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Intracellular Regulation of Cell Shape and Motility in Naegleria. First Insights and a Working Hypothesis

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Amoebae of Naegleria gruberi differentiate to temporary flagellates that have a regular, asymmetric, streamlined body contour. During the hour-long differentiation, amoeboid movement gradually ceases and as a consequence the cells round up. Subsequent elongation to flagellate shape includes the formation of a microtubular cytoskeleton. Both the loss of amoeboid motility and the formation of the flagellate shape require prior transcription and translation, suggesting the possibility that specific syntheses of RNA and protein may be required for each shape change. Flagellates can "revert" to motile amoebae within 20 sec after a suitable stimulus, indicating that the amoeboid motility system remains latent in flagellates. A cell-produced chemical factor extracted from Naegleria, ψ, triggers a reproducible sequence of rapid shape changes in flagellates when added to their environment. Cells respond to the presence of external ψ only "transiently," and the reaction of flagellates to added ψ requires extracellular Ca^{+2}. Ionophore A23187 produces shape changes in flagellates similar to those produced by ψ, supporting the conclusion that ψ is involved in the movement of Ca^{+2}. Normally ψ is intracellular, and the intracellular distribution of ψ changes during differentiation.

These results lead to and support a working hypothesis to explain the rapid changes in shape and motility in Naegleria. Four elements are postulated: Ca^{+2}; an actin-based amoeboid motility system that depends on free Ca^{+2} for functioning; a tubulin-based cytoskeleton that assembles and remains assembled only when free Ca^{+2} is low; and ψ. The factor ψ is postulated to regulate the intracellular release of Ca^{+2}. According to the hypothesis, intracellular free Ca^{+2} is constantly swept up into Ca-reservoirs. Motility of amoebae depends on local release of Ca^{+2} from these reservoirs, which in turn is caused by the intracellular release of ψ. During differentiation, ψ is "compartmentalized" as part of the developmental program, and as a consequence intracellular Ca^{+2} is swept up into Ca-reservoirs but not released. As free Ca^{+2} becomes limiting, amoeboid movement stops, and the cells round up. Subsequently, in a process that depends on low free Ca^{+2}, the microtubular cytoskeleton is assembled, and the flagellate shape is formed. During reversion of flagellates to amoebae, release of ψ from its "compartments" permits local release of Ca^{+2}, which then causes both disassembly of the flagellate cytoskeleton and immediate resumption of amoeboid movement. This testable hypothesis has implications for the study of cell shape, motility, and differentiation.

Key words: amoeboid movement, calcium ions, cell shape, Naegleria gruberi

This is the second paper in a series; the first is reference 1. A portion of this study was presented at the Fifteenth Annual Meeting of the American Society for Cell Biology (2).

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> For my part, I find it more amusing to look forward to a day when the great riddle may give up its secret.
>
> E. B. Wilson, 1925

The cells of Naegleria gruberi undergo profound changes in shape and motility during their hour-long differentiation from walking amoebae to streamlined swimming flagellates. These shape changes were first described by Schardinger nearly 80 years ago (3), and their temporal sequence was measured by A. D. Dingle and me 10 years ago (4). During the years that my collaborators and I have been studying differentiation in Naegleria, the shape changes have always challenged me as a "great riddle," but since I had no idea how to ask questions about these changes I concentrated on other aspects of the differentiation. The chance discovery of a cell-produced chemical factor, designated ψ, that causes rapid shape changes in flagellates (5), opened the way for an experimental attack on the riddle of cell shape. Our first paper on the regulation of cell shape and motility in Naegleria describes the ψ phenomenon (1). The present essay offers an overview of our present knowledge and understanding of cell morphogenesis in Naegleria. In the first portion some diverse observations, including many unpublished ones, are described as briefly as is consistent with clarity. These observations provide our first insights for thinking about cell shape and motility in Naegleria. The second portion describes a working hypothesis based on these observations. Since the Naegleria system and the ψ phenomenon have led us to an unorthodox approach to the problems of cell shape and motility, no apology is needed for the personal focus of this essay. I hope it will become evident to the reader that the possibilities for experimental analysis of cell morphogenesis are great in this system, and that answering some questions has brought into focus many unanswered but approachable questions.

CHANGES IN CELL SHAPE AND MOTILITY DURING DIFFERENTIATION

External shapes. Amoebae of N. gruberi are normally phagotrophs that feed on bacteria (reviewed in 6), but they also may be cultivated in a partially defined soluble axenic medium (7). The cells of strain NEG, with which the present research is being done, are haploid and about 15 μm in diameter when rounded (6). On a substratum, the amoebae progress by forming a single, broad, hemispherical pseudopodium of hyaline cytoplasm (Fig. 1A). They can move quite rapidly; at room temperature they move 50–100 μm, or several times their diameter, per minute (8). Amoebae can change direction abruptly by erupting new pseudopodia, but usually they are elongated in the direction of flow and have a distinct polarity. The nucleus rolls around in the cytoplasm as the amoebae move. Amoebae in suspension usually have several pseudopodia and no obvious polarity.

When amoebae are transferred from their growth environment to nonnutrient buffer, an environmental change that initiates differentiation (9), they remain actively motile for a while but then the capacity for amoeboid movement is gradually lost. Eventually the cytoplasm seems to gel, pseudopods are retracted, and the cells round up into spheres (Fig. 1B). During this period, time-lapse movies* reveal that the cell surface continually "bubbles" and abortive pseudopodia occasionally form. This surface activity declines as the rounded cells acquire a smooth contour. Flagella (usually 2) appear on the spherical cells. As the flagella elongate and become increasingly active, the cells, if

*A portion of these time-lapse movies of differentiation, filmed by Joseph Durden at the Education Development Center, is available commercially from BFA Educational Media, 2211 Michigan Avenue, Santa Monica, California 90404.

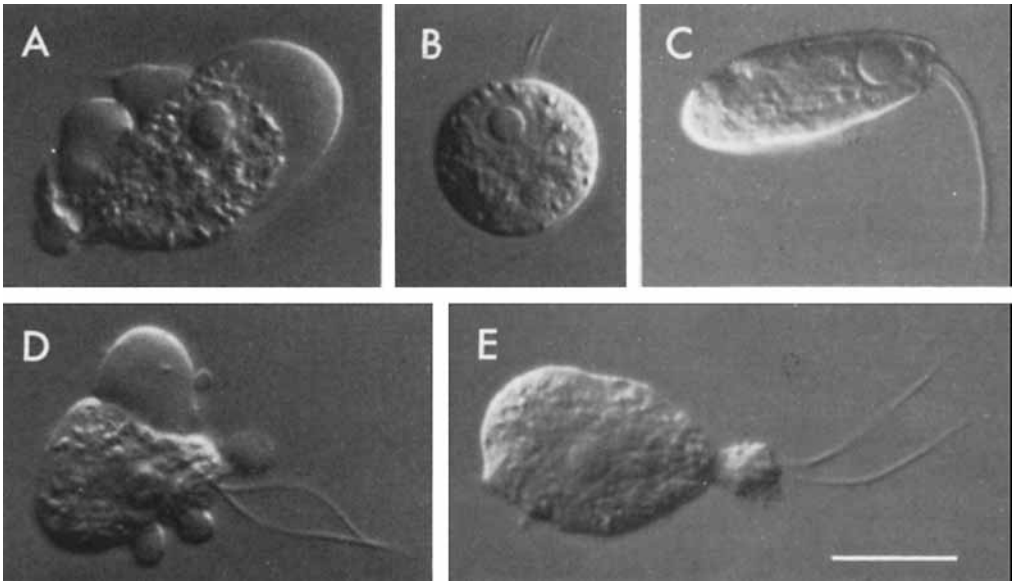


Fig. 1. Major shapes of Naegleria gruberi. These photographs, taken using Nomarski differential interference contrast optics, are of cells fixed in 1% glutaraldehyde, which gives good preservation of the shapes. A) amoeba; B) sphere; C) flagellate; D) reverting amoeba; and E) shmoo. A shows the shape of an amoeba on a substratum; D the shape of one in suspension. All the cells except A had two flagella. The bar indicates 10 μm.

sitting on a substratum, begin to quiver, and then to spin, and finally lift off the substratum and swim away, with the flagella leading. The nucleus is toward the anterior end, and the contractile vacuole toward the posterior end. While swimming, the cells elongate and form the definitive flagellate shape (Fig. 1C). The flagellates are effective swimmers; at room temperature (22–25°C) they progress at 90–140 μm per second, or nearly 100 times as rapidly as amoebae (unpublished observations).

The contoured shape of flagellates is complex, asymmetric, and, as yet, inadequately described. They are not simply "pear-shaped" or "ovoid" (previous observations are summarized in Ref. 6). The bodies of living or glutaraldehyde-fixed flagellates are more than twice as long as they are wide. The anterior end has a concave depression or groove where the flagella emerge from the cell. On one side at the anterior end there is a small, forward-projecting cytoplasmic cone, the beak or rostrum. The remainder of the cell body has a smooth, ovoid contour and "bulges" less on the side having the beak than on the opposite side (Fig. 1C). The posterior end usually is rounded but occasionally ends in a hernia of cytoplasm.

Kinetics of shape changes. During differentiation, amoebae round up to spheres, flagella form, and the flagellated spheres elongate to flagellate shape. The time course of these changes can be measured by counting fixed samples for the percentage of cells with flagella and, independently, for the percentage of cells with each body shape (amoebae, spheres, or flagellate-shaped). This counting procedure allows us to separate the sequence of shape changes, which occurs gradually as a continuum, into 2 quantal changes: amoebae to spheres and spheres to flagellate-shaped cells (4).

A sample result, obtained using the conditions for differentiation that are standard for the experiments in this study, is shown in Fig. 2. At the start of differentiation, the cells are all amoebae without flagella. The cells begin to round up at about 40 min, and 50% are spheres at 55 min. Six minutes later, 50% of the cells have flagella (T_{50} = 61 min). Flagellate-shaped cells begin to appear at 70 min, and 50% of the population is flagellate-shaped at 77 min. The "slopes" of the curves indicate the population heterogeneity in the time of differentiation; this heterogeneity is similar for all these quantal changes (4). It is convenient to use the time at which 50% of the population has a given property to measure events. These times are reproducible. For example, in well over a hundred experiments (excluding a few that had obvious problems with the cells or conditions), under these experimental conditions the T_{50} for flagella had a range of 58 to 63 min.

In our earlier study of the time course of these changes, using a different strain of Naegleria (strain NB-1), we found that the progress of differentiation was independent of cell population density (4). In strain NEG, used in the present work, formation of flagella is independent of cell population density over a range of at least 1,000-fold (from 8×10^4

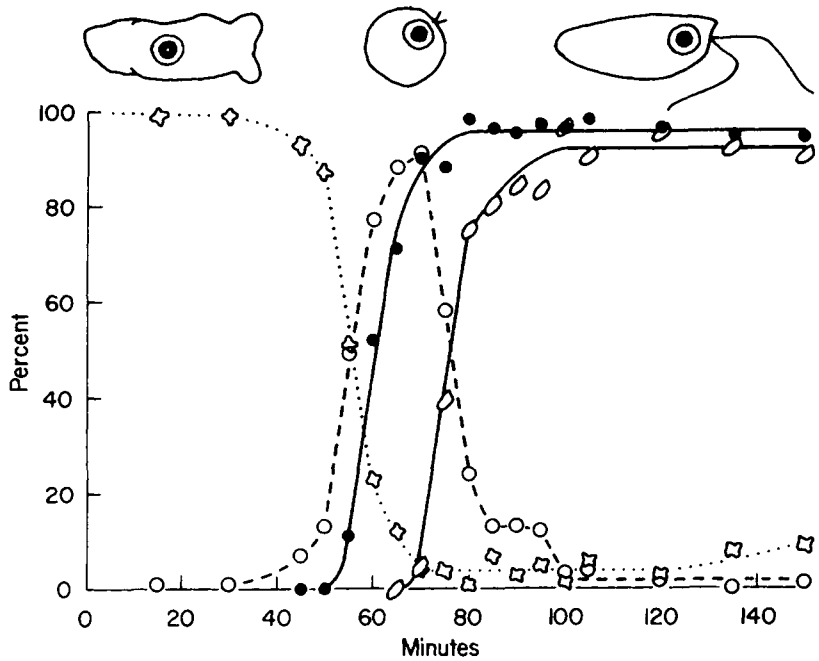


Fig. 2. Measurement of the temporal sequence of morphological changes during differentiation of amoebae to flagellates. The conditions used for obtaining differentiation in this and the other experiments in this paper have been described in detail (1, 4, 6). Amoebae of N. gruberi NEG, grown in association with Klebsiella pneumoniae on NM agar at 33–34°C, were suspended in T buffer (2 mM Tris-HCl, pH 7.2) at time zero. The amoebae were washed relatively free of bacteria by differential centrifugation at room temperature; washing was completed in 11 min. Thereafter the cells were incubated in T buffer at 25°C with gentle shaking. In this experiment the population density was 1.7×10^7/ml, and 3 ml were incubated in a 25-ml Erlenmeyer flask. Samples were taken at intervals to Lugol's iodine, a one-step fixative-stain, and two independent counts were made: one of the percentage of cells with flagella, independent of body shape (•), and the other of body shape, independent of flagella (glyphs indicate amoeba-, sphere-, and flagellate-shaped cells).

cells/ml to 8×10^7 cells/ml). However, two other parameters are influenced by population density: the formation of flagellate shape and the stability of flagellates are both favored by high population density. An example of these effects may be found in Fig. 7. Preliminary observations suggest that something accumulates in the environment of cells that favors flagellate shape and stability. This phenomenon warrants further study; in the meantime, awareness of the phenomenon allows us to manipulate conditions to influence the stability of flagellates.

Dependence of the shape changes on transcription and translation. The shape changes occur in regular procession as part of the "program" of differentiation. Do these changes require the synthesis of new macromolecules, particularly RNA and protein molecules? In collaboration with C. Walsh, I have evaluated the effects of three inhibitors on the synthesis of RNA and protein and on differentiation (10). Actinomycin D and daunomycin selectively inhibit RNA synthesis, and cycloheximide selectively inhibits protein synthesis, throughout the time required for differentiation. Any one of these inhibitors, added to cell suspensions soon after they are transferred to nonnutrient buffer, prevents differentiation. The cells remain motile amoebae though they tend to round up somewhat after an hour or so in the inhibitor. Each inhibitor, added to samples of differentiating cells at various times, ceases to block each specific event of differentiation at a reproducible time. The time at which half the cells can accomplish a specific phenotypic change in the presence of an inhibitor defines a transition point (10, 11). Inhibitors of transcription block assembly of flagella until about halfway through the temporal process from initiation to flagellum assembly, and cycloheximide blocks until three-fourths of the way through (10). In similar studies, in progress, I am determining the transition points for the shape changes (Table I). For example, samples transferred to cycloheximide at 55 min form flagella but never form the flagellate shape, whereas samples transferred after 70 min form > 75% flagellate shape. In samples transferred at successive times between 55 and 70 min, progressively fewer cells are inhibited. The cycloheximide transition point for flagellate shape is 66 min – about 5 min after the time flagella begin to be assembled and about 11 min prior to the occurrence of the change from rounded cells to flagellate shape.

Even the programmed loss of amoeboid motility that occurs during differentiation does not occur in the presence of these inhibitors if they are added early enough. The

TABLE I. Events of Differentiation Dependent on Transcription and Translation: Transition Points*

	Measured phenotypic change		
For 50% of the cells	Cells round up	Formation of flagella	Formation of flagellate body shape
	(time in minutes)		
Phenotypic change becomes insensitive to			
actinomycin D (120 μg/ml)	27	30	46
cycloheximide (100 μg/ml)	46	48	66
Phenotypic change occurs	54	61	77

*These estimates of transition points are for strain NEG differentiated at 25°C under the conditions described in the legend to Fig. 2 (from Ref. 10 and Fulton, in preparation).

transition points for capacity to lose amoeboid motility occur at or slightly prior to the time cells acquire the ability to form flagella (Table I).

The successive shape changes during differentiation are dependent on transcription and translation. Since the inhibitors, at the concentrations used, are rapidly effective in inhibiting the expected syntheses throughout differentiation (10), these experiments indicate that after the transition points RNA and protein synthesis are no longer essential. We infer, as discussed in Ref. 10, that prior to the transition points transcription and translation are essential. If this inference is correct, then RNA and protein synthesis are required to prepare the cells for both the loss of amoeboid motility and the elongation to flagellate shape. The experiments with inhibitors give a first insight into the kind of events that must be involved in the programmed shape changes during differentiation, although they do not tell us much about the specific nature of these changes.

Mutants. Strain NEG is apparently haploid and mutants may be isolated readily (6). Among mutants that were isolated in 1967 were several that formed flagella with normal kinetics but remained motile amoebae throughout the process. These mutants indicate that differentiation to "flagellates" (cells with flagella) can occur independently of the body-shape changes. It will be interesting to use such mutants to study the mechanism by which amoeboid motility is normally lost during differentiation.

Morphological correlates of the different shapes. It is generally assumed in the literature that amoebae move using an actin-based mechanochemical system at least superficially similar to that found in muscle (reviewed in 12–15). Much evidence supports this conclusion, including the presence of actin-containing microfilaments in amoebae (reviewed in 15). In amoebae of Naegleria, we have not yet seen any characteristic arrays of microfilaments, but just the "microfilament meshwork" described by others and probably due to poor preservation of microfilaments by the fixatives generally used (16). We have seen microfilaments in amoebae broken on polylysine-coated grids and negatively stained by the procedure of Clarke et al. (17), and the amoebae contain abundant actin which has been isolated and purified (J. Sellers, P. A. Simpson, and C. Fulton, unpublished observations). Much remains to be done to determine the basis of amoeboid movement in Naegleria – and in other organisms as well. Our assumption, throughout this paper, that amoebae move using an actin-based mechanochemical system, is admittedly based on tenuous evidence at present.

We assume that the cells round up during differentiation as a direct consequence of the loss of amoeboid motility. A cell, like a small oil droplet, will round up to a sphere in an aqueous environment unless other forces alter its shape. Our conclusion that rounding up in Naegleria has this simple basis is supported by the observation that many agents that interrupt amoeboid movement (such as 5 mM KCN or 50 μg/ml cytochalasin B) cause amoebae to round up (unpublished observations). However, even though the rounding up during differentiation may have this simple a basis, it should be remembered that the loss of amoeboid motility is a specific event of differentiation that undoubtedly has a very specific intracellular control.

The flagellates have an array of cortical microtubules that are numerous in the vicinity of the basal bodies and around the anterior contour and also are found parallel to the long axis of the cell (6, 18). By analogy to other cells, these microtubules presumably serve as a cytoskeleton that is at least partially responsible for the body contour of flagellates (reviewed in 19, 20, 21). In amoebae microtubules are found in the mitotic apparatus (22, 23), but so far no cortical microtubules have been found. The cortical microtubules, like those of the basal bodies and flagella (22, 24), appear to be assembled

during differentiation. The precise arrangement of the cortical microtubules, the morphogenesis of the arrangement, and the role of these microtubules in determining the flagellate shape all need to be studied.

Conclusions. During differentiation, cells of Naegleria gruberi progress, in programmed sequence, through a series of morphological changes that may be measured as quantal changes. These changes all are dependent on transcription and translation, suggesting the possibility that specific syntheses of RNA and protein may be required for each change.

The shapes are assumed to have the following bases: amoebae move using an actin-based mechanochemical system, spheres result from the loss of amoeboid motility, and flagellates have their shape because of the assembly of a tubulin-based cytoskeleton.

The challenge is to understand the basis for these shape changes: for the loss of amoeboid motility, and for the formation of the flagellate shape.

REVERSION OF FLAGELLATES TO AMOEBAE

The flagellates of Naegleria are temporary; they neither feed nor undergo fission, and eventually they revert to amoebae. The cells remain as flagellates for intervals ranging from a few minutes to several days, depending on temperature and other conditions (4 and unpublished observations). Reversion of untreated cell populations is less synchronous than differentiation (e.g., Figs. 6 and 7). In strain NB-1, reversion to amoebae is followed by redifferentiation to flagellates (4). The cells redifferentiate in the same environment, without nutrients or intervening growth. Up to four cycles of differentiation and reversion have been observed (4). Strain NEG is less able to go through repeated cycles of differentiation than NB-1; usually after reversion most of the strain NEG cells remain amoebae, and not more than 20% go through even a second differentiation.

Reversion of individual cells occurs rapidly. There is general agreement among observers that reversion of flagellates to amoebae occurs abruptly (reviewed in 6). Flagellates suddenly lose their streamlined contour and become motile amoebae, usually with flagella. Some may pass through a transient stage of being rounded, but, on the basis of many observations of living cells and kinetic studies with fixed samples, most cells seem to go directly from flagellates to amoebae without any intervening forms. In the shaking suspension cultures that we regularly use to study differentiation and reversion, the reversion to amoeba shape is normally followed by resorption of flagella (at 25°C, the loss of flagellate shape precedes the loss of flagella by 5–10 min). In living cells, the flagella cease to move, and then the axonemes are rapidly withdrawn into the cell body (6).

Mechanical or surface-tension forces can cause instant reversion of flagellates to amoebae (3, reviewed in 6). Reversion occurs more rapidly when cells are maintained in suspension by gentle agitation, as we routinely do to permit random sampling, than in undisturbed slide-coverslip preparations (6). Stronger agitation (e.g., in a rapid stirring device) can cause all flagellates to revert to amoebae within seconds. Many have made similar observations. For example, Wherry (25) observed correctly that if a drop containing flagellates is put on a microscope slide and "the coverglass is dropped on in the usual way the flagellates disappear at once."

In sealed slide-coverslip preparations in which a space is maintained (6), if flagellates simply bump into the slide they immediately walk away as amoebae, usually with flagella. In these cultures, flagellates occasionally touch down, lose their flagellate shape

but not their flagella, walk for a minute or so as amoebae while retaining their flagella, and then round up and swim away again. Four of these "touchdowns" were recorded in a time-lapse movie of differentiation; the timing of events in these is given in Table II. These limited observations indicate that cells can become amoebae within 15–20 sec after touchdown and can round up again as little as 40 sec later. The rapidity of these reversible shape changes is similar to the rapidity of changes that can be produced experimentally, described later in this paper.

Although the mechanism of spontaneous or induced reversion is not known, the properties of reversion, especially its rapidity, indicate that the capacity for amoeboid motion lies latent in the flagellates. The machinery is all there and simply needs permissive conditions to begin working immediately – within 20 sec.

The Mg^{+2} effect. In 1956 Willmer surveyed the effects of ions and other compounds on differentiation of Naegleria (26, 27). He reported that Mg^{+2} specifically inhibited differentiation and that Ca^{+2} added simultaneously reversed the effects of Mg^{+2}. When A. D. Dingle and I began to study the differentiation, we repeated these tests and obtained quite different results. Our results remained a curiosity until recently and were never published, but now in the context of the work on cell shape the insight they give is important.

Mg^{+2} ions at < 10 mM have no effect on the kinetics of differentiation of amoebae to "flagellates" (cells with flagella) (Fig. 3). In the presence of Mg^{+2}, however, the cells never cease to be basically amoeba shaped; some become rounded but none ever form the flagellate shape. In addition, as soon as the flagella elongate and cells begin to swim, the flagella are resorbed (Fig. 3). Mg^{+2} ions thus prevent the shape changes and result in unstable flagellates. Mg^{+2} does not have to be present throughout differentiation to have this effect. Addition of Mg^{+2} at any time causes loss of the flagellate shape and reversion to amoebae (data not shown). If Ca^{+2} ions are added simultaneously with Mg^{+2} (Fig. 3), or at any time prior to the formation of flagella (not shown), the effects of Mg^{+2} disappear, and the cells differentiate to stable flagellate-shaped flagellates.

One of our experiments with strain NB-1 (Fig. 4) illustrates that even though Mg^{+2} causes "flagellates" to revert as soon as they form, it does not prevent the subsequent redifferentiation of these cells to flagellates in the same environment. As in spontaneous

TABLE II. The Timing of Shape Changes in Four "Touchdowns"*

	Cell number			
	1	2	3	4
	Time (seconds)			
Touchdown	0	0	0	0
First pseudopod	20	20	15	20
Spherical again	135	60	210	140
Swam away	155	75	280	165

*The film sequence, which spanned 317 min with frames at 5 sec intervals, was taken during the differentiation of strain NB-1 in a sealed slide-coverslip culture (6) in T buffer at 25°C. Four complete touchdown cycles were observed. The cell shape at the moment of landing is unknown; when first seen these four cells look like spheres or perhaps up-ended flagellate-shaped cells.

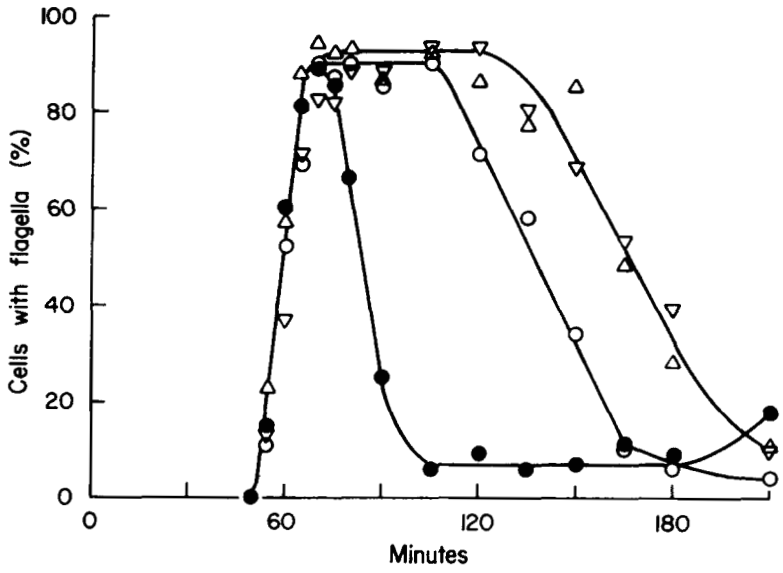


Fig. 3. Effect of Mg^{+2} and Ca^{+2} on differentiation of strain NEG. Amoebae were incubated at 25°C and 5.2 × 10^5 cells/ml in T buffer with: no additions (○), 5 mM $MgCl_2$ (●), 5 mM $MgCl_2$ plus 1 mM $CaCl_2$ (△), or 1 mM $CaCl_2$ (▽). Body shapes were also counted (not shown). None of the cells in Mg^{+2} alone formed the flagellate shape at any time; the other three cultures formed at least 75% flagellate shape and lost it as they reverted. The cultures were fixed until 300 min, but less than 20% redifferentiated to flagellates in any of them.

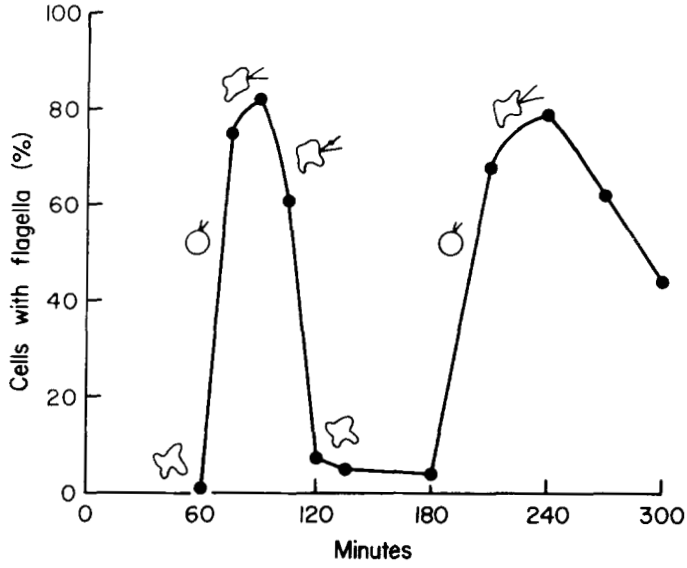


Fig. 4. Effect of Mg^{+2} on differentiation of strain NB-1. Amoebae were incubated at 25°C and 2 × 10^5 cells/ml in T buffer containing 10 mM $MgCl_2$. The time course of appearance of cells with flagella was comparable to cultures without Mg^{+2}, but unlike such cultures none of the cells formed the flagellate shape and the cells resorbed their flagella soon after they formed. [Control cells would remain flagellates to at least 180 min (4).] After reversion, the cells redifferentiated to cells with flagella again.

reversion and redifferentiation, with strain NEG fewer cells redifferentiate after reversion in Mg^{+2}.

Willmer reached the conclusion that Mg^{+2} inhibited differentiation because he made single observations to determine the extent of differentiation (26). In the experiments shown (Figs. 3 and 4), if one returned to the cultures after 2 hr, one would conclude that Mg^{+2} had prevented differentiation, whereas during that interval the cells had differentiated with normal kinetics and then reverted to amoebae.

Many other compounds, ranging from cyanide (5 mM) to the calcium ionophore A23187 (discussed later in the paper) induce rapid reversion of flagellates (unpublished). Unlike Mg^{+2}, however, the other compounds that are known to induce reversion also affect differentiation.

The important ionic species in the Mg^{+2} effect is probably Ca^{+2}. As little as 0.03 mM Ca^{+2} reverses the effects of 3 mM Mg^{+2}. EDTA (2×10^{-5} M) can mimic the effect of Mg^{+2}, although it is less effective and less reproducible than Mg^{+2}. As with Mg^{+2}, the effectiveness of EDTA is reversed by Ca^{+2}. With strain NEG at low cell population density, added Ca^{+2} (whether alone or with Mg^{+2}) actually increases the stability of flagellates (Fig. 3).

These experiments with Mg^{+2} and Ca^{+2} can be interpreted to indicate that a trace amount of environmental Ca^{+2} is required for the formation of the flagellate shape and for the stability of flagellates. An alternative view of these results is considered later.

Conclusions. The amoeboid motility system is present but latent in flagellates. During reversion the change from flagellate shape to amoeba shape occurs very rapidly and, at least sometimes, the amoeboid cells retain their flagella and quickly form the flagellate shape again. The reversion of flagellates to amoeba-shaped walking cells may or may not involve the resorption of flagella, just as the formation of flagella may or may not involve the shape changes, so the shape changes and the formation and resorption of flagella are at least partially independent. Ca^{+2} ions appear to be somehow involved in the formation of stable flagellate-shaped cells, and Mg^{+2} ions act as antagonists to Ca^{+2} in this.

The abrupt loss of the flagellate shape and simultaneous resumption of amoeboid motility during reversion is another shape change we would like to understand.

ψ: A CELL-PRODUCED FACTOR THAT CAUSES RAPID SHAPE CHANGES IN FLAGELLATES

Our interest in the shape changes was rekindled by the discovery of ψ and study of its effects. Since the ψ phenomenon has been described in detail (1), only a survey of the major results is presented here. Unsupported assertions are documented in Ref. 1.

Discovery of ψ. A chance observation led to the discovery of ψ. Some years ago I found that antisera against Naegleria flagellates induced a rapid reversion of flagellates to amoebae (6). When M. Fuller and I began to study this phenomenon, we performed a standard absorption to remove the reversion-inducing antibodies: an antiserum was incubated with a concentrated suspension of flagellates on ice for 2 hr. The supernatant of this incubation was tested on fresh flagellates, with the expectation that it would no longer induce reversion. However, the supernatant proved to be very active in inducing reversion. Upon analysis, we found that the antiserum was not relevant to the result. Instead, the concentrated flagellates, when incubated in buffer at low temperature, released a chemical substance(s) designated ψ. When this ψ-containing extract was added to a fresh sam-

ple of flagellate-shaped cells (at 25°C), it caused a dramatic series of changes in cell shape culminating in motile amoebae. This phenomenon seemed so interesting that we decided to study it. (The antiserum-induced reversion, also of interest, lies fallow as a consequence, awaiting further study.)

Reaction of flagellates to ψ . The reproducible sequence of shape changes that flagellates undergo in response to addition of ψ is outlined in Fig. 5. Upon addition of ψ, a flagellate rounds up, and herniations appear on its surface within 20 sec. Almost immediately, the disrupted surface begins to smooth out again and the cell elongates. A characteristic constriction appears, forming a wasp waist near the flagellar end (Fig. 1E). The constriction is maximal about 60 sec after the addition of ψ and disappears about 30 sec after it is formed, though the anterior end tends to remain tapered. As the constriction fades, the cell continues to elongate. Between 2 and 3 min after the addition of ψ, the cell forms one or more pseudopodia, often at the end of the cell opposite to the flagella, and walks away, dragging its flagella behind. The flagella are transiently paralyzed during the reaction to ψ , but then become active again. Gradually, most of the cells resorb their flagella and remain amoebae. About 10% of the cells, however, retain their flagella and regain the flagellate body shape.

During differentiation, the cells are sensitive to ψ only while they are flagellate shaped. Amoeba- or sphere-shaped cells, whether or not they have flagella, give no obvious morphological reaction to the addition of ψ. In addition, the proportion of flagellate-shaped cells that react to ψ is a function of ψ concentration, but the rate of the reaction is not markedly dependent on concentration. These observations suggest that there is something about flagellate-shaped cells that makes them responsive to ψ , and that a flagellate tends either to respond completely or not to respond at all — an all-or-nothing response.

The rapid shape changes produced by treatment of flagellates with ψ suggested the possibility that microfilament-containing structures might be involved. As a first test of this, we examined the effect of cytochalasin B, a drug that binds to actin as well as other

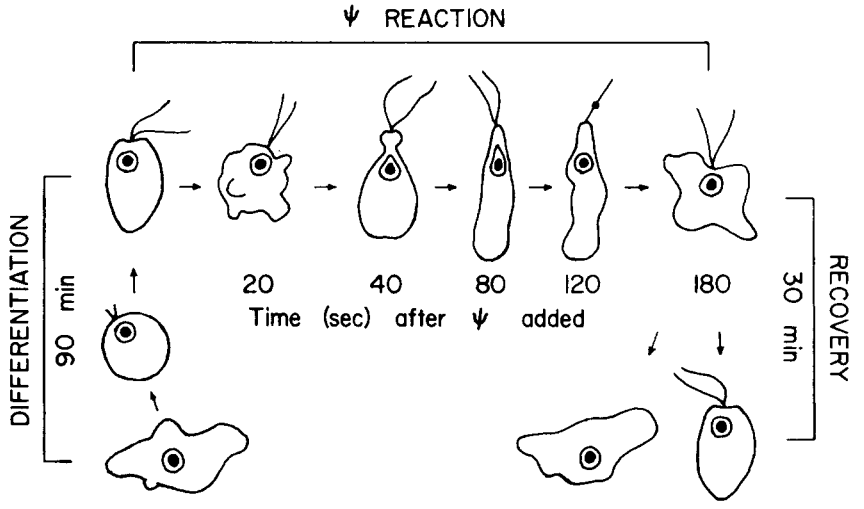


Fig. 5. Diagram of the sequence of shape changes in flagellates in response to addition of a ψ extract. For photographs, see Fig. 1E and Ref. 1.

cellular structures (e.g., 28), on the ψ reaction (5 and unpublished). Cytochalasin B, at 25–100 μg/ml, has no immediate effect on the structure of flagellates, nor does it prevent the loss of the flagellate shape in response to ψ. If cytochalasin B is added to flagellates and immediately followed by ψ, most of the flagellate-shaped cells become spheres. Cytochalasin B prevents the formation of the constriction in almost all cells. The inhibitor remains comparably effective if added as much as 20 sec after the addition of ψ. The external appearance of the constriction, and its sensitivity to cytochalasin B, suggest the possibility that the constriction might be analogous to the microfilament-containing contractile rings seen in dividing cells (reviewed in 29). This possibility remains to be explored.

Preparation, assay, and properties of ψ. We no longer prepare ψ extracts by simply incubating flagellates at 0–4°C. Instead we use the unusual procedure outlined in Table III, in which flagellates are "hard-boiled" (they retain their gross morphology) before allowing ψ to diffuse out. This procedure produces as active an extract as we have been able to obtain.

The amount of ψ activity in the extract is measured by preparing serial dilutions of the extract and assaying each dilution for its effect on flagellates. Usually we measure the percentage of cells that form "shmoos" – elongated cells with a tapered anterior end, with or without a constriction, comparable to the shapes outlined at 40, 80, and 120 sec in Fig. 5. No shmoo-shaped cells are seen in the absence of ψ. If sufficient ψ is added under optimal assay conditions, 75 to 100% of the flagellates form shmoos at the peak, which occurs between 90 and 120 sec after the addition of ψ. The proportion of flagellates affected by a ψ extract decreases rapidly with dilution. The results of an assay of ψ by dilution are shown in Fig. 9. The relative amount of ψ activity in various extracts is determined by comparing the dilutions required to produce a given percentage of shmoos.

The sensitivity of flagellates to ψ is inversely proportional to the population density at which they have differentiated. Often cells differentiated at high population density give no reaction to ψ. The cause of this has been traced to still another new factor, designated ν (unpublished observations). During differentiation, cells release ν into their environment, and in sufficient concentration ν somehow inhibits the reaction of flagellates to ψ. The ν-containing supernatant from dense cultures of untreated flagellates is effective in preventing the ψ reaction in dilute cultures, even if this supernatant is

TABLE III. Preparation of ψ Extracts

Flagellates at 1×10^7/ml in T buffer
↓
100°C, 5 min
↓
0°C, 2 hours
↓
Centrifuge*
Pellet — INACTIVE; Supernatant — ψ EXTRACT

*First a low-speed centrifugation to remove cell bodies and then 5 min at 17,000 *g* to obtain a clear supernatant.

added to flagellates just prior to the addition of ψ. Dilute cultures accumulate less ν and thus are more sensitive to ν. The ν factor, which is dialyzable and stable to boiling but otherwise awaits characterization, may be related to the factor mentioned earlier in the paper that increases the stability of flagellates in dense suspensions. Because of the existence of ν, ψ assays must be performed at a constant population density in order to obtain reproducible results.

The ψ factor itself is a small and thermostable molecule, partially purified and characterized but not yet identified. At present, all our knowledge about ψ must be gained by bioassay of ψ activity.

The ψ system. In addition to ψ, cells of Naegleria contain several other components that appear to be related to ψ (Table IV). The cells contain a macromolecular, thermolabile factor that gradually destroys ψ, which, for want of further knowledge, we designated ψase. This factor is released when flagellates are incubated on ice, and results in extracts in which ψ is unstable. Since boiling destroys ψase, we resorted to preparing ψ by the "hard-boiled flagellates" procedure of Table III. There is also a macromolecular thermolabile inhibitor of ψ activity, I_ψ. In the presence of sufficient I_ψ, flagellates are unaffected by ψ. The effect of I_ψ on ψ is reversible, in that boiling an inactive solution containing I_ψ and ψ results in the reappearance of ψ activity. Then there is the ν factor, already described. I_ψ differs from ν in size and thermolability; it differs from ψase in that it does not destroy ψ (and ψase does not, itself, inhibit the ψ reaction). In addition to these components, there also must be whatever enzymes are needed to produce ψ and ν. Each of these components is real, but since we know the other components only by their effect on the activity of ψ, we do not know whether these components are really functionally related in the cell. The putative existence of a "ψ system" suggests the possibility that such a system may play an important role in the life of Naegleria, perhaps analogous to the cyclic nucleotide systems in other cells (reviewed in 30) – although ψ is not 3′,5′-cyclic AMP or 3′, 5′-cyclic GMP!

Change in intracellular distribution of ψ during differentiation. We wished to determine whether ψ was present in amoebae, and whether the amount of ψ in cells changed during differentiation. The answers to these questions were found to vary depending on the procedure used to prepare ψ. For example, the "hard-boiled flagellates" procedure (Table III) yields comparable amounts of ψ activity from amoebae or flagellates. On a per-cell basis, amoebae yield at least 70% as much ψ activity as flagellates. Such results indicate that the amount of ψ does not change greatly during differentiation. However, some other methods of preparation yield much less ψ from amoebae than from

TABLE IV. The ψ System in Naegleria

ψ, a small, heat-stable molecule that causes rapid shape changes in flagellates (the ψ reaction);
ψase, a thermolabile macromolecule, postulated to be an enzyme, that irreversibly destroys the biological activity of ψ;
$I\psi$, a thermolabile, macromolecular inhibitor of the ψ reaction which does not destroy ψ; and
υ, a thermostable, low molecular weight inhibitor, excreted during differentiation that, like $I\psi$, masks the activity of ψ.

flagellates. For example, as mentioned earlier, flagellates release ψ if they are simply incubated at 0–4°C. This procedure yields virtually no ψ from amoebae. The procedure outlined in Table V yields about as much ψ activity from flagellates as does the procedure of Table III, yet only about 10% as much ψ activity from amoebae as from flagellates. Since the amount of ψ activity obtained from amoebae versus flagellates depends on the procedure used (Table VI), the extractibility of ψ somehow changes during differentiation.

In order to determine when the extractibility of ψ changes, we prepared ψ extracts at successive times during differentiation and compared their ψ activities. The results of one experiment are shown in Fig. 6. Differentiation was measured by counting fixed cells as usual. Samples removed from the differentiating culture were used to prepare ψ extracts by the method outlined in Table V and the amount of ψ in the extracts measured. As the results in Fig. 6 indicate, the amount of ψ activity obtained early in differentiation was about 10% of the final value. The amount of ψ activity in the extracts increased at about the time that cells formed flagella. The plateau value, shown by the line parallel to the abscissa, is uncertain because of the intrinsic imprecision of the ψ assay, but certainly the level remained relatively high until the cells had reverted to amoebae. The principle of this experiment was reproduced in several others; depending on the procedure used to

TABLE V. Another Procedure for Preparation of ψ Extracts

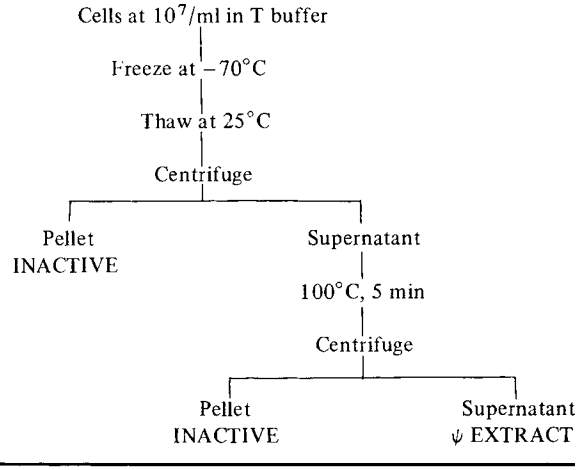


TABLE VI. Relative Amount of ψ Activity Obtained in Extracts of Amoebae and Flagellates*

Extraction procedure	Relative amount of ψ activity in extract of Amoebae	Flagellates
I. Table III	70%	100%
II. Table V	10%	100%
III. Incubate cell suspension at 0–4°C for 2 hr, test supernatant	0%	70%

*These estimates are based on repeated comparisons of extracts made using cells at the same concentration (usually about 10^7 cells/ml).

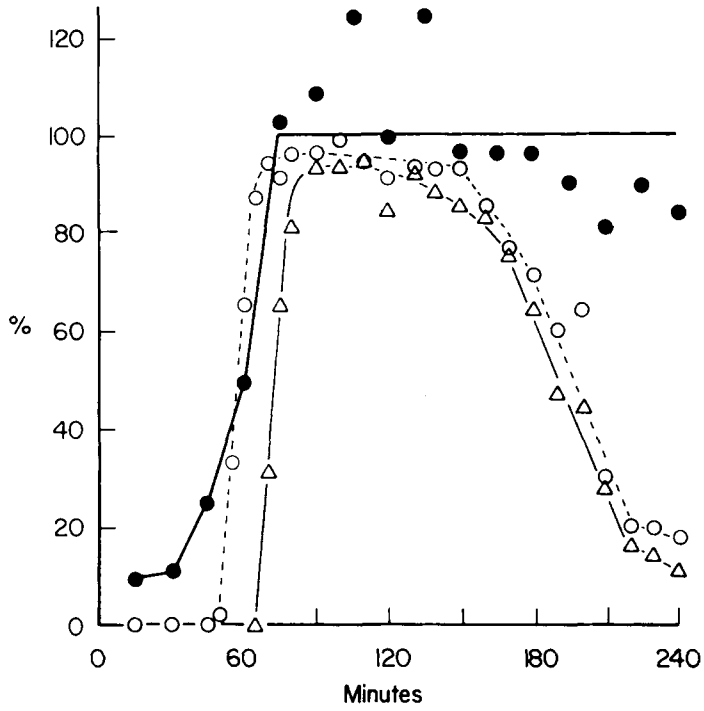


Fig. 6. The amount of ψ activity obtained from cells at various times during differentiation. Conditions for differentiation were similar to those described in the legend of Fig. 2; there were 9.1 × 10^6 cells/ml. At intervals samples of the culture were removed and immediately frozen to prepare a ψ extract using the procedure outlined in Table V. The resulting extracts were assayed for the amount of ψ activity using the dilution bioassay described in the text. The details of this experiment are described in Ref. 1. ○) cells with flagella; △) cells with flagellate body shape; •) amount of ψ activity in the extract prepared at the indicated time (as percentage of the average value of ψ activity at the plateau).

extract ψ, amoebae yielded from 0% to 70% the amount of ψ activity as did flagellates, and the apparent amount changed during differentiation at roughly the time cells formed flagella.

Since it is possible to isolate nearly as much ψ from amoebae as from flagellates, comparable amounts are present throughout differentiation. However, the extractibility of ψ changes. These results suggest an intracellular rearrangement of ψ, or some component of the ψ system, during differentiation, such that ψ becomes more extractible in flagellates. Nothing is known yet about the nature of this change in intracellular distribution, but it suggests that the ψ system may somehow be related to differentiation. It also allows the proposal, to be explored later in the paper, of a change in the "compartmentalization" of ψ during differentiation.

Normal role of ψ. The normal role of ψ in the life of Naegleria is unknown. Initially we thought ψ might somehow regulate the reversion of flagellates to amoebae, since it causes a kind of "reversion" when added to flagellates, but we have no direct evidence for this. In fact, extensive observations of the spontaneous reversion of living cells, or kinetic studies of normal reversion in fixed samples, have failed to reveal any of the shmoo shapes that form during the ψ reaction. With the finding that the intracellular distribution of ψ or some component of the ψ system changes during differentiation, we began to think that ψ might have a normal role during the differentiation process. One

possible function for ψ, which includes roles in differentiation and reversion, is explored below as part of the working hypothesis.

The ψ reaction itself is a laboratory phenomenon produced by external addition of ψ to flagellates. No detectable amounts of ψ activity are released from cells during differentiation or during reversion at 25°C. If any ψ is released, sufficient ν must also be released to mask the released ψ, or ψ must be destroyed by ψase as fast as it is excreted. Our tentative conclusion is that ψ is basically intracellular.

The concentration of ψ in cells is about 1,000-fold higher than that needed to produce the ψ reaction when flagellates are exposed to a ψ extract. This is based on a simple calculation. Cells that are 14–15 μm in diameter when rounded occupy 1.4–1.7% of the volume of a solution at 1×10^7 cells/ml. When their contents are extracted into that solution, the concentration of soluble components is reduced to 1/60–1/70 the original average intracellular concentration (if the components are compartmentalized or otherwise locally concentrated in the cells, the concentration in the extract is reduced even more). Yet ψ extracts prepared from cultures at 1×10^7 cells/ml are still very active against flagellates at 1/20 dilutions, and often show activity at dilutions as great as 1/80 (e.g., Fig. 9). At a 1/20 dilution of extract, the ψ in the extract is diluted 1/1200 to 1/1400 compared to the average intracellular concentration. It is remarkable that this small amount of added external ψ causes rapid shape changes in flagellates that themselves contain so much more intracellular ψ.

Conclusions. A small and heat-stable molecule extracted from cells of Naegleria, ψ, causes an orderly sequence of rapid shape changes in flagellates. These changes lead within 3 min to the resumption of amoeboid movement. One of the intermediate stages has a constriction that might be analogous to a contractile ring. Normally ψ is intracellular, and comparatively minute external concentrations are needed to produce the ψ reaction. In the cell ψ appears to be controlled by two macromolecules, one which destroys it and another which inhibits its activity, and by a small molecule excreted during differentiation which also inhibits its activity. The cells are sensitive to ψ only while they are flagellate shaped. During differentiation, the intracellular distribution of ψ or some component of the ψ system changes in a programmed way that suggests the possibility of compartmentalization. Cells of Naegleria thus change the intracellular distribution of ψ during differentiation, and develop the potential for giving a morphological reaction to ψ only as flagellates, suggesting the possibility that ψ may have a role during differentiation and/or reversion. The challenge for the present study is to understand the normal function of ψ, as well as the basis for the shape changes it produces when added to flagellates.

THE RESPONSE OF FLAGELLATES TO ψ IS TRANSIENT

Flagellates respond to ψ very quickly – within 25 sec after the addition of sufficient ψ all flagellate shapes are gone (1). As mentioned earlier, the rate of the morphological changes in cells that respond is relatively independent of the concentration of ψ, as if ψ either triggers a response or does not. What is the general nature of the reaction? Do flagellates react to individual molecules of exogenous ψ, or are a large number per cell required to produce a reaction? Do cells take up or destroy exogenous ψ? Do cells adapt to or otherwise become desensitized to ψ in its presence? These and many similar questions can be answered better when the identity of ψ is known, but some first results based on ψ activity are instructive.

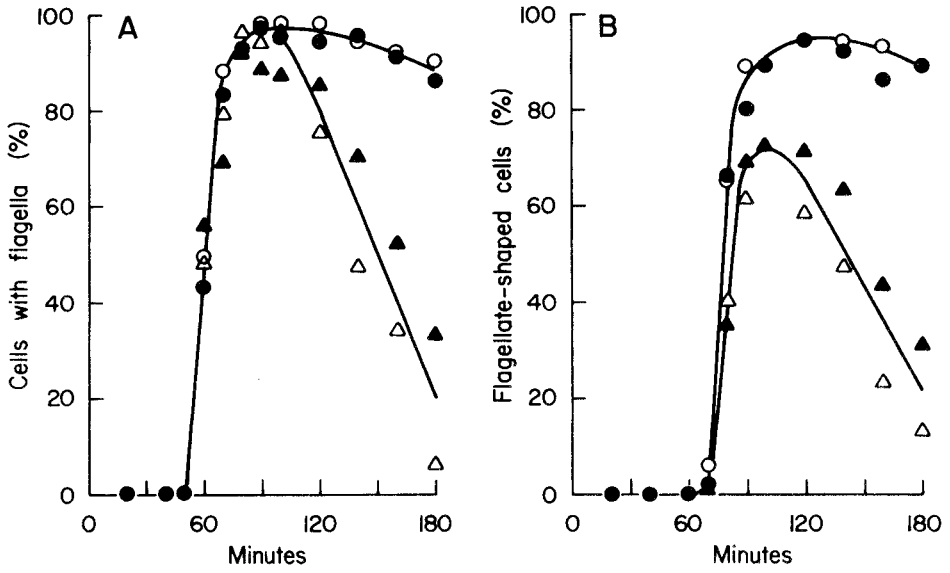


Fig. 7. Effects of cell population density and of ψ on differentiation: A) cells with flagella and B) flagellate-shaped cells. Population density: △, ▲) 2×10^5 cells/ml; ○, ●) 2×10^7 cells/ml. Addition of ψ: △,○) no addition; ▲, ●) ψ extract at 1/10 dilution. Amoebae were suspended in T buffer at time zero, washed as described in the legend of Fig. 2, and incubated at 25°C. Cell number was determined, and at 20 min the cells were inoculated into flasks containing T buffer with or without ψ extract, and incubation continued at 25°C. The ψ extract was prepared according to the protocol of Table III. Samples fixed in Lugol's iodine were counted, independently, for cells with flagella and for flagellate body shape. Where symbols are missing on the graph, the points for the open symbols were superimposed on the filled symbols. The T_{50} for cells with flagella was 61 min; for flagellate-shaped cells it was 78 min at high cell density and 83 min at low cell density.

We wished to know whether exogenous ψ would affect differentiation. A sample experiment to test this is shown in Fig. 7. Cells were allowed to differentiate at low cell population density (2×10^5 cells/ml) and at high population density (2×10^7 cells/ml) in the absence or presence of a 1/10 dilution of an active ψ extract (the same extract, assayed in a separate experiment, gave the results in Fig. 9). Cell population density had a profound effect on differentiation, as already described. Cells at high population density formed more stable flagellates, with a higher percentage of flagellate-shaped cells, than did cells at low population density. But ψ, present throughout differentiation, had little if any effect on the process. If anything, flagellates at the low population density were slightly more stable in the presence of the ψ extract. This slight difference is probably not of significance, since ions added to the differentiation environment have more pronounced effects on the stability of flagellates (e.g., Ca^{+2} in Fig. 3). Present throughout differentiation, ψ certainly does not have an adverse effect on differentiation or on the stability of flagellates. In other experiments, if ψ was added to differentiating cells at any time prior to the formation of flagellate-shaped cells, it was without significant effect on the course of differentiation.

One might imagine that ψ has no effect on differentiation because it is consumed or destroyed by the cells. To ascertain this, the supernatants at the end of differentiation were tested for ψ activity (Fig. 8). The result is clear: ψ is still present in the supernatants

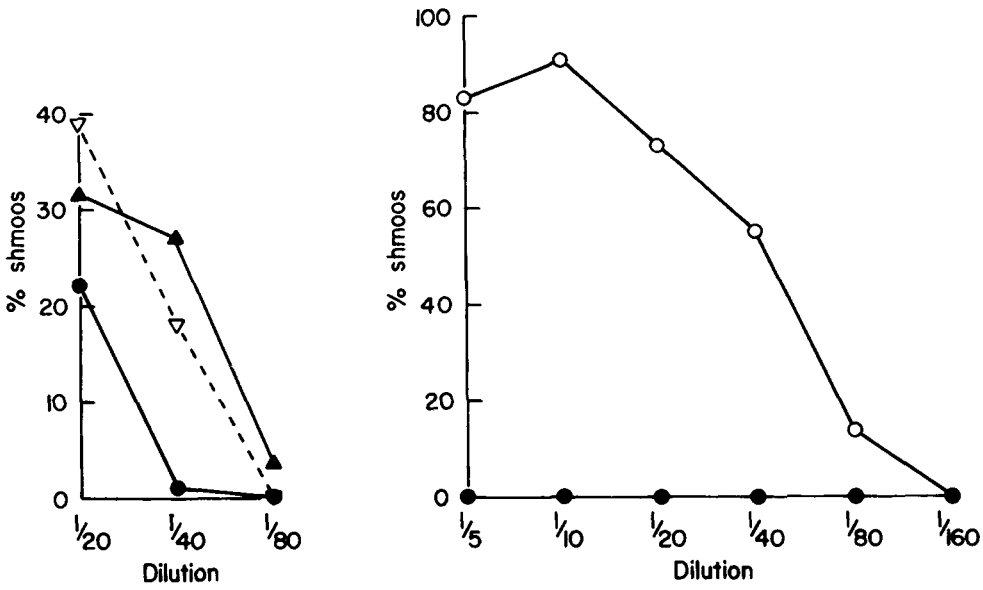


Fig. 8 (left). ψ added to the environment of cells remains there throughout differentiation. At 180 min in the experiment of Fig. 7 the cultures were centrifuged (first at low speed and then at 17,000g), and the cell-free supernatants were tested for ψ activity. While the extracts were being prepared, a second batch of cells was differentiated for the assay (at 2×10^5 cells/0.5 ml in 18 X 150 mm tubes shaken at 25°C). The ψ extract was already diluted 1/10 for the experiment, so a further 1/2 dilution gave a concentration of 1/20, the maximum that could be tested. Because preparation of the extract took longer than I anticipated, the assay cells were already reverting to amoebae by the time of the assay, and only 41-52% of the untreated assay cells were flagellate shaped when they were used (at 130 min; cf. low population density in Fig. 7). Samples of 0.5 ml of serial dilutions of culture supernatant or ψ extract were added to 0.5 ml of assay cells, and samples were fixed for counting 90 sec later (see Ref. 1). The supernatants of cultures that differentiated without added ψ (△ and ○ of Fig. 7) gave 0% shmoos, and the cells remained flagellate shaped, equivalent to buffer controls (not shown). In contrast, dilutions of the supernatant of cells that differentiated in the presence of ψ at low population density (▲) retained ψ activity comparable to the amount of ψ in the original extract at the same concentration (▽). Dilutions of the supernatant of cells differentiated in the presence of ψ at high population density had less ψ activity, but still a measurable amount (●).

Fig. 9 (right). Exposure to ψ throughout differentiation desensitizes flagellates to ψ. Flagellates were allowed to differentiate at 2.5×10^5 cells/ml, 0.8 ml per 18 X 150 mm tube, shaken at 25°C. One set of tubes served as a control (○); the other set contained a ψ extract at 1/10 dilution (●). Between 115 and 120 min, 0.2 ml samples of ψ extract or dilutions were added to the tubes with immediate mixing, and each tube fixed 90 sec later. The ψ extract used was the one used in conditioning the cells, and the same dilution series was used for both sets of tubes. At the time of the assay, untreated tubes in the control and experimental series had 87% and 89% flagellate-shaped cells, respectively. In the control series ψ abolished the flagellate shape and produced shmoos, whereas in the experimental series the flagellate shapes remained (84–96% in the dilution series), and no shmoos were formed.

of the cultures to which it was added. Virtually all the added ψ activity was recovered from the supernatant of the culture at low population density. Less was recovered from the supernatant of the culture at high population density, so perhaps some of the ψ was consumed, masked (ν), or destroyed (ψase) by these dense cell populations. However, in

several experiments, including some with sampling at intermediate times from as little as 3 min to as much as 160 min after addition of ψ, much or all of the added ψ was still present in the buffer. This indicates that ψ is not avidly removed from solution by the cells, and also that no substantial quantities of ψase are secreted by or on the exterior of the cells. Most important, it indicates that differentiation is insensitive to the continual presence of exogenous ψ for some reason other than its consumption or destruction. The results suggest the possibility that cells in the presence of ψ somehow adapt to, and become desensitized to, the external ψ.

The nature of the adaptation to external ψ can be approached another way. Are cells that differentiated in the presence of ψ less sensitive to added ψ when they are flagellates? Cells were allowed to differentiate in the absence or presence of ψ under the standard assay conditions (described in Ref. 1), and then the ψ reaction was assayed. In the sample experiment (Fig. 9), about 96% of the cells had flagella and 84–96% were flagellate shaped. The addition of ψ produced a good ψ reaction in the cells that differentiated in the absence of external ψ, but produced no reaction in those cells that had been exposed to ψ throughout differentiation. These latter cells remained flagellate shaped, as if no ψ had been added during the assay, even though excess ψ was added (the cells were differentiated in a 1/10 dilution of extract; during the assay the highest concentration was a 1/5 dilution, or a twofold excess). Such experiments make it clear that the sensitivity to external ψ is dependent on the cells' history of exposure to external ψ. In the presence of ψ, cells adapt to it and become desensitized. This desensitization is quite pronounced.

Experiments are in progress in an effort to determine how rapidly cells become insensitive to external ψ. This question has not yet been answered, but one consistently reproducible and striking experimental result is that the number of flagellates that react to added ψ is affected not only by the final concentration of the ψ extract after dilution, but also by the concentration at the moment of addition. A sample experiment is presented in Table VII. When ψ was prediluted, and then final 1/5 dilutions were made into the assay cells (the usual assay procedure, Ref. 1), this extract gave shmoos to a final dilution of 1/40. However, if the same extract was added undiluted, adjusting the volume added so the final dilutions were the same, shmoos were still found at a final dilution of 1/80. The extract appeared to be approximately twice as active when added without

TABLE VII. Influence of Transient ψ Concentration on the ψ Reaction*

	Final dilution of extract			
Dilution procedure	1/20	1/40	1/80	1/160
	% shmoos			
I (extract prediluted)	64	16	0	0
II (extract undiluted)	64	47	22	2

*A 1/4 dilution of ψ extract was added to flagellates using one of two procedures. In procedure I, the 1/4 dilution was serially diluted by successive 1:1 dilutions, and 0.2-ml aliquots were added to 0.8 ml of flagellates. In procedure II, volumes of the 1/4 dilution were added directly to flagellates; T buffer was previously added so all final cell volumes were 1.0 ml. In both procedures the assay cells were equivalent (controls had 82–87% flagellate-shaped cells) and the final concentrations of cells (2×10^5 cells/ml) and of ψ were the same. Samples were mixed immediately after the addition of extract (probably mixing was complete within one second), and were fixed after 90 sec.

prior dilution as it did when prediluted, even though the same final concentrations of ψ and cells are attained quickly in both. What does this reproducible phenomenon mean? It indicates that the proportion of cells that respond to ψ is not a simple function of the final concentration of ψ. Some cells appear to be affected, or "triggered," even before the final dilution of ψ is reached (probably mixing is complete within a second or two after addition). One possible interpretation of this result requires two assertions: 1) that more than one molecule of ψ per cell is required for the ψ reaction, and 2) that each cell must receive enough molecules to cause a response within a very short time interval, since very soon after a cell encounters a ψ molecule the cell becomes desensitized to it. The first of these assertions seems quite definite, since if one ψ molecule/cell were sufficient to induce a reaction, the number of flagellates that respond to ψ should be directly proportional to concentration and should not be affected by the concentration at the time of addition. This experiment is provocative for the first insights it provides, and the nature of the reaction of cells to ψ clearly warrants a thoughtful experimental analysis.

Conclusions. Exogenous ψ does not affect differentiation even though it remains present throughout the process. Flagellate-shaped cells that are conditioned to the presence of ψ in their environment become desensitized and give no reaction to additional exogenous ψ. All the experiments indicate that the sensitivity of flagellates to added external ψ is transient. Some preliminary experiments suggest that flagellates may become desensitized to external ψ within seconds after its addition. These experiments also indicate that more than one moleucle of ψ is required to induce the ψ reaction, and suggest that upon addition of ψ the cells either respond immediately or they rapidly become insensitive to the exogenous ψ. This all-or-nothing triggered response is reminiscent of the responses of neural cells to stimuli, and provides a challenge for further study.

ROLE OF CALCIUM IONS IN THE ψ REACTION

Because of the involvement of Ca^{+2} in amoeboid movement, contractile-ring formation, and contractility in general, we wondered if Ca^{+2} might have something to do with the ψ reaction, and in particular if ψ might cause the reaction by inducing Ca^{+2} release or uptake.

EGTA, which selectively chelates Ca^{+2} ions (31), completely inhibits the ψ reaction at concentrations $> 10^{-5}$ M (Fig. 10). In these short-term experiments, the flagellates that do not react to ψ in the presence of EGTA remain flagellate shaped. The simultaneous addition of equimolar Ca^{+2} reversed the inhibition by EGTA (Fig. 10), indicating that EGTA prevents the ψ reaction by sequestering Ca^{+2} and not by some other action. In several experiments, including the one shown in Fig. 10, EGTA at $< 10^{-5}$ M actually increased the % shmoos over the control value, suggesting that there is an optimal concentration of external Ca^{+2} for the ψ reaction. Such experiments indicate that external Ca^{+2} ions are required for the ψ reaction.

Since external Ca^{+2} is required, it is possible that exogenous ψ causes the ψ reaction by inducing an influx of Ca^{+2} into the cells. If this were so, it seemed possible that the ionophore A23187, which can transport Ca^{+2} through cell membranes (32), might induce a reaction similar to that produced by ψ. Indeed, the ionophore does produce a reaction comparable to ψ (Table VIII). It is important to emphasize that although A23187 reproducibly induces a "ψ reaction," the % schmoos obtained is not as reproducible as it is with the reaction to ψ extracts. It should also be noted that although EGTA inhibits the reaction to ψ (Fig. 10), it does not completely prevent the reaction to

TABLE VIII. Ionophore A23187 Mimics ψ*

$CaCl_2$ mM	EGTA mM	A23187 μM	Shmoos %
0	0	0	0
0	1	0	0
1	0	0	0
0	0	4	36
0	1	4	8
1	0	4	69

*EGTA and $CaCl_2$ were added to flagellates at 25°C and 1.7×10^6 cells/ml. Immediately thereafter the ionophore A23187 (Eli Lilly and Co.) was added and the cells were fixed 120 sec later.

A23187 (Table VIII). This suggests the possibility that the ionophore may cause a reaction either by facilitating an influx of Ca^{+2} from the environment or by releasing Ca^{+2} from intracellular storage depots. Schroeder and Strickland (33) found that ionophore A23187 induces rapid cortical contractions in frog eggs, and this reaction is independent of extracellular calcium. It is my impression that ψ and A23187 act somewhat differently to produce shmoos, but this requires more precise definition. Experiments also need to be done to determine whether ψ acts as a calcium ionophore to produce the ψ reaction. The fact that ionophore A23187 can mimic ψ in producing this very specific reaction supports the conclusion that ψ acts by somehow affecting the movement of Ca^{+2}.

Conclusions. Trace concentrations of extracellular Ca^{+2} ions are required for the ψ reaction. The concentration normally found in T buffer (2 mM Tris-HCl prepared in demineralized water) containing flagellates at about 0.04% of the medium volume is sufficient; that concentration is almost certainly less than 10^{-6} M. The calcium ionophore A23187 can mimic the rapid shape changes produced by ψ , including the formation of shmoos. These observations suggest that ψ acts by somehow affecting the movement of Ca^{+2}, either into the cell or intracellularly.

REPERTORY OF SHAPE CHANGES

The studies that the ψ reaction has provoked, as well as other observations, have indicated that flagellates have a repertory of rapid, reversible shape changes (Fig. 11). The changes characteristic of the ψ reaction have been described (Fig. 5). Starting with flagellate-shaped cells, each of the other changes indicated by the arrows has been observed to occur rapidly. In many cases, entire populations can be induced to change from one shape to another within 20 sec after treatment. Some of these rapid changes have been observed to occur spontaneously, as in the "touchdowns" recorded in the time-lapse movie (Table II). Many of the others can be induced by an appropriate combination of ψ, Ca^{+2}, Mg^{+2}, and/or EGTA. This repertory must be considered in any explanation of the shape changes in Naegleria.

One shape-change sequence is worth describing in detail since it gives some insight into the ψ reaction. If flagellates are treated with 10^{-3} M $MgCl_2$ and also 3×10^{-6} M EGTA (which is insufficient to inhibit the ψ reaction) for about 10 min, they lose their flagellate shape, but not their flagella, and round up to smooth spheres. This rounding

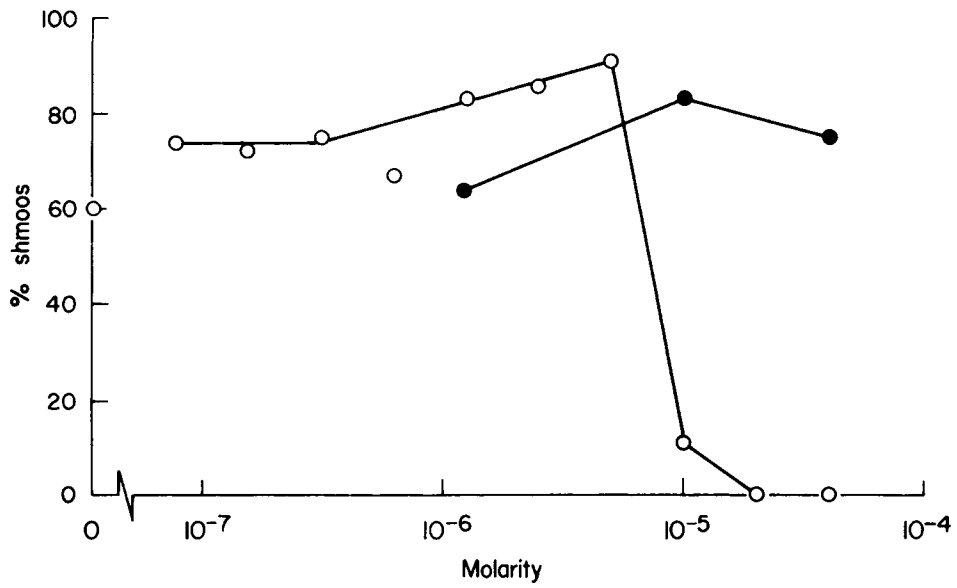


Fig. 10. Effect of the Ca^{+2} chelator ethyleneglycol-bis (β-aminoethyl ether) N,N'-tetraacetic acid (EGTA) on the ψ reaction. Cells were differentiated to 85% flagellate-shaped cells at 25°C and 3 × 10^5 cells/0.8 ml. Just prior to the time of the assay, EGTA, and in some cases also $CaCl_2$, were added to the indicated molarity. Then 0.2 ml of an active ψ extract was added (final dilution 1/5), and each tube fixed 90 sec after the addition of ψ. ○) EGTA; ●) EGTA + equimolar $CaCl_2$.

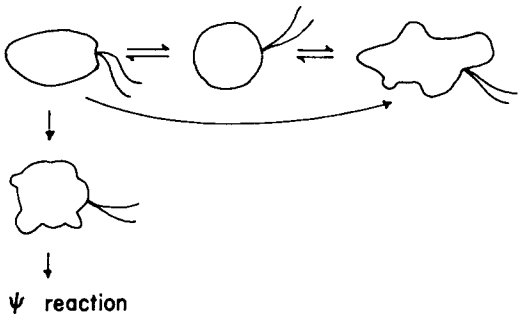


Fig. 11. Repertory of rapid shape changes, starting with flagellate-shaped cells. The ψ reaction is shown in Fig. 5. The other changes have been observed to occur without treatment, and entire populations can be induced to change from one shape to another by experimental treatments, including appropriate combinations of ψ, Ca^{+2}, Mg^{+2}, and EGTA.

up, which is perhaps related to the Mg^{+2} effect, is prevented by 10^{-4} M Ca^{+2}. What is of interest is that if the cells that have rounded up in the Mg^{+2} and EGTA are then treated with ψ, the entire population of spheres changes directly to amoebae within 60 sec. This phenomenon reveals two things about the ψ reaction. First, ψ induces these treated cells to become motile amoebae without any of the unusual intermediate shapes, the shmoos, that are characteristic of the ψ reaction. This direct conversion to amoebae is morphologically analogous to what happens in normal reversion. Second, in this experimental situation, it is sphere-shaped rather than flagellate-shaped cells that react to ψ.

During normal differentiation, as mentioned earlier, only flagellate-shaped cells react to ψ (1). The fact that these Mg-EGTA-produced spheres can react to ψ indicates that the flagellate shape itself is not necessary for a reaction to ψ . It is not known whether, in this situation, the spheres go directly to amoebae because of the ionic environment or because of their cellular architecture at the time of treatment with ψ.

Conclusions. Flagellates, once they have formed, can go through a number of rapid changes in shape and motility. They can, for example, round up to spheres, or become amoebae with flagella, and then go back, through a sphere stage, rapidly to flagellate-shaped cells again. These rapid shape changes indicate that although the streamlined flagellate shape is very regular in its contour, and takes time to construct during differentiation, once it is formed, it can be taken apart (to the point of motile amoebae) and then rapidly reformed again. Experimental manipulation of the shapes has revealed, in particular, that it is possible to create conditions where cells that are not flagellate shaped will give a reaction to ψ, and become amoebae without passing through the bizarre intermediate forms characteristic of the ψ reaction but not of normal reversion of flagellates to amoebae.

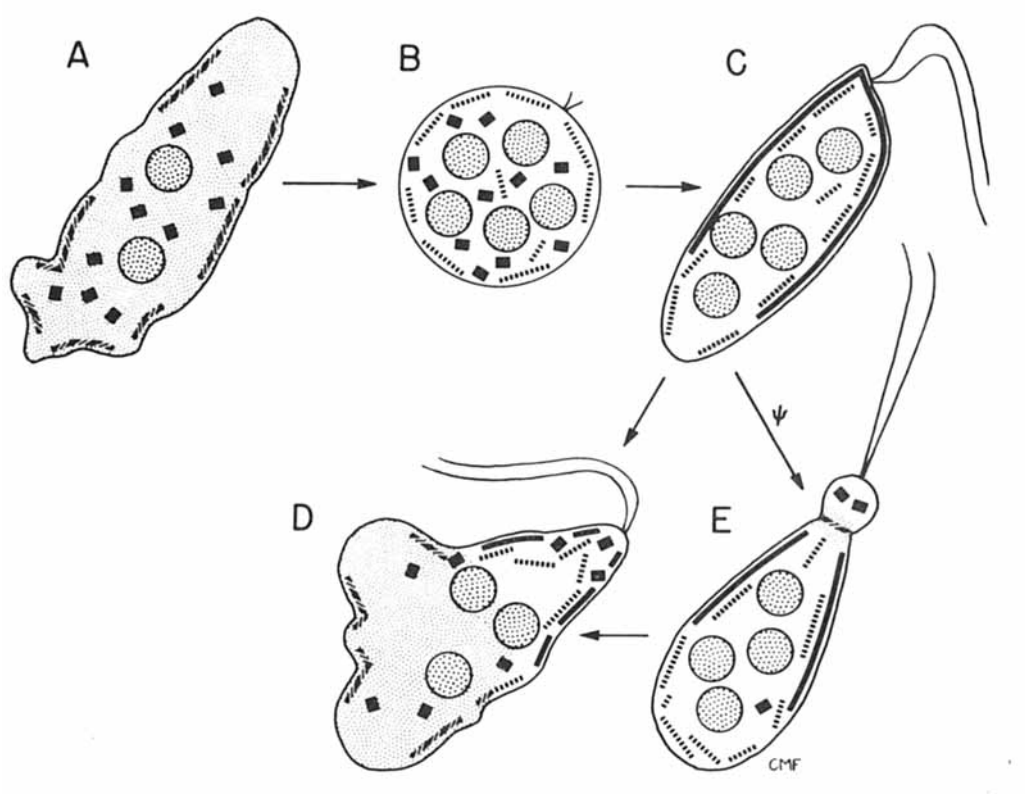
WORKING HYPOTHESIS

The experiments that have been described led to and support a working hypothesis to explain the role of ψ in regulating cell shape and motility in Naegleria. This hypothesis was first glimpsed by M. Fuller and me in September 1974.

Cell shape and motility are determined both by general factors – the intracellular availability of the components of motility and cytoskeletal systems, and intracellular environments that favor their functioning – and by the localized orchestration of these factors that causes a cell to have a specific shape and motility. Our hypothesis is concerned mainly with the general factors. All I have to do is to watch living Naegleria – whether walking amoebae as they glide along and then suddenly erupt a pseudopodium in a new direction, or streamlined flagellates as they swim gracefully, or the reproducible contortions of the same cells as they go rapidly through the ψ reaction – to realize what a mystery the orchestration is. Only 2 years ago the general factors were a mystery to me too.

Role of Ca^{+2}, actin-based and tubulin-based systems in the shape changes. Four basic elements are postulated in the hypothesis: intracellular Ca^{+2}, an actin-based amoeboid motility system, a tubulin-based cytoskeleton, and ψ. I will consider the first three and then ψ. This portion of the hypothesis is diagrammed in Fig. 12. In drawing this figure, I had to make many decisions which indicate areas of uncertainty, even within the framework of the hypothesis. For example, does the tubulin that is later assembled into cortical microtubules preexist in a cytoplasmic pool in amoebae, or is it, like flagellar tubulin, synthesized de novo during differentiation (34, 35)? The choice in that case was to postulate that the tubulin for cortical microtubules preexists. None of these small decisions affects the overall hypothesis, but they do raise questions for future experiments.

According to the hypothesis, intracellular free Ca^{+2} is constantly swept up into reservoirs. Motility of amoebae – the functioning of the actin-based mechanochemical system required for amoeboid movement – depends on local release of Ca^{+2} from these reservoirs. In the diagram (Fig. 12A), free Ca^{+2} is shown throughout the cytoplasm of the amoeba. It is recognized that for the system to work there must be local concentrations of Ca^{+2}. The shading in the diagram indicates the presence, or availability, in



	ALTERNATIVE STATES			
COMPONENT		Ameba		Flagellate
Free Ca^{+2}		Available		In reservoirs
Ameboid motility system		Functioning		Latent
Cortical microtubules		Disassembled		Assembled

Fig. 12. Regulation of cell shape in Naegleria: A hypothesis. The postulated role of ψ in controlling the shape changes by regulating intracellular free Ca^{+2} is not shown in this figure, but is described in Table IX

some parts of the cell, of sufficient free Ca^{+2} to allow the amoeboid motility system to function.

During differentiation, the free Ca^{+2} is swept up into reservoirs and not released, and as a consequence the free Ca^{+2} in the cytoplasm drops below the threshold concentration needed for functioning of the actin-based system, amoeboid movement ceases, and the cell rounds up (Fig. 12B). Subsequently, in a process that depends on continual low free Ca^{+2}, the microtubular cytoskeleton is assembled, and the flagellate shape is

formed (Fig. 12C). Sudden release of free Ca^{+2} causes disassembly of the flagellate cytoskeleton and immediate resumption of amoeboid movement (Fig. 12D).

None of the elements of this part of the hypothesis is itself original. That intracellular free Ca^{+2} is constantly sequestered into reservoirs has been shown in many types of cells (e.g., 36–38). That free Ca^{+2} is essential for amoeboid movement was suggested as early as 1926 (39), and that amoeboid movement depends on intracellular Ca^{+2} release was discussed extensively by L. V. Heilbrunn in the 1930s (e.g., 40). This notion has been refined (e.g., 12–15, 41) and is being explored experimentally in several laboratories (42–44). That amoeboid movement depends on an actin-based mechanochemical system is generally assumed, if not proved (e.g., 12–17, 42–45). The role of cortical microtubules in providing a cytoskeleton has been considered by many and is supported by much evidence (e.g., 19–21, 45, 46). That free Ca^{+2} can prevent the assembly of tubulin into microtubules and cause the disassembly of microtubules has been known since 1972 (47); since then many have discussed the idea that low free Ca^{+2} is necessary for assembly and maintenance of cytoplasmic microtubules (see below). The antagonistic role of Ca^{+2} in controlling motility versus cytoskeleton has been pointed out by others (48, 49). The originality in the hypothesis is the integration of these ideas to explain the general basis of the shape changes (Fig. 12).

Role of ψ. The completely original element of the hypothesis is ψ. ψ is postulated to regulate intracellular calcium release from the reservoirs, making free Ca^{+2} available in the cytoplasm when and where it is needed. This interpretation is supported by considerable evidence, though not as yet by any direct proof. Before discussing the postulated function of ψ, it is worth reviewing some of the main experimental results that must be explained in any hypothesis about the role of ψ.

1. Normally ψ is intracellular, so whatever normal function it has must take place inside the cell.

2. The characteristic ψ reaction is induced by a concentration of ψ that is minuscule when compared to the concentration inside the cells.

3. During differentiation ψ, or some component of the ψ system, is rearranged, as shown by the change in extractibility of ψ. This suggests that ψ has some function in relation to the differentiation process.

4. Some of the rapid morphological changes during the ψ reaction, especially the formation of an ephemeral constriction in the shmoos, are suggestive of contractile processes that involve actin-based mechanochemical systems, and these are thought to involve Ca^{+2}.

5. The possibility that the ψ reaction might involve a movement of Ca^{+2} is supported by the finding that the ψ reaction is dependent on extracellular Ca^{+2}.

6. Stronger support for the idea that ψ might control the movement of Ca^{+2} comes from the observation that ionophore A23187 can mimic ψ in causing the "ψ reaction."

7. The response of cells to ψ is transient, suggesting that when ψ is present it produces an effect for only a brief time and then is ineffective.

These experimental results lead to the general conclusions that ψ normally has some intracellular function(s), that when added externally ψ affects cell shape and contractility, that this external effect of ψ involves Ca^{+2}, and that a calcium ionophore can produce changes in cell shape and contractility that mimic those produced by ψ. It seems a reasonable extrapolation of these results to suggest that ψ regulates intracellular Ca^{+2} release.

This postulated function of ψ is quite successful in explaining the general basis of the cell-shape changes. This part of the hypothesis is not shown in the diagram (Fig. 12), but Table IX integrates ψ into the overall working hypothesis.

Motility of amoebae depends on local release of Ca^{+2}; this in turn is caused by intracellular release of ψ. We know nothing about the architecture of this system, but might conjecture that amoebae contain little packets of ψ. When one of these packets releases ψ, this causes a transient local release of Ca^{+2} from nearby reservoirs (their nature is also not specified; mitochondria, membranes, and specialized vesicles are obvious possibilities). Within a few moments the Ca^{+2} release stops, perhaps because the response to ψ is transient or perhaps because of the effects of other elements of the ψ system which are postulated to somehow regulate the functioning of ψ. The released Ca^{+2} causes a local functioning of the amoeboid motility system (here possibilies range from assembly of F-actin microfilaments to contraction and relaxation). Free Ca^{+2} stays relatively localized (38, 50, 51) and is gradually swept up into reservoirs again. At the same time, the released ψ is destroyed or repackaged. Continued functioning of the ψ-Ca^{+2} system, locally orchestrated, results in ordered amoeboid movement.

During differentiation, one programmed event is postulated to be the "compartmentalization" of ψ. The nature of this compartmentalization is quite unknown. As ψ is compartmentalized, Ca^{+2} release is blocked and the free Ca^{+2} gradually declines throughout the cytoplasm to below the threshold concentration necessary for functioning of the amoeboid motility system. As a consequence, the amoebae round up to spheres. It is conceivable, but entirely speculative at present, that this programmed "compartmentalization" requires the synthesis of new RNA and protein molecules. This could explain why the rounding up during differentiation requires prior transcription and translation.

TABLE IX. Regulation of Cell Shape and Motility in Naegleria: Summary of Hypothesis

Intracellular free Ca^{+2} is constantly swept up into "reservoirs"

Shape	Basis of shape	Intracellular free Ca^{+2}	ψ system	Local release of free Ca^{+2}
Amoeba ↓	Move using an actin-based mechanochemical system	Essential for amoeboid movement	Regulates intracellular Ca^{+2} release	+
Sphere ↓	The amoeboid motility system ceases to function, so cells round up	Limiting	Compartmentalized. Therefore intracellular free Ca^{+2} is swept up and not released	–
Flagellate ↓	Assumed fixed shape by assembling a tubulin-based cytoskeleton	Must be low for assembly of microtubules	Continues to be compartmentalized	–
Amoeba	Disassembly of microtubules and functioning of amoeboid motility system	Causes disassembly; allows movement	Released from compartments	+

ψReaction: External ψ causes a transient influx of Ca^{+2}. This disrupts cytoskeleton of flagellates and allows functioning of the actin-based mechanochemical system in the anterior region, producing shmoos with constriction. If this is followed by internal release of ψ, the shmoos become amoebae. Otherwise the Ca^{+2} is swept up and the flagellate shape returns.

Other events during differentiation, perhaps requiring the synthesis of new proteins, prepare the cell to assemble the flagellate cytoskeleton (which must include more than simply microtubules to give the flagellate its specific shape). Assembly of this tubulin-based cytoskeleton depends on continued low levels of intracellular free Ca^{+2}, which in turn depends on continued compartmentalization of ψ.

In normal reversion of flagellates to amoebae, release of ψ from its compartments permits release of free Ca^{+2}, which causes disassembly of the tubulin-based cytoskeleton and permits resumed functioning of the actin-based motility system. The amoeboid motility system is latent in the flagellate, all components ready to function but idle for want of free Ca^{+2}.

The treatment of flagellates with external ψ causes a transient influx of Ca^{+2} from the medium bathing the cells into the cortical cytoplasm. (Normally any influx of Ca^{+2} is balanced by efflux; see discussion in Ref. 38.) The increase in free Ca^{+2} in the cortical cytoplasm causes a momentary, partial disruption of the flagellate cytoskeleton, and the cells round up to bumpy spheres (bumpy, perhaps because the surface area/volume ratio is unbalanced). The major influx of Ca^{+2} occurs, for some reason, near the anterior end of the flagellates, and this influx is sufficient to cause the localized functioning of the actin-based mechanochemical system, which takes the form of a contractile ring (Fig. 12E). [Others have postulated a similar role for Ca^{+2} in the formation of contractile rings (29, 52, 53).] The Ca^{+2} that enters the cell is promptly swept up, the contractile ring fades, and some microtubules reassemble resulting in elongation of the cell. However, as a consequence of the initial perturbation, some of the intracellular ψ becomes decompartmentalized, the flagellate shape is disrupted, and amoeboid movement is resumed. In most of the cells amoeboid movement continues, but since the components for building the flagellate cytoskeleton and for compartmentalizing ψ are still present, some cells put ψ away, rebuild the cytoskeleton, and become flagellate shaped again.

The rapid, reversible shape changes in flagellates are readily explained using the hypothesis and similar arguments.

The Mg^{+2} effect requires an explanation. Mg^{+2} makes flagellates unstable and prevents formation of the flagellate shape; Ca^{+2} reverses these effects and makes flagellates somewhat more stable than they are in the absence of extracellular Ca^{+2}. At first sight this is the opposite of what is predicted by the hypothesis. Several explanations of this could be advanced, but one will serve to indicate the many possibilities. Ca^{+2} is normally bound to membranes and stabilizes them. Extracellular Ca^{+2} increases the proportion of Ca^{+2} among the cations bound to the membrane (54) but has little effect on the intracellular concentration of Ca^{+2} (38, 55). As long as there is abundant Ca^{+2} bound to the membrane, the system is relatively stable. If Ca^{+2} is removed from the membrane – e.g., displaced by a large excess of Mg^{+2} [which is a poor substitute for Ca^{+2} (54)] – the cell attempts to replace the membrane-bound Ca^{+2} by releasing Ca^{+2} from its reservoirs. As a result, the concentration of free Ca^{+2} in the cortical cytoplasm increases, and this increase is sufficient to prevent the formation of the flagellate shape and to cause instability of flagellates. This explanation is very specific and may well be incorrect. The point is that although the Mg^{+2} effect does not support the hypothesis, it is in no way inconsistent with it either.

As far as I have been able to determine, the hypothesis is permitted by all of our observations as well as by what is known about Ca^{+2}, motility, and shape in the literature. I have been able to find two possible difficulties.

First, the level of Ca^{+2} needed to prevent the assembly of, or cause the disassembly of, brain microtubules in vitro may be greater than the usual intracellular concentration of Ca^{+2}. Weisenberg (47) originally reported that 6 μm Ca^{+2} inhibited assembly, but Borisy, Olmsted, and their co-workers (56) found that under the conditions they used concentrations of Ca^{+2} in the mM range were needed to inhibit assembly. Many estimates of the intracellular concentration of free Ca^{+2} indicate that the cytoplasm contains between about 10^{-8} and 10^{-5} M (e.g., 38, 55). Thus a requirement for 10^{-3} M Ca^{+2} to inhibit microtubule polymerization would be of questionable physiological significance. Recently it has been found that the in vitro polymerization of microtubules is much more sensitive to Ca^{+2} in the presence of Mg^{+2} ions (57, 58). For example, in the presence of 5 mM Mg^{+2}, Ca^{+2} inhibits polymerization at concentrations above 10^{-5} M (57). Since cells contain free Mg^{+2} at about 2–4 mM (59, 60), as well as other ions, it remains a possibility that Ca^{+2} sometimes may be the crucial variable determining whether or not microtubules are assembled in cells. Further support comes from recent experiments with living cells in which the level of intracellular free Ca^{+2} was increased by microinjection (61) or by using ionophore A23187 (62). These experiments demonstrated that Ca^{+2} at roughly 10^{-5} M can depolymerize microtubules in vivo. The postulated role of Ca^{+2} in regulating assembly and disassembly of microtubules thus remains an attractive feature of the hypothesis.

The second possible difficulty for the hypothesis is concerned with the "compartmentalization" of ψ during differentiation. This portion of the hypothesis leads to the prediction that ψ should be "decompartmentalized" when flagellates revert to amoebae, in order to permit the amoeboid motility system to resume its functioning. The idea of "compartmentalization" of ψ is based on the observed change in the extractibility of ψ during differentiation. The prediction is that when flagellates revert to amoebae, the extractibility of ψ should change back to what it was in amoebae. So far we have made only a few measurements of the time course of the change in extractibility of ψ during differentiation and reversion (such experiments are fairly heroic). However, our best experiment (Fig. 9) does not really support the hypothesis very well. There is some uncertainty about how to draw the curve for extractibility of ψ in this experiment. It is drawn as a plateau in Fig. 9 but could also be drawn as rising to a peak and then declining. However, the extractibility of ψ had not returned to the amoeba level by the time the flagellates had returned to amoebae (and the experiment was ended). Perhaps it was declining; perhaps decompartmentalization of some ψ allows amoeboid movement. The prediction that when cells revert to amoebae the intracellular distribution of ψ should return to the configuration needed for amoeboid motility is not well supported by the experiments so far, and may not prove to be supportable.

Although the details of the mechanisms discussed are speculative and in some cases unknown, the main outlines of the hypothesis (Fig. 12 and Table IX) are not pure assumptions. The value of the hypothesis, however, is that it suggests many experiments. There are predictions to test, factors to identify, morphological correlates to observe, "reservoirs" and "packets" and "compartments" to seek, measurements of free Ca^{+2} to be done, and so forth. In addition to the tools of chemistry, biochemistry, and morphology, we also have the possibility of obtaining mutants to assist in the analysis.

SOAP BUBBLES AND THE FUTURE

We are confronted with an onrush of results, each of which suggests possibilities and demands interpretation, and inevitably it will take a while to sort out what is really

happening. This is wonderful for the experimenter since there is much detective work to do. It is also treacherous, for one tends to become enamored of the first idea, and at least some parts of the hypothesis are likely to be incorrect. If we can avoid the trap of being committed to our first insights, and, to paraphrase Oswald Avery, be the ones to burst the soap bubbles we blow, these clues should lead us toward the truth.

One hopes that the major features of this model can be generalized to cell shape and motility in organisms other than Naegleria. It is obvious to many biologists that Ca^{+2} and actin-based and tubulin-based systems play important roles in cell shape and motility, and there are hundreds of recent (and not so recent) papers in which aspects of this are considered. In many cells, such as fibroblasts, for example, amoeboid motility (the ruffled membrane) and microtubule-based cell elongation occur simultaneously (e.g., 45), somewhat as is postulated for the reverting amoeba in Fig. 12D. One of the special features of Naegleria, and one that is likely to be useful for analysis of these problems, is that the organism seems basically to approach motility and shape in a "yin-yang" fashion: amoebae with a functioning actin-based motility system and no microtubules alternating with flagellates with a microtubule cytoskeleton and no functioning actin-based motility system.

Calcium release is important in many activities of cells. It is basic to muscle contraction (36, 63), fertilization (64), cleavage (52, 53), secretion (30), adaptation of retinal cells to light (50), and so on. Yet in no one of these systems has the mechanism for calcium release been elucidated. It is perhaps in this area that the study of Naegleria may make the greatest contribution.

I began this essay with a quotation from E. B. Wilson, and it is appropriate to end with one from his colleague, T. H. Morgan. In 1932 he wrote that "it is the prerogative of science, in comparison with the speculative procedures of philosophy and metaphysics, to cherish those theories that can be given an experimental verification and to disregard the rest, not because they are wrong, but because they are useless." The beauty and promise of the Naegleria system and the ψ phenomenon are that an experimental analysis of cell shape and motility should be possible. It is not the working hypothesis that is important but rather the experiments it suggests.

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In an article of this length and breadth, it is impossible to do justice to the vast literature on calcium ions and on actin-based and tubulin-based systems. I have simply tried to give clues to this literature in the references, and I apologize for the many oversights, known and unknown.

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