FACTORS WHICH INFLUENCE THE ACQUISITION OF FLAGELLA BY THE AMOEBA, NAEGLERIA GRUBERI

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INTRODUCTION

During the course of some investigations upon the behaviour of cells isolated from sponges (Sycon sp.) by squeezing the sponge through fine silk, the behaviour of the normally flagellated collar cells, or choanocytes, aroused particular attention and curiosity. Especially was this so because of the apparent ease with which these cells could lose first their collar, then their flagellum, and finally become creeping amoeboid cells, only to reacquire the flagellum again, and sometimes the collar also, when conditions became favourable to this change. This phenomenon, which is interesting enough in itself, may also have implications of much wider significance.

In the early developmental stages of sponges, the larva can be more or less clearly subdivided morphologically into an anterior region of flagellated cells and a posterior region of amoeboid cells, or at least of cells which have no flagella. In some species (e.g. Clathrina blanca) these posterior cells may be restricted at first to a single pair of archaeocytes. In Sycon, on the other hand, the embryo is fairly evenly divided equatorially into the two classes of cells, though in this species the cells in the equatorial zone give the impression of being somewhat intermediate in character. These intermediate cells, though similar in shape to the anterior cells, are much more granular, and in this way resemble the cells of the posterior half.

This antero-posterior division of the embryo is not, however, a peculiarity of the sponges, for in many other groups of invertebrates beside the Porifera, the embryo or larval animal is clearly polarized into an anterior (animal) pole and a posterior (vegetal) pole. In some species, this polarization and subdivision into zones is as obvious as it is in the sponges, and the cells of the anterior pole are ciliated or flagellated; in others, the gradient from animal to vegetal pole only expresses itself in more subtle ways. Nevertheless, throughout a wide range of animal types, there is evidence for an axial gradient in some form or another. The difference in character between the anterior ‘animal’ cells, which primarily give rise to the external, sensory and protective layers of the organism, and the posterior, more digestive and vegetative cells which habitually enter the inner layers, is thus a very widespread and fundamental difference.

Whatever views one may hold on the origin of Metazoa from Protozoa, on the respective claims of the ciliates or flagellates as metazoan ancestors, or on the phylogenetic positions of the Porifera, Coelenterata and Turbellaria, the existence
of an antero-posterior gradient in so many organisms is a phenomenon which has been often described, but seldom explained.

Since this gradient frequently shows itself by the presence of flagellum-bearing or ciliated cells anteriorly and of more amoeboid cells posteriorly or centrally in the embryo, with often an intermediate zone of cells separating the two clear types, the nature of the difference between flagellate cells on the one hand and amoeboid cells on the other is obviously a problem worthy of investigation. More particularly does the problem become interesting when the different forms of behaviour are separated not in space, as in the two halves of the embryo as just outlined, but in time, as in isolated choanocytes or in the behaviour of certain amoebae.

Moreover, the nature of the physiological distinctions between flagellated or ciliated cells on the one hand and non-ciliated cells, which often produce 'mucin', on the other, has repercussions which may be applicable to problems of cellular differentiation even in the higher vertebrates. For example, when the perfectly uniform and normally keratinizing skin of the chick embryo is treated in tissue culture with high doses of vitamin A, the cells change both their morphological character and their physiological behaviour (Fell & Mellanby, 1953). Some of the cells acquire cilia, others secrete 'mucin'. It would be fascinating to know what are the factors operating in this change, why the change leads to a diversity of cell behaviour and how the vitamin A acts. These observations on the effects of vitamin A also raise questions as to what is the relationship between the ciliated and the non-ciliated cells in such epithelia as that of the respiratory tract, and whether there is some antithesis between the formation of flagella (or cilia) and the production of mucoprotein.

Now it has been known for many years (Schaudinn, 1896; Schardinger, 1899) that, in certain amoebae, of which Naegleria gruberi is an excellent example, the individual cell may exist in one of two forms; it may either live as a typical, creeping and phagocytic amoeba, or it may, under certain conditions, acquire one or more flagella and become free-swimming. This change, from the amoeboid to the flagellate form, almost invariably occurs when the amoebae which have been growing, for example, on an agar-meat-extract slope, are transferred to pure water.

An investigation of the nature and cause of this change from one form of activity to another seemed therefore likely to yield information of some interest, not only in its immediate relation to the life of these special amoebae, but also because of its possible application to the wider biological issues already surveyed, namely, the relationship between cells with flagella (or cilia) and those in which movement is essentially amoeboid; and, as a sequel to this, to one of the main characteristics which helps to define the antero-posterior gradient of so many invertebrate, and indeed vertebrate, embryos.

METHODS

The amoebae (Naegleria gruberi), which were originally obtained from the type collection of Protozoa, Algae, etc. in the Botany School, Cambridge (1518, 1), were kept as stock cultures on agar slopes, in test-tubes stoppered with wool and closed
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with a paper seal. The composition of the agar was as follows: 7.5 g. powdered agar was dissolved in distilled water and filtered before adding 0.5 g. 'Lemco' meat extract and 1.0 g. glucose and then making up to 500 ml. with distilled water. The tubes containing about 4 ml. of medium were sterilized in an autoclave and then cooled to form slopes. When the amoebae were in need of subculture, each slope was washed with about 5 ml. of sterile distilled water, and the amoebae spun down in a centrifuge at about 3000 r.p.m. for 3–4 min. The usual precautions against infection from outside sources were observed. These amoebae were then re-suspended in another 5 ml. of sterile distilled water and re-centrifuged. The washed amoebae were then suspended in a smaller volume (0.5–1.0 ml.) of sterile distilled water, appropriate to the cell density, and single drops from this suspension of cells were then used for seeding fresh agar–Lemco slopes. For building up the stock cultures for use in experiments it has been found better not to wash the amoebae too thoroughly. A high density of amoebae can be better maintained if they are transferred to the agar slopes with their accompanying bacteria. For experimental studies, however, more thorough washing is required.

The stock cultures normally grow freely for about 10 days at room temperature (generally 15–25° C.). During the first 3 or 4 days the amoebae are large and active; many polynucleate cells are found. After that time the cells become smaller, the growth declines and the number of amoebae in the form of cysts increases rapidly. Such cysts, if suspended on fresh agar slopes, soon produce active amoebae. For experimental purposes, cultures between the ages of 3 and 7 days have given the most uniform and satisfactory results, but there is room for a more detailed investigation into the optimal age and into the effects of culture conditions on the numbers of cells which acquire flagella when treated with water. It has been noticed that sometimes nearly all the amoebae become flagellated in a very short time, not more than 2 or 3 hr., while at other times there are never more than a relatively small percentage of flagellate forms, and these may not appear till the amoebae have been in water for six or more hours. In winter, when there is a tendency for the cells to remain in the amoeboid form in spite of their transference to distilled water, it has been found beneficial to keep the stock cultures well illuminated. Other observers (Schardinger, 1899) have noted a marked tendency for the flagellate form to increase in numbers with rise of temperature up to 34° C. One of the factors in this variability in the time of appearance may thus be the fluctuation of room temperature, but there must certainly be other factors also, since a few preliminary experiments have not so far confirmed the observations of Schardinger.

The cells normally feed on bacteria and, in healthy conditions, the populations of bacteria and amoebae seem to control each other and reach a sort of steady state. In spite of this initial necessity for bacteria, the cultures have been kept under otherwise aseptic conditions, i.e. all subculturing has been done with simple aseptic precautions; glassware and culture media have been sterilized and all tubes sealed when not in use. If these elementary precautions are not taken, the cultures quickly become contaminated with moulds, yeasts, etc., and these are then difficult
to eradicate. The presence of bacteria is, of course, a major source of difficulty and of irregularity in results, and a satisfactory sterile synthetic medium would offer enormous advantages. Brent (1954) has produced a satisfactory sterile medium for *Tetramitus* by using autoclaved bacteria, and Reich (1935) has grown *Mayorella* on a peptone medium.

In order to investigate the effects of any substance on the activity of the amoebae, the substance was first dissolved in sterile distilled water and the hydrogen-ion concentration adjusted approximately to pH 7. Serial dilutions of this solution were then made in distilled water in test-tubes which had been very thoroughly cleaned, sterilized with absolute alcohol, and rinsed out with sterile distilled water. 0.5 ml. of solution were placed in each tube and one drop of a well-mixed and uniform suspension of amoebae was then added to each tube. To make this suspension the contents of two or three culture tubes (3–7 days’ growth) were washed and centrifuged at least three times with distilled water to obtain them relatively free from bacteria, though by no means sterile. After the last washing the cells were taken up in 3 or 4 ml. of distilled water according to their density. Two culture tubes usually gave enough suspension to seed about sixty experimental tubes. Three may be necessary in winter. It has been found advantageous not to have too many amoebae present, otherwise the subsequent counting becomes difficult (about 2000 cells per cu.mm. is a convenient concentration for the original suspension).

From time to time, a sample drop of as nearly as possible constant volume (about 2 cu.mm.) was taken with a marked Pasteur pipette from each experimental tube and these drops were placed on a microscope slide, four at a time. The number of flagellates which could then be counted in a single ‘journey’ of the microscope field (⅛ in. objective) round the edge of each drop were then noted, and the population in the various tubes compared in this manner. This semi-quantitative method shows quite clearly at what concentrations of the substance under investigation the amoebae assume the flagellate form; further examination of the ‘creeping’ amoebae shows whether the solution has any differential action on the two forms of activity.

Several control tubes, containing a medium of distilled water only, were always seeded and counted at the same time, so that the behaviour of the batch of cultures as a whole could be to some extent standardized.

Estimation of pH values in the observation to be described have all been made colorimetrically with phenol-red, cresol-red, thymol-blue or B.D.H. universal indicators. Phenol-red was often incorporated in the medium without any harmful effects. The values of pH given are therefore only approximate but are considered adequate, in view of the reaction of the amoebae to this aspect of their environment. Moreover, in experiments lasting for any length of time in fluids as dilute as those used, the initial pH may differ considerably from the final pH because of the production or diffusion of CO₂ or other metabolites. In general, results obtained during the first 6 hr. or so are considered to be directly related to the medium as constituted, perhaps allowing for diffusion of CO₂, etc., but, in experiments of longer duration, subsidiary and confusing effects may be produced by bacterial growth, etc. These latter effects become particularly important in media containing
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food substances like glucose. It should, therefore, be stressed that the investigations described in this paper have been aimed more at finding the broad principles involved in the change from amoeba to flagellate and less at the detailed analysis of any one set of conditions.

MORPHOLOGICAL CHANGES

The amoebae, *Naegleria gruberi*, when growing on an agar surface in the presence of bacteria may, like other amoebae, assume almost any shape: the cells move in what appears to be a more or less random manner; though this, in fact, is probably determined by local changes in the environment. The amoebae tend to spread out and to maintain spaces between themselves, but they may cluster round a rich food supply or, if a thick population is released into a drop of fluid on a cover slip, spread centrifugally in more or less serried ranks. Rounded pseudopodia may appear at any point on the surface of the cell, and one or more contractile vacuoles come and go within the cytoplasm without any definite or obviously constant location. The nucleus also moves freely about within the cell. It contains a conspicuous nucleolus, and is surrounded by numerous highly refractile granules; these give the nuclear membrane a beaded appearance and are particularly conspicuous in preparations viewed by phase contrast. When several contractile vacuoles form they usually fuse before evacuating.

When the cells are washed almost free from bacteria and allowed to settle in distilled water on the clean glass surface of a cover-slip or slide, certain characteristic changes of morphology follow (Fig. 1). After a short time, often about an hour, a tendency towards a definite polarity develops in the cells. Rounded pseudopodia are pushed out more in one direction than another and the organism naturally tends to move in that direction. This direction is not rigidly fixed, but the amoeba is fairly clearly developing an anterior and a posterior half, the former sending out rounded pseudopodia and the latter developing a more or less definite tail-zone or 'uroid' to which numerous bacteria may often be seen to be attached possibly by a mucoid secretion (Fig. 1; 4) (cf. Hollande, 1945; Martin & Lewin, 1914). The surface of this tail-zone appears to be in some ways more stable and rigid, in the sense that pseudopodia develop less readily from it and, when they do so, they are not the usual type but take the form of fine thread-like pseudopodia generally directed backwards, approximately parallel to the antero-posterior axis (Fig. 1; 5). They sometimes appear to be the result of the cell creeping forward but leaving behind points of attachment to the glass which remain connected by ever-extending threads of protoplasm.

These filiform pseudopodia were described by Pietschmann (1929) and can be seen to come and go; they may move about slowly from side to side, or may bend sharply and fold in on themselves, thickening as they do so, to be finally reabsorbed into the cell body once more. At this stage in the progressive change of form, the contractile vacuole takes up a position near the posterior pole, and, after it has collapsed, it now reappears as a group of five or six small vacuoles very often in
the form of a rosette (Fig. 1; 6). These small vacuoles progressively enlarge, fuse together and evacuate, regularly, with a period of about 30 sec. This period remains almost constant during the subsequent transformation. In other media the frequency of evacuation may of course be different (Wolff, 1927).

Sooner or later, the precise moment presumably depending on a variety of conditions, such as the temperature, the state of health of the amoebae, their age,
the number of bacteria present, etc., a short, somewhat thicker, and definite flagellum appears at the posterior end, either among or instead of the filiform pseudopodia (Fig. 1; 7a). It is visibly different from the latter; it immediately starts to beat, and appears to be associated with a basal granule, probably derived from the nucleolus (Wilson, 1916). The relationship between the flagellum and the filiform pseudopodia reminds one of the relationship between the true cilia and the finger-like processes which are demonstrable between them by means of the electron microscope in certain ciliated epithelial cells of Metazoa. The one is a special and, in this case, a newly developed structure, the other is a modification of the cell surface.

More often than not this amoeba does not produce a single flagellum but a cluster of three or four, most commonly the latter (Fig. 1; 8, 8a). At this time the organism is, as usually observed, lying in contact with the glass of the cover-slip (Fig. 1; 7a), though the contact is not a uniform one and this amoeba, like others, may creep on leg-like processes with the ‘main body’ held somewhat away from the surface. But from now onwards the cell begins to pull itself together into a more compact structure, becoming roughly spherical or ovoid in outline, and while this is happening the point of emergence of the flagella which originated at the posterior end, near the contractile vacuole, tends to move away, over the surface, so that the flagella now beat freely into the medium (fig. 1; 8a, 9a). The contractile vacuole, however, remains roughly where it was, so that the two organelles become widely separated from each other. A similar reversal of polarity has been noted by Hollande (1942) in Tetramitus and also by Martin & Lewin (1914) in Vahlkampfia.

As the contractile vacuole fills and enlarges in each cycle the flagella have been observed to beat progressively more slowly and the beat to cease altogether as the vacuole collapses. Then after a short pause the beat starts up again, at an enhanced frequency at first, but it soon settles down once more to a steady rhythm till the cycle is resumed when the contractile vacuole is next ready to discharge. Presumably some change spreads over the surface of the cell as the vacuole breaks through it, and this change involves the flagellar apparatus also.

As the flagella move away from the area of the contractile vacuole and the cell becomes more spherical or ovoid the mean frequency of the beat increases and the cell begins to spin, slowly at first, and then with increasing speed and with wider revolutionary as well as more vigorous rotary movements, till, quite suddenly, it breaks the contact with the glass and swims freely away (Fig. 1; 10). The last point of the attachment seems to correspond to the uroid region, so that as the organism swims away the flagella lead and the contractile vacuole brings up the rear. The revolutionary movements about a fixed point suggest that the attachment to the glass at the end may be due to an extremely fine strand of protoplasm, and certainly in some electron-microscope pictures taken of amoebae (by the kindness of Dr J. R. G Bradfield) at a stage when many of them were becoming flagellate, several amoebae (with flagella) show an extremely fine long thread extending from the posterior end (Fig. 2). Similar thread-like structures have also been observed between amoebae which have been closely associated with each other and then move apart.
A stickiness between amoebae, which may be somewhat similar, has been observed by Wilson (1916).

The change from the amoeboid to the flagellate form can take place in about 20 min. but this time is naturally very variable and, as already mentioned, it may occur at almost any interval between 1 and 24 hr. after the stimulus for it has been applied. The change not only involves the morphological change between the amoeboid and the free-swimming flagellate form, but also is accompanied by the appearance or establishment of a definite cell polarity. Probably also a change in the method of feeding is involved, though on this last point there is still much to be learnt. Some species (e.g. *Tetramitus rostratus*) develop a definite cytostome in the flagellate form (Bunting, 1926), but this has not been observed in *Naegleria*.

In the experimental conditions which have been maintained in the present series of observations the flagellate phase does not last for very long and after about 24 hr. of free swimming the majority of the organisms begin to settle down as amoebae again. The conditions determining this have not yet been investigated though mechanical agitation may be a factor (Alexeieff, 1924; Wherry, 1913; Wilson, 1916).

In the introduction, emphasis was laid on the possible significance of the change from the phagocytic amoeboid form to the free-swimming flagellate in relation to animalization and vegetalization in embryos. While this may be so there is, of course, one very fundamental difference between free-living Protozoa and the cells of even the simplest metazoan. In the latter case the cells stick together and so form a coherent colony. In other words, the formation of an epithelium of adherent cells is a step which has to be taken by any unicellular organism before it can become colonial. Whether one considers that metazoan colonies have arisen by the coherence of unicellular organisms after division, as the result of random contacts among adhesive cells, or whether one thinks that they have arisen by the ultimate subdivision of the cytoplasm of a large multinucleate organism, in the end the problem remains the same; namely, how and why do the cells remain combined as a colony? This adhesiveness between cells is fundamental to the establishment of a stable metazoan organism. *N. gruberi* normally shows few signs of adhesiveness among its cells either in the amoeboid or in the flagellate form, though when the
'cells encyst these cysts usually stick together in clusters. However, one observation on the otherwise amoeboid form may be worth recording. When a drop of a thick suspension of amoebae, carefully washed so as to be nearly free of bacteria, etc., is dropped on to the surface of an agar plate in a Petri dish, the cells generally spread out quickly. On one or two occasions, however, they have formed a continuous sheet in which the cells have taken up particular positions and almost ceased to move relative to each other. Normally they creep freely over each other. When such a sheet of relatively static cells was fixed in mercuric chloride, which is a very suitable fixative for amoebae, the cells apparently remained adherent except in a few places where the sheet as a whole cracked across, presumably due to the shrinkage. The fixed material certainly gave the impression that the sheets formed in this way were composed of cells which had acquired the property of adhering to each other. Other observers have also noted a tendency for the cells to stick together under some conditions (Bunting, 1926; Wilson, 1916) and the formation of the sticky threads mentioned above may be connected with the phenomenon.

How this behaviour is related to the drying up of the colony or to the change to the cyst form has not yet been investigated. Among higher organisms it is very rarely that living cells can be found in direct contact with air, uncovered by a fluid film; thus it may be that the amoebae are also adversely affected by this condition and change their behaviour accordingly.

It appears therefore that in *N. gruberi* we have an organism which can exist at different times as isolated amoebae without definite polarity, as isolated amoebae with an antero-posterior axis, as isolated and polarized flagellates, as cysts, and possibly also as a coherent epithelium. In other words, this single organism shows at least three of the main characteristics of the cells of primitive metazoan larval forms. In the amoebae these states exist at different times; in the metazoan larva they exist simultaneously but are partly distributed in space. In the amoebae the states are certainly interchangeable, and they may often be so in the more primitive Metazoa and their larvae, though, in general, differentiation of cells among the Metazoa is well known to become progressively less and less a reversible process as the evolutionary complexity of the organisms increases.

**PHYSIOLOGICAL**

An adequate stimulus for the production of the flagellate form is to place the amoeboid form in distilled water. The first problem to be solved, therefore, concerns the means by which the distilled water exerts its effects. Some of the possibilities may, for convenience, be listed. The subdivisions are arbitrary and obviously some of the mechanisms are closely connected with one another. The list will, however, help to bring some order into the varied aspects of the problem.

1. Mechanical effects (Alexeieff, 1924; Wilson, 1916).
   1. Absence of contact with a wetable surface.
   2. Absence of proximity to, or of contacts with, neighbouring cells.
   3. Mechanical effects of currents in the fluid, etc.
(2) Changes in gaseous exchange.
   (a) Oxygen (Hollande, 1942; Wherry, 1913).
   (b) CO₂.

(3) Physicochemical changes.
   (a) Osmotic pressure (Hollande, 1942; Whitmore, 1911).
   (b) Hydrogen-ion concentration (Hollande, 1942).
   (c) Loss of essential ions (Hollande, 1942; Rafalko, 1951; Wasielewski & Hirschfeld, 1910).
   (d) Changes in the nature of the cell membrane: a, passive; b, reactive.
   (e) Loss of essential organic constituents.

(4) Nutritive effects.
   (a) Absence of bacteria (Schardinger, 1899).

Some of these possibilities will now be considered.

(a) Contact with surface

The acquisition of flagella can be followed on the microscope stage in cells which are undisturbed and which maintain their contact with the glass until they are practically free swimming. It does not, therefore, seem to be necessary for the cells to lose contact with the surface before the change can occur. The change can certainly be initiated while the cells are creeping normally.

(b) Contact with neighbouring cells

While the stock cultures are often very crowded, the amoebae normally live as more or less isolated individuals on the surface of the agar; nevertheless, in view of the well-known effects of isolation in depressing the activities of single cells of other types, e.g. those of metazoan tissue cultures or of sea-urchin eggs, the mere dilution of the amoebae could be important. That it is not a very important factor is fairly clearly shown by Figs. 3 and 4.

The figures show the effect of seeding varying numbers of amoebae into a constant volume (0.5 ml.) of distilled water on the numbers of flagellates which can be counted at various times after seeding. It seems to be clear that the proportion of amoebae which become flagellate, at least within the limits used (limits which were set by the possibility of obtaining reasonably accurate counts), is nearly independent of the number seeded. There is some tendency for more flagellates to appear in the higher concentrations, but this might well be due to secondary effects, as, for example, the population of bacteria in the cultures or multiplication of the cells (Fig. 4). In most cases the number of flagellates increases progressively and more or less linearly between the second and the seventh hours, after which time the numbers become much more constant, and then finally decline. There is no doubt that the curves, expressing numbers of flagellates against time during the period when the cells are acquiring flagella, would all in the end be more or less S-shaped. In some experiments they are more noticeably so than in others. In these cultures very few flagellates appear before about 2 hr. have elapsed after
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...seedling; sometimes, especially in winter, this interval is considerably longer. Presumably temperature may be one of the factors affecting the results (Schardinger, 1899) and the observations reported in this paper have all been made at room temperature.

In most of the experiments which are to be described the cultures have been set up in the late morning, about noon, and the flagellates counted in the evening. An obvious error arises in consequence of this, since the rising phase of the curves (Fig. 3) is still in progress at this time, and if there are many tubes to sample and count the interval between the first and the last count may be sufficient to cause a significant difference in the numbers of flagellates recorded, since the cultures thus counted are necessarily at different phases of their activity. In all the later experiments some correction, when necessary, has been made for this by first plotting the numbers found at the actual time of observation and then (using the information plotted in Fig. 3) drawing lines to connect these points to zero at 2 hr. after planting. All counts can then be approximately standardized to a given time, say 6 hr. after seeding, and so made more nearly comparable. This method, which is still only an approximation, was not immediately elaborated, and in many of the earlier experiments recorded here corrections of this kind were not made. This is not considered, however, to vitiate in any way the general results reported, though the detailed figures may be less accurate than one would like.

![Fig. 3](image)

**Fig. 3.** Rate of increase in numbers of flagellates related to the initial concentrations. The figure shows the results in two representative experiments. In the first experiment (○ — ○) initial concentrations in the proportions 8, 4, 2 and 1 were used. The numbers of flagellates did not increase much after the first 6 hr. and the curves are somewhat S-shaped. In the second (× — — — ×), the numbers of flagellates increased progressively and almost linearly during the same period.

![Fig. 4](image)

**Fig. 4.** The relationship between the numbers of flagellates formed and the relative numbers of amoebae inoculated. Combined results of three experiments. The number of flagellates formed is clearly proportional to the number of amoebae inoculated, but as the number of amoebae initially present is increased, so the proportion which becomes flagellate at any one time is somewhat greater than expected. The dotted line shows direct proportionality. This effect could be due to multiplication of amoebae during the experiment.
(c) Effect of currents in medium

The mechanical effects of currents have not been actually investigated as yet and
it will be difficult to dissociate them from the indirect action of currents in accele-
rating loss of ions, etc. There have been some indications that, in contrast to some
previous observations (Alexeieff, 1924), the more the cells are disturbed the more
flagellates appear, but so far no quantitative data have been obtained on this point.

(2) Changes in gaseous exchange

At first this was thought to be unimportant, since oxygenation is presumably
quite adequate for the amoebae on the surface of agar slopes in test-tubes. The
same must apply to the removal of CO₂. Nevertheless, when attempts were made
to follow the details of flagellum formation under the microscope in contact pre-
parations, i.e. in the fluid contained between the cover-slip and slide, where there
is likely to be restricted access of O₂ and also accumulation of CO₂, the numbers of
cells which became flagellate in a given time was definitely reduced, as Wherry (1913)
had observed, particularly towards the middle of the cover-slip. On the other
hand, in experiments in closed tubes with an air space and with a side-arm to
contain gas absorber, no effects have been noticed in the number of cells which

<table>
<thead>
<tr>
<th>Total flagellates counted</th>
<th>With CO₂ absorbent</th>
<th>Without CO₂ absorbent</th>
</tr>
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<tbody>
<tr>
<td>No. of tubes</td>
<td>1577</td>
<td>1426</td>
</tr>
<tr>
<td>Flagellates per tube</td>
<td>42</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>37</td>
</tr>
</tbody>
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became flagellate when the O₂ or CO₂ were reduced by absorption in alkaline
pyrogallol or in 40% NaOH, respectively. The absence of any effects of absorbing
the CO₂ with NaOH is shown in Table 1. As will be seen later, dissolved CO₂
and the bicarbonate ion may be important in connexion with the hydrogen-ion
concentration of the medium, and the latter is probably important in its own
right.

Obviously the respiratory metabolism of the cells will have to be investigated in
much more detail, but until the amoebae can be obtained free from living bacteria
and cultured in their absence no great advance can be made in this direction. From
the present point of view there is no evidence that conditions of oxygenation or of
CO₂ elimination are the determining factors in causing the amoebae to become
flagellate, though both may be of subsidiary importance. Some effects in restricting
the numbers of flagellates formed which were observed with methylene blue in
the medium, and which will be described in more detail in another paper, should
perhaps be considered in relation to their bearing on respiration as well as in rela-
tion to the context in which they will be discussed.
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(3) Physicochemical changes

(a) Osmotic pressure

The effects of osmotic changes, as such, can be assessed by comparing the behaviour of the amoebae in solutions of NaCl, glucose, sucrose, etc., of varying concentrations. M/2.5 solutions of these substances were therefore prepared in distilled water and serial dilutions made from them so that the concentration was halved each time. When these dilutions were seeded with amoebae, and the numbers of organisms estimated at intervals thereafter, it was found that the amoeboid form survived concentrations of M/5 glucose and sucrose for at least 24 hr. In M/2.5 solutions, the amoebae shrivelled and rounded off, though a few might remain active for a time. In sodium chloride, the highest concentration for stability was M/10 with some temporary survival in M/5 (Fig. 5). These figures, therefore, suggest

![Fig. 5. The effects of sucrose, glucose and certain cations on the behaviour of the amoebae. The curves represent the effects of different molar concentrations of the chlorides on the number of flagellates which form as compared with the number which form in parallel experiments in distilled water alone (standardized at 100). Although the actual number which form in water is unaccountably variable, it does provide a standard by which different experiments, with different cultures of amoebae, can be compared. All the salt solutions used depress the formation of the flagellate form, sucrose and glucose appear to encourage it, but this may be simply because they act as food, so that the amoebae remain in better condition, perhaps even multiplying. The maximum concentrations at which normal amoebae were observed to be active are shown by the appropriate symbols in the top left corner.](image)
that osmotic effects become too powerful for the amoeboid form at about these concentrations. The NaCl, as might be expected because of its ionization, was about twice as potent as the unionized sugars, and CaCl₂ and MgCl₂ still more so. In a preliminary experiment with urea and hexamine (hexamethylenetetramine), amoebae survived in an active form in M/2·5 in each case. The osmotic effects here can be presumed to be less, since these substances probably penetrate into the cell rather easily.

In glucose, sucrose, urea and hexamine solutions the flagellate form appeared in all concentrations up to those which were lethal to the amoeboid form, though the numbers appearing showed (except in the sucrose solutions) a tendency to fall off at the highest concentrations, thus indicating that the flagellate form is not so stable as the amoeboid form and is more easily depressed, a fact which has been abundantly confirmed throughout all the observations recorded in this paper.

In sodium chloride solutions, on the other hand, the flagellate form became less and less numerous as the concentration was increased above M/1280, and flagellates were very rare in concentrations greater than M/40.

These observations therefore indicate that osmotic pressure as such is not the determining factor in causing the change from the amoeboid to the flagellate form, though it again may exert its influence, as might be expected, on the activity of the organisms.

(b) Hydrogen-ion concentration

The amoebae can stand a very wide range of hydrogen-ion concentration; at least they are tolerant of such for a limited time. It must be remembered, however, that the effects reported here are only those observed within the first hours of seeding the amoebae into solutions of varying hydrogen-ion concentration. Nevertheless, it may be stated at once that the hydrogen-ion is not, in itself, the determining agent in causing the assumption of the flagellate form, and indeed the effects of pH are often secondary in other respects to those of the positive and negative ions present in the buffer solutions used. Flagellates may appear at any pH between 5·5 and 10, though there is generally a definite optimum and this optimum depends on the buffer solutions used (Figs. 6, 7). For example, in buffer solutions made by mixing M/160-NaH₂PO₄ and M/160-Na₂HPO₄ (Fig. 6) the maximum lies between pH 6 and 7, and definitely fewer flagellates occur in solutions on the alkaline side of neutrality. On the other hand, in mixtures of M/160-NaH₂PO₄ and M/160-Na₂CO₃ there is a definite maximum between pH 7·5 and 8, and in this solution alkalinity favours the flagellates. In mixtures of lactic acid and sodium carbonate flagellates were abundant on the alkaline side but were entirely absent at pH 6 (Fig. 8). It is thus obvious that while pH, as in all biological systems, is important it does not appear to be the critical factor in relation to the flagellate condition. Similar observations have also been made with other concentrations of the buffer solutions, and it is noticeable that as their molarity is increased so the numbers of flagellates is at first increased and then rapidly decreases again (Table 2). Fig. 7 shows the effects of pH in pure phosphate, and in phosphate-bicarbonate,
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Fig. 6. A typical experiment showing the effects of pH on the numbers of flagellates formed (each point represents the mean of six tubes) in M/160-NaH$_2$PO$_4$ + M/160-Na$_2$CO$_3$ mixtures (● — ●) and also in M/160-NaH$_2$PO$_4$ + M/160-Na$_2$HPO$_4$ mixtures (+ — +). The short lines represent the numbers which became flagellate in the relevant distilled water controls. The vertical bars represent twice the standard error of the mean.

Fig. 7. Graphs showing the influence of certain anions on the pH optima for the acquisition of flagella, at two different concentrations. Phosphate buffers produce their maxima on the acid side and phosphate-bicarbonate buffers on the alkaline side of neutrality. ● — ●, NaH$_2$PO$_4$ + Na$_2$HPO$_4$; ○ — ○ NaH$_2$PO$_4$ + NaHCO$_3$.

Fig. 8. Graph showing the results in a single experiment with cultures in mixtures of lactic acid and sodium carbonate at different pH values (means of initial and final values determined colorimetrically, and thus approximate only). Note the large numbers of flagellates in the alkaline solutions as compared with those in the distilled water controls (+).

Table 2

<table>
<thead>
<tr>
<th>Molarity of 1:1 mixture of NaHCO$_3$ and NaH$_2$PO$_4$</th>
<th>Relative no. becoming flagellate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (distilled water)</td>
<td>100</td>
</tr>
<tr>
<td>M/320</td>
<td>316</td>
</tr>
<tr>
<td>M/160</td>
<td>228</td>
</tr>
<tr>
<td>M/80</td>
<td>180</td>
</tr>
</tbody>
</table>
buffers at $M/80$ and $M/320$. With higher concentrations the pH optimum for the flagellate form seems to creep towards the acid side with the purely phosphate buffers, and definitely to the alkaline side with the phosphate-bicarbonate systems. How much the extra sodium contributes to the adverse effects of alkaline phosphates is a moot point.

(c) Loss of essential ions

In the experiment reported above on the effects of osmotic pressure and of pH various ions have been used to produce the requisite solutions, and it is abundantly clear that the nature of these ions is a matter of no little importance. The questions, in addition to those of osmotic pressure and pH, which are raised by the effects of various ions on the behaviour of these amoebae are, of course, numerous and diverse and could be tackled in a variety of ways. On the whole, biological systems are mostly dependent on a proper balance of certain inorganic salts in their environment. $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$ are important cations, $Cl^-$, $PO_4^{2-}$, $HCO_3^-$, and lactate are often important anions. A beginning has therefore been made in studying the response of *Naegleria* to some of these biologically important ions in order to see which of them act as determining factors in altering the 'phase' of the amoebae, i.e. from the amoeboid phase to the flagellate phase or vice versa. $M/5$ solutions of various salts have been made up and, from these, serial dilutions ($\times 2$) have been made and their effects on the amoebae studied. In some cases, the serial dilution modifies the hydrogen-ion concentration, so that the effects of this have to be disentangled from those caused by the ions under investigation.

A comparison has been made of the various positive ions by making serial dilutions of the chlorides. The results are summarized in Fig. 5, which also includes the results of adding lithium chloride; the interesting actions of the lithium ion will be discussed below.

All the ions suppress the formation of flagella to some extent as compared with solutions of sugars or with distilled water. There is not a very great difference between $K^+$, $Na^+$ and $Ca^{2+}$; they all begin to reduce the numbers of flagellates at concentrations greater than $M/1280$, the $K^+$ ion perhaps being the least depressing. On the other hand, magnesium is very effective in maintaining the amoeboid form and suppressing the change into the flagellate condition. Even in concentrations as low as $M/1280$ there is a very pronounced reduction in the number of flagellates as compared with that found in the distilled water controls. There is some suggestion that this action of $Mg^{2+}$ is one of delaying the onset of the change to the flagellate form, since in a few cultures examined after 24 hr. flagellates were found in considerable numbers. The amoeboid form persists in an active state in $Mg^{2+}$ concentrations up to $M/20$. This limit is somewhat lower than those for the monovalent salts (Fig. 5), but there is obviously a large range of concentrations in which the amoeboid form is stable and in which the tendency to become flagellate is suppressed. A somewhat similar state of affairs is found with lithium, but whereas the effects of magnesium gradually decrease with decreasing concentration, the effects of lithium seem to come on more suddenly at about $M/1280$. Another point about
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lithium is that at the highest concentrations, before it becomes lethal (i.e. about M/20), it causes the amoebae to assume very characteristic shapes as shown in Fig. 9. The long cylindrical process is somewhat reminiscent of the change of form which frequently occurs in the choanocytes of sponges (Sycon) when these organisms are dissociated into sea water. The significance of the change is obscure.

From the results shown in Fig. 5, it seems to be fairly clear that the presence of the usual biologically active ions in the environment opposes the formation of flagella, so that it would appear likely that the flagellate phase is perhaps an adaptive form to counteract loss of these ions, and vice versa, that the stability of the amoeboid form depends on their presence in the medium.

![Fig. 9. Diagrams to illustrate the forms commonly assumed by Naegleria gruberi in solutions of lithium salts at about M/20 concentration.](image)

In addition to those mentioned above, because of their influence on pH, the effects of some anions have also been studied by using serial dilutions of various sodium salts. The results are plotted in Fig. 10. Sodium sulphate and chloride tend to stabilize the amoeboid form and to prevent the change towards the flagellate form. The sulphate ion is particularly effective in this way, but it also proves to be somewhat toxic to the amoeboid form. On the other hand, the lactate, bicarbonate and phosphate ions all favour the production of the flagellate form when present in concentrations between M/80 and M/5120. The effects of the last two ions have, of course, to be considered in relation to the pH effects, since dilution of these salts lowers the pH. As we have seen above, alkalinity in phosphate solutions is not favourable to the flagellate form, so the beneficial effects of phosphate in the flagellate transformation are confined to very dilute solutions. On the other hand, quite high concentrations of bicarbonate solutions are consistent with the change to the flagellate form.

Some interesting results were obtained when cultures were made by adding the various salts to a buffer solution (pH approx. 8·0) consisting of:

- NaHCO₃ 0·84g. (0·01 M)
- KH₂PO₄ 0·23g. (0·00167 M)
- Distilled water 1000 cc.
Fig. 10. The effects of different concentrations of anions in favouring or hindering the assumption of the flagellate form. The ordinates express the numbers of flagellates formed in the different concentrations of sodium salts relative to the number formed in distilled water, which was standardized at 100. The isolated points (top left) illustrate the molarities at which amoeboid (as opposed to flagellate) activity is suppressed. ■ --- ■, sodium bicarbonate; ● --- ●, sodium lactate; x --- x, di-sodium hydrogen phosphate; + --- +, sodium chloride; ○ --- ○, sodium sulphate.

Fig. 11. Diagrams showing the difference in the action of sodium lactate and sodium sulphate when these salts are applied in distilled water (○ --- ○) or in a bicarbonate-phosphate buffer (pH 8.0 approx., see p. 599) (● --- ●). In each case the ordinates represent the numbers of flagellates formed as percentages of those formed in the water or buffer solution alone.
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It was then found that there was much less difference between the effects of the anions. Sodium sulphate became much less inhibitory to the formation of the flagellate type and sodium lactate less stimulating; that is to say, there was no evidence that lactate increased the numbers of flagellates over and above that found in the buffer solution alone, which was, of course, greater than in water alone owing to the presence of bicarbonate (Fig. 11). There must thus, as in other situations, be considerable interaction between the ions in determining how the cells behave. It would seem that the bicarbonate, the lactate and, in dilute solutions, the phosphate ion all have similar effects; all encourage the formation of the flagellate form while the sulphate ion, on the other hand, depresses the metamorphosis, but its action is largely annulled by the presence of bicarbonate. When lithium salts were studied in this way, instead of the sodium salts, it was again found that the sulphate became less inhibitory to the change to the flagellate form when it was applied in bicarbonate buffer solution; but so also did the lactate, in contrast to the behaviour of sodium lactate (Fig. 12). In buffer solution there was indeed little to choose between the actions of lithium chloride, lactate and sulphate; all were less inhibitory than they were when applied in water and the difference in the case of the sulphate was very large.

DISCUSSION

The capacity of Naegleria gruberi to change from one form of cell function to another in response to changes in its environment offers a splendid opportunity to investigate in isolation one of the types of change in cellular activity which occurs among metazoan cells both during embryonic development and also in cases of metaplasia later in life.

On the addition of water, Naegleria changes from being a more or less non-polar cell which creeps about, mostly by means of lobose pseudopodia and without any clear orientation, to become a highly polarized cell, progressing by means of flagella.
Almost nothing is known of the nature of this change. Since it occurs when the cells are placed in water it may be an attempt by the cell to counteract the loss of important constituents. It seems likely enough that in distilled water the amoeba should lose salts, and that to counteract this loss it may assume the flagellate form, perhaps with a different mechanism for maintaining water and salt balance, and almost certainly with a modified cell surface. Thus, temporarily or perhaps permanently stabilized, it swims away to find some more suitable environment. From an ecological point of view this may perhaps be considered as an adaptation, in that, when water is abundant in the environment of the amoeba, the animal may benefit from it to extend the range of the species more quickly than it could do in the amoeboid form.

Magnesium, which in many cells is known to decrease permeability, may act by checking this loss of essential ions, and so rendering unnecessary the compensating change of form. Bicarbonate and lactate ions, on the other hand, encourage the metamorphosis and favour the flagellate form. The action of the magnesium ion in stabilizing cell matrices in higher animals and its action in binding proteins and polysaccharides may be significant for the amoeba. The trails of protoplasm and the somewhat glutinous uroid of the polarized amoeba should perhaps be investigated from this point of view.

It was mentioned earlier that the behaviour of *Naegleria* might throw light on the nature of the gradient which exists in many embryos and larvae and which may manifest itself by the larva possessing flagellate cells in the anterior (or animal) half, and cells of a more phagocytic character in the posterior (or vegetal) half. It is well known that this gradient can be upset by the presence of lithium salts, and it is of particular interest therefore to find that lithium salts are among the effective agents which favour the amoeboid form of *N. gruberi* and suppress the flagellate form. It is true that on many marine embryos the lithium salts are effective in the presence of sea water, while in the case of *Naegleria* the presence of even small amounts of sodium bicarbonate and potassium phosphate lessens their effect; nevertheless, the action of lithium salts is similar in the two cases in that it suppresses the flagellate form. It would be interesting to know more of the effects, if any, of magnesium salts on the vegetalization of embryos.

The two other changes of form which *Naegleria* shows in response to changes in the environment may also be important in relation to the formation of colonies of cells and the early differentiation of Metazoa. Under unfavourable conditions of food supply (e.g. after about 10 days on the agar-Lemco culture slope) *Naegleria* encysts, and the cysts have that capacity for sticking together which is such a necessary preliminary to colonial organization. A similar tendency for the cells to stick together has also been shown by the amoeboid form itself when plated out in large numbers on to the relatively dry surface of an agar gel in a Petri dish; under these conditions the amoebae were observed to form what appeared to be an epithelial sheet.

It thus appears that *N. gruberi* may prove to be an important and readily available organism for the experimental study of some of the primary manifestations of cellular differentiation and perhaps also of colony formation.
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SUMMARY

1. When placed in distilled water *Naegleria gruberi* changes from an amoeboid organism, with little evidence of polarity, to a highly polarized free-swimming flagellate. The details of this metamorphosis are described. The change is reversible.

2. Alteration of osmotic pressure is not in itself the direct cause of the metamorphosis, though the loss of certain ions is clearly important.

3. The metamorphosis is favoured by the presence of low concentrations (less than \(\frac{M}{80}\)) of sodium bicarbonate, sodium lactate and sodium phosphate.

4. The flagellate form probably occurs most frequently in conditions of neutrality; but, in the presence of phosphate, acid conditions tend to be more favourable to the flagellate form, while in the presence of bicarbonate the optimum pH is nearer pH 8.0.

5. The metamorphosis to the flagellate form is suppressed by a variety of agents including lithium salts, magnesium chloride and the sulphate ion under some conditions. These all act at concentrations which leave the amoeboid form in full activity. In some cases their action is decreased by the presence of bicarbonate in the medium.

REFERENCES


