CHEMOTAXIS OF BRACKEN SPERMATOZOIDS

THE ROLE OF BIMALATE IONS

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Fern spermatozoids respond chemotactically to concentration gradients of malic or maleic acid salts (Pfeffer, 1884). They respond weakly to salts of a few other dicarboxylic acids, but do not respond to succinate or fumarate (Shibata, 1911; Rothschild, 1956). Previous workers concluded, without adequate justification, that the malate ion, and not the undissociated malic acid molecule, is chemotactically active; but no consideration has been given to the possibility that the bimalate ion may be the active species.

A malate-dependent aggregation of bracken spermatozoids in a pH gradient has been briefly reported (Brokaw, 1957). In this paper, this phenomenon and several related observations are examined in greater detail.

MATERIAL AND METHODS

Spores of bracken, *Pteridium aquilinum* (L.) Kuhn, were sown thickly on agar plates made up with approximately 1% agar in a Knop's solution (0·1% KNO_3, 0·05% NaCl, 0·05% $CaSO_4$, 0·05% $MgSO_4$, 0·05% $Ca_3(PO_4)_2$). A dense mat of small prothalli was obtained after about 3 months' growth. When a piece of this mat is removed, placed in a small quantity of water, and gently squashed with a glass rod to ensure thorough wetting, spermatozoids emerge from the antheridia. After about 20 min. the suspension of spermatozoids may be removed with a pipette.

A cleaner and/or more concentrated sperm suspension can be obtained by taking advantage of the chemotactic reactivity of the spermatozoids. A drop of sperm suspension is placed on a glass plate under a low-power microscope and small droplets of sodium L-malate solution are carefully injected into the drop of sperm suspension. After 3–5 min., the aggregations of spermatozoids in these droplets may be removed with a fine-tipped pipette. This procedure may be repeated to obtain specially clean sperm suspensions for dark-ground photomicrography.

The crude sperm suspension obtained from the prothalli is contaminated with substances which act as pH buffers and with traces of chemotactically active substances. When a low activity of malate is required in the sperm suspension, or when accurate pH control is necessary with minimum concentrations of buffer, the spermatozoids must be washed several times by centrifugation and resuspension in buffer. This procedure causes some damage to the spermatozoids, which must not be confused with the effects of low malate concentration.

DISSOCIATION OF MALIC ACID

The dissociation of malic acid can be represented by

$$H_2M \rightleftharpoons HM^- + H^+ \quad pK_1 = 3{\cdot}41,$$
$$HM^- \rightleftharpoons M^{2-} + H^+ \quad pK_2 = 5{\cdot}05.$$

The relative concentrations of malic acid, H_2M, bimalate ion, HM^-, and malate ion, M^{2-}, existing at any pH can be calculated and are shown by the curves in Fig. 1.

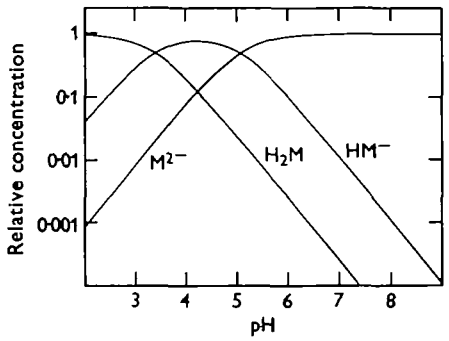

Fig. 1. Relative concentrations of malate species (logarithmic scale) at various pH's

There are two forms of bimalate ion, depending on which carboxyl group is ionized. Consideration of the pK's of α- and β-hydroxy-butyric acids (3·9 and 4·5) indicates that bimalate will consist of about 80% of the form shown in Fig. 2*a* and about 20% of the form shown in Fig. 2*b*. In either form, the possibility of hydrogen bonding between the dissociated and undissociated carboxyl groups will tend to favour the *cis*-configuration. A hybrid structure (Fig. 2*c*) may best represent the bimalate ion, although there is no evidence that it is appreciably stabilized. On the other hand, where the two carboxyl groups are both neutral, as in the malic acid molecule (Fig. 2*d*), or both negatively charged, as in the malate ion (Fig. 2*e*), the *trans*-configuration will be strongly favoured, so that the two groups will be separated from each other as much as possible.

RESULTS

A drop of 0·1 M hydrochloric acid was placed on a glass slide in contact with a drop of sperm suspension containing 0·005 M *tris*-(hydroxymethyl) aminomethane—hydrochloric acid buffer, pH 8·1; 10^{-4} M sodium L-malate; and a small amount of pH indicator. Under these conditions the acid diffuses into the sperm suspension, neutralizes the buffer, and changes the colour of the pH indicator. A sharp colour change and, therefore, a sharp pH change occur at the junction of the region in which the buffer is completely neutralized with that in which it is not yet neutralized. This junction moves slowly across the drop of sperm suspension as the acid diffuses into the drop. A narrow aggregation of spermatozoids forms in this region of sharp

pH change (Brokaw, 1957). Dark-ground track photographs of the spermatozoids in this aggregation show them swimming actively within a narrow band about 75 μ wide and turning sharply back into the band from both sides.

The same phenomenon was observed with sperm suspensions buffered with 0·01 M sodium citrate, sodium phosphate, or sodium borate buffers, at pH's ranging from 5·6 to 8·7. With borate buffer, pH 8·7, and either cresol red indicator (pK 8·3) or brom cresol purple indicator (pK 6·3), the band of spermatozoids was located in

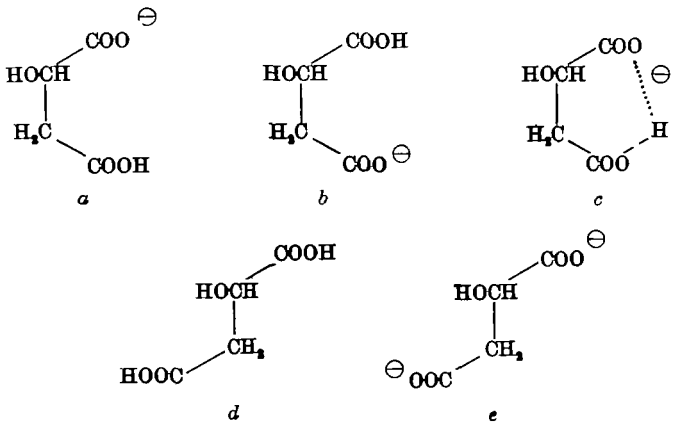

Fig. 2. Configurations of malate species. For details see text.

the same position as the colour change of the indicator, which confirmed the existence of a steep pH gradient in the region of sperm aggregation. With methyl-orange indicator (pK 3·5), the colour change was located slightly on the acid side of the band of spermatozoids. These experiments locate the region 'preferred' by bracken spermatozoids between pH 3·5 and 5·6.

When the spermatozoids are repeatedly washed by centrifugation and re-suspended in buffer solution without malate, no aggregation occurs when a pH gradient is established as described above. If 10^{-5} M sodium L-malate is added to a repeatedly washed sperm suspension, the spermatozoids again aggregate in a band when a pH gradient is established.

Fig. 1 shows that when a pH gradient extending from pH 2 to pH 8 is established in a solution of uniform malate concentration, there is a maximum concentration of the bimalate ion at pH 4·2 and bimalate gradients on either side of this point. The chemotactic response of bracken spermatozoids to these bimalate gradients could cause them to aggregate around pH 4·2, and explain the results of the above experiments.

It has not been possible to calculate precisely the pH gradient obtained when hydrochloric acid diffuses into a dilute buffer solution, as in the above situation. A very rough estimate of the resulting bimalate gradients indicates that they are sufficient to cause the observed aggregation, but this interpretation must be tentative until a more exact calculation of the gradients is achieved.

An alternative suggestion, which is more complex yet consistent with these observations, is that sensitivity to the undissociated malic acid molecule, plus an independent negative chemotaxis to low pH's, might produce this aggregation. Although there is some indication of negative chemotaxis to low pH's in washed sperm suspensions, the phenomenon of electrochemical orientation of bracken spermatozoids (Brokaw, 1957) suggests that the spermatozoids are sensitive to a charged ion.

Chemotaxis of bracken spermatozoids from a drop containing 10^{-4} M sodium L-malate and 0·015 M sodium phosphate buffer, pH 7·5, into one containing 10^{-4} M sodium L-malate and 0·015 M sodium phosphate buffer, pH 6·2, has been observed. The response is comparable to that observed when a drop containing 10^{-3} M sodium L-malate is placed in contact with one containing 10^{-4} M sodium L-malate, both drops containing the same buffer.

The gradient of the logarithm of concentration is the important variable in fern sperm chemotaxis (Pfeffer, 1884). Since Fig. 1 shows that the gradient of the logarithm of the bimalate ion concentration will be nearly equal to the gradient of pH at pH's above 6, the bimalate hypothesis predicts that bracken spermatozoids should be as sensitive to a pH gradient as to a malate gradient. This agrees with the above result, as the gradients set up by the diffusion of sodium phosphates will be of the same order of magnitude as the gradients set up by the diffusion of sodium malate.

Bracken spermatozoids orientate to a voltage gradient only if the sperm suspension contains a chemotactically active compound (Brokaw, 1957). The amount of sodium L-malate which must be added to obtain this electrochemical orientation is less at pH 6·8 than at pH 8·1 by approximately an order of magnitude. Fig. 1 shows that the malate concentration required to give a given concentration of bimalate ions will be twenty times less at pH 6·8 than at pH 8·1. The hypothesis that bimalate ions are required to sensitize spermatozoids to a voltage gradient is, therefore, in approximate agreement with the above result.

If the sperm suspension contains sodium maleate instead of sodium L-malate, addition of a drop of hydrochloric acid leads to the formation of a broader band of spermatozoids than when malate is used, and when the pH of the sperm suspension is low (6·2) there is no sharp aggregation. These observations suggest that in this case, too, the singly ionized ion, bimaleate, is the active species, and are consistent with the higher pK_2 of maleic acid (6·6).

DISCUSSION

Shibata (1911) suggested that malate might have a *cis*-configuration, since he, and Pfeffer, found maleate (*cis*) to be chemotactically active for many pteridophyte spermatozoids while fumarate (*trans*) was inactive. However, the malate ion, which predominates in solutions of pH above 6, is not likely to exist in the *cis*-configuration, while the bimalate ion will have a *cis*-configuration. Shibata also found that spermatozoids of *Equisetium arvense* L. and of *Salvinia natans* Allioni were positively chemotactic to solutions of dilute acids. He found that the sensitivity to a gradient of H^+ ions was just as strong as the sensitivity to a gradient of malate, and that the

effects of H^+ and malate concentrations were not additive. Since he did not show that the response to H^+ required the presence of malate, he concluded that the sensitivities to H^+ ions and to malate were independent. My experiments demonstrating the chemotactic interrelationship of malate and pH suggest that these spermatozoids are sensitive to the bimalate ion and not to the malate ion or the malic acid molecule, in agreement with all the above observations.

Shibata also studied the spermatozoids of *Isoetes japonica* A. Braun, which show positive chemotaxis to gradients of malate, fumarate, and OH^- ions. The above considerations suggest that in the case of *Isoetes* spermatozoids, the malate ion, rather than the bimalate ion, may be active. However, it is difficult to see how a pH gradient in solution above pH 6 could produce significant gradients of malate ion (see Fig. 1). It would be interesting to know if *Isoetes* spermatozoids are attracted by OH^- ions in the absence of malate.

SUMMARY

1. Bracken spermatozoids are chemotactically attracted by malic acid salts and in a pH gradient aggregate between pH 3·5 and 5·6.

2. This response to pH occurs only when the sperm suspension contains malate.

3. At higher pH's, the response to an H^+ gradient is as strong as the response to a malate gradient.

4. These observations suggest that bracken spermatozoids are sensitive to the bimalate ion rather than the malate ion. This is consistent with an earlier suggestion that chemotactic activity is associated with the *cis*-configuration, for the bimalate ion will have this configuration, while the malate ion and the malic acid molecule will have the *trans*-configuration.

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