Further Observations on the ‘Metaplasia’ of an Amoeba, *Naegleria gruberi*

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The development of flagella by *Naegleria gruberi*, which occurs when a culture of organisms in the amoeboid form is treated with distilled water, has been shown (Willmer, 1956) to be a phenomenon probably related more to the ionic balance between the organism and its external environment than to the simple movement of water in and out of the cell in response to total osmotic changes. From Text-fig. 1 it may be seen that with respect to the response of the amoeba to different concentrations of the various salts investigated there are for each

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Text-fig. 1. Action of various kations on the numbers of amoebae which become flagellate as percentage of the number becoming flagellate in distilled water \([=100]\).

Ordinates: percentage which become flagellate in experimental solution.
(Number in distilled water \(=100\).)

Abscissae: molar concentrations of solutions.

The arrows represent the K and Na content of *Spirostomum* as determined by Carter (1957). The crosses with appropriate symbols represent the concentrations at which the amoeboid form is damaged, and rounds off.

- \(\text{NaCl}\)
- \(\text{CaCl}_2\)
- \(\text{KCl}\)
- \(\text{MgCl}_2\)
salt two somewhat critical concentrations. In the case of NaCl solutions in distilled water these turning points occur at about 30 mM. and 1 mM., and in the case of KCl solutions at about 50 mM. and 6 mM. Above the higher of these concentrations in each case the organisms always assume the amoeboid form, and below the lower they become flagellate at least as frequently as they do in distilled water.

As with some other protozoa (e.g. *Paramecium* (Hayes, 1930); *Spirostomum* (Carter, 1957)) there may well be some concentration of cations within the cell which can be considered as an equilibrium concentration and which can be maintained with the minimum expenditure of energy or which is, for some other reason, 'preferred' by the cell. This concentration may not be rigidly fixed but may perhaps vary to a limited extent according to the external conditions. Indeed, in some other species of amoebae (e.g. *Amoeba* (*Mayorella*) *lacerata* (Hopkins, 1946), in *Paramecium* (Kamada, 1935, 1936) and in the peritrich ciliate *Vorticella* (Mast & Bowen, 1944), which maintains an osmotic pressure difference of about 0.33 atmospheres above its surroundings, there is evidence that the organisms can adapt themselves in this way to changed osmotic conditions, though the part that ions play in this adaptation cannot at present be distinguished from that of other osmotically active substances. Sooner or later, however, such organisms, if they are to preserve their equilibrium, must adopt means of forcibly maintaining the internal concentration against further gain or loss of ions or of water.

There are unfortunately very few measurements of the actual ionic, as distinct from osmotic, concentrations within protozoan cells. In the recent paper by Carter (1957), however, figures are given for the potassium and sodium content of the ciliate *Spirostomum ambiguum*. In this species the internal potassium is regulated at about 7 mM. and the sodium between 1.01 and 1.49 mM. in spite of an almost fourfold increase in external sodium. It will be noticed first that, in *Spirostomum*, potassium is in excess and second that these figures correspond approximately to the lower figures for K+ and Na+ mentioned above for *Naegleria* at which these amoebae tend to assume the flagellate form. In some preliminary estimations by flame photometry of the sodium and potassium of *Naegleria* itself, carried out in collaboration with Dr. Whittam, the sodium content has been found to be higher than the potassium content in a ratio of about 4:1. The absolute concentrations are, however, at present uncertain. The estimates indicate that they may be considerably higher than those for *Spirostomum*, but the actual quantities being measured by the methods employed may be different in the two cases and so the figures may not be strictly comparable; for the present then the issue must remain undecided.

On the basis of the results shown in Text-fig. 1 it could be suggested with some justification that *Naegleria* assumes the amoeboid form when the external cation concentration exceeds the internal, and the flagellate form when the situation is reversed. The flagellate form may indeed be an adaptation towards cation
conservation, while in the amoeboid form the cell is more concerned with preventing the entry of cations. Water movements in the opposite direction may, of course, also be involved; i.e. the flagellate form needs to eject water while the amoeboid form may need to conserve it. In both forms there is a contractile vacuole.

There are many estimations, by a variety of methods, of the total osmotic concentration of the cytoplasm of different freshwater protozoa, though these are obviously not all relevant to the particular problem, for the internal osmotic

<table>
<thead>
<tr>
<th>Organism</th>
<th>Internal concentration (m.osmols/l.)</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chaos chaos.</td>
<td>94</td>
<td>Vapour pressure determination</td>
<td>Løvtrup &amp; Pigon (1951)</td>
</tr>
<tr>
<td>Amoeba proteus</td>
<td>20</td>
<td>Electrical conductivity</td>
<td>Gelfan (1928)</td>
</tr>
<tr>
<td>Chaos diffuens</td>
<td>5</td>
<td>Size changes in lactose solutions</td>
<td>Mast &amp; Fowler (1935)</td>
</tr>
<tr>
<td>Amoeba mira*</td>
<td>200 (approx.)</td>
<td>Vacuolar activity suddenly increases in dilutions of sea-water below 20 per cent.</td>
<td>Mast &amp; Hopkins (1941)</td>
</tr>
<tr>
<td>Amoeba lacerata*</td>
<td>200 (approx.)</td>
<td>Vacuolar activity becomes almost constant in concentrations of sea-water above 20 per cent.</td>
<td>Hopkins (1946)</td>
</tr>
<tr>
<td>Stentor</td>
<td>70</td>
<td>Electrical conductivity</td>
<td>Gelfan (1928)</td>
</tr>
<tr>
<td>Euplotes</td>
<td>75</td>
<td>Electrical conductivity</td>
<td>Gelfan (1928)</td>
</tr>
<tr>
<td>Oxytricha</td>
<td>100</td>
<td>Electrical conductivity</td>
<td>Gelfan (1928)</td>
</tr>
<tr>
<td>Spirostomum teres</td>
<td>77</td>
<td>Electrical conductivity</td>
<td>Gelfan (1928)</td>
</tr>
<tr>
<td>S. ambiguum</td>
<td>7 mM. K</td>
<td>Tracer technique</td>
<td>Carter (1957)</td>
</tr>
<tr>
<td>S. ambiguum</td>
<td>1 mM. Na</td>
<td>Tracer technique</td>
<td>Carter (1957)</td>
</tr>
<tr>
<td>S. ambiguum</td>
<td>52</td>
<td>Vapour pressure</td>
<td>Picken (1936)</td>
</tr>
<tr>
<td>Paramecium</td>
<td>120</td>
<td>Electrical conductivity</td>
<td>Gelfan (1928)</td>
</tr>
<tr>
<td>Paramecium</td>
<td>62</td>
<td>Contractile vacuole activity (in 25 mM. NaCl)</td>
<td>Kamada (1936)</td>
</tr>
<tr>
<td>Paramecium</td>
<td>50</td>
<td>Contractile vacuole activity (in distilled water)</td>
<td>Kamada (1936)</td>
</tr>
<tr>
<td>Paramecium</td>
<td>48</td>
<td>Minimal O₂ consumption</td>
<td>Hayes (1930)</td>
</tr>
<tr>
<td>Frontonia</td>
<td>106</td>
<td>Electrical conductivity</td>
<td>Gelfan (1928)</td>
</tr>
<tr>
<td>Vorticella</td>
<td>12</td>
<td>Size changes in lactose solution</td>
<td>Mast &amp; Bowen (1944)</td>
</tr>
<tr>
<td>Vorticella</td>
<td>50</td>
<td>Swelling of cyanide-treated organism</td>
<td>Kitching (1938)</td>
</tr>
</tbody>
</table>

* These two species can both adapt themselves to fresh water or sea-water.

concentration may be maintained not only by salts, but also by sugars, fatty-acids, amino-acids, proteins, &c. Nevertheless, from Table 1 it is clear that most of the figures for the osmotic concentration lie within, or only just outside, the critical range of concentrations mentioned above for Naegleria. Among them there are wide variations in the estimates, some of which may be due to the different methods employed which measure different things, some to technical difficulties in measuring such important things as, for example, the volume of an amoeba. If, however, the series of figures obtained by Gelfan (1928) for the
electrical conductivity are studied, it will be seen that *Amoeba proteus* has a much lower conductivity than any of the ciliates. If, therefore, the optimal internal ionic concentration of amoebae in general is initially low, the organism, in order to preserve this low concentration, is likely to be more often concerned with ejecting or preventing the entry of incoming salts than with concentrating them. If that of the ciliates is initially higher, then these organisms must normally be concerned with preventing the loss of ions, with actively accumulating ions from the surroundings or with the elimination of water drawn in by the higher osmotic pressure. As mentioned above, both *Paramecium* (Kamada, 1936) and *Vorticella* (Mast & Bowen, 1944), appear to be able to maintain a constant excess of internal concentration over the external concentration in spite of quite large changes in the latter.

So far as they go, therefore, these figures are all consistent with the idea already suggested that the flagellate form of *Naegleria* is concerned with ionic conservation and the amoeboid form with preventing the accumulation of ions. In *Naegleria*, for example, water appears to be always passing into the cell in all concentrations of NaCl in the external medium up to approximately 200 mM., for it is not until this value is reached that the contractile vacuole ceases to function (Wolff, 1927). Much the same is true for *Amoeba (Mayorella) lacerata* (Hopkins, 1946). In other freshwater species the water intake, or its production by metabolism, is connected with cell movement (Hogue, 1923) and the contractile vacuole may go on ejecting water when the organism is subjected to external media which are probably distinctly hypertonic to the internal contents of the cell.

With these considerations in mind, the experiments to be described in this paper have been designed to investigate the truth or otherwise of the hypothesis that the flagellate form of *Naegleria* has an inwardly-directed cation pump while the amoeboid form has an outwardly directed pump, and that the organism can turn on the one or the other in order to maintain its ionic content within certain limits, more or less independently of the external medium.

Some of the various substances known to alter the rate of sodium or cation transport in other situations, either directly or indirectly, have been examined for their action on the behaviour of *Naegleria*. Some attention has also been paid to the relative importance of sodium and potassium, and the available evidence indicates that in *Naegleria* the one may substitute for the other, though this does not mean that the amoeba has no ‘preference’ for the one or the other, when both are available.

**METHODS**

The methods employed have, in general, been similar to those described in connexion with the earlier series of experiments (Willmer, 1956). All observations have been made by counting the flagellate cells in a single circuit of standard-sized (0.01 ml.) drops plated out on a microscope slide, four at a time, and
examined under a 3/4 in. lens always keeping the edge of the drop in the field. All test solutions have been used in 0.5 ml. aliquots contained in thoroughly cleaned test-tubes and each inoculated with a single drop from a well-stirred suspension of amoebae previously washed as free from bacteria as possible (see below). Counts of those in the flagellate form have been made either on amoebae inoculated into serial dilutions (generally × 2) of the substance under investigation or into quadruplicate series of tubes containing the experimental media at particular concentrations.

The use of glass-distilled water, by itself, as a standard medium, for evoking the flagellate form, has been largely abandoned in favour of an extremely dilute phosphate buffer solution, and this change has led to much more uniform results. The buffer solution \( (B_2) \) was made up so that the final concentrations were as follows:

\[
\begin{align*}
0.2 \text{ mM} & \quad \text{KCl} \\
0.2 \text{ mM} & \quad \text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} \\
0.2 \text{ mM} & \quad \text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}
\end{align*}
\]

This solution has the advantage of preventing large pH changes in response to such random variables as small changes in CO\(_2\) content, and yet its salt content is not sufficient to cause any appreciable lessening of the numbers of amoebae which initially become flagellate when transferred to it, though, as will be seen, it probably hastens their ultimate return to the amoeboid condition.

In some experiments in which only the sodium ion or the potassium ion was required in the medium, the appropriate changes were made in this basic solution.

In the previous paper the rate at which the flagellate cells appeared after the amoebae were placed in experimental media was briefly discussed. In the buffer solution \( (B_2) \) the flagellate forms generally appear after about 2 hours, and the numbers increase for about another 3–4 hours, after which they remain more or less stationary for a time and then decline. This observation which is consistent with the view that the flagellate form is concerned with picking up Na\(^+\) (or other positive ion) has led to the practice of following the time-course of most experiments instead of making counts only at one time, e.g. after 4–6 hours; this in its turn has led to a better appreciation of the behaviour of the amoebae. For example, the effects of the age of the initial culture have become more obvious. In general, the amoebae from 4-day-old cultures, produce the flagellate form quickly and then revert quickly to the amoeboid form. Such amoebae are mostly large and active and very few are present as cysts. Five-day-old cultures have slightly smaller cells which react rather more slowly and are less regular in size. Still older cultures react even more slowly and irregularly, and more and more of the amoebae are found in the cyst condition.

There is still much that is not understood about the conditions of culture and their effects on the subsequent behaviour of the amoebae, for the amoebae from different stock cultures, in spite of rigid experimental conditions, are somewhat
variable in the way in which they respond to changes in their environment. Different preparations of amoebae, which appear to be closely similar, often give different results when subjected to apparently the same set of experimental conditions. This variability has always to be borne in mind in any interpretation of the results described. Some of the variability must arise from the manner in which the amoebae are grown on beef-extract-agar slopes with a somewhat unknown bacterial flora, and their state of nutrition may well influence the way in which the cells respond to the methods used for freeing them from bacteria for experimental purposes.

Two methods have been used for washing the amoebae and sometimes a combination of both was necessary. The amoebae have generally been washed by repeated centrifugation from relatively large volumes of buffer solution (B₂) but occasionally by allowing the cells to settle out from suspensions in B₂ on to the bottom of a clean and sterile Petrie dish about 1½ in. in diameter. After about 20 min. in the dish, by which time the majority of the cells were actively creeping on the glass surface, the cells were very gently washed with frequent changes of B₂ till most of the bacteria were removed. This latter method is convenient when the bacteria from the stock culture tubes happen to be easily thrown down with the amoebae in the centrifuge. It is very satisfactory for obtaining clean, active, and more or less uniform amoebae, though the wastage is somewhat greater than by the centrifuge method. When the amoebae are thoroughly cleaned they can be readily dislodged from the glass by more vigorous washing and then concentrated by gentle centrifugation.

The effects of these 'washing' procedures on the amoebae may also contribute to the variability of behaviour. Many metazoan cells lose ribosenucleoproteins, and phosphates when washed in simple salt solutions, and there may be many other properties of the amoebae which change when the organisms are washed free from their normal growth medium and their usual bacterial population. Moreover, the importance of these losses or changes may well vary with the initial state of nutrition of the amoebae. Clearly, there would be enormous advantages in being able to keep Naegleria in a standard synthetic medium, and using the cultures at standard ages.

The pH of all solutions has been adjusted to pH 6·8, and within the duration of the experiments there has not been found to be any significant departure from this value. Appropriate adjustments have been made in all control solutions for alterations in the concentrations of other ions, caused by correction of pH.

**EFFECTS OF CHOLINE AND RELATED COMPOUNDS**

The investigations of Koch (1954) on the active uptake of sodium by the gills of the crab, together with the association of cholinesterase and acetyl choline with ciliated membranes (Bülbring, Burn, & Shelley, 1953; Kordik, Bülbring, & Burn, 1952) and in various ciliate (Bayer & Wense, 1936) or flagellate organisms
(Bülbring, Lourie, & Pardoe, 1949) suggested that choline and similar compounds might be of importance in relation to the ionic balance of these amoebae, particularly of those in the flagellate state. It is interesting, for example, that a trypanosome produces acetyl choline, but the more amoeboid malarial parasite does not (Bülbring, Lourie, & Pardoe, 1949). Moreover, the observations of Thomas (1936) and of Chèvremont & Chèvremont-Comhaire (1945) on the action of choline and related compounds in favouring the amoeboid (macrophage) form of cells in tissue cultures suggested that choline and quaternary ammonium compounds might favour the amoeboid form of *Naegleria*.

For these and similar reasons amoebae were treated with serial dilutions of choline chloride after washing with several changes of distilled water or buffer-solution (*B*₂). The results are shown in Text-fig. 2 and it is evident that choline chloride acts on the amoeba in much the same way as does NaCl. It is not immediately toxic to the organism in the amoeboid form till a concentration of about 200 mM. is reached, but the flagellate form begins to be suppressed at concentrations higher than 0.5 mM. and flagellates are practically absent above about 25 mM.
When flagellates were placed in solutions of choline chloride (20 mM. and 6 mM.) there was an almost immediate reduction in their numbers as compared with controls simultaneously placed in distilled water (Table 2).

Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Test solution (mM/l.)</th>
<th>Control medium</th>
<th>Duration of test (hours)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline</td>
<td>6.25</td>
<td>Distilled water</td>
<td>2</td>
<td>37.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Distilled water</td>
<td>5</td>
<td>17.5</td>
</tr>
<tr>
<td>Tetramethylammonium</td>
<td>10</td>
<td>$B_2$</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Tetraethylammonium</td>
<td>6.25</td>
<td>$B_2$</td>
<td>4</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>$B_2$</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Pentamethonium</td>
<td>10</td>
<td>$B_2$</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>$B_2$</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Hexamethonium</td>
<td>10</td>
<td>$B_2$</td>
<td>8</td>
<td>25</td>
</tr>
</tbody>
</table>

This reduction, without complete suppression, is somewhat difficult to interpret. It may indicate that the flagellate form is altered back to the amoeboid form by the choline, but that not all the cells are affected by the concentrations used, or it may be that there is normally some cycling between the amoeboid and the flagellate states by cells approaching equilibrium, and that choline prevents those cells which revert to the amoeboid form from again becoming flagellate.

These results with choline suggested that perhaps acetyl choline would be more active than choline itself. However, this was not found to be so. Acetyl choline caused the amoebae to round up and become inactive at concentrations of about 0.5 mM. and higher, but in the experiments here described there was no indication of any differential action on the two forms at lower concentrations. Moreover, 1/3,000 acetyl choline added to a suspension of flagellates did not have any apparent and immediate effect.

Nevertheless, in view of Koch’s observations on crabs’ gills, the action of anticholinesterases was also investigated. The action of eserine sulphate (10⁻³–10⁻⁶M) was first tried. The results were anomalous. In three experiments fairly high concentrations of eserine (1: 4,000 to 1: 32,000) produced more than twice as many flagellates as the controls, but later repetitions (4 experiments) failed to show any such action. Concentrations of 1: 1,000 and higher were toxic. In any case eserine never had any action at those concentrations in which, in other situations, it is known to poison cholinesterase (i.e. about 10⁻⁵M), so that whatever may have been the cause of the anomalous results at concentrations around 10⁻⁴M it was probably concerned with factors other than cholinesterase, and possibly with other esterases.
Similar results were obtained with DFP (diisopropylfluorophosphonate, kindly supplied by Dr. B. C. Saunders) for this inhibitor of cholinesterase showed little selective action on either the amoeboid or flagellate form. It became toxic to both at concentrations greater than about $10^{-4}$ M and flagellates occurred in normal numbers until concentrations greater than $10^{-3}$ M were used.

It may be concluded, therefore, that under the conditions of these experiments acetyl choline and cholinesterase are not immediately concerned with the change of form in the amoebae.

It appeared from these results that the action of choline was not augmented by its acetylation, and other substances related to choline were therefore examined.

These included betaine, ammonium chloride, trimethylamine oxide, tetramethyl ammonium iodide and bromide, tetraethyl ammonium bromide and iodide, tetrabutylammonium chloride, pentamethonium iodide, hexamethonium iodide, and decamethonium iodide, as well as one or two other substances, e.g. cetyl trimethyl ammonium bromide containing quaternary ammonium groups. Ammonium chloride was found to behave very similarly to sodium chloride and to choline (Text-fig. 3). So also did the tetramethyl, tetraethyl, and tetrabutyl ammonium compounds. Trimethylamine oxide and betaine were both relatively
inactive. Cetyl trimethyl ammonium bromide was rather toxic even at 0.001 mM., but at great dilutions it showed some selective action in favour of the amoeboid form. The methonium compounds containing two quaternary nitrogen groups were very potent in suppressing the flagellate form and maintained the cells as amoebae over a very wide range of concentrations (100 mM.–1 mM.; Text-fig. 3). Like choline, these compounds when applied directly to the cells in the flagellate form also reduced the numbers of flagellates, but, as before, it was not clear whether this was a direct action or was due to reverting amoebae being fixed in the amoeboid form (Table 2).

All the quaternary ammonium compounds which suppressed the flagellate form also produced interesting morphological changes in the amoebae. In concentrations between 25 mM. and 3 mM. there was a great tendency for the amoebae to contain large vesicles, which often resulted in the cells becoming spherical and inactive. This inactivity, however, was not due to the toxicity of the compounds for at higher concentrations of the drugs the organisms again became actively amoeboid, though they then showed a sharp distinction between a granular endoplasm and wide hyaline ectoplasmic pseudopodia. This phenomenon was particularly noticeable with the methonium compounds, and is perhaps connected with a change in some of the cell proteins to a more coiled or contracted form (Marsland, 1956). Perhaps relevant to this are some unpublished observations by Mr. T. Vickers on the effects of decamethonium iodide and tetraethylammonium chloride on certain cells in both fish and rats. He found that these compounds produced well-developed intracellular canals in the exocrine cells of the pancreas and in the cells of the liver and kidney tubules, and that the oxyntic cells of the gastric mucosa showed large vesicles. A similar development of large vesicles occurs on treatment of the amoeba Chaos chaos, with 5 mM. adenosine triphosphate (Kriszat, 1950), on feeding Paramecium with thyroid (Shumway, 1917) and on treating Paramecium with adrenalin or with anterior pituitary extract (Flather, 1919).

The quaternary ammonium compounds were applied to the amoebae either as chlorides, bromides, or iodides. It was therefore necessary to investigate the actions of bromides and iodides to ascertain that the observed actions were not due to these anions rather than the cations. Bromides of sodium and potassium were found to behave very like the chlorides. The action of the iodides was, however, interesting in that not only could the iodide ion not be held responsible for the complete inhibition of the flagellate form, e.g. at 3 mM., as occurs with the methonium compounds, but in some experiments it actually caused an acceleration of the change to the flagellate form, particularly at concentrations more dilute than 1.6 mM. These results, however, like those with eserine were by no means uniform and there is still more to investigate in connexion with both these substances. It is perhaps of interest, however, to note that iodide is one of the substances which under some conditions promote ‘animalisation’ of developing embryos and its action on amoebae in favouring the flagellate form
may thus be similar and is to be contrasted with that of the 'vegetalizing' agent lithium which suppresses the flagellate form of amoebae.

Koch (1954) in his work on sodium transport in crabs' gill tissue observed that certain basic dyes, some of which contain quaternary ammonium groups act like the simpler quaternary ammonium compounds and prevent the uptake of sodium into the blood. Among these dyes were methylene blue, brilliant cresyl blue, and neutral red. All three of these have been found to be strongly inhibitory to the change from the ameboid to the flagellate form when applied in distilled water to *Naegleria gruberi*. Under these conditions methylene blue (Gurr's vital)...
did not stain either the amoebae or the flagellates, apart from a few cytoplasmic granules which stained intensely, till applied in concentrations greater than 1.2 mM., at which point the amoeba became round and inactive. On the other hand, the cysts became very quickly and intensely stained in even the most dilute solutions. The flagellate form was rarely seen in concentrations of methylene blue higher than 0.02 mM. (Text-fig. 4).

The physiological actions of methylene blue are certainly various, but it is of interest that this action in suppressing the flagellate form of the amoeba could well be similar to the action on sodium transport in the gills of the crab. In the gills the sodium uptake is inhibited by the presence of methylene blue on the outside of the gill membrane. When applied to Naegleria in the flagellate phase it causes an immediate change to the amoeboid form, i.e. it suppresses the form in which sodium accumulation is proceeding. Perhaps it actually blocks the sodium mechanism.

There is, however, another side to the action of methylene blue on the amoeba. When it was applied, not in water, but in a buffer solution containing NaHCO₃ (see Willmer, 1956) it became toxic to the amoeboid form in much lower concentrations, so that it had very little differential action in suppressing the flagellate form. It will be remembered (Willmer, 1956) that the action of the bicarbonate ion was also important in determining the behaviour of the amoeba in response to the presence of lithium salts and to sodium salts other than the bicarbonate. Possibly these differences may be caused by the penetration of the cell by CO₂ from the bicarbonate solution and the consequent change to acidity on the part of the cell contents, though it is not immediately clear why this should increase the toxicity of methylene blue to the amoeboid form and somewhat decrease its effectiveness on the flagellate form. It could be suggested that the methylene blue might be more ionized in the acid cell contents and that its toxic effects are dependent on its action as a cation.

As already mentioned, neutral red behaves in a manner somewhat similar to methylene blue and brilliant cresyl blue. It accumulates to some extent in a few cytoplasmic granules, rather more when the cell is in the amoeboid form than in the flagellate form but it is not as actively accumulated as it is for example by some other living cells, e.g. macrophages in tissue cultures of vertebrate tissues. It often accumulates strongly in the uroid region of those cells which have developed a definite polarity, in a manner similar to that described by Goldacre (1952) for the limax form of Chaos chaos.

ON THE METABOLISM OF THE AMOEBAE IN RELATION TO THEIR FORM

If, as the hypothesis outlined above suggests, the change of form of the amoeba is related to the accumulation or excretion of cations, then at all concentrations except the equilibrium concentration the cell must either become relatively impermeable or must do work to maintain its ionic balance. Sub-
stances which affect biological membrane permeability and those which alter metabolic rate are therefore both likely to have effects on the 'metaplasia' of amoebae. For example, an amoeba placed in distilled water presumably tends to lose ions and it changes to the flagellate form to counteract this. Anything which decreases permeability must delay this change by decreasing the loss. The action of Mg$^{++}$ may be a case in point, for this ion definitely delays the change and in other situations, e.g. certain nerve-cells (del Castillo & Engbaek, 1954) where it is known to prevent the release of acetyl choline, it certainly causes a decrease in permeability. It may, of course, have other actions also. In bacteria, for instance, it affects the synthesis of amino acids which in itself may have far-reaching effects on the ionic content of the cell. In amoebae the addition of 3.1 mM MgCl$_2$ to cells in the flagellate form virtually stops their swimming within half an hour. The effect, however, is not immediate as in the case of methylene blue.

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Flagellates</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 mM. MgCl$_2$</td>
<td>3</td>
</tr>
<tr>
<td>3.1 mM. CaCl$_2$</td>
<td>22</td>
</tr>
<tr>
<td>3.1 mM. MgCl$_2$</td>
<td>30</td>
</tr>
<tr>
<td>3.1 mM. CaCl$_2$</td>
<td>54</td>
</tr>
<tr>
<td>3.1 mM. KCl</td>
<td>12</td>
</tr>
</tbody>
</table>

The action of Mg$^{++}$ on nerve-cells can be reversed by the simultaneous presence of Ca$^{++}$ (del Castillo & Engbaek, 1954) and this has also been found to be true for its action on amoebae (Table 3).

Quaternary ammonium compounds have also been shown to prevent the liberation of acetyl choline from nerve cells (Paton & Perry, 1953), so these substances also may be acting on amoebae by virtue of some similar action on permeability. On the other hand, there are many other ways in which both these groups of substances could be involved, and apart from indicating a certain similarity between amoebae and some nerve-cells in the manner in which they each respond, the observations by themselves throw little light on how the ionic regulation of the amoeba is carried out.

When amoebae were placed in buffer solution ($B_3$) the flagellate form began to appear in increasing numbers after about 2 hours, and then, after about 6 or 7 hours their numbers again began to fall off (Text-fig. 5). With concentrations of NaCl up to 2 mM. added to the buffer at the beginning of the experiment there was little difference to be noted in the response of the amoebae until after about 4 hours, nearly as many flagellates being formed as in the buffer solution.
alone. From that time onwards, however, the number of flagellates became definitely less than in the buffer solution. This result would follow if the flagellate cell were able to concentrate the sodium ions within itself, or if the sodium ions after penetration helped the cell to pick up potassium ions from the buffer solu-

![Text-fig. 5](image)

**Text-fig. 5.** Summary of experiments showing the effect of glucose and sodium chloride on the numbers of cells in the flagellate form during the first 7 hours after the addition of these substances, in buffer solution (B₂) to freshly washed amoebae. All figures have been expressed as a percentage of the numbers of flagellates in the buffer solution after 5 hours.

- **□** — **□** Buffer solution (B₂)
- **○** — **○** +6.7 mM. glucose
- **●** — **●** +6.7 mM. NaCl
- **○** — **○** +6.7 mM. glucose +6.7 mM. NaCl

Note the more immediate action of the NaCl and the delayed action of the glucose.

tion, which contains 0.2 mM. KCl, as they do in other similar situations. The former is the simpler hypothesis and as yet there is little to suggest that this amoeba discriminates, except quantitatively, between these cations. It may be that it normally accumulates whichever monovalent cation is available, or, if more than one is available, the proportions of each which enter may be partly determined by ionic size or mobility in the membrane.
At concentrations of NaCl higher than 6.7 mM, the numbers of amoebae which initially turned flagellate became noticeably reduced (Text-fig. 6). At this stage the loss of cations to the external medium presumably becomes insignificant and the necessity to 'switch on' the concentrating mechanism is reduced. This would be consistent with a total cation content of about 8 mM, which is approximately that found by Carter (1957) for the ciliate but is probably rather lower than that in *Naegleria* if the preliminary estimates are confirmed.

If instead of NaCl, glucose (final concentration 6.7 mM) was added to the buffer solution at the beginning of the experiment an exactly similar result followed (Text-fig. 5). There was no initial difference in the numbers which turned flagellate, but after about 4 hours the tubes containing glucose showed progressively fewer and fewer swimming forms. When glucose was added in distilled water it had no apparent effect on the numbers of cells which were flagellate after 6 hours (Willmer, 1956) so it is unlikely that these effects of glucose are purely osmotic.

Thirdly, the simultaneous addition of NaCl (final concentration 2 mM to 6.7 mM) and glucose (final concentration 6.7 mM to 10 mM) to the buffer solution enhanced the delayed effect still further.

These results, which are illustrated in Text-figs. 5 and 6, strongly suggest that the flagellate form of the cell has an active mechanism for capturing positive ions and this is assisted by further supplies of glucose. While the possibility remains that the action of the glucose is concerned with preventing the loss of ions, rather than with actively assisting in their accumulation, the time-scale of
events points to the latter, and it may therefore be tentatively concluded that the flagellate form has an inwardly directed cation pump.

Certain similarities with nerve-cells have already been mentioned and some nerve-cells are known (Krebs & Eggleston, 1949; Weil-Malherbe, 1950) to have a mechanism dependent on glutamic acid and glucose for the accumulation of their intracellular potassium. Thus the possibility of a 'glutamate mechanism' for maintaining the cation content of *Naegleria* seemed to be worth investigating.

When sodium *l*-glutamate was added to a glucose-containing medium it was found to have a much greater action than the corresponding concentration of NaCl in hastening the return of the flagellates to the amoeboid form (Text-fig. 7). Thus the similarity with nerve-cells appeared to be capable of further extension. However, the action of the glutamate was not found to be so specific for amoebae as it apparently is for nerve-cells, because it can be replaced very effectively with sodium *a*-ketoglutarate (Text-fig. 8), which is not so for nerve-cells.

The addition of glutamine to the amoebae in buffer solution generally produced similar results to those produced by sodium glutamate and these were dependent on the simultaneous presence of glucose (Text-fig. 9). The action of glutamine was, however, not so regular or predictable as that of sodium glutamate and, indeed, in certain experiments it appeared to have no action at all. Possibly it has to be converted to glutamic acid before it is effective and, in support of this, there was some indication that freshly made solutions were less effective than those which had been stored at about 3°C for 24 hours and in which bacteria could have caused some conversion. Alternatively the amoebae may sometimes be so well nourished as to make additions of this sort from the

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**Text-fig. 7.** Summary of experiments comparing the effects of sodium chloride and sodium *l*-glutamate on the numbers of cells in the flagellate form in the first 7 hours after the addition of these substances to freshly washed amoebae in buffer solution (B). All figures as percentages of numbers of flagellates at 5 hours in buffer solution (B).

© Buffer solution (B) + 67 mM. glucose + 67 mM. NaCl

© © " " + 67 mM. glucose + 67 mM. Na *l*-glutamate
outside quite ineffective. There was, in fact, a general correlation between the age of the culture used and the magnitude of the effect produced by glutamine. The very well-fed cells of the 4-day culture were less influenced by added glutamine and glucose than those from 6- or 7-day cultures.

Asparagine was found to behave like glutamine so that again there is probably very little specific about these compounds in relation to the metabolism required for the 'cation pump'. Nevertheless, the fact that these compounds affect the working of the pump makes it fairly clear that an active process is involved in causing the cells to revert to the amoeboid form.

As already pointed out, these substances were not so effective immediately as they became after the lapse of some hours; that is to say they did not lengthen the lag period before flagellates appeared nor, in the lower effective concentrations at least, did they at first greatly alter the rate at which the amoebae became flagellate. This was particularly true for the mixtures of glutamine and glucose (Text-fig. 9). In the case of sodium glutamate the position has to be controlled with reference to the effects of the added sodium. When, however, allowance
was made for this by comparison with additions of sodium chloride (6.7 mM.) then the glutamate at the same concentrations was seen to have very little immediate effect on the transformation to the flagellate form (Text-fig. 7), and the lag period was not lengthened any more than by the sodium added.

Text-fig. 9. Summary of experiments showing the effect of glutamine on the numbers of cells which become flagellate in the first 7 hours after its addition to amoebae in various media. All figures are percentages of the number of flagellates after 5 hours in buffer solution (B).

- Buffer solution (B)
- + 6.7 mM. glutamine
- o 6.7 mM. glucose
- © 6.7 mM. glucose + 6.7 mM. glutamine
- X 6.7 mM. glucose + 6.7 mM. NaCl

Note that only in the medium containing NaCl is there any significant immediate effect.

In contrast to its lack of action on the amoeboid form, sodium glutamate when added to the amoebae after they had already become flagellate had an immediate action in reversing the change (Text-figs. 10–13). There are certain technical difficulties about preparing large numbers of cells in the flagellate form for inoculating into series of experimental tubes in a manner which allows comparable results to be obtained, so in these experiments the amoebae (as such)
were inoculated into the experimental tubes each containing 0·5 c.c. of $B_2$ only; then, after about 4 hours, when the number of flagellates was still increasing but nearly at the maximum, another 0·5 c.c. of experimental solution of twice the required strength was added. Counts of amoebae were made immediately before the addition and as often as possible afterwards. The method is not entirely satisfactory since, because of the time consumed in counting them, the amoebae cannot always be caught at the same state of the tide of change to the flagellate form and this makes the data somewhat difficult to standardize. For example, a tube caught early in the process towards flagellum formation may actually
show more flagellate cells after the addition of an inhibitor than before, while another control tube taken after the turn of the tide may show fewer flagellate cells than before the addition of the control $B_2$ solution. Controls therefore have to be used continually throughout the experiment. While this method is clearly not ideal, the accompanying figures show that definite results can be obtained by its use.

Text-fig. 10 shows the results of a particular experiment in which the actions of glutamine, sodium glutamate, and sodium chloride have been compared. Glucose was present in all cases. It will be seen that after the addition of glutamine the numbers of flagellates at first followed a course very similar to that of the controls in buffer solution alone. Only later did the curve diverge from the $B_2$ curve and fall appreciably below it. On the other hand, with both sodium chloride and, particularly, sodium glutamate the number of flagellates was immediately reduced. Repetition of similar experiments led to the results depicted in Text-fig. 11, and it is evident that sodium glutamate had an immediate and direct action but glutamine had none or only a very delayed one either in the presence or absence of NaCl. This again can be taken as an argument that glutamine only becomes effective after some hydrolysis, or that it cannot act immediately on the flagellate form but only after penetrating the cell in the amoeboid form.
In these experiments the sodium glutamate reduced the numbers of flagellates almost immediately and continued to do so, while the chloride, though sometimes causing an initial drop, acted in general much more slowly. Text-figs. 12 and 13 illustrate the results obtained from a long series of experiments in which NaCl and Na l-glutamate were added to amoebae in the flagellate form and the

**TEXT-FIG. 12.** Summary of all experiments in which 2 mM. NaCl and 2 mM. Na l-glutamate have been added to amoebae in the flagellate form. All figures expressed as percentage of the number of flagellates at the time of addition of experimental medium.

- □ Buffer solution
- NaCl x Na l-glutamate

Vertical lines represent twice the standard error of the mean and the figures the numbers of readings. Each point represents the mean for all readings within the hour.

Different timing of the effects of the two substances is clearly seen. This difference may simply be the result of different rates of penetration but it is exactly what would be expected if the entry of sodium was normally proceeding slowly in accordance with its availability in the external medium, and if the uptake could be further accelerated by a metabolic process involving in this case the use of glutamic acid and glucose. These results were all obtained with 2 mM. concentrations of chloride and glutamate and if the concentrations were increased to 10 mM the difference between the chloride and the glutamate practically disappeared. Presumably the ionic balance of the cells no longer required the
active collection of sodium ions from the external medium at that concentration. The conclusion seems inevitable therefore that in the flagellate form *Naegleria gruberi* has a metabolic mechanism, involving glucose and glutamic acid or other similar constituents of the metabolic pool, for the accumulation of cations from the medium. In general, there seems to be little discrimination between Na\(^+\) and K\(^+\). A pure K-phosphate buffer was found to give results which did not differ significantly from those obtained either in a pure Na-phosphate buffer or in a mixed Na-K-phosphate buffer. In one or two experiments in which both Na\(^+\) and K\(^+\) were present the amoebae became flagellate somewhat earlier than in solutions with either cation alone, and they returned more quickly to the flagellate form. The results, however, were not statistically significant.

**DISCUSSION**

As emphasized in a previous paper, *Naegleria gruberi* is an interesting organism for the study of differentiation in that it changes its morphology under the influence of changed external conditions, and consequently can be used as a test
It now seems to be certain that a primary cause of the 'metaplasia' from amoeba to flagellate is a drop in the cation content of the surrounding medium and the change can be inhibited by raising the ionic content of the medium.

Naturally not all cations are equally effective; Li+ and Mg++ ions, for example, tend to inhibit the change to the flagellate form in very low concentrations. Na+, K+, and Ca++ on the other hand, while showing some individual differences act over rather similar concentration ranges but at a higher level than Mg++. Probably there are definite interactions between these ions, as there are for nearly all other organisms, but only the reduction of the Mg++ effect by Ca++ has so far been studied. Furthermore, it has been shown that the presence of certain anions in the environment, e.g. bicarbonate, phosphate, and lactate, may favour the change towards the flagellate form, while others, e.g. sulphate, have exactly the opposite action.

The actions of these various ions may of course, be many and various, e.g. on surface charges, on membrane permeability, on colloidal state, on internal pH, on chemical composition, on enzymic activity, and on the orientation of protein particles, to mention only a few. It is unwise therefore to expect, at this stage, to be able to sort out exactly what is happening in the amoeba as the external ionic content is being changed.

Nevertheless, if a cell contains a certain amount of protein, and other complex ions will of course enter into this category also, then the state of that protein must depend on the balance of positive and negative ions in its immediate surroundings, because these affect the numbers of charged groups on the proteins themselves and no doubt the state of the protein, i.e. as a coiled or extended chain, may affect the numbers of groups which could bind other ions. It is easy to imagine, therefore, that any particular cell, in order to carry out its normal functions should have a definite requirement for its internal ionic content. In the case of Spirostomum this appears to centre round K+ at 7 mM./l. and Na+ at 1 mM./l. If the cell were to synthesize more protein or accumulate amino acids these figures might have to be raised; conversely a forced change in ionic content could well lead to alteration in the amount, distribution, or state of aggregation of the cell proteins. A cell, then, in order to preserve its status quo, should be expected to have some sort of ion-regulating mechanism, and, conversely, changed ionic content of the medium, if it causes changed internal content also, should be expected to cause changed morphology. In Naegleria gruberi these correlations seem to be borne out, and the amoeboid form with its lobose pseudopodia, random shape, relative lack of orientation, roving contractile vacuole, highly granular cytoplasm with hyaline ectoplasmic processes is characteristic of the state in which the external cation concentration probably exceeds the internal. In this state one would expect water to be drawn out of the cell, and the contractile vacuole to be unnecessary. However, the vacuole
continues to function till much higher concentrations are reached, and it seems possible that an active engulfing of water may have to take place to prevent dehydration, and that excess ions simultaneously imbibed are then eliminated through the contractile vacuole. Something of this sort may lie at the root of the somewhat mysterious process of pinocytosis, which could then be seen as a mechanism by which a cell could retain an ionic balance in a situation in which the external medium is more concentrated than that required for the equilibrium of the cell.

The flagellate form of *Naegleria*, with its definite orientation and polarity, its contractile vacuole in a fixed position, its more rigid surface and its inability to form pseudopodia, is on the other hand characteristic of the conditions imposed by an external cation concentration lower than the internal. The flagellate cell may perhaps have its proteins in a more fibrous form, as suggested by the ability to form flagella and filamentous rather than lobose pseudopodia. These fibrous proteins would be expected to have more exposed polar groups capable of binding ions. When these proteins change to the globular form they drop these ions. It seems possible to believe then that in a given environment the amoeboid form would have a lower ionic content than the flagellate form. It would also have a mechanism for eliminating ions while the flagellate, requiring more ions, has a mechanism for their accumulation. These pumps could well be based on the fibrous-globular proteins themselves as has been suggested by Goldacre (1952).

Since the flagellate state can be brought about by the presence in the environment of anions which easily penetrate cells, e.g. $\text{HCO}_3^-$ which can penetrate as $\text{CO}_2$, lactate and low concentrations of phosphate, it is possible that an increase in internal hydrogen ion concentration may result and this then lead to the assumption of the flagellate form, while on the other hand increase in the internal $\text{OH}^-$ concentration as by the penetration of $\text{NH}_3$ would by the same token favour the amoeboid form.

The experiments with glucose, glutamic acid, and sodium chloride indicate that ionic regulation is not a passive process in these amoebae and that the flagellate form actively absorbs cations from the surroundings. The fact that ketoglutaric acid is an effective substitute for glutamic indicates that there is nothing very specific about the glutamic acid as there is in the case of nerve-cells. In all these experiments, however, the amount of reserves available within the cell is an unknown quantity and the fact that ketoglutaric acid helps the pumping mechanism does not necessarily mean that glutamic acid is not involved. Indeed, the way in which glutamine behaves shows that glutamic acid is probably a key substance and its concentration in the cell may be maintained from either internal or external sources.

Into this picture the quaternary ammonium compounds could be fitted in several places and there is as yet little evidence as to which is most likely. They could alter the surface and prevent the loss of ions, and the formation of vesicles
in the higher concentrations perhaps suggests some interference with the normal excretion processes of the amoeboid form. Alternatively, if they enter the cell they presumably raise the cation content and so help to maintain it above the critical value. Since methylene blue penetrates the cell and acts in the same way as the other quaternary ammonium compounds this is at least a possibility, but against this it should be noted that the methylene blue is under these circumstances and everywhere except in a few granules reduced to the leucoform in which it is no longer ionic or a quaternary ammonium compound.

While the experiments described in this paper provide grounds for believing that the flagellate form of *Naegleria* has an inwardly-directed 'cation pump' which depends on active metabolic processes, there is nothing in them which indicates definitely that in the amoeboid form there is any comparable process acting in the opposite direction. It seems probable that there is, but experimental evidence is needed. A contractile vacuole is present in both states, and there may be more than one in the amoeboid form, but, so far, there is nothing known about the contents. Data on the ionic composition of *Naegleria* in its two states, comparable to those obtained by Carter (1957) on *Spirostomum*, would be invaluable.

Finally, it will be remembered that these experiments on *Naegleria* were initiated in order to explore the possibility that in this organism there was present at different times the same type of differentiation as is found at different places among the cells of the embryos of the most primitive metazoa, e.g. in the 'animal' and 'vegetal' poles of the various forms of amphiblastulae. A few comments on the results from this point of view may not therefore be out of place.

In an amphiblastula a cavity is formed within a single layer of cells, and, within limits, this cavity neither swells nor shrinks. When tissue cultures are made of portions of the mesonephros of chick embryos the pieces of nephric tubules tend to form little closed vesicles, and in the normal tissue culture medium those from the proximal tubules tend to swell up with increasing amounts of contained fluid, while those from the distal tubules, however, tend to collapse to solid masses of cells (Chambers & Kempton, 1933). This would happen if the proximal tubules tended to expel material or fluid into the cavity and the distal tubule cells tended to extract materials from the cavity. Since the amphiblastula neither shrinks nor swells, and if it is a closed vesicle like those formed by the nephric tubules, there are two possibilities. Either the cells contribute nothing and take away nothing from this cavity, which seems inherently incredible, or else they regulate its volume very accurately, and could well do this by opposing two mechanisms. It is worth considering then whether the flagellate anterior cells of the amphiblastula of the sponge contribute towards ionic stability by accumulating ions from the environment, while the posterior granular cells eliminate any excess ions which may arise. There is, of course, one very large difference between the state of affairs in the amoeba and that in
the amphiblastula. In the latter, the external surface of all the cells is exposed to one environment while the internal surface may meet another; in the amoeba the whole surface of the cell is exposed to one environment only. In the amphiblastula, therefore, the activities of both 'faces' of the epithelial cells have to be considered separately, but there is no reason to believe that this necessarily detracts from the idea that the amphiblastula is a stable structure because it combines cells, not only with different feeding mechanisms, and with different methods of locomotion, but also with differently orientated mechanisms for regulation of their ionic content and that of the fluids bathing their surfaces. If this is so, then it may be a principle which is capable of extension into other fields, e.g. to various epithelia which contain two types of cell and which are concerned with regulating the composition of the fluids on either side of them.

The recent observations of Tuft (1957) on the fluid accumulation in the cavities of the Xenopus embryo speak strongly in favour of ionic regulation and forcible movements of fluid. Indeed, it may be a useful concept in relation to embryonic development in general, that particular groups of cells can by their orientation of water- or ion-pumping mechanisms create local environments of specific ionic content for cells in their immediate neighbourhood. For example, cells lying below part of the ectoderm which is accumulating, say K⁺, from the environment may well be forced to assume the form in which they prevent K⁺ ions entering them excessively. The movement of water through cells may also produce a similar state of affairs and in such cases morphogenetic movements may actually be induced by the pressure changes involved.

SUMMARY

1. Further observations have been made on the effects of various substances on the change of form from amoeba to flagellate and vice versa in Naegleria gruberi.

2. Mg²⁺ reduces the numbers of flagellates formed from amoeba and favours the conversion of flagellates to amoebae. Its action can be partly offset by the addition of Ca²⁺.

3. Methylene blue and other basic dyes suppress the change towards the flagellate form, particularly in distilled water, less so in a bicarbonate-buffer.

4. Substances containing a quaternary NH₃⁺ generally suppress the change to the flagellate form. Methonium compounds are particularly effective. At certain concentrations these substances cause vesicles in the cytoplasm.

5. l-glutamine and Na l-glutamate applied to the amoebae reduce the numbers of flagellates which would otherwise form after a few hours in buffer solution.

6. Asparagine and Na a-ketoglutarate have similar effects.

7. Na l-glutamate (but not glutamine) applied to the flagellate form immediately reduces the numbers of flagellates. An equivalent amount of sodium chloride acts much more slowly.
8. It is suggested that the flagellate form of the amoeba has a mechanism for maintaining a stable internal cation concentration. This mechanism requires metabolic energy. Reduced permeability and more active expulsion of water are probably not adequate to explain the facts and an inwardly-directed cation pump is suggested for the flagellate form.

9. The application of ideas engendered by this work to other fields of cell differentiation are briefly considered; in particular, the concept of ionic regulation by the amphiblastula stage of invertebrates is discussed.

REFERENCES


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