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Enflagellation of Naegleria Fowleri

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Enflagellation of Naegleria fowleri

By

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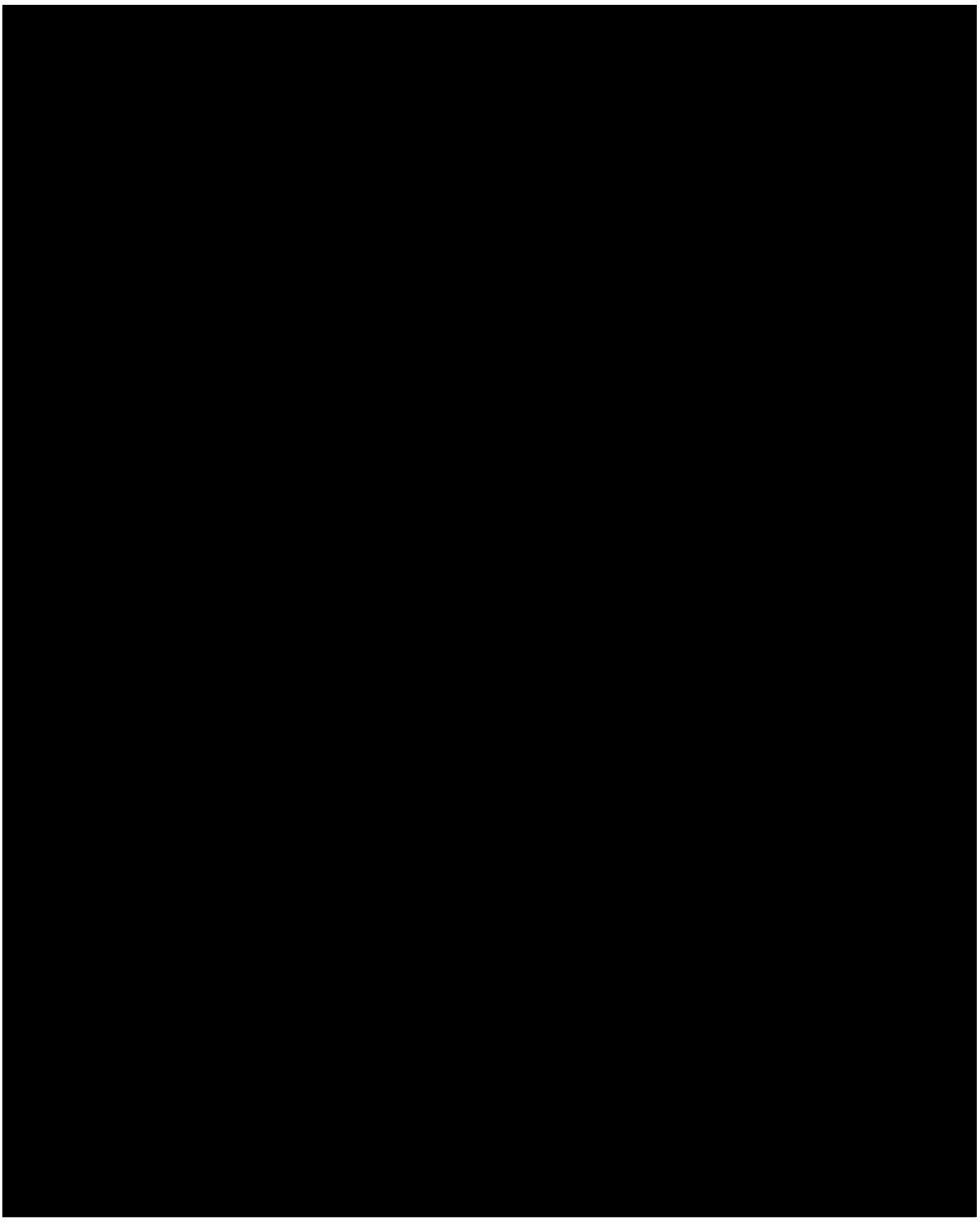

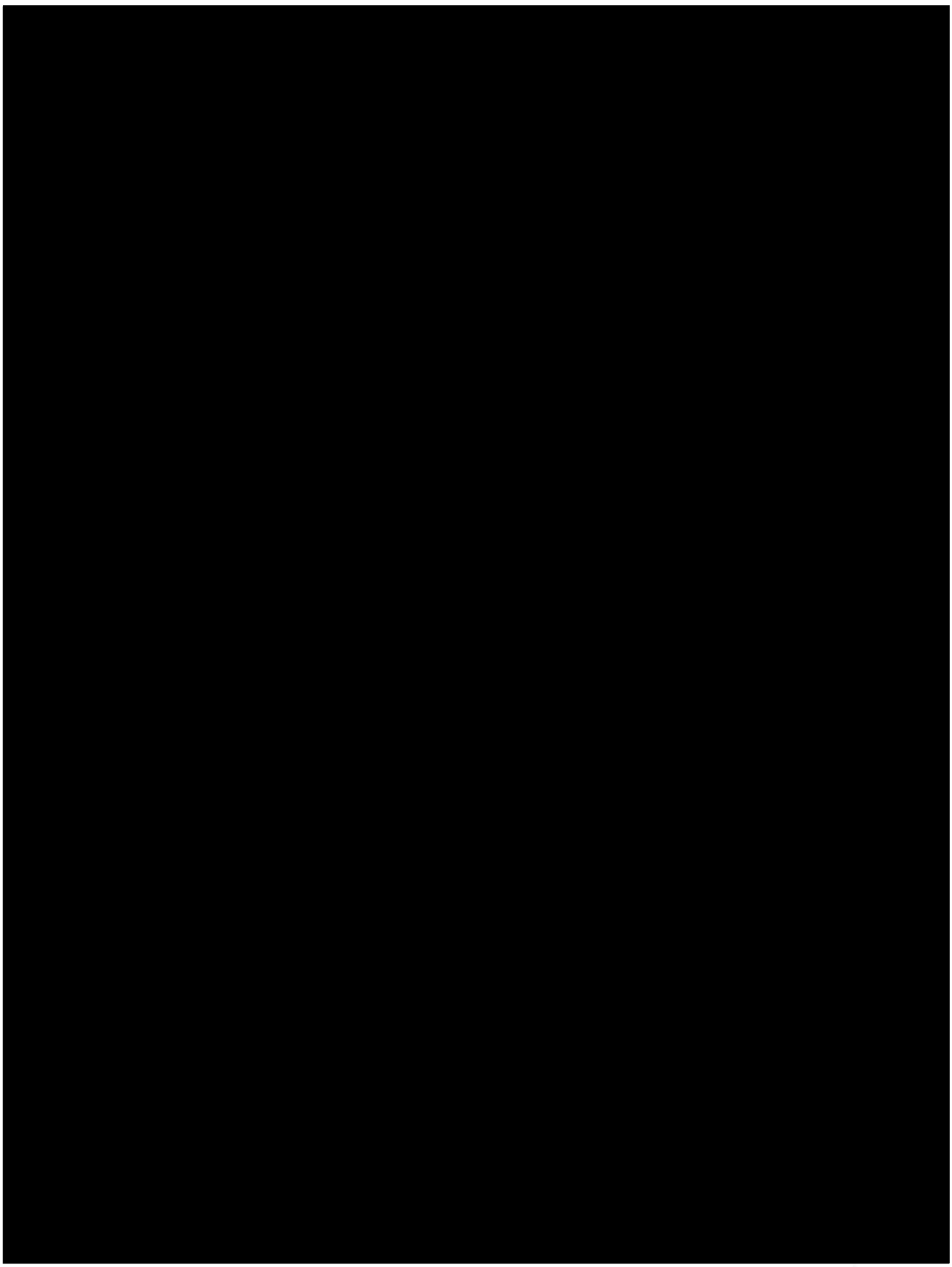

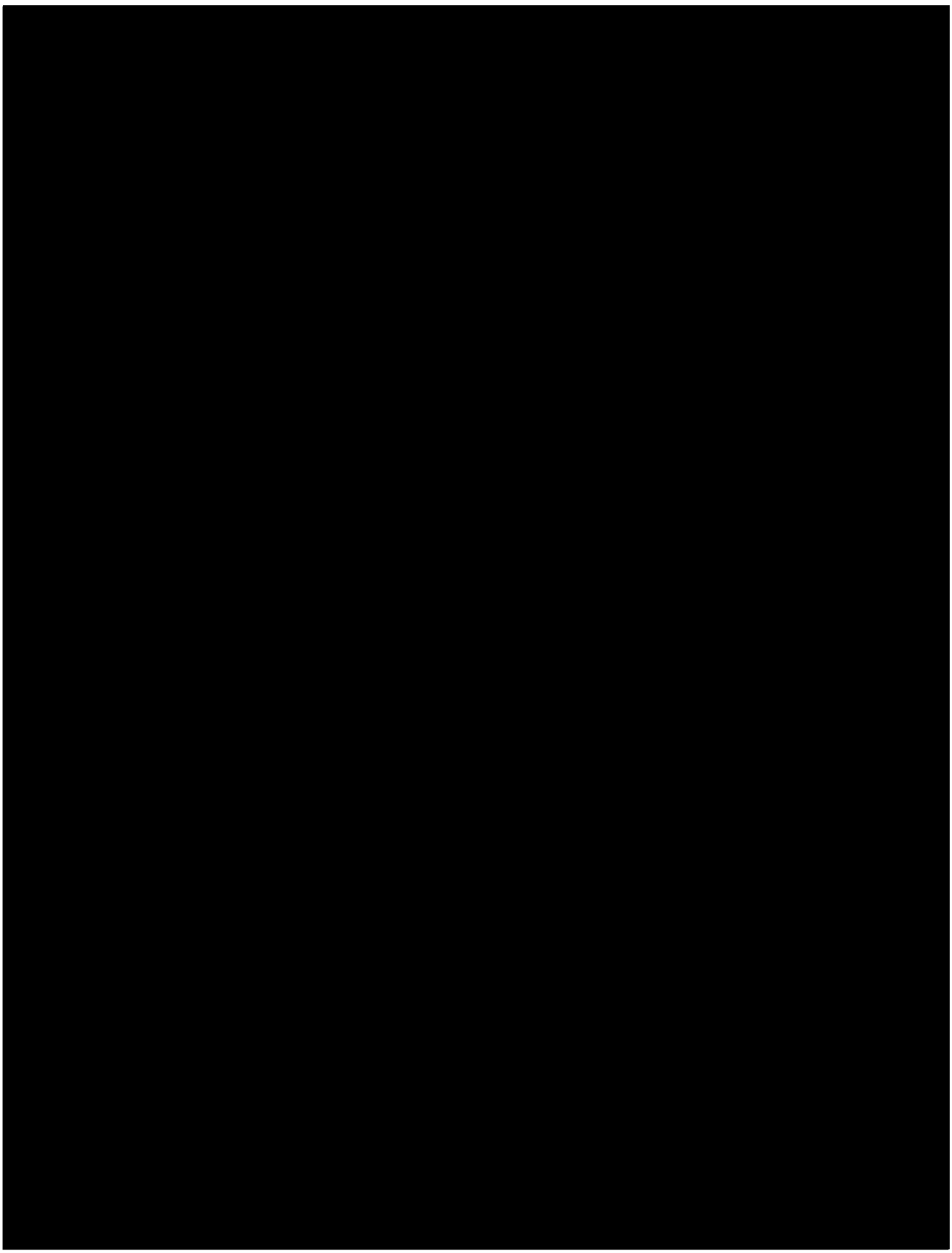

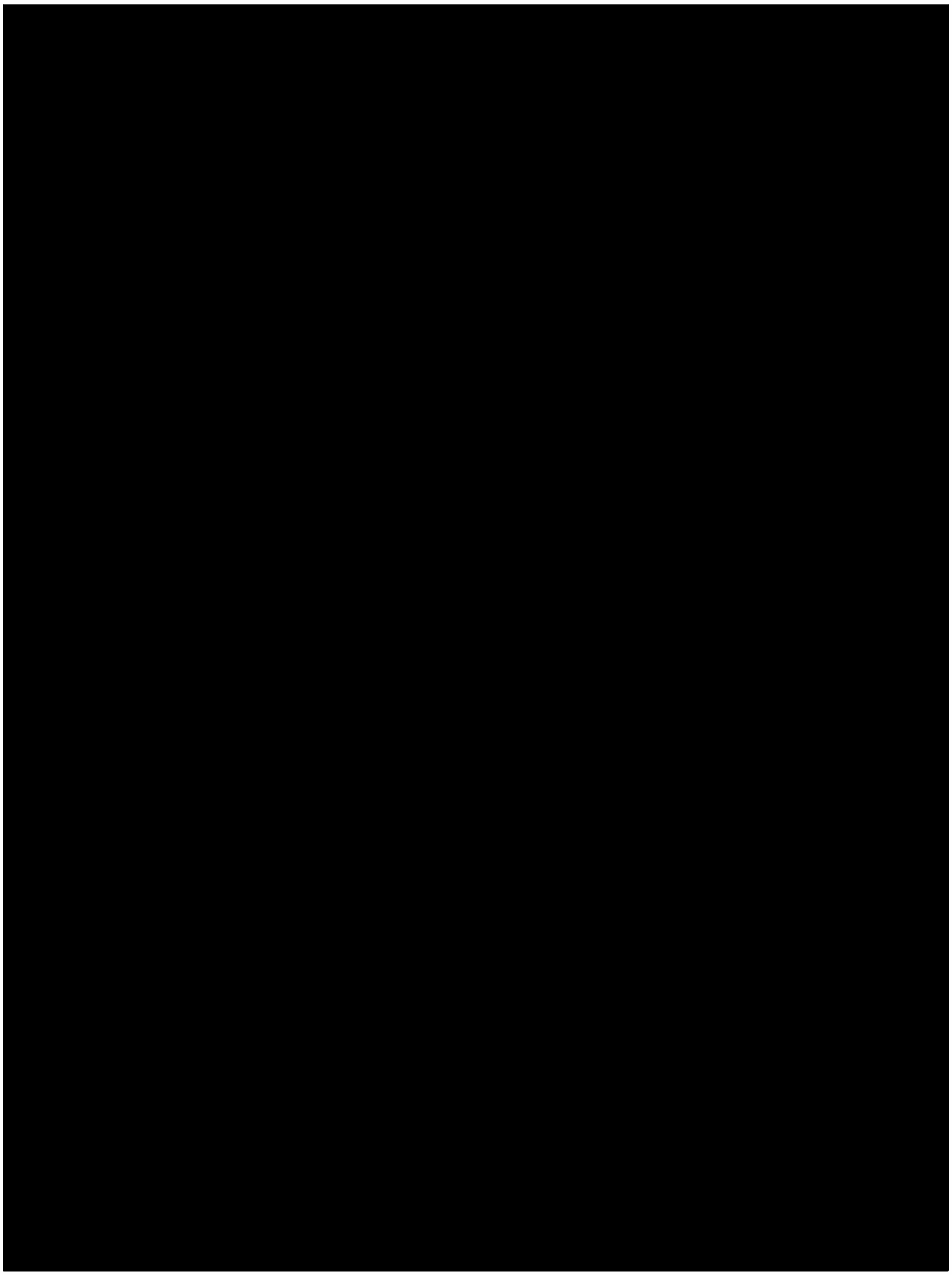

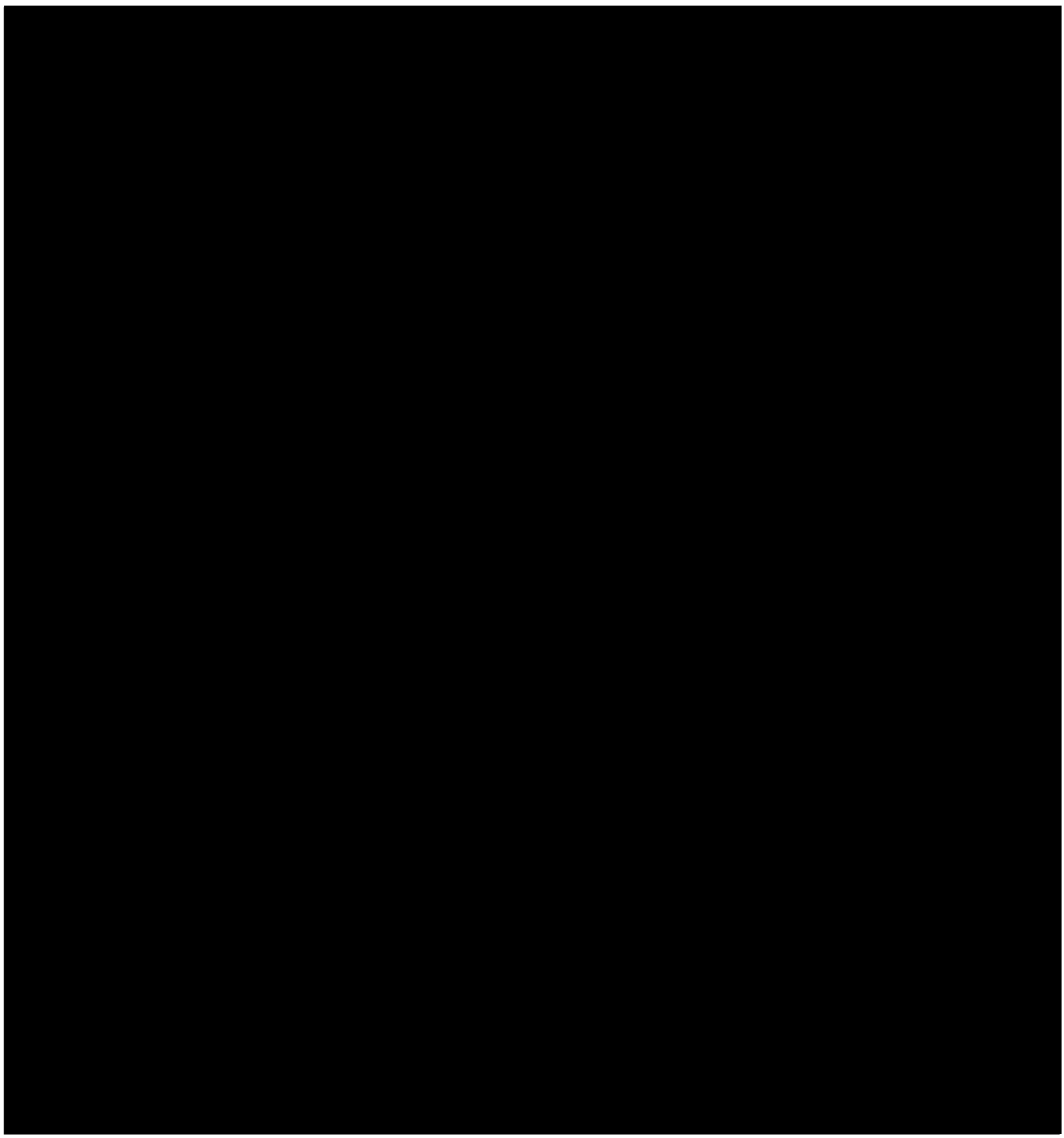

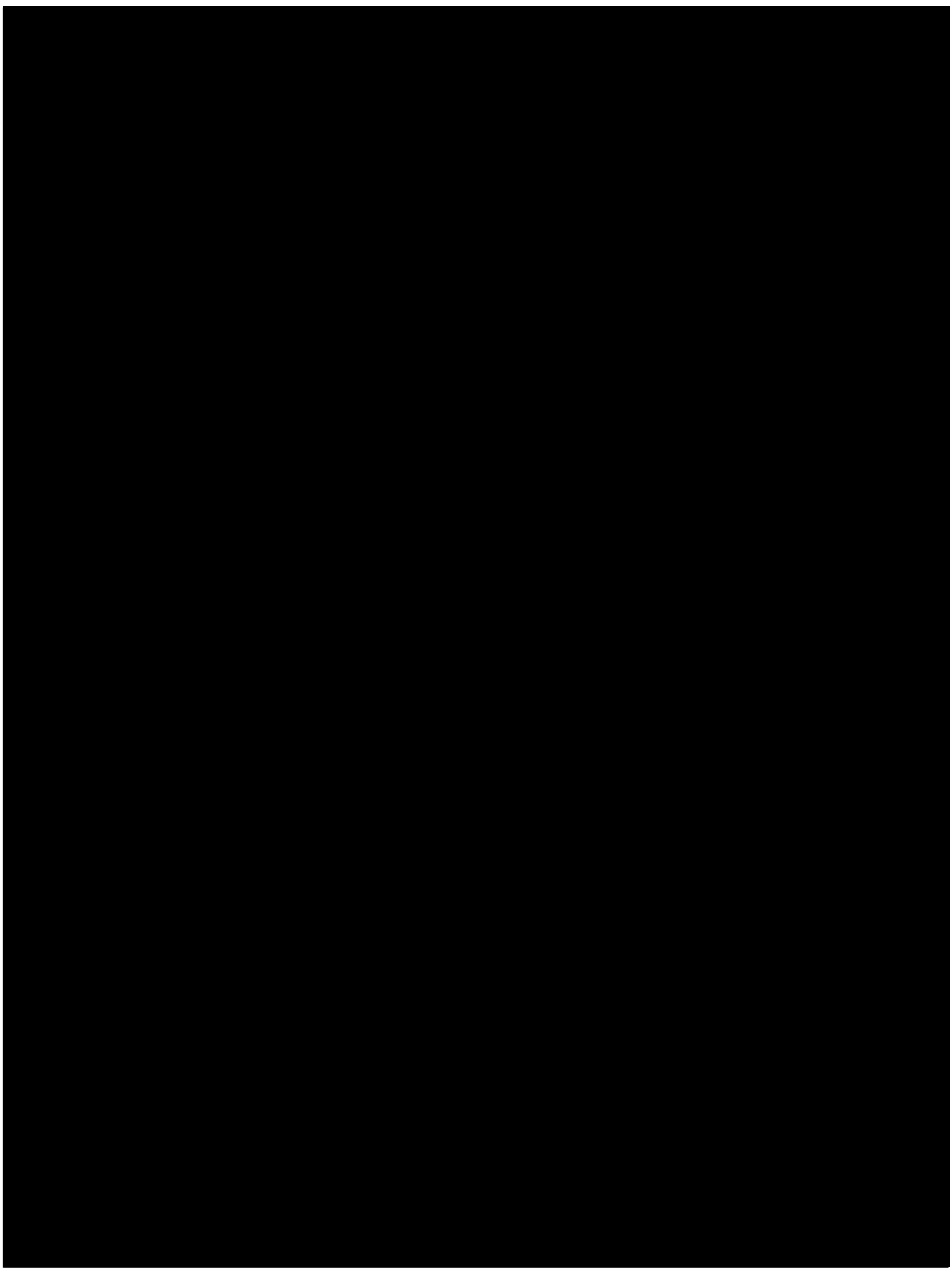

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ABSTRACT

ENFLAGELLATION OF NAEGLERIA FOWLERI

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Organisms of the genus Naegleria differentiate from feeding, dividing amebae into temporary swimming flagellates when deprived of nutrients. Factors critical for enflagellation of the pathogenic species, N. fowleri, were delineated and optimal conditions established for evoking reproducible conversion of N. fowleri populations. N. fowleri enflagellation differed from that of N. gruberi in its dependence upon growth phase, population density during enflagellation and in the timing and extent of the conversion. N. fowleri amebae from stationary phase cultures grown at 37°C, washed free of medium and suspended in nutrient-free ameba saline, acquired flagella and converted to mature flagellates in a synchronous manner beginning 90 minutes after subculture to ameba saline. No prolonged intermediate round phase occurred during N. fowleri enflagellation such as has been seen with N. gruberi. Enflagellation of N. fowleri was prevented by actinomycin D and cycloheximide added at the time of subculture to non-nutrient medium. Delayed additions of

the same inhibitors caused hastened reversion of flagellates, suggesting a requirement for continued synthesis. Ultrastructural changes during N. fowleri enflagellation generally paralleled those observed in N. gruberi except that flagellum outgrowth in the former occurred on cells while still ameboid. An extensive complement of cell polypeptides continued to be synthesized at a reduced level during enflagellation; no limited number of species was made in exceptional abundance. A moderate number of qualitative and quantitative changes were detected in the amounts of individual polypeptides resolved by two-dimensional electrophoresis when amebae enflagellated, some of which also occurred in a non-enflagellating N. fowleri strain and may therefore be related to starvation. Quantitative, computer-assisted densitometric analyses of polypeptide autoradiograms revealed that larger and more acidic proteins were relatively more abundant in amebae than in flagellates and that a correlation existed between molecular size and charge of N. fowleri polypeptides.

Several observations in both N. fowleri and N. gruberi enflagellation implicate regulatory mechanisms in addition to, or coordinated with, de novo protein synthesis. Enflagellation of Naegleria species provides a useful paradigm in which to study the contributions of various regulatory processes upon the expression of a differentiated state in an eukaryotic cell.

INTRODUCTION

The regulatory mechanisms and programs by which eukaryotic cells accomplish the complex changes involved in cellular differentiation have been the subjects of many studies in a number of "model systems." Each system has usually been most instructive in one or a few specific aspects of cell differentiation which the attributes of that system made especially amenable to study. A system which is being studied increasingly as a model of cell differentiation is that of the ameba to flagellate conversion by members of the genus Naegleria (36,45,48,145). This genus is characterized by unicellular organisms which feed, grow and divide as small amebae but which are capable, upon suitable stimulation, of changing into a quite distinct, temporary flagellate stage which swims actively by means of two (occasionally more) anterior flagella, but which does not feed or reproduce (21,45,103).

The ameba to flagellate conversion, first described in 1899 by Schardinger (45,113), is known by a number of different names, the merits of which have been previously discussed (48). The original term given to the process by Schardinger, 'transformation' (Umgestaltung), has generally been avoided to prevent confusion with the changes induced in cultured cells by virus or other tumorigenic agents. The ameba to flagellate conversion is referred to in this

dissertation as 'enflagellation,' a protozoological term which is descriptive of the major new distinguishing structure formed, prior trivial objections not withstanding (48). Occasionally, the conversion will be referred to as 'differentiation.' Naegleria enflagellation certainly qualifies as such if differentiation is defined as a process "in which a whole constellation of characteristics change in a temporally and spatially programmed sequence, culminating in a differentiated cell that has specific macromolecules but also has a different morphology and behavior" (48).

Literature Review

Enflagellation has been brought under precise, impressive control in the laboratory with the ubiquitous soil/water ameba, Naegleria gruberi (36,45,48,50). Large quantities of amebae can be cultivated and stimulated, at the experimenter's desire, to convert almost quantitatively to flagellates. The conversion is characterized by a synchronous, rapid (<90 minutes) and reproducible sequence of events (45,49,50). The ability of experimenters to achieve such control, although partly the result of refined methodologies for growth and for stimulation of the change (50), is largely the result of a number of important attributes of the Naegleria gruberi system. These attributes have been thoroughly described (36,48,50) and include: a) lack of dependence on cell cycle or population

growth phase; b) individual cell response, independent of population density; and c) uniform response (population heterogeneity less than 20 minutes in many cases) to an experimenter-controlled signal which d) precisely defines the starting point for the sequence of changes leading to the flagellate phenotype. The combination of these attributes and the control which they permit for this system has resulted in the promotion of the ameba to flagellate conversion of N. gruberi as a particularly valuable tool for studies on a variety of aspects of cell differentiation (7,36,48,119,125).

The second major species of Naegleria, N. fowleri, is a pathogenic ameba which causes a fatal human disease, primary amebic meningoencephalitis (18,22,25,40,41,91,141) and has been extensively studied in that respect (1,17,26, 28,30,65,66,73,76,89,128). N. fowleri can be distinguished from N. gruberi by a number of characteristics (reviewed in 48 and 67) including temperature tolerance (64), cyst morphology (102,116), immunochemical characters (4,31,130, 142), agglutination with concanavalin A (79), growth in various axenic media (29,136,137), effects on cell cultures (27,89) and pathogenicity for mice (26,45,66). The two species are related by shared antigens (4,130,142) and by those properties of intranuclear karyokinesis, general morphology, and ability to enflagellate which classifies each as a member of the genus Naegleria (21,45,103).

Conditions for isolation and axenic laboratory cultivation of Naegleria fowleri have been developed and optimized (67,68,77,78,95,136). Identification of isolates as Naegleria fowleri has required that enflagellation be seen, yet the methods for these tests have been described poorly, if at all, in most instances and the appearance of flagellates to any extent was considered a positive diagnosis (15,17,19,21,22,40,41,101,103). Most investigators have merely suspended the organisms in water and periodically examined the suspension for appearance of flagellates. Precise experimental control of the enflagellation process in N. fowleri to an extent similar to that achieved with N. gruberi has not been previously reported. It is not known, therefore, whether or not N. fowleri demonstrates the same attributes which permit the level of control achieved with N. gruberi enflagellation.

Optimal conditions for enflagellation of N. gruberi from cultures grown in association with bacteria (45,50) and from axenic cultures (47) have been developed. The stimulus for enflagellation, which has historically been removal of amebae from growth environment and suspension in non-nutrient solutions, has been investigated, critical factors determined, and conditions optimized. The effects of ions (36,46,74,80), osmotic pressure (36,46,145), temperature (45,50,145), aeration (45), agitation (45,50, 145), and ultraviolet light (145) on the initiation and

progress of enflagellation have been delineated. The development of a non-particulate axenic medium (47) led to the discovery of a dialyzable factor in yeast extract that is determinative for enflagellation. This component of the growth medium, individually removed from the medium, permits enflagellation and individually added to non-nutrient suspensions of enflagellating cells, prevents enflagellation (46). Control of N. gruberi enflagellation by synergistic combinations of electrolytes, temperature, agitation and the yeast extract factor has been shown (36,46) and studies using various of these alternate stimuli have shown that enflagellation events can be separated from those of nutritive starvation (48). The results of these studies have been consistent with proposals that cell differentiation cues may take the form of relatively minor alterations in extracellular ion concentrations (9,36) and that physiological determinants may play crucial roles in expression of differentiated states (4,36,48, 49,107). Critical factors for evoking enflagellation in N. gruberi include growth conditions in axenic cultures (47), electrolyte concentrations (<80 mM, 46) incubation temperature during differentiation (50), agitation (36,48), and removal of the specific factor from the growth medium.

N. gruberi enflagellation is especially amenable to studies of the timing of events during differentiation

because synchronous changes in the cell population occur at reproducible times from the experimenter-controlled start signal (36,48,50). Using an approach based upon the ability to evaluate quantitatively changes in individual cells during enflagellation and then assign times to those changes by using measures of central tendency for the population, a number of specific events have been ordered sequentially during enflagellation (36,48,49,56) including times of shape changes, flagella appearance, insensitivity to inhibitors and synthesis of specific molecules. The method of measurement of enflagellation (45,50) and the validity of using times based on 50% change in the population (T_{50}) as measures of relative timing have received considerable analysis (45,49,50) and have been given credibility by the reproducibility and the internal and external consistency of results one obtains in N. gruberi experiments run under a variety of conditions (36). One of the major points of the temporal sequences obtained with different N. gruberi strains, different growth conditions and different enflagellation conditions is that relative proportionalities between times for specific events is maintained (36,56).

The high degree of synchrony and relative completeness of N. gruberi enflagellation permitted detailed, electron microscopic study of the progress of enflagellation on the morphological level (37). Enflagellating N.

gruberi undergoes major rearrangements of cellular organelles (114), which assume fixed positions in flagellate cells. The entire flagellar apparatus is assembled de novo (37), including basal bodies (51), flagella and a rootlet fiber which associates with the nucleus and which may be involved in contractile processes effecting flagella movement (36,37,118). N. gruberi cells progress through a reproducible series of shape changes, from ameboid to spherical cells to elongated, streamlined cells with smooth, fixed contours (49). The shape changes occur in individual cells in a normal distribution with respect to time as revealed by probability analysis (50) and have been used as a model on which to base mathematical simulation approaches to differentiation (108). Unusual, rapid shape changes can be induced in flagellates by external addition of cell-produced factors extracted from flagellates (49). An entire system of internal controls regulating cell shape has been partially delineated as a result of studies using this factor in combination with varying cationic solutions (46,49). A hypothesis for the regulation of cell shape and motility during N. gruberi enflagellation has been postulated (49).

Enflagellation of N. gruberi is prevented by inhibitors of ribonucleic acid and protein synthesis (56,63, 109,146). Addition of inhibitors at appropriate concentrations inhibits enflagellation if added before, but not

after, specific times (transition points) during enflagellation, even though the expected syntheses are still inhibited at the later times (48,56). Shape changes are inhibited at different times than is the appearance of flagella (56). Organelle assembly and requisite syntheses are temporally separate, thus assembly can be studied in the absence of continued synthesis (36,46,56,133). Perturbation of N. gruberi enflagellation by heat shock has shown that the control of organelle number and assembly can also be assigned to a specific time period during enflagellation and is sensitive to the effects of RNA and protein synthesis inhibitors (35,133). Flagellar outer doublet tubulin is different from that present in N. gruberi amebae and is synthesized de novo during enflagellation (52-54,83,84) as are central pair tubulin, dynein, and the flagellar rootlet protein (48,56). A proportionally higher amount of messenger-like ribonucleic acid rather than other RNA types is synthesized during enflagellation (70,75,132,134). In particular, in vitro translatable mRNA for flagellar tubulin cannot be detected in N. gruberi amebae but appears in differentiating cells at times correlating to the appearance of new flagellar tubulin (86) and the appearance of this mRNA can be prevented by actinomycin D (55).

Evidence is accumulating from studies with several model differentiation systems that sequential phenotypic

changes involve processes in addition to transcription and translation of new messenger RNA to form new cell proteins. Coordinated changes in phenotype may also involve modification of existing structural proteins and enzymes (39,43, 110,139), translational control by modification of ribosomes or aminoacyl-transfer RNA synthetases (57,72,85,96, 111,120), selective removal of proteins no longer required (and perhaps detrimental) for the new phenotype (5,123,124), and coordination of rates of protein degradation with those of synthesis (44,92,104,105,121). Developmental changes involving loss of proteins which must occur rapidly or in slow- to non-growing cells are dependent upon either compartmentalization (81), secretion (124) or some form of degradation (62,71) as opposed to the dilution-by-division capability in rapidly growing microorganisms. Single deficiencies in a proteolytic enzyme can result in arrested differentiation, as has been demonstrated by the isolation of sporulation mutants which lack an intracellular protease (23) and of temperature-sensitive variants with a demonstrated lesion in the structural gene of an intracellular protease (82).

Cell number, total ribonucleic acid and total cell protein remain constant during enflagellation of N. gruberi (56); therefore, the de novo synthesis of tubulin and other required proteins must utilize intracellular precursor pools or be balanced by degradation of pre-existing

proteins. Fulton (48) has reported that differentiation can occur, under certain conditions, without starving N. gruberi and that a general increase in protein degradation is not observed under those conditions. Enflagellation of N. gruberi in non-nutrient buffer, however, is accompanied by protein degradation measured at approximately 6% of total protein per hour and enflagellation can be prevented by serine protease inhibitors which reduce the rate of degradation to 1% per hour (48). Thymidine kinase activity has been shown to decrease during N. gruberi differentiation and it has been postulated that the decrease is due to a cycloheximide-inhibitable activation of a degradation mechanism (11). The decrease in thymidine kinase activity is due to loss of enzyme and occurs when cells differentiate in the presence or absence of nutrients (24). The level of enzyme and activity increases when flagellates revert to amebae and resume growth in nutrient medium (24). Selective protein degradation as well as protein synthesis is therefore an integral part of Naegleria enflagellation.

The enzymatic mechanisms and features of protein structure which influence the selectivity of protein degradation are still largely unknown, although correlations have been observed between degradative rates and subunit molecular size (33,60), isoelectric point (32,34, 61,62), hydrophobicity (10) and rate of spontaneous

denaturation (12,71). Some of the factors which have been correlated with degradative rates may not be independent variables of the polypeptides. Relationships between subunit size and isoelectric points of polypeptides have been observed by several workers using different eukaryotic models (42,99) and the potential interrelatedness of size, charge and turnover characteristics has been recognized (42). Furthermore, those proteins in eukaryotic cells that degrade most rapidly seem to have synthetic rates that change dramatically, whereas the proteins with slow degradative rates appear to have relatively constant rates of synthesis (62,92,104). Accordingly, the steady-state levels in eukaryotic cells of proteins that are synthesized or degraded rapidly are most rapidly responsive when new conditions are imposed (14,104). The enflagellation of N. fowleri appears to be a useful model in which to study relationships between inherent properties of proteins and their regulation during a differentiation event.

Statement of the Problem

The research reported in this dissertation has endeavored to meet several objectives. One objective has been to determine those factors which are critical for evoking amebae of Naegleria fowleri to convert to flagellated cells. Optimized conditions of growth and enflagellation which result in synchronous, near complete

conversion of amebae into flagellates in a temporally reproducible manner have been sought. Standardization of optimum conditions for obtaining reproducible enflagellation is needed for possible studies on regulation during enflagellation in addition to providing a more systematic method for evaluating whether or not clinical isolates are amebo-flagellates.

Means to arrest or alter the course of enflagellation of N. fowleri are needed in order to gain insight into those processes required for enflagellation and to assist in delineating stages of the process. Incorporation data have been used to determine the nature of syntheses occurring during enflagellation. Ultrastructural studies, in conjunction with the biochemical approaches, are required in order to establish reference points during N. fowleri enflagellation.

The contribution which regulation of pre-existing proteins makes to the differentiation of Naegleria has been studied previously only with respect to total cell protein (48) or a single enzyme (11,24). A description of regulatory changes in the levels of a substantial fraction of the cell's individual polypeptide species is needed to make a more accurate assessment of this contribution to Naegleria enflagellation. Information on physical properties of these individual polypeptides is needed to

determine if their regulation is related to any inherent qualities of the polypeptides.

Rationale

The effects of a variety of growth and enflagellation conditions were tested with respect to enflagellation of Naegleria fowleri. Axenic conditions, rather than bacteria-associated cultivation, were desired for cultivation of amebae to be used in enflagellation experiments in order to eliminate the contributions of the bacterial structures, macromolecules and products to analyses of enflagellation. Experiments with Naegleria gruberi were performed to validate that several variables, previously described as critical or non-critical for its enflagellation, had the same effects under our laboratory conditions. Differences in the response of N. fowleri enflagellation to those factors could then be attributed to differences in the two systems rather than differences due to laboratory conditions. By identifying critical factors for enflagellation, optimal conditions for enflagellation of some, but not all, strains of N. fowleri were determined and utilized for remaining experiments. An alternative method of enflagellation initiation, in which amebae on the surface of unagitated tissue culture flasks were washed free of nutrients without centrifugation, was developed and used for several reasons. First, washing of the amebae by the rapid centrifugation method (Method C, Ref. 45, see

Materials and Methods) requires a number of manipulations of cells which extends the time between removal of amebae from the incubator and their suspension and agitation in non-nutrient buffer. This time delay is a factor which should be kept to a minimum for optimum enflagellation of amebae (45,50). Second, amebae are damaged by incubation as pellets (45,48,50) and this could be avoided completely by the use of the alternate procedure. Third, the use of the new protocol made it possible to carry out all of the washing and suspension steps in the original culture vessels, minimizing the number of physical manipulations of amebae and simplifying handling of radiolabeled cells.

The effects of inhibitors of RNA or protein synthesis on N. fowleri enflagellation were tested as potential means to arrest differentiation and the incorporation of selected radiolabeled precursors was assayed during enflagellation.

The finding that a number of strains of N. fowleri maintained in our laboratory would not enflagellate under conditions optimal for other strains (nor any other conditions tried) enabled use of these strains as aids to distinguish between effects resulting from nutritional deprivation and those more directly pertaining to morphogenesis of N. fowleri. Electron microscopic studies of the enflagellation process were therefore performed using an enflagellating strain and a non-enflagellating variant.

In order to determine the extent to which changes in the amounts of individual N. fowleri polypeptides occur during enflagellation, advantage was taken of the development of two-dimensional electrophoretic methods which permit resolution of hundreds to thousands of individual cell polypeptides from whole cell preparations (97,99,100). Two-dimensional electrophoresis has been used to study individual polypeptide changes occurring during differentiation and development in a variety of model systems (2,3,38,69,98,126). Additionally, these two-dimensional techniques, utilizing isoelectric focusing in the first dimension and sodium dodecyl sulfate gel electrophoresis in the second dimension, resolve polypeptides by isoelectric charge and subunit molecular size; the position of resolved polypeptides may therefore be used to gain information on the relationships of size and charge parameters and their potential correlation to regulatory phenomena.

Proteins of enflagellating and non-enflagellating strains of Naegleria fowleri amebae were radiolabeled during growth and whole cell extracts of these amebae separated by two-dimensional electrophoresis. Similarly, whole cell extracts of flagellates or of the non-enflagellated amebae, derived when these growth-labeled amebae were subjected to enflagellation conditions, were also resolved on two-dimensional gels. A visual comparison of autoradiograms of these gels permitted identification

of differences in the polypeptide patterns of amebae and flagellates and, by using the non-enflagellating ameba patterns in a comparison similar to that of Trew et al. (126), allowed tentative discrimination of those differences related to starvation effects. A comparison of prelabeled phosphorylated proteins in amebae and flagellates was also performed by two-dimensional electrophoresis.

Finally, autoradiograms of amebae and flagellate gel-resolved polypeptides were analyzed to characterize and compare the polypeptides with respect to molecular size, charge, number of species and relative amounts. Computer assistance in analyses of complex, changing polypeptide patterns such as those described herein is a rapidly developing technology (13,58,59,88) and has been used in these analyses to speed, organize and simplify the characterizations of N. fowleri ameba and flagellate polypeptides.

MATERIALS AND METHODS

Strains

Naegleria fowleri strains nN68, Lovell, KUL, nN69-1, HB-5, nN69-2, nN67, 0359, GJ, HB-4, NF66, and NF69 were obtained from Dr. David T. John in whose laboratory they had been maintained with bi-weekly transfers in unagitated axenic cultures in Nelson medium (95,136, see below) with 2% (v/v) calf serum. Sources and references for the strains are presented in Table 1. *Naegleria fowleri* has been identified as the causative agent in cases of primary amebic meningoencephalitis (15,18,22,26), therefore certain precautionary measures were taken even though cases are rare and no laboratory infection has ever been reported. Masks were used when handling large numbers of amebae or when aerosols were likely to be generated and all used biological materials were decontaminated and autoclaved. Strain nN68, used in the majority of these studies, was formerly designated LEE (41,136) and has been deposited in the American Type Culture Collection as ATCC-30894.

The strain of *Naegleria gruberi* used in these studies was NEG-M, also obtained from Dr. John, and by him from Dr. Chandler Fulton (Brandeis University, Waltham, MA). Strain NEG-M was chosen for its ability to grow on an axenic medium, M7 (47), which is less complex than the

Table 1

Strains of Naegleria fowleri Used, Histories and References

Strain	Isolator	Location and Date of Isolation	Reference
nN68 (formerly Lee, deposited as ATCC-30894)	E.C. Nelson	Virginia, 1968	(41)
Lovell	S.L. Chang	Florida, 1974	(29)
KUL	E. van der Driessche	Belgium, 1973	(128)
nN69-1 (formerly TY)	E.C. Nelson	Virginia, 1969	(41)
HB-5	G.S. Visvesvara	Texas, 1977	None
nN69-2 (formerly WM)	E.C. Nelson	Virginia, 1969	(41)
nN67 (formerly CJ)	E.C. Nelson	Virginia, 1967	(41)
0359	J.B. Jadin	Belgium, 1970	(73)
GJ	C. Baro	Florida, 1972	(140)
HB-4	R.B. Finley	North Carolina, 1977	None
NF66	R.F. Carter	Australia, 1966	(17)
NF69	M. Fowler	Australia, 1969	(17)

Balamuth medium generally used for axenic maintenance and cultivation of N. gruberi in the laboratory (137). Strain NEG-M is a variant of strain NEG which is capable of growth in M7 medium. Strain NEG was clonally derived from an environmental isolate, EG, obtained by F.L. Schuster (45,115). Clonal strains of Naegleria gruberi NEG and NEG-M were deposited by Fulton in the American Type Culture Collection as 30223 and 30224, respectively.

Media

Amebae of Naegleria fowleri were maintained and cultivated axenically in Nelson medium (68,95,136) which contained 2% (v/v) calf serum (Gibco, Grand Island, NY), 0.1% (w/v) glucose, 0.1% w/v liver digest (Panmede, Harrisons and Crosfield, Bronxville, NY) in ameba saline. Routinely, ameba saline (10l) containing 120 mg NaCl, 142 mg Na_2HPO_4, 136 mg KH_2PO_4, 4 mg $MgSO_4$-$7H_2O$ and 4 mg $CaCl_2$-$2H_2O$ per liter of deionized water was prepared and stored in large quantities for general use. One liter batches of Nelson medium (glucose and liver digest in ameba saline) were prepared without the serum component, distributed in 100 ml aliquots to clean serum bottles and autoclaved. The media were cooled, allowed to equilibrate for at least two days before use (136) and stored at room temperature. Sterile calf serum, as supplied (Gibco, Grand Island, NY), was distributed into 2.5 ml portions and stored frozen at -20°C. One tube of serum was thawed per 100 ml bottle

of medium required for each experiment and 2.0 ml of serum added per bottle of medium before distribution to culture vessels. The final pH of the medium was approximately 6.5, previously determined to be the optimum pH for unagitated culture initiation of N. fowleri (136). When amebae were to be radiolabeled with [^{32}P]-inorganic phosphate, they were grown in Nelson medium made in ameba saline in which 2 mM MOPS buffer (morpholinopropanesulfonic acid, Calbiochem, La Jolla, CA) replaced the phosphate salts.

Two major types of media were used for maintenance and cultivation of Naegleria gruberi amebae. Balamuth medium (6) consisted of 0.5% (w/v) glucose, 0.5% (w/v) yeast extract (Difco, Detroit, MI), 1.0% (w/v) liver digest (Panmede, see above) and 1.0% (w/v) proteose peptone (Difco) in ameba saline (101). One liter of medium was routinely made, pH adjusted to 6.55, distributed to 100 ml serum bottles and autoclaved. The medium was cooled, allowed to equilibrate at least two days (137) at room temperature and stored at room temperature. Cultivation of N. gruberi strains in Balamuth medium required addition of hemin to a concentration of 1.0 µg/ml (8). Hemin was made in a stock solution of 1.0 mg/ml in 1.0 N NaOH, filter sterilized (Millipore HA, pore size 0.45 µm, Millipore Corporation, Bedford, MA) and stored at 4°C. Hemin was added aseptically to the correct concentration at the time of culture inoculation. The final pH of

Balamuth medium after autoclaving was 6.5, previously determined (137) to be the optimum pH for growth initiation of N. gruberi cultures.

M7 medium was used for cultivation of N. gruberi NEG-M and contained 0.5% (w/v) yeast extract (Difco Labs, Detroit, MI), 0.03 M glucose, 0.3 mM L-methionine and 7 mM KH_2PO_4/Na_2HPO_4 buffer (47). Fulton's original formulation contained 10% (v/v) high molecular weight component of fetal calf serum as prepared by dialysis and 35,000 x g clarification (47). The component was replaced in these studies by commercial dialyzed calf serum (Gibco, Grand Island, NY). Routine preparation of M7 medium without the serum component was performed as previously described (47). Bottles of sterile M7 medium without serum were stored at 4°C. Dialyzed calf serum (Gibco), as supplied, was distributed aseptically in 8 ml amounts into sterile 15 ml test tubes and frozen at -20°C. These tubes were thawed as needed prior to use in culture preparation.

One set of experiments (Fig. 10) required the use of a hybrid medium consisting of equal parts of Balamuth medium (without hemin) and Nelson medium (prior to addition of serum) to which was added hemin (to 1 µg/ml) and calf serum to 2% (v/v) prior to inoculation. This medium, termed (1:1 Nelson/Balamuth), was used to grow N. gruberi and N. fowleri.

Cultivation

Naegleria fowleri strains were maintained at 30°C in unagitated stock cultures in Nelson medium with 2% calf serum as previously described (77,136). Naegleria gruberi strains were maintained at 25°C in unagitated stock cultures in 25 cm^2 tissue culture flasks (Falcon Plastics, Oxnard, CA) with 10 ml Balamuth medium containing hemin. Stock cultures of N. gruberi NEG-M were also maintained in unagitated stock cultures at 32°C in 25 cm^2 tissue culture flasks in 10 ml M7 medium with 8% dialyzed calf serum.

Amebae of N. fowleri and N. gruberi were grown axenically in agitated and unagitated cultures for experiments. Agitated cultures were grown in siliconized 50 ml Erlenmeyer culture flasks with screw-cap closures containing 10 ml of the appropriate medium. Amebae were inoculated at 2 x 10^4 amebae/ml into pre-warmed medium and the culture flasks were agitated on a gyrotory incubator shaker (New Brunswick Scientific Company, New Brunswick, NJ) operating at 130 rpm and 32°C. The large majority of experiments were performed using amebae grown in unagitated cultures on the surface of tissue culture flasks. Amebae of N. gruberi were inoculated at 2 x 10^4 amebae/ml in 5 ml (Figs. 10 and 11, Table 6) or 10 ml medium in 25 cm^2 tissue culture flasks (Falcon 3012, Oxnard, CA) and were incubated without agitation at 32°C or 25°C as indicated. Amebae of N. fowleri strains were inoculated at

10^5 amebae/culture in 25 cm^2 tissue culture flasks (Falcon 3012) containing 5 ml Nelson medium and were incubated without agitation at 37°C, unless noted otherwise. In several experiments (Table 9, Figs. 40 to 51, Table 11, Fig. 54), larger numbers of N. fowleri amebae were grown in 75 cm^2 tissue culture flasks (Falcon 3024) containing 10 ml Nelson medium, inoculated at 3 x 10^5 amebae/flask and incubated at 37°C without agitation.

Cell Counting

Cell counts were performed with an electronic particle counter (Coulter Counter, Model ZB, Coulter Electronics, Hialeah, FL) as previously described (135,136). Prior to counting, Coulter cuvettes containing the samples diluted in formalin-electrolyte counting solution were inverted several times to suspend settled cells and were read after the bubbles dispersed. Three successive counts were taken for each cuvette. For counts of 10^4 or higher, a coincidence correction chart was consulted for the adjusted true count. This corrected for aggregates of cells and coincident passage of cells through the aperture. Occasionally, cell counts were performed on very concentrated suspensions or on small volumes such that 0.2 ml samples were too large. In those cases, 20 µl of suspension was added to 180 µl of electrolyte solution and then 9.80 ml of electrolyte solution added to make the final dilution 10-fold higher than normally used. Counts

from the Coulter counter were then multiplied by a factor of 1,000 to give amebae/ml.

Enflagellation

The enflagellation of amebae growing in agitated cultures was evoked by the rapid centrifugation/suspension method described previously (Method C, 45). Amebae growing in agitated cultures were transferred to sterile 50 ml polypropylene screw-capped centrifuge tubes and rapidly sedimented by centrifugation for 45 seconds in a clinical centrifuge (Model CL, International Equipment Company, Needham Heights, MA) at the highest setting (∿ 3,100 rpm, ∿ 1,500 x g). The rotor was brought quickly to a stop, the growth medium decanted and the amebae quickly suspended in 10 ml TK buffer (2 mM Tris HCl, 10 mM KCl pH 7.2, 47) at 25°C. The amebae were centrifuged again, suspended in a second 10 ml of TK buffer and sedimented once more. The pellets from the last wash were suspended in 6 ml TK buffer, transferred to sterile, siliconized 50 ml Erlenmeyer culture flasks and agitated at 120 rpm on a water bath shaker (Model G76, New Brunswick Scientific, New Brunswick, NJ) at 25°C. All steps were completed within 8 to 10 minutes from the time at which the amebae were first suspended in non-nutrient buffer. In all experiments reported in this dissertation, times during enflagellation were measured from the time at which cells were first exposed to non-nutrient solutions, designated

time zero (t_o). In one experiment (Fig. 5), amebae of an unagitated culture of N. gruberi were suspended in the overlying medium, then transferred to a centrifuge tube, washed, and agitated in TK buffer as described above.

In the remainder of the experiments, enflagellation of amebae grown in unagitated cultures was evoked routinely by a procedure designed to eliminate the centrifugation steps and to speed and simplify the initiation of enflagellation (see Rationale). In the new procedure, detailed in scheme 1, growth medium and suspended cells were decanted from amebae growing on the surface of unagitated tissue culture flasks. At time zero of enflagellation, the amebae were washed twice with 5 ml non-nutrient buffer pre-warmed to the desired temperature and finally suspended in the desired volume of non-nutrient buffer by vigorous agitation of the culture flasks. The amebae were kept in suspension by agitation of the suspension (6 ml) in 25 cm^2 tissue culture flasks (Falcon 3012) turned upright and fastened by clamps onto the platform of a water bath shaker (Model G76, New Brunswick Scientific, New Brunswick, NJ), which was operated at 180 rpm and the desired temperature. When amebae were grown in 25 cm^2 tissue culture flasks (Falcon 3012), suspensions of amebae in buffer could be agitated in the original culture vessel. When amebae were grown in 75 cm^2 tissue culture flasks, rinse volumes were increased proportional to surface area and

Scheme 1

Amebae growing in unagitated 25 cm^2 tissue culture flask.

↓

Medium and suspended cells decanted (into decontamination fluid).

↓

5 ml of non-nutrient buffer (ameba saline, M7 buffer, TK buffer, etc.), pre-warmed to desired enflagellation temperature, added along top of flask (away from adherent cells), then rocked gently over the cells at time zero.

↓

First rinse decanted, repeat with a second 5 ml aliquot, decant.

↓

Suspended attached amebae in a fresh volume of non-nutrient buffer by vigorous agitation.

↓

Amebae kept in suspension by agitation in tissue culture flasks (Falcon 3012) fastened into clamps on water bath shaker platform operated at enflagellation temperature desired.

the final suspensions of cells were transferred to the same type of 25 cm^2 tissue culture flask (Falcon 3012) so that the same relationship of suspension volume, agitation and aeration was maintained in all experiments. When large numbers of amebae were required, the washed amebae from a number of flasks (either 25 cm^2 or 75 cm^2) were suspended in a single small volume of solution to give a concentrated cell suspension which could then be diluted as appropriate into various test vessels to give the desired concentrations of amebae and any other test substance in 6 ml of non-nutrient buffer.

Several non-nutrient buffers were used for enflagellation of N. gruberi or N. fowleri. N. gruberi NEG-M was generally enflagellated in M7 buffer (7 mM phosphate, pH 6.8, equivalent to M7 medium without nutrient sources), but TK buffer (see above), ameba saline (see above), deionized water and several variations of M7 medium (Table 6) were used in some experiments.

Amebae of Naegleria fowleri were generally enflagellated in Page ameba saline, although experiments were done in which the amebae were suspended in TK buffer, deionized water or variations of Nelson medium (Table 6). In one set of experiments (Table 7), enflagellation was performed in variations of Page ameba saline lacking various cations (see following page). Amebae to be labeled with [^{32}P]-inorganic phosphate were enflagellated in

Page Ameba Saline

Stock Solution	Stock Recipe (g/100 ml Deionized H_2O)	ml of Stock / 100 ml Page Saline	Final Concentration in Ameba Saline
NaCl (50 mM)	0.288	4.1	2.05 mM
$MgSO_4$ (1 mM)	0.025 ($-7H_2O$)	1.74	1.74×10^{-5} M
$CaCl_2$ (1 mM)	0.015 ($-2H_2O$)	2.72	2.72×10^{-5} M
Na_2HPO_4 (0.1 M)	1.42	1.0	1 mM
KH_2PO_4 (0.1 M)	1.36	1.0	1 mM

Page minus Na^+

- 50 mM KCl (0.186 g/50 ml) substituted for NaCl stock.
- 0.1 M K_2HPO_4 (0.87 g/50 ml) substituted for Na_2HPO_4 stock.
- $[K^+]$ concentration increased by 5-fold.

Page minus K^+

- 0.1 M NaH_2PO_4 (0.69 g 1-hydrate/50 ml) substituted for KH_2PO_4 stock.
- $[Na^+]$ concentration increased x 1.25.

Page minus Ca^{++}

- 1 mM $MgCl_2$ (10.2 mg 6-hydrate/50 ml) substituted for $CaCl_2$ stock.
- $[Mg^{++}]$ concentration increased x 2.5.

Page minus Mg^{++}

- 1 mM $(NH_4)_2SO_4$ (6.6 mg/50 ml) substituted for $MgSO_4$ stock.
- $[NH_4+]$ concentration increased from zero to 3.5×10^{-5} M.

ameba saline containing 2 mM MOPS buffer replacing the phosphate salts.

Assay of Enflagellation

The progress of enflagellation was monitored essentially as previously described (45,50). Samples were removed from the agitating suspensions with Pasteur pipets and 1 drop of suspension was added directly to 2 drops of Di Anton iodine (1 g KI and 1.5 g powdered iodine crystals per liter deionized water) which immediately fixed the cells. In order to simplify assay of the enflagellation, the iodine fixative stain was placed into numbered, flat-bottomed wells of a 96-well microtiter test plate (Falcon 3042 Microtest II) and the timed samples from the different test suspensions were added in sequence to these wells containing fixative. After the experiments, the flat-bottomed wells allowed direct microscopic observation of the fixed samples in each well. Both inverted light and phase-contrast microscopes were used. At least 100 cells in each fixed sample were counted and scored as either amebae, round cells or flagellate-shaped cells by light microscopy as previously described (45,49,50). When the appearance of flagella on cells was the assayed property, phase-contrast microscopy was used as previously described (45,50). Validation and quantitative assessment of the methods of measurement of enflagellation have been previously described (50). The definition, derivation and use

of reference time points during enflagellation (e.g. T_{50}, TP_{50}) have also been reported previously (36,45,50,56).

The progress of enflagellation was also checked during the experiments by quickly removing flasks containing the suspensions from the shaker platforms and observing the suspensions through the tissue culture vessels with an inverted light microscope. The suspensions were then immediately returned to the shaker so as not to influence the enflagellation adversely. This procedure verified times at which cells began to spin or swim with directed motility in the suspensions. Qualitative comparisons between flasks in an experiment could be made upon the basis of these quick visual observations but estimates of the percentage of flagellates present in suspension by visual observation were generally higher by a small margin than were the quantitative assays.

Mutant Isolation Attempts

Attempts were made to isolate non-enflagellating variants of Naegleria gruberi NEG-M by taking advantage of procedures outlined previously (45) for the cloning of single amebae. Potentially mutant cells were selected from suspensions of N. gruberi NEG-M which had achieved maximum enflagellation (90 to 100 minutes after t_o). Ameboid cells were carefully removed from dilute suspensions using very fine drawn-out Pasteur pipettes observed under the light microscope. Single drops of suspension

containing less than 20 such ameboid cells were placed on dilute agar plates (NM, 45) with 0.1 ml of bacterial suspension (Klebsiella pneumoniae, 48) and were distributed over the surface of the plate with a sterilized glass spreader. The plates were incubated at 32°C. Bacterial lawns appeared within hours; within 48 hours small plaques could be observed in the lawns. These were observed to be colonies of amebae ingesting the bacteria. Ameba clones from each of the plaques were cultivated individually by punching out plugs of the plaques with sterile Pasteur pipettes and placing the plugs into 15 ml test tubes with 1 ml M7 medium. Penicillin G (500 units) and 500 µg Streptomycin sulfate (Microbiological Associates, Walkersville, MD) were included to prevent growth of bacteria. Amebae were suspended from the plugs into the medium by vortexing and then were allowed to grow on the side of the test tube incubated on a horizontal slant at 32°C. After an initial lag period of 1 to 2 days, amebae from the plugs proliferated and could be used to initiate unagitated cultures in 25 cm^2 tissue culture flasks with M7 medium containing no antibiotics. These cultures were grown and tested for ability to enflagellate using the routine procedures.

Inhibitors

Cycloheximide (Sigma Chemical, St. Louis, MO) and actinomycin D (Sigma Chemical, St. Louis, MO) were prepared

in 200 µg/ml stock solutions in M7 buffer (when used in N. gruberi studies) or in Page ameba saline (for N. fowleri studies). Stock solutions were filter sterilized (Millipore HA 0.45 µm pore size, Millipore Corporation, Bedford, MA) and stored at 4°C. Sterile portions of these solutions were pre-warmed to appropriate corresponding temperatures prior to addition to cultures or to enflagellating suspensions. All solutions containing actinomycin D were covered with aluminum foil to prevent light inactivation of the inhibitor.

Electron Microscopy

Samples of Naegleria fowleri amebae from growing cultures or from suspensions in ameba saline were fixed by adding an equal volume of cold 4% glutaraldehyde. The glutaraldehyde was prepared in Sorensen phosphate buffer (100 mM), pH 7.2, containing 0.85% NaCl (576 mOsmol). Cells were immediately sedimented by centrifugation, suspended in 2% glutaraldehyde, and stored at 4°C overnight. The fixed cells were rinsed twice with cold buffer and then treated with cold, buffered 2% osmium tetroxide for 90 minutes. After two rinses with buffer, the samples were dehydrated through a graded series of ethanol and then transferred to propylene oxide. Similar volumes of cells in propylene oxide and of an Epon 812-Araldite 502 resin formulation (94) were equilibrated for 2 hours. Samples of biological material were then transferred to embedding

mixture (94) for overnight equilibration. Samples were transferred to embedding molds and polymerized at 60°C for 2 days. Ultrathin sections were stained with saturated aqueous uranyl acetate followed by lead citrate (112) and examined in an RCA EMU-3F or an Hitachi HU-12 electron microscope operating at 100 and 75 kV, respectively.

Radiolabeling Enflagellating Suspensions

Amebae of N. fowleri were grown in unagitated cultures at 37°C in Nelson medium and stimulated to enflagellate by washing and suspension in ameba saline by the routine methods described above. Radiolabeled precursors were either present in the final volume of suspending ameba saline or were added to flasks immediately after suspension of amebae into ameba saline. Amebae were washed and suspended in ameba saline containing MOPS buffer (see Enflagellation) when radiolabeling with [^{32}P]-inorganic phosphate.

Radiolabel Incorporation Assays

Samples (5 µl) of cell suspensions in non-nutrient buffer or growth media were removed immediately after addition of isotopes and deposited on filter discs (Whatman GF/A glass fiber discs, Clifton, NJ) which were dried and counted by liquid scintillation spectrometry in order to determine actual amounts of precursor present in the suspensions. Incorporation of radiolabeled amino acids into

trichloroacetic acid (TCA) precipitable material was measured. Samples of cell suspensions (50 µl) were removed into 12 x 75 mm test tubes containing 1.0 ml of a solution consisting of 0.05 N NaOH, 100 µg/ml bovine serum albumin (BSA) as carrier protein, and 10 mg/ml of the corresponding unlabeled amino acid. Tubes were incubated at 37°C for 15 minutes to hydrolyze charged transfer RNA (86,133) after which macromolecules were precipitated by addition of 1.0 ml of 25% TCA at 4°C, mixing and incubation on ice for 1 hour. Precipitates were collected by filtration onto glass fiber filters (Whatman GF/A, Clifton, NJ) pre-wetted with cold 5% TCA. The filters were washed three times with 5% TCA at 4°C and once with 95% ethanol. The filters were dried for 15 minutes in a hot oven and counted by liquid scintillation spectrometry. When glucose was used as the radiolabeled precursor, unlabeled glucose was included in the NaOH/BSA solution. When radiolabeled purines, pyrimidines, nucleosides or nucleotides were used as precursors, samples (50 µl) of cell suspensions were added to 0.05 N NaOH with 100 µg/ml BSA and precipitation of nucleic acids was effected by addition of 1.0 ml 25% TCA at 4°C without prior incubation at 37°C; the remaining steps were as described above.

Incorporation of [^{32}P]-inorganic phosphate was measured by removing samples (50 µl) of cell suspensions into each of two 12 x 75 mm tubes containing 1.0 ml 7 mM phosphate

buffer (40 mg KH_2PO_4 and 116 mg $Na_2HPO_4.7H_2O$ per 100 ml deionized water) with 500 µg/ml BSA. One tube was treated by addition of 1.0 ml 40% TCA at room temperature followed by incubation at 90°C for 30 minutes and precipitation on ice for a subsequent 30 minutes. The second sample per time point was treated by the addition of 40% TCA at 4°C and incubation on ice for 1 hour. Both samples were collected on glass fiber filters, washed, dried, and counted as described above.

Radiolabeling of Growing Amebae

N. fowleri amebae were grown axenically in 10 ml Nelson medium in unagitated 75 cm^2 tissue culture flasks (Falcon Plastics 3024, Oxnard, CA). The proteins of growing N. fowleri were radiolabeled by addition of L-[^{35}S]-methionine (970 to 1095.1 Ci/mmole, New England Nuclear, Boston, MA) at 10 to 50 µCi per ml at the mid-logarithmic phase of growth. Cells were incubated with radiolabeled amino acid for 24 hours (approximately 3.5 generations); during this period, the culture grew to a late logarithmic population density of 1.6 to 2.5 million amebae/ml. Incorporation of radiolabeled methionine into protein and the actual radiolabel concentration in the growth medium were measured (described in the previous section) at the time of label addition (background value), and twice more during 24 hours of labeling.

N. fowleri amebae were also labeled with [^{32}P]-inorganic phosphate during growth in Nelson medium containing MOPS buffer but otherwise as described above. Carrier-free [^{32}P]-orthophosphate (New England Nuclear, Boston, MA) was neutralized with sterile NaOH and added to mid-logarithmic phase cultures (18 hours) at approximately 50 μCi/ml. The cultures were incubated with radiolabel for 24 hours as before. Assays of radioactivity in the medium and incorporation into hot- or cold-TCA insoluble material were performed as described in the previous section at the time of radiolabel addition (background) and several times during 24 hours of labeling.

Preparation of Radiolabeled Cells for Electrophoresis

Radiolabeled amebae were harvested, washed free of medium, and prepared for electrophoresis or subcultured to ameba saline (101) and shaken to evoke enflagellation (106). The flagellates, formed during 3.5 hour incubation of N. fowleri at 42°C in a gyrotory water bath, were separated from untransformed amebae. The remaining amebae attached to the culture vessel within a few minutes once agitation was stopped, leaving only flagellates in the supernatant fluid. Similarly, flagellates labeled during differentiation were separated from untransformed amebae and harvested.

Radiolabeled flagellates or growing amebae were washed thoroughly with Page saline (101), warmed to 37°C. The washed cells were sedimented by centrifugation and suspended in 0.5 ml ice-cold disruption buffer (10 mM trishydroxymethyl-aminomethane, 50 µg deoxyribonuclease I/ml, 50 µg ribonuclease A/ml, 5 mM $MgCl_2$, pH 7.4). Amebae or flagellates were ruptured by three cycles of freezing and thawing; the resulting extracts were lyophilized. The lyophilized material was suspended in lysis buffer I at a concentration of 10^7 cell-equivalents per 250 µl. Lysis buffer I contained 0.5% sodium dodecyl sulfate (SDS), 9.5 M urea, 0.2% ampholytes (pH 3-10), 5% 2-mercaptoethanol), supplemented with 10 mM lysine (69, 100). The two known proteolytic activities of N. fowleri (106) were inactivated by lysis buffer I. After 5 minutes at 25°C, an equal volume of lysis buffer II (9.5 M urea, 2% Nonidet P-40, 1.5% ampholytes [pH 5-7], 0.4% ampholytes [pH 3-10], 5% 2-mercaptoethanol, supplemented with 10 mM lysine [97,100]) was added. The samples were held at -20°C until subjected to isoelectric focusing on the same day.

Two-Dimensional Electrophoresis

Isoelectric focusing (IEF) was achieved with mixed ampholytes (1.6% pH 5-7, 0.4% pH 3-10) in 12.5 cm 3% polyacrylamide cylindrical gels containing 9.5 M urea

as previously described (97). Sample volumes were adjusted so that approximately equal numbers of cell-equivalents were loaded for each preparation. Assay of radioactivity in each final protein sample (100) was made by removing 2 µl of sample in the lysis buffers into 0.5 ml of a 0.05 N NaOH solution at room temperature containing 100 µg/ml bovine serum albumin (BSA) and 10 mg/ml unlabeled methionine, addition of an equal volume of 10% TCA (also at room temperature) and precipitation at room temperature for 1 hour followed by collection of precipitates on filters and counting as described above (Radiolabel Incorporation Assays).

Isoelectric focusing was performed using a Hoeffer model PS 1200 power supply (Hoeffer Scientific Company, San Francisco, CA) operated at constant voltage at 400 V for 17 hours, followed by hyperfocusing at 800 V for 1 hour (100). Isoelectric focusing gels were equilibrated immediately after removal from the tubes in preparation for running in the second dimension as previously described (97).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with uniform 12% acrylamide gels, but otherwise as described previously (97). Molecular size standards (myosin, 200 kd, β-galactosidase, 130 kd, phosphorylase b, 94 kd, bovine serum albumin, 68 kd, ovalbumin, 45 kd, carbonic anhydrase, 30

kd, soybean trypsin inhibitor, 21 kd, and lysozyme, 14.3 kd were electrophoresed from a well on the side of each SDS-PAGE gel.

Ampholytes, SDS, acrylamide, N,N'-methylene-bis-acrylamide and molecular size standards were obtained from Bio-Rad Laboratories, Richmond, CA. Urea (ultrapure grade) was obtained from Schwartz-Mann, Orangeburg, N.Y. Tris-hydroxymethylaminomethane, deoxyribonuclease I, and ribonuclease A were from Sigma Chemical Company, St. Louis, MO. Nonidet P-40 was obtained from Bethesda Research Laboratories, Rockville, MD.

Gel Staining

Following electrophoresis, gels were fixed and stained in 10% acetic acid, 25% isopropanol, 0.04% Coomassie blue R-250 (Sigma Chemical Company, St. Louis, MO), followed by destaining in several changes of 10% acetic acid, 20% methanol. In some cases, gels were stained by a modification of the silver stain method of Merrill et al. (93). Gels were fixed for 2 hours with 200 ml of fresh 50% methanol, 10% acetic acid in a clean staining tray agitated gently on a shaker platform. This solution was aspirated from the gel to avoid leaving fingerprints (which stain by the method), replaced with a fresh 200 ml of 50% methanol, 10% acetic acid and shaking was continued overnight. The gels were then rinsed in 200 ml 10% ethanol, 0.5% acetic acid for 1 hour with shaking followed by

2 successive 10 minute washes in fresh 200 ml portions of the same solution. The last of these rinses was aspirated and 200 ml of an oxidizing solution (3 mM $K_2Cr_2O_7$ and 0.00032 N HNO_3) added. The gels were shaken for 10 minutes in this solution while protected from light. Gels were then rinsed 3 times with deionized water and 250 ml of silver nitrate solution (0.01 M $AgNO_3$ containing 0.5 mg/liter benzotriazole [Kodak antifog #1, Rochester, NY]) was added to the gels. The gels were shaken in this solution for 20 minutes while protected from light. Most of the silver solution was aspirated, the gels were transferred to clean trays and without rinsing, 300 ml of freshly made developer solution (0.28 M Na_2CO_3, 0.5 ml/liter commercial formalin [Fisher], and 0.6 mg/liter benzotriazole) was added. The trays were agitated by hand for 15-20 seconds until a brown precipitate appeared, then the solution was aspirated and a second 300 ml portion of developer was added. After 30 seconds, the second solution was discarded and replaced with a third 300 ml of developer solution and the gels were shaken in this for 10-15 minutes while protected from light until the spots appeared on the gels and before background staining was significant. Development was stopped by replacing the developer with 200 ml of 0.2 N acetic acid and agitating for 5 minutes. The acetic acid step was repeated and the gels then rinsed twice in deionized water and stored in sealed plastic bags.

Autoradiography

The gels were dried; Kodak X-OMAT R film was exposed to the dried gels for several time periods. Comparisons of polypeptide patterns of amebae and flagellates were made on autoradiograms of gels exposed for time periods such that the product of radioactivity loaded and exposure time was equivalent to 820,000 count min^{-1} for 21 days.

Densitometric Analyses

Autoradiograms were scanned systematically using a digital drum-scanning microdensitometer (model C4100 Optronics International, Chelmsford, MA) operated at a raster setting of 4 and at 200 μm resolution. One record represented a 200 μm wide zone in which readings were taken during a single revolution of the scanning drum. Optical densities of 200 μm long segments of a record, ranging continuously from 0.0 to 2.0, were stored sequentially on magnetic tape as eight bit digital numbers (integer range 0 to 255) by means of an analog to digital converter (88). Each stored datum therefore represented the optical density of a 200 μm square "frame" in the autoradiogram.

Processing of the data for the quantitative analyses presented was accomplished by means of computer programs developed and implemented by Dr. William E. Keefe of the Microbiology Department at MCV. Film background density levels were eliminated by means of a histogram program

(HISTO) that determined the most often recorded density readings, and calculated the mean and standard deviation of the background density distribution. The background mean plus one standard deviation was subtracted from each datum and the corrected values printed in their original relative position by means of program (MCVPLOT), which assigned a value of zero to all non-positive values obtained after background subtraction. The density values for autoradiogram spots were easily distinguished against the corrected "0" background and were brought into relief by contouring manually the film density readings on these printouts. The maximum peak densities of spots on a two-dimensional autoradiogram were determined to facilitate resolution of multiple spots in very close proximity on a gel. The contour patterns were compared with the spots observed visually on the autoradiograms. The resulting contour patterns corresponded to the patterns of polypeptide spots observed on the autoradiograms.

Quantitative analysis of autoradiogram spot densities in various size and charge regions of the two-dimensional separations was performed by apportioning the data, by means of program (MCVPLOT 2), into intervals along both the IEF and SDS-PAGE dimensions for enumeration and computer quantitation of the contoured peaks. The IEF dimension was divided arbitrarily into 6 mm sections corresponding to the width of the printouts, which was 30 record columns.

Similarly treated IEF gels were cut into sections, the sections placed into deionized water, and the pH of the extract measured in order to relate the arbitrary divisions to the pH gradient in the IEF gel. The SDS-PAGE dimension was organized into intervals of 20 kilodaltons by the computer program (MCVPLOT 2) that calibrated the vertical scan coordinate against size standards (14.3 to 200 kilodaltons) that were run in the SDS-PAGE dimension with each sample. The data were sorted into 270 areas (9 molecular size divisions x 30 IEF divisions) and the density values in each area were summed which were greater than or equal to the center peak value of the least intense contoured spot. The latter value was entered into the program along with the histogram-program derived background corrections, the size standard positions, and the reference starting point for the IEF intervals.

The results of these computations were displayed in a matrix listing a) the density sum in each area, b) subtotals per molecular size interval across all IEF divisions, c) subtotals per IEF interval through all molecular size divisions, and d) a total per autoradiogram. The computer-derived areas were then used to enumerate the contoured spots within the different areas and similar subtotals for size intervals and IEF intervals were computed.

The data presented were based upon three independent experiments, two-dimensional polyacrylamide gel electrophoretic separations, and computer-assisted analyses.

RESULTS

Naegleria gruberi NEG-M exhibited biphasic logarithmic growth at 32°C in 10 ml agitated cultures in Balamuth medium containing 1 μg/ml hemin. Cultures inoculated at 2×10^4 amebae/ml grew to a maximum cell density of 2.7×10^6 amebae/ml at 70 hours with a minimum mean doubling time (9 experiments) of 7.4 hours (Fig. 1). N. fowleri nN68 was grown in 10 ml agitated cultures in Nelson medium with 2% (v/v) calf serum at 32°C. Cultures inoculated at 2×10^4 amebae/ml grew to a maximum cell density of 1.15×10^6 amebae/ml at 92 hours with a minimum mean doubling time of 11 hours (12 experiments) (Fig. 1). Amebae of both strains in late growth phase (70 hours, NEG-M; 92 hours, nN68) were harvested, washed, and suspended in 6 ml TK buffer at 25°C. The suspensions were shaken and the progress of enflagellation monitored. Under these conditions, 80% of the N. gruberi amebae converted to actively swimming flagellates by 90 minutes after subculture to non-nutrient buffer (Fig. 2). The time at which 50% of the cells had become flagellates, defined as the T_{50} (36,50) was 59 minutes. By contrast, none of the N. fowleri amebae became flagellates during the first 70 minutes, nor were more than 5% observed through 120 minutes. Similarly, few or no flagellates were observed when amebae from 70 hour (9.4×10^5 amebae/ml) or 120 hour (5×10^5 amebae/ml) cultures of N. fowleri

were washed and suspended in TK buffer at 25°C (data not shown). Suspension of N. fowleri amebae in distilled H_2O at 25°C or agitation of cultures remaining in their growth medium at lower incubation temperatures did not result in conversion of amebae to flagellates (data not shown).

N. gruberi NEG-M amebae were were also grown in tissue culture flasks without agitation in Balamuth medium with hemin and also in a less complex medium (M7) reported to support good growth of NEG-M amebae capable of synchronous differentiation to flagellates (47). M7 medium was modified for the experiments in all studies reported here by the inclusion of commercial dialyzed calf serum instead of the dialyzed fetal calf serum preparation described originally (47) because of the inability to achieve growth greater than 2×10^5 amebae/ml with such a preparation (data not shown). Agitated cultures of NEG-M in M7 medium grew poorly (doubling time 16 hours versus 8-10 hours reported, 47) and to a low yield of approximately 1×10^6 amebae/ml (versus $2\text{-}4 \times 10^6$/ml) (Fig. 3). In contrast, unagitated cultures of N. gruberi NEG-M in tissue culture flasks grew equally well in Balamuth and M7 media. Amebae inoculated at 2×10^5 amebae/10 ml medium in 25 cm^2 tissue culture flasks grew at 32°C with mean generation times of 11 hours (8 experiments) in Balamuth and 10 hours (6 experiments) in M7 until the amebae formed a near confluent sheet on the flask (attached cell density approximately

4 x 10^5 amebae/cm^2) in approximately 50 hours for both media (Fig. 4). Maximum growth of NEG-M in M7 medium was achieved at 32°C, as reported (47). Attempts at further simplification of M7 medium to facilitate studies of initiation signals and radiolabeling were not successful. Hemin, at concentrations up to 10 µg/ml, did not replace serum for optimal growth of NEG-M in M7 (Table 2, 48). A reduction in the concentration of dialyzed calf serum from 10% (v/v) to 8% (v/v) yielded equivalent growth of NEG-M in M7, but lower concentrations resulted in less growth (Table 2).

Stationary phase cells of agitated (120 hours) and unagitated cultures of N. gruberi NEG-M in M7 medium at 32°C were harvested, washed and agitated in TK buffer at 25°C as described above. Amebae from unagitated, more rapidly growing cultures differentiated more quickly, more synchronously and to higher yields of flagellates than did those from the agitated cultures (Fig. 5). The T_{50} for the unagitated culture amebae was 68 minutes, compared to a T_{50} of 76-80 minutes reported by Fulton (47) for axenically grown (M7, 32°C) NEG-M amebae differentiated under the same conditions.

Enflagellation of N. gruberi amebae by a different protocol (Materials and Methods) resulted in near complete conversion of the amebae to flagellated cells. N. gruberi NEG-M in early stationary (69-72 hours) unagitated cultures

Figure 1. Agitated cultivation of Naegleria gruberi NEG-M and N. fowleri nN68.

Amebae of N. gruberi NEG-M (●) were inoculated in 10 ml of Balamuth medium containing 1 µg/ml hemin in 50 ml Erlenmeyer culture flasks. Amebae of N. fowleri nN68 (▲) were inoculated in 10 ml of Nelson medium containing 2% (v/v) calf serum in 50 ml Erlenmeyer culture flasks. All cultures were incubated with agitation on a gyrotory shaking incubator operated at 130 rpm and 32°C. Cell counts were made on 0.2 ml samples using a Coulter counter as described in Materials and Methods. The curves shown are the average of 9 growth curves for N. gruberi and 12 curves for N. fowleri.

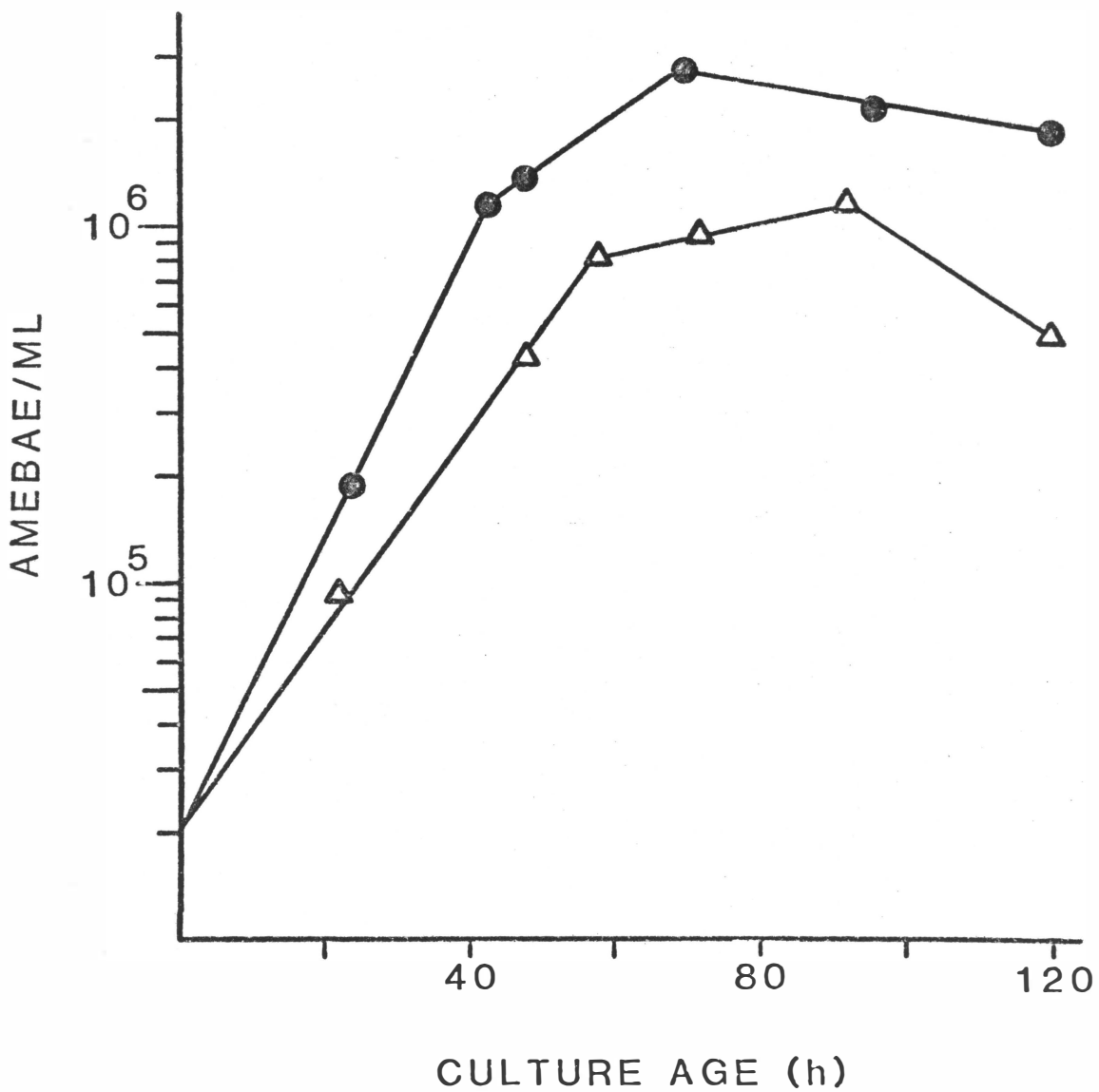

AMEBAE/ML
10^6
10^5
40
80
120
CULTURE AGE (h)

Figure 2. Enflagellation of amebae of Naegleria gruberi and Naegleria fowleri grown in agitated cultures.

Amebae from cultures of N. gruberi NEG-M (70 hours, ●) and N. fowleri nN68 (92 hours, ■) grown as in Figure 1 and nearing maximum cell density were harvested and washed free of medium by three cycles of centrifugation and suspensions (Method C, Fulton 1970 - see Materials and Methods) in 10 ml TK buffer at 25°C and were finally suspended in 6 ml TK buffer at 25°C. The suspensions were maintained in suspension by gentle agitation in 50 ml Erlenmeyer culture flasks in a waterbath shaker operated at 120 rpm and 25°C. Enflagellation was monitored by removing samples into an iodine fixative stain at regular timed intervals and determining the number of enflagellated cells in a field of at least 100 cells counted per sample. Zero time was the time of first suspension in TK buffer at 25°C.

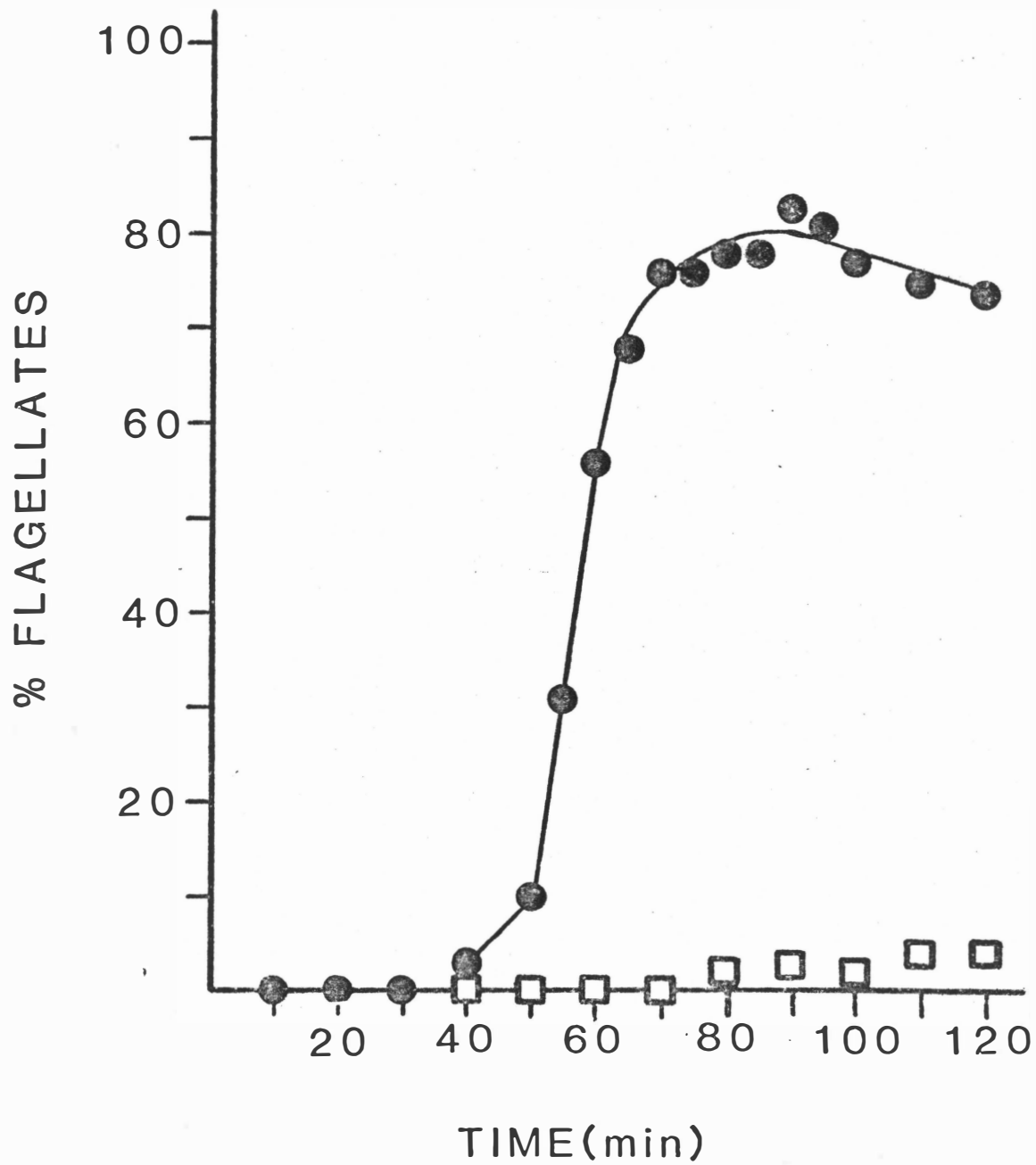

100
80
60
40
20
% FLAGELLATES
20
40
60
80
100
120
TIME(min)

Figure 3. Agitated growth of N. gruberi NEG-M in M7 medium.

N. gruberi NEG-M amebae from stock cultures in Balamuth medium were inoculated at 2 x 10^4 amebae/ml in 10 ml of M7 medium containing 8% (v/v) dialyzed calf serum in 50 ml Erlenmeyer culture flasks shaken at 130 rpm and 32°C. Cell counts were made as described in Materials and Methods. The curve drawn represents the average of 15 such growth curves.

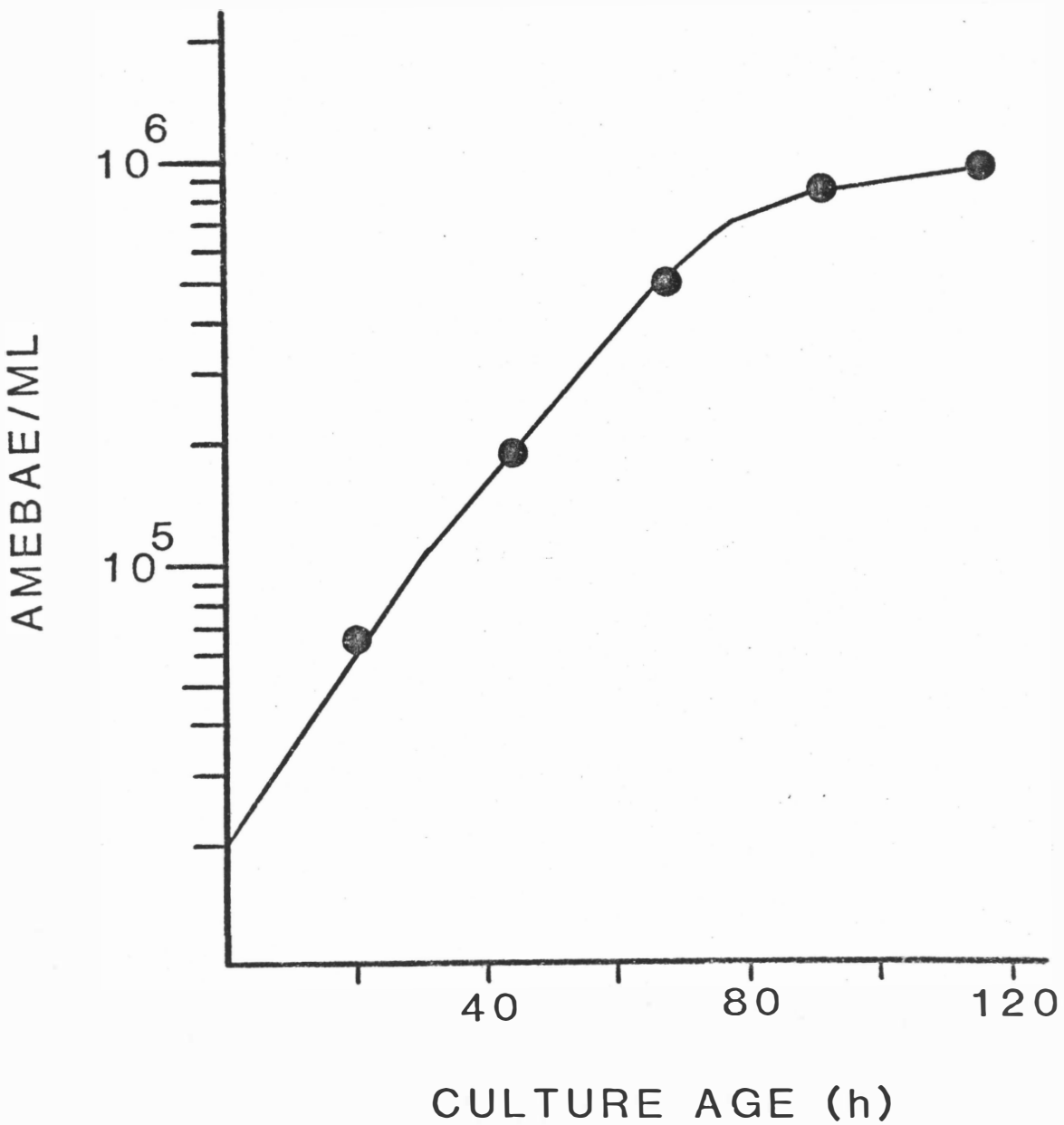

AMEBAE/ML
10^6
10^5
40
80
120
CULTURE AGE (h)

Figure 4. Unagitated growth of Naegleria gruberi NEG-M in Balamuth and M7 media at 32°C.

N. gruberi NEG-M amebae were inoculated at 2×10^5 amebae per 10 ml medium in 25 cm^2 tissue culture flasks in either Balamuth medium containing 1 µg/ml hemin (●) or M7 medium with 8% (v/v) dialyzed calf serum (■). Cultures were incubated at 32°C without agitation. At sampling times, amebae growing on the flask surface were suspended in the growth medium by agitation and the density of the cell suspension determined by Coulter counter as described in Materials and Methods. The curves represent the average of 8 growth curves (Balamuth) and 6 growth curves (M7).

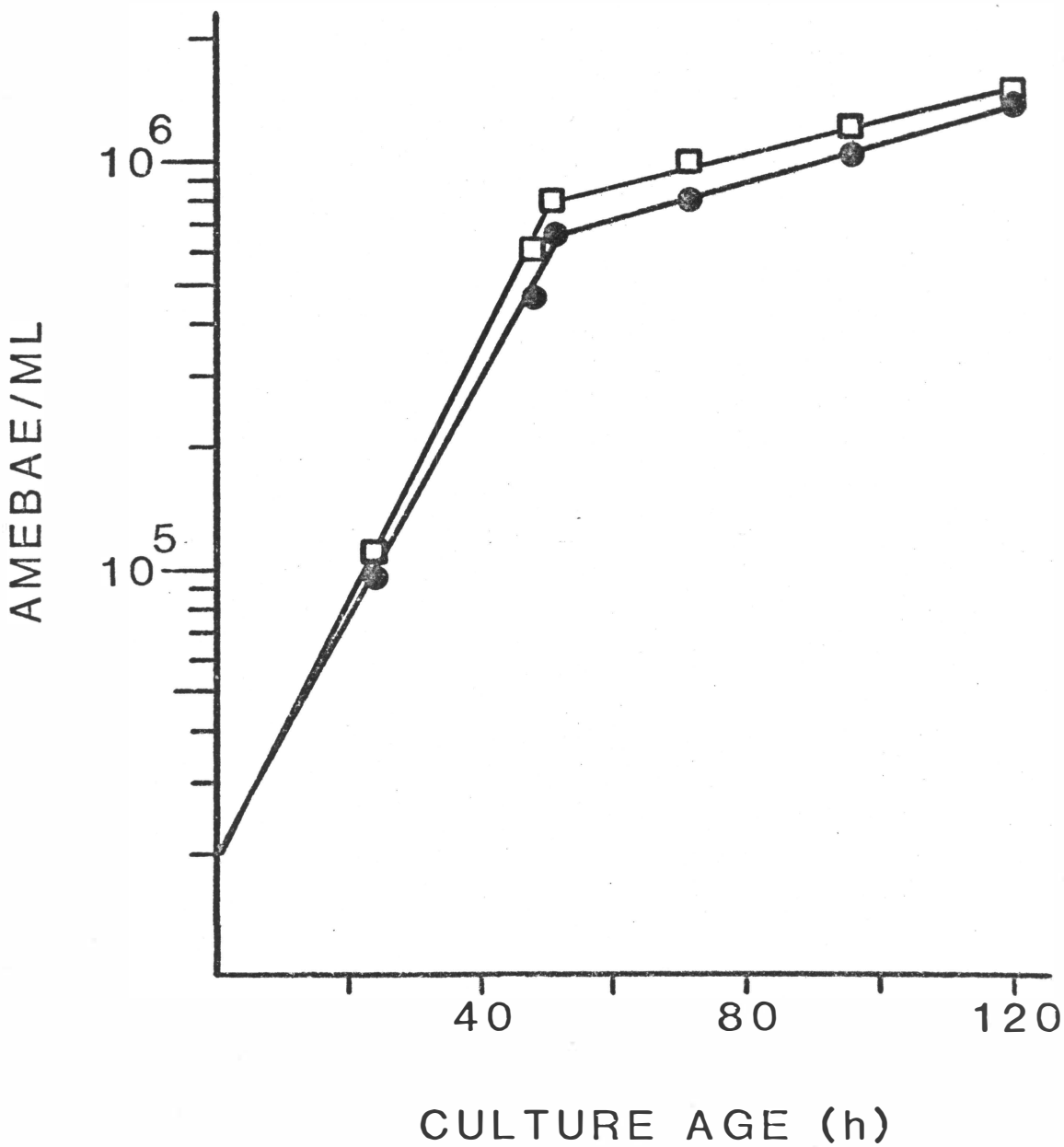

AMEBAE/ML
10^6
10^5
40
80
120
CULTURE AGE (h)

Table 2

Effects of Varying M7 Medium Constituents Upon

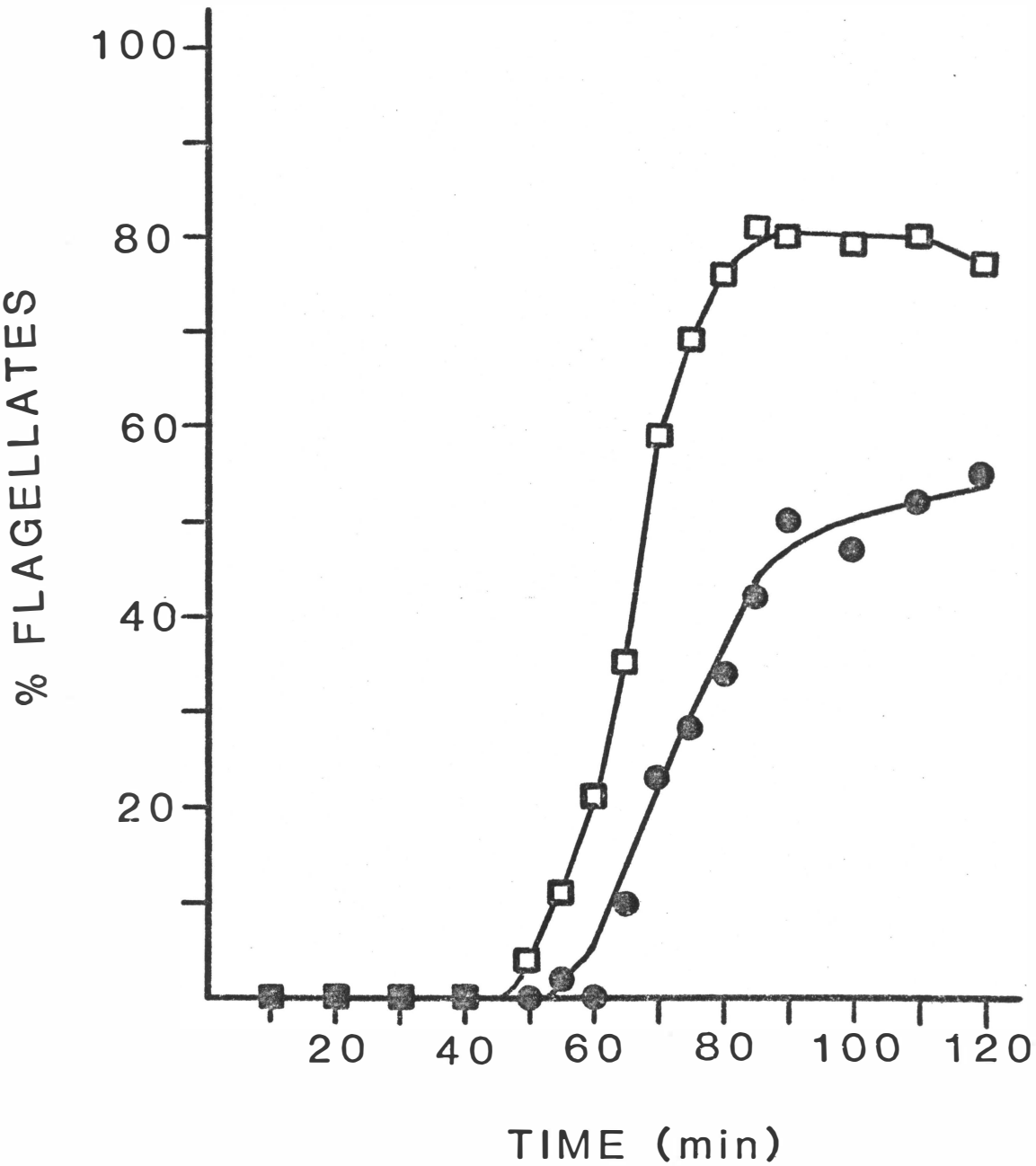
Growth of *Naegleria gruberi* NEG-M

Serum Concentration (% v/v)	Hemin (μg/ml)	Maximum Cell Density (Amebae/10 ml Culture)	Time (h)
10	0	9×10^6	70
8	0	9×10^6	70
6	0	8×10^6	70
4	0	7×10^6	70
2	0	5×10^6	100
0	10	3.5×10^5	40

N. gruberi NEG-M growing in M7 medium in mid-logarithmic phase was inoculated at 10^5 amebae/culture into 25 cm^2 tissue culture flasks containing 10 ml (final volume) of M7 medium with the indicated amounts of dialyzed calf serum or of hemin. The cultures were incubated at 32°C and cell counts were made daily for 5 days as described for Figure 4. The time at which the highest cell density was reached is shown for each case.

Figure 5. Enflagellation of Naegleria gruberi NEG-M grown in agitated or unagitated cultures in M7 medium.

Stationary phase amebae (120 hours) of agitated (●) and unagitated (□) cultures of N. gruberi NEG-M grown at 32°C in M7 medium were harvested, washed and suspended in TK buffer at 25°C. The suspensions were agitated at 25°C and enflagellation was assayed as described for Figure 2.


100
80
60
40
20
% FLAGELLATES
20
40
60
80
100
120
TIME (min)

were washed while attached to the flask, suspended in non-nutrient buffer (7 mM phosphate buffer, pH 6.8) at 25°C and agitated. In 10 experiments, an average of 86% (range 80 to 95) of the amebae became flagellated cells by 90 minutes after t_o (Fig. 6, dark circles). The T_{50} for cells with flagella in these experiments averaged 72 $\pm$ 6 minutes.

The enflagellation of N. gruberi NEG-M by this method followed the general scheme of events previously reported for N. gruberi differentiation (48,50). Very little morphological change was observed during an initial 30 minute "covert period" following the stimulus. A substantial change in the population morphology occurred between 40 minutes and 80-90 minutes after t_o in which the majority of ameboid cells converted to very regular spherical cells. Beginning at about 55 minutes, as flagella appeared on the cells, an increasing number of the cells were seen to begin spinning around their centers without any directional motion. From 80 minutes onward, a striking conversion of spinning circular cells into the streamlined, teardrop-shaped, directionally swimming mature flagellate forms occurred. The change from round to flagellate shape occurred very quickly for individual cells, with a substantial number of additional, fully mature flagellate-shaped cells appearing in successive timed samples fixed in iodine stain and counted for percentages of cells with

the different shapes (49,50). The progress of enflagellation as assayed by cell shape changes (Fig. 6, open symbols) demonstrates the conversion of amebae into round cells and then of round cells into elongated flagellate-shaped cells. The T_{50} for conversion of amebae to round cells was 63 minutes. As round cells began to swim and lose their spherical shape, a significant number did not assume the characteristic flagellate shape, or did so and reverted quickly to amebae and were, therefore, counted as amorphous amebae when assays according to cell shape were done. Accordingly, a maximum of only 50% flagellate-shaped cells was seen in 3 hours even though the number of cells with flagella remained nearly constant and a number of swimming cells without streamlined shape were noted (Fig. 6).

A marked dependence of enflagellation upon prior growth temperature had been reported for N. gruberi NEG-M grown axenically in M7 medium, but not when grown in association with bacteria (47). Amebae grown axenically at 25°C were found to enflagellate more slowly (T_{50} fl = 85 minutes) than did the amebae grown at 32°C (T_{50} fl = 74 minutes) (Table 3). The temperature of the differentiating suspension has been reported to be a critical factor for differentiation of N. gruberi regardless of the conditions of growth (axenic or bacterial) (45,48,50). Increasing the temperature of the differentiation

Figure 6. Enflagellation of Naegleria gruberi NEG-M grown in unagitated cultures, washed and suspended in non-nutrient buffer without centrifugation.

Amebae of Naegleria gruberi NEG-M, growing in M7 medium with 8% (v/v) dialyzed calf serum in unagitated 25 cm^2 tissue culture flasks at 32°C, were allowed to grow to late logarithmic phase (near-confluent monolayer of cells, 4 x 10^5 amebae/cm^2). Enflagellation was initiated by decanting the growth medium and rinsing the attached cells (beginning at t = o) twice with non-nutrient M7 buffer (7 mM phosphate butter, pH 6.8) at 25°C. The washed amebae were suspended in 6 ml of the same buffer at 25°C and were agitated in tissue culture flasks (turned upright and fastened into a waterbath shaker) at 120 rpm and 25°C to keep the amebae in suspension. Samples were removed into iodine fixative and 100 cells per time point were counted for percentage of cells having:

(●) flagella visible under phase contrast optics, regardless of shape;

(○) round shape, regardless of flagella;

(△) flagellate shape, regardless of flagella;

(⬡) ameboid shape, regardless of flagella.

The points represent the average of 10 enflagellations.

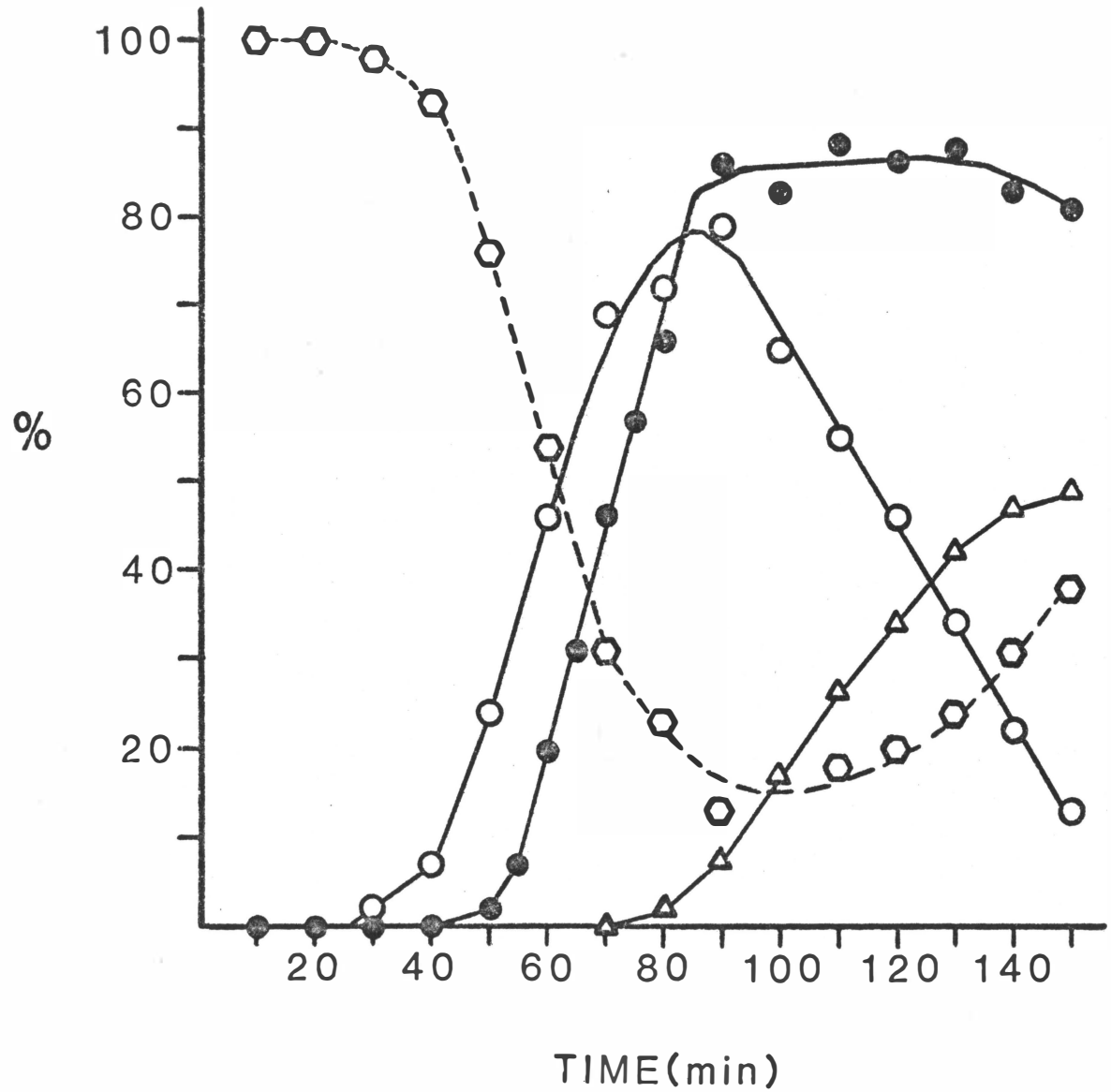

100
80
60
40
20
%
20
40
60
80
100
120
140
TIME(min)

Table 3

Effect of Growth Temperature of Axenically Cultured *Naegleria gruberi* Upon Enflagellation at 25°C

Growth Temperature	Cell Density	T_{50}fl (min)	Maximum % Flagellates
25°C	9.5×10^5/ml	85	80
32°C	9.8×10^5/ml	74	53

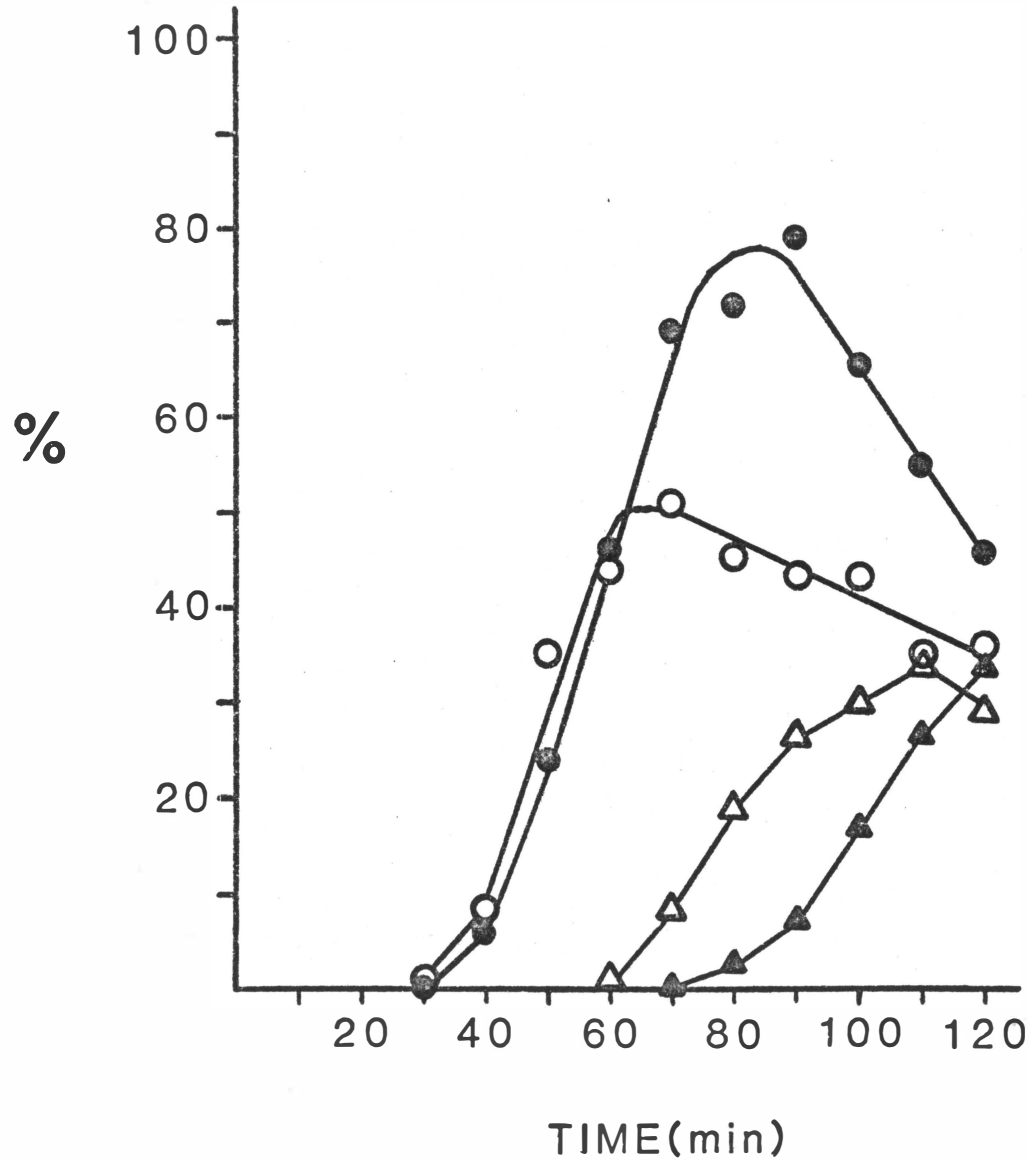
N. gruberi NEG-M amebae were grown in unagitated 25 cm^2 tissue culture flasks in 10 ml M7 medium with 8% (v/v) dialyzed calf serum. Amebae were inoculated at 2×10^5 amebae per culture and incubated at the indicated temperature for 70 hours (32°C) and 85 hours (25°C) until amebae reached the stationary phase cell density. Enflagellation was evoked as described for Figure 6 and fixed samples were assayed for cells with flagella. The time at which half of the population were scored with flagella was the T_{50}fl. The maximum % flagellates scored in 100 min is shown.

Figure 7. Enflagellation of Naegleria gruberi NEG-M amebae at 32°C and 25°C.

Amebae of N. gruberi NEG-M were grown at 32°C in M7 medium in unagitated cultures as described for Figure 6. Half of the cultures were washed and suspended in non-nutrient M7 buffer at 25°C (closed symbols) as described in the legend to Figure 6. The other half of the cultures were similarly washed and suspended in M7 buffer at 32°C (open symbols). The suspensions were agitated at 120 rpm in waterbaths maintained at the same temperature as the suspension buffer.

(●, ○) round cells.

(▲, △) flagellate-shaped cells.


100
80
60
40
20
%
20
40
60
80
100
120
TIME(min)

suspension (and water bath) from 25° to 32°C resulted in a quicker conversion of rounded cells to flagellate shape; however, the stability of flagellates and of the flagellate shape was less than at 25°C and the yield of mature flagellate-shaped cells was concomitantly less (Fig. 7).

The effects of varying growth and differentiation temperatures upon N. gruberi enflagellation prompted a test of N. fowleri enflagellation under temperatures more closely approaching its normal growth temperature range. Initially, a survey for enflagellation of a number of N. fowleri isolates maintained in the laboratory of D.T. John was conducted. Axenically grown N. fowleri strains in unagitated cultures grown at 32°C were washed and suspended in non-nutrient ameba saline and agitated at 32°C. At this temperature, a number of strains enflagellated to high percentages, others to lesser extents, and a few strains not at all (Table 4). Enflagellation occurred, however, only after a delay of 4 to 5 hours after suspension in Page saline at 32°C. Since a temperature downshift had been found to be important in the extent and kinetics of differentiation of axenically grown N. gruberi NEG-M, the effects of growth temperature and that of enflagellation were studied with N. fowleri nN68, the best enflagellating strain in the first survey. Amebae of N. fowleri nN68 exhibit very similar growth kinetics at 32°C and 37°C (77) but grow less well at 42°C. Growth at the

optimum growth temperature, 37°C (78,136), resulted in faster transformation of amebae to flagellates at all differentiation temperatures (Fig. 8). Amebae grown at 32°C and shifted up in temperature for differentiation enflagellated nearly as fast and to equivalent extents as did amebae grown at 37°C and enflagellated at the same (37°C and 42°C) temperatures. None of the cultures showed enflagellation over 8 hours when incubated in Page saline at 25°C. N. fowleri nN68 amebae grown at 42°C enflagellated to a lesser extent at all enflagellation temperatures than did the amebae grown at 32°C or 37°C. N fowleri nN68 amebae grown at any of the three temperatures differentiated quicker, more synchronously, and to higher maximum yield of flagellates when transformed at higher temperatures than at the lower temperatures (Fig. 9). The enflagellation temperature was a more important variable for enflagellation than growth temperature for cultures grown between 32°C and 37°C. Although enflagellation at 42°C occurred with synchrony (∿ 60% completing change from amebae to flagellate shape within 30 minutes, Fig. 8c) comparable to that with N. gruberi, in no case were greater than 5% flagellates observed before 100 minutes after suspension in non-nutrient ameba saline.

A second survey of N. fowleri strains, conducted using amebae grown at 37°C and incubated at 37°C in Page saline, resulted in improvement in the maximum extent of

Table 4

Strains of Naegleria fowleri Tested for Ability to Enflagellate in Non-Nutrient Buffer

Strain	% Enflagellation	
	32°C	37°C
nN68	62	52
Lovell	35	65
KUL	22	53
nN69-1	22	22
HB-5	17	14
nN69-2	15	17
nN67	8	12
0359	3	2
GJ	0.3	<1
HB-4	0	0
NF66	0	0
NF69	0	0

N. fowleri strains (see Table 1) were grown in Nelson medium containing 2% calf serum at 32°C or 37°C. At 72 hours, the medium was removed and the adherent amebae were rinsed with Page saline, then covered with Page saline to give $\sim 10^6$ amebae/ml. The cultures were chilled at 5°C for 5 minutes to facilitate release of the amebae and then shaken at the same temperature at which they were grown (32°C or 37°C) in a gyrotory shaker. Flasks were examined hourly for 8 hours by placing samples in iodine fixative and counting the percentage of flagellates. The % enflagellation given is the maximum obtained at any time during 8 hour incubation.

[a]Unpublished results, D.T. John, Oral Roberts Univ., Tulsa, OK 1981-1982.

Figure 8. Effect of prior growth temperature upon enflagellation of N. fowleri nN68. Growth temperatures were (■) 32°C, (●) 37°C, (▲) 42°C. Enflagellation performed at A) 32°C, B) 37°C, C) 42°C.

Figure 9. Effect of suspension temperature upon enflagellation of N. fowleri nN68. Enflagellation temperatures were (■) 32°C, (●) 37°C, (▲) 42°C. Growth temperatures were A) 32°C, B) 37°C, C) 42°C.

Amebae of Naegleria fowleri nN68 were grown in unagitated cultures with 5 ml Nelson medium containing 2% calf serum. Six flasks each were incubated at 32°C, 37°C, and 42°C without agitation until near confluent cell layers were obtained. A pair of flasks from each growth temperature were drained of growth medium, rinsed with Page ameba saline pre-warmed to 32°C, suspended in 5 ml 32°C Page saline, and agitated in a shaker bath at 32°C and 120 rpm. Similar rinsing and suspension with ameba saline pre-warmed to 37°C and 42°C were performed with flasks grown at all temperatures and the suspensions were agitated at the corresponding temperature. Assay of enflagellation was performed as described in the legend to Figure 6. Each data point is the average of the % flagellate-shaped cells (per 100 cells counted) in the fixed samples from a pair of flasks.

FIGURE 8

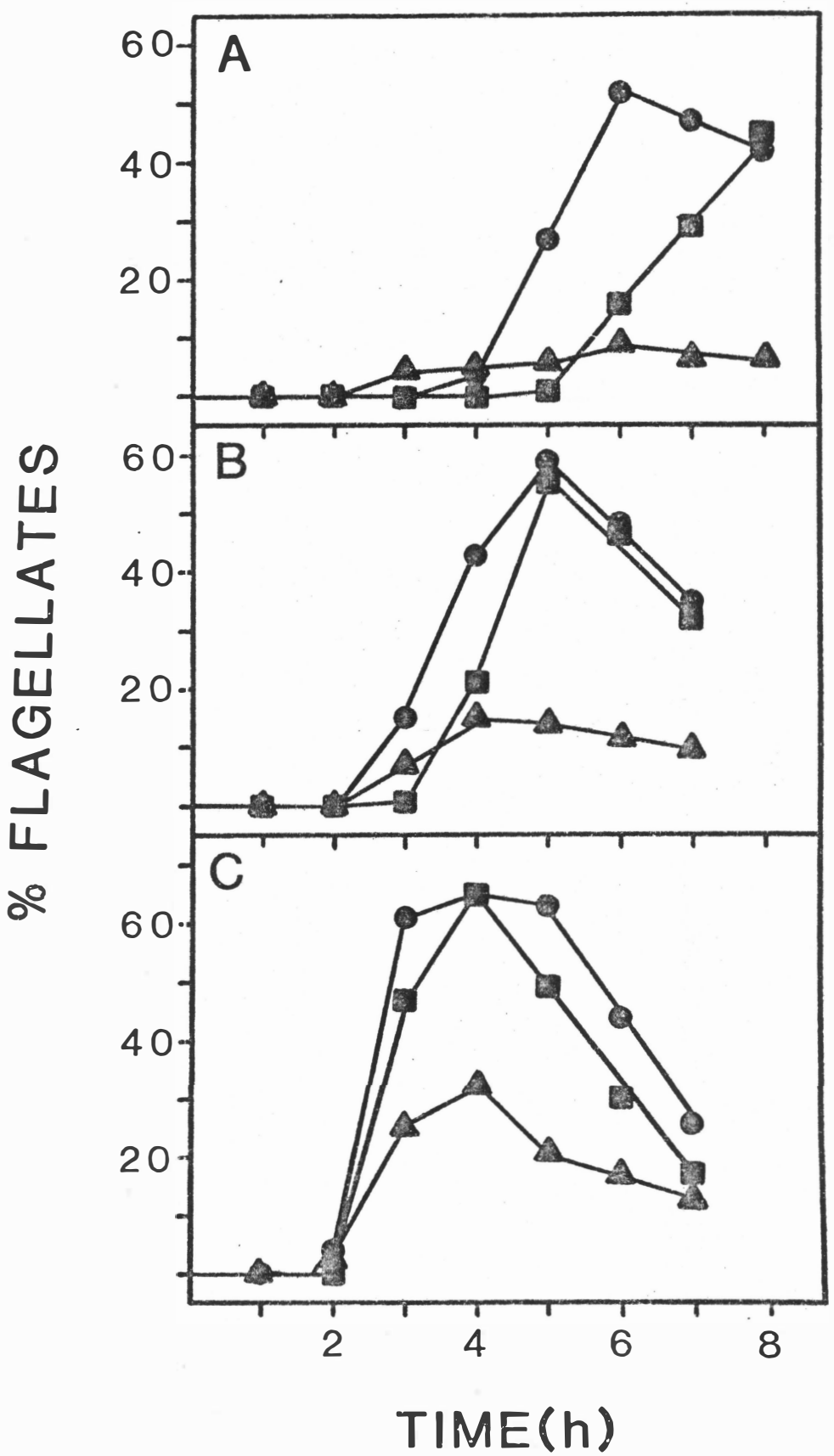
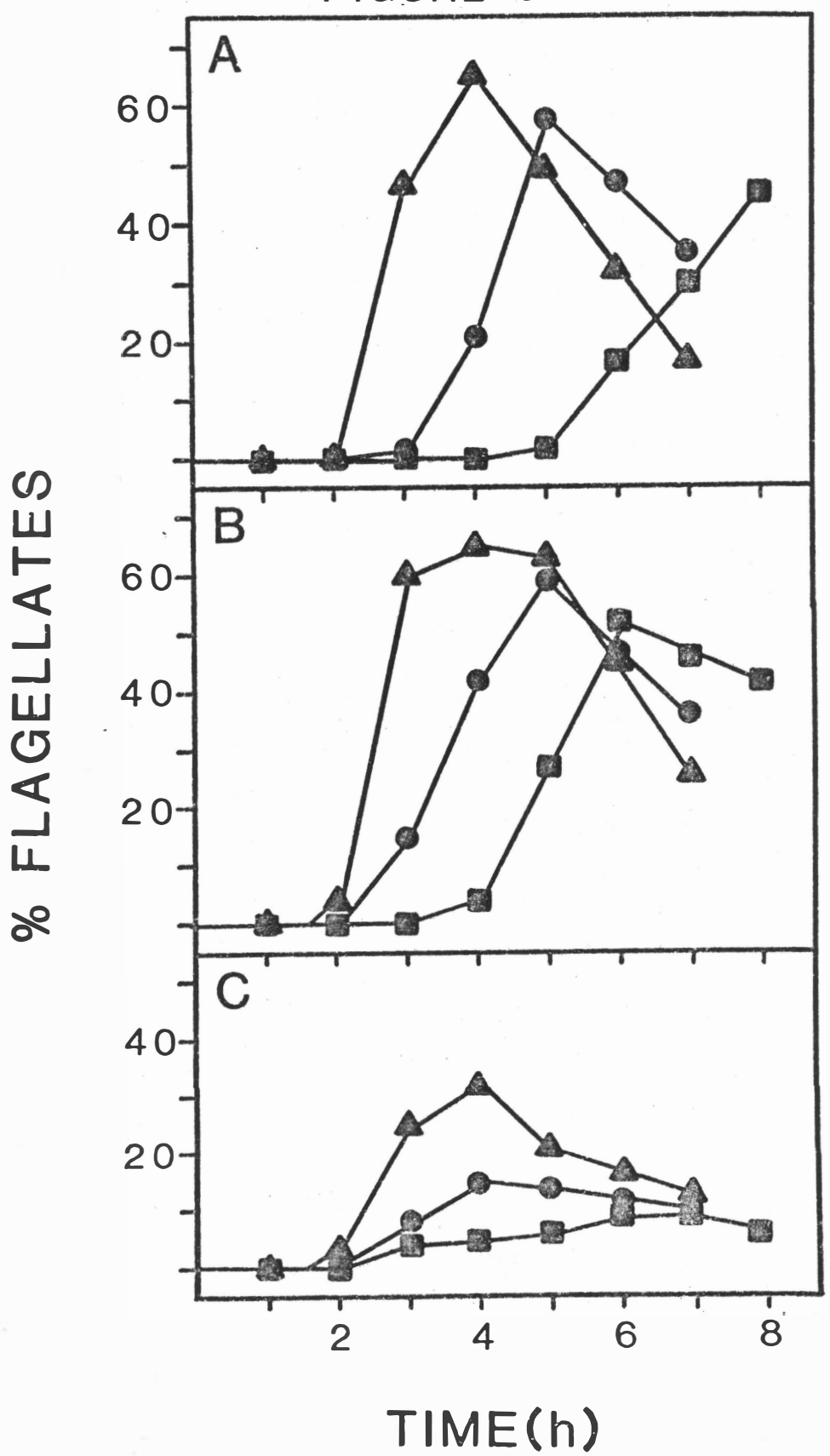


FIGURE 9



enflagellation of a few strains, particulary Lovell and KUL, but in general did not greatly change the results seen previously with 32°C (Table 4). Results obtained were comparable between our laboratory and that of D. John (performed at Oral Roberts University, Tulsa, OK). Even at optimal temperature conditions established for N. fowleri nN68, Lovell and KUL strains, there were no flagellates observed during 8 hours in Page saline for strains HB-4, NF66 and NF69. The three non-enflagellating strains (NF66, NF69, and HB4) were mixed in pair-wise combinations. No flagellates were observed in the combinations or the individual suspensions. The non-enflagellating strains were individually mixed with the enflagellating strain nN68. Enflagellation in these pair-wise mixtures was same as that for a comparable number of enflagellating nN68 alone. Strain nN68 was selected for subsequent studies on conditions affecting enflagellation. The reasons for the inability to evoke enflagellation of some strains were not known, but the possibility that these strains could be used to compare events occurring in enflagellating amebae with those occurring in amebae similarly deprived of nutrients, but not undergoing morphogenesis, made these strains of significant interest.

An attempt was made to isolate mutants of N. gruberi NEG-M unable to enflagellate by selective removal of non-enflagellated amebae from extensively enflagellated cultures

and clone plating of these cells by the methods outlined by Fulton (45) (Materials and Methods). Selective removal of amebae from such enflagellating suspensions and good growth of the amebae to yield clone plaques was achieved; however, screening of these clones for the ability to differentiate in non-nutrient buffer always resulted in populations with variable, but high, proportions of flagellates.

The difference in the time course for enflagellation of N. fowleri from that seen with N. gruberi, as well as the degree of variability seen between strains of N. fowleri with respect to enflagellation ability, demonstrated that the two systems behaved quite differently under the conditions tested. Furthermore, although a distinct rounding stage was observed for N. gruberi, amebae of N. fowleri appeared to acquire flagella and elongate without a rounding stage (Figs. 26-31). N. fowleri and N. gruberi grown in a common medium at 32°C and induced to enflagellate under the same conditions continued to exhibit these differences. Growth of amebae of N. fowleri and N. gruberi in a hybrid medium (1:1 Nelson:Balamuth) at 32°C was comparable to that obtained by the amebae in their respective growth media at the same temperature (data not shown). N. gruberi amebae grown in this medium and induced to enflagellate began conversion to flagellates beginning at approximately 1 hour *after*

suspension whereas N. fowleri amebae treated similarly did not enflagellate until after 3.5 hours (Fig. 10).

N. gruberi amebae from cultures in mid-logarithmic to mid-stationary phases of growth in M7 medium began rounding up and converting to flagellates at approximately the same time after suspension in non-nutrient buffer and to equivalent extents (Fig. 11). In contrast, use of N. fowleri amebae from different stages of the population growth curve markedly influenced the nature of the enflagellation response obtained. Actively growing cultures of N. fowleri enflagellated only after a delay of 3 to 4 hours following subculture to ameba saline. Extensive enflagellation was achieved in a shorter period and with greater synchrony using amebae in mid-to-late stationary phase (Fig. 12). However, even the most synchronous enflagellation obtained with N. fowleri amebae never began sooner than 90 minutes after suspension in ameba saline. Replenishment of the medium on older cultures with fresh medium for 18 hours impaired the capability to enflagellate upon subculture to ameba saline, whereas incubation of sparsely populated, growing cultures in expended medium for 18 hours augmented their capability to enflagellate (Table 5). Maximum enflagellation by control amebae from a mid-stationary phase culture and by amebae from a mid-logarithmic phase culture incubated in expended medium occurred 2 to 3 hours after subculture to ameba saline.

The critical component of M7 medium, which when removed individually can stimulate enflagellation of N. gruberi NEG-M, is yeast extract (46, Table 6). Similarly, subculture of N. fowleri amebae from Nelson medium to Nelson medium lacking liver digest is alone sufficient to evoke enflagellation (Table 6).

N. gruberi NEG-M amebae grown in M7 medium progressed through rounding and conversion to flagellated cells without distinguishable difference in M7 buffer, TK buffer, Page ameba saline, or deionized water (Fig. 13). Some variation was seen in the kinetics and extent of appearance of the flagellate shape in these axenically grown cells. Amebae in the M7 buffer converted to flagellate shape somewhat more quickly than those in the other buffers (Fig. 13).

Enflagellation of N. fowleri nN68 in Nelson medium and suspended in Page ameba saline, TK buffer or deionized water at 32°C did not occur prior to 90 minutes in any case (Table 7). Enflagellation in a number of variations of Page saline lacking various cations did not result in a larger proportion of enflagellating cells nor were flagellates observed to appear in a shorter time period (Table 7).

Classically used inhibitors of ribonucleic acid (RNA) and protein synthesis could prevent enflagellation of Naegleria. Growth of N. gruberi NEG-M was retarded by

Figure 10. Enflagellation of Naegleria fowleri nN68 and Naegleria gruberi EGB Grown in (1:1 Nelson/ Balamuth) Medium.

Amebae of N. fowleri nN68 (▲) and N. gruberi EGB (●) were inoculated at 2 x 10^4 amebae/ml in 5 ml of (1:1 Nelson/Balamuth) medium in 25 cm^2 tissue culture flasks and incubated without agitation at 32°C for 65 hours. Attached cells of the cultures were washed, suspended and agitated in Page ameba saline at 32°C as described for Table 4. Data plotted are the average of two experiments.

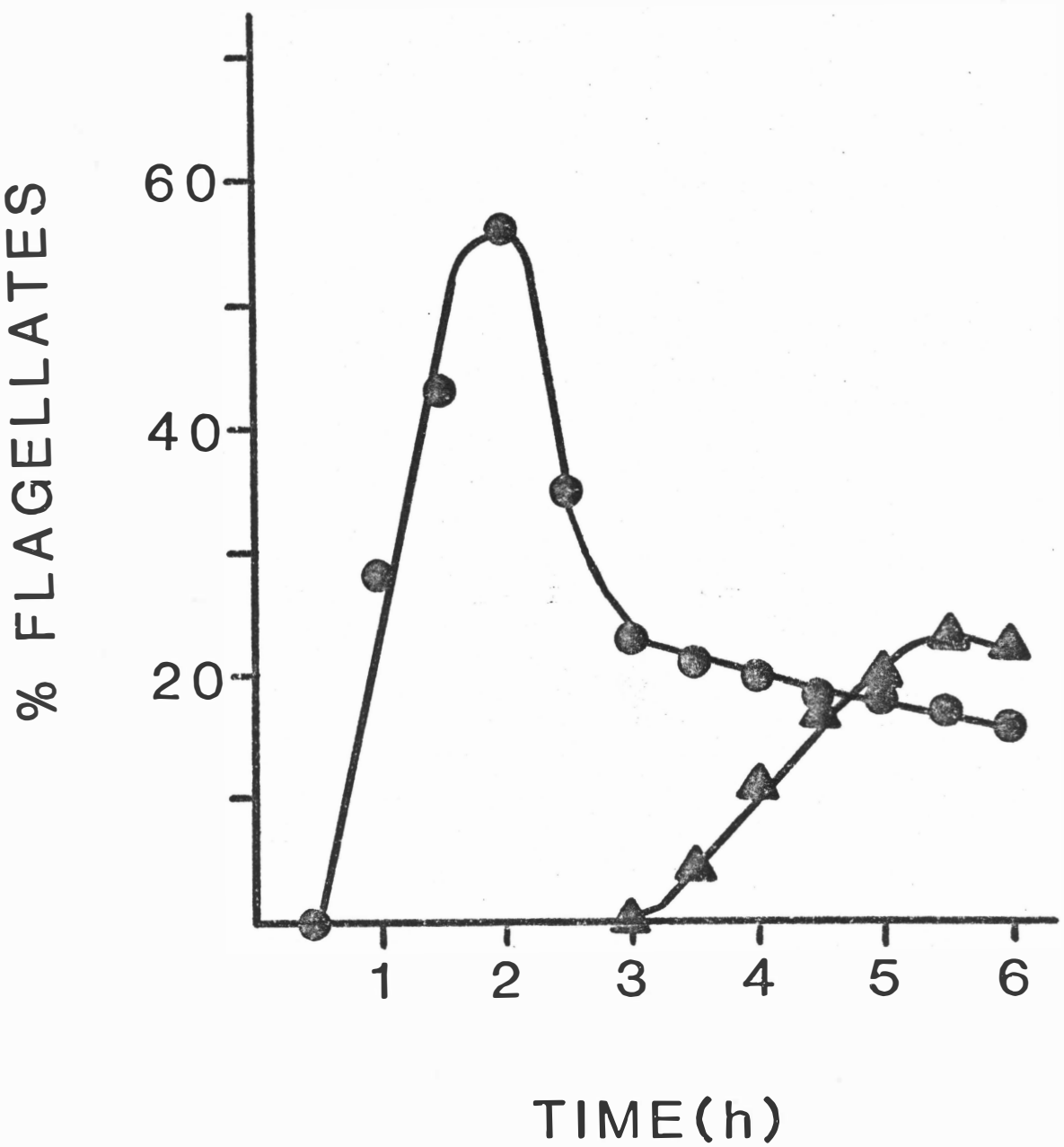

% FLAGELLATES
60
40
20
1
2
3
4
5
6
TIME(h)

Figure 11. Effect of culture age of Naegleria gruberi NEG-M upon enflagellation.

Amebae of N. gruberi NEG-M were inoculated at 1 x 10^5 amebae/culture in 5 ml unagitated cultures in M7 medium on successive days and incubated at 32°C. On the fifth day, amebae were at the following densities (amebae/ml): (○,●) 24 hours, 2.4 x 10^5; (□,■) 72 hours, 8.0 x 10^5; (△,▲) 96 hours, 1.2 x 10^6; (◇,◆) 120 hours, 1.24 x 10^6. Two flasks at each age were washed, suspended, agitated in M7 buffer at 25°C and assayed for cell shape changes as described in the legend to Figure 6. Round cells (open symbols), flagellate-shaped cells (closed symbols).

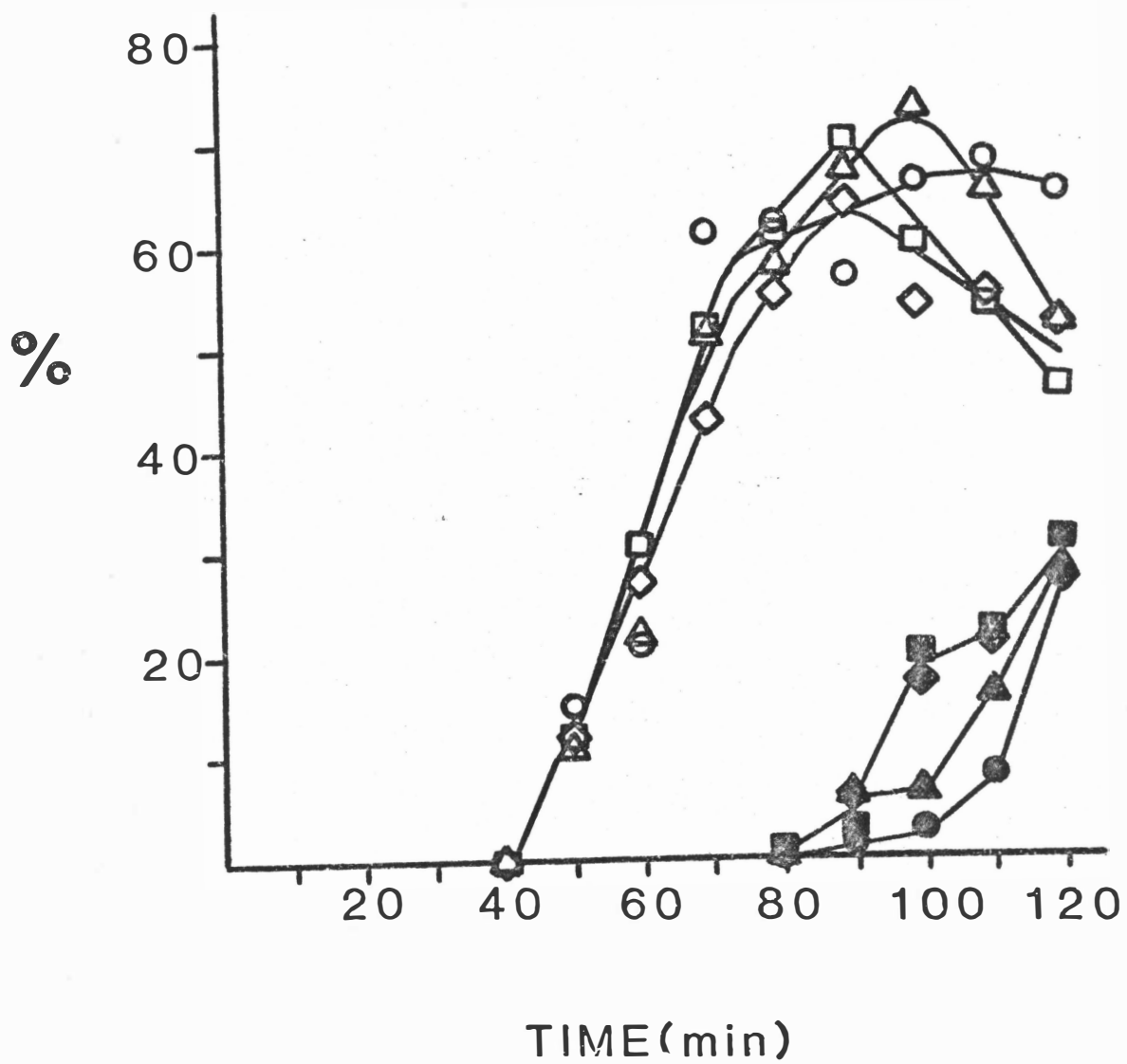

80
60
40
20
%
20
40
60
80
100
120
TIME(min)

Figure 12. Effect of growth phase on the capability of axenic cultures of Naegleria fowleri nN68 to enflagellate.

N. fowleri nN68 amebae were grown in unagitated cultures at 37°C in Nelson's medium with 2% (v/v) calf serum to the indicated points on the growth curve and then were washed and suspended in ameba saline at equivalent densities and agitated at 180 rpm and 37°C in a waterbath shaker. Assay of enflagellation was as described in the legend to Figure 9. Relative enflagellation compared to that achieved by 90 hour cells (48%) is shown.

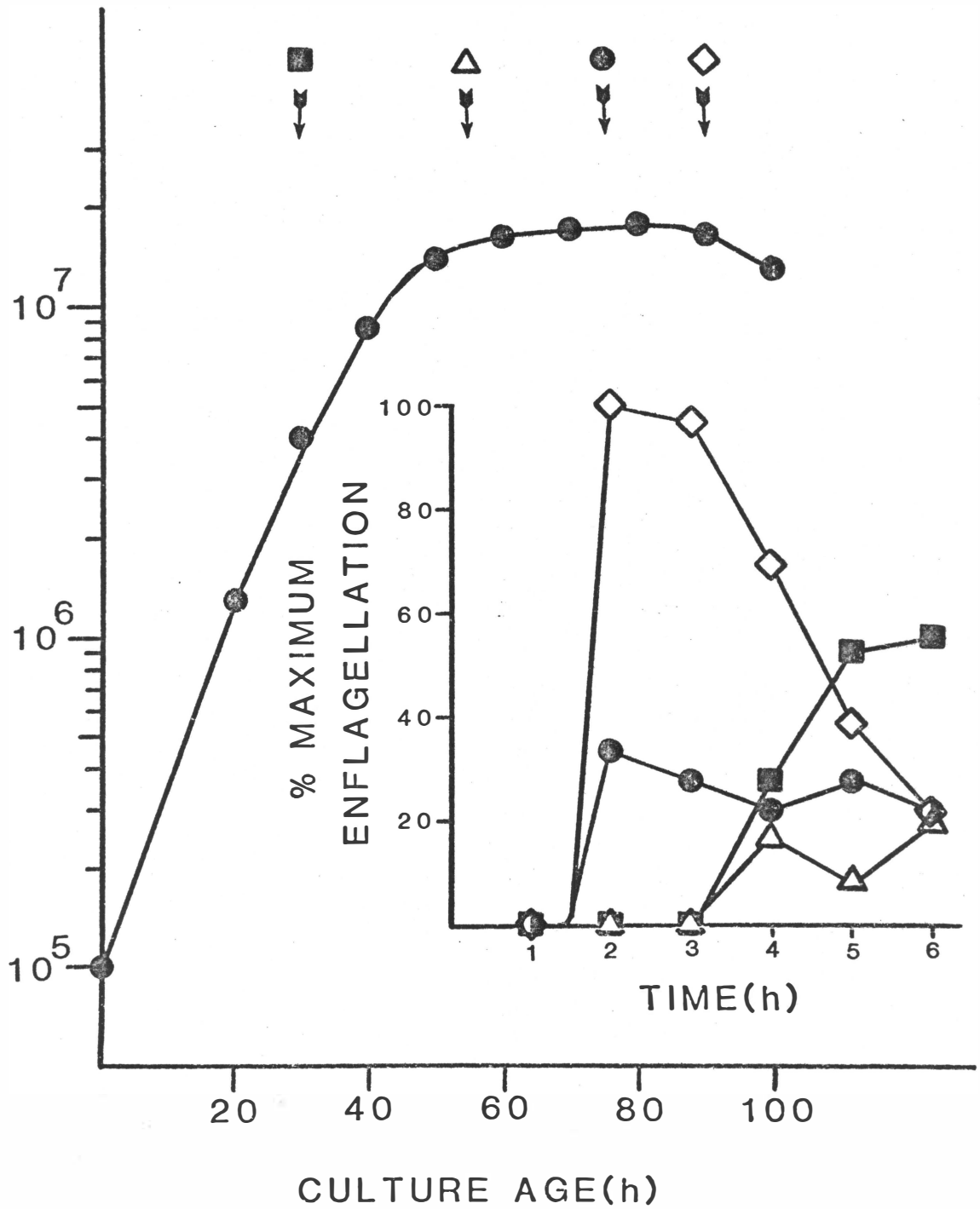

10^7
10^6
10^5
20
40
60
80
100
CULTURE AGE(h)
% MAXIMUM ENFLAGELLATION
100
80
60
40
20
1
2
3
4
5
6
TIME(h)

Table 5

Alteration of the Capability of Naegleria fowleri to Enflagellate by Replacement of Growth Medium With Expended or Fresh Nutrient Medium

Stage of Growth	Medium Replacement	Maximum Enflagellation	Time of Maximum Enflagellation (h)
Mid-logarithmic	none	7%	6
Mid-logarithmic	expended medium	42%	2
Stationary	none	44%	3
Stationary	fresh medium	5%	6

Cultures of N. fowleri nN68 were grown at 37°C to the mid-logarithmic (24 hours) or early stationary (74 hours) phase of growth. Fresh Nelson medium with 2% (v/v) calf serum or 74 hour cell-free culture supernatant ("expended medium") was added as indicated and cultures were incubated at 37°C for an additional 18 hours. The amebae were washed and suspended in ameba saline at a population density of 2×10^5 amebae/ml and shaken at 37°C. Enflagellation was assayed for 6 hours as described in the legend to Figure 9.

Table 6

Effect of Medium Constituent Removal Upon Enflagellation of *Naegleria gruberi* and *Naegleria fowleri*

Strain	Enflagellation Environment	Maximum Enflagellation (%)
nN68	Nelson medium	4
nN68	Nelson minus glucose	2
nN68	Nelson minus serum	8
nN68	Nelson minus liver digest	58
nN68	Page saline	60
NEG-M	M7 medium	10
NEG-M	M7 minus serum	12
NEG-M	M7 minus methionine	8
NEG-M	M7 minus yeast extract	52
NEG-M	M7 buffer	82

Amebae of *N. gruberi* NEG-M and *N. fowleri* nN68 were grown in unagitated cultures at 32°C in M7 medium or Nelson medium, respectively. Stationary phase cultures were washed and suspended in the indicated variations of the growth media and agitated at 32°C. Assays of enflagellation were performed by the standard method and the maximum % enflagellation in 120 minutes (NEG-M) or 6 hours (nN68) noted.

Figure 13. Enflagellation of Naegleria gruberi NEG-M in different non-nutrient buffers.

N. gruberi NEG-M amebae in 3-day unagitated cultures in M7 medium at 32°C were washed and suspended in different non-nutrient buffers at 25°C by the method described for Figure 6. Flasks with amebae suspended in Page saline (◇,◆), TK buffer (△,▲), M7 buffer (□,■), or deionized H_2O (○,●) were agitated at 120 rpm and 25°C and assayed for cell shape changes as described for Figure 6. Round cells (open symbols), flagellate-shaped cells (closed symbols).

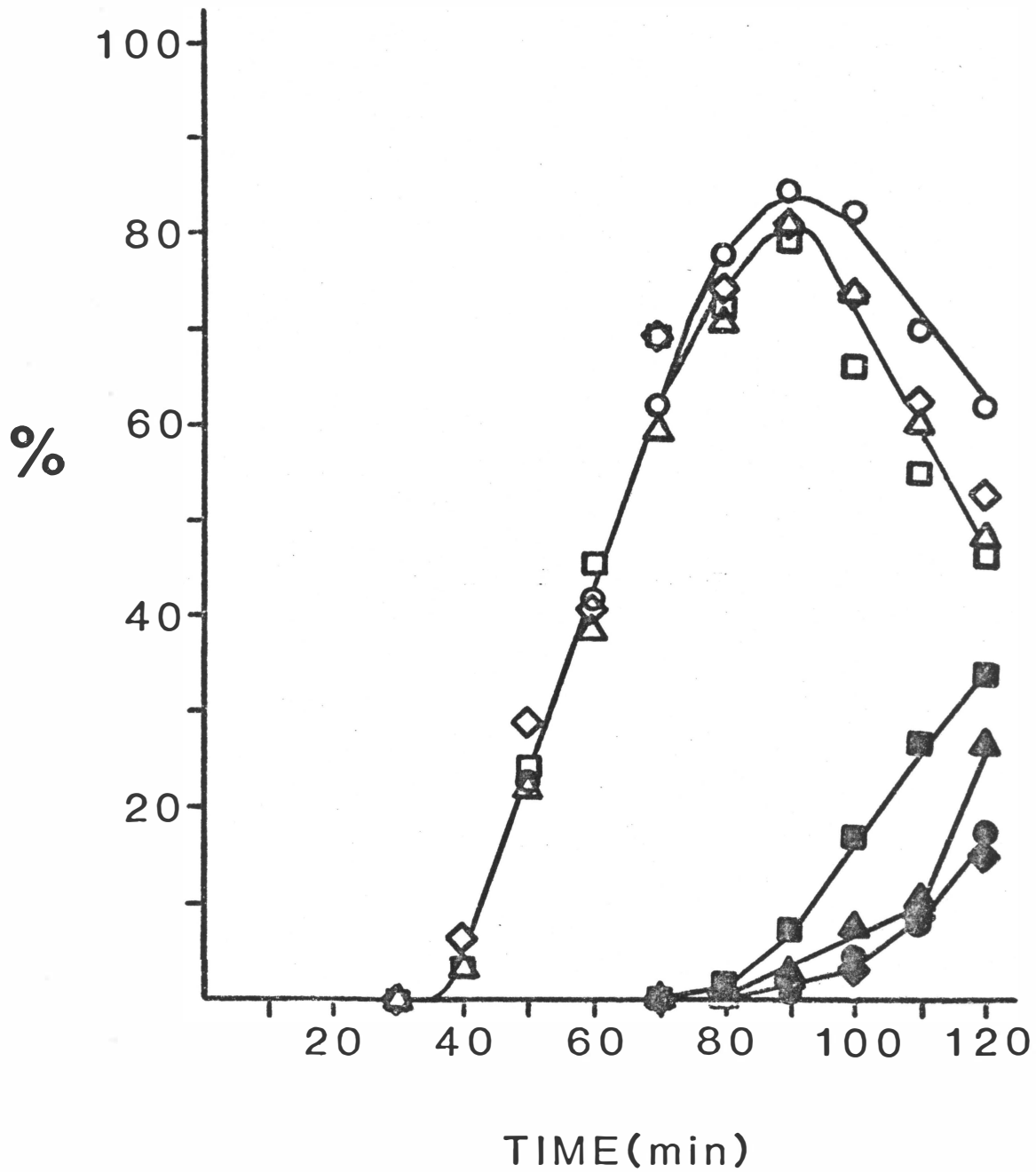

100
80
60
40
20
%
20
40
60
80
100
120
TIME(min)

Table 7

Enflagellation of Naegleria fowleri nN68 in

Different Non-Nutrient Environments

Enflagellation Environment	First Flagellates Observed (h)	Maximum Enflagellation (%)	Time of Maximum Enflagellation (h)
Page ameba saline	2	58	3.5
TK	2	35	4.5
Page minus K^+	2	49	4
Page minus Na^+	2	44	4.5
Page minus Ca^{++}	2	46	3
Page minus Mg^{++}	2	48	3.5
dH_2O	3.5	18	4.5

Naegleria fowleri nN68 amebae were grown in unagitated cultures at 32°C in Nelson medium with 2% (v/v) calf serum. Stationary phase cultures (96 h) were washed and suspended in the various non-nutrient buffers indicated at 32°C. Compositions of the Page ameba saline variations are listed in Materials and Methods. The suspensions (5 ml) were shaken at 32°C at 180 rpm and assayed for enflagellation as described for Figure 9.

1 µg cycloheximide/ml or 1 µg actinomycin D/ml and markedly inhibited by 5 µg/ml of either inhibitor (Fig. 14). Cycloheximide at 1 µg/ml completely inhibited enflagellation when added at the initiation time (Fig. 15), whereas actinomycin D up to 10 µg/ml inhibited formation of the flagellate shape but not loss of ameboid shape and rounding (Fig. 16).

Addition of cycloheximide at 45 minutes allowed a plateau value of approximately 50% of the cells to round up (Fig. 17); thus, the cycloheximide transition point ($TP_{50}CH$, 56) for rounding was 45 minutes. By a similar analysis, the $TP_{50}CH$ for flagella appearance was found to be approximately 50 minutes. A true $TP_{50}CH$ for appearance of flagellate shape was difficult to measure in enflagellating suspensions of the axenically grown cells due to the variable and low proportion of cells acquiring that shape (Fig. 6 and Fig. 17). The addition of cycloheximide at 60 minutes prevented conversion to flagellate shape but allowed conversion of 95% of the amebae to round cells (nearly all with flagella) by 100 minutes. Addition at 75 minutes and 85 minutes allowed an increasing proportion of cells to assume the flagellate shape and concurrently less of an "overshoot" was observed in the percentage of cells rounding in 85-minute treated cultures (Fig. 17).

Growth of Naegleria fowleri nN68 was retarded by 1 µg cycloheximide/ml or 15 µg actinomycin D/ml and markedly

Figure 14. Effects of different concentrations of cycloheximide and actinomycin D upon growth of Naegleria gruberi NEG-M in M7 medium.

Naegleria gruberi NEG-M amebae were inoculated at 2×10^5 amebae per 10 ml culture in M7 medium with 8% (v/v) dialyzed calf serum and incubated at 32°C. Cultures contained: no drug (●), 1 µg/ml of actinomycin D (◇) or cycloheximide (△), or 5 µg/ml of actinomycin D (□) or cycloheximide (○). Growth was monitored by Coulter counting samples as described in Materials and Methods. Each datum is the average from two experiments.

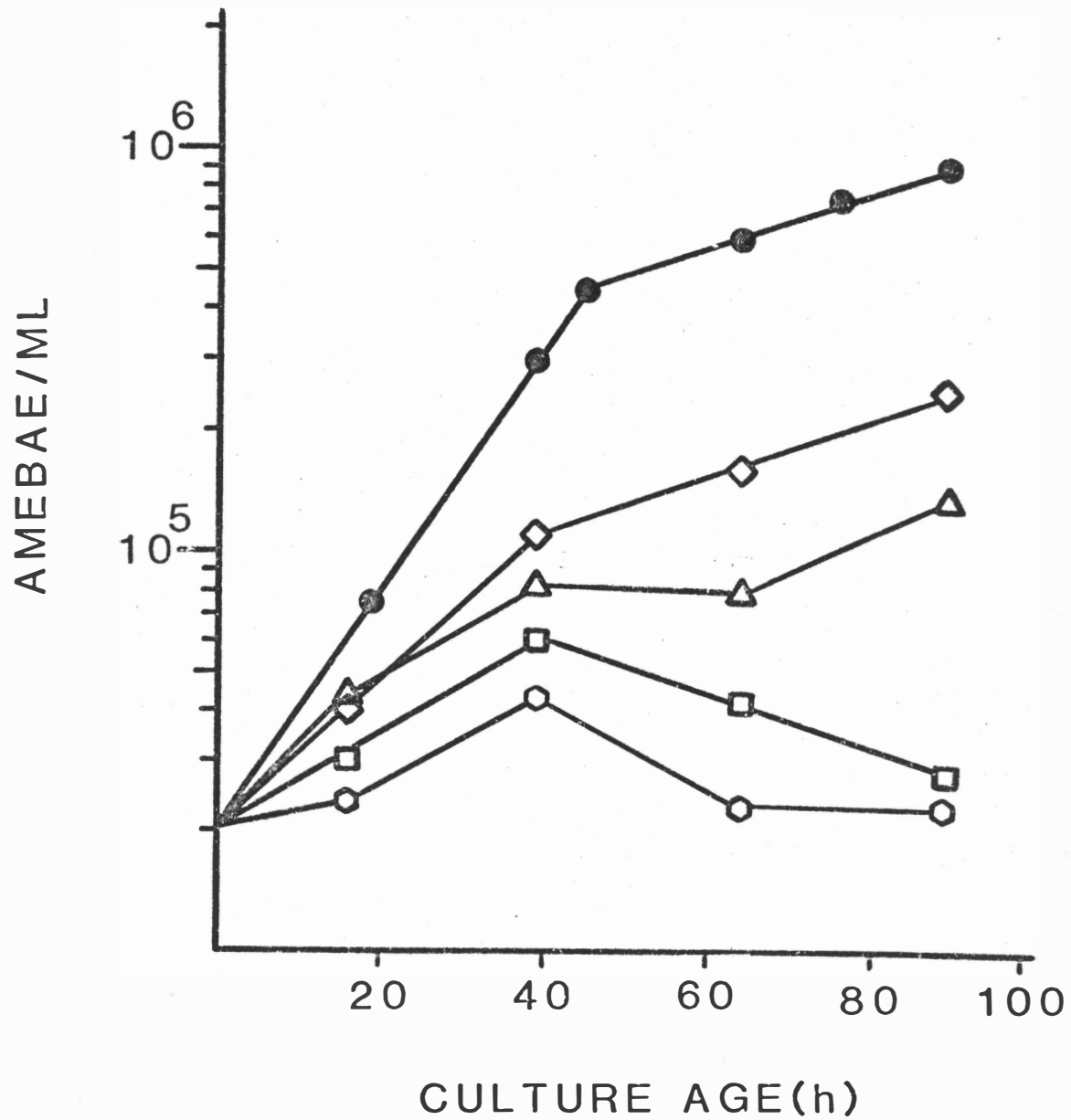

AMEBAE/ML
10^6
10^5
20
40
60
80
100
CULTURE AGE(h)

Figure 15: Effect of different concentrations of cycloheximide upon enflagellation of Naegleria gruberi NEG-M.

N. gruberi NEG-M amebae grown at 32°C in unagitated cultures in M7 medium for 72 hours were washed and suspended in M7 buffer containing cycloheximide at: 0 µg/ml (■), 0.5 µg/ml (○), 1 µg/ml (△), 2 µg/ml and 5 µg/ml. The suspensions (5 ml) were agitated at 120 rpm at 25°C and cell shape changes were monitored as described for Figure 11. No change in the amebae suspended in M7 buffer containing cycloheximide at 2 or 5 µg/ml was detected. The data are the average of two experiments. Round cells, main graph; flagellate-shaped cells, inset.

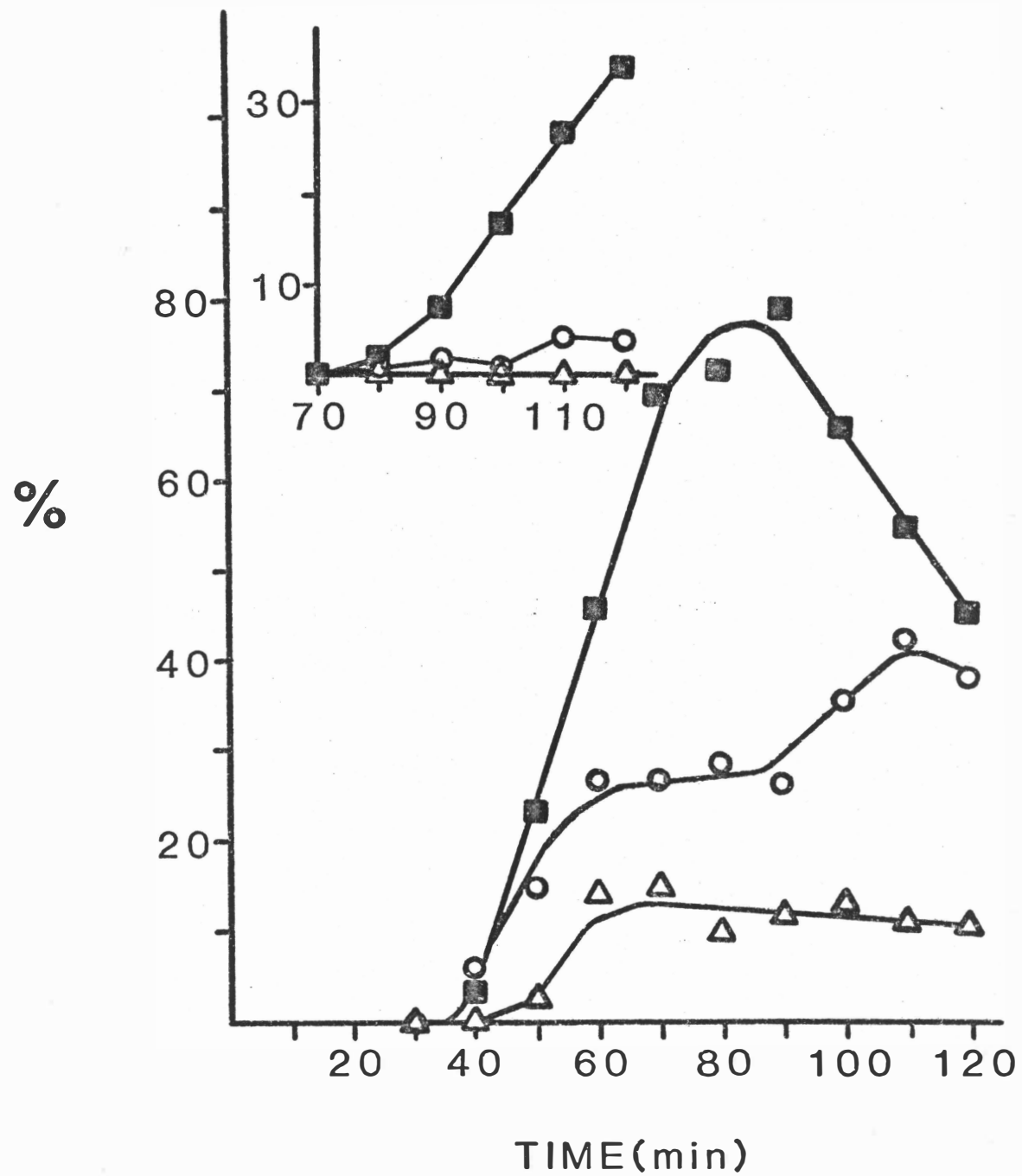

%
30
10
80
60
40
20
70
90
110
20
40
60
80
100
120
TIME(min)

Figure 16. Effect of different concentrations of actinomycin D upon enflagellation of *Naegleria gruberi* NEG-M.

N. gruberi NEG-M amebae were grown as described for Figure 15. Cultures at 70 hours were washed and suspended in M7 buffer at 25°C containing actinomycin D at: 0 µg/ml 0.5 µg/ml, 1.0 µg/ml, 5 µg/ml (□), and 10 µg/ml (◇). Suspensions (5 ml) were agitated at 120 rpm at 25°C and enflagellation monitored as described for Figure 11. Amebae suspended in buffer containing 0.5 and 1.0 µg/ml actinomycin D enflagellated like the controls. Control amebae without inhibitor converted to round cells (●) and then to flagellate-shaped cells (■) as indicated. All solutions of actinomycin D and flasks containing suspensions for enflagellation were covered with aluminum foil to prevent light inactivation of the drug. The data are the average of two experiments.

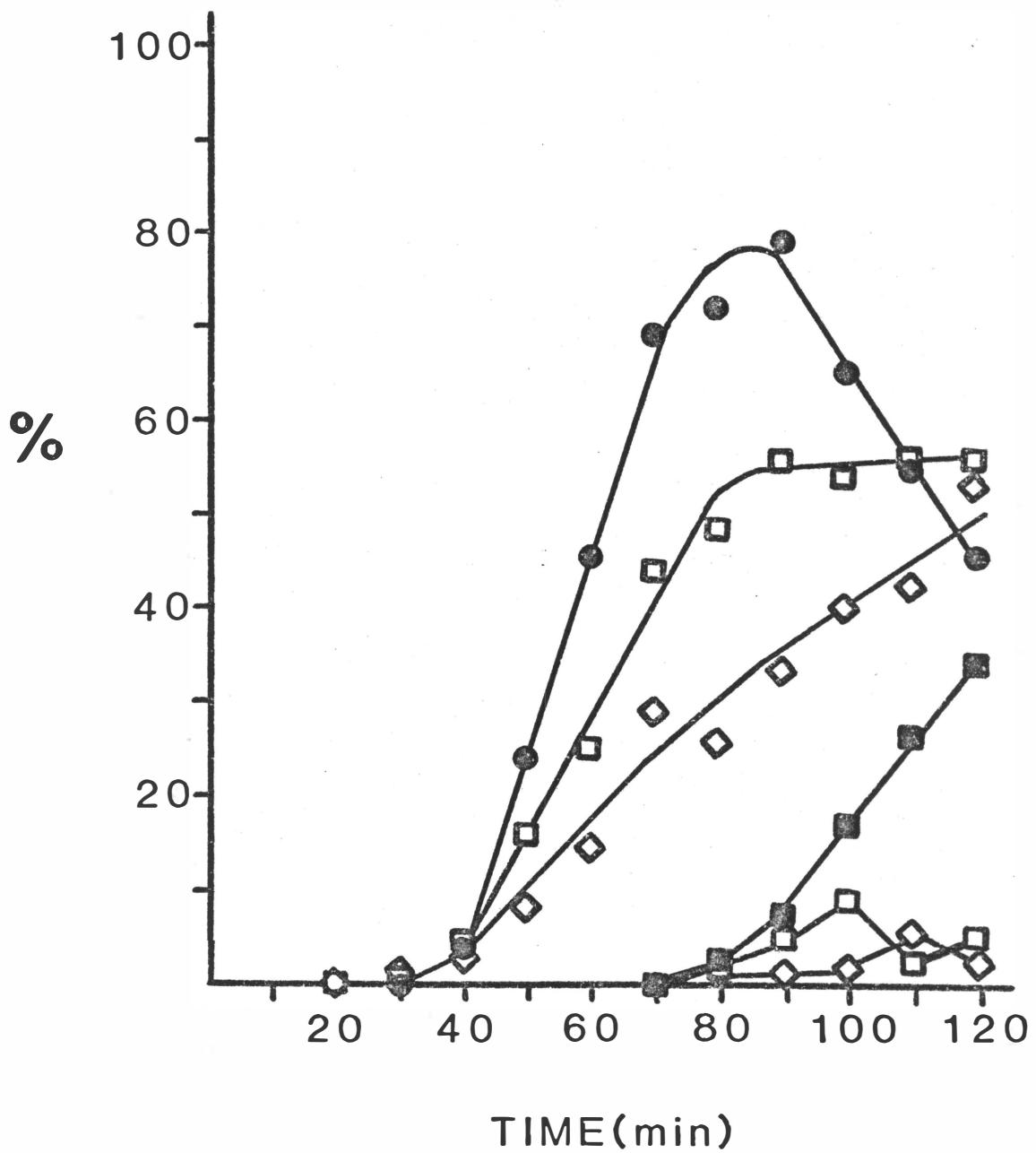
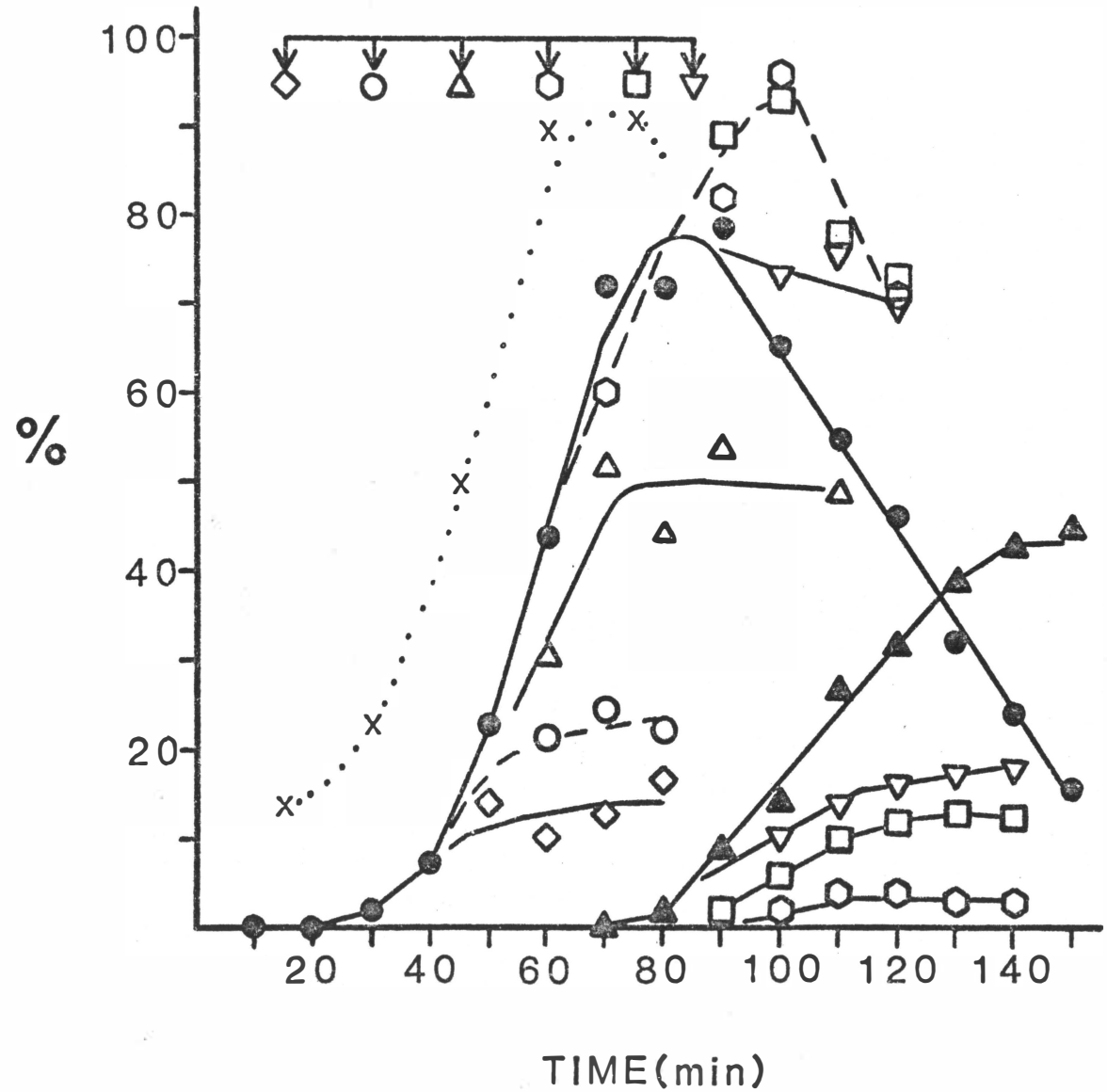

100
80
60
40
20
%
20
40
60
80
100
120
TIME(min)

Figure 17. Effect of delayed addition of 5 µg/ml cycloheximide upon enflagellation of Naegleria gruberi NEG-M.

Cultures of N. gruberi NEG-M grown at 32°C in unagitated cultures in M7 medium for 72 hours were washed and suspended in M7 buffer at 25°C as described for Figure 6. Suspensions (5 ml) were agitated at 120 rpm at 25°C and monitored for cell shape changes as described for Figure 11. At zero time (no enflagellation seen), 15 minutes (◇), 30 minutes (○), 45 minutes (△), 60 minutes (⬡), 75 minutes (□), and 85 minutes (▽), cycloheximide was added to separate suspensions to a final concentration of 5 µg/ml and agitation of the suspensions continued. The "plateau percent" round cells observed in cultures treated at those times are plotted (✕) as a function of time of addition of cycloheximide to give an "onset of insensitivity" curve (56). Control amebae without additions converted to round cells (●) and then flagellate-shaped cells (▲) as indicated. Data are the average of two independent experiments.


100
80
60
40
20
%
20
40
60
80
100
120
140
TIME(min)

inhibited by 5 µg cycloheximide/ml or 25 µg actinomycin D/ml (Fig. 18). Cycloheximide at 0.5 µg/ml and actinomycin D at 25 µg/ml completely prevented enflagellation when added at the time of initiation (Fig. 19). Delayed additions of cycloheximide produced quite different results in Naegleria fowleri than had been obtained for N. gruberi. Cycloheximide added as late as 60 minutes after subculture to ameba saline completely suppressed the normal onset of enflagellation at 2 hours (Fig. 20). Cycloheximide added after 120 minutes not only prevented cells from progressing to flagellates but caused reversion of those flagellates present. Similarly, cycloheximide added after 240 minutes caused a more rapid reversion of flagellates to ameboid cells.

Actinomycin D at 25 µg/ml added as late as 60 minutes after subculture to ameba saline completely suppressed normal onset of enflagellation (Fig. 21). Actinomycin D added at 90 minutes retarded enflagellation, but the number of flagellates continued to increase for 30 to 60 minutes thereafter. Addition at 120 minutes prevented a further increase in percentage of flagellates and addition after 180 minutes accelerated the rate at which existing flagellates reverted to ameboid cells (Fig. 21). Light microscopic observation of enflagellating cultures confirmed that the flagellates reverted to ameboid cells and

Figure 18. Effects of cycloheximide and actinomycin D upon growth of Naegleria fowleri nN68 in Nelson medium.

Amebae of N. fowleri nN68 were grown at 37°C in unagitated cultures in Nelson medium in the presence or absence of cycloheximide (panel A) or actinomycin D (panel B). Concentrated cycloheximide stock solution was added at final concentrations of 0.5 (◇), 1.0 (△), and 5.0 µg/ml (□) to growing cultures at 20 hours; other cultures were treated with equivalent amounts of Page saline (●). Actinomycin D at concentrations of 10 (○), 15 (◇), or 25 µg/ml (△) was included in the growth medium at time of inoculation. Untreated controls received equivalent amounts of Page saline (●). Actinomycin D flasks were covered with aluminum foil. Cell counts were performed as described in Materials and Methods.

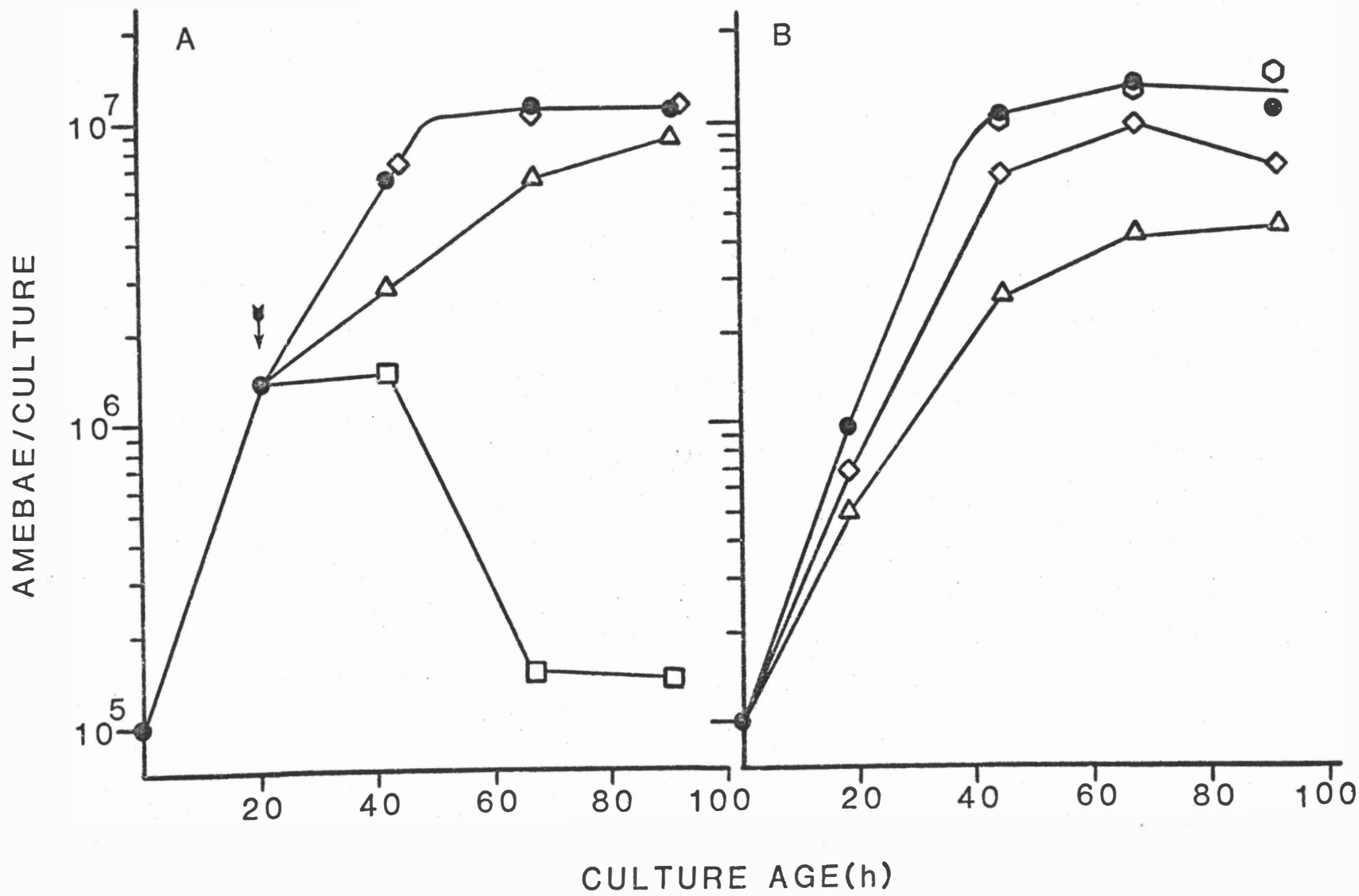

A
B
10^7
10^6
10^5
AMEBAE/CULTURE
20
40
60
80
100
CULTURE AGE(h)

Figure 19. Effect of different concentrations of cycloheximide and actinomycin D upon enflagellation of Naegleria fowleri nN68.

Amebae of Naegleria fowleri nN68 were grown at 37°C in unagitated cultures in Nelson medium. Amebae from cultures at 92 hours were washed, then suspended in a small volume of ameba saline at 37°C and diluted into aliquots of ameba saline containing sufficient amounts of inhibitor to give the desired final concentrations of drug and a cell density of 2×10^5 amebae/ml. Enflagellation was monitored as described for Figure 9.

Panel A. Cycloheximide at 0.1 (○), 0.25 (▽), and 0.5 µg/ml (◇) was present in the enflagellating suspensions. Untreated control (●).

Panel B. Actinomycin D at 5 (□), 10 (○), 15 (◇), and 25 µg/ml (△) was present in enflagellating suspensions. Untreated control (●). All flasks were covered with aluminum foil.

Maximum control enflagellation was 36%. Values plotted are the percentages of the maximum control value and are the averages of two replicate flasks per point.

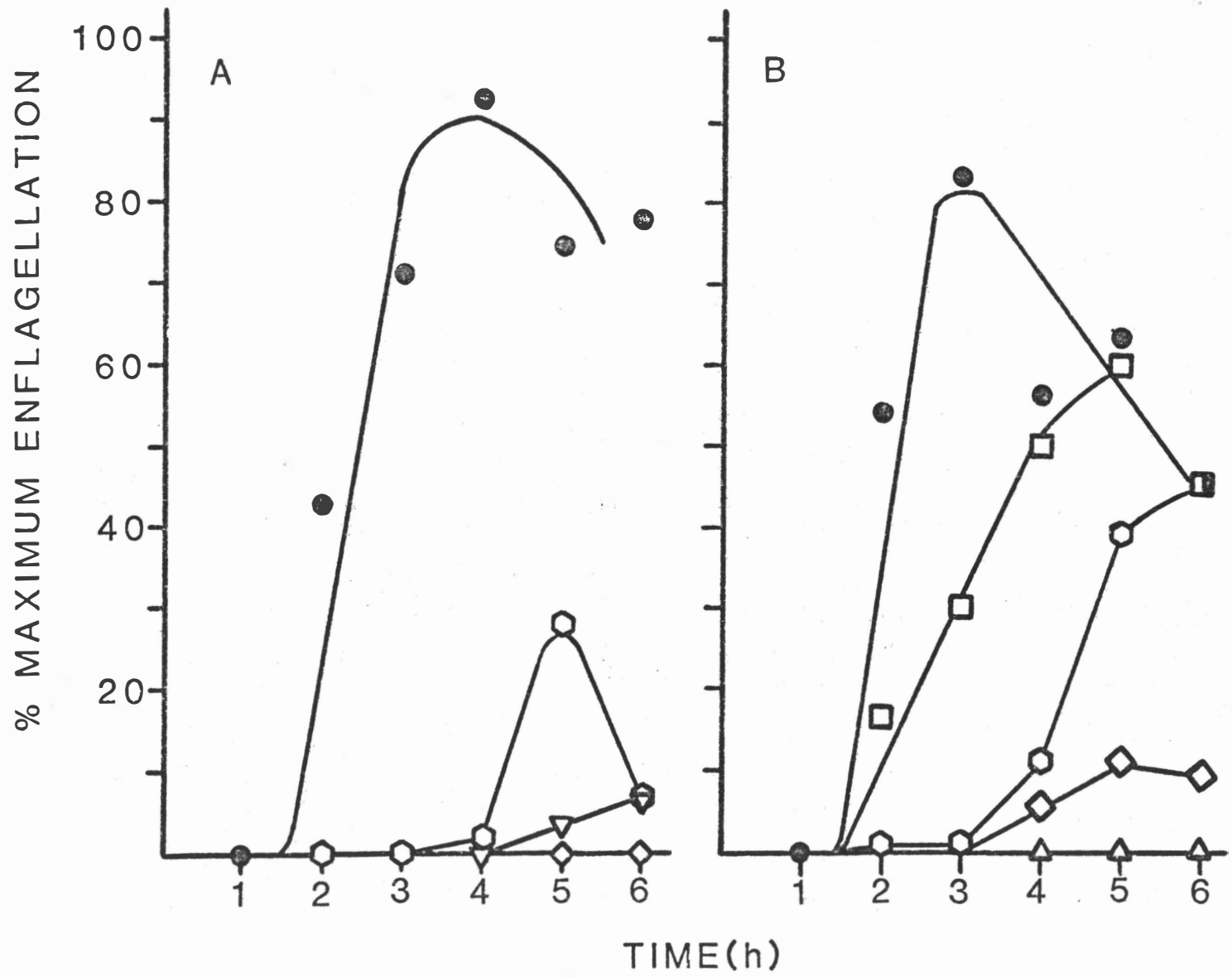

% MAXIMUM ENFLAGELLATION
100
80
60
40
20
A
B
1
2
3
4
5
6
TIME(h)

Figure 20. Effect of delayed addition of cycloheximide upon enflagellation of Naegleria fowleri nN68.

Naegleria fowleri nN68 amebae were grown in unagitated cultures at 37°C in Nelson medium. Amebae from cultures at 94 hours were washed, concentrated by suspension in a small volume of ameba saline at 37°C and immediately diluted into flasks containing ameba saline to give 2 x 10^5 amebae/ml. Two flasks contained a final concentration of 0.5 µg/ml cycloheximide at zero time. Additional flasks were treated with concentrated cycloheximide stock solution to give a final concentration of 0.5 µg/ml at the times indicated. Flasks treated with an equivalent amount of ameba saline at zero time (●) or given no additional treatment enflagellated equally well. Enflagellation was monitored as described for Figure 9. The maximum percent flagellates scored in control flasks was 50%. Values plotted are percentages of the maximum control value and are the average from two identically treated flasks per time point. No flagellates were observed in flasks treated at zero time, 30 minutes or 60 minutes after initiation.

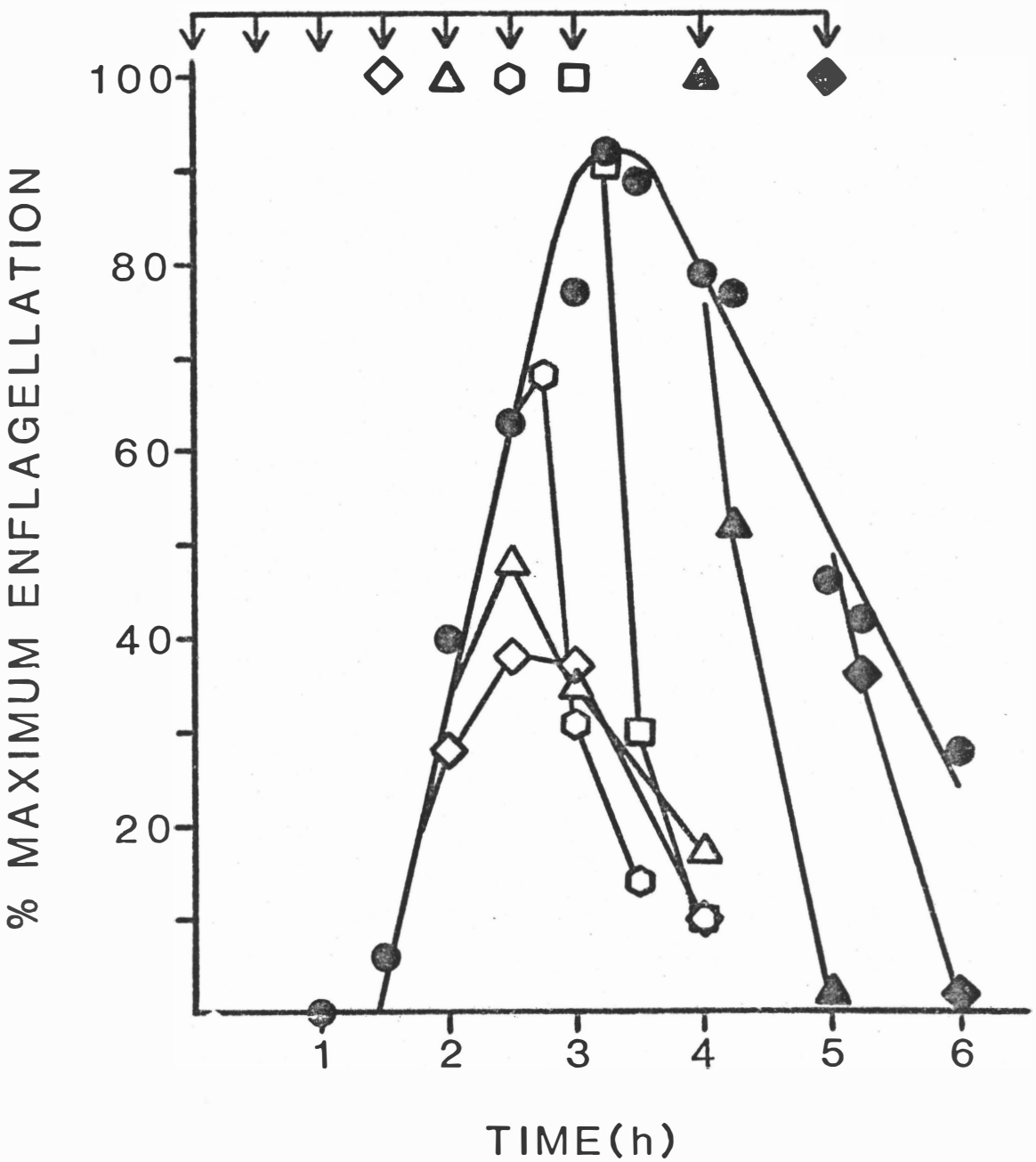
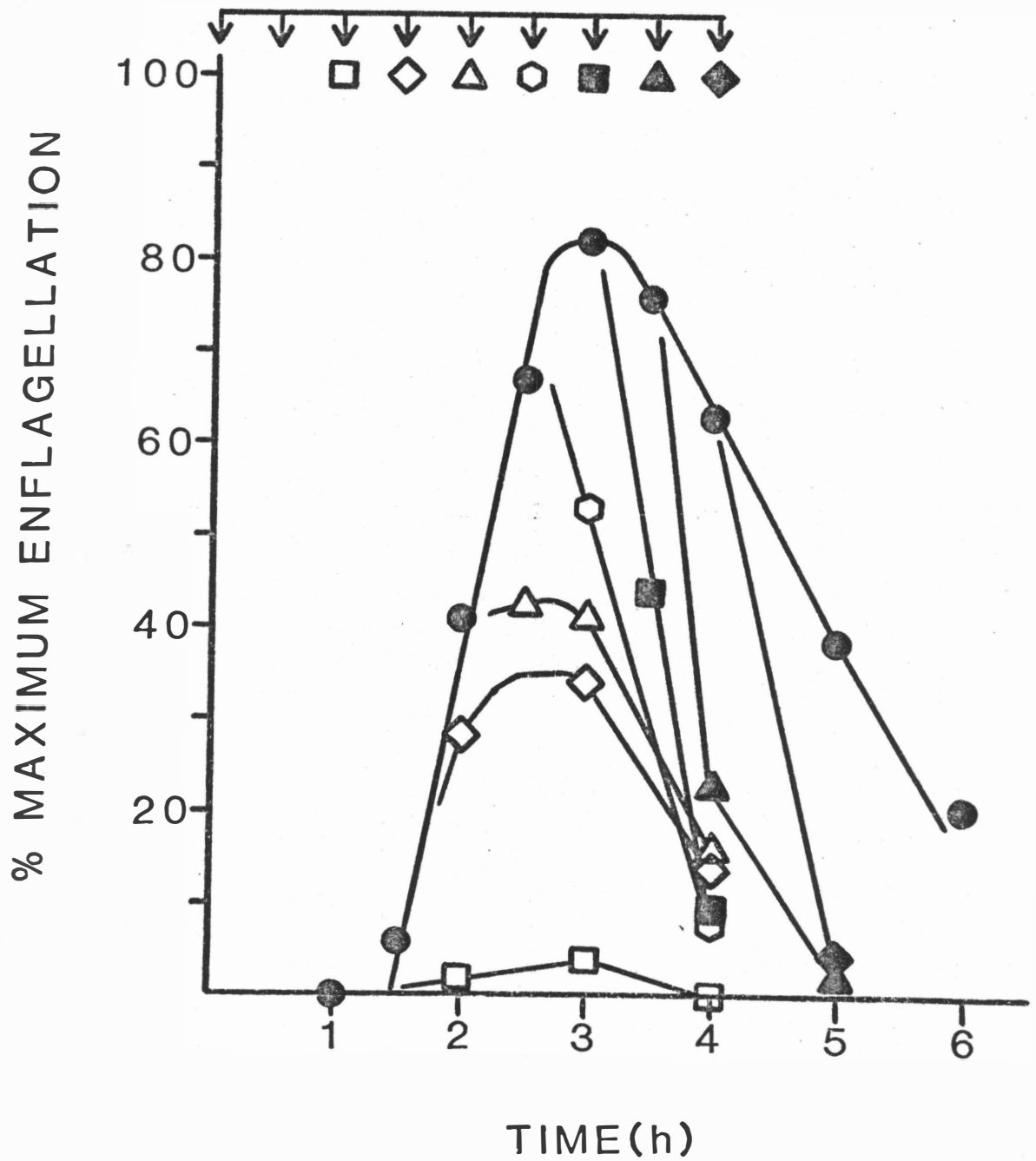

% MAXIMUM ENFLAGELLATION
100
80
60
40
20
1
2
3
4
5
6
TIME(h)

Figure 21. Effect of delayed addition of actinomycin D upon enflagellation of Naegleria fowleri nN68.

Amebae of N. fowleri nN68 were grown, washed, suspended and distributed to shaking suspensions at 4 x 10^5 amebae/ml as described for Figure 20. Two flasks contained actinomycin D at a final concentration of 25 µg/ml in the suspension at zero time. Additional pairs of flasks were treated with concentrated actinomycin D stock solution to give a concentration of 25 µg/ml at the times indicated. Flasks treated with equivalent amounts of ameba-saline at zero time (●) or given no additional treatment enflagellated equally well. All flasks were covered with aluminum foil throughout enflagellation except an additional pair of untreated controls which enflagellated like the covered controls. Enflagellation was monitored as in Figure 9. The maximum percent flagellates scored in control flasks was 49%. Values plotted are percentages of the maximum control value and are the average obtained from two identically treated flasks per time point. No flagellates were observed in flasks treated at zero time or 30 minutes after initiation.


% MAXIMUM ENFLAGELLATION
100
80
60
40
20
1
2
3
4
5
6
TIME(h)

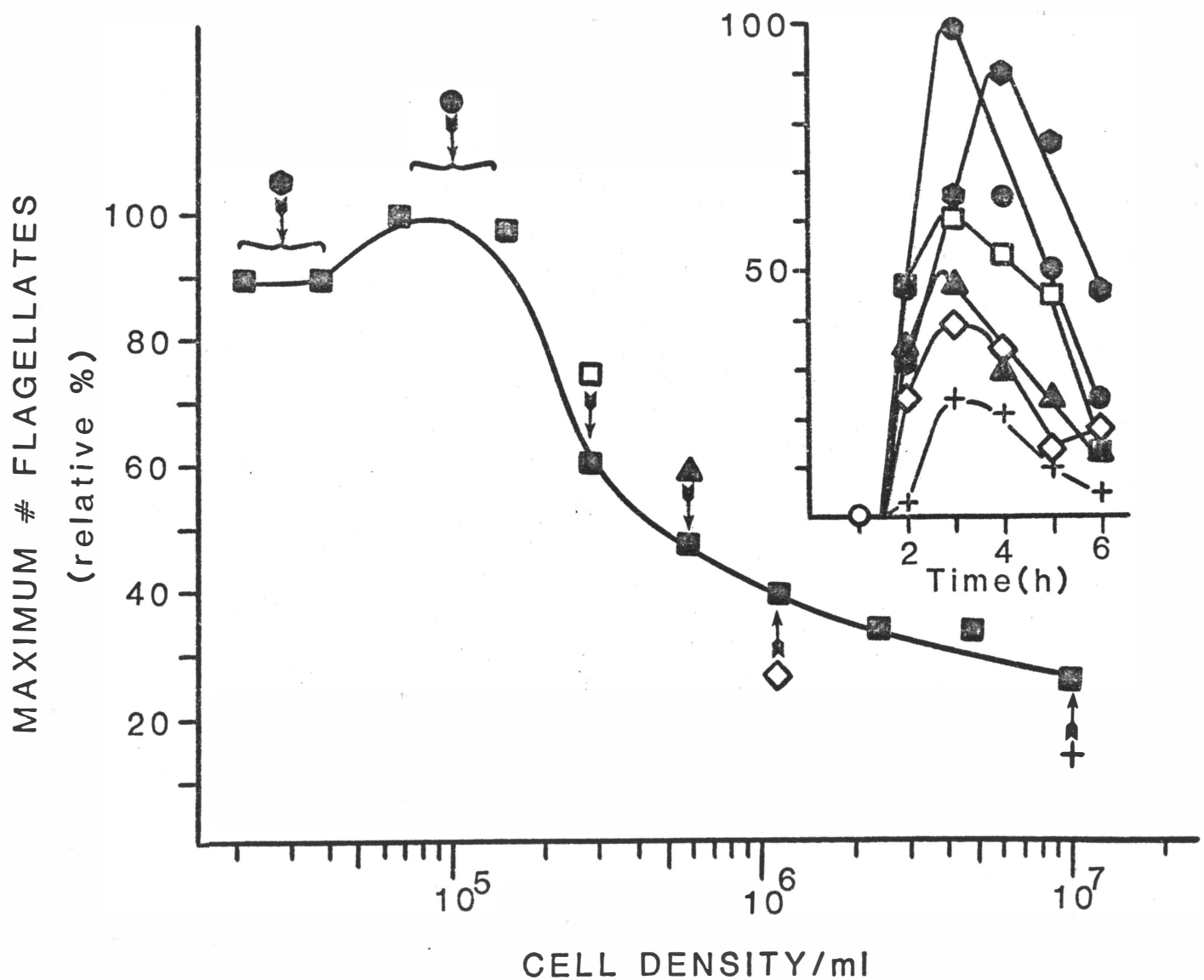
that neither inhibitor caused lysis of the differentiating cells at the concentrations used.

Several additional factors that could affect enflagellation of N. fowleri were examined. The number of rinses with ameba saline (from 2 to 5) (not shown), nor the volume of the rinses (2.5 ml to 20 ml) markedly altered either the yield of flagellates or the rate of flagellate formation. Delays between rinsing and suspension of cells in ameba saline adversely affected the yield and rate of enflagellation of N. fowleri. N. fowleri could be concentrated by releasing amebae from several flasks into small volumes of ameba saline by chilling the cultures at 5°C for 10 minutes. The amebae could be pipetted vigorously and dispersed by vigorous agitation using a vortex mixer without adversely affecting their capacity to enflagellate. The extent of enflagellation by strain nN68 was suppressed at population densities greater than 5×10^5 amebae/ml. Population densities of less than 2×10^5 amebae/ml enflagellated to approximately the same extent (Fig. 22). Population density did not appear to affect the time of first flagellate appearance nor the time at which the maximum percentage of flagellates was observed except for cells at the lowest two densities which were less synchronous in enflagellating (Fig. 22).

The capability of enflagellating N. fowleri amebae to incorporate selected radionuclides during incubation in

Figure 22. Effect of population density upon enflagellation of Naegleria fowleri nN68.

N. fowleri nN68 were grown in unagitated cultures at 37°C in Nelson medium. Amebae from cultures at 96 hours were washed twice with ameba saline and suspended in a single aliquot of ameba saline at 37°C to give a concentrate density of 1.1×10^7 amebae/ml. Serial dilutions of the suspension into ameba saline blanks at 37°C were made, the dilutions transferred to tissue culture flasks and then shaken at 37°C. Cell densities of the suspensions were ascertained by counting samples of each dilution as described in Materials and Methods. Enflagellation was monitored as described for Figure 9. Maximum enflagellation scored during 6 hours in each of the suspensions is plotted relative to that achieved by cells at 6.8×10^4 amebae/ml, 40% (assigned as 100%). Inset, kinetics of enflagellation for suspensions at the densities indicated by the symbols.


MAXIMUM # FLAGELLATES
(relative %)
100
80
60
40
20
10^5
10^6
10^7
CELL DENSITY/ml
100
50
2
4
6
Time(h)

ameba saline for 4 hours at 37°C was assessed. Adenine, leucine, methionine, and inorganic phosphate were incorporated into trichloroacetic acid precipitable material (Table 8). Most, but not all, of the ^{32}P label was soluble in hot 5% TCA. Little, if any, radioactivity was incorporated into trichloroacetic acid precipitable material during a 4-hour period when [^{14}C]-glucose or [^{3}H]-thymidine was supplied to the enflagellating cells. Incorporation of ^{35}S-methionine into enflagellating amebae was at least 20-fold less than that achieved in amebae growing in Nelson medium (Table 9) and remained low even when a 4- to 10-fold increase in concentration of radiolabel was used. However, long exposure autoradiograms of flagellates radiolabeled during differentiation demonstrated widespread distribution of [^{35}S]-methionine among the several hundred polypeptides resolved by two-dimensional electrophoresis (Fig. 23).

Autoradiograms of flagellates radiolabeled with inorganic phosphate during differentiation also showed incorporation into a variety of polypeptides (Fig. 24). Some of the [^{32}P]-labeled polypeptides could be identified with ^{35}S-labeled polypeptides by matching the patterns but many of the [^{32}P]-labeled polypeptides did not appear to co-migrate with ^{35}S-label.

A morphological study of the events occurring during the enflagellation of Naegleria fowleri was done.

Table 8

Incorporation of Selected Radiolabeled Precursors into Macromolecules of Enflagellating Naegleria fowleri

Precursor	Input (μCi/ml)	Incorporation[a]
[8-^{14}C]-adenine	6.7	4.0×10^4
[U-^{14}C]-leucine	7.4	3.6×10^4
[^{35}S]-methionine	(1) 54	(1) 5.7×10^4 (0.21)[b]
	(2) 200	(2) 2.8×10^5 (1.28)
[^{32}P]-inorganic phosphate	113	1.7×10^5

N. fowleri amebae from stationary phase cultures were rinsed and suspended in ameba saline at a density of 5×10^5 amebae/ml and shaken at 37°C. The indicated radiolabeled precursors (with specific activities of 41 Ci/mole for adenine, 320 Ci/mole for leucine, and 1095 Ci/mmole for methionine; the ^{32}Pi was carrier-free) were added at time of suspension. Incorporation of precursors into trichloroacetic acid insoluble material was measured as described in Materials and Methods.

[a]Counts per minute per 10^6 cells, incorporated during 4 h of enflagellation, corrected for background.

[b]Numbers in parentheses are picomoles [^{35}S]-methionine incorporated per 10^7 cells per 4 h (compare to Table 9).

Table 9

Incorporation of [^{35}S]-Methionine into Naegleria fowleri nN68 During Growth in Nelson Medium

Experiment	Input Label (μCi/ml)	Label Period (h)	Incorporation pmole/10^7 Cells
1	35	6	4.94
2	20	5	2.68

N. fowleri nN68 amebae growing in unagitated cultures in Nelson medium at 37°C were labeled by addition of [^{35}S]-methionine (1095.1 Ci/mmole, New England Nuclear) to growing cultures at the indicated final activity concentrations. Incorporation of radioactivity into trichloroacetic acid precipitable material was measured as described in Materials and Methods. Incorporation is expressed as the picomoles of [^{35}S]-methionine incorporated per 10^7 amebae during the label period, corrected for background.

Figure 23. Autoradiogram of newly synthesized [^{35}S]-methionine-labeled polypeptides in flagellates of N. fowleri.

The proteins were labeled with [^{35}S]-methionine during differentiation in non-nutrient buffer, prepared and resolved by two-dimensional electrophoresis as described in Materials and Methods.

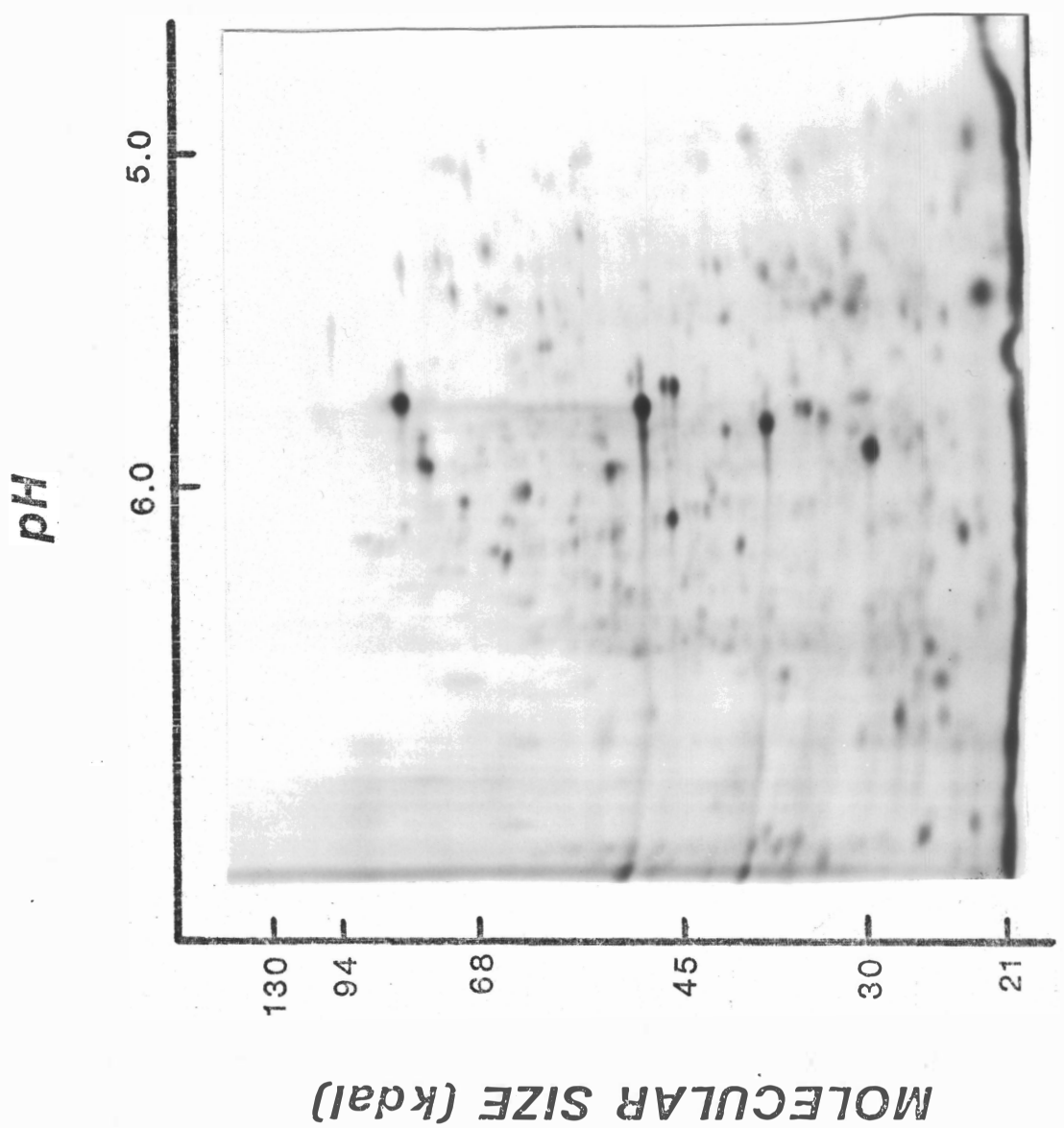
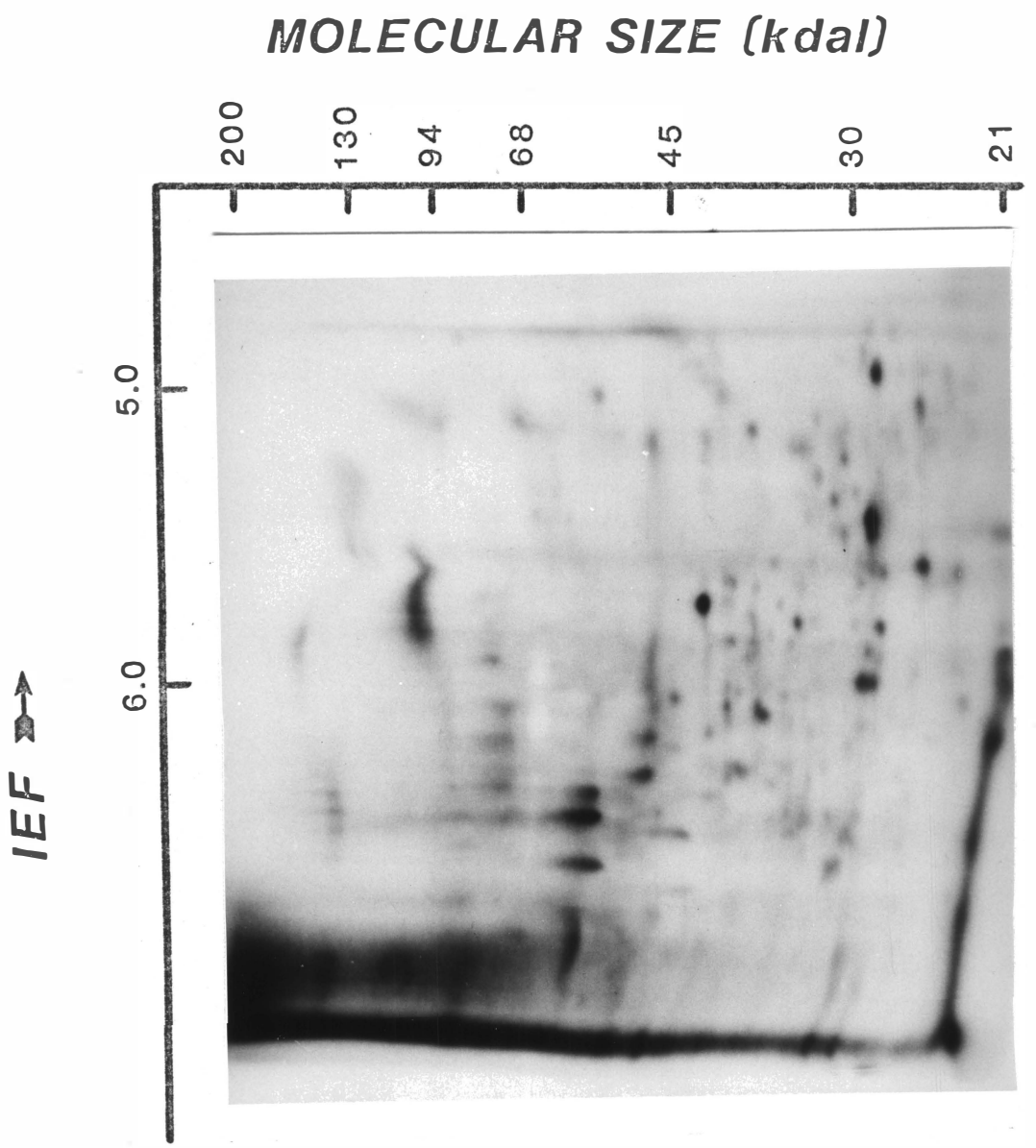

pH
6.0
5.0
130
94
68
45
30
21
MOLECULAR SIZE (kdal)

Figure 24. Autoradiogram of polypeptides of flagellates of Naegleria fowleri labeled with [^{32}P]-orthophosphate during enflagellation.

N. fowleri amebae were grown at 37°C in unagitated cultures in Nelson medium. Stationary phase cultures at 75 hours were washed with ameba saline containing 2 mM MOPS (morpholinopropane sulfonic acid) buffer in place of phosphate buffer. Amebae were suspended in MOPS-buffered ameba saline at 37°C containing 500 μCi/ml [^{32}P]-orthophosphate. Enflagellation was carried out at 37°C agitating at 180 rpm. Samples of flagellates were washed in cold ameba saline with phosphate and prepared for electrophoresis as described in Materials and Methods. The sample containing 235,000 cpm was subjected to two-dimensional electrophoresis and autoradiography as described in Materials and Methods.


MOLECULAR SIZE (kdal)
200
130
94
68
45
30
21
5.0
6.0
IEF

A non-enflagellating strain was also studied to determine if it was arrested at some morphologically identifiable stage of enflagellation and to aid in distinguishing effects resulting from nutritional deprivation from those pertaining more directly to the morphogenesis (106).

Amebae of N. fowleri nN68 and NF69 were grown and stimulated to enflagellate by washing and suspension in non-nutrient ameba saline. Under the optimized conditions, N. fowleri nN68 amebae began to enflagellate at approximately 120 minutes after transfer, and a yield of 65% to 70% transformed cells was achieved in the subsequent 60 minutes (Fig. 25). Under those conditions, N. fowleri NF69 amebae did not become mobile flagellates nor did they assume the elongated flagellate body shape (Fig. 25).

Electron microscopic examination of N. fowleri confirmed that it is a typical eucaryotic protist. Numerous membrane-bound cytoplasmic vacuoles were observed in both strains; these vacuoles contained a variety of materials, including membranous structures, aggregates of electron-dense fibrillar material and loosely arranged, lightly stained fibrillar material (Fig. 26 and 27). After subculture to Page saline, the number of vacuoles decreased within 2 hours in the enflagellating strain nN68 (Fig. 28 and 29). The progressive loss of vacuoles was somewhat slower in the non-enflagellating strain NF69 (Fig. 30).

Concomitant with the observed decrease in number of vacuoles in the amebae, an increase in the amount of membranous structures and aggregated electron-dense fibrillar material was noted in the culture medium.

Three types of inclusions were observed within the cytoplasm of N. fowleri. First, small electron-dense particles approximately 83 nm in diameter were present in both enflagellating and non-enflagellating strains, including mature flagellates (Fig. 26-30); when viewed at high magnification they appeared to be membrane bound. Second, numerous electron-translucent droplets approximately 500 nm in diameter, not limited by a membrane, were observed in all stages of the enflagellating strain (Fig. 26, 28, and 29) but were not seen in the non-enflagellating variant (Fig. 27 and 30). The number and morphology of the droplets remained relatively constant in all stages of enflagellation. And third, other inclusions consisted of large, dense membrane-bound granules approximately 1.7 μm in diameter. The latter were seen in the enflagellating strain nN68 for up to 60 minutes after transfer to Page saline (Fig. 26). These structures were not observed in the non-enflagellating strain NF69 (Fig. 27 and 30).

Rough endoplasmic reticulum and free ribosomes were recognized in both strains (Fig. 28 and 31). Apparently spherical or spheroidal mitochondria were observed in the

Figure 25. Time course of enflagellation in N. fowleri.

Amebae of the enflagellating strain nN68 (●) and the non-enflagellating variant NF69 (▲) were shaken in Page saline at 42°C. The proportion of elongated flagellated cells was determined by light microscopic examination of fixed samples.

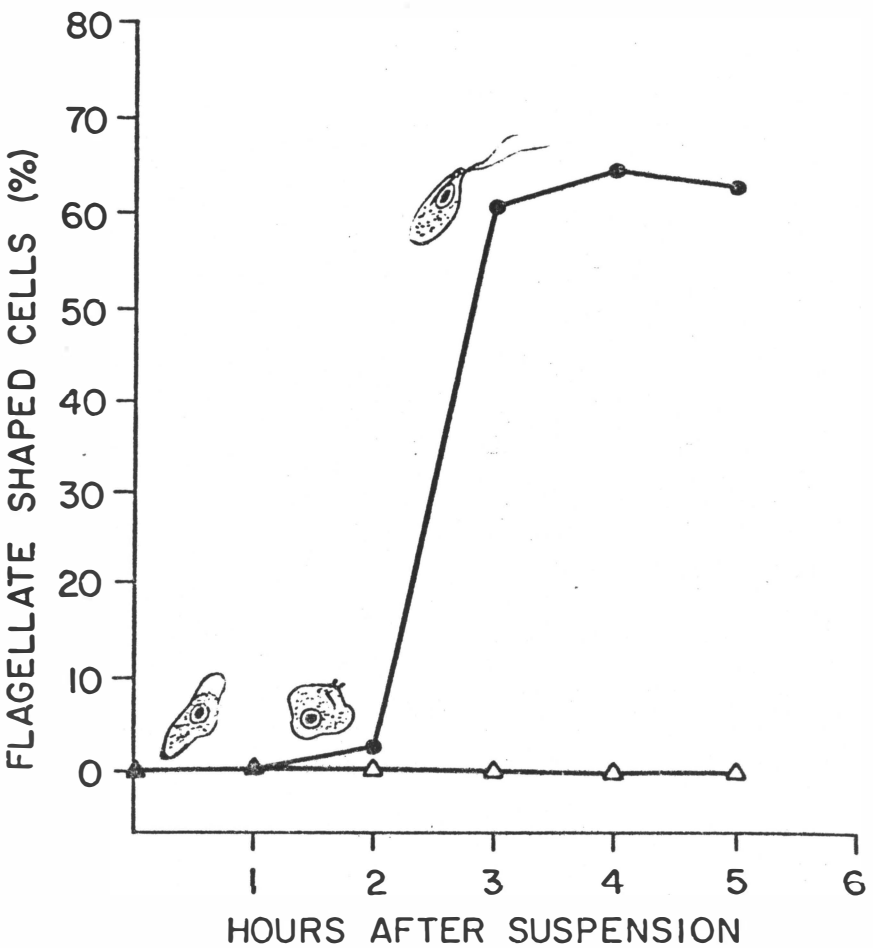

80
70
60
50
40
30
20
10
0
FLAGELLATE SHAPED CELLS (%)
1
2
3
4
5
6
HOURS AFTER SUSPENSION

Figure 26. Ultrastructure of an ameba of N. fowleri nN68 grown in Nelson medium and fixed immediately after transfer to Page saline.

N, nucleus; V, vacuole; TD, translucent droplet; DG, dense granule. Scale marker: 1 μm.

Figure 27. Ultrastructure of an ameba of N. fowleri NF69 grown in Nelson medium and fixed immediately after transfer to Page saline.

N, nucleus; NL, nucleolus; V, vacuole. Scale marker: 1 μm.

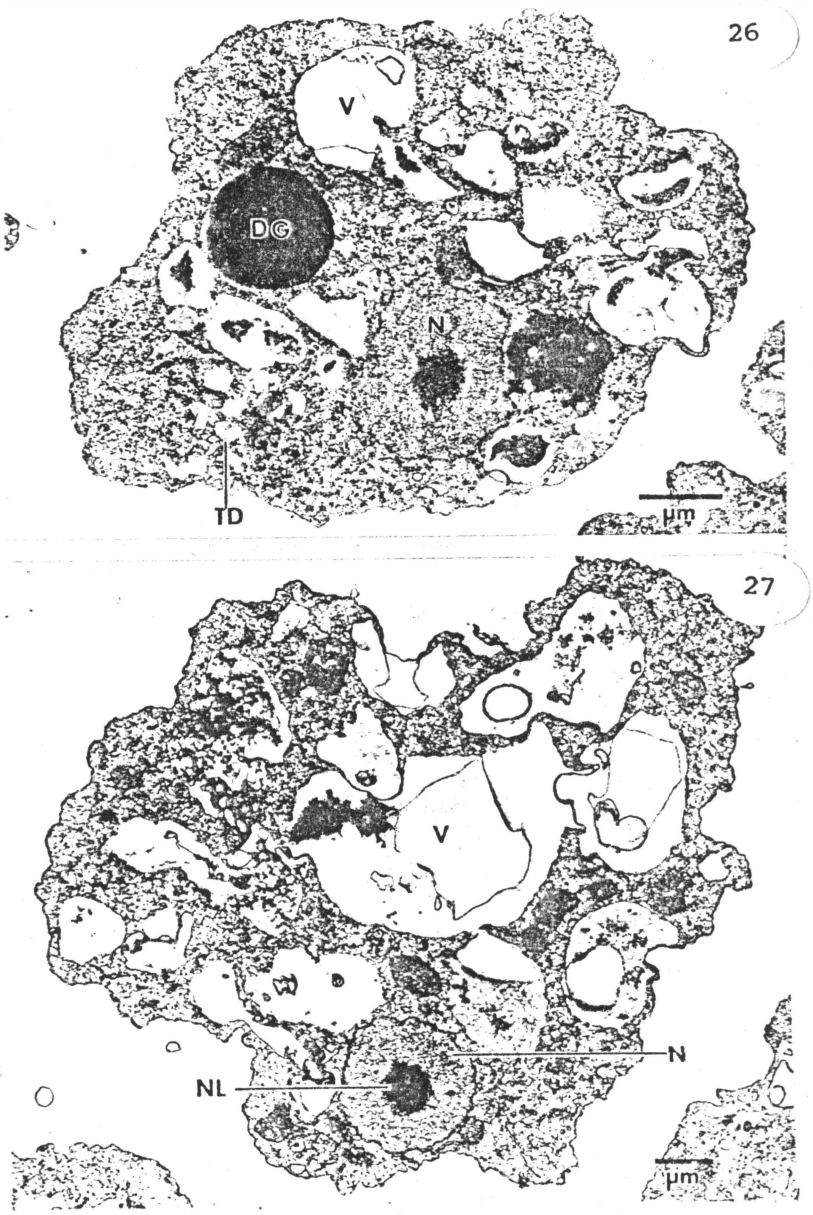

26
V
DG
N
TD
µm
27
V
N
NL
µm

Figure 28. Ultrastructure of an ameba of N. fowleri nN68 after 120 minutes of incubation in Page saline.

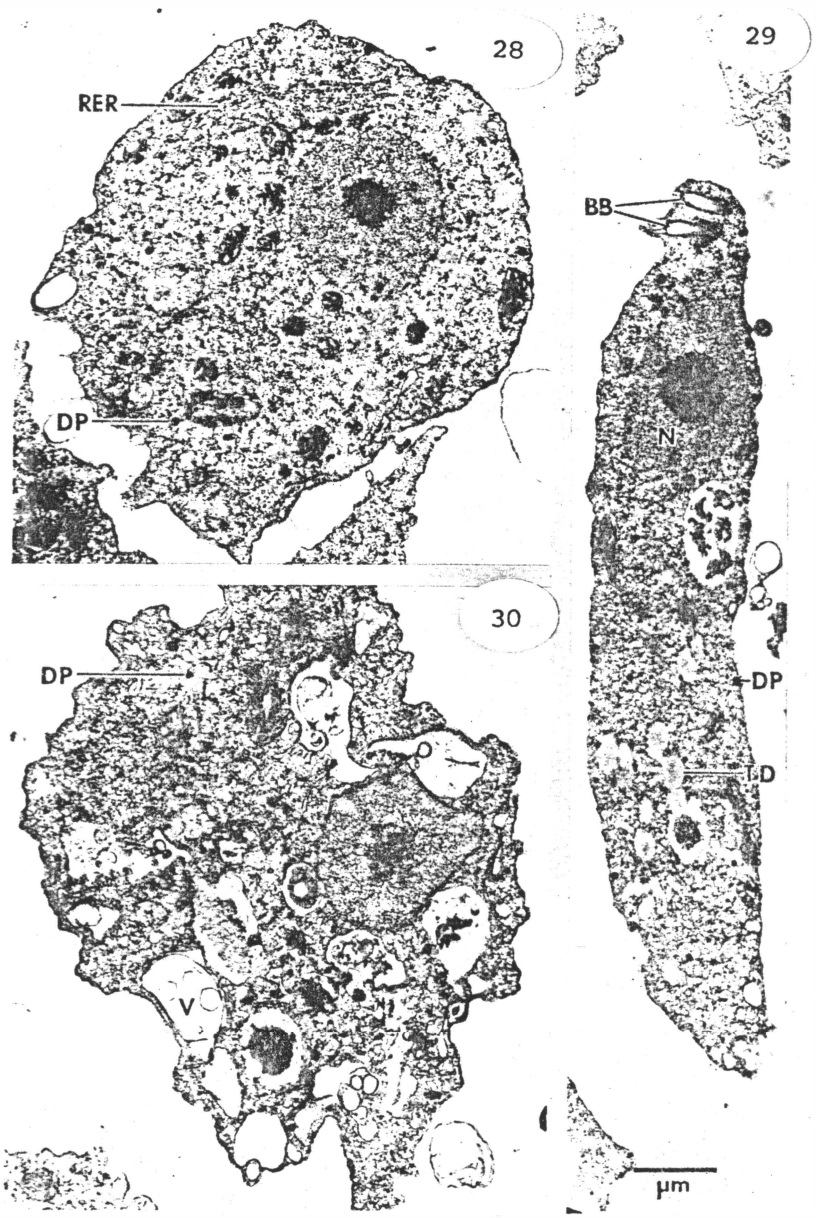
RER, rough endoplasmic reticulum; DP, dense particle. See Figure 29 for scale marker.

Figure 29. Ultrastructure of an elongated flagellated cell of N. fowleri nN68 after 210 minutes of incubation in Page saline.

BB, basal bodies; TD, translucent droplets; DP, dense particles; N, nucleus. Scale marker: 1 μm.

Figure 30. Ultrastructure of an ameba of N. fowleri NF69 after 210 minutes of incubation in Page saline.

DP, dense particle; V, vacuole. See Figure 29 for scale marker.


28
RER
DP
29
BB
N
DP
D
30
DP
V
µm

cytoplasm of amebae (Fig. 31). In contrast, dumbbell-shaped mitochondria were prevalent in elongated cells (Fig. 32 and 33). Nuclei within the amebae exhibited a homogeneous nucleoplasm which surrounded a central dense nucleolus. Ribosomes were observed in association with the outer membrane of the nuclear envelope (Fig. 31).

The basal bodies, rootlet, and flagella arose quickly after 90 minutes of incubation in Page saline. Under the light microscope, it was clear that the flagellar apparatus was partially developed in amebae before motility or change in cell shape (Fig. 25 and 31). The rootlet extended into the cell perpendicular to the basal body and the emerging flagellum. Initially, the developing rootlet was not associated with the nucleus but they became associated subsequently at the leading end of the elongated cell (Fig. 31 and 32). In elongated cells, the rootlet lay in a furrow or groove extending the length of the nucleus (Fig. 32-34). In oblique sections, the rootlet was seen in section within the groove of the cup-shaped nucleus (Fig. 33). A mitochondrion was usually located close to the distal end of the rootlet (Fig. 32).

The flagella and basal bodies were located in a protuberance at the leading end of the elongated cell (Fig. 29 and 32). Flagella of N. fowleri exhibited the typical 9 + 2 arrangement of filaments and were surrounded by a sheath which was continuous with the cytoplasmic membrane

Figure 31. Ultrastructure of an enflagellating ameba of N. fowleri nN68 after 100 minutes of incubation in Page saline.

F, flagellum; RT, rootlet; M, mitochondrion, RB, ribosomes. Other abbreviations are defined in the legends to Figure 26 through 29. Scale marker: 1 μm.

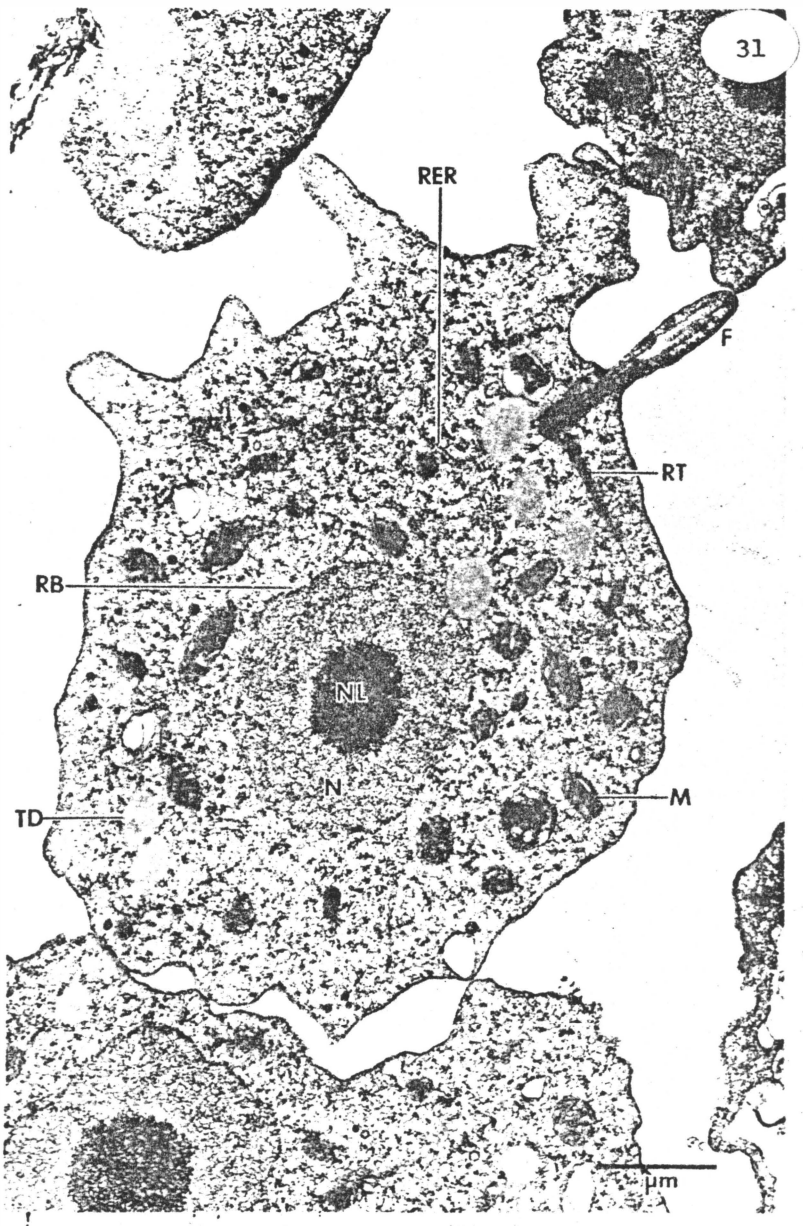

31
RER
F
RT
RB
NL
N
M
TD
µm

Figure 32. The flagellar rootlet embedded in the nuclear groove of an elongated cell of N. fowleri nN68 after 210 minutes of incubation in Page saline.

Abbreviations are defined in the legends to Figure 26 through 29 and 31. See Figure 34 for scale marker.

Figure 33. The rootlet nestled in the groove of a cup-shaped nucleus of N. fowleri nN68 210 minutes after subculture to Page saline.

See Figure 34 for scale marker.

Figure 34. The nuclear groove extending the length of the nucleus of N. fowleri nN68 210 minutes after subculture to Page saline.

Arrows indicate the proximal and distal ends of the nuclear groove. Scale marker: 1 μm.

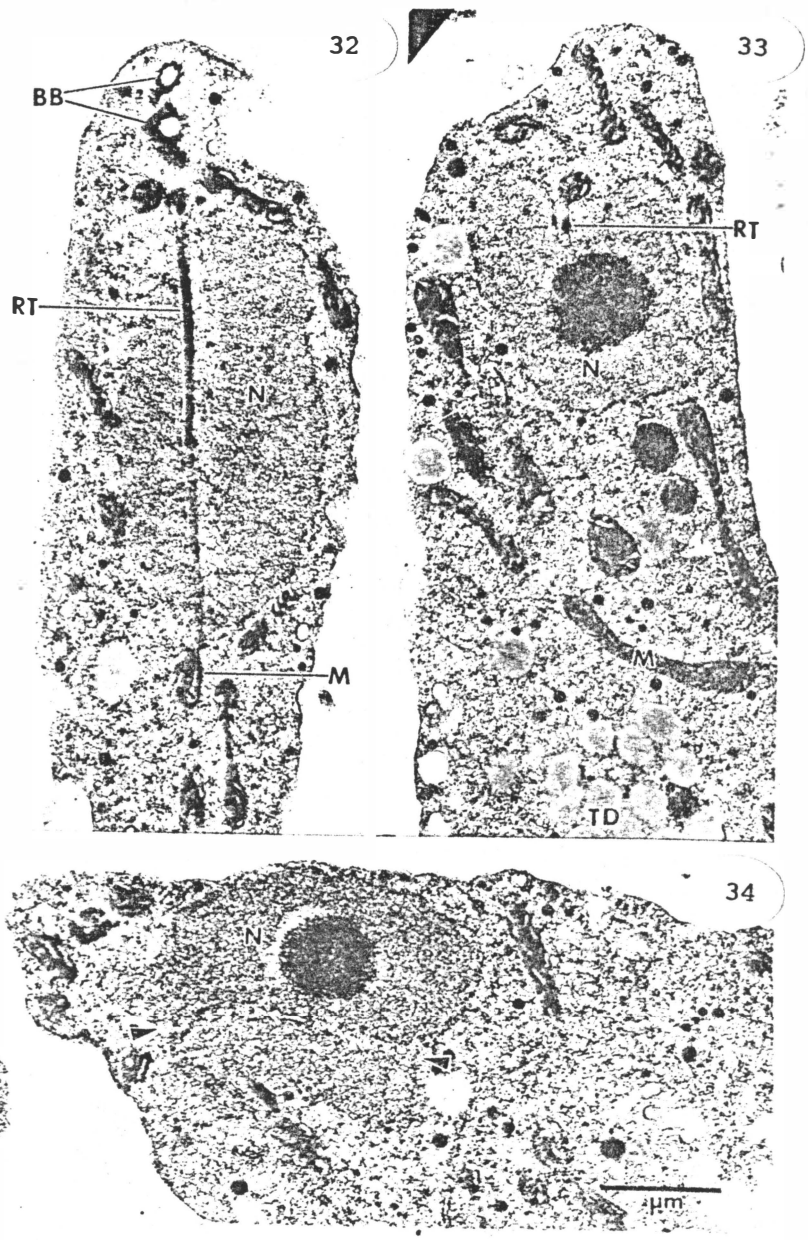

32
BB
RT
N
M
33
RT
N
M
TD
34
N
μm

Figure 35. Continuity of the basal body with the flagellar shaft in N. fowleri nN68 210 minutes after subculture to Page saline.

Numbers indicate the approximate locations of the cross-sections shown in Figure 36 to 38. BP, basal plate; FS, flagellar sheath; CM, cytoplasmic membrane. See Figure 39 for scale marker.

Figure 36. Cross-section of the shaft of a flagellum from N. fowleri nN68 210 minutes after subculture to Page saline.

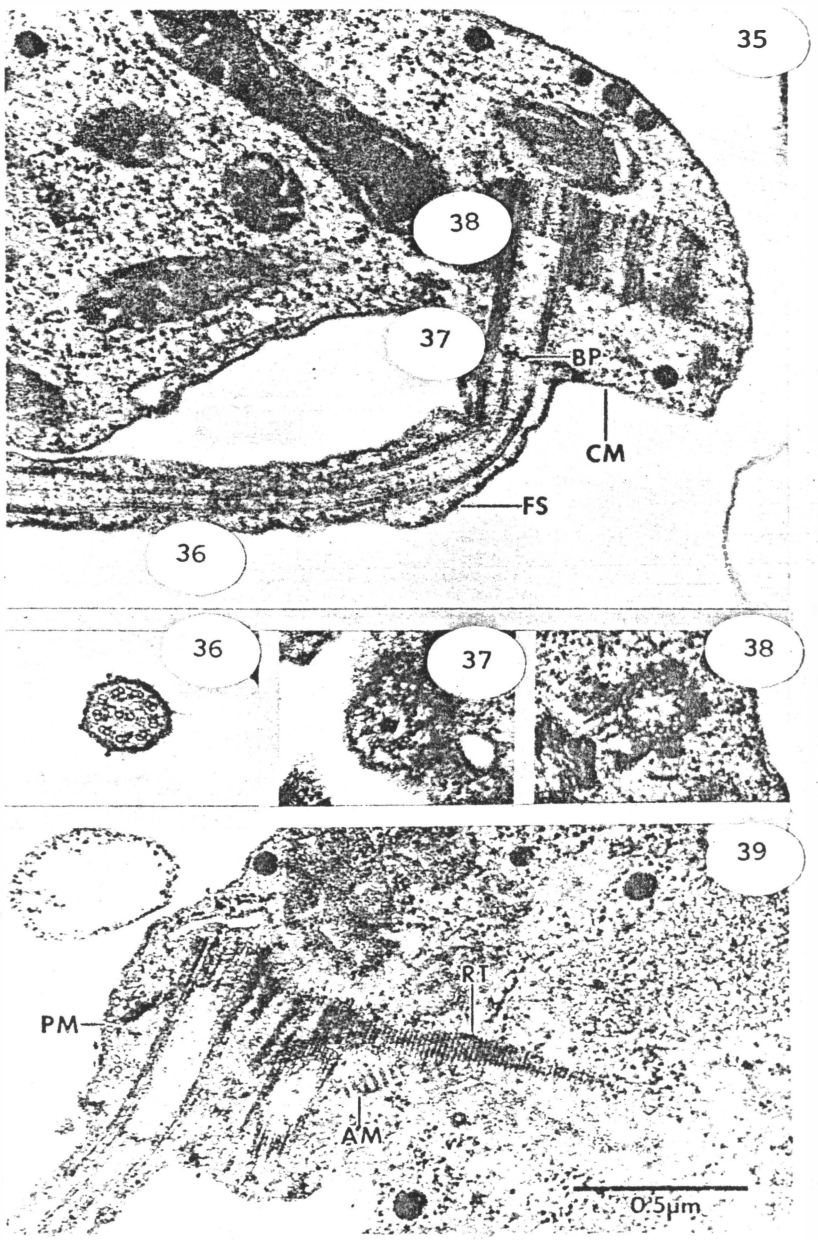
Figure 37. Cross-section in the vicinity of the basal plate of N. fowleri nN68 210 minutes after subculture to Page saline.

Figure 38. Cross-section at the proximal end of a basal body from N. fowleri nN68 210 minutes after subculture to Page saline.

The outer nine triplets form a "pinwheel." There is no central pair of filaments. See Figure 39 for scale marker for Figures 36-38.

Figure 39. Continuity of the rootlet with the basal bodies of N. fowleri nN68 210 minutes after subculture to Page saline.

AM, anchoring microtubules between the basal body and the rootlet. Scale marker: 0.5 μm.


35
38
37
BP
CM
FS
36
36
37
38
39
RT
PM
AM
0·5µm

(Fig. 35 and 36). The outer circle of flagellar doublets was continuous with the cylinder of nine triplet filaments which made up the basal body (Fig. 35-38). In contrast, the central pair of filaments terminated at the basal plate which was located at the juncture between the flagellum and the basal body (Fig. 35 and 39). Groups of anchoring microtubules were seen around the basal bodies; still other microtubules were aligned along the periphery of elongated cells. The rootlet was connected to the basal bodies by an intricate series of parallel and transverse microtubules (Fig. 35 and 39). The rootlet consisted of alternating light and dark bands which extended from the basal bodies through the nuclear groove (Fig. 32 and 39). The width of the light band was approximately 4.5 nm, and the width of the dark band was approximately 11.5 nm. The rootlet was not enclosed within a membrane.

The polypeptides of amebae and flagellates of Naegleria fowleri nN68 were characterized and compared. Amebae of N. fowleri nN68 incorporate [^{35}S]-methionine during growth in Nelson medium (Table 9). The polypeptides of amebae radiolabeled during growth and those of flagellates developed from a sample of the same pre-labeled amebae were resolved by two-dimensional electrophoresis. Several hundred polypeptides of N. fowleri were detected in autoradiograms of these gels (Fig. 40). During the process of enflagellation, the complement of the polypeptides detected

on autoradiograms was not markedly altered but a relatively small number of qualitative and quantitative differences were noted. A comparison of these differences to those detected between autoradiograms of amebae of non-enflagellating strain NF69 before and after incubation in non-nutrient conditions (Fig. 41) revealed that some of these changes occurred in both the enflagellating strain and the non-enflagellating variant after subculture to non-nutrient buffer, whereas others were observed only when amebae and flagellate preparations were compared (Table 10). The largest number of polypeptide changes observed were enflagellation-related increases. Few polypeptides were detected anew in flagellates which had not been detected in amebae. The relative concentration of some polypeptide species decreased in both the enflagellating and the non-enflagellating strain after subculture to non-nutrient buffer; a similar number of decreases were specific to the enflagellating cells. Eight polypeptides displayed unexpected changes; they were present in growing amebae of the non-enflagellating strain and in both strains after subculture to non-nutrient buffer but absent in growing amebae of the enflagellating strain.

A quantitative analysis of the polypeptides detected on autoradiograms of amebae and flagellates was undertaken. Autoradiographs were scanned systematically using a digital drum scanning microdensitometer. Histograms of the recorded

Figure 40. Autoradiograms of [^{35}S]-methionine-labeled polypeptides of amebae (A) and flagellates (B) of Naegleria fowleri nN68.

The proteins were pre-labeled in amebae and, after various manipulations, resolved by isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the Materials and Methods. Positions of size standards run in the same gels are shown. Qualitative changes are indicated by solid arrows (spot present) and dashed outlines (spot not detected); quantitative changes are indicated by open arrows. Is denotes an increase related to starvation; Ie, increase related to enflagellation; Ds, decrease related to starvation; De, decrease related to enflagellation; Vs, polypeptides that vanished only during enflagellation; Ae?, polypeptides appearing in flagellates but are also present in amebae of the non-enflagellating strain before and after incubation in non-nutrient buffer. Indicated changes are examples of those enumerated in Table 10.

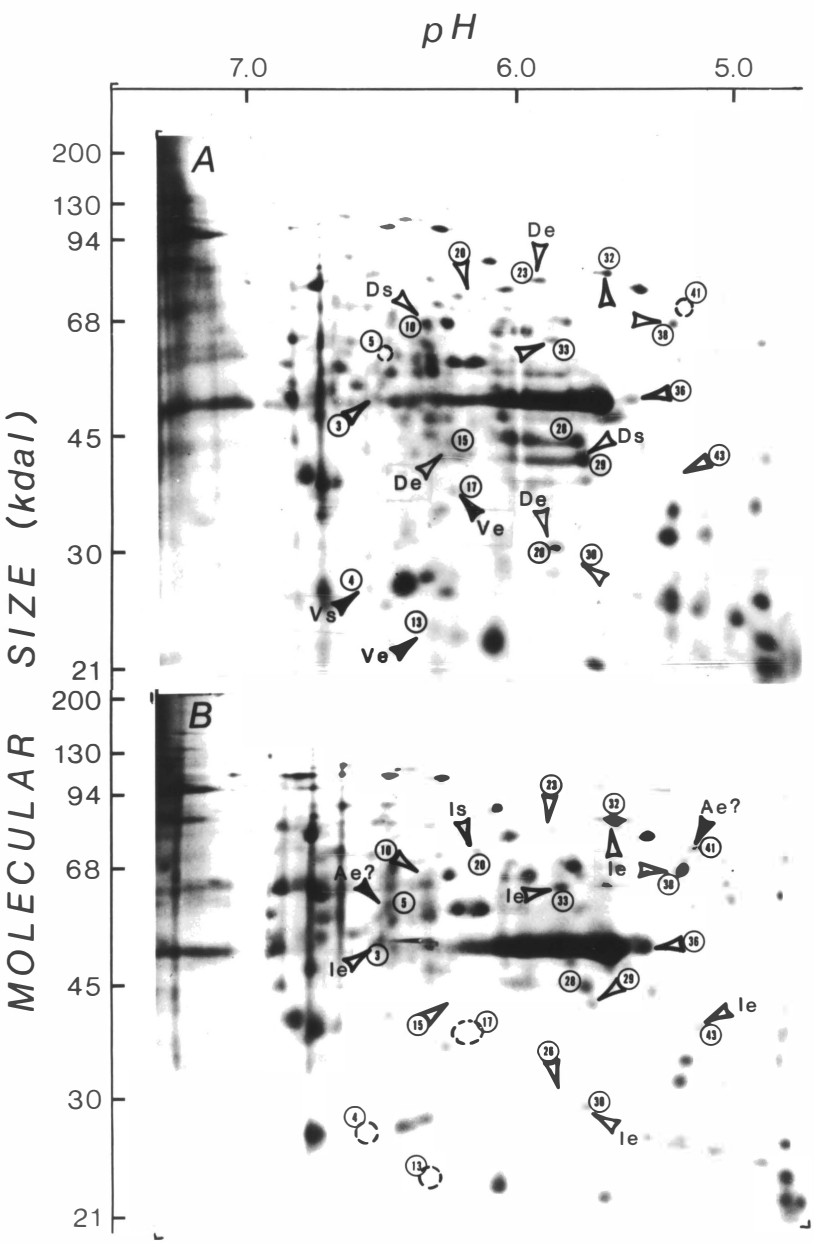

pH
7.0
6.0
5.0
MOLECULAR SIZE (kdal)
200
130
94
68
45
30
21
A
B
De
Ds
Ve
Vs
Is
Ie
Ae?

Figure 41. Autoradiogram of [^{35}S]-methionine-labeled polypeptides of amebae of Naegleria fowleri NF69 during growth (A) and after being washed, suspended and incubated in ameba saline as described for enflagellation of strain nN68 (B).

Symbols are described in the legend to Figure 40.

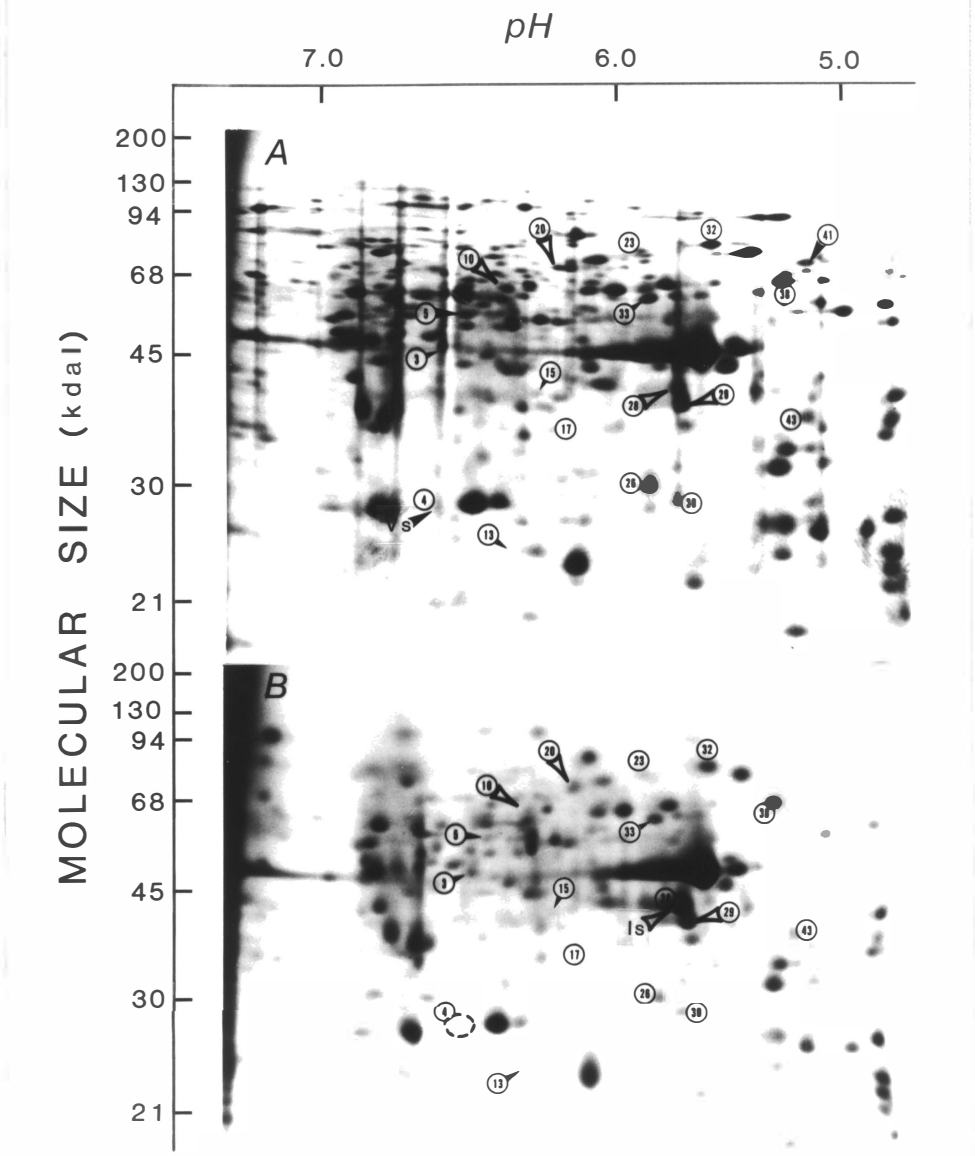

pH
7.0
6.0
5.0
MOLECULAR SIZE (kdal)
200
130
94
68
45
30
21
A
B
Vs
Is

Table 10

Changes in Pre-labeled Polypeptides of

Naegleria fowleri Upon Enflagellation

Nature of Change[a]	Polypeptide No.[b]	~M_r[c]	~pI[d]
1. Qualitative-appearance			
a. enflag. related			
b. see footnote[e]	5[f]	58	6.5
	16[f]	44	6.1
	35	26	5.6
	36	51	5.6
	41	68	5.4
	42	68	5.4
	44	71	5.0
	47	67	4.4
c. starvation related	19[g]	30	6.1
	31[f]	24	5.7
	48	53	4.4

Continued

Table 10 (Continued)

Nature of Change[a]	Polypeptide No.[b]	~M_r[c]	~pI[d]
2. Qualitative-disappearance			
a. enflag. related	7	44	6.5
	9	90	6.4
	13	24	6.2
	17	38	6.2
	27	55	5.8
b. starvation related	4	27	6.6
	11	58	6.4
	18[g]	30	6.1
3. Quantitative increase			
a. enflag. related	1	117	7.1
	2[f]	56	6.7
	3	52	6.5
	6[f]	49	6.5
	24	51	6.0

Continued

Table 10 (Continued)

Nature of Change[a]	Polypeptide No.[b]	~M_r[c]	~pI[d]
a. enflag. related (continued)	25	35	5.9
	30	30	5.8
	32	82	5.6
	33	62	5.7
	38	65	5.4
	40	44	5.4
	43[f]	38	5.4
	45	54	5.1
	46[f]	37	4.4
b. starvation related	20	71	6.1
	28[f,g]	44	5.8
4. Quantitative Decrease			
a. enflag. related	12	45	6.2
	14	72	6.1
	15	44	6.1
	21	55	6.0
	23	79	5.9
	26	32	5.8

Continued

Table 10 (Continued)

Nature of Change[a]	Polypeptide No.[b]	~M_r[c]	~pI[d]
b. starvation related	8	34	6.4
	10	65	6.5
	22[f]	45	6.0
	29[g]	42	5.7
	34	48	5.6
	37[f]	23	5.5
	39	62	5.5

The polypeptides seen in Figures 40 and 41 were compared to determine apparent differences between those changes seen between amebae and flagellates of N. fowleri nN68 and those between amebae of N. fowleri NF69 before and after exposure to the same non-nutrient conditions. More than 300 visibly detectable spots were compared.

[a]A change was related to enflagellation if it occurred in nN68 but not in NF69. A change was related to starvation if it occurred in both nN68 and NF69.

[b]Numbers refer to Figures 40 and 41. Not all changes listed in the table are visible in photographs of the autoradiograms.

[c]Relative mobility from a standard curve derived using positions of molecular size standards run with each gel in the second dimension. Standards used are listed in Materials and Methods.

Continued

Table 10 (Continued)

[d]Values indicate the relative positions of spots in the pH gradient of the isoelectric focusing dimension as determined by measuring the pH of sections of an isoelectric focusing gel run at the same time as the samples in Figure 40.

[e]A number of spots were observed to appear anew in nN68 flagellates which were present in amebae of NF69 both before and after incubation in non-nutrient conditions.

[f]The change appears to involve a change in pI.

[g]The change appears to involve a change in Mr.

integer density readings demonstrated both the uniformity of the film background and the relatively even distribution of frames with the remaining density values (Fig. 42). The film background peak was confined to a small range of integer values. It was possible to subtract the background mean density (43) plus one standard deviation (4) in order to remove film background without losing information. The faintest visually observable spots (Fig. 40 and 41) were compared with the corresponding regions in the background corrected printouts. Spots were detected at a level as low as 4 or 5 integer density units above "0" background. By manual contouring, it was possible to differentiate between true spots and random high background readings for spots in the lower density range. Even the faintest observable spot appeared as a distribution of density readings which could be contoured from lower to higher density readings with a distinct central peak, whereas "noise" could not be contoured due to its random nature. The small number of densitometric data points (frames) with values at the upper limit (0.7% relative to the total number of data points having values above background) confirmed that the exposure chosen (product of radioactivity in becquerels and time in seconds = 2.5×10^{10} disintegrations) did not unduly exceed the film capacity. The rate of increase of density in units/day was a linear function of the spot intensity

Figure 42. Frequency distribution of the film densities in 200 μm square frames in an autoradiogram of a two-dimensional gel separation of the polypeptides of Naegleria fowleri nN68.

The peak representing background density (43 ± 4 units) is unimodally distributed. Approximately 85,000 out of 420,000 frames had film densities above background.

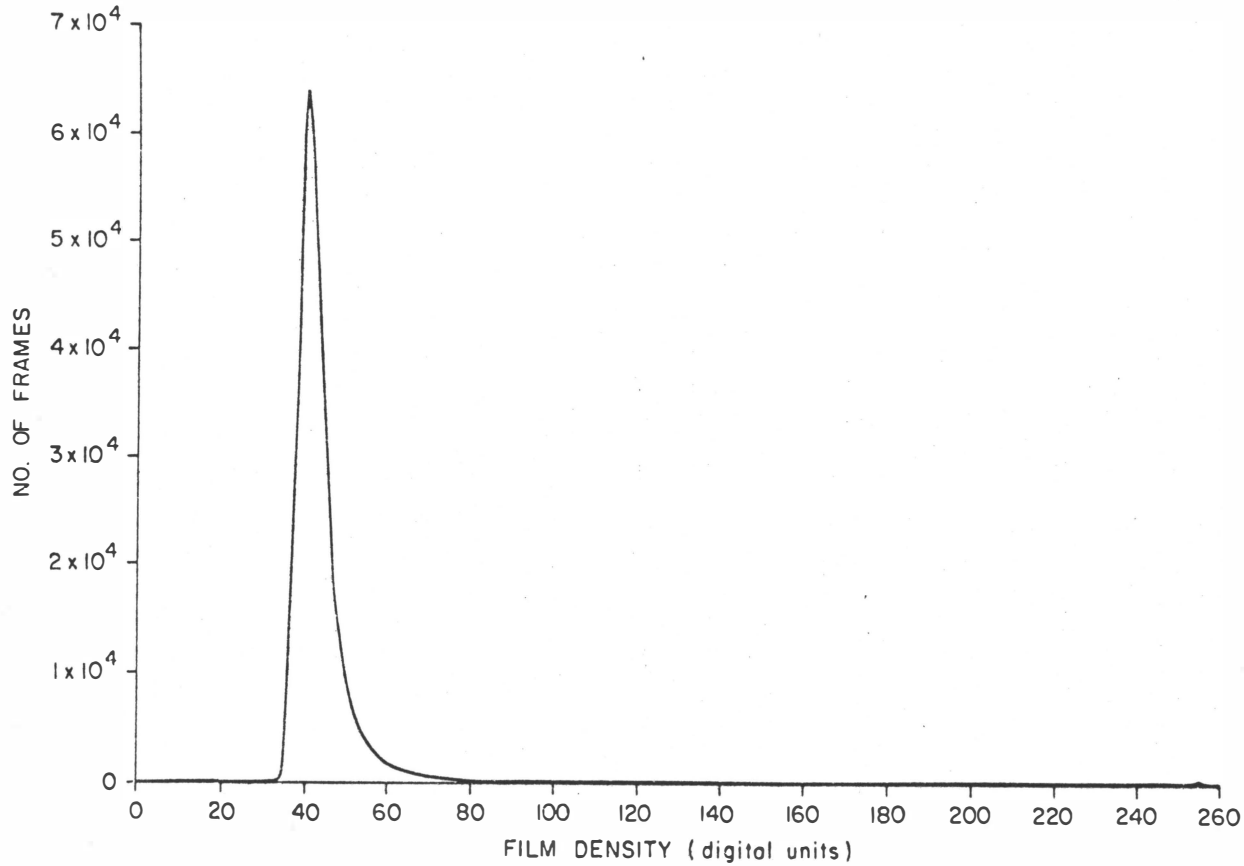
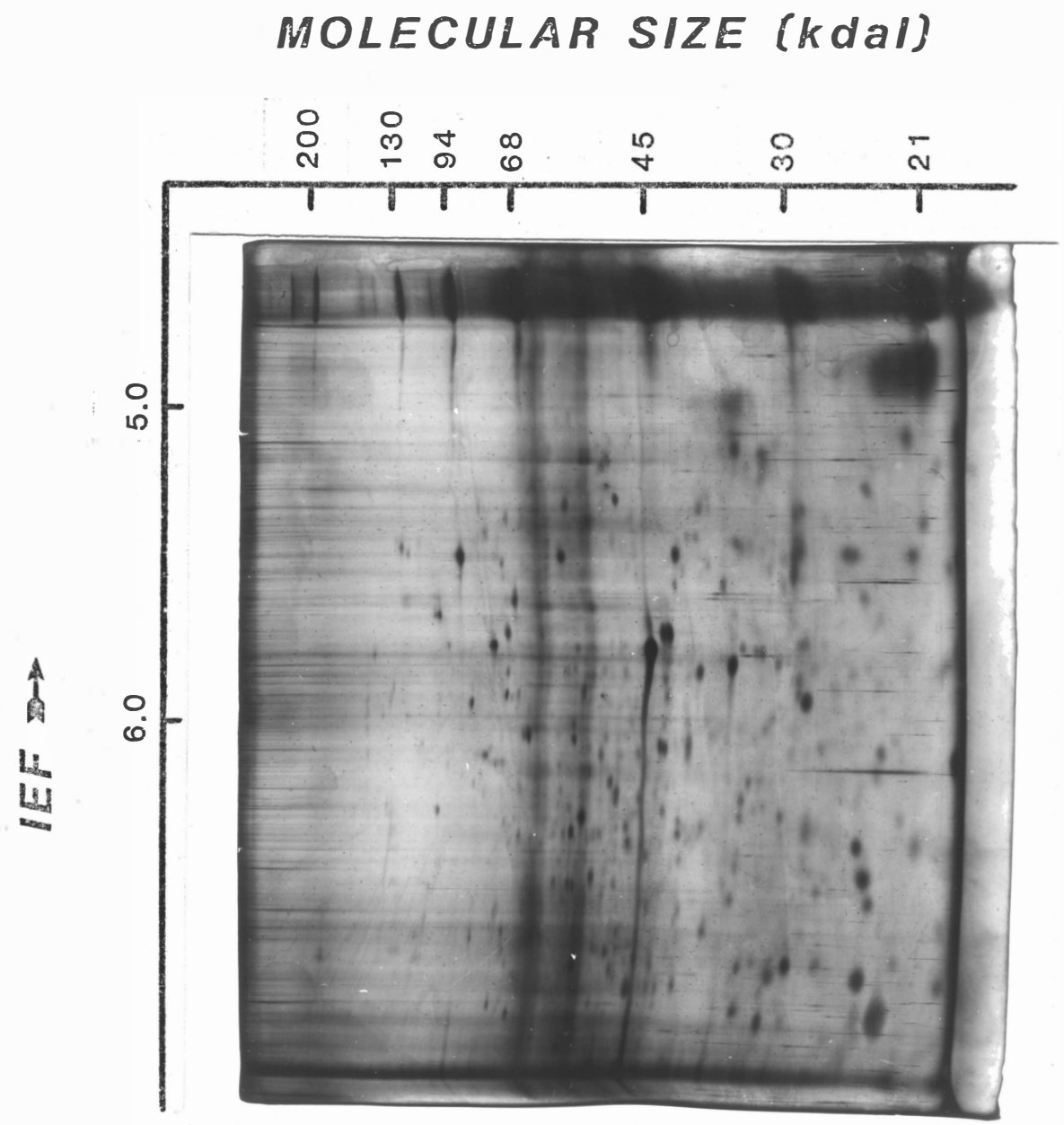

7 x 10^4
6 x 10^4
5 x 10^4
4 x 10^4
3 x 10^4
2 x 10^4
1 x 10^4
0
NO. OF FRAMES
0
20
40
60
80
100
120
140
160
180
200
220
240
260
FILM DENSITY (digital units)

Figure 43. Silver-stained polypeptides of amebae of N. fowleri resolved by isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.


MOLECULAR SIZE (kdal)
200
130
94
68
45
30
21
5.0
6.0
IEF

(data not shown). Furthermore, the distribution of polypeptides revealed in autoradiograms was similar to that revealed by a sensitive silver stain method (Fig. 43), both with respect to positions within the gel and relative intensities of the spots. Comparisons of spot intensities within the limits of film capacity therefore could be used to gauge the relative amounts of radiolabeled polypeptides.

The amount of radiolabeled polypeptide varied widely among the individual spots (Fig. 44). The faintest detectable polypeptide spot had a sum of 6 digital units (143); the most abundant polypeptide in Figure 44 had a sum of 183,000 digital units. The amount of the most abundant radiolabeled polypeptide on this gel therefore was 20,000-fold greater than the amount of a polypeptide at the lower limit of detection (Fig. 44). By using several different exposure times to allow quantitation of more dense and less dense spots, the range in polypeptide amount was extended to 10^5-fold.

The greatest number of radiolabeled polypeptides in ameba and flagellate extracts had molecular sizes in the range of 20 to 40 kilodaltons, and the majority of the polypeptides (60%) were 20 to 60 kilodaltons (Fig. 45). The largest amount of radiolabeled polypeptide in extracts of amebae and flagellates (63% and 46%, respectively) was present in species of 40 to 60 kilodaltons (Fig. 46). About 92% of the radioactivity of polypeptides in amebae,

Figure 44. Frequency distribution of the amount of polypeptide for individual species. The most abundant polypeptide is present at an estimated 10^8 molecules/ameba (52,136, see Discussion).

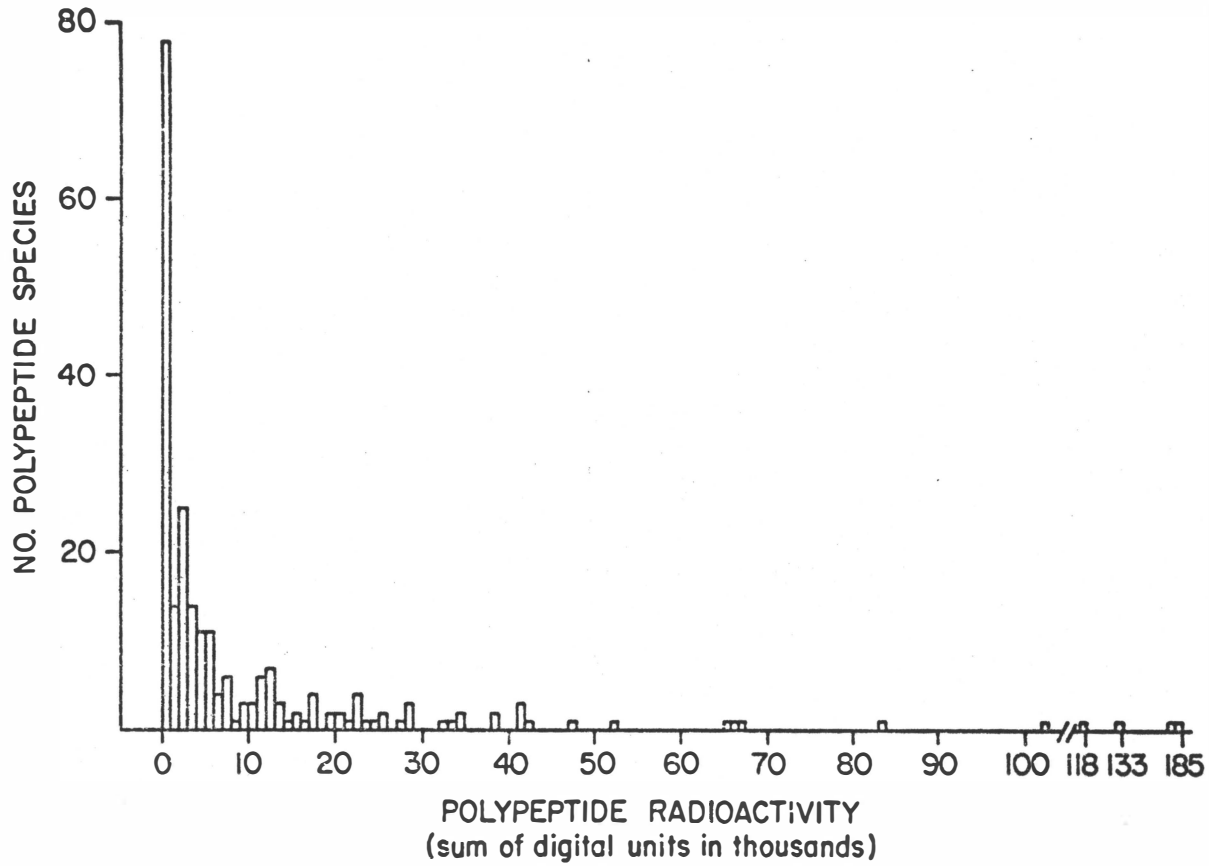

80
60
40
20
NO. POLYPEPTIDE SPECIES
0
10
20
30
40
50
60
70
80
90
100
118
133
185
POLYPEPTIDE RADIOACTIVITY
(sum of digital units in thousands)

Figure 45. Distribution of the number of polypeptide species of amebae and flagellates of Naegleria fowleri nN68 by molecular size.

Amebae: shaded bars; flagellates: open bars.

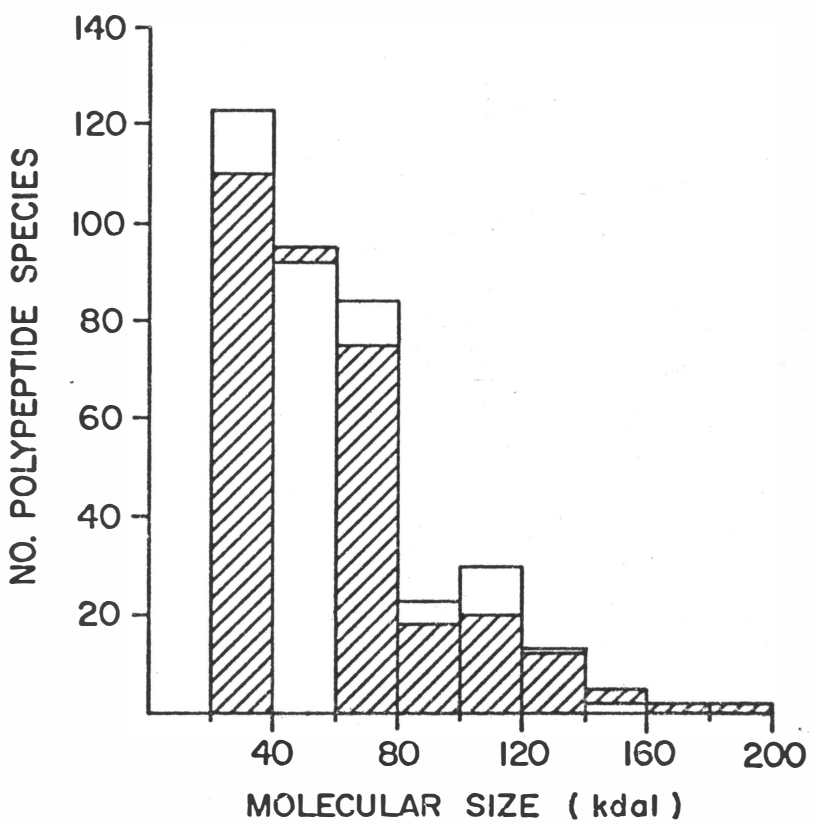

140
120
100
80
60
40
20
NO. POLYPEPTIDE SPECIES
40
80
120
160
200
MOLECULAR SIZE (kdal)

Figure 46. Distribution of the amount of polypeptides of amebae and flagellates of Naegleria fowleri nN68 by molecular size.

Amebae: shaded bars; flagellates: open bars.

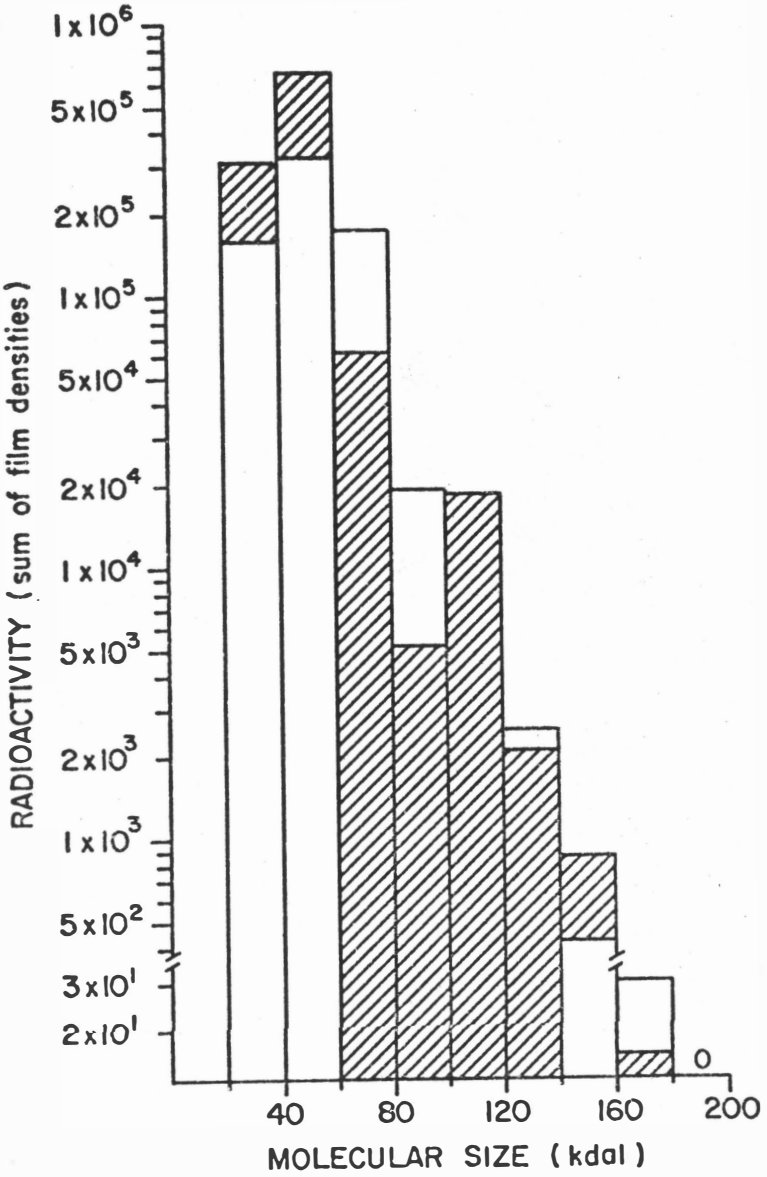
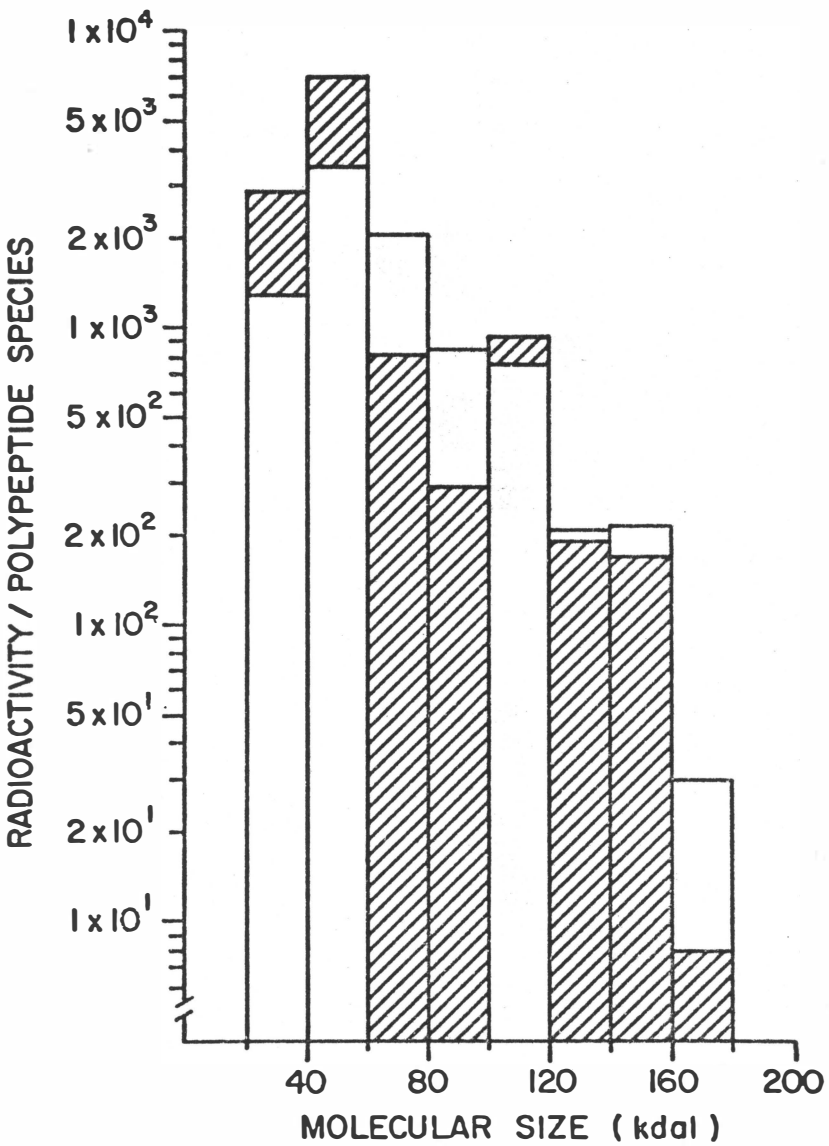

1x10⁶
5x10⁵
2x10⁵
1x10⁵
5x10⁴
2x10⁴
1x10⁴
5x10³
2x10³
1x10³
5x10²
3x10¹
2x10¹
0
RADIOACTIVITY (sum of film densities)
40
80
120
160
200
MOLECULAR SIZE (kdal)

Figure 47. The mean amount of polypeptide per species in amebae and flagellates of Naegleria fowleri according to molecular size.


1 x 10^4
5 x 10^3
2 x 10^3
1 x 10^3
5 x 10^2
2 x 10^2
1 x 10^2
5 x 10^1
2 x 10^1
1 x 10^1
RADIOACTIVITY / POLYPEPTIDE SPECIES
40
80
120
160
200
MOLECULAR SIZE (kdal)

but only 69% of that of flagellates, was in the size range of 20 to 60 kilodaltons. Only 6% of the polypeptide radioactivity of amebae was in the size range of 60 to 100 kilodaltons, whereas 28% of that of flagellates was in this size range (Fig. 46). The mean radioactivity per polypeptide species in the molecular size range of 20 to 40 kilodaltons was less than the mean amount in the range of 40 to 60 kilodaltons for both amebae and flagellates (Fig. 47). From 40 to 200 kilodaltons, the mean amount of polypeptide tended to decrease with increasing molecular size for both amebae and flagellates. There was relatively a greater amount of polypeptide per spot for amebae than for flagellates in the size range of 20 to 60 kilodaltons, whereas the converse was true in the range of 60 to 100 kilodaltons (Fig. 47).

The distribution of polypeptide species along the IEF dimension was similar for amebae and flagellates. A greater number of radiolabeled polypeptides in ameba and flagellate extracts had isoelectric positions between pH 6 and pH 7 than in more acid portions of the gels (Fig. 48). The number of polypeptides in extracts of both amebae and flagellates decreased as the pH of the isoelectric focusing gradient decreased. The amount of polypeptide in extracts of amebae or flagellates was not markedly different at various positions in the pH gradient. There was relatively a lesser amount of polypeptide in the gradient

below pH 6.3 for flagellates than for amebae (Fig. 49). Similarly, there was relatively a lesser amount of polypeptide per spot for flagellates than for amebae at pH values below 6.3 (Fig. 50).

The overall correlation for both amebae and flagellates between subunit charge and size, using the median polypeptide as the indicator of central tendency, was that the isoelectric point increased as molecular size increased (Fig. 51a). Similarly, for both amebae and flagellates, the median amount of polypeptide in smaller species had lower pI values than did the median amount in species of larger size (Fig. 51b).

An analysis of the size and charge characteristics of those polypeptides in which differences were noted between amebae and flagellates (Table 10) was done. The number of polypeptides exhibiting increases or decreases in amount within each of the general size or charge categories delineated in the quantitative analysis (20 to 60 kd, 60 to 100 kd; pI >6.3 or pI <6.3) was generally the same and could not, by numbers alone, account for the differences found in the quantitative analyses.

The state of phosphorylation of proteins of amebae and flagellates of N. fowleri was examined. Labeling of phosphoproteins during growth of N. fowleri nN68 was made possible by substitution of the phosphate buffer in Page ameba saline with 2 mM MOPS (morpholinopropanesulfonic

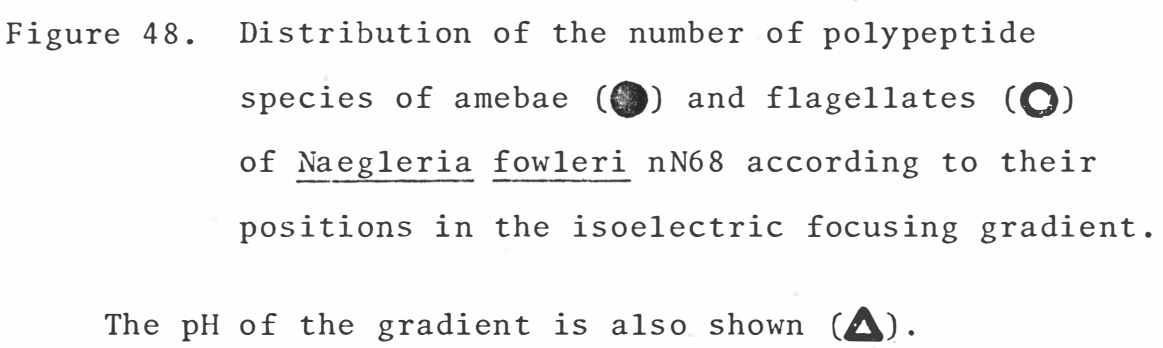


Figure 48. Distribution of the number of polypeptide species of amebae (●) and flagellates (○) of Naegleria fowleri nN68 according to their positions in the isoelectric focusing gradient.

The pH of the gradient is also shown (△).

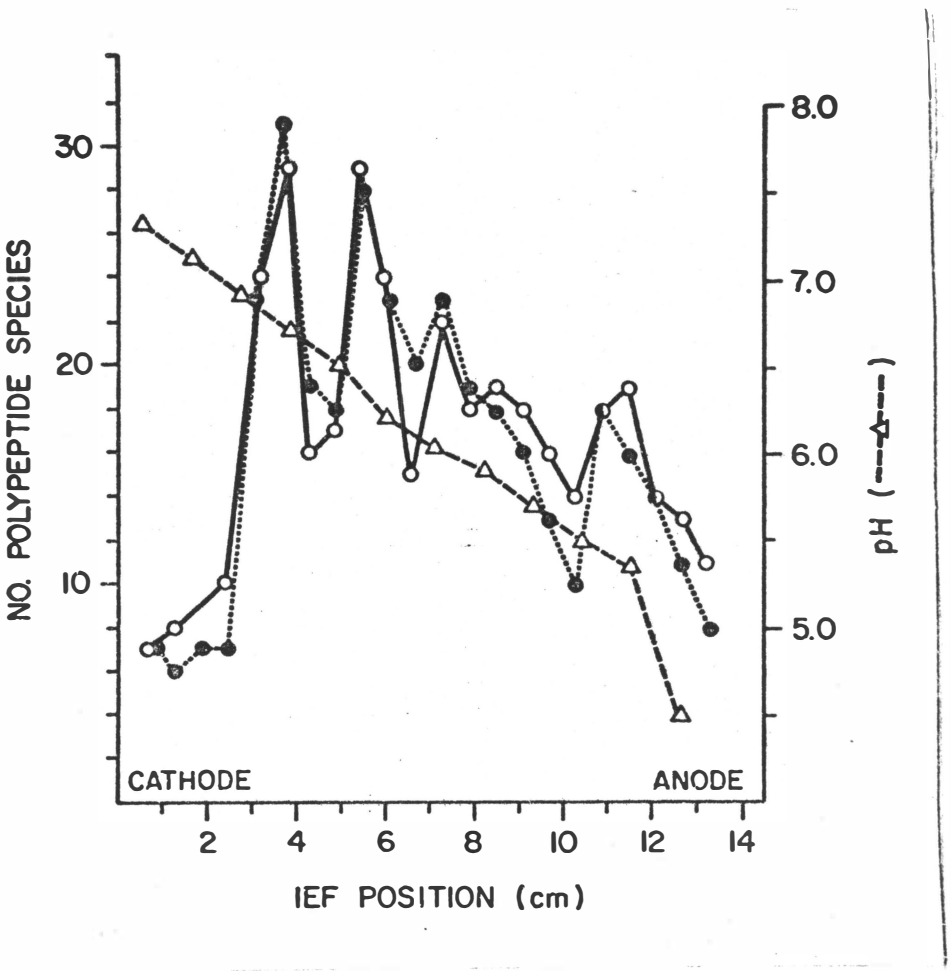

NO. POLYPEPTIDE SPECIES
30
20
10
8.0
7.0
6.0
5.0
pH (—△—)
CATHODE
ANODE
2
4
6
8
10
12
14
IEF POSITION (cm)

Figure 49. Distribution of the amounts of polypeptide species of amebae (●) and flagellates (○) of Naegleria fowleri nN68 according to their positions in the isoelectric focusing gradient. The pH of the gradient is also shown (△).

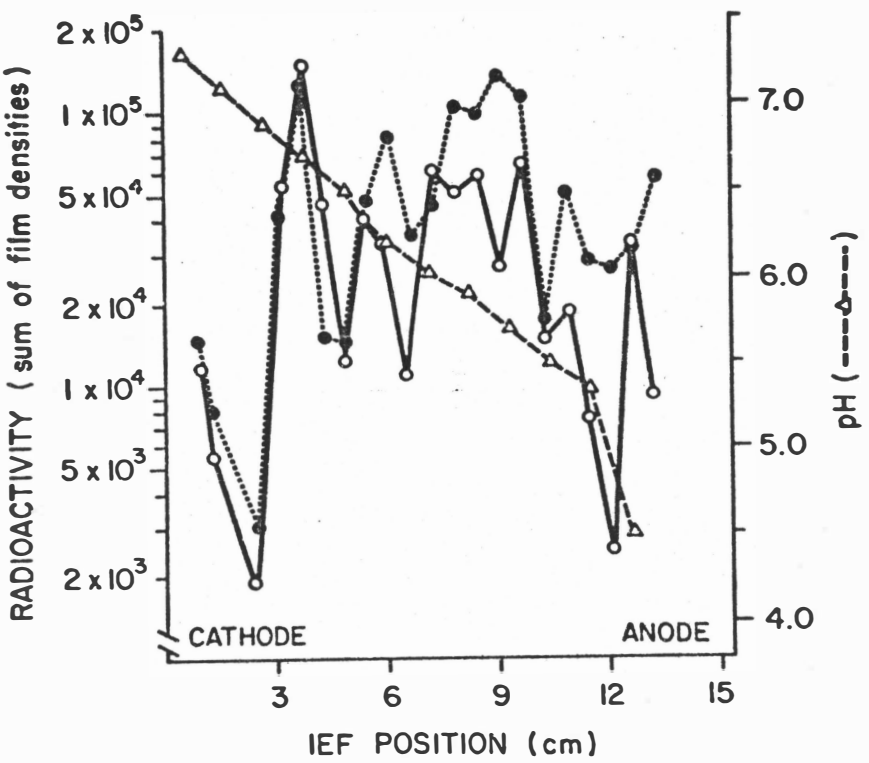

RADIOACTIVITY (sum of film densities)
2 x 10^5
1 x 10^5
5 x 10^4
2 x 10^4
1 x 10^4
5 x 10^3
2 x 10^3
CATHODE
ANODE
3
6
9
12
15
IEF POSITION (cm)
7.0
6.0
5.0
4.0
pH (---△---)

Figure 50. The mean amount of polypeptide per species according to position in the isoelectric gradient for amebae (■) and flagellates (□) of Naegleria fowleri nN68.

The pH of the gradient is also shown (△).

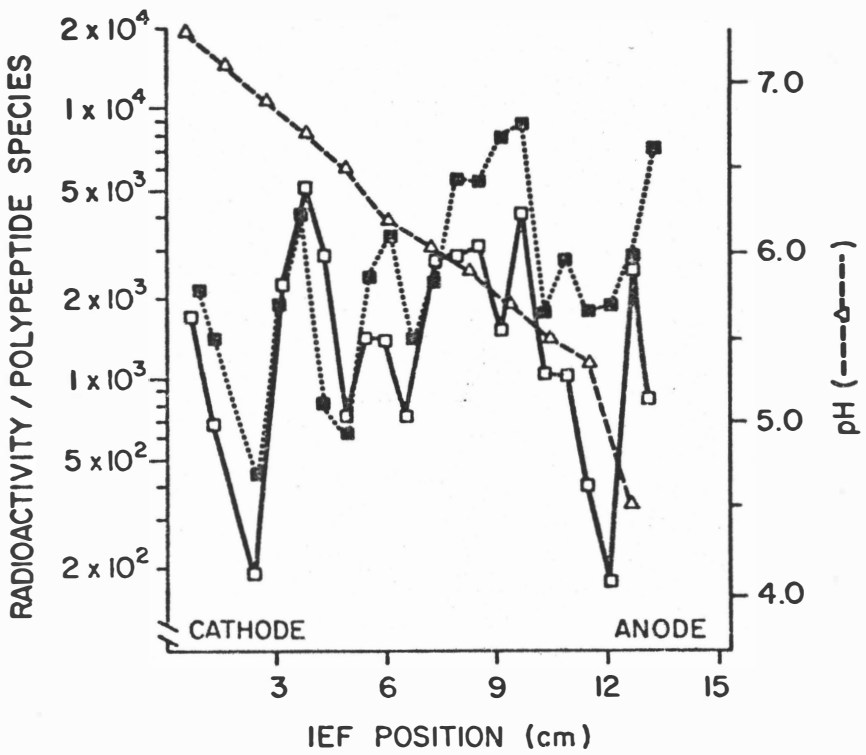
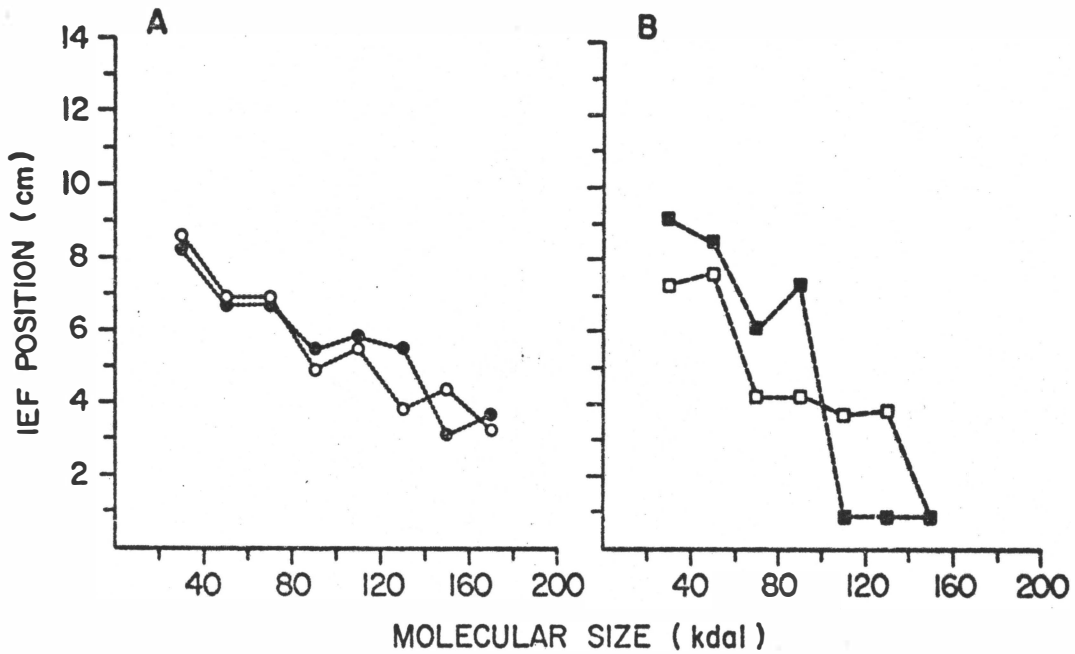

RADIOACTIVITY / POLYPEPTIDE SPECIES
2 x 10^4
1 x 10^4
5 x 10^3
2 x 10^3
1 x 10^3
5 x 10^2
2 x 10^2
CATHODE
ANODE
3
6
9
12
15
IEF POSITION (cm)
7.0
6.0
5.0
4.0
pH (---△---)

Figure 51. Relationship between molecular size and charge of polypeptides of amebae (closed symbols) and flagellates (open symbols).

Each point represents the position in the isoelectric focusing gradient at which the median species (A) or median amount of labeled polypeptide (B) is located within each of the molecular size ranges. The relationship between IEF position and the pH of the gradient is similar to those shown in Figure 48 to 50.


A
B
14
12
10
8
6
4
2
IEF POSITION (cm)
40
80
120
160
200
MOLECULAR SIZE (kdal)

acid) buffer and growing amebae in Nelson medium prepared with the modified saline. Growth of amebae of N. fowleri in MOPS-buffered Nelson medium was equivalent to that obtained in phosphate-buffered Nelson medium (Fig. 52). Enflagellation of amebae grown in the modified medium was similar to that of amebae grown in phosphate-buffered Nelson medium (Fig. 53). N. fowleri nN68 growing in MOPS-buffered Nelson medium incorporated ^{32}P-labeled inorganic phosphate into macromolecules (Table 11). Approximately 7% of the incorporated label was insoluble in hot trichloroacetic acid and included phosphoproteins.

Autoradiography of polypeptides of these phosphate-labeled cells resolved by two-dimensional electrophoresis revealed that a variety of polypeptides had incorporated small amounts of labeled phosphate and a few polypeptides had incorporated a significant amount of ^{32}P-label (Fig. 54a). Many, but not all, of the ^{32}P-labeled polypeptides co-migrated with ^{35}S-labeled polypeptides. Phosphoproteins were distributed throughout the gels without any distinct size or charge bias. A number of changes in the distribution of ^{32}P-label occurred when labeled amebae were enflagellated. The most prominent change observed was a major shift of ^{32}P-label from the doublet spot seen in the vicinity of the ^{35}S-spot 205 position to a more basic position, designated P3, which did not appear to co-migrate with any of the ^{35}S-labeled polypeptides seen in

Figure 52. Growth of Naegleria fowleri nN68 in modified Nelson medium with MOPS buffer.

Amebae of N. fowleri nN68 were grown in unagitated cultures at 37°C in 25 cm^2 tissue culture flasks containing 5 ml of either Nelson medium in Page ameba saline (2 mM phosphate buffer) (●) or of Nelson medium in ameba saline containing 2 mM MOPS buffer (□). Both media contained 2% (v/v) calf serum and the pH of both media at inoculation was 6.45. Growth was monitored by cell counts as described in Materials and Methods.

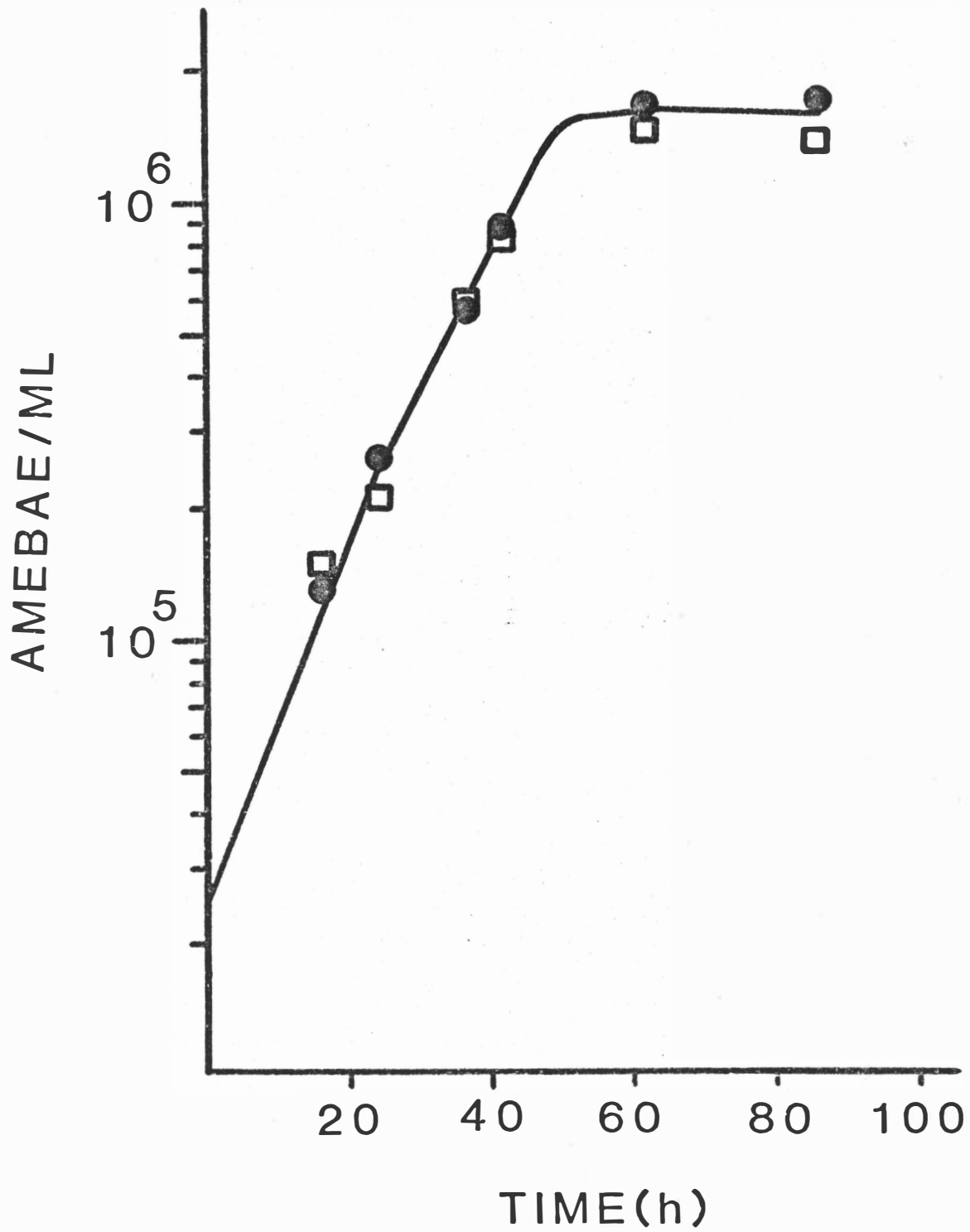

AMEBAE/ML
10^6
10^5
20
40
60
80
100
TIME(h)

Figure 53. Enflagellation of Naegleria fowleri nN68 grown in Nelson medium or in MOPS-buffered Nelson medium.

N. fowleri nN68 amebae were grown at 37°C in unagitated cultures containing either Nelson medium in Page ameba saline (●) or Nelson medium prepared in MOPS-buffered ameba saline (■). Late logarithmic phase cultures of amebae in the different growth media were washed, suspended and agitated in ameba saline with MOPS buffer at 37°C. Enflagellation was monitored as described for Figure 9.

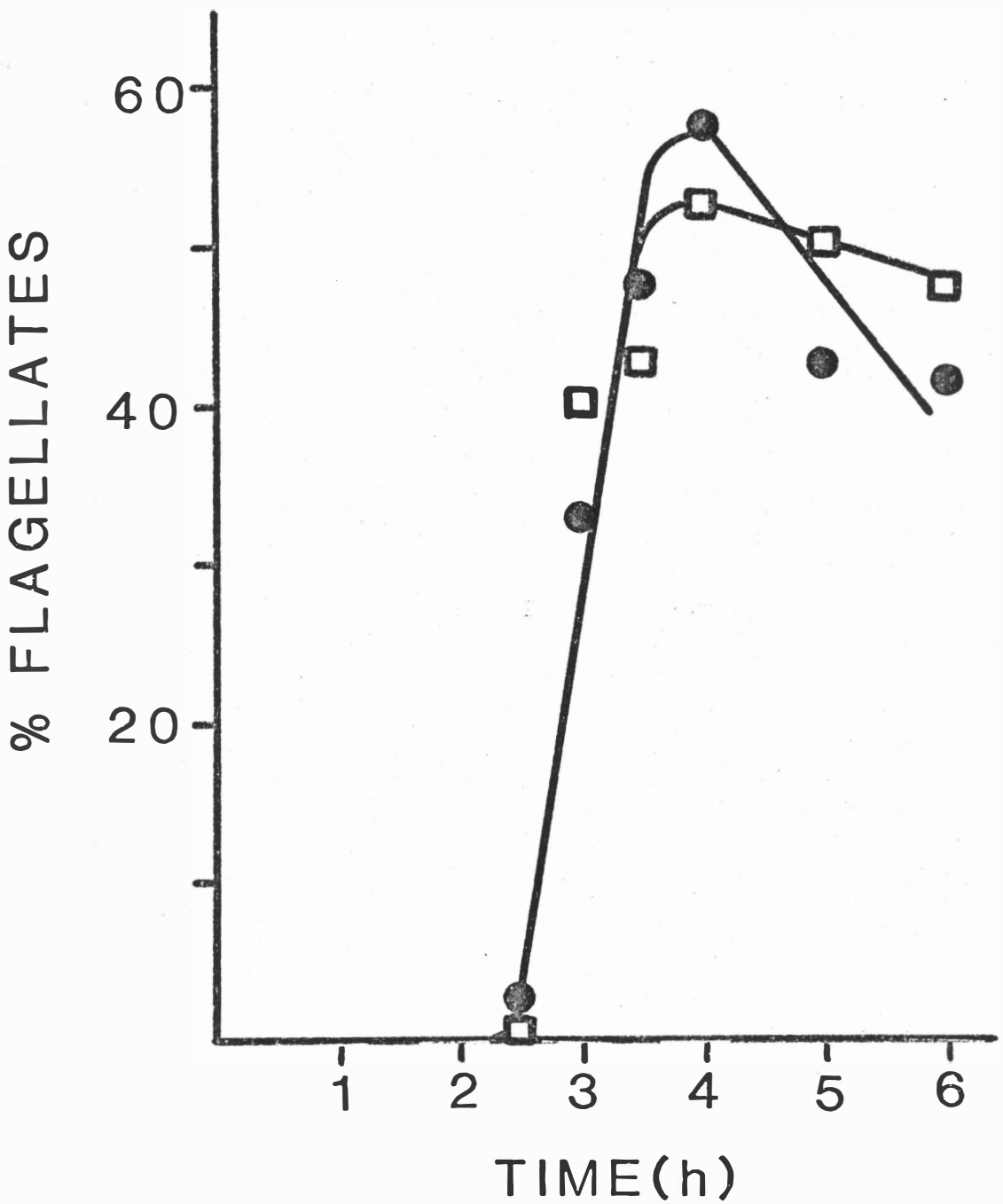

% FLAGELLATES
60
40
20
1
2
3
4
5
6
TIME(h)

Table 11

Incorporation of [^{32}P] Inorganic Phosphate During Growth of *Naegleria fowleri* nN68 in MOPS-Buffered Nelson Medium

Experiment	Input Label (μCi/ml)	% Utilization of Medium Isotope	Incorporation[a]	
			Cold TCA	Hot TCA
1	54	19	6.7×10^6	4.7×10^5
2	55	21	7.2×10^6	4.3×10^5

Amebae of *N. fowleri* nN68 were grown at 37°C in unagitated cultures in 75 cm^2 flasks containing 10 ml of Nelson medium with MOPS buffer and 2% calf serum. Carrier-free [^{32}P] inorganic phosphate was neutralized and added to mid-logarithmic phase cultures. Cultures were incubated for 24 hours with label. Incorporation into trichloroacetic acid (hot or cold) insoluble material was measured as described in Materials and Methods.

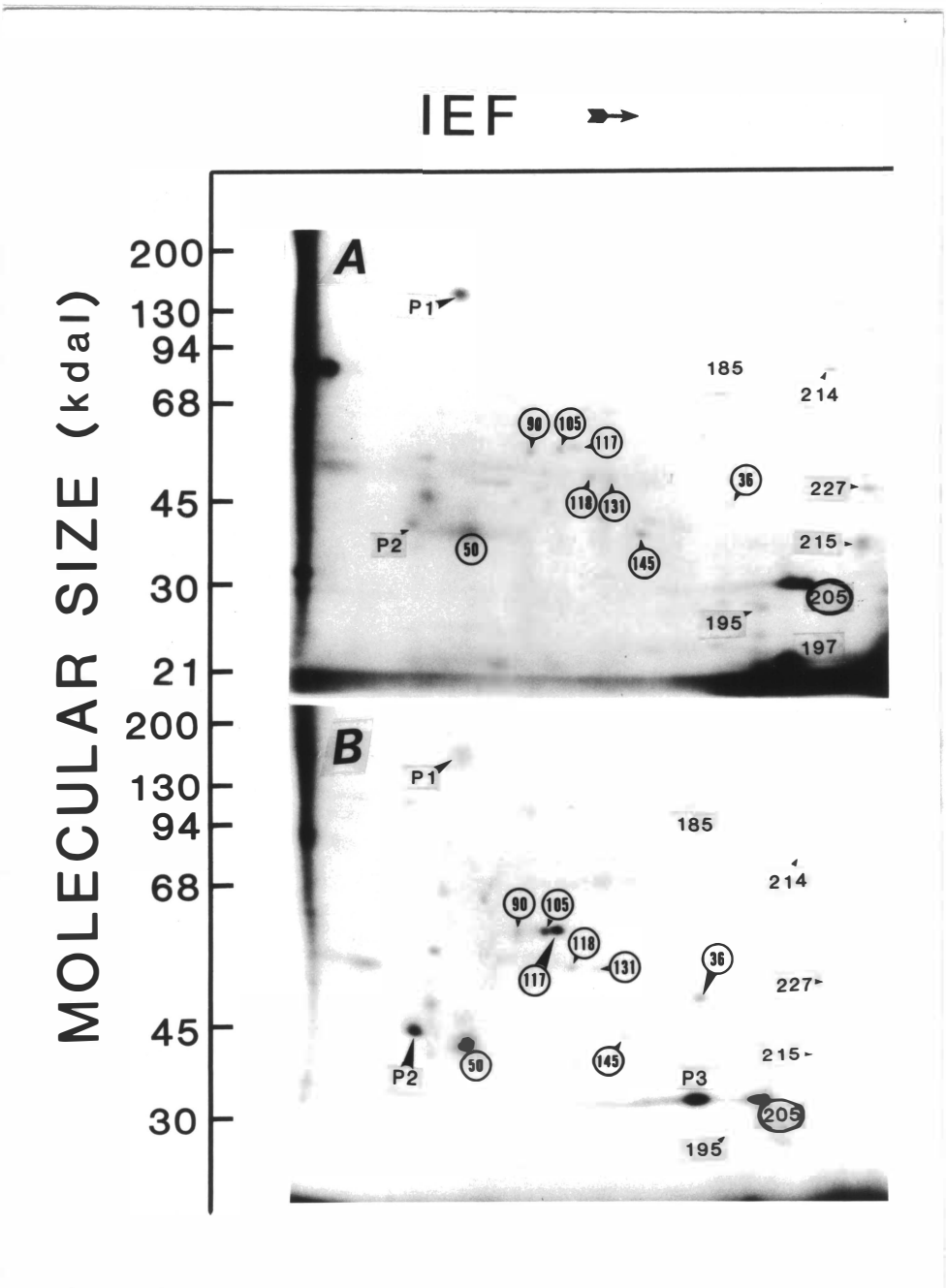
[a]Incorporation expressed as the counts per minute per 10^6 cells in trichloroacetic acid insoluble material, corrected for background.

Figure 54. Autoradiograms of [^{32}P]-labeled polypeptides of Naegleria fowleri nN68 amebae and flagellates resolved by two-dimensional electrophoresis.

Amebae of N. fowleri nN68 were labeled for 24 hours with [^{32}P]-inorganic phosphate during growth at 37°C in MOPS-buffered Nelson medium. Half of the labeled amebae were washed and suspended in ameba saline for enflagellation. Whole cell preparations of growing amebae and of flagellates formed after 3 hours in ameba saline were resolved by two-dimensional electrophoresis and detected by autoradiography as described in Materials and Methods. Numbered spots indicate label co-migrating with ^{35}S-methionine-labeled polypeptides as detected in Figure 39 and 40. Large arrows denote major changes during enflagellation.

A. Growing amebae. The sample contained 340,000 cpm in approximately 200 µg protein. P1 (∿ 150K, pI ∿ 6.8) and P2 (∿ 40K, pI ∿ 7.0) are major phosphorylated spots observed to change during enflagellation and which did not co-migrate with ^{35}S-labeled polypeptides.

B. Flagellates after 3 hours. The sample contained 95,000 cpm in approximately 60 µg protein. P3 (∿ 32K, pI ∿ 5.6) did not co-migrate with any ^{35}S-labeled spot seen in amebae or flagellates.


IEF
MOLECULAR SIZE (kdal)
200
130
94
68
45
30
21
A
P1
185
214
90
105
117
36
227
118
131
P2
50
215
145
205
195
197
B
P1
185
214
90
105
118
117
131
36
227
P2
50
145
215
P3
205
195

amebae or flagellates (Fig. 54b). The intensity of two spots increased during enflagellation (dark arrows, Fig. 54b) and one new spot (36) was detected. One spot (P1) declined noticeably in intensity, whereas several others (including 214 and 227) were no longer detected in the flagellates. The changes in distribution of ^{32}P-label did not correlate with those changes observed previously with ^{35}S-methionine-labeled polypeptides except for spot 36, which exhibited increases in relative amount in both ^{32}P and ^{35}S-labeled polypeptide patterns of flagellates arising from pre-labeled amebae (Fig. 40 and 54).

DISCUSSION

Growth characteristics of N. gruberi NEG-M at 32°C in agitated cultures are very similar to those reported for N. gruberi EGB under like conditions at 28°C (137) and the growth of N. fowleri nN68 at 32°C is only slightly slower than previous results obtained at 37°C under otherwise similar conditions (136). When the amebae in late logarithmic phase of growth are washed and suspended in non-nutrient buffer by the methods established previously (45), most of the N. gruberi amebae convert to flagellates by 90 minutes after subculture to buffer but very little enflagellation of N. fowleri amebae is observed. The T_{50} for N. gruberi enflagellation, 59 minutes, is almost identical to that reported at 25°C for bacterial-grown N. gruberi amebae (T_{50} = 60 minutes, Ref. 48) and is somewhat less than that reported previously for amebae grown axenically in M7 medium (79 minutes, Ref. 47,48). Axenic growth of N. gruberi NEG-M in the simplified M7 medium is equivalent to that in Balamuth medium under unagitated culture conditions (Fig. 4, Ref. 137), and the amebae grown in M7 medium and stimulated to differentiate by the same method again demonstrate synchronous, high yield enflagellation with a T_{50}, 68 minutes, again less than reported previously (47).

The lower yield of flagellated cells in some experiments may be attributable to damaging effects of the centrifugation/washing protocol as has been noted with N. gruberi (45). An alternate protocol for enflagellation initiation which eliminates centrifugation as well as simplifying and shortening the process (Rationale, Materials and Methods), results in conversion of up to 95% of M7 grown amebae into flagellated cells with an average T_{50}, in many experiments, of 72 minutes (Fig. 6). The enflagellation of axenically cultivated N. gruberi amebae in the experiments performed here is very similar to that reported elsewhere (47,48,50) by all criteria of timing, synchrony, extent of change, and the nature of the morphological changes from ameba to round cells to the elongate flagellate form. Furthermore, the results of experiments demonstrating the effects on N. gruberi differentiation of growth temperature (Table 3, Ref. 47), temperature of enflagellation (Fig. 7, Ref. 45,50), culture age (Fig. 11, Ref. 48), different non-nutrient buffers (Fig. 13, Ref. 45,49), removal of M7 medium constituents (Table 6, Ref. 46) and inhibitors of ribonucleic acid (RNA) or protein synthesis (Figs. 14-17, Ref. 56,63,109,146) are all consistent with the literature on N. gruberi differentiation.

Naegleria fowleri nN68 amebae do not enflagellate well under the conditions employed for optimal N. gruberi enflagellation. The variability in extent of enflagellation

among strains grown and induced to enflagellate at 32°C was first thought to be an indication of differences in optimal conditions for each strain. Indeed, the extent of enflagellation of two strains (Lovell and KUL) is markedly increased by changing the temperature of growth and differentiation to 37°C but the extent of enflagellation of several strains remain remarkably similar under a wide range of test conditions. At least one strain (NF69) does not enflagellate under any of the variety of conditions tested with strain nN68. The non-enflagellating strains do not produce a diffusible substance that inhibits enflagellation by an enflagellating strain nor do enflagellating strains release an activator that promotes enflagellation by non-enflagellating strains. Although it cannot be ruled out that favorable conditions for extensive enflagellation by the poor-to non-enflagellating strains do exist, it seems possible that the capability of N. fowleri strains to enflagellate efficiently is determined by genetic, as well as physiologic, factors.

A number of factors influence the enflagellation of Naegleria fowleri. The temperatures of growth and differentiation are important variables for N. fowleri enflagellation, as they are for differentiation of axenically grown N. gruberi amebae. However, N. gruberi amebae enflagellate extensively when subcultured to the same or lower temperature than that of growth (47,50), whereas N.

fowleri nN68 amebae enflagellate with higher yields and greater synchrony when subcultured to ameba saline at the same or a higher temperature than that of growth.

An important determinative factor for N. fowleri enflagellation, but not for N. gruberi, appears to be population density during growth and differentiation. Amebae from stationary phase cultures of N. fowleri nN68 which have achieved near confluency on the unagitated tissue culture flask surface differentiate with greater synchrony and to higher measured yield than do those from actively growing, sparse cultures. Changes occurring in the culture medium during growth also affect the extent of enflagellation after subculture to conditions eliciting enflagellation. Expended culture medium may prime amebae for enflagellation as a consequence of nutrient deprivation, depletion of an inhibitor or production of a differentiation factor. The critical component of the medium which when individually removed evokes enflagellation is the most complex constituent; that is, Panmede liver digest. Perhaps some critical factor in this component prevents amebae from enflagellating and cells normally become competent to enflagellate only after the level of this substance is reduced late in culture growth. These amebae, however, do not usually enflagellate spontaneously in old cultures in Nelson medium. Amebae of N. fowleri adapted to Balamuth medium, containing yeast extract and

proteose peptone in addition to the components of Nelson medium, can spontaneously enflagellate in old cultures (Mike Cline, unpublished results). N. fowleri nN68 amebae passaged in mice enflagellate spontaneously when they are recovered and cultivated in Nelson medium with serum. These results seem to agree with more exact results obtained with N. gruberi NEG-M in which the relative concentrations, in growth and enflagellation media, of a factor from yeast extract is the important variable with respect to enflagellation (46). N. gruberi NEG-M amebae cultivated in M7 medium with added factor enflagellate well when transferred to M7 medium without excess factor, whereas amebae grown in M7 medium without added factor require the removal of yeast extract for enflagellation to occur.

N. fowleri amebae enflagellate to a greater extent when suspended at densities less than 2×10^5 amebae/ml than at those greater than 5×10^5/ml. This may also be due to residual amounts of a substance on the cells which, when present above a certain concentration, inhibit enflagellation. Neither population density during differentiation nor growth stage appears to be a critical variable for N. gruberi enflagellation (48), although some correlations have been observed between the pH of late logarithmic phase axenic cultures of N. gruberi and the extent of enflagellation (47).

The experimental cues for enflagellation are suspension, dilution and agitation of amebae in non-nutrient ameba saline. Electrolyte concentrations have substantial influence upon the enflagellation of N. gruberi and specific cations can regulate both the initiation of enflagellation (36,46,48) and the interconversion of cells into the various morphological stages of N. gruberi enflagellation (49). Differences in the formation of the flagellate shape during enflagellation of N. gruberi in M7 buffer, TK buffer, Page ameba saline or deionized water (Fig. 13) are probably attributable to differences in such ionic factors. N. fowleri amebae, under the conditions tested, acquire flagella and assume the elongated flagellate shape without a distinctive rounded intermediate stage such as that regularly observed during N. gruberi differentiation (49,50,106). Enflagellation of Naegleria fowleri in a series of variations of Page ameba saline lacking K^+, Na^+, Ca^+ and Mg^+ (Materials and Methods, Table 7) does not result in a large difference in the extent of enflagellation but enflagellation in TK buffer (Tris buffer and 10 mM K^+) or deionized water resulted in significant reduction in the extent of enflagellation. Enflagellation also occurs similarly in ameba saline with phosphate buffer or with MOPS buffer (Fig. 53). A significant reduction in the level of incorporation of [^{35}S]-methionine and [^{32}P]-orthophosphate into macromolecules is evident in enflagellating N. fowleri as compared to growing amebae, even

though incorporation does occur during enflagellation (Tables 8, 9, and 11). Further studies are necessary for both N. fowleri and N. gruberi to elucidate the effects of physiological factors on both the initiation of enflagellation and on the progress of events during enflagellation.

Optimal conditions for enflagellation of axenically cultivated N. fowleri, based upon the results of the studies on factors critical to enflagellation, include: a) cultivation of amebae under nutritional conditions, environment and temperature favoring most rapid growth of amebae; b) use of amebae in mid-to-late stationary phase of the growth curve; c) removal of growth medium nutrients (or other factors preventing enflagellation); d) rapid completion of washing steps for amebae under conditions in which the cells are not subjected to damage by centrifugation; and e) suspension and agitation of amebae at a cell density less than 5×10^5 amebae/ml in nutrient-free buffer, optimally ameba saline, maintained at a temperature equal to or higher than the growth temperature.

The extent to which N. fowleri enflagellates under the optimized conditions is significantly less than that observed for Naegleria gruberi. This has been noted previously when N. fowleri isolates were induced to enflagellate at the same time and under similar conditions as N. gruberi (21). It is not known with certainty whether the highest percentages of flagellates scored (65-70%)

reflects an inability of 35% of the cells to enflagellate at that time or whether the numbers represent the maximum value resulting from the contribution of enflagellation and simultaneous reversion of earlier differentiated cells. Similar percentages of flagellates are obtained from cultures grown from either flagellates or amebae purified from enflagellated suspensions, arguing against the former alternative. The synchrony of appearance of the mature flagellate shape under the best conditions obtained is such that at least 60% of the amebae become flagellate-shaped cells within 30 minutes (Fig. 8C), synchrony rivaling that of N. gruberi (45,50). The decline in the percentage of flagellates in suspensions of synchronously enflagellated N. fowleri occurs much sooner and over a much shorter relative time span than is normally seen in N. gruberi enflagellation. Flagellates of N. gruberi, however, revert much faster at higher temperatures of enflagellation than at 25°C (Fig. 7, Ref. 45,49, 50) and show an increased susceptibility to reversion in the presence of inhibitors following heat-shock treatment at 38°C (133). It seems probable, then, that the quick reversion of N. fowleri flagellates prevents observation of near 100% flagellates at any one time.

Enflagellation of N. fowleri is generally slower than that seen with N. gruberi, even under conditions resulting in the most synchronous enflagellation with a high measured

yield of flagellates. It seemed possible that the differences in timing were due as much to the differences in the axenic growth media and enflagellation environments used as they were to program differences in the two species. However, N. fowleri and N. gruberi amebae grown in a common medium and stimulated to enflagellate in ameba saline continued to exhibit the same difference in timing of flagellate appearance (Fig. 10).

The results of inhibition studies on N. fowleri differentiation may provide insight into possible reasons for both the prolonged time of N. fowleri enflagellation and the quick reversion of N. fowleri flagellates. RNA and protein syntheses are required for N. fowleri differentiation, as judged by incorporation of radiolabeled precursors (Table 8), incorporation of radiolabel into a wide spectrum of polypeptides (Fig. 23) and inhibition of enflagellation by actinomycin D and cycloheximide (Fig. 19) at concentrations which do not markedly affect morphology or growth of N. fowleri amebae (Fig. 18). The interesting results, different from those observed with N. gruberi, are the effects of delayed additions of these inhibitors to differentiating N. fowleri cells. Addition of inhibitors at certain times during the enflagellation of N. gruberi at 25°C allows limited, but continued, enflagellation due to the conversion of cells which had completed the requisite synthesis. The flagellates which

form from these non-inhibited cells are stable at 25°C and, therefore, different 'plateau values' of flagellates are observed depending on the time of inhibitor addition (Fig. 17, Ref. 48,49,56). The stable 'plateau percentages' of flagellates have permitted a description of transition points, analogous to the T_{50} measurement, at which time 50% of the cells have completed the requisite synthesis for enflagellation and are, therefore, insensitive to the inhibitor (56,133). In contrast, addition of actinomycin D and cycloheximide at delayed times during N. fowleri enflagellation not only reduces the final maximum percentage of flagellates which form but also can prevent further enflagellation and cause reversion of existing flagellates (Figs. 20 and 21). The inability to measure 'plateau percentages' of non-inhibited flagellates in N. fowleri prevents derivation of transition points analogous to those measured for N. gruberi and suggests that continued RNA and protein syntheses are required for maintenance of the flagellate morphology. N. gruberi amebae which have been subjected to 38.2°C heat-shock demonstrate increased susceptibility to reversion in the presence of actinomycin D or cycloheximide (133). It has been shown that the completion of essential RNA synthesis for N. gruberi enflagellation is delayed, although limited progress continues, during the interval of heat-shock (133). It has further been postulated that the effects of

heat-shock on the delay in completion of N. gruberi enflagellation may be due to delay in the production of a temperature-sensitive protein required for assembly of the flagellar apparatus (133). The delay in appearance of N. fowleri flagellates, the apparent requirement for continued synthesis, and the quicker reversion of N. fowleri flagellates may be the result of the need for continued production of just such a labile component required for the flagellate morphology, although temperature lability does not seem likely in view of the suspension temperature effects on N. fowleri enflagellation. Differing lability of the essential protein might provide a basis for differences in the capability of N. fowleri strains to enflagellate. Among the candidates for the labile component would be components of degradative mechanisms which provide precursors for the necessary syntheses for enflagellation in the absence of exogenous nutrients or which function in removal of specific enzymes, such as thymidine kinase, during enflagellation.

The major ultrastructural changes which occur during enflagellation of Naegleria fowleri closely resemble those seen in N. gruberi. Some of the alterations unique to enflagellation have been identified by comparing events in a non-enflagellating strain of N. fowleri with those in enflagellating N. fowleri nN68. The ultrastructural changes related to enflagellation occur within a relatively

short period of approximately 60 minutes. In both species, large vacuoles appear to be expelled from the cell. It is not clear whether the loss of these vacuoles reflects the cessation of endocytosis in non-nutrient medium, preventing formation of food vacuoles, or active expulsion of the contents of the vacuoles into the medium or both. Although some of these vacuoles are reminiscent of phagocytic vacuoles, the cell may not be secreting hydrolases (106). Similar vacuoles, which are prominent in growing amebae of N. gruberi, also disappear from the cells during enflagellation (37). Apparently, the loss of vacuoles is not an enflagellation-specific process.

The small electron-opaque bodies observed in both ameboid and flagellated stages are similar to those in other species of Naegleria (122). It has been proposed that these bodies may be secretory granules (122) or may represent virus-like particles (117). The electron-translucent droplets seen in the enflagellating strain at all stages, but not in the non-enflagellating variant, have been identified as lipid globules by several investigators (16,129). The large electron-opaque granules in amebae of strain nN68 have not been reported previously in any Naegleria species. The function of these inclusions is presently unknown.

Strains of N. fowleri that form flagella do so while they are still ameboid. Ameboid flagellated cells lacking

directional motility apparently proceed directly to the elongated form. In contrast, N. gruberi becomes spherical before the appearance of flagella (48,49). Rounded cells become enflagellated and commence spinning without apparent directed motility. The round stage of N. gruberi is postulated to be merely a transitional phase representing the normal response of cells to surface tension forces when loss of ameboid motility occurs (49). The loss of ameboid motility is, however, a specific, defined event during N. gruberi enflagellation which requires prior RNA and protein synthesis (49). The lack of a distinct round phase of observable duration during N. fowleri enflagellation may be explained in a number of ways; one explanation is that the longer time between initiation and flagellar assembly in N. fowleri permits sufficient synthesis of materials needed for production of the microtubular cytoskeleton so that the flagellate shape is assumed shortly after assembly of the flagellar apparatus. The important difference between the two species appears to be that the loss of ameboid motility always occurs prior to assembly of the flagellar apparatus in N. gruberi, whereas the ameboid shape of N. fowleri persists, in some manner, throughout assembly of the basal bodies, flagella and rootlet. It seems clear from the results with N. fowleri that the prolonged round stage observed during N. gruberi enflagellation is not a required event for cells to undergo

the change from an actin-based ameboid motility system (48,87) to the microtubular cytoskeleton of flagellates (35,37, 45,49). It may be, however, that those factors which allow the ameboid shape to persist (under the conditions used herein for optimum N. fowleri enflagellation) also present some degree of impediment for the establishment of the flagellate cytoskeleton, perhaps by the postulated psi-factor-regulated Ca^{+2} system (49), and therefore contribute to less than complete conversion of N. fowleri amebae to mature flagellates and faster reversion. A number of other factors influence the stability of the flagellate shape, however, as evidenced by the incomplete conversion of rounded N. gruberi amebae into flagellate-shaped cells under a variety of conditions (Fig. 6, Ref. 49,56). Whatever the reasons for acquisition or loss of flagellate shape, the difference in whether or not loss of ameboid shape occurs before assembly of the flagellar apparatus could be considered as one argument that N. fowleri enflagellation is not merely a protracted version of that in N. gruberi.

The rapid assembly and appearance of the components of the flagellar apparatus in N. fowleri is similar to that observed in N. gruberi. The speed of its assembly and the rapidity with which shape changes may occur in cells of Naegleria have both been emphasized as phenomena which provoke testable hypotheses and promise new insights

into the regulation of cell shape, motility and organelle assembly (49,51). An association between the developing rootlet and the nucleus appears to be required for flagellar function. N. gruberi rootlets demonstrate variable periodicities in banding patterns when observed in sections of fixed flagellates, whereas the periodicities are constant in isolated rootlets (118). This difference suggests a functional role of the rootlet in flagella movement, additional evidence for which is the fact that the isolated rootlet protein is collagen-like in its properties (36,48). The periodicities observed for dark and light bands of N. fowleri rootlets were within the ranges reported for those measured in N. gruberi rootlets observed in sections (118). It is not known whether the proximity of the rootlet with the nucleus provides a favorable topography for mitochondria to align along the rootlet, serves as an anchor for the flagella, or reflects some other relationship. The morphologies of the N. fowleri flagellar axonemes, interconnections between flagella, basal bodies and rootlet, continuity of the cellular and flagellum membranes, and appearance of the cortical microtubules lining the anterior flagellate periphery were identical to those observed in N. gruberi flagellates (37,45,106,114).

Based upon the electron microscope study, the morphogenesis of the flagellar apparatus in N. fowleri appears

to proceed along the following steps: i) de novo formation of a pair of basal bodies 90 minutes after subculture to non-nutrient medium; ii) extension of the flagella and rootlet from the basal bodies while the cell remains ameboid in shape; iii) migration of the nucleus to the vicinity of the developing flagellar apparatus while elongation of the flagella and rootlet are in progress; and iv) completion of flagellar extension and of the association between the nucleus and the rootlet.

The appearance of a number of organelles during Naegleria enflagellation, for which no evidence exists for the prior existence in amebae of the intact structures or of precursors (36,51), suggests these as candidates for required new syntheses of polypeptides. Since the conversion of amebae to flagellates occurs in the absence of exogenous nutrients, the formation of new cell structures probably requires a coordination of new protein synthesis, degradation of stored materials and macromolecules, and modification of cellular components such as cytoskeletal elements. Although new protein synthesis is required for enflagellation, essentially a full complement of cellular proteins is being synthesized during enflagellation, indicating that preferential new synthesis of one or a few proteins is not occurring and that the nutritional deprivation used to initiate this differentiation process does not result in a general shutdown of protein synthesis.

Undoubtedly, however, there are new protein species among the multitude being synthesized during enflagellation of N. fowleri, probably including those structural polypeptides for which de novo synthesis of protein has been demonstrated in N. gruberi [outer doublet and central pair flagellar tubulins (52-54,83), dynein (56) and rootlet protein (48)].

Similar profiles of polypeptides are observed in two-dimensional gels of Naegleria fowleri proteins, whether visualized by sensitive silver staining (Fig. 43) or by autoradiograms of the polypeptides labeled with [^{35}S]-methionine during several generations of growth. These results indicate that the polypeptides of N. fowleri have similar methionine contents and that estimates of amount of polypeptide based upon radiolabeled methionine are useful (143). Several polypeptide species are abundant in N. fowleri, being present at an estimated concentration of 10^8 molecules/ameba. Such an estimate may be calculated from the fact that the median polypeptide has a molecular weight of 5×10^4 g/mole and represents approximately 10% of total cell protein. N. fowleri nN68 cells in stationary phase contain approximately 180-200 pg of protein per ameba (138). Therefore, 10% of the protein, or 20 pg, represents 2.4×10^8 molecules of 50,000 MW (5×10^4 g/6×10^{23} molecules = 2×10^{-11} g/2.4×10^8 molecules). The most abundant polypeptides are probably structural in

nature and might be expected to be relatively stable. For example, the cytoskeletal ameboid motility system remains present in N. gruberi flagellates inasmuch as reversion to ameboid form and motility may occur almost instantly under various conditions (49). The majority of the polypeptides detected in the gels are present at an estimated 10^3 to 10^4 molecules/ameba as calculated from the range in amounts of individual species, the estimated percentage of radioactivity present in the most abundant species, and the amount of total protein present in N. fowleri amebae (138).

The practice of loading approximately constant numbers of cell equivalents to the first-dimension gel, followed by exposure of the completed slab gels for varying time periods to yield a constant product of radioactivity x time, aids in normalizing for changes in radiospecific activity which might affect all polypeptides equally (69). Comparisons of autoradiograms may thereby identify selective changes in polypeptide amounts which occur in addition to any general effects. Most of the polypeptides of N. fowleri, previously radiolabeled with [^{35}S]-methionine, are not perceptibly changed during enflagellation with respect to subunit size and charge, and relative amount (144). These results indicate that most of the Naegleria proteins examined are relatively stable during enflagellation and that they are not covalently modified extensively during differentiation.

Particular polypeptides, however, may be modified in both amebae and flagellates by phosphorylation, amidation, methylation, dephosphorylation, etc. (39,111, 139). The steady-state level of a polypeptide is generally lower for proteins whose degradation is relatively rapid (92,104, 105), and it is these polypeptides which are most responsive to environmental changes (14). Many enzymes and regulatory proteins, particularly those critical to differentiation, may be present at concentrations less than 10^3 molecules/ameba, and therefore may not have been detected in this study.

A few polypeptides do decrease in amount, or vanish, during enflagellation. Presumably these proteins are no longer needed by cells in a non-nutritive environment and can be degraded to supply the precursors and energy required for new macromolecular syntheses. A candidate for one of these polypeptides is thymidine kinase, which has been demonstrated to decrease in amount as well as activity during enflagellation in N. gruberi (11,24). A few polypeptides appear in enflagellating and non-enflagellating strains of N. fowleri, presumably as a consequence of nutritional deprivation. It is not known whether these new polypeptides represent newly synthesized polypeptides or modification of pre-existing proteins. The lack of any preferential high level of synthesis of specific polypeptides during enflagellation argues against the former

alternative, particularly since the new species are observed in flagellates formed from growth-labeled amebae in the absence of additional radiolabel. No new enflagellation-related polypeptides have been detected, indicating that a number of changes associated with differentiation in Naegleria, as in Saccharomyces (126), may involve regulatory processes other than extensive synthesis of unique proteins, even though some new proteins may be necessary; for example, quantitative shifts (92,104), redistribution within the cell (81), or altered supramolecular interactions, partial enzymatic cleavage of polypeptides (39,90), covalent modifications of protein species (43,57,85), or some combination of these possibilities. It is also conceivable that the non-enflagellating variant is capable of producing most of the polypeptides necessary for differentiation but is blocked at some critical step in subunit interaction. The presence of some polypeptides in amebae of the non-enflagellating strain and in flagellates, but absent in amebae of the enflagellating strain, may indicate that the differentiation program is perturbed in the variant strain.

Most of the variability in time required for differentiation of N. gruberi (due to culture conditions, temperatures, etc.) has been ascribed to the early stages of differentiation (56), prior to the time at which essential RNA synthesis for enflagellation is completed. *Perturbation*

of N. gruberi enflagellation by heat-shock (131,133) or differentiation under different conditions of growth and differentiation has shown that the macromolecular syntheses maintain very fixed temporal relationships to one another and that the essential RNA and protein syntheses for structural proteins occur relatively late in the time course of enflagellation (48,49,56,131). The processes other than extensive macromolecular synthesis may therefore be a substantial part of the enflagellation response in Naegleria. For example, covalent modification may be responsible for the reversible compartmentalization of molecules such as that postulated for psi-factor in controlling the regulation of cell shape and motility in N. gruberi (49). Preliminary studies with phosphate-labeled N. fowleri amebae have shown that a number of changes in distribution of pre-labeled phosphoproteins on two-dimensional gels occurs during enflagellation. Although many of the labeled phosphoproteins co-migrated with [^{35}S]-labeled polypeptides, not all did and several of the major changes cannot be correlated with changes in [^{35}S]-methionine-labeled proteins. Perhaps these changes reflect those in crucial regulatory molecules present in too low an abundance to be seen in the [^{35}S] patterns. Clearly, additional study is needed to elucidate the contributions of covalent modification processes to the enflagellation process in N. fowleri.

Quantitative analysis of autoradiograms of samples from amebae and flagellates discloses correlations among charge, size and amount of polypeptide that are not evident from visual examination of the autoradiograms. Experience gained from contouring the printouts of spots on many gels has been invaluable in guiding the selection of minimum values below which the computer program does not include data for summation. Manual contouring also allows for reproducible enumeration of faint spots without the inevitable differences in viewer detection during visual examination of autoradiograms. Occasionally autoradiograms of gels are observed to have visually noticeable differences in film background which result in broadened histogram background peaks or doublet peaks. In these instances, faint spots in areas of lighter background have density readings which are equal to, or less than, the background in the darker areas. These autoradiograms are unsuitable for analysis by our methods because mean background subtraction will delete data for faint spots in lighter areas and will fail to eliminate the background in darker areas. For the most part, uniform backgrounds in autoradiograms are obtained and accurate discrimination values for background and for quantitation purposes are easily defined. Only autoradiograms meeting these criteria have been used in our analyses.

Polypeptides having molecular sizes between 60 to 100 kilodaltons and pI values above 6.3 become relatively more abundant during differentiation than those 20 to 60 kilodaltons in size and with pI values below 6.3. Possible explanations for the observed results include a) an increase in the rate of synthesis of larger, more neutral polypeptides relative to that of smaller, more acidic polypeptides; b) a decrease in the rate of degradation of larger, more neutral polypeptides relative to that of smaller, more acidic polypeptides; and c) an enrichment of larger, more neutral polypeptides as a result of selective export of smaller, more acidic polypeptides during enflagellation, perhaps by the vacuole expulsion observed in the morphological studies. In addition, the amount of polypeptide per spot in the size range of 40 to 60 kilodaltons is greater in both amebae and flagellates than other molecular size classes. It is not known at this time whether the higher levels achieved by particular polypeptide classes reflect faster rates of synthesis, slower rates of degradation or both.

Our data indicate that size and charge are not entirely independent variables as proposed by some workers (32, 34,62). A correlation between molecular size and charge has also been reported previously by other investigators using several different eukaryotic models (42, 99). The direction of the correlation observed for

whole-cell Naegleria polypeptides is the opposite of that observed for soluble proteins in mammalian cells. In Naegleria, large polypeptides tend to have neutral pI values and small polypeptides tend to have acidic pI values, whereas the opposite is true for soluble liver proteins (42,99). These differences may reflect differences in determinative properties for degradation which exist in different functional compartments of the cell; for example, hydrophobic membrane environments versus hydrophilic soluble compartments (127).

Summary

The results of these studies with N. fowleri enflagellation have given additional insights and posed significant questions regarding those mechanisms by which these eukaryotic protists accomplish the dramatic conversion from amebae to flagellates. The nature of the cues which stimulate enflagellation are in part known, but differences between the effects of population age and cell density upon N. fowleri versus N. gruberi enflagellation suggest that the cells not only respond to a decline in concentration of some factor(s) present in the growth medium but may require prior achievement of a state of enflagellation - "competence." N. fowleri amebae in latter stages of the growth phase exhibit this competence and substances in their culture medium can activate sparse, growing cultures to enflagellate, whereas N. gruberi

amebae appear to be competent throughout growth. The nature of such competency factors, their effects on cells prior to nutrient removal and their apparent differential expression in N. gruberi and N. fowleri await further studies which might provide interesting clues as to which mechanisms are involved in the response to the differentiation cues.

Once the competent amebae have been washed free of nutrients, a substantial number of physiological changes ensue, many of which no doubt pertain to the starvation situation, others which begin to direct the expression of those genes and gene products required for the conversion of cell metabolism, shape and motility to that of the flagellate state. To an extent, those changes which ensue from starvation may be largely, and naturally, intertwined with those required for enflagellation. The various processes of macromolecular modification, compartmentalization, secretion and degradation may serve the cell's energy needs as well as eliminating unnecessary/harmful enzymes and synthesizing more useful products for the new state. An entire complement of cellular polypeptides, very similar to that existing in growing amebae, continues to be synthesized during enflagellation of N. fowleri. The synthesis, during N. gruberi enflagellation, of the major structural proteins of the flagellar apparatus and microtubular cytoskeleton has been well studied. These

changes, however, might be among the few large scale changes in specific protein syntheses, other necessary events being accomplished by changes in activity or location brought about by the aforementioned processes. The lack of detection of new polypeptides specifically related to enflagellation is possibly due to the fact that new polypeptides present in sufficient abundance to be observed in this survey are likely structural and may very well be synthesized as well in non-enflagellating amebae as in flagellates. The defect in the non-enflagellating cells is then postulated to reside in either an inability to produce a low abundance (non-detected) polypeptide necessary for proper assembly of the synthesized structural elements or in some other of the regulatory processes contributing to enflagellation. Experiments which determine the extent of different types of protein modifications occurring in different strains, or in different "competency" states of the same strain, may provide clues to the relevant defects in non-enflagellating amebae and thus increase the understanding of the normal enflagellation. The parallel observed between N. fowleri and heat-shocked N. gruberi with respect to the extended time for enflagellation and the quick reversion of flagellates suggests that a search be made for the postulated "labile component," which may also be the source of variability in enflagellation of different N. fowleri strains. It seems

appropriate that the study of enflagellation in Naegleria should be made an interspecies study, with the ability to compare almost identical morphological changes occurring under apparently differing regulation. For example, the morphological sequences of N. fowleri enflagellation apparently provide evidence showing that the prolonged round stage of N. gruberi is not a requisite for cells to change from the ameboid motility system to the microtubular cytoskeleton of flagellates. The synchronous and rapid completion, in a population of biochemically manipulable cells, of such coordinated sequences of organellar assemblies in the absence of exogenous nutrients offers an excellent opportunity for studies of coordinated regulation of existing macromolecules and new gene expression. These processes are the essential components of any scheme proposed to account for cellular differentiation; therefore, the enflagellation of Naegleria species, as a group, should be considered an excellent model system for eukaryotic cell differentiation.

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