

# KINETIC STUDIES OF THE FLAGELLAR MOVEMENT OF SEA-URCHIN SPERMATOOZOA

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## INTRODUCTION

In previous papers (Holwill & Silvester, 1965, 1967) results have been presented which support the hypothesis that the frequency of beating of a flagellum (hereinafter referred to simply as 'frequency') is identical with, or at least proportional to, a rate constant of the first-order chemical reaction which limits the frequency. If, as seems probable (e.g. Bishop, 1962; Brokaw, 1962) adenosine triphosphate (ATP) is directly involved in the supply of energy to an active organelle, then the experimental evidence indicates that the rate-limiting reaction is the breakdown of an ATP-ATPase complex.

It has been found possible to treat certain flagella with solutions containing glycerol in such a way that these appendages can be reactivated by the addition of ATP, the frequency being controlled by the ATP concentration (e.g. Hoffmann-Berling, 1955; Brokaw, 1961, 1967). Earlier workers (e.g. Bishop, 1958; Bishop & Hoffmann-Berling, 1959) reported that the re-activated appendages exhibited only local oscillations but more recently Brokaw (1962, 1963) and Gibbons (1965*c*) have prepared 'models' in which all the features of normal wave propagation are preserved. The relation between frequency and ATP concentration is found to be similar in form to the Michaelis-Menten law of enzyme kinetics (Brokaw, 1967; Brokaw & Holwill, 1967). The glycerinated models appear to provide an externally controllable system in which all the machinery for wave propagation is preserved and are potentially suitable for further studies of the thermodynamic aspects of flagellar movement. In this paper the reactions of sea-urchin spermatozoa are investigated in an attempt to characterize further the enzymatic reactions which underly flagellar motility.

## REACTION KINETICS

### *Briggs-Haldane kinetics*

For an enzymic reaction of the type



where  $E$ ,  $S$  and  $P$  represent the enzyme, substrate and products,  $ES$  is an enzyme-substrate complex and  $k_1$ ,  $k_{-1}$ ,  $k_2$  are the rate constants for the individual reactions, Briggs & Haldane (1925) derived the following general relation for the overall velocity,  $V$ , of the reaction:

$$V = \frac{k_2 K [E_0] [S]}{1 + K [S]}. \quad (2)$$

In this equation,

$$K = \frac{k_1}{k_{-1} + k_2},$$

$[E_0]$  is the total concentration of enzyme and  $[S]$  is the substrate concentration.

For glycerinated flagella

$$V = \alpha f \quad (3)$$

(Brokaw, 1967; Brokaw & Holwill, 1967), where  $\alpha$  is a constant with suitable dimensions and  $f$  is the frequency. At sufficiently high substrate concentrations  $K[S] \gg 1$  so that, from equations (2) and (3), we have

$$\alpha f_{\max} = k_2 [E_0], \quad (4)$$

where  $f_{\max}$  is the frequency which the flagellum approaches as the substrate concentration is raised.

Combining equations (2), (3) and (4) and re-arranging we have

$$\frac{1}{f} = \frac{1}{f_{\max}} + \frac{1}{f_{\max} K [S]}. \quad (5)$$

Thus a plot of  $1/f$  against  $1/[S]$  should be linear and allow the calculation of both  $f_{\max}$  and  $K$  from the intercept and slope.

This treatment follows closely Lineweaver & Burke's (1934) analysis of the Michaelis-Menten equation.

#### *Effects of temperature on reaction rates*

A statistical treatment of reaction rates yields the following equation relating a first-order rate constant  $k$  and the absolute temperature  $T$ :

$$k = \frac{kT}{h} \exp \left( -\frac{\Delta G^\ddagger}{RT} \right). \quad (6)$$

Here  $k$ ,  $h$  and  $R$  are the Boltzmann, Planck and gas constants respectively while  $\Delta G^\ddagger$  is the molar change in free energy that accompanies the activation of the chemical reaction. A similar relation holds for equilibrium constants, but in this case the free energy change  $\Delta G$  corresponds to the difference between the activation energies of the reaction in the forward and reverse directions. The activation free energy can be expressed in terms of an activation enthalpy ( $\Delta H^\ddagger$ ) and an activation entropy ( $\Delta S^\ddagger$ ) as follows

$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger. \quad (7)$$

From (6) and (7) we obtain

$$\ln \left( \frac{k}{T} \right) = \ln \left( \frac{k}{h} \right) - \frac{\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R}, \quad (8)$$

so that an experimental plot of  $\ln(k/T)$  against  $1/T$  should be linear. Values of  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$  can be calculated from the slope and intercept of the line. (In previous papers (Holwill & Silvester, 1965, 1967) the frequency in intact organisms has been identified with a rate constant.)

#### *Effects of pH on reaction rates*

Laidler (1955*a, b*) has developed an extensive theory pertaining to the effects of pH change on the rates of enzyme reactions. If the rate of the enzyme reaction passes

through a maximum as the pH is varied, it is necessary to assume that the enzyme contains an acidic and a basic group, both of which take part in the reaction. It is possible that one, both or neither of these groups is involved in complex formation, but if the reaction passes through a maximum both groups must be involved in the breakdown of the complex. Laidler derives equations relating the reaction rate to the hydrogen-ion concentration for each of the four possible ways in which the activated complex may be formed. In each case, on the acidic side of the optimum pH he predicts that a graph of  $V_{\max}/V$  ( $V_{\max}$  and  $V$  are the velocities of the reaction at the optimum pH and the given pH respectively) against the hydrogen-ion concentration  $[H^+]$  should be linear. On the basic side of the optimum pH, it is predicted that in all cases except where complex formation involves the basic group of the enzyme only, a plot of  $V_{\max}/V$  against  $1/[H^+]$  should be linear; for the exception a linear variation of  $V_{\max}/V$  with  $[H^+]$  is predicted.

The slopes of the graphs are related to equilibrium constants for reactions involving the enzyme, enzyme-substrate complex and the hydrogen ions. The way in which the constant  $\bar{K}$  varies with pH change is also dependent on the type of complexing involved in the reaction, as is the position of the pH optimum at different substrate concentrations.

#### *Effect of dielectric constant on reaction rates*

The force between two ions in solution depends on the dielectric constant of the solution. The work done during the relative movement of two ions, which we will assume are capable of reacting with each other, will also depend on the dielectric constant, which will therefore appear in any formulation of the electrostatic contribution to the increase in free energy occurring when the two ions react with each other. If the reacting ions are considered as charged spheres widely separated before the reaction and just touching after the reaction (the double-sphere model of the activated complex, e.g. Laidler, 1965) then the free energy of activation per molecule is

$$\frac{\Delta G^\ddagger}{N} = \frac{\Delta G_{\text{nes}}^\ddagger}{N} + \frac{Z_a Z_b e^2}{\epsilon d_{ab}}. \quad (9)$$

In this expression  $\Delta G^\ddagger$ ,  $\Delta G_{\text{nes}}^\ddagger$  are the total and non-electrostatic molar free energy increases,  $N$  is Avogadro's number,  $Z_a e$ ,  $Z_b e$  are the charges on the reacting ions ( $e$  being the electronic charge and  $Z_a$ ,  $Z_b$  whole numbers),  $d_{ab}$  is the separation of the ions while  $\epsilon$  is the dielectric constant of the medium. Substituting this expression for  $\Delta G^\ddagger$  in equation (6) and taking logarithms we obtain

$$\ln k = \ln k_\infty - \frac{Z_a Z_b e^2}{\epsilon d_{ab} kT}, \quad (10)$$

where  $\ln k_\infty$  has been written for

$$\ln \frac{kT}{h} - \frac{\Delta G_{\text{nes}}^\ddagger}{RT}.$$

$k_\infty$  is thus the rate constant in a medium of infinite dielectric constant. According to this simple treatment, a plot of  $\ln k$  against  $1/\epsilon$  should be linear (in the present study  $k$  will be identified with a frequency).

More complex treatments involving the interaction of dipoles also predict a linear dependence of  $\ln k$  on  $1/\epsilon$  but in this case the slope of the graph involves the dipole

moments of both reactants and of the resulting complex. Further analysis of the effect of the dielectric constant on reaction rates is undertaken in the discussion.

### *Effects of ionic strength on reaction rates*

Using the Debye-Hückel theory, Laidler (1965) obtains the following relation for the rate constant of a reaction occurring in an aqueous solution of ionic strength  $u$ :

$$\log_{10} k = \log_{10} k_0 + 1.02 Z_a Z_b \sqrt{u}, \quad (11)$$

where  $k_0$  is the rate constant at zero ionic strength. The equation predicts a linear relation between  $\log_{10} k$  and  $\sqrt{u}$ .

### MATERIAL AND METHODS

Spermatozoa from the sea-urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* were obtained and prepared using the methods described by Brokaw (1965, 1966, 1967). Frequency determinations were made stroboscopically, the organisms being viewed under conditions of dark-field illumination.

The temperature of the preparation was varied by passing heated or cooled water (containing a small percentage of ethylene glycol to prevent freezing at low temperatures) through the specially modified microscope stage. Measurement of the temperature of the preparation was achieved by using a thermocouple inserted in a hole bored through the centre of a microscope slide; a potentiometer arrangement allowed a null-point detection method to be used and suitable resistances were chosen to permit the temperature to be read directly from the dial of a helical potentiometer (Holwill & Silvester, 1967). The calibration technique employed (Holwill & Silvester, 1967), introduces a maximum error of  $0.3^\circ \text{C}$ . in temperature over the range used. The scale could be read to within  $\pm 0.1^\circ \text{C}$ .

Variations in the dielectric constant of the medium were made by adding small amounts of methanol to the re-activation solution, while the ionic strength was altered by changing the potassium chloride concentration.

### OBSERVATIONS

#### *Effects of temperature on frequency*

The frequencies of both intact and re-activated glycerinated spermatozoa were measured at a variety of temperatures between  $5$  and  $30^\circ \text{C}$ . For the glycerinated spermatozoa observations were made over a range of ATP concentrations from  $10^{-4}$  to  $10^{-3} \text{ M}$ . For each frequency determination 20 spermatozoa were selected at random and an average frequency was obtained; at a given temperature the frequency of the population was constant to within 10% of the average value. Since the frequency is known to change with altering viscosity all frequencies were corrected for the viscosity change which occurs with changing temperature using the results of Brokaw (1966) and M. E. J. Holwill (unpublished results). The corrected frequencies are those expected in a medium of viscosity 1 centipoise.

Figure 1 is a plot of  $\ln f/T$  against  $1/T$  for intact *Lytechinus* spermatozoa. The curve is linear over the range  $5$ – $21^\circ \text{C}$ ., but above  $21^\circ \text{C}$ . there is no correlation between the two parameters. A similar curve was obtained for *Strongylocentrotus* spermatozoa.

From the slope and intercept of the calculate regression line of the straight region of each curve average values for  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$  were obtained (Table 1). At temperatures above 21° C. the preparations appeared to deteriorate rapidly and afforded no opportunity to investigate the movement on lowering the temperature.

The straight line of Fig. 2 is the regression line of  $\Delta H^\ddagger$  upon  $\Delta S^\ddagger$  previously derived from observations on large numbers of cilia and flagella (Holwill & Silvester, 1967). The points derived for sea-urchin spermatozoa are also plotted in Fig. 2 and, within the limits of experimental error, lie on the regression line.

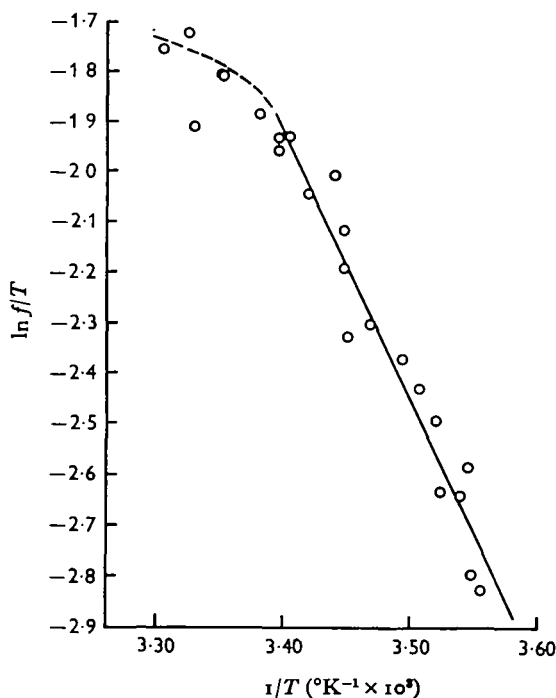


Fig. 1. Relation between frequency ( $f$ ) and absolute temperature ( $T$ ) for living spermatozoa of the sea-urchin *Lytechinus pictus*.

Table 1. Values for the change in entropy ( $\Delta S$ ) and enthalpy ( $\Delta H$ ) derived from observations on sea-urchin spermatozoa

Measured parameter	<i>Lytechinus pictus</i> sperm (L)		<i>Strongylocentrotus purpuratus</i> sperm (S)	
	$\Delta H$ (Kcal/mole)	$\Delta S$ (entropy units)	$\Delta H$ (Kcal/mole)	$\Delta S$ (entropy units)
Frequency, live sperm (L)	$10.5 \pm 0.3$	$-15.4 \pm 0.5$	$6.7 \pm 0.3$	$-28.3 \pm 1.0$
$f_{\max}$ sperm models (M)	$9.2 \pm 0.3$	$-20.1 \pm 0.7$	$7.0 \pm 0.8$	$-28.0 \pm 1.7$
Frequency at low ATP concentration, sperm models (N)	$13.7 \pm 0.5$	$-8.7 \pm 0.5$	$7.4 \pm 0.5$	$-29.1 \pm 2.0$
$\bar{K}$ sperm models (K)	$6.5 \pm 0.5$	$-21.0 \pm 2.0$	$7.6 \pm 0.6$	$-12.9 \pm 1.0$

(Letters in parentheses are the key to abbreviations used in Fig. 2: thus LM in Fig. 2 refers to activation parameters derived from measurements of  $f_{\max}$  on *Lytechinus* sperm models.)

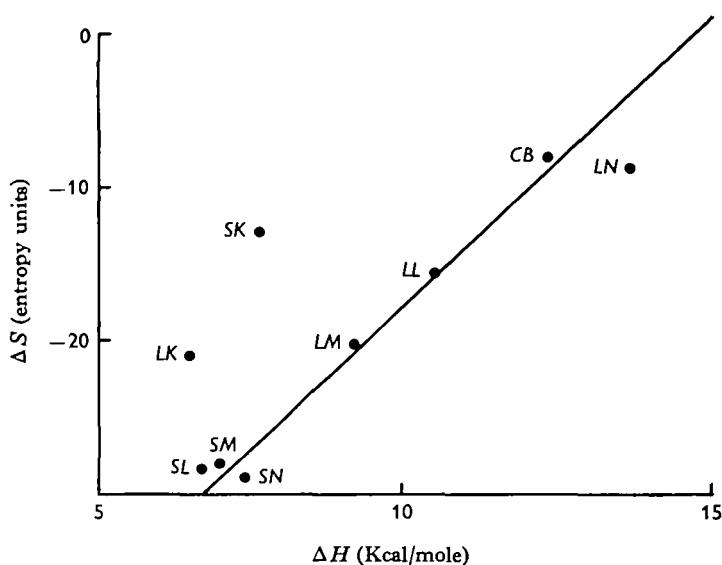


Fig. 2. Variation of the change in entropy ( $\Delta S$ ) with the change in enthalpy ( $\Delta H$ ) derived from the temperature dependence of various parameters. CB corresponds to the activation enthalpy and entropy for the ATP-myosin system. The key to the other abbreviations is given in Table 1. The straight line is the regression line derived from the activation parameters calculated from observations of several cilia and flagella (Holwill & Silvester, 1967).

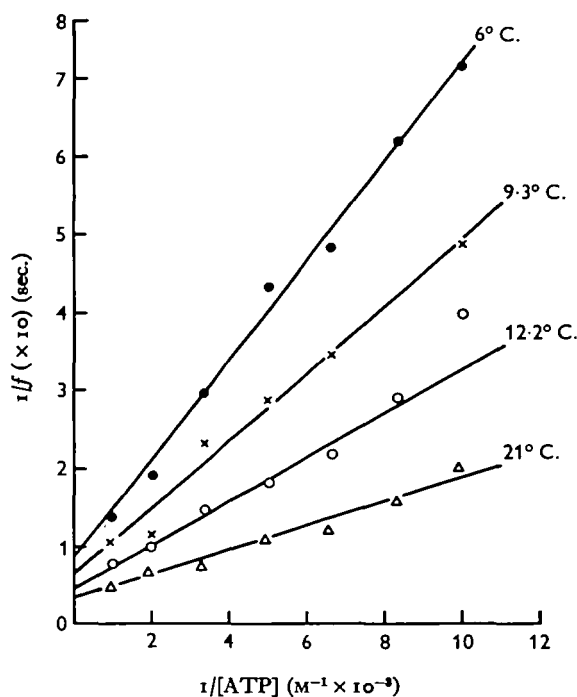


Fig. 3. Relation between frequency ( $f$ ) and ATP concentration (ATP) at several different temperatures for glycerinated spermatozoa from the sea-urchin *L. pictus*.

The variation of the reciprocal of frequency with the reciprocal of the ATP concentration for glycerinated models of *Lytechinus* spermatozoa at different temperatures is shown in Fig. 3. (Similar results were obtained for *Strongylocentrotus* spermatozoa.) At each temperature a linear relationship was obtained, showing that equation (5) holds. From the regression lines values of  $f_{\max}$  and  $\bar{K}$  (equation (5)) were obtained at each temperature.

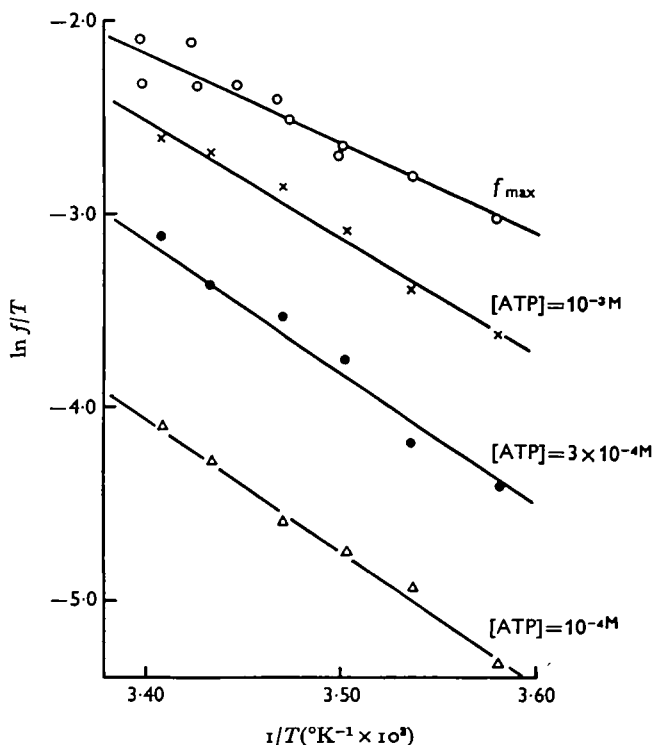


Fig. 4. Variation of frequency ( $f$ ) with absolute temperature ( $T$ ) at various ATP concentrations for glycerinated spermatozoa from the sea-urchin *L. pictus*. The variation with temperature is also shown for the frequency ( $f_{\max}$ ) which is approached as the ATP concentration is increased.

In Fig. 4 are plotted the variations with  $1/T$  of  $\ln f_{\max}/T$  and of  $\ln f/T$  at various ATP concentrations for spermatozoa of *Lytechinus*. (Graphs of similar character were obtained for *Strongylocentrotus* spermatozoa.) The curves for  $f_{\max}$  and low ATP concentrations ( $3 \times 10^{-4}$  M) are linear, and allow the calculation of activation entropies and enthalpies (see Table 1) while the graph at a 1 mM concentration shows some curvature. A graph of  $\ln \bar{K}/T$  against  $1/T$  for spermatozoa of *Lytechinus* is shown in Fig. 5. This curve, and the corresponding one for *Strongylocentrotus* spermatozoa, are both approximately linear over the range of temperatures in which studies were made, thus permitting the calculation of activation parameters (see Table 1) whose significance will be discussed later. For comparison with other results all the thermodynamic data calculated here are plotted on Fig. 2.

*Effects of pH on frequency*

Glycerinated spermatozoa from *Strongylocentrotus* were examined over a wide range of pH values at high ( $10^{-3}$  M) and low ( $10^{-4}$  M) ATP concentrations. The results are presented in Fig. 6 in which frequency is plotted against pH. At each concentration

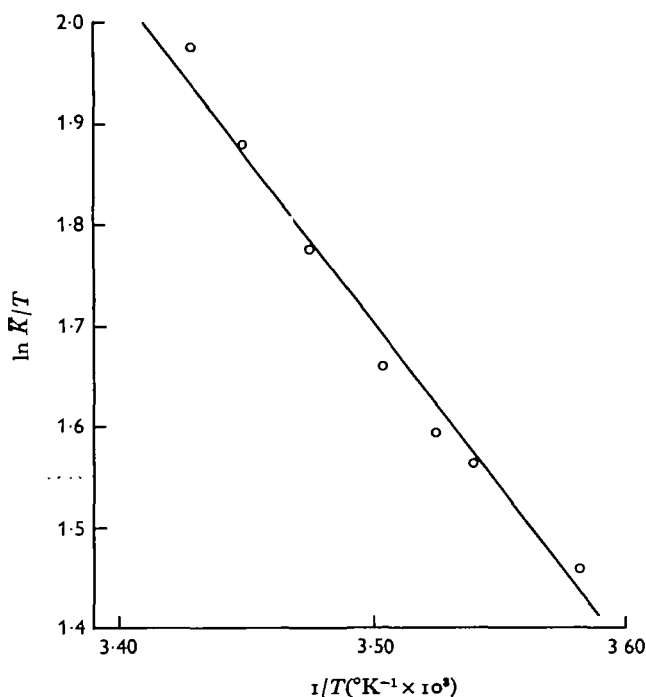


Fig. 5. Variation of the constant  $K$  (see equation (2)) with absolute temperature ( $T$ ) for glycerinated spermatozoa from the sea-urchin *L. pictus*.

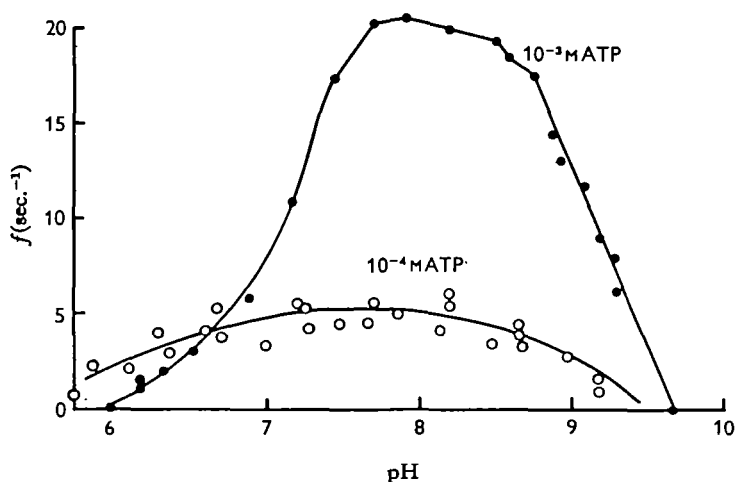


Fig. 6. Relation between the frequency ( $f$ ) and pH at high ( $10^{-3}$  M) and low ( $10^{-4}$  M) concentrations of ATP for glycerinated spermatozoa from the sea-urchin *Strongylocentrotus purpuratus*.



of ATP there is an optimum pH range centred at about pH 8.0. In the range pH 7.0—9.0 all motile organisms maintained progressive waves on their flagella. The number of motile organisms was about 40–50 % of the total population, as is normally seen in preparations of this nature. In the regions below pH 7.0 and above pH 9.0 the number of moving spermatozoa fell rapidly to about 5 % of the total population and the movements observed were mainly localized oscillations.

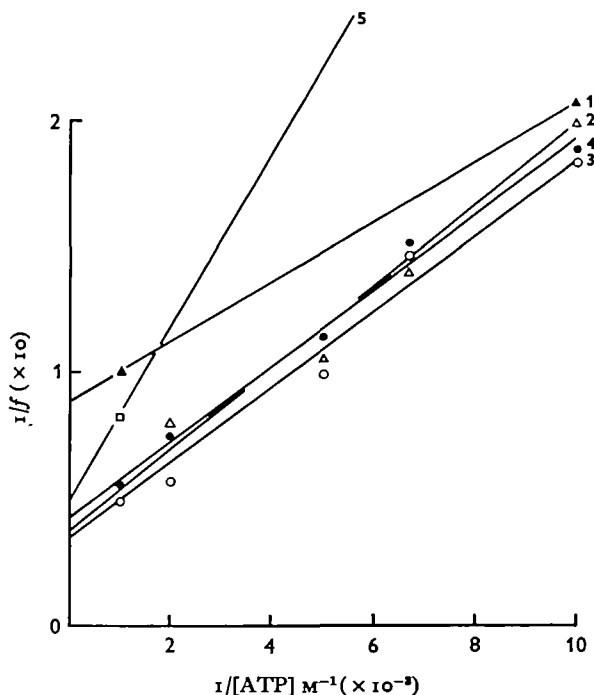


Fig. 7. Relation between the frequency ( $f$ ) and ATP concentration (ATP) at various values of pH for glycerinated spermatozoa from the sea-urchin *S. purpuratus*. Key: 1, ▲, pH 7.0; 2, ●, pH 7.5; 3, ○, pH 8.0; 4, Δ, pH 8.5; 5, □, pH 9.0. The line for pH 9.0 was extrapolated to allow a reasonable scale to be used.

Table 2. Values of  $f_{\max}$  and  $K$  at various pH for spermatozoa from *Strongylocentrotus purpuratus*

pH	$f_{\max}$ (sec. <sup>-1</sup> )	$K$ (M <sup>-1</sup> × 10 <sup>-3</sup> )
7.0	11.2	7.5
7.5	23.8 ± 2.0	2.7 ± 0.3
8.0	28.6 ± 3.0	2.4 ± 0.4
8.5	27.0 ± 3.0	2.4 ± 0.4
9.0	20.4	1.4

Frequency measurements were also made on *Strongylocentrotus* spermatozoa at several ATP concentrations while the pH was maintained at 7.5, 8.0 and 8.5. At each pH graphs were drawn of the reciprocal of the frequency against the reciprocal of the ATP concentration (Fig. 7). The two results obtained in the previous study at both pH 7.0 and pH 9.0 are also plotted on this graph. Values of  $K$  (from the slope) and  $f_{\max}$  from the intercept are given in Table 2. Although the values for  $f_{\max}$  show some

variation, the values of  $K$  are equal within the limits of experimental error, except at the high and the low pH values.

Figure 8 shows the variation of  $f'/f$  with  $[H^+]$  on the acidic side of the pH optimum ( $f'$  is the value of the frequency at the optimum pH) while Fig. 9 is a plot of  $f'/f$  against  $1/[H^+]$  on the basic side of the pH optimum. Linear relationships are obtained in all cases, but whereas the slopes of the lines at the two ATP concentrations in Fig. 9 are roughly equal, in Fig. 8 they are certainly different.

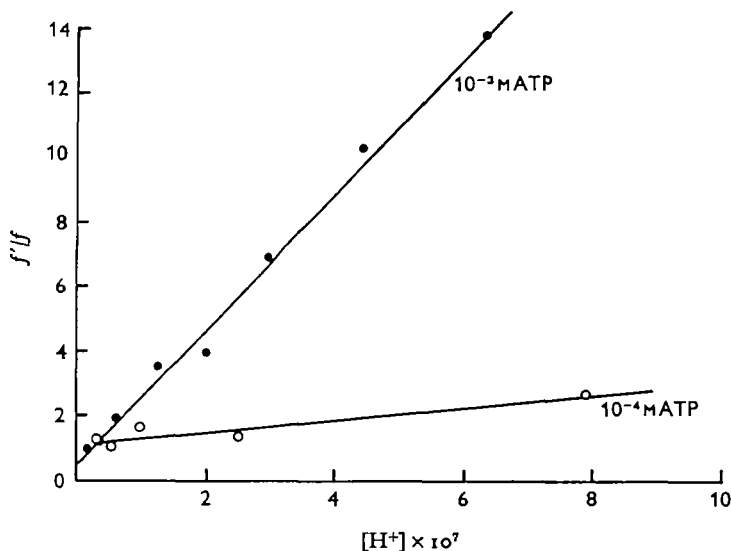


Fig. 8. Relation between the frequency ( $f$ ) and the hydrogen-ion concentration ( $[H^+]$ ) at low ( $10^{-4}$  M) and high ( $10^{-3}$  M) concentrations of ATP on the acidic side of the pH optimum for glycerinated spermatozoa from *S. purpuratus*.  $f'$  is the frequency at the pH optimum in Fig. 6.

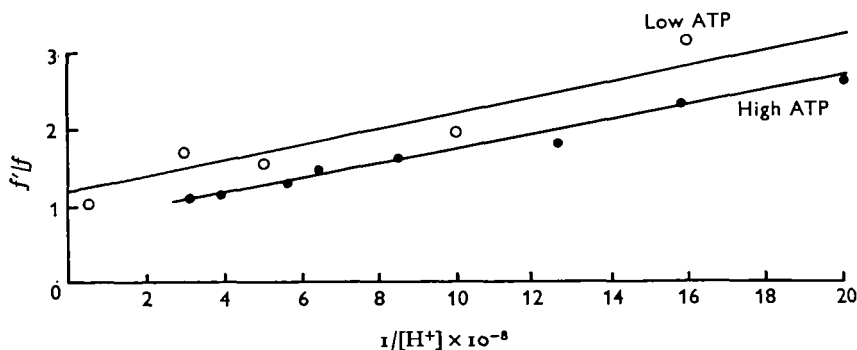


Fig. 9. Relation between the frequency ( $f$ ) and the hydrogen-ion concentration ( $[H^+]$ ) at low ( $10^{-4}$  M) and high ( $10^{-3}$  M) concentrations of ATP on the basic side of the pH optimum for glycerinated spermatozoa from *S. purpuratus*.  $f'$  is the frequency at the pH optimum in Fig. 6.

The variation of frequency with pH was also examined for glycerinated *Lytechinus* spermatozoa at an ATP concentration of  $10^{-3}$  M. The relationship is qualitatively similar to that for *Strongylocentrotus* spermatozoa, but the optimum value of the pH is about 8.5. Abnormalities were again noticed in the movements of spermatozoa outside

the range pH 7.0–9.0. Linear relationships were obtained between  $f'/f$  and  $[H^+]$  on the acidic side of the optimum pH and between  $f'/f$  and  $1/[H^+]$  on the basic side, although some deviation occurs near the optimum pH in the latter case.

#### Effects of dielectric constant on frequency

Suspensions of glycerinated spermatozoa from *Strongylocentrotus* were subjected to varying concentrations of methanol in an attempt to discover the effects of changing the dielectric constant of the medium on the frequency. The ATP concentration was

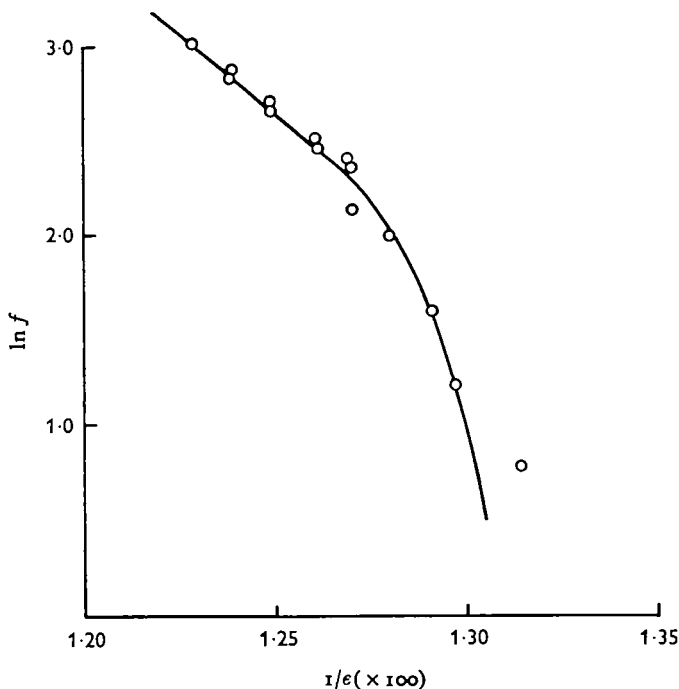


Fig. 10. Relation between the frequency ( $f$ ) and the dielectric constant  $\epsilon$  for glycerinated spermatozoa from *S. purpuratus*. The ATP concentration was  $10^{-3}$  M.

maintained at  $10^{-3}$  M. Figure 10 is a graph of the logarithm of the frequency against the reciprocal of the dielectric constant ( $\epsilon$ ). For  $1/\epsilon$  in the range  $1.23 \times 10^{-2}$  to  $1.28 \times 10^{-2}$  the graph is linear with a slope of  $-1.88 \pm 0.2 \times 10^3$ . Further examination of these results will be deferred to the discussion.

#### Effects of ionic strength on frequency

Figure 11 depicts the variation of the frequency of glycerinated spermatozoa from *Strongylocentrotus* with the ionic strength ( $u$ ) of the medium. It can be seen that the frequency passes through a maximum in the region  $u = 0.5$ . At low ( $< 0.1$ ) and high ( $> 0.5$ ) ionic strengths the number of motile organisms was much less than normal, and the movements observed were erratic and irregular, often consisting of localized oscillations. Figure 12 shows a graph of  $\log_{10} f$  against  $\sqrt{u}$  for the region of ionic strength in which normal movement was observed. The graph is curved, in contrast to the prediction of equation (11) and the results of Hoffmann–Berling for locust spermatozoa.

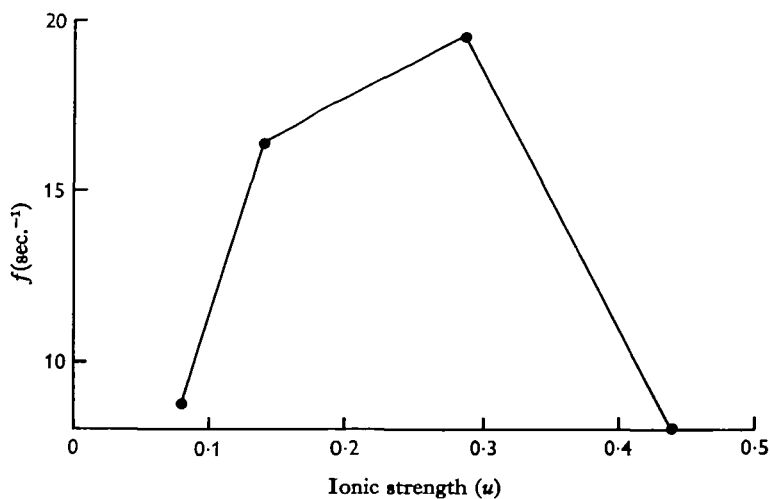


Fig. 11. Variation of frequency ( $f$ ) with ionic strength ( $u$ ) for glycerinated spermatozoa from the sea-urchin *S. purpuratus*. The ATP concentration was  $10^{-8}$  M.

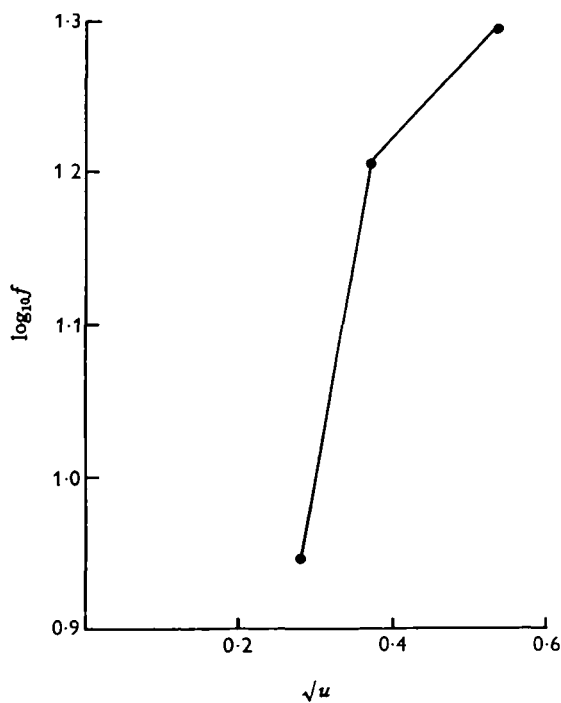


Fig. 12. Another presentation of the relation between the frequency ( $f$ ) and the ionic strength ( $u$ ) for glycerinated spermatozoa of the sea-urchin *S. purpuratus*. The ATP concentration was  $10^{-8}$  M.

## DISCUSSION

The results summarized by Fig. 1 confirm an earlier subjective impression (Holwill & Silvester, 1967) that at the higher temperatures the frequencies are lower than those required to give a consistent linear relationship between  $\ln f/T$  and  $1/T$ . The behaviour shown in Fig. 1 is typical of the temperature dependence of the rate of enzyme reactions, the curvature being attributed to denaturation of the enzyme. For the spermatozoa of *Lytechinus* and *Strongylocentrotus* and for the spermatozoa from several marine organisms (M. E. J. Holwill, unpublished results) the curvature (and presumably the denaturation of the enzyme which participates in the reaction providing energy for the motile process) occurs at a lower temperature than for other organisms (Holwill & Silvester, 1967). This observation could result from one of two situations. In the first, the enzymes differ slightly in structure from one organism to another so that the number or type of bonds which need to be broken for denaturation to occur is variable among organisms; consequently the denaturation energy (and hence effect of temperature) could change from one species to another. In the second, the enzymes are identical in all organisms but the environmental conditions (e.g. pH, ionic strength, etc.) are variable and are such as to favour denaturation at lower temperatures in the spermatozoa of marine organisms than in others. The effect could be associated with the fact that marine organisms normally encounter lower environmental temperatures (in the region of 16° C.) than many of the organisms previously examined by Holwill & Silvester (1967). For example, mammalian spermatozoa and ciliated lung tissue are normally exposed to temperatures of the order of 37° C.

The results obtained from the variation of frequency with temperature for both live and glycerinated sea-urchin spermatozoa conform to the linear pattern of activation parameters obtained earlier for a number of flagellates and ciliates (see Fig. 2 and Holwill & Silvester (1967)), thereby suggesting that the chemical reaction which limits the frequency of the sperm tails is similar to that in other flagellates. It may be significant that for the two species of sea-urchin the thermodynamic parameters characteristic of the rate-limiting step in the live spermatozoa are the same as those in the glycerinated models within the limits of experimental error. This observation strongly suggests that the reactions *in vitro* are identical with those *in vivo*, but further work on a variety of glycerinated flagellates is needed to test this conclusion.

Since the relation between frequency and ATP concentration appears to follow Briggs-Haldane kinetics, the variation of  $f_{\max}$  with temperature essentially represents the variation of  $k_2$  with temperature, since the total enzyme concentration is constant (see equation (4)). Since  $k_2$  is the rate constant applicable to the breakdown of an enzyme-substrate complex the activation parameters derived from a plot of  $\ln k_2/T$  against  $1/T$  are those corresponding to this breakdown. It is necessary to examine the constants derived from the  $f_{\max}$  plots to see how they are related to those of complex breakdown. Since  $\Delta H^\ddagger$  is determined by the slope of the graph, its value is independent of the magnitude of the constant  $[E_0]/\alpha$  (equation (4)), whereas this is not true for  $\Delta S^\ddagger$ , which is obtained from the intercept of the line; it is thus possible that the entropies derived are not those of the chemical reaction which limits the frequency but contain an additional term that is constant for all organisms. If each enzyme molecule is used once per beat (the available experimental evidence does not contradict this (Brokaw,

1967)), then the constant  $E_0/\alpha$  is unity. In any event the linearity of the  $\Delta S^\ddagger$  against  $\Delta H^\ddagger$  plot indicates that the constant  $E_0/\alpha$  is the same for all flagella so far examined, and is of such a value as to allow the point corresponding to the breakdown of an ATP-myosin complex to fall close to the line. The close correspondence of the entropies and enthalpies obtained from the live spermatozoa and the  $f_{\max}$  of glycerinated spermatozoa supports the earlier conclusion (Holwill & Silvester, 1967) that the rate-limiting step within a flagellum is the breakdown of an ATP-ATPase complex.

The activation parameters derived for glycerinated spermatozoa may be discussed in terms of the enzymic reaction (equation (1)) used to derive the Briggs-Haldane equation (equation (2)). It must be pointed out, however, that equation (1) may not be truly representative of the chemical reactions occurring within the flagellum; for

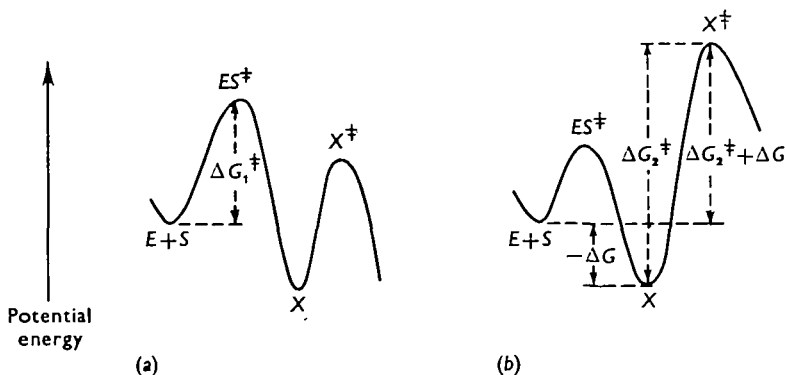


Fig. 13. Schematic energy diagrams for enzyme reactions which may be represented by equation (1): (a) when  $k_2 \gg k_{-1}$  and (b) when  $k_{-1} \gg k_2$ .  $[ES]^\ddagger$ ,  $X$  and  $X^\ddagger$  are three forms of the enzyme substrate complex, the superscript  $\ddagger$  indicating the activated state.

example, there might be intermediate processes which would necessitate the introduction of further rate constants. The presence of such intermediate reactions would not necessarily alter the form of the Briggs-Haldane equation, but would certainly alter the significance of the constant  $\bar{K}$  and possibly also  $k_2$  (see, for example, Laidler (1958)).

At low substrate concentrations equations (2) and (3) reduce to

$$V = \alpha f = k_2 \bar{K} [E_0] [S]. \quad (12)$$

Since  $[E_0]$  and  $\alpha$  are constants the variation of  $f$  at a single low substrate concentration reflects the variation of the complex constant  $k_2 \bar{K}$ . The relation between  $\ln f/T$  and  $1/T$  at  $10^{-4}$  M-ATP is linear, suggesting that  $k_2 \bar{K}$  is a rate constant, an equilibrium constant or the product of two such constants, since it is only under these special conditions that linearity is to be expected. Since  $\bar{K} = k_1/(k_{-1} + k_2)$  it is evident that if either of the inequalities  $k_{-1} \ll k_2$  or  $k_{-1} \gg k_2$  obtains, then two of the special conditions noted above are possible. If  $k_{-1} \ll k_2$  then  $\bar{K} = k_1/k_2$  and  $k_2 \bar{K}$  is the rate constant  $k_1$ . On the other hand, if  $k_{-1} \gg k_2$ ,  $\bar{K}$  is the equilibrium constant  $k_1/k_{-1}$ . Because the energy relationships are different in the two cases (see Fig. 13 and Laidler (1958)) it is important to establish which is applicable to the flagellum. On the basis of the present results the distinction cannot be made and further experiments designed to investigate more fully the type of reaction responsible for flagellar motility are evidently necessary

before the real nature of the reaction can be resolved. One such experiment would involve the use of reagents to block the reaction at certain parts of its cycle while another might involve stop-flow calorimetry.

On the basis of simple Briggs-Haldane kinetics,  $k_{-1}$  should be larger than  $k_2$  to be consistent with the results, leading to the conclusion that the limiting chemical reaction in flagella is the breakdown of an enzyme-substrate complex.

The general nature of the effects on the frequency of changing the hydrogen-ion concentration is similar to the variation in ATPase activity of glycerinated flagella

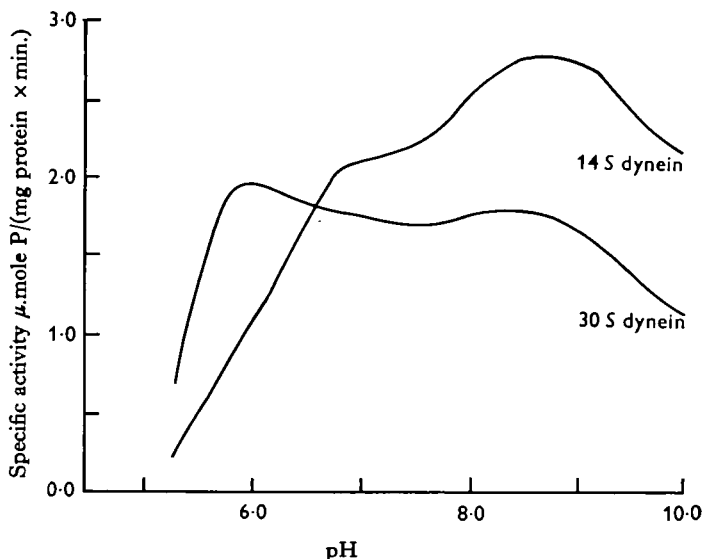


Fig. 14. Variation of the specific ATPase activity with pH for 14S and 30S dynein. (From Gibbons, 1966.)

subjected to similar treatment (e.g. Tibbs, 1957, 1959) in that the activity in both cases passes through a maximum. However, as pointed out by Gibbons (1966), no direct comparison can be made with the earlier results since the enzymic activity of glycerinated flagella is probably due to a mixture of ATPase components, whereas the frequency presumably derives from a single component (but see later). It is of greater interest to compare the frequency results with those obtained for dynein (Gibbons, 1966), the ATPase protein which has been isolated from *Tetrahymena* cilia and which is believed to play an important role in flagellar movement. Dynein is found in two forms (Gibbons, 1965*a, b*; Gibbons & Rowe, 1965), one sedimenting at 14S and the other at 30S in the ultracentrifuge. The enzymic and physico-chemical properties of the two forms of dynein are consistent with the idea that the 30S protein is a polymer of the 14S form (Gibbons, 1966; Gibbons & Rowe, 1965).

The ATPase activity of 30S dynein varies little in the range of pH from 6.0 to 9.0, whereas for 14S dynein an optimum pH occurs, with a fairly broad maximum, in the region of pH 9.0 (Fig. 14). The different behaviour of the two types of dynein is attributed to the more unstable nature of 14S dynein under acid conditions.

The variations of frequency with pH for glycerinated spermatozoa at high concentrations of ATP (Fig. 6) are much closer to the curve for 14S dynein than to that for 30S

dynein, although graphs similar to the observed frequency variations could be obtained by combining the two dynein curves in suitable proportions.

Caution must, of course, be exercised in the interpretation of the frequency results in terms of the activity of dynein *in vivo* since: (a) the dynein employed by Gibbons was obtained from *Tetrahymena* cilia while the observations of frequency were made on sea-urchin spermatozoa; (b) the results for dynein were obtained from experiments on extracted protein, whereas the frequency results derive from a physiologically intact mechano-chemical system; and (c) Gibbons did not use KCl or urea in his assay solutions, whereas these compounds were both present in the solutions used here to re-activate the glycerinated flagella.

Although these facts must be borne in mind, the results lead one to speculate that dynein in the 14S form is the more likely of the two types of protein to be involved in the motile process *in vivo*. Other work (Gibbons, 1965*a, b*) has shown, however, that 30S dynein is the more physiologically active of the two types. Perhaps it is not unreasonable to suppose that, in the glycerinated organisms, both types of dynein contribute in some way to the bending of the flagellum and their combined ATPase activities manifest themselves in terms of the frequency. More confidence could be placed in this interpretation if it were shown that dynein extracted from sea-urchin spermatozoa had a variation of ATPase activity with pH similar to that of the dynein extracted from *Tetrahymena* cilia.

It is, of course, possible, as suggested by C. J. Brokaw (personal communication), that the variations in frequency reflect the behaviour of an ATPase responsible for initiating the propagation of waves along the flagellum rather than that of an ATPase (dynein) distributed throughout the flagellum. If this is shown to be the case the preceding argument will be clearly invalid. The results of further experiments are necessary to test this idea.

Most of the results pertaining to the pH studies are consistent with the conditions derived by Laidler (1955*b*) for an enzyme in which both the acidic and basic groups of the enzyme participate in the reaction but where complexing with the substrate occurs at neither of these sites. Specifically, these conditions are: (a) the rate of reaction should pass through maxima at low and high substrate concentrations, (b) the pH optimum should be independent of the substrate concentrations, (c) the constant  $K$  should be independent of pH, and (d) on the acidic side of the pH optimum  $V_{\max}/V$  should be proportional to  $[H^+]$ , while on the basic side it should be proportional to  $1/[H^+]$ , the proportionality constants in each case being independent of the substrate concentration.

For flagellar movement  $V = \alpha f$  (equation (3)), so that the variation of  $f'/f$  essentially represents that of  $V_{\max}/V$ . The investigations of the effect of pH changes on the movement of glycerinated sea-urchin spermatozoa are not comprehensive, but only one exception to the above conditions has so far been observed. This is in the plots for *Strongylocentrotus* spermatozoa of  $f'/f$  against  $[H^+]$ , on the acidic side of the pH optimum, where a difference occurs in the proportionality constants at low and high ATP concentrations. The discrepancy may be associated with the errors of measurement of the low frequencies found at low ATP concentrations, since considerable scatter was observed in the readings, particularly on the acid side of the pH optimum (Fig. 6).

The linear character of Figs. 8 and 9 supports the idea that the substrate does not change its state of ionization over the range of pH investigated (Laidler, 1955*a, b*).



This is in accord with a report by Phillips, George & Rutman (1966), who found that in the presence of excess magnesium the ionization constant of ATP is so altered that above pH 5.8 more than 80% of the total ATP is in the form of  $\text{Mg-ATP}^{2-}$ . Consequently, in the range of pH under discussion, the substrate will not sensibly change its state of ionization.

It is of interest to note that the values for  $K$  obtained here ( $\sim 2.5 \times 10^3 \text{ M}^{-1}$ ) are somewhat lower than that ( $\sim 10^5 \text{ M}^{-1}$ ) obtained for dynein but correspond closely to the value derived elsewhere for glycerinated spermatozoa of *Lytechinus pictus* (Brokaw, 1967). The difference between the two figures is evidently significant and may be associated with the different ionic conditions of the two systems.

When considering the results of variations in dielectric constant and ionic strength, the conclusion that the frequency is limited by the breakdown rather than the formation of an enzyme-substrate complex leads to a different inference from the one which was drawn in an earlier paper (Holwill & Silvester, 1965). In the earlier work examination of the results of Hoffmann-Berling (1955) relating to ionic strength led to the conclusion that the limiting reaction within the flagellum is one between ions of like sign. The arguments which lead to this deduction, however, are based on a model for complex formation and are derived from a consideration of the approach of two ions (see REACTION KINETICS, p. 205).

In the case where the dielectric constant is varied, equations will be obtained which are similar in character for either the breakdown of a complex or its formation. However, the electrostatic forces between ions are such that the conclusion to be drawn from a particular result will, in the case of breakdown, be directly opposite to that which would follow if one were considering complex formation.

Thus, in the case of the variation of dielectric constant, during breakdown of an enzyme-substrate complex, the molecules will separate from each other and the following equation would replace equation (10):

$$\ln k = \ln k_{\infty} + \frac{Z_a Z_b e^2}{\epsilon d_{ab} kT}. \quad (13)$$

The form of the appropriate graph is thus unchanged, but the conclusion to be drawn therefrom is different. In these circumstances the results described in an earlier section lead us to conclude that the breakdown of the complex involves the separation of unlike ions. Equation (13) applies to the separation of two ions but it is likely that the disruption of the complex involves at least three products, e.g.



where  $P_i$  represents inorganic phosphate. The separation of several ions would obviously lead to a more complex relationship than equation (13), but the reciprocal dependence of  $\ln k$  on  $\epsilon$  would be preserved. To extend equation (13) to the reaction specified by equation (14), consider the simultaneous separation to infinity of three charges  $Z_a e$ ,  $Z_b e$  and  $Z_c e$ . The work done during the separation is

$$W = -\frac{1}{2} \sum_{j=1}^3 q_j \phi_j, \quad (15)$$

where  $\phi_j$  is the potential at the charge  $q_j$  due to the other two charges. If the assumption is made that the three charges are each separated by the same distance  $d$  (this is

obviously incorrect since the products have varying sizes, but it will serve to provide a measure of the order of magnitude of the separations) then

$$W = \frac{-e^2}{d}(Z_a Z_b + Z_b Z_c + Z_c Z_a), \quad (16)$$

leading to

$$\ln k = \ln k_\infty + \frac{e^2}{\epsilon d k T}(Z_a Z_b + Z_b Z_c + Z_c Z_a). \quad (17)$$

According to this equation, the slope of Fig. 10 is

$$\frac{e^2}{d k T}(Z_a Z_b + Z_b Z_c + Z_c Z_a),$$

so that the value for

$$\frac{Z_a Z_b + Z_b Z_c + Z_c Z_a}{d} \text{ is } -3.4 \times 10^8 \text{ cm}^{-1}.$$

Table 3 indicates the values of  $d$  for various values of  $(Z_a Z_b + Z_b Z_c + Z_c Z_a)$ . Since the slope of Fig. 10 is negative, the sum in brackets must also be negative. To obtain a reasonable value for  $d$  the sum of the products of the charges must be in the region of  $-4$  or less. Such a value could be obtained by setting (say)  $Z_a = -1$ ,  $Z_b = -2$  and  $Z_c = 2$ , which are not unreasonable values in the present situation. It is interesting to note that, since the products—Mg-ADP and inorganic phosphate—have negative charges, the enzyme must be considered as having a positive charge. Since, from the pH studies, both the acidic and basic groups on the enzyme participate in the breakdown of the complex, the basic group must possess numerically more charges than the acidic group.

Table 3. *Values for the interionic separation ( $d$ ) computed for integral values of  $(Z_a Z_b + Z_b Z_c + Z_c Z_a)$  using Fig. 10 and equation (17)*

$Z_a Z_b + Z_b Z_c + Z_c Z_a$	$d(\text{\AA})$	$Z_a Z_b + Z_b Z_c + Z_c Z_a$	$d(\text{\AA})$
1	0.29	5	1.47
2	0.59	6	1.77
3	0.88	7	2.30
4	1.18		

The foregoing analysis presupposes that the only effect of the introduction of methanol into the medium is to change the dielectric constant of the flagellar environment. It is possible that other effects contribute to the change in frequency when various concentrations of methanol are introduced. Attempts to use other liquids, e.g. dioxane, as a substitute for the methanol resulted in the death of the organisms and it has not been possible to establish whether the only factor that changes in this case is the dielectric constant. These comments must be considered in relation to the foregoing analysis.

The results obtained from varying the ionic strength of the medium are more difficult to interpret in terms of complex breakdown since the simple Debye-Hückel theory, upon which equation (11) is based, fails when the concentration of ions becomes high. In the present case the 0.25 M concentration of KCl is apparently too high for the simple theory to apply and probably accounts for the non-linearity of Fig. 12. If the more extensive Debye-Hückel theory is applied to the flagellar motion, equations are obtained that cannot be usefully interpreted in terms of the results

obtained. The results are generally in agreement with those obtained by Hoffmann-Berling (1955), but it is apparent that a different interpretation is to be placed on these results from that discussed previously (Holwill & Silvester, 1965).

It is possible that the effects observed when the ionic strength was changed were due not only to the change in ionic strength produced by the addition of potassium chloride but also to the presence of the potassium chloride *per se*. Gibbons (1966) found that the activity of 30S dynein was enhanced when the KCl concentration was increased, while that of 14S dynein was decreased. Again, as in the results from the pH studies, to obtain a curve from the dynein results that is similar in form to the frequency results (which pass through a maximum at an ionic strength of about 0.5 (Fig. 11) corresponding to a KCl concentration in the region of 0.2 M) it is necessary to assume that both 14S and 30S dynein participate in the process responsible for flagellar movement.

The fact that fairly extreme departures from the normal re-activation medium prepared for the flagellar models restricted the ability of an organelle to sustain a progressive wave suggests that these treatments (high or low pH, low ionic strength, high KCl concentrations) in some way affect the mechanism responsible for wave propagation. Since the membrane is modified in some way by the procedures of glycerination, the ions introduced into the medium have access to all components of the flagellum, so that it is not possible to assign the function of propagation to any particular structure on the basis of the present results.

Evidence has been presented here that further supports the conclusion that the reaction which limits the frequency is the breakdown of an enzyme-substrate complex, and preliminary studies have been made to characterize more fully the enzymic reaction itself. These studies need to be extended in many ways, and it would be useful if studies could be made of the ATPase activity of dynein from the same organism that is used for frequency measurements.

#### SUMMARY

1. The movements of intact and glycerinated spermatozoa from two species of sea-urchin were examined in the range of temperature between 5 and 30° C. The variations of the frequency of the glycerinated spermatozoa with changing pH, dielectric constant and ionic strength were examined.

2. From the temperature studies values for activation entropies and enthalpies were obtained that pertain to the chemical reaction which limits the frequency.

3. Within the limits of experimental error the activation parameters are identical for the intact spermatozoa and for the glycerinated model of the same species. The results are consistent with the hypothesis that the rate-limiting reaction is the breakdown of an enzyme-substrate complex.

4. The pH studies suggest that neither the acidic nor the basic part of the enzyme is involved in complex formation but that both participate in the breakdown of the complex.

5. The results obtained from the studies of dielectric constant are interpreted in terms of a model for the breakdown of the enzyme-substrate complex involving the separation of several spherical charges.

6. The studies of pH and ionic strength suggest that both 14S and 30S dynein participate in the mechano-chemical process responsible for bending the flagellum.

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