Deuterium Oxide-induced Reversion of *Naegleria gruberi* Flagellates

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SUMMARY

A bead column technique is described for the separation of *Naegleria gruberi* flagellates from amoebae and cysts. It is shown that D₂O induces a rapid reversion of these flagellates to amoebae, and the behaviour and structure of the reverting cells is described. The amoebae are viable in high concentrations of D₂O, and when returned to suspension in H₂O readily retransform to the flagellate stage. The reversion of flagellates to amoebae is insensitive to cycloheximide, but the subsequent retransformation to flagellate can be prevented by both actinomycin D and cycloheximide.

INTRODUCTION

The soil protozoon *Naegleria gruberi* (Schardinger) can exist in three states: as an amoeba which feeds and divides; as a flagellate which swims strongly for a few hours but does not feed or divide; and as a cyst. The transformation from amoeba to flagellate can readily be controlled in the laboratory and provides a particularly amenable model for the study of morphogenesis in unicellular eukaryotes (Fulton, 1970). The process is temperature dependent and takes about 100 min at 27 °C; it can be prevented by inhibitors of RNA and protein synthesis (Balamuth, 1965; Preston & O'Dell, 1971; Yuyama, 1971). The reversion of flagellate to amoeba is very much faster and, as will be described here, shows no comparable sensitivity to these inhibitors. Spontaneous reversion of individual flagellates, which occurs sporadically and can be increased by, for example, applying mild shearing forces or adding certain fixatives, can create difficulties for the experimentalist. In this paper, methods are described which enable the easy production and fixation of populations consisting entirely of flagellates or amoebae, and it will also be shown that deuterium oxide is an inducer of a general and rapid reversion to the amoeboid stage. These techniques have enabled a number of observations to be made on the reversion process and the transformation back to flagellate.

METHODS

Protozoa

Naegleria gruberi (stock 1518/1C, Cambridge Culture Collection) was maintained at 25 °C in monoxenic culture with *Klebsiella aerogenes* on 0.2 % (w/v) peptone agar plates. When cells were required in the amoeboid phase cultures were harvested within 20 h of inoculation as previously described (Preston & O'Dell, 1971). Extension of the incubation period beyond 48 h resulted in encystment of most of the amoebae; a pure, viable cyst suspension could be readily secured if residual trophic cells were removed by mild detergent lysis with Triton X100, 0.05 % (v/v) in 2 mM-tris (Fulton, 1970) before harvesting. Incubation of amoebae suspended in 2 mM-tris, pH 7.5, in a shaking water bath at 27 °C resulted

Fig. 1 Glass bead column used in the separation of flagellate *Naegleria gruberi* from other stages. For details of assembly, see text. Scale marker represents 5 cm.

in their transformation to the flagellate stage. The synchrony of this change was high (80 to 90 %) but not perfect. Therefore in order to obtain pure suspensions of flagellate cells other procedures were tested.

Flagellate separation methods

Density gradient centrifugation. Continuous gradients of 0 to 20 % (w/v) Ficoll in 2 mM-tris were prepared and samples of populations of mixed stages carefully layered on top. After brief periods of centrifugation at 500 to 2000g at room temperature, samples were drawn off the gradients and examined by phase-contrast microscopy.

Capillary tube migration. The capillary region of a Pasteur pipette was drawn into zigzags and sealed distally. Amoebae and flagellates were suspended in 2 mM-tris and introduced into the wide end which was then sealed and the whole stood pointing upwards. At intervals the pipette was examined by dark ground microscopy. The contents of the capillary region could be isolated by snapping the pipette in the transition region.

Glass bead column (Fig. 1). A 10 ml plastic syringe without the piston was used to support a column of 2 mm diameter glass beads. A 5 ml syringe was inserted into the top of this to act as a reservoir. Layers of parafilm provided an airtight seal between the two barrels.

Flow rate of liquid through the column was controlled by a burette clip on silicone rubber tubing leading off a 21-gauge needle attached to the larger syringe barrel. Such a column had an average void volume of 3.5 ml and a glass surface area of the order of 15 cm². For an experiment the column was thoroughly washed with 2M M-tris, then 3 to 4 ml of harvested cells (about 3×10^6 cells/ml) were layered on to it. These cells were carefully drawn into the bead matrix, and then fluid flow stopped for 15 min to allow the amoebae to attach to the glass surface. The column was then carefully eluted with buffer flowing gently under gravity for an hour to remove any unattached cells and bacteria, the eluent being monitored microscopically. Elution was then discontinued and the column allowed to stand until the buffer above the bead bed became turbid – usually after a period of 150 min at room temperature. Then 5 ml of buffer were drawn off the column and checked microscopically to estimate the percentage phenotype distribution of the cells. A sample of this suspension was fixed by dilution 1:10 with commercial formalin (approx. 40 % formaldehyde), and used after further dilution 1:25 with isotonic saline to estimate cell number in a Coulter Counter, Model ZB 1 (140 μ m aperture, 500 μ l sampling volume, aperture current 4 mA and amplification 2).

Electron microscopy of reverting cells

Flagellates were drawn off a glass bead column into two 10 ml graduated centrifuge tubes. Deuterium oxide (99.7 % minimum isotopic purity, B.D.H. Chemicals Ltd, Poole, Dorset) was added to these to final concentrations of 38 and 78 % (v/v) respectively. Ten minutes later the cells were fixed by addition of 25 % (v/v) glutaraldehyde (Taab Laboratories, Reading, Berkshire) to give a final concentration of 2.5 % (v/v). After 18 h at 20 °C the fixative was removed by thorough washing in 2 mM-tris at pH 7.5. The cells were postfixed for 14 h in 1 % (w/v) OsO₄ in 2 mM-tris, pH 7.5, then immersed in 0.5 % (w/v) aqueous uranyl acetate for 48 h. Dehydration was effected with graded water–ethanol mixtures and the cells were subsequently embedded in araldite resin. Sections showing silver/grey interference colours, prepared on a Huxley ultramicrotome, were collected on uncoated copper grids and stained in lead citrate. They were then examined in an AEI EM6B electron microscope operated at an accelerating voltage of 60 kV with an objective aperture of 50 μ m. Images were recorded on Ilford N60 plates and nominal instrument magnifications were corrected to a standard by means of a diffraction grating replica (2.16⁻¹ μ m interval). Measurements on individual electron micrographs were made by means of a microdensitometer (Joyce–Loebl, MK IIIc) calibrated against the diffraction grating image recorded at the same magnification.

Light microscopy and filming

Routine microscopy was carried out with both Leitz and Zeiss (Oberkochen) equipment. Phase-contrast micrographs were recorded on Ilford HP4 emulsion using Zeiss $\times 100$, N.A. 1.3 and $\times 40$, N.A. 1.0 objectives, a 547 nm interference filter being inserted into the optical path below the condenser. An electrically driven Bolex 16 mm camera loaded with Ilford Mark V emulsion was used to film through a Zeiss photomicroscope bearing a $\times 6.3$ N.A. 0.2 objective. Filming speed was 16 frames/s and the microscope was fitted with an interference heat filter. For each trial D₂O was added to a flagellate suspension and gently mixed. A drop was withdrawn and placed on a microscope slide without the addition of a coverslip. By adjustment of the height of the condenser below the specimen stage, good dark ground illumination was produced by the field stop of a suitable phase annulus. With practice filming could be commenced within 30 s of the addition of D₂O to the flagellates. Films were analysed by plotting the tracks of individual cells from frame to frame.

Fixation of flagellates

Preliminary experiments showed that some fixatives were unsatisfactory, as flagellates were able to revert at least partially to amoebae during the brief period before fixation was complete. For example, when a culture consisting entirely of flagellates was mixed with an equal volume of 12 % acrolein, cells could be seen to alter their shape on contact with the fixative and reverting cells were found in the fixed material. Chang (1958) reported that silver nitrate fixed flagellates, but in the present study zinc ions were tested, following the observation that they stabilize some membranes during cell fractionation procedures (Warren, Glick & Nass, 1966). Accordingly, 0.001 M-ZnCl₂ was applied to *Naegleria* flagellates for 15 to 30 min, and good preservation resulted, without emission of pseudopodia or other signs of reversion to the amoeboid form. The cells became, however, rounder than unfixed flagellates.

RESULTS

Separation techniques

In preliminary trials, attempts were made to separate the cyst, flagellate and amoeba stages of *Naegleria gruberi* by centrifugation at low speeds in a Ficoll density gradient. This method was never entirely satisfactory; first, because there was too much overlap in density between the three stages; secondly, because the flagellates tended to revert during the process; and thirdly, because of some cell loss by lysis of the motile stages.

The glass pipette technique enabled flagellates, by virtue of their much greater mobility, to migrate away from untransformed amoeboid cells. Phenotype separation by this method was perfect and distally the capillary contained only flagellates, but the yield was low.

The glass bead column method, by exploiting the difference in adhesion properties between the two motile stages of *Naegleria gruberi*, proved to be a far more efficient procedure for the isolation of flagellates. The success of this technique depended on gentle elution of the column. Too high a flow rate during harvesting resulted in amoebae detaching from their glass substrate and being drawn off with the flagellates. Provided that elution of the column after the layering on and settling of the cell suspension was thorough, any cysts present were either washed clear or became firmly trapped in the bead bed. A suitable flow rate for our column was 2 ml/min. Using a glass bead column of the given dimensions and starting with 10⁷ cells a yield of 10⁶ flagellates could be routinely obtained and contamination of this by other stages was difficult to detect. Preparations of cells were initially contaminated with *Klebsiella aerogenes* from the culture plates, but the bacteria were readily eliminated by chloramphenicol treatment. Since our strain of *N. gruberi* is impermeable to this drug unless treated with agents such as polymyxin B (Preston & O'Dell, 1971), it was possible to treat a mixture of amoebae and bacteria selectively with chloramphenicol.

The effects of deuterium oxide

Induction of flagellate reversion. After harvesting from the column, control flagellates showed a variable degree of activity; most moved rapidly and swam flagella foremost in a straight line or with frequent changes of direction. An occasional cell showed the first sign of reversion, a change of locomotory pattern from linear progression to constant movement along a circular path, or rotation about its axis.

The overall effect of adding D₂O to the flagellates was to cause a general and rapid reversion to the amoeboid form. In high concentrations (50 or 75 %), within 30 s half or more of the cells were spinning, although a decreasing proportion maintained typical

flagellate locomotion for a little longer. By 2 min, virtually all cells demonstrated stages of reversion such as the change in swimming pattern, the emission of pseudopodia and attachment to the substrate. As far as could be seen D₂O did not introduce any new characters into the reversion process, but merely induced its rapid onset.

Twenty-five per cent D₂O was less effective in causing reversion. After 30 s, only a few cells were obviously reverting and even after 5 min there was still a roughly equal mixture of reverting cells and apparently normal and active flagellates. Reversion, when it occurred, seemed to follow the standard pattern.

Viability of amoebae in D₂O. Suspensions of flagellates without amoebae or cysts were pipetted into D₂O–H₂O mixtures at final D₂O concentrations of 25 to 95 %, and the flasks left for 2 days at room temperature. After this time viable amoebae, with active contractile vacuoles, were found in all concentrations of D₂O. In 90 % or 95 % D₂O, however, the amoebae were unable to attach normally to a glass substrate, although streaming movements could be seen in the cytoplasm and active pseudopodia were formed. Occasionally, minor adhesions were made to the substrate and filopodia trailed behind. When these amoebae were plated out under our standard conditions, the growth of normal cultures demonstrated the lack of any permanent lesion induced by D₂O.

The reversion process in deuterium oxide-treated flagellates

Observations on living cells. Active streaming in the cytoplasm was suppressed in the flagellate stage, but recommenced rapidly after the administration of D₂O causing symmetry to be lost as small pseudopodia erupted at any point on the cell surface (Fig. 3, 4). At this stage if the cell collided with the glass substrate it adhered briefly. Eventually the organism adhered to the glass strongly enough to nullify the resultant of the flagellar strokes. This marked a transition point in the cell's locomotory behaviour, as although the flagella continued to beat, the cell now moved by amoeboid locomotion. Two further events could be seen as reversion approached completion. In the active flagellate the rhizoplast (Fig. 5, 7) inserted at the basal bodies transmitted slight waves from the flagella to the nucleus causing this organelle to undergo small displacements. As reversion was completed and amoeboid movement commenced, the nucleus was still restrained by its rhizoplast connexion from being drawn freely into the path of cytoplasmic flow, and this antagonism between the rhizoplast anchorage and the cytoplasmic streaming could deform the nucleus into a conoid (Fig. 5). A further stage in the process of phenotype reversion was marked when the connexion broke between the rhizoplast and the nucleus so that the latter could be moved freely by the flowing cytoplasm. Those mitochondria which in flagellates were aligned along the rhizoplast (Fig. 2) became dispersed in the cytoplasm.

The other and more readily observable event was flagellar loss, which usually occurred in one of two ways. Either a bleb appeared at the distal end of the flagellum and, as it increased in size, the length of the flagellum decreased until the bleb became incorporated in the cytoplasm of the cell; or, during a posterior stroke, a flagellum collided with the cell membrane, rapidly penetrated it and continued to beat for a short time within the cytoplasm (Fig. 6).

Ultrastructure. Schuster (1963) and Dingle & Fulton (1966) have given a general account of the fine structure of the developing flagellate stage; attention here was confined to changes in the mastigont system during reversion. The only difference between the flagellate samples fixed after 10 min in 38 % or 78 % (v/v) D₂O concerned the distribution of their flagella. Cells treated with the lower concentration of D₂O had normal, external flagella, whereas those exposed to the higher concentration had at least one flagellum, if not both (or all in

the case of occasional multiflagellate cells), withdrawn into their cytoplasm. Furthermore the flagella, on entering the cell during D₂O-induced transformation, lost their enclosing membrane (Fig. 9). No complete internal flagellum was seen; only exposed axonemes were present within the cytoplasm even in cells that had not completed the intake of their flagellar complement (Fig. 8). There were indications of a loss of spatial organization within some of these axonemes although the basal body-rhizoplast complex was still intact (Fig. 11).

The close spatial relationship existing in *Naegleria gruberi* flagellates between the rhizoplast and the nuclear envelope, evident by light microscopy, was confirmed by the electron microscope (Fig. 10), but the increase in resolution did not reveal the structural basis for the mechanical strength of this association. Optical density profiles along rhizoplasts in cells treated with D₂O revealed a major period of 19 nm in the cross-striations. This period was the same whether flagella were external or internal.

Cycloheximide: lack of effect on reversion. Flagellates were treated with D₂O in the presence of 10 µg/ml cycloheximide, an inhibitor of protein synthesis which penetrates *Naegleria gruberi* amoebae without difficulty and inhibits the initial transformation into flagellate (Preston & O'Dell, 1971). Reversion proceeded as before, with the onset of streaming, eruption of pseudopodia and loss of flagella.

Retransformation of reverted cells. When flagellates had reverted to amoebae, the latter on removal from D₂O retransformed to flagellates as readily and under the same conditions as untreated amoebae did, and showed similar susceptibility to antibiotics. Under our standard transformation conditions (Preston & O'Dell, 1971) actinomycin D (50 µg/ml) and cycloheximide (10 µg/ml) both inhibited the production of flagellates.

DISCUSSION

The ability of *Naegleria gruberi* to survive in an active amoeboid stage in 95 % D₂O is not unique but is none the less notable, as sudden immersion in this concentration is toxic for most protozoa (see reviews by Thomson, 1963; Flaumenhaft, Bose, Crespi & Katz, 1965; Katz & Crespi, 1970). In this concentration the amoebae were abnormal in that they displayed a reduced ability to attach to a glass substrate, but the contractile vacuole continued to function under circumstances which reversibly inhibit that of *Paramecium*, *Blepharisma*, *Discophrya* and *Carchesium* (Gaw, 1936; Kitching & Padfield, 1960). Cytoplasmic streaming

Key to symbols: *ax* = axoneme, *b* = basal body, *f* = flagellum, *m* = mitochondrion, *n* = nucleus, *nu* = nucleolus, *ps* = pseudopodium, *rh* = rhizoplast, *v* = contractile vacuole.

Fig. 2. to 7. *Naegleria gruberi*. Phase-contrast micrographs of unfixed normal and reverting flagellates. All scale markers represent 5 µm.

Fig. 2. Normal flagellate. The flagella, out of the plane of focus, are inserted in an apical depression (arrowed) from which a line of mitochondria lead posteriorly adjacent to one side of the nucleus.

Fig. 3. Binucleate flagellate, and Fig. 4, the same cell 10 s later at the onset of cytoplasmic streaming and pseudopodia production (marked by large arrowheads).

Fig. 5. Reverted flagellate progressing along the substrate in amoeboid fashion. The nucleus, anchored by the rhizoplast (small arrowheads) to the basal bodies, is being drawn into a conoid by the flowing cytoplasm of the leading pseudopodium.

Fig. 6. The flagella have been withdrawn into the amoeboid organism and the mastigont system has now lost its physical connection with the cell membrane. A flagellar axoneme (arrowed) is still visible within the cytoplasm.

Fig. 7. The same cell some minutes later lysed to demonstrate the intimate association between the rhizoplast and the nuclear envelope.

continued, as it does in *Amoeba proteus* (Marsland, 1964). Although our experiments gave no evidence of multiplication in high concentrations of D₂O, no significance should be attached to this, as the cells were not fed. The most remarkable effect of heavy water on *Naegleria* was its action on interconversions between the active phenotypes, so that a mixed population of amoebae and flagellates could be converted to amoebae alone and kept in that form.

That D₂O would induce a rapid reversion from the flagellate stage was unforeseen when the experiments were started. In a number of circumstances D₂O enhances the stability of transient cytoplasmic structures based on microtubules, such as the mitotic spindle (Inoué & Sato, 1967), axopods (Tilney, 1968; Marsland, Tilney & Hirschfield, 1971) and *Amoeba* gel structures (Marsland, 1964), and heavy water was first employed by us in an attempt to reduce the lability of *Naegleria* flagellates. Why instead it greatly increased this lability is not clear, but the action presumably reflects the differing structural basis for the two forms. The shape of the flagellate is fairly constant and in the absence of a substantial microtubular corset, such as encountered for example in *Trypanosoma* spp. (Rudzinska & Vickerman, 1968), it would seem to be maintained in part by the state of the cytoplasm. The quiescent cytoplasm of the flagellate contrasts strikingly with the active streaming found in the amoeba and raises intriguing questions about the disposition and cellular control of the actomyosin-like components (Lastovica & Dingle, 1971) in these two phenotypes.

Although the flagellate stage will revert spontaneously and is sensitive to a number of stresses such as temperature shock, shearing forces, or high pressure (Todd, 1972), as far as we know no agent has yet been reported which will give a reversion as synchronous as that induced by D₂O. At a cytological level, three effects may be relevant to the reversion process. The first is the change in locomotory pattern, which might be effected either by an action on the flagella or on their co-ordinating system. There seems to be a second effect, on the surface membrane of the cell. Several changes are noticed which are manifestations of this: the protrusion of pseudopodia; the ability to settle and move on the substrate; and the permission of penetration of the surface by flagella which happen to strike it. A third change in the cell is the onset of cytoplasmic streaming which accompanies the formation of pseudopodia. Whether any of these visible changes is the initial event is not entirely certain, but the first sign of reversion is the alteration of locomotory pattern.

The glass bead column technique for the production of flagellates gave a suitably homogeneous population for the study of the reversion process. Pittam (1963) reported that on reversion, flagella could enter the cell, and from writhing movements within the cytoplasm he was able to infer that the flagellar beat continued for some time. Dingle & Fulton (1966) encountered occasional intracytoplasmic axonemes in their study of the developing masti-

Fig. 8 to 11. Electron micrographs of *Naegleria gruberi* flagellates fixed 10 min after exposure to the stated doses of D₂O. Scale markers represent 0.5 μ m.

Fig. 8. Seventy-eight per cent D₂O. This cell at an early stage of reversion possesses two flagella, one of which is external; the other, devoid of its surrounding membrane, exists as a naked axoneme visible within the cytoplasm at two points in the section.

Fig. 9. Seventy-eight per cent D₂O. The axonemes of formerly external flagella are embedded now in the cytoplasm.

Fig. 10. Thirty-eight per cent D₂O. Note the close spatial relationship between the rhizoplast and the nuclear envelope.

Fig. 11. Seventy-eight per cent D₂O. Here at a late stage in reversion the flagella have been withdrawn and the basal bodies, no longer located at the cell periphery, lie deep in the cytoplasm. The structural integrity of the axonemes is breaking down.

gont system, but considered that this represented a stage in flagellar reversion, rather than in assembly as had been suggested earlier by Schuster (1963). In our studies on uniform populations of reverting flagellates, both light and electron microscopy confirmed the presence of intracytoplasmic axonemes as a stage in the loss of the kinetic apparatus. As we encountered only naked intracytoplasmic axonemes, it seems likely that during their lateral resorption flagellar membranes fused with and became incorporated into the cell membrane. This presumably reflected a recent membrane change, since there was no fusion on random contact between the flagellar and surface membranes of an active flagellate.

The nature of deuterium isotope effects on biological macromolecules is complex and several different modes of action may be distinguished. There is a solvent effect, in which the composition of isotopes in the medium is changed. With D_2O , this also means that the medium is more structured than ordinary water (Némethy & Scheraga, 1964), which can affect the aggregation of proteins (Khalil & Lauffer, 1967). Primary and secondary isotope effects will occur where 2H has replaced 1H elsewhere. If a C-D, O-D or N-D bond is formed near the active centre of an enzyme or the labile portion of its substrate, the effect is primary; if the substitution is not near the centre, it is termed secondary although it may still exert some influence on reactions involving that centre (Collins & Bowman, 1970). Since, however, biological macromolecules have a great deal of exchangeable hydrogen, it is not always easy to distinguish between solvent and other effects, especially in an intact organism. Many properties of proteins will be altered by deuteration; for example, if 2H replaces 1H in a dissociating group, the pK value will be changed. Nor can ready predictions be made about changes in protein configuration, as Tomita, Rich, de Lozé & Blout (1962) have pointed out in their discussion on the stability of the deuterated α -helix. We can say little at this stage, therefore, about the exact mode of action of deuterium oxide on *Naegleria gruberi*.

As reverted amoebae are able to retransform only when suspended in H_2O , D_2O is a useful agent for the easy manipulation of the mobile phenotypes of *Naegleria*. Combined with availability of homogeneous populations of flagellates from the column, the biochemistry of reversion is now amenable to investigation. The lack of an effect of cycloheximide suggests that there is no requirement for protein synthesis during this rapid process. In contrast, when reverted cells are induced to retransform back to flagellates, they are as sensitive as fresh amoebae to antibiotic inhibitors of RNA and protein synthesis, which would seem to indicate that there is no large-scale storage of flagellar components and that the production of at least some of these depends on *de novo* synthesis.

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