

2.5 Cell Counting

Cell concentrations were determined using the Coulter Counter, Model F (Coulter Electronics Inc., Hialeah, Florida). An aliquot of cells was diluted in an electrolyte (0.4% NaCl), vortexed gently to disrupt clumps and counted at attenuation 2, aperture current 64 and threshold 20. Two counts per sample were taken and the average, corrected for the dilution factor, gives the cell concentration of the sample. Occasionally, cell concentrations were measured with a hemacytometer.

2.6 Amoeba to Flagellate Differentiation

Amoebae were washed free of bacteria by suspending the cells in 10 ml buffer, 2 mM tris-HCl (tris (hydroxylmethyl) aminomethane) pH 7.4 or 2.5 mM imidazole-HCl pH 6.25 at 2°C. The amoebae were removed from the agar surface with the aid of a bacterial spreader. The bacteria-amoebae suspension was decanted into a 50 ml centrifuge tube. The plate was washed again and the 20 ml combined suspension was centrifuged in a table top clinical centrifuge (model CL, International Equipment Co., Needham Hts., Mass.) at rheostat setting 6 for 45 sec. The cell pellet was suspended in 20 ml buffer and the centrifugation repeated twice. The final pellet was resuspended in 15 ml buffer and transferred to a 125 ml Erlenmeyer flask. Differentiation was initiated by inserting the flask into a reciprocal

shaking water bath at 100 2.5 cm strokes per min at 25°C. The usual time period between initial suspension of the cells and placement of the flask in the water bath was 5 to 6 min. Zero time (t_0) was defined as the time at which the flask was inserted into the water bath.

At specific time intervals, samples were removed from the flask, fixed in Lugol's Iodine (4 g I_2 and 6 g KI in 100 ml DW) and observed with Leitz phase contrast optics. The percentage of cells with flagella at any time interval was determined from a sample size of 100. The mean number of flagella per flagellate was determined using three independent 100 cell counts once the cell population had reached greater than 90% flagellates. Flagellum lengths were measured using a filar micrometer.

In some experiments, developing flagellates were subjected to a sublethal temperature shock (37.9°C) for various time intervals and the differentiation completed at 21 or 25°C. In these experiments, water bath temperatures were controlled to within ± 0.1°C. To produce means of approximately 2, 3, 4 or 5 flagella per flagellate, the temperature shock intervals were 0-18, 30-48, 10-46 or 30-66 min post-initiation, respectively. All differentiations were completed at 25°C. The maximum mean number of flagella per flagellate (5.7 to 6.0) was produced by subjecting amoebae to 37.9°C from 35 to 71 min post-initiation and completing the differentiation at 21°C.

Harvesting of large quantities of amoebae grown on PM in pyrex trays required 20 to 25 min. Therefore, amoebae from each tray were

washed twice from the agar surface in a total of 100 ml 2 mM tris-HCl pH 7.4 plus 100 mM NaCl which inhibits the initiation of differentiation (Fulton, 1972) at 2°C. All subsequent washings were done in iced buffer alone. The final pellet was resuspended in 35 ml buffer and transferred to a 250 ml Erlenmeyer flask. Differentiation was initiated by insertion of the flasks into a shaking water bath at 25°C.

2.7 Flagellar Apparatus Isolation

Flagellar apparatuses were released from intact mature flagellates by gentle agitation after the addition of Triton X-100 (Sigma) to a final concentration of 0.1%. The lysing buffer consisted of 30 mM tris pH 8.0 plus 3 mM $MgSO_4$.

2.8 Rootlet Isolation

At 120 min after the initiation of cell differentiation, flagellate suspensions were passed through four layers of cheesecloth and collected by spinning for 1 min at rheostat setting 7 in a table top clinical centrifuge. Flagella were removed from flagellates according to the method of Kowit and Fulton (1974b). The flagellate pellet was resuspended in 10 ml buffer consisting of 10 mM sodium acetate pH 3.7, 150 mM sucrose, 1 mM EGTA (ethylene glycol-bis-(β-aminoethylether)N,N'-tetraacetic acid), 2 mM $MgSO_4$ and 0.005% PMSF (phenymethyl sulfonyl fluoride) at 1-3 x 10^7 cells/ml. The suspension was shaken vigorously for 10 sec and the pH adjusted to 8 by the addition of 0.5 ml of 0.5 M tris-HCl pH 8.25. The cell bodies,

minus flagella, were collected by centrifuging for 1 min at rheostat setting 7 in a table top centrifuge. Cell bodies were resuspended in lysing buffer consisting of 30 mM tris-HCl pH 8.0, 50% glycerol, 3 mM $MgSO_4$, 1 mM EGTA, 1 mM dithiothreitol, 10 mM ε-amino-n-caproic acid and 0.005% PMSF at a concentration of 5-10 x 10^6 cells/ml. Cells were lysed by adding Triton X-100 to a final concentration of 0.5% and immediately vortexing at top speed for 20 sec. The suspension was adjusted to 25% glycerol by adding lysing buffer minus glycerol and the nuclei were pelleted by spinning for 2 min at 10,400 g in an HB-4 swinging bucket rotor. Potassium iodide was added to the supernatant to yield a final concentration of 0.1 M KI and the rootlets were washed once in lysing buffer minus glycerol plus 0.1 M KI and were pelleted by spinning for 10 min at 16,300 g. A, 109

2.9 Rootlet Solubilization

The final rootlet pellet was suspended in a solubilizing solution (see below) for 1 h at room temperature. The effect of this solution was monitored by phase microscopy and electron microscopy after uranyl acetate staining. The suspension was then centrifuged at 16,300 g for 10 min, a speed which pellets intact rootlets. The resulting supernatant and pellet were termed the "soluble" and "insoluble" fractions, respectively. The proteins of each fraction were identified by separation on sodium dodecyl sulphate (SDS) polyacrylamide gels. A protein was considered soluble when the majority (>75%) of it was lost from the pellet (insoluble fraction).

after centrifugation as judged by SDS polyacrylamide gel electrophoresis.

The solubilizing solutions consisted of 20 mM tris-HCl pH 8.0 plus one of the following: 0.1% Sarkosyl, 0.4 M KI, 50 mM EDTA (ethylene diaminetetraacetic acid), 2 M or 8 M urea. The buffer composition of the test pH solutions was 20 mM citrate-phosphate pH 2.0 or 20 mM glycine→NaOH pH 10.0.

2.10 Microscopy

The purity of rootlet preparations was monitored using Leitz phase contrast optics at 500 x magnification. For electron microscopy, one drop of a rootlet suspension was applied and allowed to settle for 30 sec on a Parlodion-carbon coated grid, rinsed with water or solubilizing agent and stained with 2% uranyl acetate for 30 to 45 sec. Fluorescent images were observed with a Leitz Orthoplan ultraviolet microscope using specific fluorescein excitation filters.

2.11 Gel Electrophoresis

The protein composition of various cell fractions was analyzed by SDS polyacrylamide gel electrophoresis using three different buffer systems: the Weber-Osborn (1969) sodium phosphate pH 7.0 buffer system, the tris-glycine pH 8.3 buffer system described by Stephens (1975b) and Laemmli's (1970) discontinuous (pH 6.8-8.8) buffer system.

The Weber-Osborn SDS phosphate polyacrylamide gel system was used 1) to analyze proteins of various rootlet fractions, especially if the samples contained high salt concentrations, and 2) to determine the molecular weights of various proteins. Tube gels (60 x 5 mm) consisted of 5% acrylamide (BioRad), 0.13% N,N'-methylenebisacrylamide (Eastman), 0.1% ammonium persulphate, 0.05% TEMED (N,N,N',N'-tetramethylenediamine), 0.1% SDS (Sigma, recrystallized from 95% ethanol) and 100 mM sodium phosphate pH 7.0. The chamber buffer consisted of 100 mM sodium phosphate pH 7.0 and 0.1% SDS.

Protein samples for electrophoresis in this system were suspended in sample buffer so that the final solution consisted of 100 mM sodium phosphate pH 7.0, 2% β-mercaptoethanol, 2% SDS, 0.005% PMSF, 20% glycerol and 1% bromphenol blue (indicator dye). Samples were incubated at 100°C in a boiling water bath for 3 min, cooled to room temperature and 10 to 50 µl layered onto the tops of gels using a microsyringe. Electrophoresis was carried out at 8 mA per gel until the bromphenol blue band had migrated approximately 95% of the total gel length.

The tris-glycine pH 8.3 buffer system described by Stephens (1975b) was used for routine analysis of proteins in various rootlet fractions because of the short electrophoresis running time (45 to 60 min). This buffer system was also used on a preparative scale (10 x 1 cm tube gels) for purification of the major rootlet protein for the same reason. The compositions of the tube gels and the chamber buffer were identical to those described above for the phosphate SDS buffer system except that the buffer was replaced by 25 mM tris-glycine pH 8.3. The final concentration of the tris-glycine pH 8.3 buffer in the sample buffer was 2.5 mM. Electrophoresis was carried out at 3 mA per gel.

Total cellular proteins were separated by SDS polyacrylamide slab gel electrophoresis using the discontinuous buffer system described by Laemmli (1970). The running gel consisted of 7.5% acrylamide, 0.2% N,N'-methylene-bisacrylamide, 0.05% ammonium persulphate, 0.02% TEMED, 0.1% SDS, and 375 mM tris-HCl pH 8.8. The stacking gel consisted of 4% acrylamide, 0.1% N,N'-methylene-bisacrylamide, 0.1% ammonium persulphate, 0.1% TEMED, 0.1% SDS and 62.5 mM tris-HCl pH 6.8. Protein samples for electrophoresis were resuspended in 6 M urea, 5% SDS, 0.005% PMSF, 2% β-mercaptoethanol and 62.5 mM tris-HCl pH 6.8. Samples were incubated in a boiling water bath for 3 min and then at 50°C for 30 min. Samples were centrifuged for 5 min at 3,000 g in a table top clinical centrifuge. Samples (2 to 20 µl) were immediately layered into the wells of the stacking gel. The chamber buffer consisted of 52.5 mM tris-glycine pH 8.8, 0.1% SDS and 1% bromphenol blue.

Electrophoresis was conducted at 100 volts for approximately 8 h or until the indicator dye had migrated 95% of the total gel length. In those experiments in which the amount of rootlet protein in cells was quantitated, electrophoresis was continued for 90 to 120 min after the dye front had left the running gel.

Molecular weight standards were mouse myosin, actin and tropomyosin, bovine serum albumin, pig brain tubulin and chick ovalbumin. Flagellar microtubules from Naegleria flagellates were isolated according to the method of Fulton and Simpson (1976). Myofibrils were prepared by teasing apart the psoas muscle from rabbit and washing several times in 50% glycerol. Immunoglobulins, used for glycoprotein standards, were isolated from rabbit serum (globulin fraction, section 2.13).

High concentrations of ions were removed from protein samples by acetone precipitation prior to electrophoresis. Each protein sample was resuspended in 1 ml DW and 9 ml acetone added. After 10 min on ice, the 90% acetone precipitate was collected by centrifuging for 10 min at 16,300 g in a HB-4 rotor. The precipitate was resuspended in 10 ml iced acetone, left for 10 min on ice and pelleted as described above. After the acetone precipitate was dried under vacuum, each protein sample was resuspended in electrophoresis sample buffer.

The power source used for the electrophoresis was a Heathkit regulated high voltage power supply, model IP-17.

Following separation by SDS polyacrylamide gel electrophoresis, proteins were stained by one of four methods. Routinely, tube gels were incubated at 50°C with 0.25% Coomassie Brilliant Blue R 250 in 50% methanol and 7.5% acetic acid for 1 h. Initial destaining was accomplished by one change in 50% methanol and 7.5% acetic acid for 30 min and this was followed by several changes of 5% methanol and 7.5% acetic acid. Gels were stored in 7.5% acetic acid.

Alternatively, proteins were stained quantitatively with fast green FCF as described by Fulton and Simpson (1976). This procedure involved incubation of gels in a 50-fold excess of 25% isopropanol and 10% acetic acid for 90 min to reduce the SDS concentration. Gels were stained with two changes (90 min each) of 0.1% fast green FCF in 50% methanol and 7.5% acetic acid and destained with several changes of 5% methanol and 7.5% acetic acid. All steps were done at 50°C with continuous agitation.

Glycoproteins were detected using a specific staining method modified after Fairbanks et al. (1971) and Glossman and Neville (1971). This procedure, performed at room temperature, involved incubation of gels in a 50-fold excess of 25% isopropanol and 10% acetic acid for 24 h with continuous agitation. Gels were immersed in 5% acetic acid containing 1% periodic acid (freshly prepared) for 1 h, washed four times, 15 to 20 min each, in 7.5% acetic acid and stained with Schiff's reagent for 1 h. Schiff's reagent was prepared by mixing 2.5 g basic fuchsin, 5 g sodium metabisulphite, 50 ml 1N HCl and

500 ml DW for several hours. The suspension was decolorized with activated charcoal (2 g) and filtered. The filtrate was stored in the dark at 4°C. Following treatment with Schiff's reagent, gels were washed 3 to 4 times, 30 min each with 0.5% sodium metabisulphite in 0.1 N HCl. Gels were counterstained with Coomassie Brilliant Blue as described above.

Total cellular proteins, separated by SDS polyacrylamide slab gel electrophoresis, were stained quantitatively using a procedure based on methods described by Fishbein (1972) and Kahn and Rubin (1975). Gels were incubated in 10% TCA (trichloroacetic acid) for 16 h, stained with Coomassie Brilliant Blue in 50% methanol and 7.5% acetic acid for 2 h and destained with several changes of 10% TCA for 48 h at room temperature. B,]09

Staining patterns were recorded by photography or microdensitometry. Gels were placed on a lightbox and photographed using Kodak Ortholith film with a green filter. The film was developed with Kodal D-19 (½ dilution) for 3.5 min. Alternatively, the stained patterns were recorded with a Gilford microdensitometer (model 2400) at wavelengths of 560 and 660 nm for Coomassie Brilliant Blue and fast green FCF, respectively. For quantitation, gels were scanned at a gel speed of 1 cm/min and recorded at a chart speed of 2.5 cm/min. For quantitation of specific proteins bands in slab gels, four readings of each gel section were recorded. The gel was scanned, then a 1.5 to 2 mm longitudinal section was removed and the gel rescanned. This procedure was repeated twice. The peak area corresponding to specific

protein bands was either traced, cut out and weighed or the area was measured with a planimeter.

In order to determine the distribution of radioactivity in gels stained quantitatively with fast green FCF, gels were cut into 1 mm slices with a Joyce-Loebl gel slicer. Slices were dissolved in 0.2 ml 30% hydrogen peroxide at 60°C for 5 h and counted in 3 ml Aquasol (New England Nuclear Corp.). Radioactivity was measured in a Beckman LS-250 scintillation counter.

2.12 Rootlet Protein Purification

To obtain the necessary quantities of rootlet protein to elicit antibody production in rabbits, rootlets were isolated from approximately 5×10^9 flagellates as described in section 2.8. The proteins of this rootlet fraction were solubilized in 2.5 ml tris-glycine pH 8.3 sample buffer and separated on 5 preparative polyacrylamide tube gels (see section 2.11 for details). The banding position of the major rootlet protein was determined by staining a slice from each tube gel with Coomassie Brilliant Blue. The remaining gel sections were kept on ice. The appropriate gel sections were chopped with a razor blade and the protein was eluted from the gel in a 50-fold excess of 10 mM ammonium bicarbonate, 0.005% PMSF and 0.1% SDS at 34°C for 24 h. The suspension was passed through Whatmann #1 filter paper and the resulting filtrate lyophilized. The yield of rootlet protein from 5×10^9 flagellates was 20 to 30 µg. This protein concentration was estimated by a comparison to the staining intensity with Coomassie Brilliant Blue of the protein standard, bovine serum

albumin, following SDS polyacrylamide gel electrophoresis.

2.13 Preparation of Antibodies to Rootlet Protein

Antibodies directed against the major rootlet protein were obtained from sera from white New Zealand rabbits, initially weighing 1700 g. Each of three rabbits was initially injected (day 0) with 2 ml of a suspension containing approximately 30 µg rootlet protein, emulsified with an equal volume of Freund's complete adjuvant (Difco). Rabbits were injected intramuscularly in the hip region and subcutaneously in the shoulder region. All subsequent injections contained the rootlet protein in DW only. Rabbits were injected on day 20 and 34 with approximately 30 µg rootlet protein. For each subsequent injection, the protein level was decreased to 20 µg (day 48, 62, 76 and 119). Rabbits were bled one week following each injection and at weekly intervals between days 76 and 119. Rabbits were killed by cannulation on day 138.

Serum was collected by incubation of blood samples at 37°C for 20 to 30 min to ensure clotting, contraction of the blood clot by incubation at 2°C for 2 h and removal of the blood clot and red blood cells by centrifuging twice for 10 min at 2,000 g in a table top clinical centrifuge. The serum was incubated at 56°C for 30 min to inactivate serum proteases and stored at -70°C. Preimmune sera were obtained from the same animals prior to immunization.

The globulin fraction was prepared by ammonium sulphate precipitation of serum. An equal volume of saturated ammonium sulphate was slowly added to the serum and mixed for 10 min. The precipitate was

collected by centrifuging for 15 min at 16,300 g. The pellet was resuspended in PBS (phosphate buffered saline - 0.2 g KH_2PO_4, 11.5 g $Na_2HPO_4 \cdot 7\ H_2O$, 8.5 g NaCl and 1 g NaN_2 per 1, pH 7.1) to the original serum volume and the ammonium sulphate precipitation was repeated. The final pellet was resuspended in PBS to ½ the original serum volume and dialyzed for 24 h at 2°C against 2 l PBS.

2.14 Antigen-Antibody Assays

The production of antibody directed against the rootlet protein was assayed by Ouchterlony (1973) double diffusion test. The agar support medium contained 1% bacto agar (washed extensively with DW containing 15 mM NaN_2), 0.5% Triton X-100, 0.1% SDS and 3% polyethylene glycol 6000 (Baker). The medium was solubilized by incubation in a boiling water bath, cooled to 60°C and 2 ml aliquots applied to microscope slides (75 x 25 mm) using a pipette to control the flow rate. Wells (2 mm in diameter) were punched into the agar support medium 5 to 8 mm apart. Routinely, test antigens contained 2% SDS. All immunodiffusion tests were performed at 37°C in a humid atmosphere for 20 to 24 h. Results were recorded by contact prints on Kodak Ilfobrom #5 paper.

The specificity of the antibody was tested using a double antibody-enzyme technique described by Olden and Yamada (1976). Following separation of proteins by SDS polyacrylamide gel electrophoresis, gels were incubated at 37°C in the following solutions for 24 h each: 25% isopropanol and 10% acetic acid, 10% isopropanol and 10% acetic acid, 10% acetic acid and then 7% acetic acid. After washing

in several changes of PBS for 48 to 72 h, each gel was treated with rabbit antiserum to rootlet protein (1/10 dilution) for 16 h at 37°C in a humid atmosphere. Following extensive washing with several changes of PBS for 48 to 72 h, gels were incubated with 1/10 diluted goat anti-rabbit immunoglobulin coupled with horseradish peroxidase (Miles) for 16 h at 37°C in a humid atmosphere. Unreacted serum was removed by washing in PBS for 48 to 72 h. The enzyme was localized by incubating gels in 10 ml PBS containing 5 mg diaminobenzidine (Polyscience) plus 5 µl 30% hydrogen peroxide for 1 min and then washing gels extensively in PBS. Alternatively, the same procedure was followed as described above except that the first serum was preimmune serum or the second antibody was unconjugated sheep anti-rabbit immunoglobulin (McMaster Univ. Central Resource for Immunological Reagents, Hamilton, Ontario).

2.15 Immunofluorescence

At various times during the differentiation period, samples (1-2 x 10^6 cells) were fixed for 20 sec at room temperature with an equal volume of fixative solution such that the final concentrations were 0.75% paraformaldehyde, 0.005% nonidet P-40 (Bethesda Research Lab., Rockville, Md.) and 25 mM sodium phosphate pH 7.4. The cell suspension was centrifuged for 45 sec at rheostat setting 6 in a table top clinical centrifuge. The supernatant was decanted and the cell pellet resuspended in 0.3 ml of 25 mM sodium phosphate pH 7.4 plus 0.015% nonidet P-40. The cell suspension was applied to microscope slides and air dried. Slides were rinsed twice with PBS for

10 min. Samples were dehydrated by rinsing in methanol and then acetone for 10 min each at 2°C and air dried. C,]09

Prior to treatment of cells with antisera, samples were rehydrated by immersion in PBS for 2 min. After draining excess buffer, samples were treated with 30 µl antiserum to rootlet protein (diluted 1:10) and incubated at 37°C for 30 min in a humid atmosphere. Samples were washed three times for 10 min each with PBS and treated with 30 µl fluorescein-conjugated sheep anti-rabbit immunoglobulin (Gibco) diluted 1:10 as described above. Samples were rinsed three times for 10 min each with PBS and dehydrated with methanol for 10 min at room temperature. Cells were mounted in 50% glycerol in PBS prior to microscopic examination. The fluorescent images were recorded using Kodak Tri-X film with an exposure time of 30 sec. The film was developed with undiluted Kodak D-19 for 15 min. Rootlet lengths were measured by projecting negatives onto a white screen and tracing the images.

Antiserum to rootlet protein and fluorescein-conjugated anti-rabbit immunoglobulin was absorbed with acetone dried powders of amoebae to reduce non-specific staining of the fluorescein-conjugated serum (Nairn, 1976). Acetone dried powders were prepared by adding 10 ml acetone to a pellet of amoebae. The cell suspension was left on ice for 10 min and then centrifuged for 5 min at 3,000 g in a table top clinical centrifuge. The supernatant was decanted and the acetone wash was repeated twice. The final cell pellet was dried under vacuum and stored at -20°C. Prior to use, the acetone dried powder was

rehydrated in PBS and mixed with serum at a concentration of 100 mg acetone dried powder per ml serum. The resulting suspension was left at 2°C for 16 h with occasional mixing. The absorbing material was removed by two 10 min centrifugations at 3,000 g in table top clinical centrifuge.

2.16 Isotope Dilution Experiment

The laboratory strain of the amoebo-flagellate Naegleria gruberi, NEG, was originally isolated by Schuster in California and cloned by Fulton (1970). Strain NEG-4c was adapted to axenic culture in a semi-defined growth medium (Fulton, 1974). Naegleria amoebae NEG-4c were used for the isotope dilution experiment for two reasons: 1) their growth in medium M7, a semi-defined growth medium, is dependent on methionine (Fulton, 1974) and 2) the incorporation of the radioactive amino acid, ^{35}S-methionine, is direct rather than via a bacterial intermediate. Amoebae NEG-4c were grown in medium M7 which consisted of 3 mM L-methionine, 30 mM dextrose, 7 mM sodium phosphate pH 6.8, 0.5% yeast extract (Difco), 10% dialyzed fetal calf serum (Gibco). M7 minus dialyzed fetal calf serum was stored sterilized at 2°C. Prior to use, dialyzed fetal calf serum was added. Fetal calf serum was dialyzed for 48 h at 2°C against DW, sterilized by filtration and stored at -20°C. Cultures were maintained in 20 ml medium M7 in 125 ml DeLong flasks at 32°C and shaken at 80 3.8 cm strokes per min in a reciprocating water bath shaker. New cultures were inoculated with 2-7 x 10^4 amoebae/ml, reaching stationary phase in 48 to 72 h at which time new cultures were initiated.

Amoebae for differentiation were taken at early stationary phase, at a density of 3-4 x 10^6 cells/ml. To initiate differentiation, amoebae were washed free of culture medium and resuspended in 2 mM tris-HCl pH 7.2 plus 20 mM KCl at room temperature. Time zero in this procedure was the point of introduction of the buffer to the first cell pellet. Washing of amoebae was continued as described for strain NB-1, section 2.6. For the isotope dilution experiments, differentiation proceeded in the presence or absence of 50 mM methionine. D,]09

Chapter 3

The Flagellar Rootlet: Isolation, Morphology and Composition

3.1 The Flagellar Apparatus

When amoebae of Naegleria are subjected to a decrease in the nutritional supply, they lose their characteristic amoeboid shape and form spheres, at which time two short flagella appear extruding from the cell surface. By 120 min after the initiation of differentiation, cells have developed the characteristic fusiform flagellate shape with two long flagella at the anterior of each cell. At 25°C the first appearance of flagella in populations of developing flagellates occurs between 60 and 65 min post-initiation (Fig. 2). By 70 min, at least 50% of the cell population is flagellated. Flagella appear on greater than 90% of the cell population within a 25 min interval.

Gentle lysis of mature flagellates with dilute (0.1%) Triton X-100 results in the release of an intact flagellar apparatus (Fig. 3a). The rootlet and basal bodies are tightly attached since violent mechanical agitation such as being vortexed at top speed for 2 min does not dissociate the basal body-rootlet complex. In addition, chemical treatment of isolated flagellar apparatuses with various detergents (Sarkosyl, digitonin), extremes in pH (2 or 10) or high salt (0.4 m KI) does not cause dissociation of the basal body-rootlet complex without solubilizing the rootlet. In contrast, flagella can be readily detached from their basal bodies by gentle vortexing or

Figure 2

Time of appearance of the flagellate phenotype in populations of amoebae differentiating at 25°C. T_{50} for the appearance of flagella in this experiment is 69 min. Each point represents the number of cells with flagella from a sample size of 100.

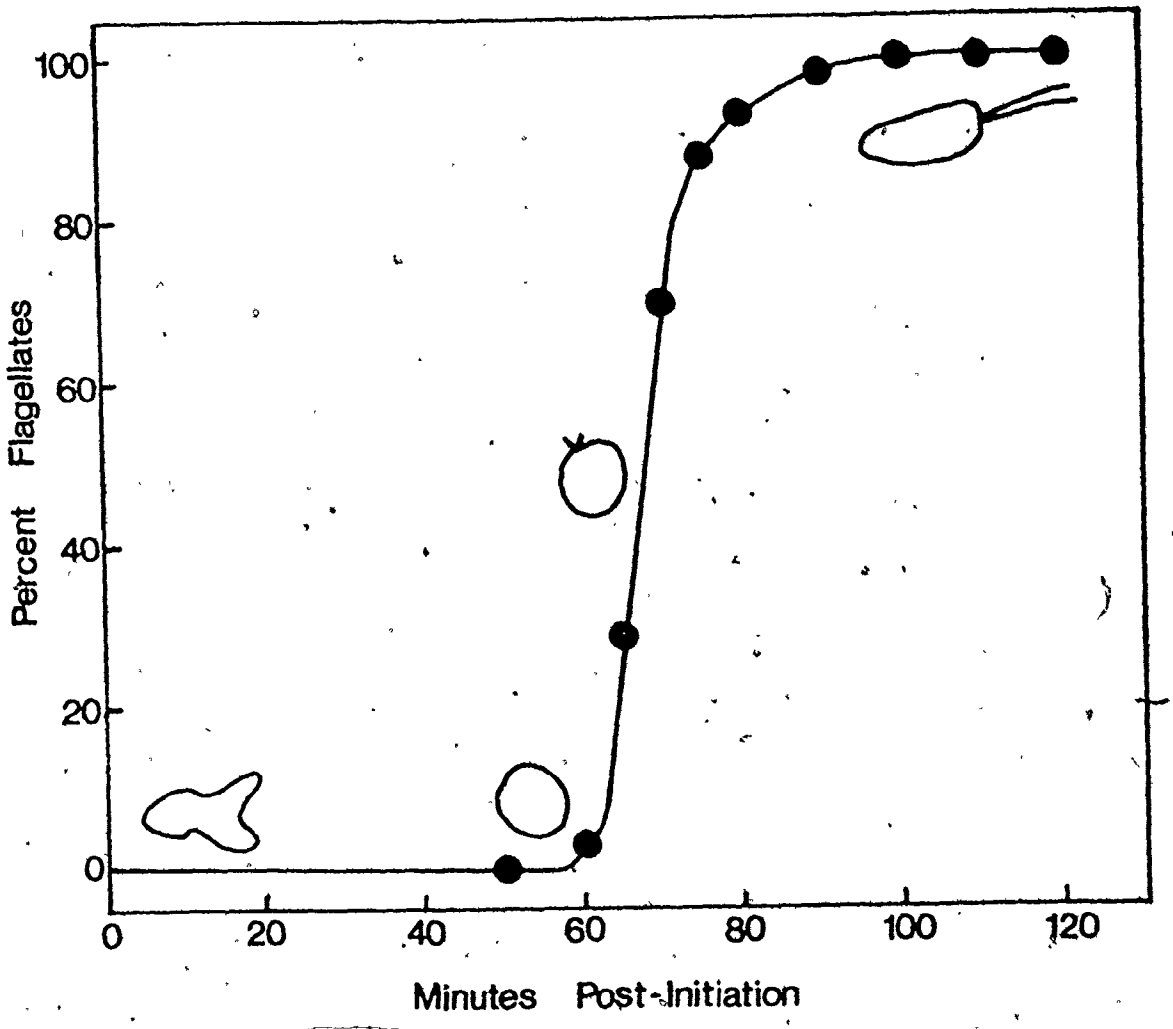

100
80
60
40
20
0
Percent Flagellates
0
20
40
60
80
100
120
Minutes Post-Initiation

Figure 3

Morphology of Isolated Rootlets

A - a complete flagellar apparatus consisting of two long flagella, approximately 16 μm in length, and a long, straight rootlet, 13 μm in length. Phase contrast, X 1,000.

B - a field of rootlets isolated by differential centrifugation. The rootlets are long and thin and the basal body complex at the proximal end of the rootlet appears as a small black dot. Phase contrast, X 500.

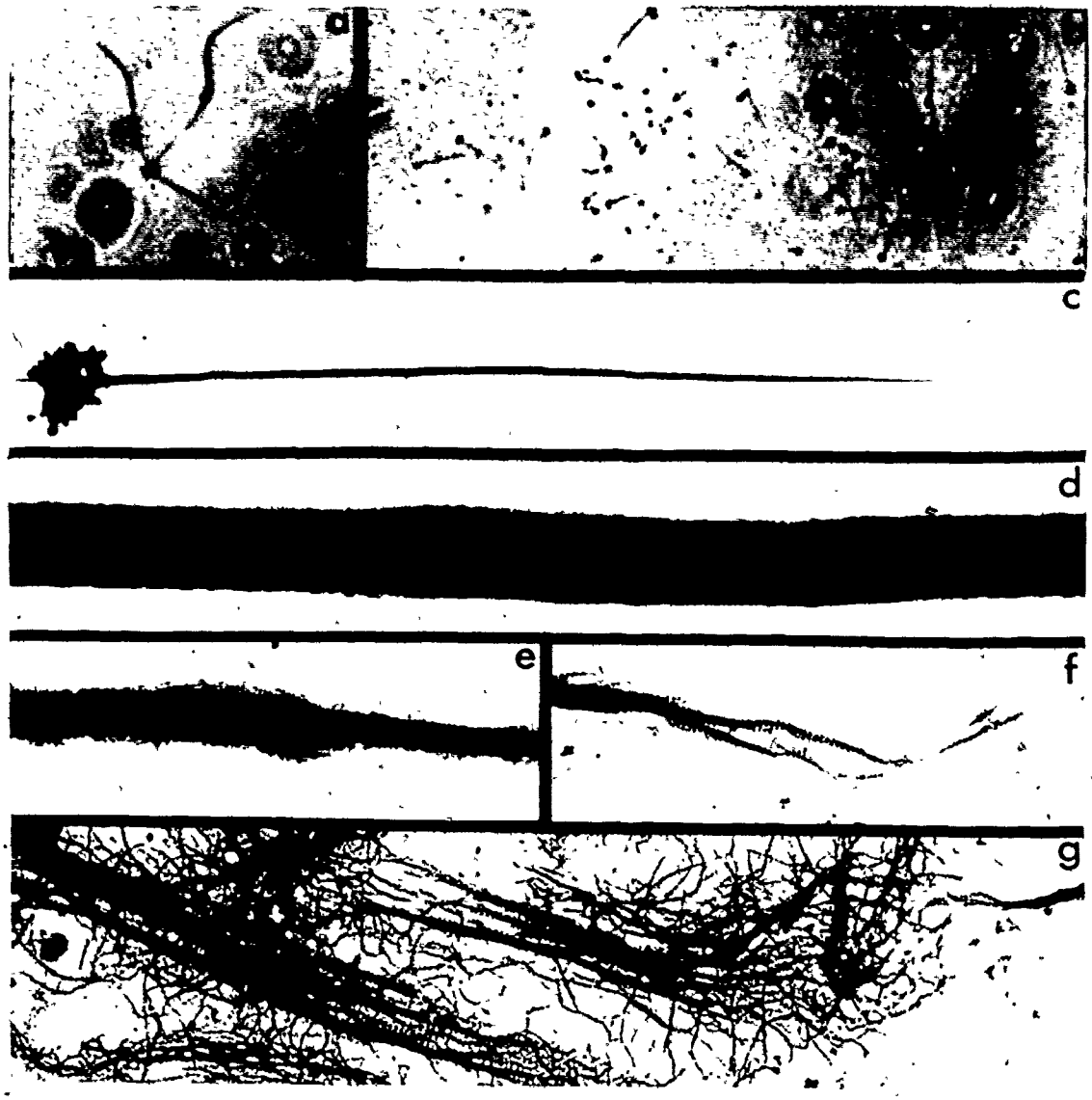
C - an isolated rootlet illustrating the tapering nature of the organelle and the basal body complex at the proximal end. Electron microscopy, X 10,000.

D - an isolated rootlet at a higher magnification illustrating the alternating electron opaque and electron lucent pattern of the organelle. Finer bands are also seen within each major band. Electron microscopy, X 137,000.

E - a partially disrupted rootlet illustrating the filamentous nature of the organelle and the disruption of the banding pattern. Electron microscopy, X 40,000.

F - distal rootlet tip illustrating the filamentous nature of the organelle and the presence of the banding pattern in fibres 15 to 20 nm in diameter. Electron microscopy, X 40,000.

G - urea-treated rootlets dissociated into thin filaments approximately 5 nm in diameter. Occasionally the periodicity is still present. Electron microscopy, X 40,000.


a
c
d
e
f
g

by shaking in the presence of a low pH buffer (sodium acetate pH 3.7).

3.2 Isolated Rootlets

In order to study the morphology and composition of rootlets a procedure has been developed for obtaining a purified rootlet fraction. Briefly, this procedure involves removal of flagella by shaking cells in the presence of a low pH buffer and then lysis of the cell bodies with Triton X-100 in the presence of magnesium ions to prevent disruption of nuclei. Glycerol is also present in the lysis buffer because it prevents the aggregation of particulate cytoplasmic constituents and aids in the separation of nuclei such that more than 98% can be removed from the cell lysate in one low speed centrifugation with a loss of less than 10% of rootlets. In the absence of glycerol the loss of rootlets increases to 20 - 30%. Rootlets are then removed by centrifugation from the low speed supernatant. A phase photomicrograph of rootlets isolated by this procedure is shown in Fig. 3b. In isolation mature rootlets have a mean length of 12.8 μm with a range of 8.3 to 15.4 μm.

An electron micrograph of an isolated rootlet stained with uranyl acetate illustrates that the rootlet tapers as it extends from the basal bodies (Fig. 3c). Close examination of the basal body complex isolated by this method does not reveal any associated microtubules because the potassium iodide used in the isolation procedure solubilizes flagellar and cytoplasmic (but not basal body) microtubules. At higher magnifications (Fig. 3d) the constant 20 nm

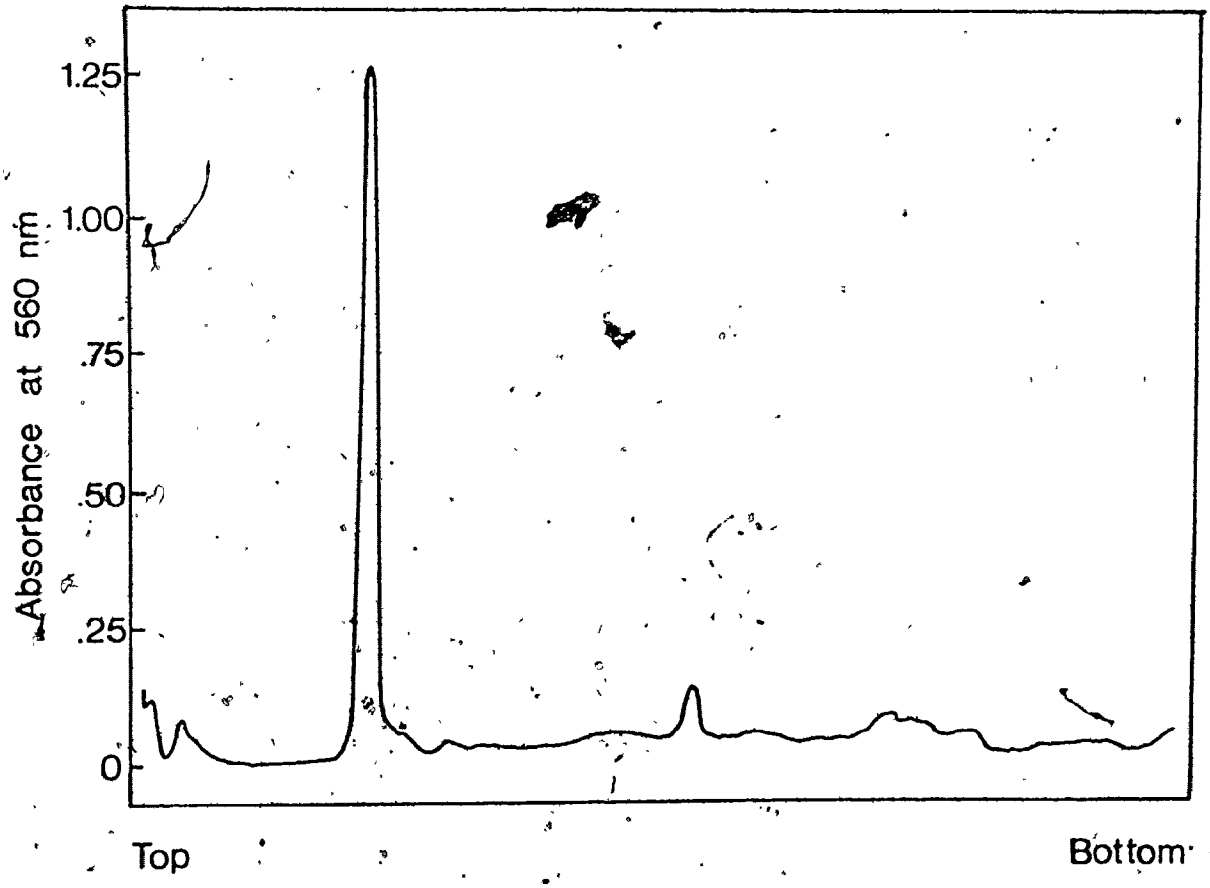
periodicity of isolated rootlets is apparent. A filamentous nature is suggested by the occasional finding of a partially disrupted rootlet (Fig. 3e). The banding pattern is not lost until the rootlet breaks down into individual filaments of approximately 5 nm diameter. In addition, at the tip of the rootlet where the diameter decreases to 15 - 20 nm (Fig. 3f), the banding pattern extends over the length of only a few filaments. The filamentous nature of the organelle is further suggested by observations of the effects of brief exposure of rootlets to 1 M urea: although the fundamental period is retained, rootlets are disrupted laterally into thin filaments which again are approximately 5 nm in diameter (Fig. 3g).

3.3 Composition of the Rootlet

The proteins of a typical rootlet preparation such as is shown in Fig. 3b have been identified by SDS polyacrylamide gel electrophoresis using the Weber-Osborn phosphate buffer system (1969). The microdensitometer gel scan of stained proteins (Fig. 4) demonstrates that the most abundant protein comprises 40 - 50% of the total protein by densitometry. During the development of the rootlet isolation procedure this predominant protein increased in relative proportions as the purity of the rootlet fractions also increased. This protein has been identified as the major rootlet protein by a series of rootlet solubilization experiments (see below and Table 1) and will be referred to as "the rootlet protein" throughout the remainder of this thesis.

Figure 4

Electrophoretic protein pattern of isolated rootlets. The proteins of a typical purified rootlet fraction were separated by SDS polyacrylamide gel electrophoresis using the Weber-Osborn (1969) phosphate buffer system. The gel was stained with Coomassie Brilliant Blue and scanned at 560 nm with a Gilford microdensitometer. The predominant band is the major rootlet protein. The small protein band migrating to a central position in the gel is tubulin.


1.25
1.00
.75
.50
.25
0
Absorbance at 560 nm
Top
Bottom

Other minor proteins are present in rootlet fractions (Fig. 4). Since alpha and beta tubulin have the same molecular weight but differ in charge, they have been identified in rootlet fractions by comparing their mobility in two different SDS polyacrylamide gel buffer systems using tubulin standard isolated from Naegleria flagellar microtubules. In addition to having the same R_f values as alpha and beta tubulin in the tris-glycine pH 8.3 buffer system (Stephens, 1975b) these two proteins migrate as one in the Weber-Osborn phosphate buffer system (protein migrating to the central position in the gel, Fig. 4), identical to the tubulin standard. The tubulin present in these fractions is probably a component of basal body microtubules because (1) no cytoplasmic microtubules were observed associated with isolated rootlets, either stained with uranyl acetate or in thin sectioned preparations, and (2) thin sections of isolated rootlets showed that basal body structure was retained throughout the isolation procedure.

The series of solubility studies summarized in Table 1 was undertaken for two reasons: (1) to confirm that the major protein species present in rootlet fractions is a rootlet component and (2) to determine the solubility characteristics of rootlets as a preliminary step for reconstitution studies of the striated fibre. It should be emphasized that the rootlet is an unusually stable organelle, e.g., a purified rootlet fraction is stable for several days on ice in 30 mM tris pH 8 and 3 mM $MgSO_4$. In addition, although Naegleria amoebae actively phagocytoze bacteria and therefore must contain high levels

Table 1

The solubility characteristics of isolated rootlets and the resulting fate of the major protein component of these fractions (designated the rootlet protein) following treatment with various chemicals.

Chemical Treatment[a]	Solubility of Rootlet	Solubility of Rootlet Protein[b]
Sarkosyl (0.1%)	rootlets disappear immediately	soluble
pH 2	rootlets disappear within 1 - 2 min	soluble
pH 10	most rootlets disappear by 30 min no rootlets present at 60 min	insoluble
KI (0.4 M)	rootlets disappear immediately	soluble
EDTA (50 mM)	many rootlets still present at 30 min no rootlets present at 60 min	insoluble.
Urea (2 M)	rootlets disappear within 5 min	soluble
(8 M)	rootlets disappear immediately	soluble

[a] Rootlet fractions were treated for one hour at room temperature with the various solubilizing solutions

[b] Solubility judged by loss of majority (>75%) of rootlet protein from pellet after centrifugation (16,300 g for 10 min) as determined by SDS polyacrylamide gel electrophoresis.

of proteases, rootlets appear intact after 24 h on ice even in an unfractionated cell homogenate. Rootlets can be solubilized, however, by a variety of treatments such as detergent, extremes in pH, high salt or urea (Table 1). When rootlets are exposed to 0.1% Sarkosyl, or to pH 2, to 0.4 M KI or to 2 M urea, they disappear immediately as evidenced by phase microscopy. Close examination of the solubilized organelles by electron microscopy demonstrated that after such chemical treatment for 60 min the rootlets merely dissociated into a fibrous mat. Following exposure to either low pH, KI or urea for 60 min the treated rootlet fractions were separated into "soluble" and "insoluble" fractions (as defined in Materials and Methods 2.9). Following any of these chemical treatments the rootlet protein is rendered soluble. The low percentage (10 - 20%) of rootlet protein which remains insoluble after treatment with low pH, KI or urea is due to the aggregation of thin filaments forming a mat or to adherence of the filaments to the insoluble basal body complexes during the low speed centrifugation. Higher concentrations of urea (8 M) solubilizes the rootlet protein completely.

In contrast to the immediate solubilization of rootlets exposed to Sarkosyl, low pH, KI or urea, rootlets treated with EDTA or pH 10 disappeared slowly over a period of one hour as evidenced by phase microscopy, but the rootlet protein remained "insoluble". This apparent discrepancy has been resolved by electron microscopic examination of rootlets treated with EDTA or high pH for 60 min. The rootlet is only partially intact: the characteristic cross-banded structure is present over

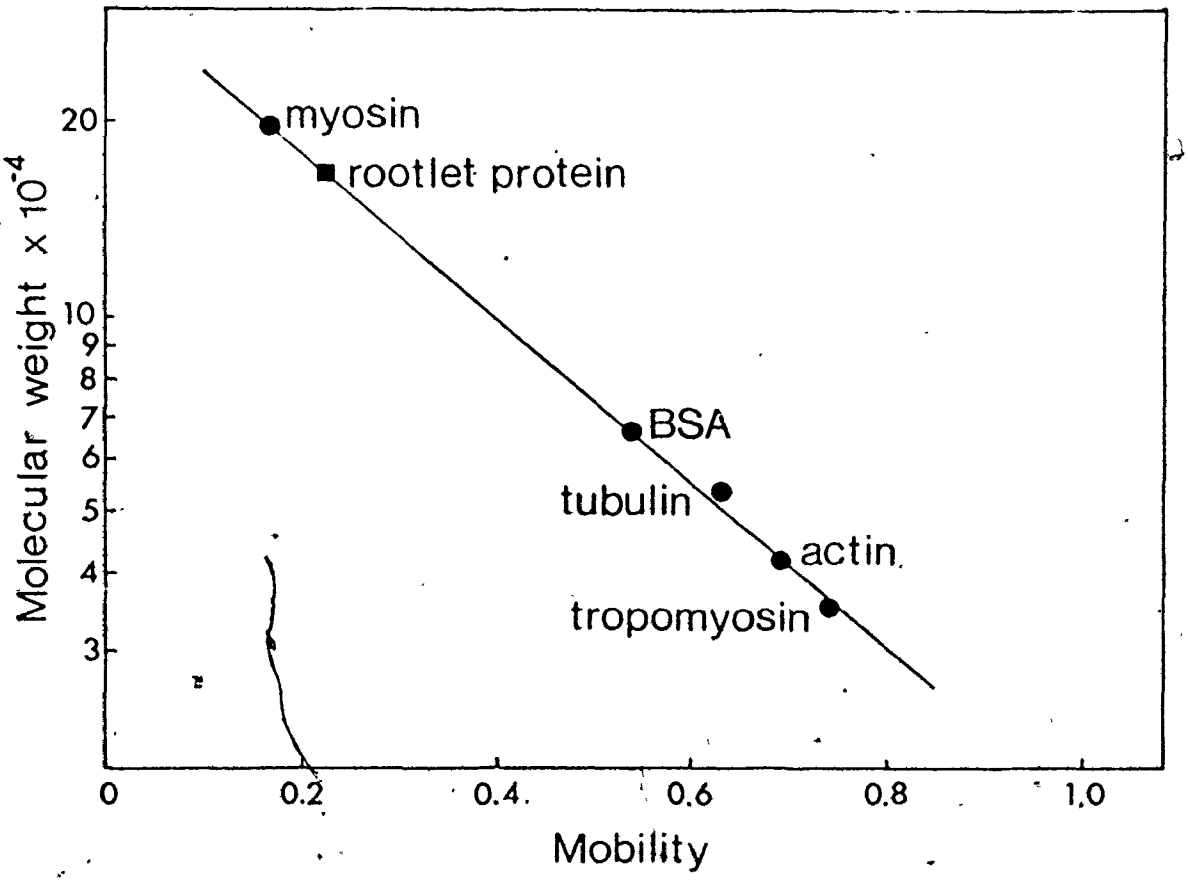
almost the entire length of the extremely thin filamentous rootlet.

Electrophoretic methods were used to determine the subunit molecular weight of the rootlet protein and to indicate whether or not it contains a carbohydrate moiety. The subunit molecular weight of the rootlet protein was determined by SDS polyacrylamide gel electrophoresis using the Weber-Osborn phosphate buffer system (1969) in which the distance a protein migrates is proportional to the log of its molecular weight. To establish a calibration curve relating molecular weight to migration distance, five protein standards with molecular weights ranging from 42,000 to 200,000 daltons were used (Fig. 5). On the basis of this relationship, the subunit molecular weight of the rootlet protein was 170,000 daltons. In three determinations the subunit molecular weight differed by less than 5,000 daltons, i.e., 165,000, 170,000 and 170,000 daltons.

After separation of proteins of a typical rootlet fraction by SDS polyacrylamide gel electrophoresis, gels were stained for glycoproteins with periodic acid-Schiff's reagent and counterstained with Coomassie Brilliant Blue, a protein stain. The rootlet protein only stained with Coomassie Brilliant Blue and therefore, based on this method, is not a glycoprotein. However, four of the minor components of rootlet fractions stained positive for glycoproteins. Whether these are minor constituents of the rootlet or contaminants is presently unknown.

Figure 5

Molecular weight determination of the rootlet protein. Rootlet protein and five standards were separated on the Weber-Osborn (1969) SDS polyacrylamide phosphate gel system. The position of the protein bands was determined by staining with Coomassie Brilliant Blue.


myosin
rootlet protein
BSA
tubulin
actin
tropomyosin
Molecular weight x 10^{-4}
20
10
9
8
7
6
5
4
3
0
0.2
0.4
0.6
0.8
1.0
Mobility

Chapter 4

Synthesis of the Rootlet Protein During Flagellate Differentiation

Since, during the differentiation, rootlets as well as flagella and basal bodies are assembled de novo, and the tubulin assembled into flagellar microtubules has been shown to be synthesized de novo (Kowit and Fulton, 1974b), it is of interest to question whether the rootlet protein is also synthesized de novo. This question was investigated by means of an isotope dilution experiment as follows. Naegleria amoebae, strain NEG-4c rather than NB-1, were used for this experiment because their growth in a semi-defined growth medium is dependent on methionine (Fulton, 1974) and the incorporation of ^{35}S-methionine is direct rather than via a bacterial intermediate. Amoebae were prelabelled by growing them in the presence of ^{35}S-methionine for at least five generations to uniformly label their protein and amino acid pools. Amoebae were then washed free of culture medium and differentiated in (1) non-nutrient buffer (control) and (2) buffer containing unlabelled methionine at the highest concentration (50 mM) that did not interfere with differentiation (chased).

A comparison of differentiation in the presence and absence of methionine demonstrated that there is no parameter of differentiation which differs significantly between the two flagellated populations (Fig. 6 and Table 2). Not only do flagellates first appear at approximately 70 min post-initiation in control and chased differentiations but the shape of the curves for the appearance of flagellates

Figure 6

The appearance of the flagellate phenotype in populations of Naegleria amoebae prelabelled with ^{35}S-methionine and differentiated in the presence and absence of 50 mM methionine. Each point represents the number of cells with flagella from a sample size of 100. ●——● , no methionine; ■——■ , methionine.

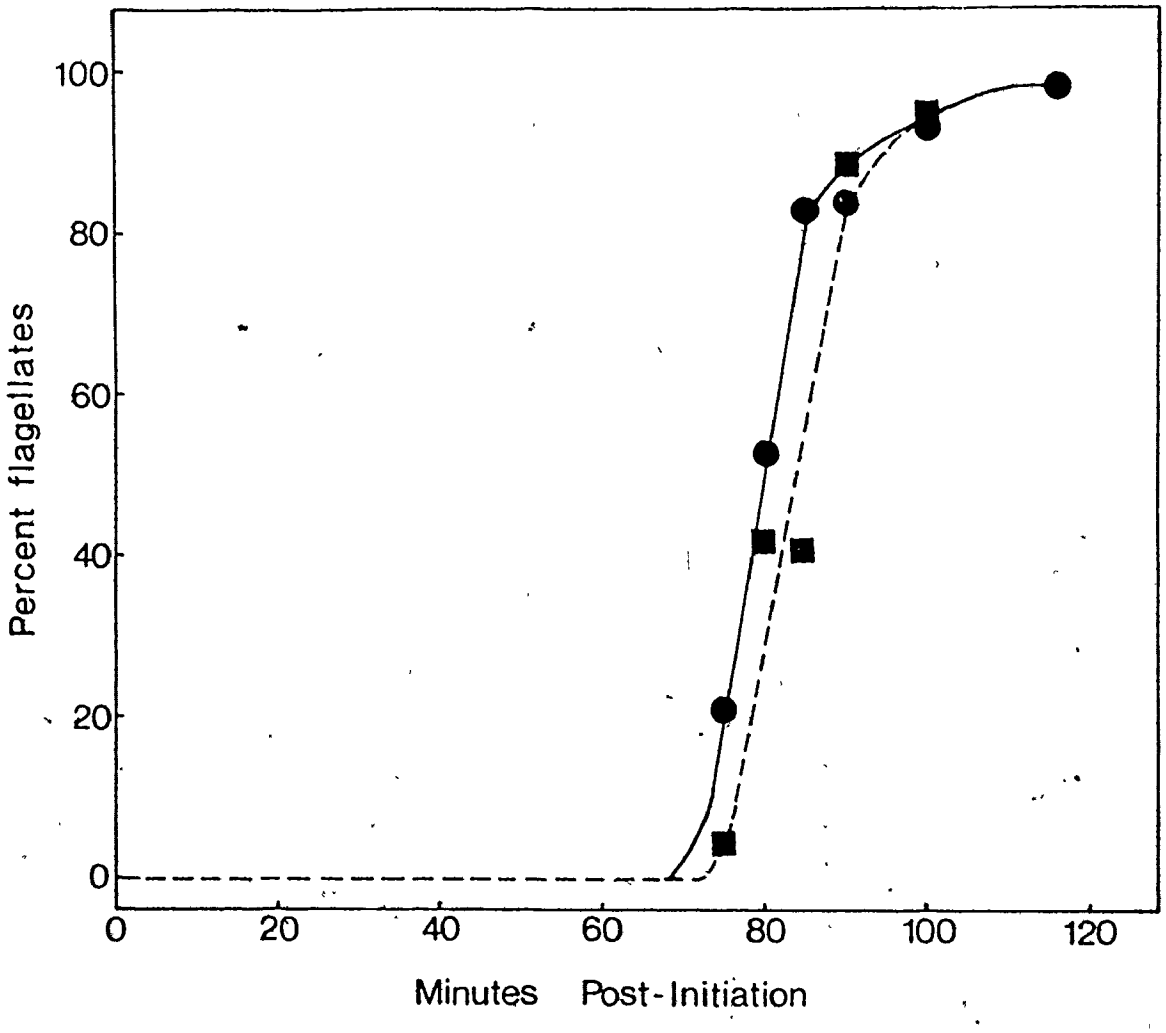

Percent flagellates
100
80
60
40
20
0
0
20
40
60
80
100
120
Minutes Post-Initiation

Table 2

A comparison of parameters of flagellate differentiation in the presence and absence of methionine. T_{50} represents the time required for 50% of the population to become flagellated.

Parameter	no methionine	50 mM methionine
T_{50}	80	85
Percent Flagellates[a]	98	98
Mean Flagellum Length[a]	13.67 μm	13.62 μm
Mean Number of Flagella Per Flagellate[a]	3.53	3.61

[a]measurements were made at 110 min after the initiation of differentiation.

is the same (Fig. 6). T_{50} for the appearance of flagella in the presence and absence of methionine is 85 and 80 min, respectively. The mean flagellum length (13.62 vs 13.67 µm; sample sizes of 50 flagella) and the mean number of flagella per cell (3.61 vs 3.53; sample sizes of 100 flagellates) were the same in both situations. Although the T_{50}s differ by 5 min, considering the other parameters of the flagellate populations (percent, number, length), the differentiations in the presence and absence of methionine were the same.

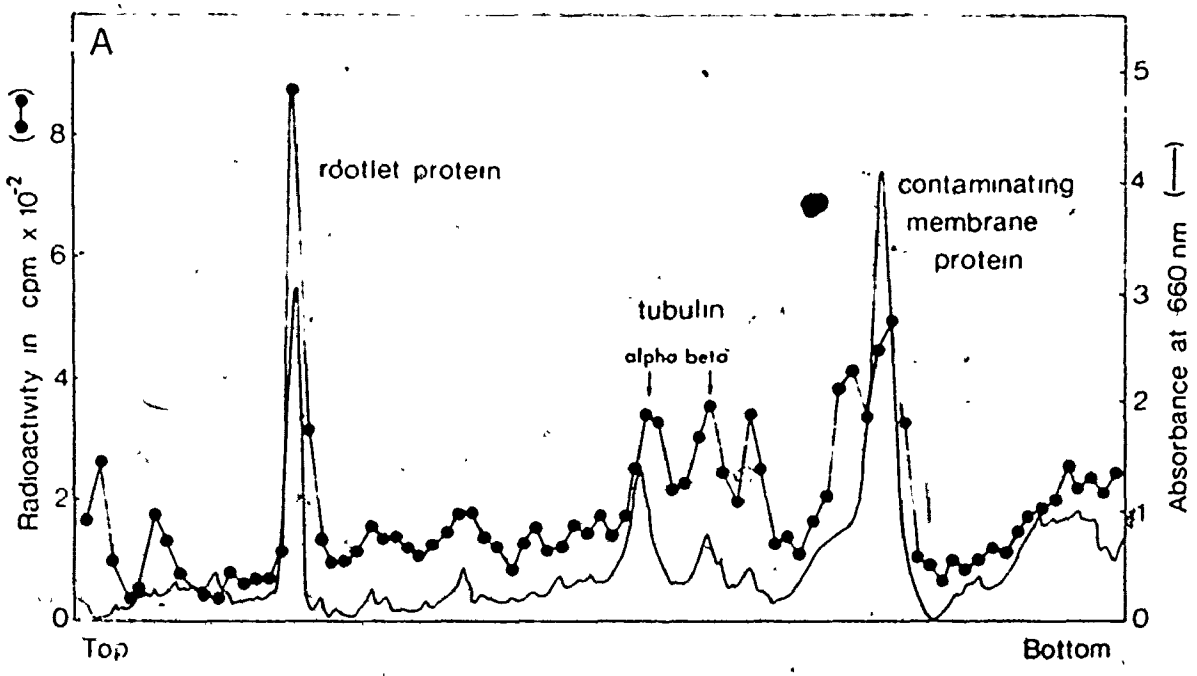
A comparison of the specific activity of the rootlet protein following differentiation of prelabelled amoebae in the presence and absence of methionine yields a quantitative estimate of the fraction of assembled protein which is synthesized de novo. Since, in the absence of methionine, differentiation occurs in non-nutrient buffer, the only source of methionine for protein synthesis is the endogenous radioactive amino acid. If differentiation occurs in the presence of exogenous, unlabelled methionine and the cells incorporate this methionine into proteins, then the amount of radioactive methionine incorporated into newly synthesized protein will decrease relative to the control situation, where all available methionine is radioactive. This method yields a precise measurement of the extent of de novo synthesis - provided the chase is 100% effective, i.e., if only unlabelled methionine is incorporated into proteins synthesized during the differentiation period. Otherwise a minimal estimate of the fraction of rootlet protein synthesized de novo is obtained.

Rootlets were isolated from flagellates at 115 min post-initiation (at which time cells were 98% flagellated). The proteins of the rootlet fractions were separated by SDS polyacrylamide gel electrophoresis. The gels were stained quantitatively, scanned for absorbance and the distribution of radioactivity measured. As shown in Fig. 7, although the two protein patterns are the same, indicating that identical levels of protein were layered onto the gels, there are significant differences in the distribution of radioactivity. In the presence of methionine the radioactivity associated with the rootlet protein had decreased by 61% compared to the control. A similar pattern (54% decrease) is observed for tubulin, though there may be other proteins that band at this position of the gel. Since the level of radioactivity associated with the protein bands identified as tubulin was low, only one measurement of the decrease in radioactivity was determined. The other major peak of absorbance represents contaminating membrane proteins (Fig. 7). The level of radioactivity associated with this peak in the presence and absence of methionine is the same, suggesting that only a few specific proteins, and particularly the rootlet protein, present in the rootlet fractions are synthesized de novo during the two hour differentiation period.

The specific activity of the rootlet protein (control and chased) was calculated for three different gel loadings (Fig. 8 and Table 3). Both the peak area corresponding to the rootlet protein and the amounts of radioactivity per peak were proportional to the volume layered onto the gels (Fig. 8). The average specific

Figure 7

Protein and radioactivity distributions of rootlet fractions isolated from flagellates following differentiation in the presence and absence of methionine. Following separation of proteins by SDS polyacrylamide gel electrophoresis, gels were stained quantitatively with fast green FCF, scanned for absorbance at 660 nm and cut into 1 mm slices to determine the distribution of radioactivity. A - no methionine present during the differentiation; B - 50 mM methionine present during the differentiation; (———), absorbance; ●——●, radioactivity, no methionine; ■——■, radioactivity, 50 mM methionine.


A
rootlet protein
tubulin
alpha beta
contaminating membrane protein
Radioactivity in cpm x 10^{-2} (●)
Absorbance at 660 nm (—)
Top
Bottom

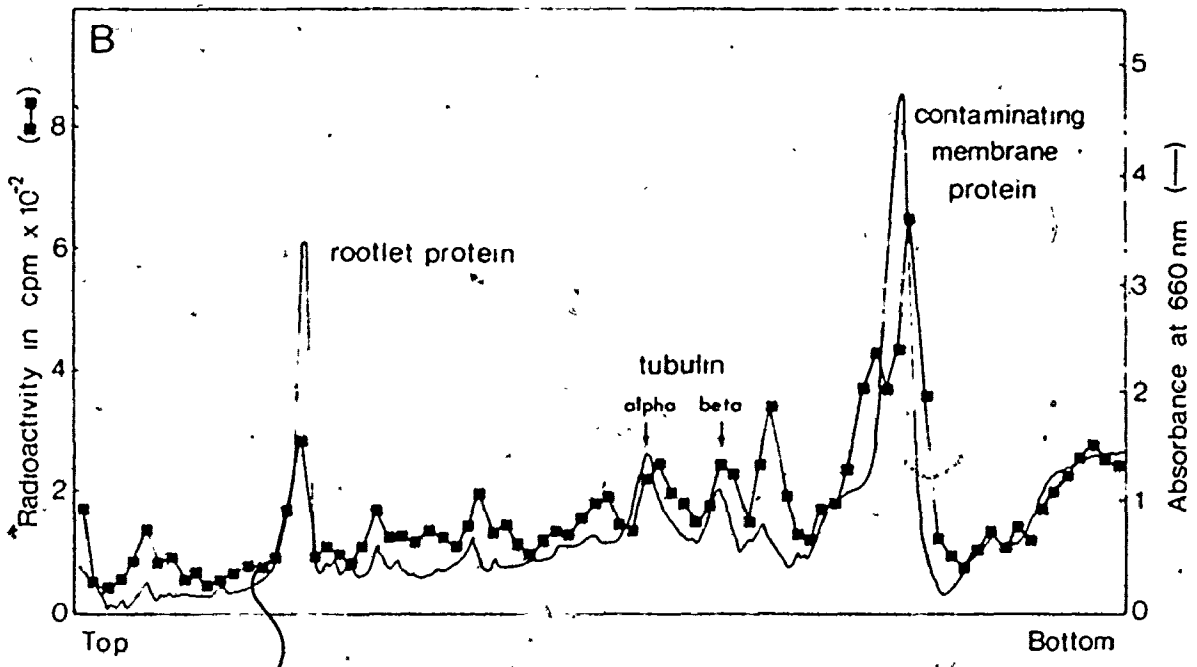

B
contaminating membrane protein
rootlet protein
tubulin
alpha beta
Radioactivity in cpm x 10^{-2} (■)
Absorbance at 660 nm (—)
Top
Bottom

Figure 8

Quantitation of peak area and radioactivity associated with the rootlet protein. Proteins of rootlet fractions isolated from flagellates differentiated in the presence or absence of methionine were separated by SDS polyacrylamide gel electrophoresis and stained quantitatively with fast green FCF. Gels were scanned at 660 nm with a Gilford microdensitometer and the area associated with the rootlet protein peak measured with a planimeter. Radioactivity associated with this peak was also measured. This procedure was performed for three different gel loadings (10, 20 and 40 µl). ■——■ , radioactivity, no methionine; □——□ , radioactivity, methionine; ●---● , area, no methionine, ○---○ , area, methionine.

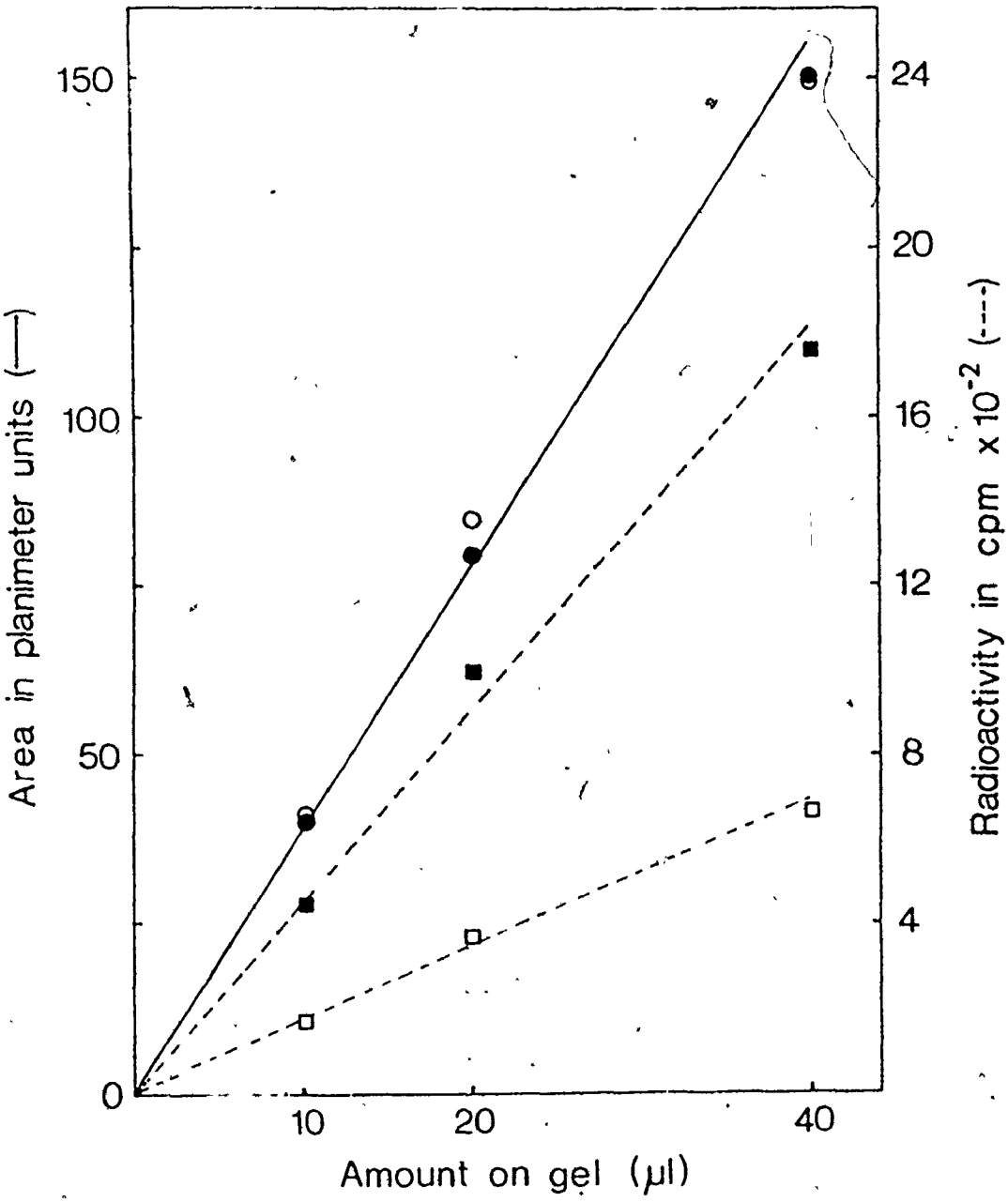

150
100
50
0
24
20
16
12
8
4
10
20
40
Area in planimeter units (—)
Radioactivity in cpm x 10^{-2} (----)
Amount on gel (µl)

Table 3

The specific activity of the rootlet protein following synthesis in the presence and absence of exogenous cellular methionine.

Volume of Samples Layered on gel (µl)	Specific Activity[a]	
	no methionine	50 mM methionine
10	11.7	4.5
20	11.6	4.5
40	11.3	4.1
average	11.5	4.3
percent	100	38

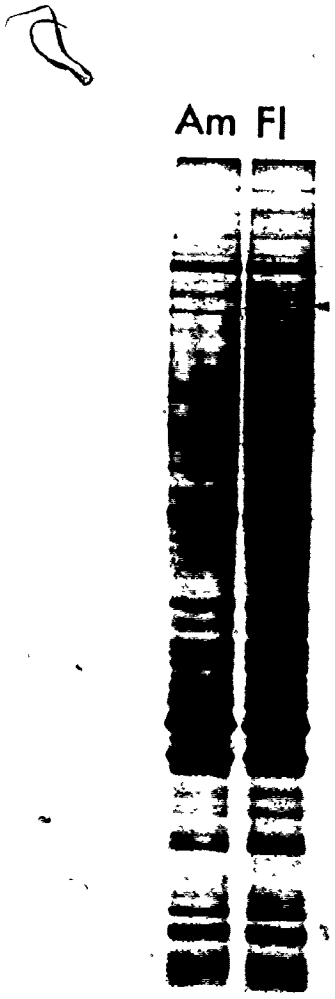
[a] The specific activities were calculated as radioactivity/area; cpm/planimeter units.

activities for control and chased rootlet protein were 11.5 and 4.3 cpm/planimeter units, respectively (Table 3). On a relative basis, 38% of the radioactivity associated with the rootlet protein remained. Thus at least 62% of the rootlet protein was synthesized de novo during the amoeba to flagellate differentiation.

Since the results from the isotope dilution experiment described above suggest that only 62% of the rootlet protein is synthesized de novo during the differentiation, either the methionine chase is not 100% complete or some rootlet protein pre-exists in amoebae. To answer this latter question, total cellular proteins were separated by SDS polyacrylamide gel electrophoresis. As shown in Fig. 9, the rootlet protein is present in flagellates but not amoebae. By this method, the rootlet protein was first detected in developing flagellates 50 min after the initiation of differentiation. Therefore, most likely a larger percentage of the rootlet protein is synthesized de novo and the methionine chase in the isotope dilution experiment is not complete. The protein distribution in amoebae and flagellates is very similar (Fig. 9) suggesting that there is not a major shift in the pattern of protein synthesized during the amoeba to flagellate differentiation.

Figure 9

Electrophoretic patterns of total cellular proteins of amoebae and flagellates. Total cellular proteins of amoebae and flagellates (120 min post-initiation) were separated by SDS polyacrylamide gel electrophoresis using the discontinuous buffer system described by Laemmli (1970) and stained quantitatively with Coomassie Brilliant Blue. The rootlet protein band (arrow) was identified by co-migration with the rootlet protein of isolated rootlets.


Am Fl

Chapter 5

Development of the Flagellar Rootlet

5.1 Preparation of a Rootlet Protein Antibody

The sequence of rootlet development was elucidated by indirect immunofluorescence. This procedure involves localization of the organelle *in situ* using an antibody which specifically recognizes a structural protein. Then, using a fluorescent tag, the position of the antibody can be detected by light microscopy.

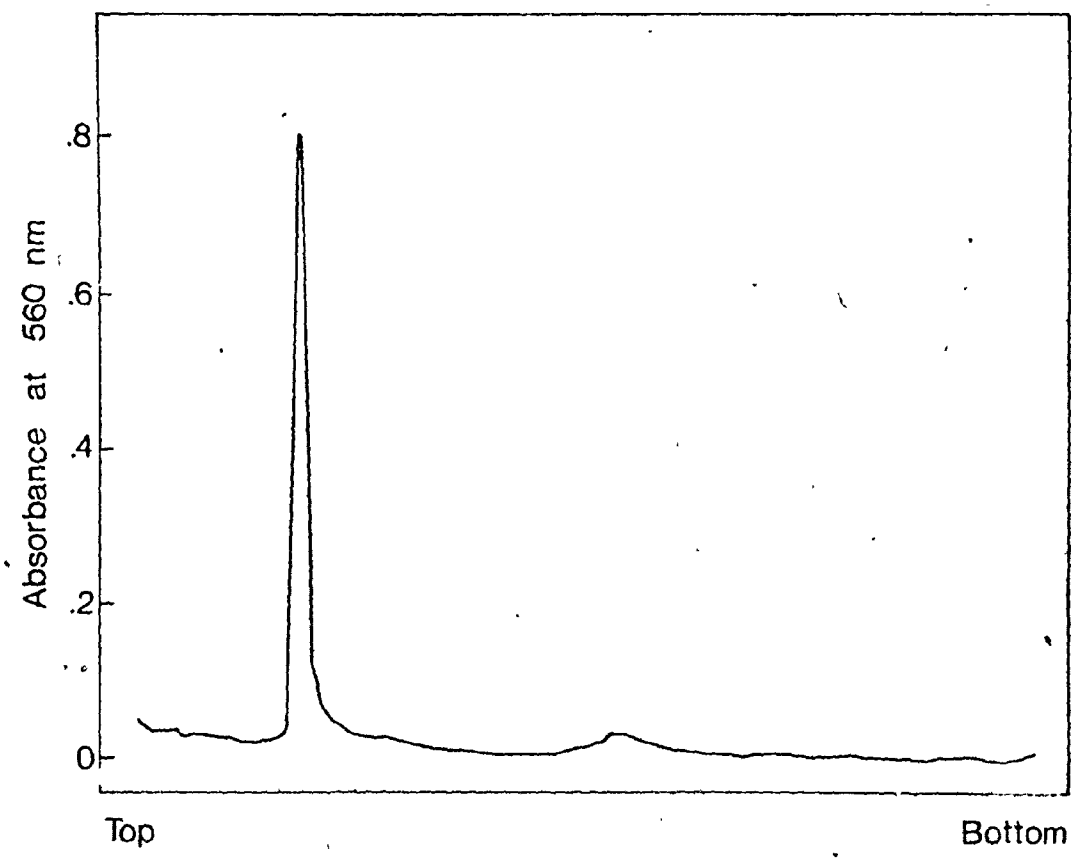
The first step required purification of the rootlet protein. As described in Materials and Methods, the major steps involved were large scale isolation of rootlets, separation of the proteins of a rootlet fraction by preparative polyacrylamide gel electrophoresis, localization of the rootlet protein by staining a gel slice and elution of the rootlet protein from the corresponding unstained gel section. A microdensitometric gel scan of the purified rootlet protein used for injection is shown in Fig. 10.

An antibody to the rootlet protein was obtained by injecting rabbits with the purified protein. The production of antibody was assayed by the Ouchterlony (1973) double diffusion test. In this assay the antigen and antibody are placed in separate wells in an agar matrix. The proteins diffuse through the matrix and for each antibody-antigen system present, a precipitin line is formed.

of an antibody to the rootlet protein was tested against all the

Figure 10

Electrophoretic profile of purified rootlet protein. The purified rootlet protein was separated by SDS polyacrylamide gel electrophoresis using the Weber-Osborn phosphate buffer system (1969). The gel was stained with Coomassie Brilliant Blue and scanned at 560 nm with a Gilford microdensitometer. One protein species is present on the gel.


Absorbance at 560 nm
.8
.6
.4
.2
0
Top
Bottom

proteins of a typical rootlet fraction (Fig. 4), one precipitin line was detected (Fig. 11a). No reaction occurred with preimmune serum. Since the purification of the rootlet protein involved SDS polyacrylamide gel electrophoresis, protein-bound SDS was also injected into the rabbit and the antibody produced may have been directed against the SDS moiety. However, when the serum from the immunized rabbit was tested against other proteins containing bound SDS (muscle proteins), no precipitin line was detected (Fig. 11a).

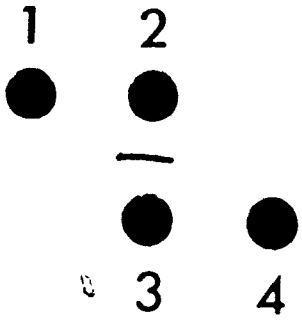
The specificity of the rootlet protein antibody was demonstrated by localization of the rootlet protein by antibody binding in polyacrylamide gels. In this method the proteins of a typical rootlet fraction were separated by SDS polyacrylamide gel electrophoresis, the SDS eluted and the gel incubated with immunized serum. The bound antibody is detected by exposing the gel to goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase. The enzyme is then localized by treatment of the gel with diaminobenzadine which can serve as a substrate. As shown in Fig. 11b, only one band comigrating with the rootlet protein is detected using this method, even though there are many proteins present on the gel. No precipitate is formed if preimmune, rather than immunized, serum or unconjugated sheep anti-rabbit immunoglobulin is used.

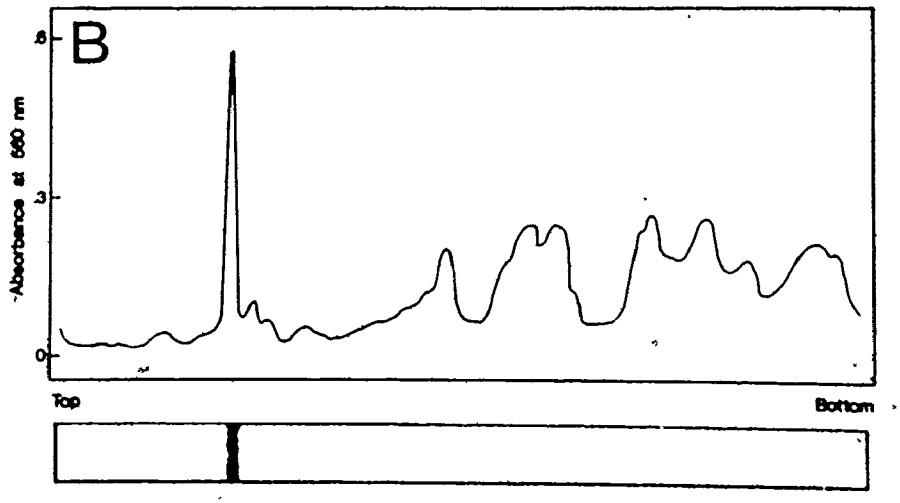
Figure 11

A. Diagrammatic representation of an Ouchterlony immunodiffusion test of antiserum to rootlet protein. The antiserum to rootlet protein (well 2) was assayed against all the SDS-solubilized proteins of a typical rootlet preparation (well 3) or SDS-solubilized muscle proteins (well 1). Well 4 contained preimmune control serum.

B. Specificity of the antiserum to rootlet protein. Proteins of a typical rootlet fraction were separated by SDS polyacrylamide gel electrophoresis. Following fixation and removal of SDS, the gel was incubated with antiserum to rootlet protein and then washed extensively with PBS. The gel was incubated with rabbit anti-goat immunoglobulin conjugated to horseradish peroxidase and finally reacted with diaminobenzidine. The diagram demonstrates that one protein was located with the enzyme reaction. The microdensitometric gel scan is of an identical gel stained with Coomassie Brilliant Blue.

A





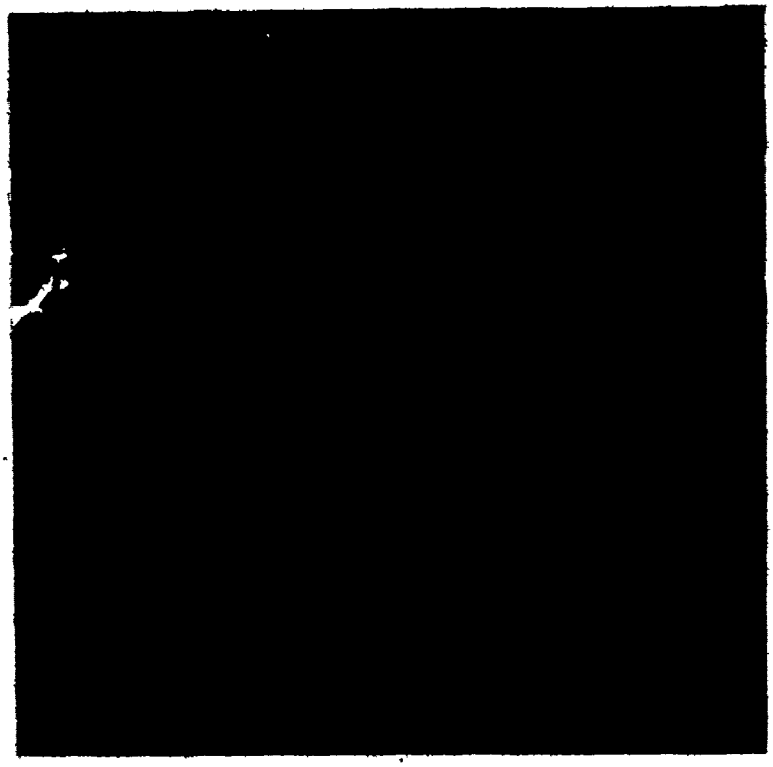
5.2 The Sequence of Rootlet Appearance

In order to localize developing rootlets during flagellate differentiation it is necessary to demonstrate that the rootlet protein antibody was specifically recognizing the rootlet. Thus, rootlets were isolated by differential centrifugation, fixed in buffered paraformaldehyde and stained by indirect immunofluorescence. As shown in Fig. 12, rootlets stained by indirect immunofluorescence appear as long, thin tapering structures similar to those observed with phase optics (cf Fig. 3b). At the proximal end of the rootlet there is an aggregation of rootlet protein. In the fully developed rootlet this area is more fluorescent than the rest of the organelle. The specific fluorescent staining of the rootlet is not detected when cells are incubated either with preimmune rather than immunized serum or with unconjugated sheep anti-rabbit immunoglobulin prior to treatment with the fluorescein-conjugated serum. The former control demonstrates that the antibody directed against the rootlet protein is binding to the organelle whereas, in the latter, the fluorescein-conjugated sheep antiserum is shown to specifically recognize the rootlet protein antibody.

At 5 min intervals throughout the flagellate differentiation period, cell samples were fixed in buffered paraformaldehyde. Developing rootlets were observed by fluorescent microscopy following localization by indirect immunofluorescence. The rootlet is first detected in cell samples 65 - 70 min after the initiation of

Figure 12

Isolated rootlets stained by indirect immunofluorescence. Rootlets were isolated by differential centrifugation and fixed in buffered paraformaldehyde. Following staining by indirect immunofluorescence, rootlets were observed by fluorescent microscopy. X1,000.



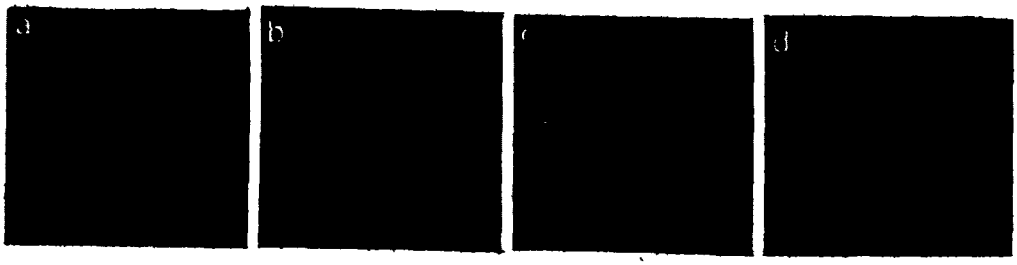
differentiation as a highly fluorescent dot, generally near the periphery of the cell (Fig. 13a). Even at the earliest stage the fluorescent dot generally contains a small, less fluorescent, finger-like projection, the total structure being 1 to 2 μm in length. The individual cells can be distinguished by the faint fluorescent background. The rootlet develops by rapid elongation of this projection towards the nucleus such that by 75 min post-initiation at least 50% of the cells contain a small, immature rootlet ranging in length from 2 - 10 μm (Fig. 13 b and c). By 90 min more than 90% of the cells contain rootlets. These reach an average maximum length of 13 μm by 110 - 120 min (Fig. 13d and 14). At these later stages the rootlets, as they extend through the cytoplasm, are usually in close association with the nucleus. Rootlets have never been found in amoebae using this specific staining method.

5.3 A Comparison of Rootlet and Flagellum Development

Since the complete flagellar apparatus consisting of two flagella, two basal bodies and a flagellar rootlet is assembled de novo during the amoeba to flagellate differentiation, it was of interest to compare the time of appearance and rate of assembly of rootlets relative to flagella. The appearance of rootlets in developing flagellates was monitored by fluorescent microscopy using indirect immunofluorescence to detect the organelles and flagellum appearance was monitored by phase microscopy of Lugol's iodine-fixed cells. During the initial appearance of the rootlet (65 - 70 min post-initiation) the earliest developing rootlet scored appeared as a

Figure 13

Sequence of flagellar rootlet development. Cell samples, fixed at 5 min intervals during flagellate differentiation, were observed by fluorescent microscopy following localization of the developing rootlets by indirect immunofluorescence. Fig. 13a, earliest stage of rootlet development observed, 65 min post-initiation. Fig. 13b and c, developing rootlets, 75 min post-initiation. Fig. 13d, fully developed rootlet, 110 min post-initiation. The nucleus is located adjacent to the rootlet and the cell periphery is a faint white, definitely seen in Fig. 13a. X 1,400.

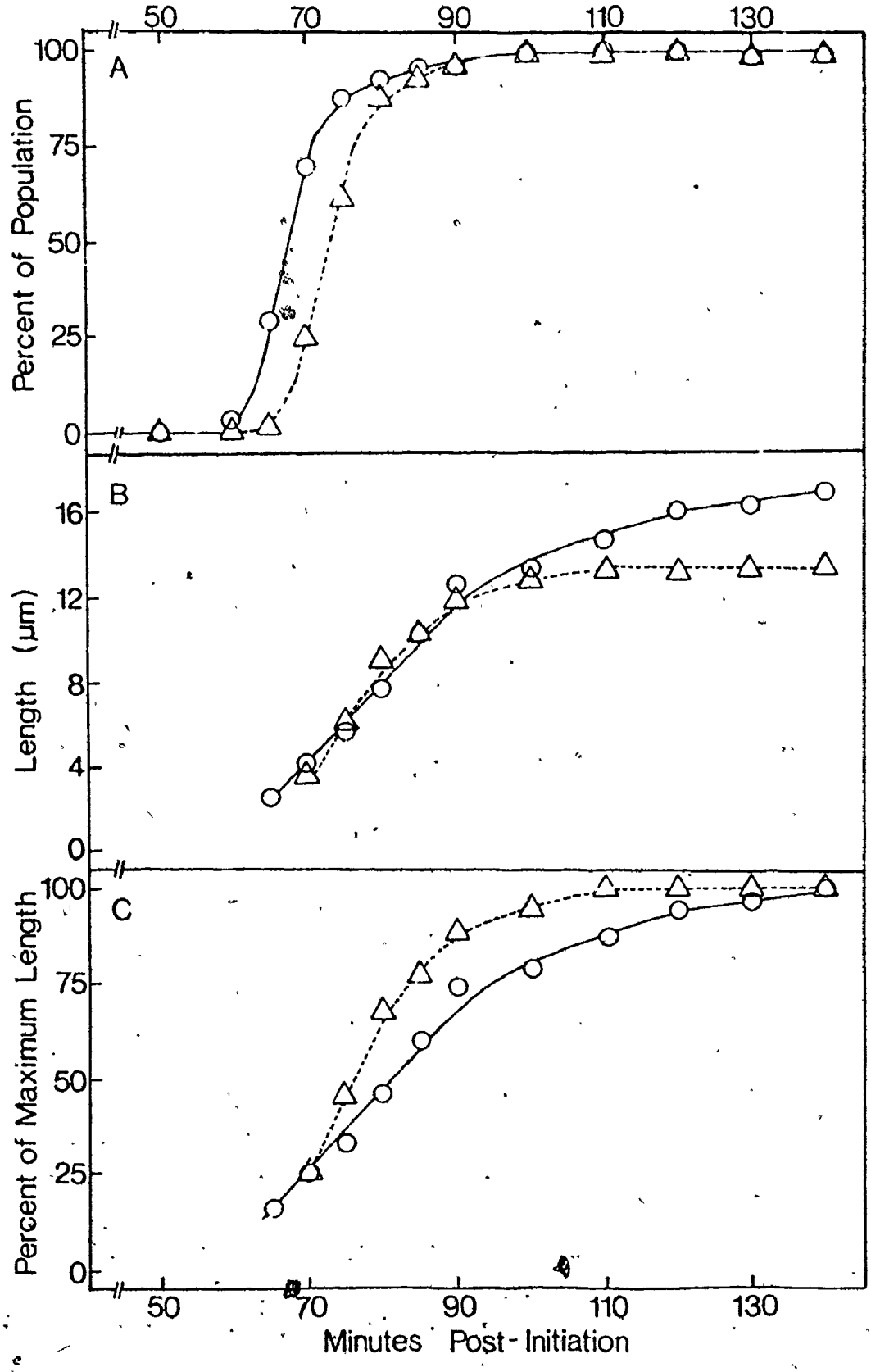

a
b
c
d

highly fluorescent dot with a small projection (Fig. 13 a and b). The assay systems for rootlets and flagella detect both organelles at a minimum length of 1.5 to 2 μm. Although flagella first appeared 60 min after initiation, rootlets were not detected until 65 min (Fig. 14a). Not only do both organelles appear in more than 90% of the cells within a 25 min time interval, but the shape of the curves for the appearance of flagella and rootlets is the same, the latter being shifted to the right by 5 - 6 min. In this experiment, T_{50} for the appearance of flagella is 67.5 min whereas for rootlets, it is 73.5 min. A consistent difference of 5 - 6 min between the T_{50}s was observed in four experiments.

The mean lengths of flagella and rootlets were measured at 5 min intervals, beginning at 65 or 70 min, respectively, to 160 min after the initiation of differentiation. As shown in Fig. 14b, flagella assemble at a slower rate than do rootlets. As the mean maximum length of 17.0 μm is approached, this rate further decreases. In contrast to the 80 min required for complete growth of flagella, rootlets reach their mean mature length of 13.2 μm within 40 min after first being detected. Since the mean maximum lengths of the two organelles are different, the data have been presented in terms of percent total length versus time after initiation (Fig. 14c). When expressed in this fashion, the data show that rootlets assemble at a faster rate than do flagella but the rate of assembly decreases as the full length is approached, similar to the kinetics of flagellum assembly.

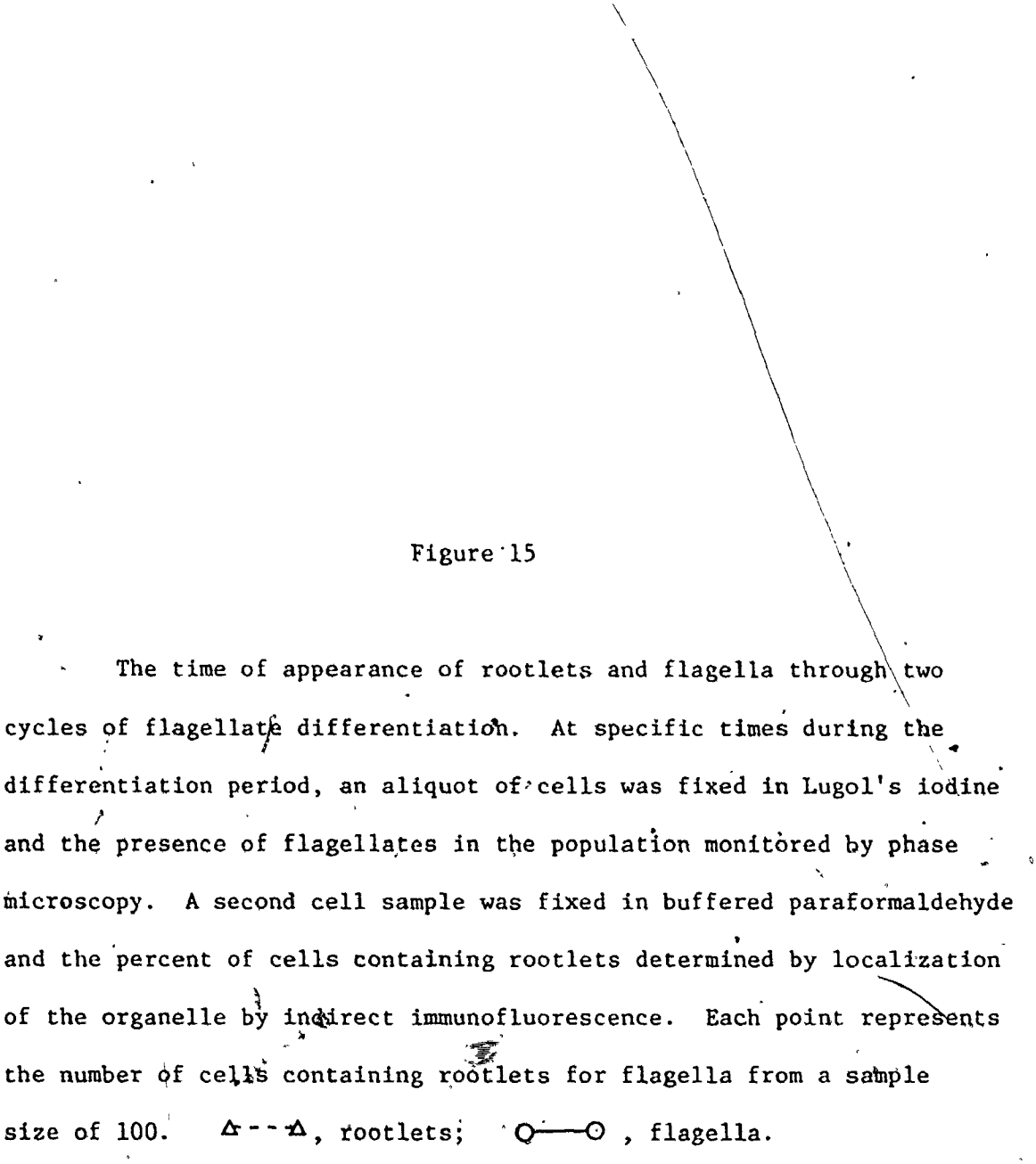
Figure 14

A comparison of the time of appearance of flagella and rootlets during flagellate differentiation. At specific time intervals during the differentiation period, an aliquot of cells was fixed in Lugol's iodine and the presence of flagellates in the population monitored by phase microscopy. Flagellum lengths were measured with a filar micrometer. A second cell sample was fixed in buffered paraformaldehyde and the percent of cells containing rootlets determined by localization of the organelle by indirect immunofluorescence. Rootlet lengths were measured by projecting negatives onto a white screen and tracing the image. Fig. 14a, number of cells containing rootlets or flagella from a sample size of 100. Fig. 14b. Each point represents the mean length (μm) of rootlets and flagella throughout the differentiation. The standard error for each point is less than 0.1 μm (sample size 100). Fig. 14c, Since the mean maximum lengths of the two organelles are not the same, the data in Fig. 14b has been expressed as the percent of the mean maximum length attained at various times during the differentiation. Δ- - -Δ ,rootlets; O——O , flagella.

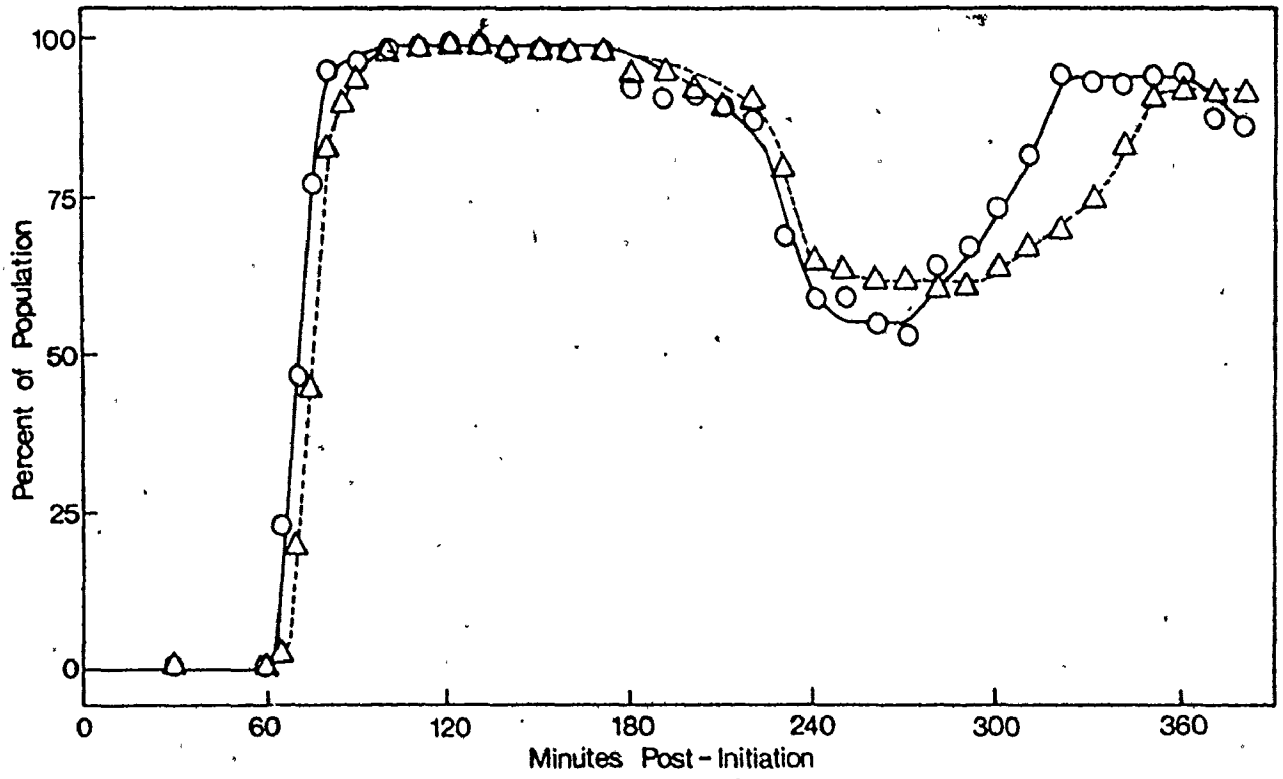

50
70
90
110
130
100
75
50
25
0
A
Percent of Population
B
16
12
8
4
0
Length (μm)
C
100
75
50
25
0
Percent of Maximum Length
50
70
90
110
130
Minutes Post-Initiation

The flagellate phenotype is transitory and is lost within 3 to 4 h after the initiation of differentiation (Fig. 15). Following the less synchronous reversion, the amoebae retransform. Rootlets appear just after flagella develop (cf Fig. 14a), disappear after flagella during reversion to amoebae and reassemble during the subsequent retransformation, maintaining a constant relationship in relative time of assembly, disassembly and reassembly.

Figure 15



The time of appearance of rootlets and flagella through two cycles of flagellate differentiation. At specific times during the differentiation period, an aliquot of cells was fixed in Lugol's iodine and the presence of flagellates in the population monitored by phase microscopy. A second cell sample was fixed in buffered paraformaldehyde and the percent of cells containing rootlets determined by localization of the organelle by indirect immunofluorescence. Each point represents the number of cells containing rootlets for flagella from a sample size of 100. △--△, rootlets; ○—○, flagella.


100
75
50
25
0
Percent of Population
0
60
120
180
240
300
360
Minutes Post - Initiation

Chapter 6

Regulation of Organelle Distribution in Naegleria Flagellates

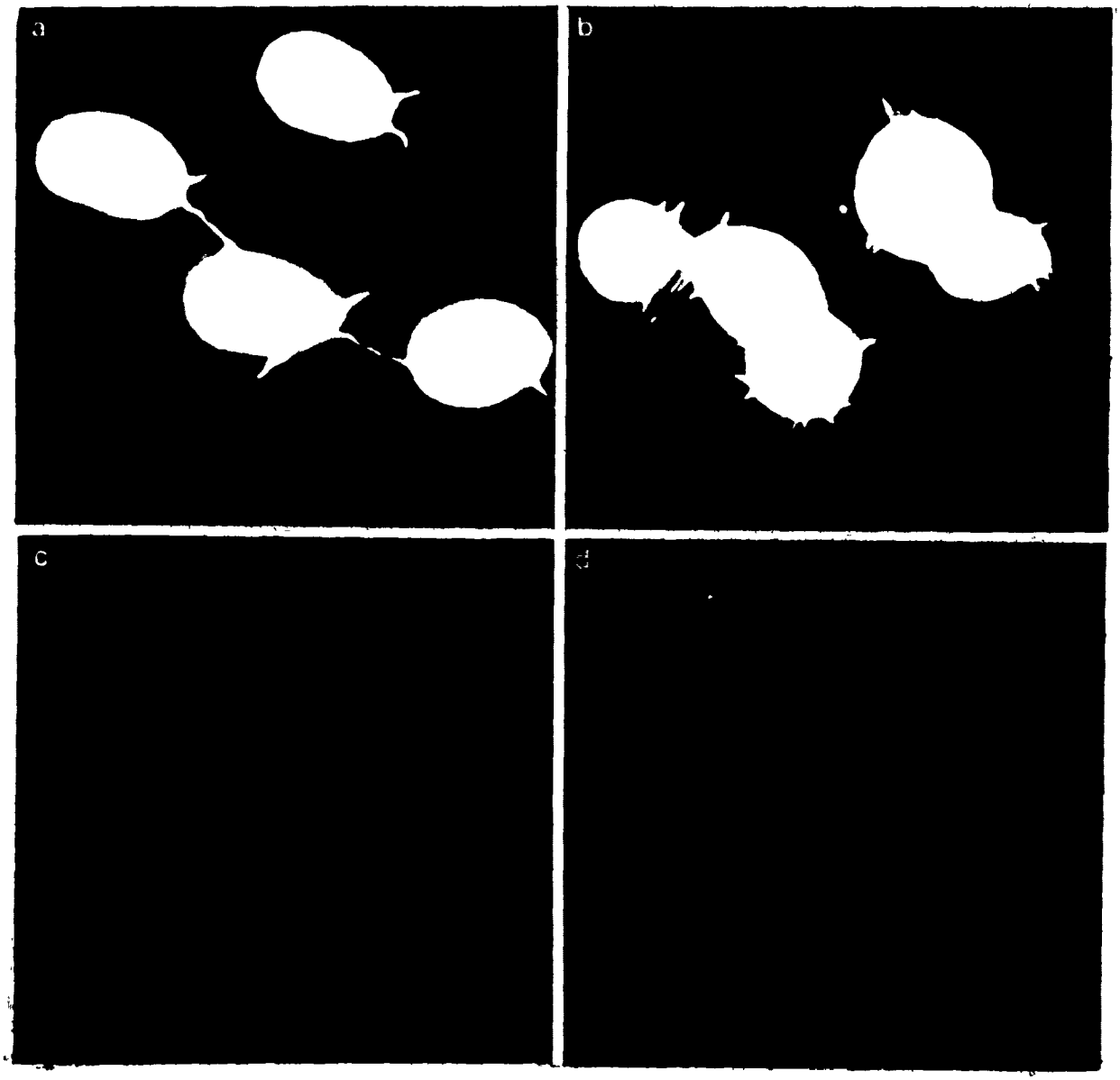
6.1 Rootlet Development in Multiflagellates

When amoebae are subjected to a sublethal temperature shock (37.9°C) at a specific time interval during differentiation, the resulting flagellate population develops a mean of nearly 6 flagella per cell (Dingle, 1977). The number of flagella per cell is not constant, ranging from 1 to 12, and occasionally a cell may contain more than 20. The flagellum length on any one cell is not constant (Fig. 16 a and b). In addition, the characteristic fusiform flagellate shape is often lost in cells with excess flagella.

From an electron microscopic study of multiflagellates, Dingle (1970) observed that the flagellar rootlet is occasionally doubled. Since the sublethal temperature shock may result in an altered rootlet organization which would be reflected in the characteristic organelle periodicity, the mean periodicity of rootlets isolated from multiflagellates and stained with uranyl acetate was compared to that of rootlets from flagellates which had not been subjected to the sublethal temperature shock. The mean periodicities are 20.8 and 21.7 nm, respectively, demonstrating that the rootlet organization in multiflagellates is not changed. It was of further interest to characterize the rootlet distribution in multiflagellates in order to determine whether there is a coordinate assembly of flagella, associated basal bodies and rootlet into some basic flagellar apparatus unit.

Figure 16

The appearance of flagella and rootlets in multiflagellates. Note the difference in flagellate shape, the increase in flagellum number and the decrease in flagellum length in the multiflagellated cells (B) compared to the control biflagellated cells (A). Rootlets are similar in appearance but longer in biflagellated (C) than multiflagellated cells (D). There is also an increase in rootlet number in multiflagellates, emanating from a common centre. A, B, darkfield; C, D, fluorescent microscopy. all X 1,100.

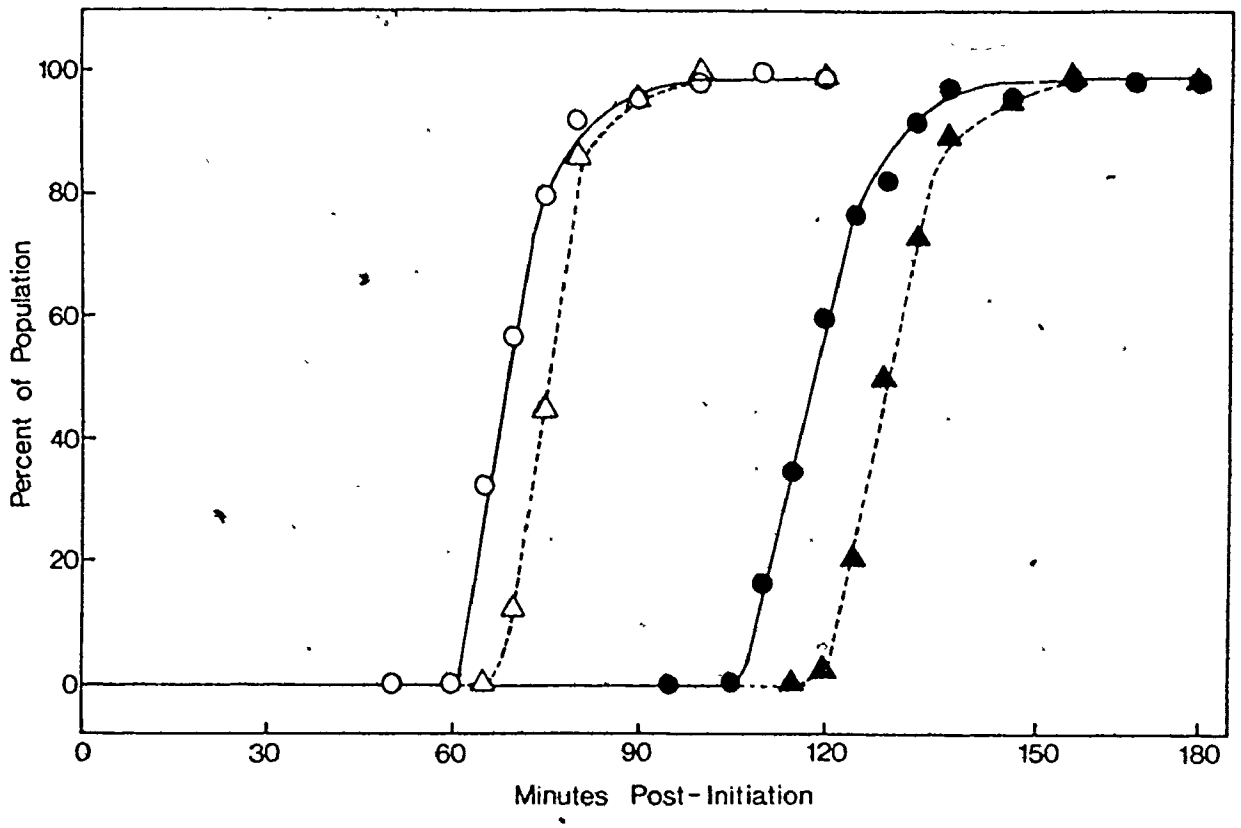

a
b
c
d

The sequence of rootlet development in multiflagellates is similar to that observed in biflagellates. In multiflagellates, rootlets first appear at the cell periphery as highly fluorescent dots each containing a small projection which rapidly elongates into a recognizable rootlet. Usually 2 or 3 rootlets are found in these multiflagellated cells in which the average number of flagella per cell is 5.4. Even though multiple rootlets may be present in a flagellate, all rootlets in the cell are usually at approximately the same stage of development. If a cell contains more than one rootlet, the organelle length is apparently decreased since the rootlets do not usually span the entire cell as do rootlets of biflagellates (Fig. 16 c and d). Occasionally 2 or 3 rootlets are attached at a common proximal end (Fig. 16d).

A comparison of the time of appearance of rootlets and flagella in biflagellated and multiflagellated cells is shown in Fig. 17. Following a temperature shock at 37.9°C from 35 to 71 min post-initiation, flagellates do not appear for 105 - 110 min as compared with 60 - 65 min in the control population. However, flagella are seen on greater than 90% of the cells within a 25 - 30 min period regardless of the temperature shock indicating that the population heterogeneity is identical. T_{50}s for the appearance of flagella in this experiment are 69 and 118 min for biflagellates and multiflagellates, respectively. Rootlets are first visible 5 - 10 min after flagella are first seen in both flagellated populations. T_{50}s for the appearance of rootlets in biflagellated and multiflagellated

Figure 17

The time of appearance of flagella and rootlets in multiflagellates compared to the control biflagellates. Developing flagellates were subjected to a sublethal temperature shock (37.9°C) from 35 to 71 min post-initiation, resulting in a mean number of flagella per cell of 5.4. The control biflagellated population was maintained at 25°C throughout the differentiation period. The appearance of flagella was detected by phase contrast optics whereas rootlet appearance was monitored by specifically localizing the organelle by indirect immunofluorescence. For each time point the sample size was 100 cells. In this experiment T_{50} for the appearance of flagella and rootlets in the control biflagellated population was 69 and 76 min, respectively. T_{50} for the appearance of flagella and rootlets in the multiflagellated population was 118 and 131 min, respectively. ○ , flagella, control; △ , rootlets, control; ● , flagella, temperature shock; ▲ , rootlets, temperature shock.


100
80
60
40
20
0
Percent of Population
0
30
60
90
120
150
180
Minutes Post-Initiation

cells are 76 and 131 min, respectively. On a relative time scale of 1.0 which is equal to the T_{50} for flagellum appearance, the T_{50} for rootlet appearance is 1.1 for both biflagellates and multiflagellates. Therefore, rootlets appear at essentially the same time relative to flagella in the two populations.

6.2 Organelle Distribution in Multiflagellates

To determine whether the increase in rootlet number corresponds to the increase in flagellum number of multiflagellates, differentiating amoebae were subjected to a sublethal temperature shock for various times so that the resulting flagellate populations would have a mean number of flagella per cell of approximately 2, 3, 4, 5 or nearly 6. Initial observations suggested that if a flagellated cell contained more than one rootlet, there was a decrease in the length of individual rootlets (Fig. 16 c and d). A similar pattern for flagellum number and length has been observed (Fig. 16 a and b). Therefore, in the experiment described below, the numbers and lengths of both flagella and rootlets have been measured. Due to the various temperature treatments, the T_{50}s for the appearance of flagella differed, ranging from 70 to 120 min, e.g., T_{50} for the control biflagellated cells was approximately 70 min, but to obtain the maximum number of flagella per cell, differentiating amoebae were subjected to 37.9°C from 35 to 71 min post-initiation resulting in a T_{50} of approximately 120 min. Therefore all organelle lengths were measured at 60 min past the T_{50} for flagellum appearance for each treatment.

In flagellated populations in which the mean number of flagella per cell is greater than 2, flagellates are present which have a typical flagellar apparatus (two flagella, two basal bodies and one rootlet). However, the lengths of the flagella and rootlets may be altered by the temperature shock. To determine the effect of temperature on organelle length in biflagellated cells, differentiating amoebae were subjected to 37.9°C from 0 to 18 min post-initiation. The resulting flagellate population has a mean number of flagella and rootlets per cell of 2.2 and 1.1, respectively, similar to the flagellate population not subjected to the temperature shock. In addition the mean flagellum and rootlet lengths varied by less than 0.4 μm between the two populations. Therefore, the temperature shock does not affect organelle length in a typical (biflagellate) flagellar apparatus.

In multiflagellates the increase in the mean number of rootlets per flagellate is directly related to the corresponding increase in flagellum number (Fig. 18). The slope of the curve is approximately 2 and therefore demonstrates that the two flagella to one rootlet relationship of a typical (biflagellate) flagellar apparatus is maintained following the sublethal temperature shock.

As the mean number of flagella per flagellate increases, there is a corresponding increase in the total length (μm) of flagellum assembled per flagellate (Fig. 19a). There is, however, a decrease in the mean flagellum length as the number of flagella per flagellate increases. As shown in Fig. 20 and Table 4, the mean flagellum length of a biflagellated cell is 14.5 μm (range 7 to 19 μm). This length

Figure 18

Increase in mean number of flagella relative to increase in mean rootlet number in multiflagellates. To produce the increase in the mean number of organelles per cell, developing flagellates were subjected to 37.9°C for specific time intervals during the differentiation period. Each flagellum and rootlet distribution was determined from a sample size of 300 cells. Data are pooled from two separate experiments.

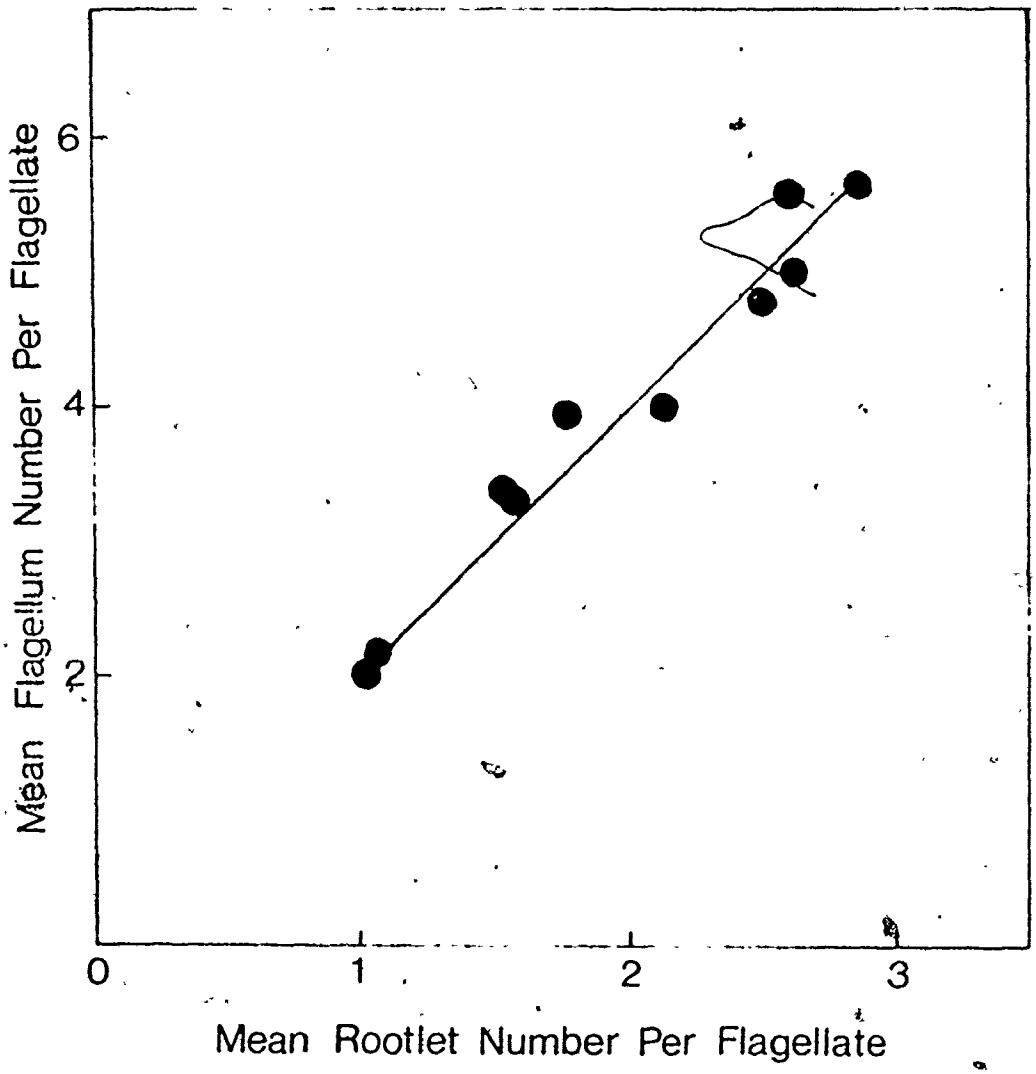
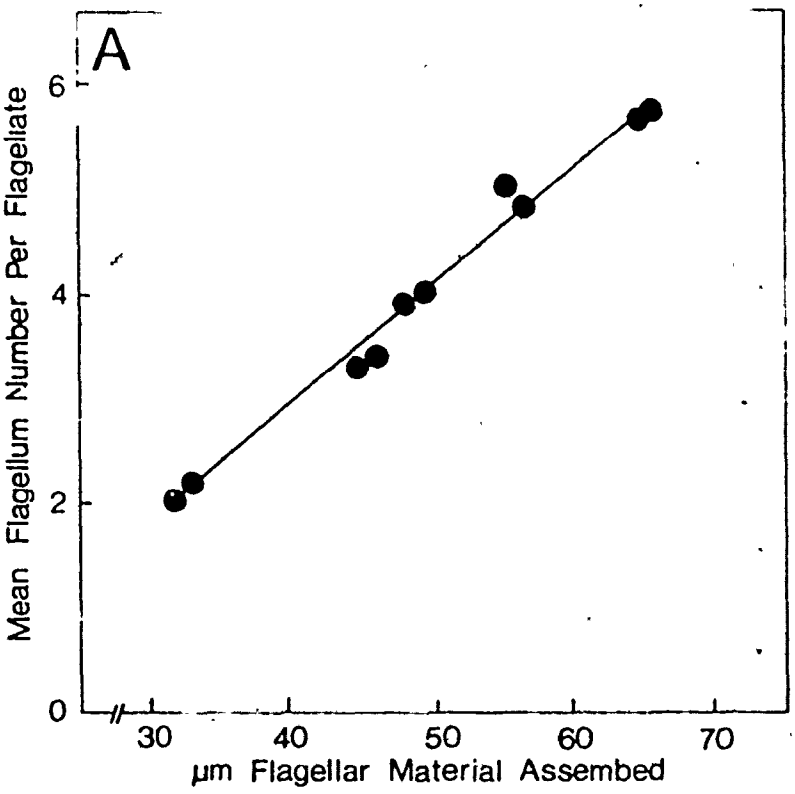

Mean Flagellum Number Per Flagellate
6
4
2
0
1
2
3
Mean Rootlet Number Per Flagellate

Figure 19

Increase in total length of flagellum and rootlet assembled in multiflagellates. To determine the total flagellum lengths, the distribution of flagellum number was determined for a sample size of 300 cells and all flagella from fifty representative flagellates were measured with a filar micrometer using phase contrast optics. Similarly, to determine rootlet lengths, the rootlet distribution in 300 cells was determined using indirect immunofluorescence to localize the organelles and then all rootlets in fifty representative flagellates were measured from tracings of projected negatives of fluorescent rootlets. Data are pooled from two separate experiments.


A
Mean Flagellum Number Per Flagellate
0
2
4
6
30
40
50
60
70
µm Flagellar Material Assembled

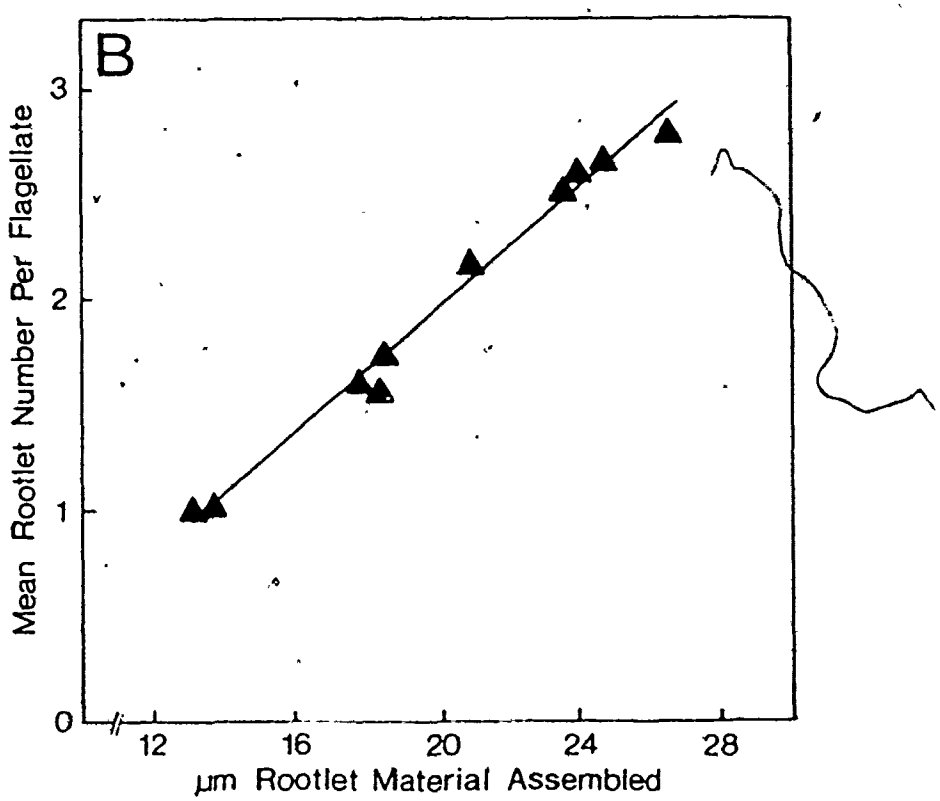

B
Mean Rootlet Number Per Flagellate
0
1
2
3
12
16
20
24
28
µm Rootlet Material Assembled

Figure 20

Frequency distribution of flagellum lengths for populations having from 2 to 6 flagella per cell. To produce the increase in the number of flagella per cell, developing flagellates were subjected to 37.9°C for specific time intervals during the differentiation. Flagellum lengths were measured with a filar micrometer. The data have been expressed on a frequency distribution from sample sizes ranging from 183 to 484 flagella (see Table 4). In a representative experiment the frequency of two flagella per cell in populations with a mean number of flagella per cell of approximately 2, 3, 4, 5, and 5.7 is 84, 35, 16, 7 and 2%, respectively. In contrast, the frequency of six flagella per cell in populations with a mean number of flagella per cell of approximately 2, 3, 4, 5 and 5.7 is 0, 2, 10, 20 and 25%, respectively. Data are pooled from two separate experiments.

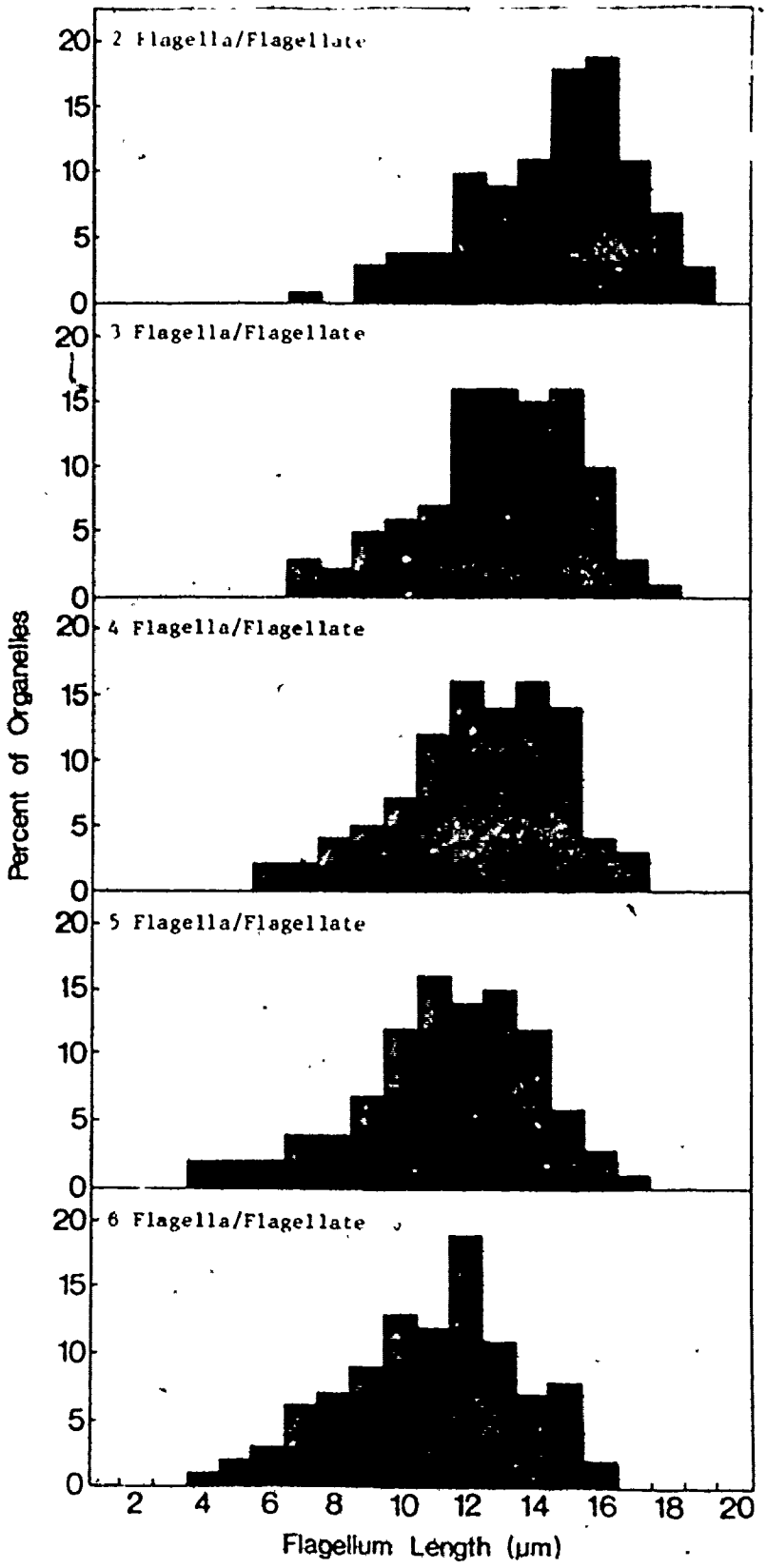

2 Flagella/Flagellate
3 Flagella/Flagellate
4 Flagella/Flagellate
5 Flagella/Flagellate
6 Flagella/Flagellate
Percent of Organelles
0
5
10
15
20
2
4
6
8
10
12
14
16
18
20
Flagellum Length (µm)

Table 4

The relationship between organelle number and length for both rootlets and flagella.

	Number of Organelles/Flagellate	Mean Length (μm)	Sample Size	Std. Error (μm)
Rootlets	1	12.9	245	.02
	2	10.3	338	.02
	3	8.8	306	.02
	4	8.2	116	.02
Flagella	2	14.5	346	.02
	3	13.0	183	.02
	4	12.5	484	.03
	5	11.4	365	.03
	6	10.9	384	.03

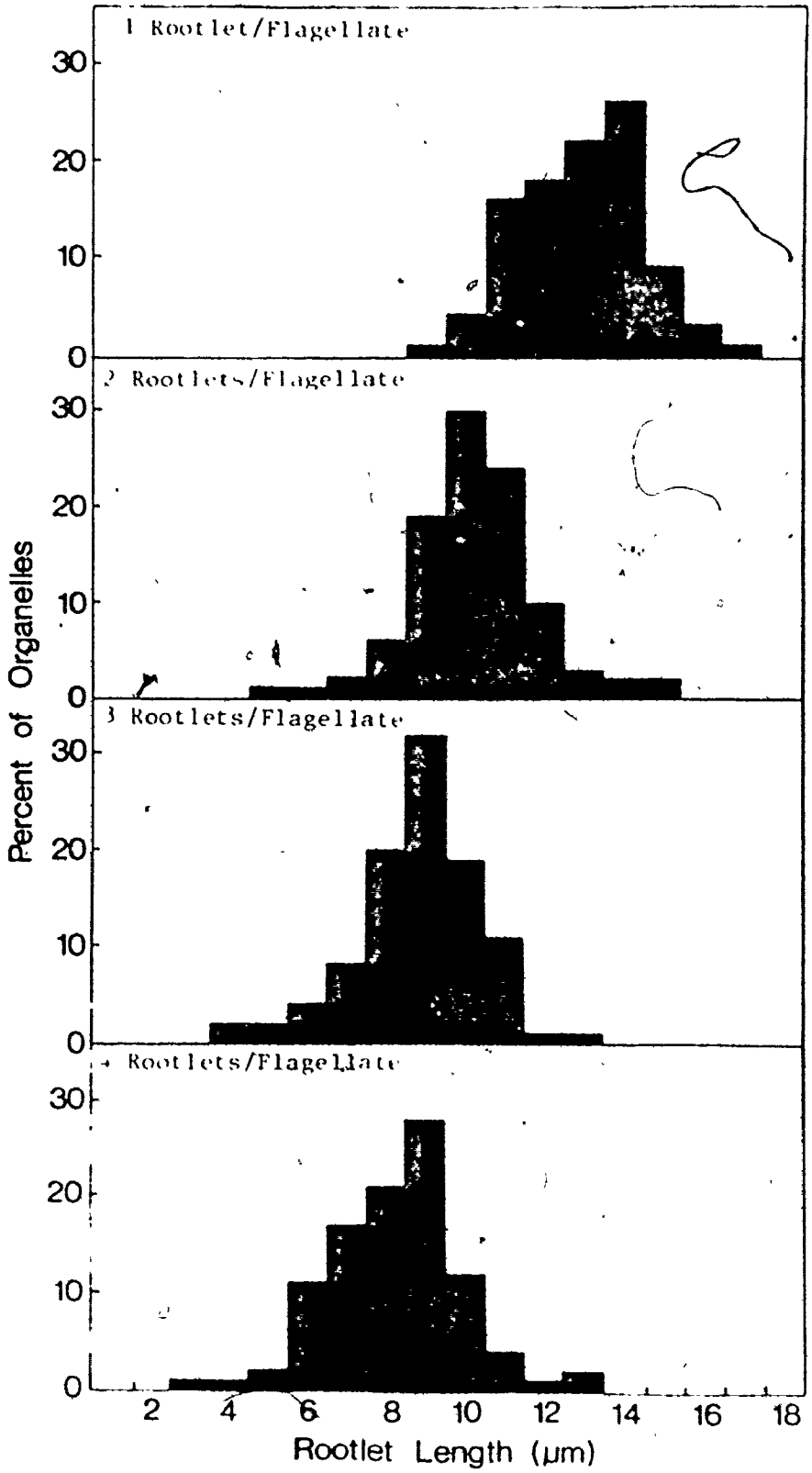
The population means of flagellum and rootlet lengths are all significantly different at $\rho = .005$ according to the 't' test for differences between sample means.

decreases to 10.9 μm (range 4 to 16 μm) in flagellates containing six flagella. The histograms shown in Fig. 20 illustrate the shift in distribution of flagellum lengths to shorter organelles as the number of flagella per cell increases from 2 to 6. The lengths of flagella from a flagellated cell with six flagella are similar regardless of whether the cells are from populations with a mean number of flagella per flagellate of 2, 3, 4, 5 or nearly 6. The same is true for 2, 3, 4 and 5 flagella per flagellate.

The change in length distributions of rootlets in multiflagellates is similar to that of flagella. As the mean number of rootlets per cell increases, there is a corresponding increase in the total length (μm) of rootlet assembled per flagellate (Fig. 19b). However, as shown in Fig. 21 and Table 4, the mean rootlet length decreases as the number of rootlets per flagellate increases. For example, the mean length of one rootlet per flagellate is 12.9 μm (range 9 to 17 μm). In a flagellate containing four rootlets the mean length decreases to 8.2 μm (range 3 to 13 μm). The histograms shown in Fig. 21 illustrate the shift in distribution of rootlet lengths to shorter organelles as the number of rootlets per cell increases. The length distributions of rootlets from flagellates containing 1, 2, 3 or 4 rootlets is similar regardless of whether the cells are from populations with a mean number of flagella per flagellate of 2, 3, 4, 5 or nearly 6.

Figure 21

Frequency distribution of rootlet lengths for populations having from 1 to 4 rootlets per cell. To produce the increase in the number of rootlets per cell, developing flagellates were subjected to 37.9°C for specific time intervals during the differentiation. Rootlets were localized by indirect immunofluorescence and the negatives of fluorescent images were projected on a white screen, traced and measured. The data have been expressed on a frequency distribution from sample sizes ranging from 116 to 306 rootlets (see Table 4). In a representative experiment the frequency of one rootlet per cell in populations with a mean number of flagella per cell of approximately 2, 3, 4, 5 or 5.7 is 96, 60, 40, 10 and 8% respectively. In contrast, the frequency of three rootlets per cell in populations with a mean number of flagella per cell of approximately 2, 3, 4, 5 and 5.7 is 0, 4, 16, 34 and 40%, respectively. Data are pooled from two separate experiments.


1 Rootlet/Flagellate
2 Rootlets/Flagellate
3 Rootlets/Flagellate
4 Rootlets/Flagellate
Percent of Organelles
0
10
20
30
2
4
6
8
10
12
14
16
18
Rootlet Length (μm)

6.3 Synthesis of the Rootlet Protein in Multiflagellates

Since the rootlet protein is synthesized de novo during the amoeba to flagellate differentiation (Table 3) and there is an increased length of rootlet assembled in multiflagellates (Fig. 19b), the question arises whether there is also an increase in the total cellular amount of rootlet protein? In other words is the increase in the total length of assembled rootlet a result of increased synthesis of the rootlet protein or is it a result of increased utilization of the available unassembled rootlet protein? In order to answer this question, the total amount of rootlet protein was measured in flagellated populations in which the mean number of rootlets per flagellate ranged from the minimum to the maximum values. Total cellular proteins were separated by SDS polyacrylamide gel electrophoresis and stained quantitatively with Coomassie Brilliant Blue. The area associated with the rootlet protein band (arrow 1, Fig. 22), measured by densitometry, was the same in the various flagellated populations. Therefore, the increase in the number of rootlets in multiflagellated cells does not involve an increase in synthesis of the rootlet protein but rather an increase in utilization of the available rootlet protein.

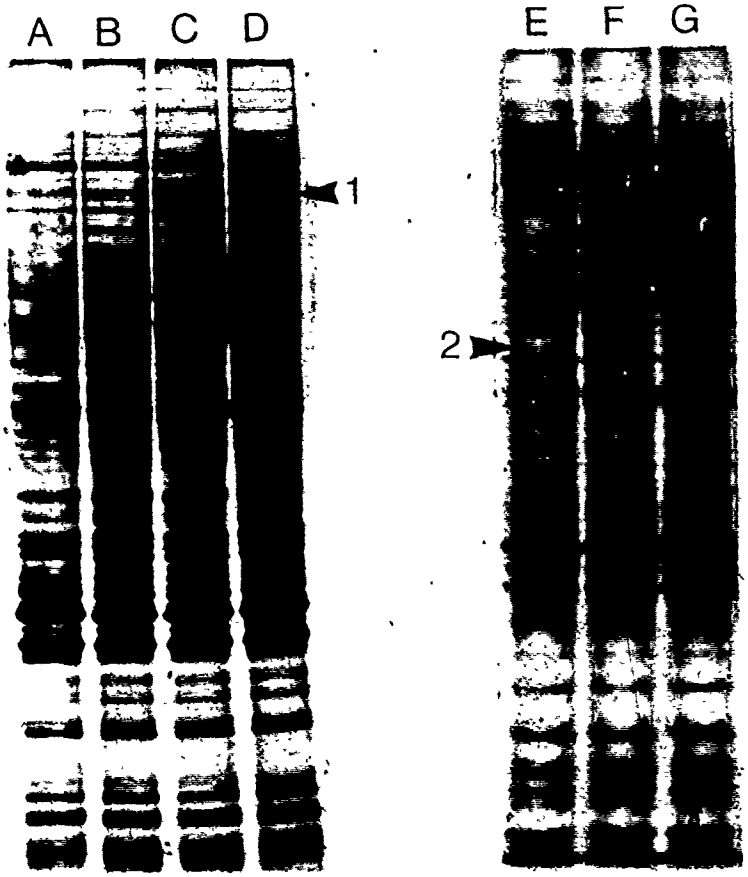
The protein distributions shown in Fig. 22 are similar regardless of the extent of flagellation. One band difference, however, is present. In multiflagellates one minor protein band appears while an adjacent minor protein band (arrow 2, Fig. 22) present in the control flagellate population disappears. The function of this protein

resulting from the sublethal temperature shock is unknown.

Figure 22

Electrophoretic patterns of total cellular proteins of multiflagellates. Total cellular proteins of flagellates with a mean number of rootlets per cell of 1.1, 2.2 and 2.7, separated by SDS polyacrylamide gel electrophoresis, are shown in channels B, C and D, respectively. Channel A represents total cellular proteins of amoebae. Following quantitative staining of proteins with Coomassie Brilliant Blue, the area associated with the rootlet protein was measured by scanning with a Gilford microdensitometer at 560 nm. The rootlet protein (arrow 1) is absent in amoebae (channel A).

Channels E, F and G are identical to B, C and D, respectively. In this electrophoretic protein separation the minor protein band (arrow 2) present in the control biflagellates disappears in the multiflagellated populations; an adjacent protein band appears in channels F and G.


A
B
C
D
E
F
G
1
2

Chapter 7

Discussion

7.1 The Flagellar Apparatus

The morphology of the isolated flagellar apparatus of Naegleria has been previously described by both Schuster (1963) and Dingle and Fulton (1966) but no biochemical analyses of the structure were undertaken. The flagellar apparatus can be easily released intact by gentle lysis of flagellates with dilute Triton X-100 but the basal body-rootlet complex cannot be dissociated without solubilization of the rootlet. In situ, the rootlet is attached to the basal bodies by a palisade of microtubules (Schuster, 1963; Dingle and Fulton, 1966) which may account for the tight organelle association. In contrast, the flagella are easily freed from either the isolated flagellar apparatus or flagellates by gentle vortexing or by shaking in the presence of a low pH buffer (Kowit and Fulton, 1974b), probably due to breakage at the basal plate (Blum, 1971).

7.2 Isolation, Morphology and Composition of the Flagellar Rootlet

Previous attempts by Simpson (1970) and Green (1974) to purify rootlets from Naegleria flagellates were not successful due to the non-specific adherence of the organelle to other cellular structures. In the present study, however, a procedure has been developed for obtaining a purified rootlet fraction which led to the identification of the major rootlet protein component. Although minor cellular contaminants are present in these fractions, the only identifiable contaminants were

basal bodies and membranes, the latter being greatly reduced in quantity but not completely eliminated. The purity of the rootlet fractions is confirmed by the limited number of protein components as demonstrated by SDS polyacrylamide gel electrophoresis. The major rootlet protein represents approximately 50% of the total protein by densitometry in these fractions. As the purity of the rootlet fractions increased, this major protein component increased proportionately. A number of minor protein bands are also present. One of the low molecular weight proteins has been identified as tubulin by its co-migration with tubulin standard in two different gel electrophoresis buffer systems. It is likely, inasmuch as the basal body, but not other cellular microtubules, are maintained during the isolation procedure, that this is basal body tubulin.

The identity of the major rootlet protein has been confirmed by differential solubilization of the rootlet. Exposure of the rootlet to low pH results in the solubilization of the organelle and the release of its protein constituents into the soluble fraction. The predominant protein present in the supernatant is the major rootlet protein. A small percentage, less than 10%, of this protein remains in the insoluble fraction probably due to the breakdown of the rootlet into short filaments which either form a mat or adhere to the insoluble basal body complexes. Solubilization of the rootlet with sarkosyl, high salt (0.4 M KI) or urea substantiate the identification of the major rootlet protein.

The subunit molecular weight of the major rootlet protein of the Naegleria flagellar rootlet is 170,000 daltons which is significantly different from that of the only two other rootlet systems characterized to date. Through a detailed series of solubilization experiments Stephens (1975a) has identified two major proteins (subunit molecular weight of 230,000 and 250,000 daltons) of the basal apparatus isolated from the ciliated gill epithelium of Aequipecten irradians. Although Stephens has not unequivocally demonstrated that basal rootlets are composed of these proteins (e.g., by immunofluorescent localization of the proteins in the organelle or by reconstituting rootlet-like structures from the purified proteins), his differential solubilization results provide good evidence that the two high molecular weight proteins are at least major constituents of the rootlets. He has proposed the term "ankyrin", referring to the presumed anchoring function of rootlets, for these and like proteins which might constitute similar anchoring fibres. It seems inappropriate to refer to the 170,000 dalton rootlet protein of Naegleria as an ankyrin at this time, not just because of the considerable difference in subunit molecular weight, but also because there are significant differences in the solubility properties of the two rootlets. Although one would like to see evidence develop for some evolutionary relatedness among major rootlet proteins isolated from diverse organisms, there is no obvious link between the Aequipecten and Naegleria rootlet proteins.

Rubin and Cunningham (1973) tentatively identified a 21,000 dalton protein as the structural component of the striated fibres of the ciliate Tetrahymena. That identification was based solely on solubilization of the striated fibres with one reagent, phosphotungstate. Phosphotungstate, which presumably solubilized the rootlet through its low pH, is an unusual reagent to use for studying the solubility characteristics of the rootlet or, for that matter, of any protein. It does not solubilize the Naegleria flagellar rootlet. It is questionable whether the 21,000 dalton protein is a major component of the Tetrahymena striated fibre, whether it is a breakdown product of a higher molecular weight component of the striated fibre, or whether it is a contaminant of the heterogeneous basal apparatus fractions. It is clear that this protein has no counterpart in the Naegleria rootlet.

The filamentous nature of the flagellar rootlet of Naegleria is suggested by the appearance of filaments in partially disrupted rootlets and in the tapering distal tip of the organelle. Dissociation of the rootlet by brief treatment with urea, however, demonstrated that the organelle is composed of filaments 5 nm in diameter associated in a linear fashion. This conclusion is supported by brief treatment with other solubilizing agents such as EDTA and high pH. In contrast, in a similar study Lillie et al. (1977) used the denaturing agents urea and guanidine-HCl and demonstrated that the collagen filaments were arranged in a helical fashion. Other studies with native collagen

fibrils using freeze-fracture, negative staining and shadow cast techniques (Bouteille and Pease, 1971; Rayns, 1974) have also suggested that the collagen filaments were assembled in a helical organization.

The filamentous nature of the Naegleria rootlet is consistent with the general structure of rootlets in other systems, e.g., the ciliated gill epithelium (Gibbons, 1961; Stephens, 1975a), the ciliate Tetrahymena (Munn, 1970) and the ciliated tail fin cells of larval amphioxus (Flood, 1975). The loss of the characteristic banding pattern following dissociation of the rootlet into constituent filaments shown here support the suggestion by Munn (1970) that the banding pattern reflects the arrangement of the filaments in the organelle. Since both urea and low pH disrupt hydrogen bonding, the arrangement of filaments in the rootlet is probably a result of hydrogen bonding in addition to van der Waal's forces between the constituent protein component(s). If the Naegleria rootlet is composed of essentially one protein, then the filaments may represent linear aggregations of this protein. The resulting periodicity of the organelle would then reflect the arrangement of the amino acids in the aligned protein molecules. Alternatively, additional, perhaps regulatory, proteins, may be integral components of the protein matrix.

Reconstitution studies with the major rootlet protein of the Naegleria flagellar rootlet may elucidate the bonding arrangement of the filaments which results in the typical cross-banded structure of the native fibre. Reconstitution of soluble collagen under various

environmental conditions such as pH, ionic strength and the presence of adenosine triphosphate or glycoprotein resulted in the formation of three ordered aggregation states (native-type, fibrous long spacing and segment long spacing), suggesting that the collagen fibril was composed of tropocollagen molecules aggregating in a staggered fashion with gaps and overlaps and connected by hydrogen and other bonding forces (Gross et al., 1954; Hodge and Schmitt, 1960). Reconstitution experiments with paramyosin from molluscan catch muscles suggested a bonding pattern similar to that of collagen (Cohen et al., 1971). In contrast, both tropomyosin (Cohen, 1975) and fibrinogen (Tooney and Cohen, 1977) bond in an end-to-end fashion. Similar reconstitution experiments with the Naegleria rootlet protein cannot be performed using the purified protein isolated from preparative SDS polyacrylamide gels due to the presence of protein-bound SDS. Although the SDS can be removed by electrodialysis, the method requires the use of milligram quantities of rootlet protein which are not easily attainable. The rootlet protein, however, can be purified using the solubility characteristics of the organelle which would permit the reconstitution studies to be carried out.

7.3 Development of the Flagellar Rootlet

During the amoeba to flagellate differentiation the assembly of the three major organelles (flagella, basal bodies and rootlet) into a functioning flagellar apparatus is closely coordinated. Basal bodies

are first seen 50 - 55 min after the initiation of differentiation, prior to the appearance of flagella in the population. The kinetics of appearance of both basal bodies and flagella are similar, with the T_{50} for the appearance of flagella 10 min later than for basal bodies (Fulton and Dingle, 1971). The present study has demonstrated that rootlets are first detected 5 min after flagella. The kinetics of rootlet appearance are similar to those of flagella and thus also basal bodies. The T_{50} for rootlet appearance is consistently 5 - 6 min later than for flagella. Thus, all organelles of the flagellar apparatus appear in developing flagellates within a 15 min period. On a time scale in which T_{50} for the appearance of flagella is defined as 1.0, the T_{50}s for basal body and rootlet appearance are 0.86 and 1.1, respectively.

In this study the T_{50} for rootlet appearance is 1.1 relative to flagella which is slightly but significantly different from 1.0 reported by Green (1974) who scored for the presence of rootlets by cell lysis with detergent. In contrast, the method used in this study evaluated the presence of rootlets in developing flagellates by a specific staining method. My attempts to repeat Green's measurements were unsuccessful since it is very difficult to lyse the cells slowly enough to permit observation of rootlets and evaluating the presence of developing rootlets within the dense cytoplasm with the large nucleus thus becomes extremely subjective. Green may have confused developing axonemes with developing rootlets which would lead to a shift to an earlier T_{50} for

rootlet appearance. This apparent contradiction might be resolved by specifically staining the developing axoneme by indirect immunofluorescence using an antibody specific for tubulin. Since flagella elongate at such a rapid rate, the appearance of the developing axoneme below the cell membrane would closely parallel flagellum appearance.

The sequence of rootlet development has been elucidated by specific localization of the organelle using the technique of indirect immunofluorescence with employs an antibody specific for the rootlet protein. By this method the organelle is first seen in developing flagellates 65 min after the initiation of differentiation. It appears as a highly fluorescent dot, 1 μm in diameter, generally near the periphery of the cell. Within the next 10 min there is a rapid elongation of a small finger-like projection such that the length of the rootlet in the cell varies from 2 - 10 μm. By 100 - 110 min post-initiation rootlets have reached their mean maximum length of 13 μm.

A comparison of the appearance of rootlets detected by indirect immunofluorescence and by thin sectioning of flagellates demonstrates that there is an accumulation of rootlet protein at the proximal end (fluorescent dot) not seen in the electron micrographs. This accumulation of rootlet protein appears to surround the basal bodies and is not assembled into the characteristic rootlet banding pattern. In the quadriflagellate alga Polytomella, the amorphous material associated with the flagellar rootlets functions as cytoplasmic microtubule organizing centre (Stearns et al., 1976). Whether the aggregation of rootlet protein surrounding the basal bodies in Naegleria also serves as an

organizing centre for cytoplasmic microtubules *in vivo* is not known.

The mechanism by which rootlets assemble into a completed organelle is unknown. It is clear, however, that rootlets do not develop by the laying down of a few filaments along the entire length of the organelle and subsequent lateral addition of filaments. On the contrary, rootlets develop by rapid elongation from the basal bodies while the diameter of the organelle remains essentially constant. Whether the developing rootlet elongates by proximal, as opposed to distal, addition of subunits is not known. One way to determine this would be to develop an *in vivo* or *in vitro* method for the study of the assembly of rootlet protein components analogous to those used for studying microtubule elongation of *Chlamydomonas* axonemes (Rosenbaum and Child, 1967; Rosenbaum *et al.*, 1975; Whitman, 1975). In both of these studies developing axonemes were treated with labelled precursors and the site of addition determined by autoradiography. Most of the label was located at the distal end of the microtubules suggesting distal, rather than proximal, addition of subunits.

A comparison of the rates of assembly of flagella and rootlets demonstrates that although flagella are present in developing flagellates prior to rootlets, rootlets assemble at a faster rate than do flagella. By 75 min after the initiation of differentiation the mean lengths of both flagella and rootlets are the same (approximately 3 μm) suggesting that rootlets may elongate at a faster rate because a minimum organelle length is required for rootlet function. During continued elongation of both flagella and rootlets the two organelle

lengths remain approximately the same. This apparent coordination in size of the two organelles suggests that they are functionally interrelated. By 100 - 110 min post-initiation the rootlets have reached their maximum length. In contrast flagella continue to elongate up until 140 min post-initiation, at a much slower rate.

The present study has demonstrated that at least 62% of the rootlet protein assembled into the organelle has been synthesized de novo during the amoeba to flagellate differentiation. Less than 100% is attained presumably since the methionine chase is not complete. The quantity of methionine used in these experiments was the maximum concentration that permitted flagellate differentiation. Tubulin assembled into flagella is also synthesized de novo (Kowit and Fulton, 1974b). Similarly, dynein, the high molecular weight flagellar ATPase, is newly synthesized (Simpson et al., 1978). The present study has also demonstrated that basal body tubulin is probably synthesized de novo, although an accurate value of the extent of de novo synthesis cannot be determined since it could be possible that other proteins band in this region of the gel. In contrast, actin, a major structural protein of the cytoplasm of amoebae but not of the flagellar apparatus, is not synthesized de novo. Thus, although not all of the proteins of the flagellar apparatus have been identified, a sufficient number of distinct proteins have been investigated to conclude tentatively that all the proteins of the flagellar apparatus are likely synthesized de novo during the amoeba to flagellate differentiation.

Since flagellar tubulin is synthesized de novo despite an excess of tubulin in amoebae, Fulton and Simpson (1976) have suggested that an organsim may contain several distinct tubulins produced by different genes. These tubulins differ slightly in amino acid composition and are used selectively for the assembly of different microtubule-containing organelles. This "multi-tubulin hypothesis" implies the existence of a number of discrete tubulin pools within a cell. In support of this hypothesis, a comparison of the amino acid composition between basal body tubulin and the other tubulins of the flagellum, cytoskeleton and other microtubule-containing organelles should demonstrate a few, distinct amino acid differences. Since basal bodies are present in rootlet fractions, the basal body tubulin can be easily purified and used for this amino acid comparison.

Tubulin is a structural protein of basal body and flagellar microtubules as well as of the cytoplasmic microtubules found in Naegleria amoebae and flagellates. Thus, it is not suitable to use tubulin as an indicator molecule for monitoring the assembly and disassembly of the flagellar apparatus because of the significantly high background levels of tubulin in any analysis. The rootlet protein, however, is not present in amoebae but appears during the differentiation and is probably only a strucutral protein of the Naegleria flagellar rootlet. Therefore, the rootlet protein can be used as an indicator molecule for monitoring the assembly and disassembly of the flagellar apparatus. Monitoring flagellate differentiation by scoring

for the presence of either rootlets or flagella in the population demonstrates that in the initial flagellate differentiation, rootlets appear just after flagella. In the subsequent reversion to amoebae rootlets are lost just after flagella. And in the following flagellate differentiation rootlets appear just minutes after flagella. Thus, it appears that the assembly and disassembly of the two organelles follow closely during the two cycles of flagellate differentiation, lending support to the use of the rootlet protein as an indicator molecule for monitoring the assembly and disassembly of the flagellar apparatus.

7.4 Regulation of Organelle Distribution in Naegleria Flagellates

Following a sublethal temperature shock during the amoeba to flagellate differentiation, there is an increase in the number of rootlets and flagella produced. This study has demonstrated that the sequence of rootlet development in the multiflagellated population is similar to that observed in control biflagellates. Flagellum development is also not altered (Dingle, 1970). The kinetics of rootlet and flagellum appearance in multiflagellates are also the same. T_{50} for rootlet appearance is 1.1 relative to the appearance of flagella, a value identical to that measured for rootlet appearance in the control biflagellated populations. Thus, the ordered sequence of organelle development has not been altered by the temperature shock.

In multiflagellated cells, the increase in the number of rootlets assembled is proportional to the increase in flagellum number such that the two flagella to one rootlet relationship of a typical (biflagellate)

flagellar apparatus is maintained. Thus the temperature effect which leads to uncontrolled basal body and flagellum assembly simultaneously deranges whatever controls govern the assembly of rootlets.

As the mean number of flagella and rootlets assembled per cell increases in multiflagellates, there is a corresponding increase in the total length (μm) of assembled flagellum and rootlet per cell. Regardless of the mean number of flagella per cell of the population, the ratio of the total length of assembled flagellum to rootlet is constant. The fact that the two to one organelle relationship is maintained and that there is a constant ratio of flagellum to rootlet assembled suggests that these two parameters are coordinately controlled and implies that this relationship between organelle number and length is important for proper functioning of the flagellar apparatus. As the number of both flagella and rootlets per cell increases, there is a shift to shorter organelles. Moreover, there is a direct relationship between the number of organelles per cell and the corresponding organelle lengths, regardless of the mean number of flagella per cell of the population. For example, the lengths of rootlets in a cell containing three rootlets are similar regardless of whether the cell was present in a flagellated population in which the mean number of flagella per cell was two, three, four, five or nearly six. Likewise, the lengths of flagella on a cell containing six flagella are similar, regardless of whether the cell was present in a flagellated population in which the mean number of flagella per cell was two, three, four, five or nearly six. Thus, despite the apparent lack of precise control

of the organelle number displayed by the control flagellated population, there appears to be a precise coordinate control of the number of organelles per cell and the corresponding organelle length.

What is seen throughout this study of rootlet and flagellum development in multiflagellated populations is the regulation of organelle distribution, such that a two:one relationship of flagella to rootlet is maintained. One visualizes individual cells controlling the synthesis and assembly of the diverse molecular components so as to maintain this relationship. It is important, therefore, to point out that the simultaneous measurement of both flagella and rootlets is not technically possible. The relationships described are based upon population means derived from independent determinations of the two organelles.. Despite this reservation, the degree of coordination observed is highly impressive, and it seems reasonable to view it as a reflection of coordinating mechanisms operating within individual cells.

The increase in total length of assembled flagellum and rootlet in multiflagellated cells may be a result of increased synthesis or utilization of the available, unassembled rootlet protein, or of both. To determine if there was an increase in the amount of rootlet protein synthesized in multiflagellates, total cellular proteins were separated by SDS polyacrylamide gel electrophoresis and stained quantitatively. The area associated with the rootlet protein band in the flagellated populations with a mean number of rootlets per cell ranging from 1 to nearly 3 were measured and shown to be the same. Thus the increase

in total length of assembled rootlet resulting from high temperature shocks is not a result of increased synthesis of rootlet protein but rather an increased utilization of the normal amount of rootlet protein.

The parallel question concerning total cellular amount of flagellar tubulin in the various flagellated populations was not posed due to the existence of other tubulins in the cell which would interfere with a simple determination of total amount. The increase in total length of flagellum assembled in multiflagellates is also probably a result of increased utilization of the normal amount of flagellar proteins for three reasons. Tubulin and dynein, two of the major flagellum proteins, are synthesized de novo during the differentiation, similar to the rootlet protein. Secondly, the time of organelle appearance and the development of both flagella and rootlets are not altered by the temperature shock. Finally, the ratio of the total length of flagellum to rootlet assembled is constant, suggesting coordinated control over the amount of flagellum and rootlet assembled.

The production of excess flagella following the sublethal temperature shock is transitory since following reversion to amoebae, flagellate differentiation results in the assembly of the same number of flagella as in the control flagellated populations (Dingle, 1970). Since the temperature shock is not thought to be affecting the synthesis of the structural proteins of the flagellar apparatus, it may be altering the synthesis of key components involved in controlling the assembly process. In this context, one peripheral observation made

during this study has become highly intriguing: amongst the multitude of proteins which were resolved on polyacrylamide gels of whole cells, there is a change in only one minor protein band in the multiflagellated populations. Though the idea is admittedly highly speculative, the possibility is raised that this protein plays some role in the initiation or regulation of assembly of the flagellar apparatus.

Summary

1. The flagellar rootlet has been isolated from Naegleria flagellates by differential centrifugation and the major rootlet protein component has been identified by solubilization of the organelle in urea, high salt, detergents or extremes in pH. The rootlet protein has a subunit molecular weight of 170,000 daltons and has been purified by separation on and elution from polyacrylamide preparative gels.

2. At least 62% of the rootlet protein assembled during the amoeba to flagellate differentiation is synthesized de novo as measured by means of an isotope dilution experiment. Since the rootlet protein is absent in amoebae but present in developing flagellates prior to the appearance of rootlets, most likely the rootlet protein is completely synthesized de novo.

3. The sequence of flagellar rootlet development has been elucidated by indirect immunofluorescence using an antibody specific to the rootlet protein. Rootlets, 1 to 2 μm in length, first appear in developing flagellates 65 - 70 min after the initiation of differentiation and reach their mean maximum length of 13 μm 40 min later. The appearance of rootlets during the differentiation closely follows that of flagella, but is delayed by about 5 min. The rates of rootlet and flagellum assembly are similar. The appearance and disappearance of rootlets through two cycles of flagellate differentiation closely follows that of flagella.

4. The sublethal temperature shock during the differentiation which leads to uncontrolled basal body and flagellum assembly simultaneously deranges whatever controls the number of rootlets assembled. The increase in the number of flagella and rootlets assembled in multiflagellates results in a shift to shorter organelles. There is, however, a net increase in the total length of organelle assembled. Since the total cellular amount of rootlet protein is constant regardless of the extent of flagellation, the increase in rootlet assembled is a result of increased utilization, but not increased synthesis, of the available rootlet protein.

5. The two flagella to one rootlet relationship of a typical biflagellate flagellar apparatus is maintained following the temperature shock and despite an increase in the total length of flagellum and rootlet assembled, the ratio of flagellum to rootlet assembled remains constant. This suggests that these two parameters are coordinately controlled and implies that this relationship between organelle number and length may be important for proper functioning of the flagellar apparatus.

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Appendix 1

Additions to Materials and Methods

A. The concentration of rootlets in various fractions was determined with a Petroff-Hauser bacterial counter.

B. The staining of the rootlet protein in polyacrylamide slab gels is quantitative. Following scanning at 560 nm with a Gilford microdensitometer, the area associated with the rootlet protein band is directly proportional to the amount layered onto the gel.

C. To stain isolated rootlets by immunofluorescence, rootlets were isolated as described in Section 2.8 and the final rootlet pellet resuspended in buffered paraformaldehyde. After applying rootlet suspensions to slides and allowing to dry, samples were processed as described in Section 2.15.

D. In the isotope dilution experiment ^{35}S-methionine, specific activity 450 Ci/mol (New England Nuclear), was used at a concentration of 5 μCi/ml.

Appendix 2

Negative Control for Imunofluorescence

The figure illustrates the typical appearance of a flagellate in which no specific fluorescent staining is present, following incubating cell samples with preimmune serum followed by fluorescent anti-rabbit immunoglobulin. If samples of amoebae or developing flagellates prior to the appearance of rootlets are incubated with immunized serum followed by fluorescent anti-rabbit immunoglobulin, the same background fluorescence is observed. The film exposure and print development were identical to those in which specific fluorescence is observed (c.f. Fig. 13). X 1,400.

Appendix 3

Rootlet Protein Purification: A Sample Calculation

The yield of purified rootlet protein can be calculated as follows:

Assume a rootlet is a solid cylinder of protein, 10 μm in length and 0.2 μm in diameter, the volume of protein = 0.314 μm^3.

Assume the rootlet protein is an α-helix with a molecular weight of 170,000 daltons and the average molecular weight of an amino acid is 115 daltons. Since an α-helix has 3.6 amino acid residues per 5.4 A, the length of the rootlet protein molecule is 220 nm.

Assume the diameter of an individual filament in the rootlet, 5 A, is also the diameter of the rootlet protein molecule.

Therefore, the volume of the rootlet protein molecule is 4.3×10^{-7} μm^3 and the number of rootlet protein molecules per rootlet = 7.3×10^5.

Since one mole of rootlet protein contains 6.023×10^{23} molecules weighing 170,000 g, 7.3×10^5 molecules weighs 2.06×10^{-13} g.

The rootlet protein was purified from 5×10^9 flagellates or rootlets.

Thus, the starting quantity of rootlet protein was 1.03 mg.

Since 20-30 μg rootlet protein is purified from this starting material, the yield is 2-3%.