THERMODYNAMIC ASPECTS OF FLAGELLAR ACTIVITY

BY M. E. J. HOLWILL AND N. R. SILVESTER

Department of Physics, Queen Elizabeth College, London, W.8

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INTRODUCTION

Despite recent advances in the fields of cytochemistry and electron microscopy the processes which are responsible for flagellar movement, and their chemical foundations, cannot be described with any certainty. In a previous communication (Holwill & Silvester, 1965) we described the variation with temperature of the frequency of the flagellar beat in *Strigomonas oncopelti* and deduced thermodynamic parameters which referred to the rate-limiting chemical reaction underlying the flagellar motion. The present paper represents an extension of the previous work to the cilia and flagella of a fairly wide variety of organisms. For each organism one can evaluate an activation enthalpy and an activation entropy that are characteristic of that particular motile system, and later we shall discuss the possible significance of these values in terms of a published model of flagellar action (Silvester & Holwill, 1965). Similar activation parameters may be calculated for some muscles and it is of interest to compare these values with those obtained for flagella.

The fine structure of cilia and flagella has been well established by electron microscopy (see Holwill, 1966, for references) although the functions of the several components are the subject of discussion. The reactivation of glycerol-extracted cilia and flagella by ATP (e.g. Brokaw, 1962, 1963; Satir & Child, 1963) has led to the suggestion that the dislocation of the terminal phosphate bond in adenosine triphosphate (ATP) may be the source of energy for their movement. The way in which the chemical energy is released in a controlled manner is uncertain, but it is accepted that a motile flagellum must possess elements for contraction and compression and, further, a means of propagating waves along its length.

Cytochemical techniques in conjunction with electron microscopy have been used in attempts to discover the presence and the site of ATPase activity within the flagellum. Positive results have been obtained in some instances, but the location of the ATPase appears to vary according to the organism examined. Thus, in the cilia of a rotifer, activity is found in two well-defined sites outside the axoneme (Lansing & Lamy, 1961) while for rat spermatozoa the nine fibrils surrounding the axoneme are found to have ATPase activity (Nelson, 1962). In *Tetrahymena* cilia, Gibbons (1963) has found that the activity is associated with the presence of 'arms' on the peripheral fibrils of the axoneme.

The sustained amplitude of beat which is observed in the flagella of many organisms implies that chemical energy is available throughout the length of the flagellum (e.g. Gray, 1955; Machin, 1958). The rate of expenditure of this energy is dependent on the ambient temperature and thus, as we have previously remarked (Holwill &

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Silvester, 1965), the thermal dependence of flagella activity may provide information about the molecular events which occur during the contraction cycle.

A contractile system of an apparently different character operates in muscle, but it has been demonstrated (Cain, Infante & Davies, 1962) that the supply of chemical energy needed for a muscle to perform external work is directly provided by ATP. The involvement of this energy-rich molecule in both muscular and flagellar mechanisms may provide an interesting link between the two systems. For a muscle which exhibits an exponential decay with time of either length or tension, the activation entropy and activation enthalpy can be deduced from the observed variation of the decay constant with temperature (Burge & Elliott, 1963). Such information, although little is available, may be compared directly with the thermodynamic parameters we have found for flagella.

THERMODYNAMICS

If one assumes that wave propagation in a flagellum is accomplished by repeated activation of successive contractile units along its length, it can be shown (Holwill & Silvester, 1965) that the relation between the rate of activation of contractile units and the number of units present resembles the equation which defines a first-order chemical reaction (Hinshelwood, 1940). The frequency of the flagellar beat (f) can be identified with the rate constant (usually given the symbol k) of the slowest chemical reaction involved in the contraction-relaxation cycle.

A statistical treatment of reaction rates (Glasstone, Laidler & Eyring, 1941) gives the equation

$$k = \frac{kT}{h} \exp\frac{-\Delta G^{\ddagger}}{RT}, \tag{1}$$

in which k and h are respectively the Boltzmann and Planck constants, R is the gas constant per mole, T the absolute temperature and $\Delta G^{\ddagger}$ the molar change in free energy that accompanies the activation of the chemical reaction. 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:

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}. \tag{2}$$

From equations (1) and (2), on equating the frequency with the rate constant, we obtain

$$\ln\left(\frac{f}{T}\right) = \left[\frac{\Delta S^{\ddagger}}{R} + \ln\left(\frac{k}{h}\right)\right] - \frac{\Delta H^{\ddagger}}{RT} \tag{3}$$

so that if experimental results are plotted as a graph of $\ln(f/T)$ vs. $1/T$ the points should lie on a straight line. Values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ can be calculated respectively from the slope and intercept of the line.

If a chemical reaction in solution is investigated under varied conditions (e.g. of pH, ionic strength, type of solvent) there is a general tendency for the free energy of activation to remain constant or at least change very little. Laidler (1965) explains this as a compensation effect in which, although $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ may both be influenced by solvent–solute interactions, the variations are frequently such that a change in $\Delta H^{\ddagger}$ is accompanied by a similar change in $T\Delta S^{\ddagger}$, so that the free energy is unaffected (see equation (2)). In such a case there is a linear correlation between values of $\Delta H^{\ddagger}$

and of $T\Delta S^{\ddagger}$ obtained for the same reaction under different conditions. Conversely, if such a straight line relationship is obtained between the values found for a given process in different organisms (in our case, for flagellar activity) one may be able to infer something about the underlying chemical reaction. Some care is necessary in the interpretation of such apparently linear relationships, as they can arise solely from errors of measurement (Exner, 1964; see also Discussion).

MATERIALS

The ciliated and flagellated cells used in this investigation were obtained from several sources which are acknowledged elsewhere in this paper. Some remarks on the preparation and use of individual specimens are made below.

Each of the three *Trypanosoma* species was obtained in culture form, as were also the ochromonad flagellate and *Tetrahymena pyriformis*. No dilution of these cultures was made before examination.

Chlamydomonas reinhardii, *Naegleria gruberi*, *Euglena viridis* and *E. gracilis* were all grown on nutrient agar. To obtain motile organisms the cultures were washed off the agar slopes and suspended in distilled water for varying periods of time.

Spores of the water-mould, *Blastocladiella emersonii*, were obtained in suspension by flooding scrapings of the mould with sterilized distilled water and, after a period of between 15 and 20 hr., pouring the suspension on PYG agar (0·125 % peptone, 0·125 % yeast extract, 0·3 % glucose, 2·0 % agar; Cantino, Lovett, Leah & Lythgoe, 1962). After a day in this condition motile spermatozoa were present in the preparation.

The boar semen was maintained at room temperature for about 18 hr. after ejaculation before being observed in undiluted form. Approximately 9 hr. elapsed between ejaculation and observation of the bull spermatozoa. The semen in this case was diluted tenfold with physiological saline immediately before observation.

Cilia from the lungs of man and guinea-pig were obtained in the form of tissue cultures prepared on specially designed microscope slides.

METHODS

Cilia and flagella were observed under phase-contrast conditions with a Zeiss W.L. research microscope whose stage was maintained at a controlled temperature. Frequency measurements were made stroboscopically except in the case of lung cilia, where the frequencies were so low as to make this method impracticable. The lung cilia were filmed with a Stalex high-speed cine camera at 200 frames per sec. (Holwill, 1964) and the frequencies were obtained by analysis of the film, which bore timing marks at 0·01 sec. intervals.

Temperature control and measurement. A Perspex box of internal dimensions 7·5 cm. × 12·5 cm. × 3·5 cm. and wall thickness 5 mm. was machined so as to fit on the mechanical stage of the microscope and move in response to the usual stage controls. The floor of the box (7·5 × 12·5 cm.) carried an ordinary microscope slide, but it was necessary to cut away the floor beneath the slide itself to allow the close approach of the substage condenser lens. The lid of the box (17 × 13 cm.) was larger than the box

itself and had a central hole through which the objective nose-piece penetrated. The lid remained stationary when the box was traversed beneath it.

A heater of about 20 turns of bare resistance wire was mounted in the box on the side farthest from the microscope body and worked at 6·3 V. a.c., 12 W. The other three sides of the box were lined with copper plates soldered to $\frac{1}{8}$ in. o.d. copper tubing which connected outside the box to a liquid cooling system. The coolant (ethyl alcohol) was circulated by a peristaltic pump through a copper heat-exchanger coil contained in a Dewar flask filled with alcohol and solid CO_2. The pump and stage-heater were controlled by the thermostatic relay system described below.

Microscope slides were adapted for temperature measurements by drilling through them a central hole of diameter 1 mm. A copper-constantan thermocouple junction entered the hole from below and was flush with the upper surface. The junction wires led to a miniature jack-plug socket at the side of the slide and the components were cemented in position. The thermocouple circuit was continued through a jack-plug connected to miniature coaxial cable in which the central core had been replaced by insulated constantan wire. The ice-point junction was simply made at the end of the coaxial cable (outside the box) by soldering the constantan core to the copper braid.

The e.m.f. in the circuit was found by interrupting the outer braid of the cable and balancing the e.m.f. across the interruption against a potentiometer voltage. The potentiometer system was a mercury cell in series with a 100 Ω calibrated helical potentiometer and a 50 kΩ variable resistance which was adjusted to make the potentiometer dial read directly, at the point of balance, in °C. The null detector was a Pye 'Scalamp' reflected-spot galvanometer of sensitivity 31 mm./μA. The potentiometer was made direct reading when the 'hot' junction was in sodium sulphate at the transition point, 32·38° C., and the thermocouple characteristic was then assumed to be linear over the range of temperature measurements. The assumption introduces errors which are zero at 0° C. and 32·4° C., and never more than 0·3° C. in the range 0–37° C. Temperatures could be read to within ±0·05° C.

Control of the temperature at the microscope slide was directly effected by means of the measuring system itself. Four cadmium sulphide photoconductive cells were mounted inside the galvanometer so that the light-spot fell on them successively as it traversed the scale (zero was at the centre of the scale). The outer cells, nearest the two ends of the scale, operated 'on' relays so that as the spot was deflected past one of them (due to a difference between the real temperature and the potentiometer setting) the heater or the cooling pump was put in action to restore the chosen temperature. As the temperature returned to the chosen value the spot passed one of the inner cells which switched off the previously activated relay. In this way temperature could be controlled to within ±0·5° C. The temperature latitude is relatively unimportant, as temperature readings were taken within approx. 10 sec. of each measurement of frequency.

Stroboscopic measurement of frequency. The stroboscope comprised a rotating disk with two open sectors of angle approx. 11°, which was mounted on the shaft of a variable-speed electric motor and periodically interrupted the illumination incident on the substage mirror of the microscope. The centre of the disk bore a small permanent bar magnet with the magnetic axis normal to the axis of rotation. The magnet rotated between the soft-iron poles of a magnetic circuit which threaded a high-

resistance coil ($2 \cdot 5$ kΩ). The alternating current thus produced in the coil was rectified, smoothed and measured with a microammeter. The current reading provided an indication of the speed of rotation of the stroboscope and the system was calibrated by viewing the disk with a flashing-neon lamp controlled by a conventional signal generator. Microammeter readings could then be converted directly into the equivalent cyc./sec. The stroboscope was mounted in a shock-absorbing system with a period of approx. $\frac{1}{3}$ sec. to prevent vibration from impairing the resolution of the microscope.

The stroboscope flash-rate could be determined to within $\pm 0 \cdot 25$ cyc./sec. and the stationary appearance of the flagella occurred within approximately the same frequency limits.

Analysis of data. For each species, measurements were made at six or seven temperature levels over the range 5–35° C. At each level the frequency and temperature was measured for each of three to six organisms chosen at random. (In the case of lung cilia, four films were taken at each level.) The set of data for each species was treated statistically to obtain the regression line of a graph of ln (f/T) *vs.* $1/T$, values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, and their computed errors. The experimental points were compared with the regression line to see whether the graph showed any significant curvature.

<div align="center">RESULTS</div>

Within the limits of our experimental method all the graphs obtained of ln (f/T) *vs.* $1/T$ were found to be essentially linear, showing that the thermodynamic relation of equation (3) holds for the organisms examined, in the range 5–35° C. A subjective impression, that the frequencies at the two ends of the range tended to be slightly lower than expected, would need statistical confirmation with a much larger number of observations. In any case, the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ obtained from the graphs must be regarded as average values for the quoted temperature range.

A typical graph obtained for one species is shown in Fig. 1. The activation enthalpies and entropies are recorded, with their computed standard deviations, in Table 1. For completeness, the activation parameters derived previously (Holwill & Silvester, 1965) are also included.

A graph which was plotted of $\Delta H^{\ddagger}$ *vs.* $\Delta S^{\ddagger}$, using the results of Table 1, was found to be approximately linear (Fig. 2) with a regression line of the form,

$$\Delta S^{\ddagger} = 3 \cdot 25 \Delta H^{\ddagger} - 50 \cdot 75.$$

It is necessary to demonstrate that the straight-line relationship has real significance and is not a fortuitous result of the kind of errors attributed to such isokinetic relationships by Exner (1964). The demonstration was accomplished (see Discussion and Fig. 3) by plotting a graph of ln f_{10} *vs.* ln f_{30} where f_{10} and f_{30} are the interpolated frequencies of a given species at 10 and 30° C. The graph showed a positive linear correlation.

Since we were primarily interested in the frequencies, no attempt was made in this work to measure any changes with temperature of amplitude or wavelength. Nevertheless, it may be stated that any such changes were very small and could not be detected by visual observation alone.
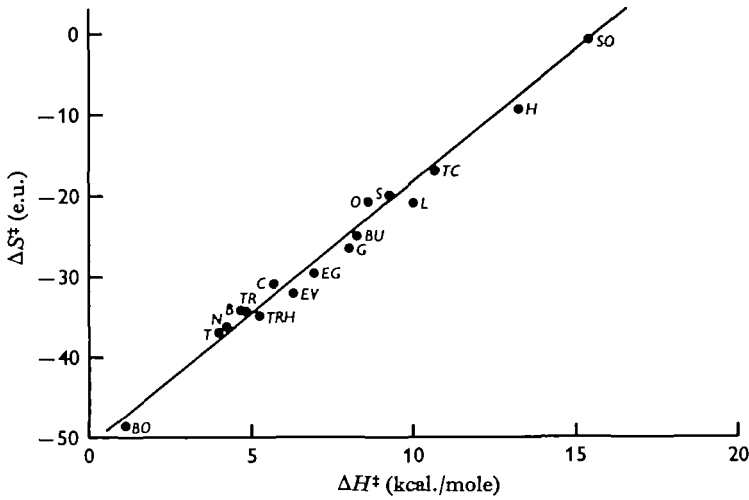
Fig. 1. The relation between flagellar frequency (*f*) and absolute temperature (*T*) in the case of *Chlamydomonas reinhardii*. The results are typical of those obtained for the organisms of Table 1. The numbers indicate how many readings gave an identical result.

Table 1. *The activation parameters of cilia and flagella of various organisms*

	Organism	$\Delta H^{\ddagger}$ (kcal./mole)	$\Delta S^{\ddagger}$ (e.u.)
(C)	*Chlamydomonas reinhardii*	$5\cdot70\pm0\cdot11$	$-31\cdot03\pm0\cdot40$
(EV)	*Euglena viridis*	$6\cdot30\pm0\cdot16$	$-32\cdot16\pm0\cdot58$
(EG)	*E. gracilis*	$6\cdot95\pm0\cdot25$	$-29\cdot62\pm0\cdot88$
(TR)	*Trypanosoma raiae*	$4\cdot85\pm0\cdot10$	$-34\cdot33\pm0\cdot35$
(TC)	*T. conorrhini*	$10\cdot68\pm0\cdot24$	$-17\cdot03\pm0\cdot86$
(TRH)	*T. rhodesiense*	$5\cdot27\pm0\cdot06$	$-34\cdot81\pm0\cdot24$
(N)	*Naegleria gruberi*	$4\cdot23\pm0\cdot08$	$-36\cdot12\pm0\cdot28$
(O)	Ochromonad flagellate	$8\cdot60\pm0\cdot31$	$-20\cdot84\pm1\cdot09$
(SO)	*Strigomonas oncopelti**	$15\cdot4\pm0\cdot7$	$-0\cdot9\pm2\cdot5$
(B)	*Blastocladiella* spermatozoa	$4\cdot67\pm0\cdot07$	$-34\cdot19\pm0\cdot26$
(BO)	Boar spermatozoa	$1\cdot14\pm0\cdot11$	$-48\cdot54\pm0\cdot39$
(BU)	Bull spermatozoa	$8\cdot27\pm0\cdot22$	$-25\cdot13\pm1\cdot24$
(L)	Locust sperm model†	10	-21
(T)	*Tetrahymena pyriformis*	$3\cdot97\pm0\cdot25$	$-36\cdot81\pm0\cdot88$
(S)	*Stentor‡*	$9\cdot3$	-20
(G)	Guinea-pig lung cilia	$8\cdot03\pm0\cdot70$	$-26\cdot6\pm2\cdot4$
(H)	Human lung cilia	$13\cdot27\pm0\cdot82$	$-9\cdot5\pm2\cdot8$

* Holwill & Silvester (1965).
† Glycerol-extracted models of locust sperm; calculated from the results of Hoffmann Berling (1955).
‡ Calculated from the results of Sleigh (1956).
The abbreviations in parentheses refer to points in Fig. 2.

Fig. 2. The activation entropy ($\Delta S^{\ddagger}$) plotted against activation enthalpy ($\Delta H^{\ddagger}$) for the organisms listed in Table 1. (See this table for abbreviations.) The line is a weighted regression line of $\Delta S^{\ddagger}$ on $\Delta H^{\ddagger}$.
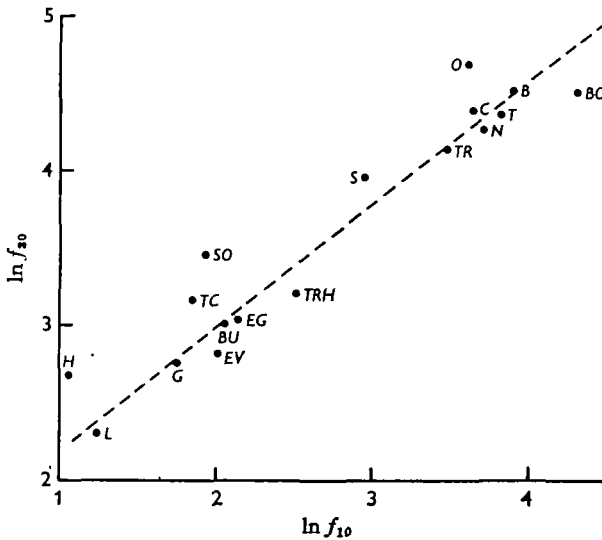

Fig. 3. A graph to test the significance of the linear relationship of Fig. 2 (see Discussion). f_{10} and f_{30} are corresponding beat frequencies of an organism at 10 and 30° C.

DISCUSSION

Although the temperature measurements in this work were straightforward it may be worth pointing out a difficulty which was experienced in the interpretation of stroboscope frequencies. Since the flagellar wave motion is in most cases sinusoidal or helical, the view in projection, when the motion is apparently arrested by the stroboscope, is of either a single or a double sine wave. (The double wave is a super-position of two images of the sine wave, with a phase difference of 180°.) The single

wave appears when the ratio of stroboscope frequency over beat frequency is 1, 1/2, 1/3, 1/4, etc., and the double wave when the ratio is 2, 2/3, 2/5, 2/7, etc. It is easy to confuse the ratios unless the stroboscope is taken through the whole range of frequencies to determine the true situation.

A possible criticism of the interpretation of the frequency measurements is the possibility that the variations in frequency can be attributed wholly to a change with temperature of the viscosity of the medium. We discounted this effect in a previous paper (Holwill & Silvester, 1965). That variation of viscosity with temperature which does occur will affect the values of the thermodynamic parameters to a small extent, but the published changes of frequency with viscosity for *Strigomonas* (Holwill, 1965) and some spermatozoa (Brokaw, 1966) indicate that, in the majority of organisms examined, the error involved will lie within the quoted experimental error. Exact corrections cannot be made until the variation of frequency with viscosity is known for each organism.

Over a variety of organisms and a range of frequencies from about 110 to 3 cyc./sec. the linearity of the graphs of $\ln (f/T)$ *vs.* $1/T$ appears to show that first-order reaction kinetics can be applied, at least within a limited temperature range, to the flagellar system. The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ obtained from the graphs (Table 1) vary between 1 and 15 kcal./mole, and between -50 and 0 e.u., respectively, and there is a good correlation between corresponding values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (Fig. 2). An unwary acceptance of such apparently isokinetic relationships has been criticized by Exner (1964) who points out that the errors involved in determining $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ from the thermal dependence of a rate constant are not independent of one another. ($\Delta S^{\ddagger}$ arises from the intercept of a graph, which depends on the slope of the chosen straight line; the slope of the line also determines $\Delta H^{\ddagger}$.) The result is that the error ellipse for a point ($\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$) on a graph such as Fig. 2 is a very narrow one with its long axis directed along a line of slope, $1/T'$, where T' is the average temperature of the experiment. A succession of experiments, even on a single system with constant values of $\Delta H^{\ddagger}$ and of $\Delta S^{\ddagger}$, could thus result in a series of points ($\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$) spread out along a line with slope $1/T'$, giving the appearance of a linear relationship. This spurious appearance will arise when the errors involved have the same order of magnitude as the range of measured values.

One way to determine whether the correlation in Fig. 2 is significant would be to plot error ellipses on the graph and show that they were much shorter than the extent of the line itself. The test proposed by Exner (1964) is much more sensitive: he plots $\ln k_1$ *vs.* $\ln k_2$, where k_1 and k_2 are the corresponding reaction rates at two temperatures T_1 and T_2 for a given reaction. If a series of reactions gives a linear correlation between $\ln k_1$ and $\ln k_2$ he admits the existence of a real isokinetic relationship. In our case we took frequencies as the rate constants, and used the frequencies at 10 and 30 °C. for each organism. The graph (Fig. 3) shows a correlation between $\ln f_{10}$ and $\ln f_{30}$, which we take to show that a significant isokinetic relationship exists between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$.

Two inferences may be drawn from this relationship between the activation parameters of the various organisms. First, the fact that the points ($\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$) lie on a regular line rather than in some random array seems to indicate that the chemical reactions which limit the flagellar activity in each organism are similar, and form a

reaction series with a common mechanism (Exner, 1964; Laidler, 1965). The reactions could be homologous ones, in which one reactant is varied from one organism to another by substitution (e.g. slight alterations in the structure of the ATPase molecule). The variations in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ from one reaction to another would be due, in this case, to steric and charge effects. Possibly a more likely hypothesis is that there is only one, identical, reaction in each organism. In this case the variations of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ can be explained as the effects of solvent–solute interactions. For instance, the local environment inside a flagellum may alter as from one organism to another, so that the limiting reaction takes place under different conditions of pH, ionic strength, dielectric constant, etc. Significant deviations from the line of Fig. 2 may arise in the case of some unusual factor in the constitution of the flagellum.

The second inference from the graph is that, since $\Delta S^{\ddagger}$ increases as $\Delta H^{\ddagger}$ increases, a 'compensation effect' (Laidler, 1965) is occurring in which changes in one parameter tend to balance changes in the other, so that the change in activation free energy, $\Delta G^{\ddagger}$, is minimized. The regression line of Fig. 2, which was obtained by weighting each point with regard to its computed variance, has a slope of $307 \pm 17^{\circ}$ K. so that at this temperature ($34 \pm 17^{\circ}$ C.) the changes in $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$ exactly balance, with the result that $\Delta G^{\ddagger}$ is constant from one organism to another. This value of $\Delta G^{\ddagger}$, obtained from the intercept on the $\Delta H^{\ddagger}$ axis, is 15·6 kcal./mole.

Since the energy-rich molecule, ATP, is believed to be involved in the mechanisms of flagellar movement, it is interesting to compare the activation parameters of flagella with those which have been obtained for the formation and breakdown of ATP-ATPase complexes. The values quoted by Laidler (1958) are respectively $\Delta H^{\ddagger} =$ 20·4 kcal/mole, $\Delta S^{\ddagger} = 44$ e.u. and $\Delta H^{\ddagger} = 12·4$ kcal./mole, $\Delta S^{\ddagger} = -8$ e.u., taken from Ouellet, Laidler & Morales (1952). The ATPase concerned is myosin, at pH 7·0. These values are plotted on the graph of Fig. 4, which essentially duplicates Fig. 2 but contains additional information. It can be seen that the point corresponding to the breakdown of the ATP-ATPase complex lies close to the experimentally derived line, while that corresponding to complex formation is a significant distance from the line. If we continue with the supposition that ATP is, in fact, concerned in the rate-limiting reaction which controls frequency, then from the above it seems likely that the *breakdown* of enzyme-substrate complex is the limiting step.

A positive identification of this breakdown, and consequent hydrolysis of the ATP, as the limiting factor would provide evidence in favour of one of the three models of flagellar action which we have proposed (Silvester & Holwill, 1965). Each model takes as its origin the hypothesis that the nine peripheral fibrils of the axoneme contain contractile units evenly distributed throughout their length. Stimuli, which may take the form of passive deformation of successive units (Machin, 1958, 1963; Brokaw, 1966) or some more active signal (Miles & Holwill, in preparation), induce the units to contract in an order that results in a smoothly propagated wave on the flagellum. The stimulus to contraction may operate in at least three ways:

(*a*) The stimulus enables the contractile unit to interact with ATP molecules in its local environment, and use the energy of dephosphorylation in changing its shape.

(*b*) The unit already forms a complex with ATP and the stimulus merely initiates the breakdown, dephosphorylation and consequent contraction.

(*c*) The stimulus raises the unit over a potential energy barrier so that it is able to

contract to a state which is physically at a lower energy level; ATP is only used afterwards, in the relaxation stage, to extend the unit to its previous metastable condition.

The effect of stimulation in mode (a) depends on a collision/diffusion process for the interaction with ATP molecules and this mode can be discounted (Silvester & Holwill, 1965) because the diffusion process does not allow ATP to be exchanged in the contraction phase with sufficient rapidity to sustain a wave along the flagellum. The other two modes possess the common feature that they rely on an energetic stimulus to initiate the contraction process itself, rather than depend on the statistical effects of thermal energy to overcome the activation barrier, as in (a). Teleologically we may argue that this has to be so if the flagellum is to work efficiently, in a co-ordinated fashion, for if the contractile units depended for their activation on the random supply of energy by thermal processes there would be little chance of units contracting successively and generating a coherent wave.

The differences between (b) and (c) are that in (b) the breakdown of complex is stimulated during contraction, and complex *formation*, in the relaxation phase, is the rate-limiting step; in (c) both complex formation and breakdown take place in the relaxation phase. In the latter case, since breakdown has the higher activation free energy, (c. 10 kcal./mole as opposed to 7·2 kcal./mole) the *breakdown* of complex would be the rate-limiting step. Formerly, making a comparison with the behaviour of F-actin (Asakura, Taniguchi & Oosawa, 1963) we favoured an interaction such as (b) where the ATP is bound to the contractile unit before contraction occurs. However, the suggestion of Fig. 4 is that, of the two processes, breakdown rather than formation of the enzyme-substrate complex could be the rate-limiting activity, so that we must now consider mode (c) for flagellar action as a distinct possibility. In this mode the interaction of the contractile unit with ATP occurs entirely in the relaxation phase, when the energy of dephosphorylation is used to re-extend the unit. Clearly it would be useful to study the behaviour of ATP-ATPase complexes under conditions which one believes to approximate to those in the flagellum.

In this connexion it is interesting to note that a protein having ATPase activity has been isolated from *Tetrahymena* cilia (Gibbons, 1965, 1966; Gibbons & Rowe, 1965). The protein, called dynein because it is considered to be the enzyme directly associated with the contractile process, forms the arms on the nine peripheral fibrils of the axoneme and, when isolated, has two forms which are believed to be related as monomer and polymer. Since the enzyme is unlikely to be far removed from the region in which the forces are developed, the proximity of the enzyme to the fibrils lends support to the suggestion that the fibrils contain the active contractile units necessary for flagellar movement. Gibbons (1966) has shown that a dynein monomer hydrolyses between 13 and 35 molecules of ATP per second. Although dynein probably occurs as a polymer *in vivo*, it is probably significant that this rate of dephosphorylation is of the same order as the frequency of ciliary and flagellar beating in living organisms (see above, Discussion, and, for example, Holwill, 1966). It would be interesting to compare the thermodynamic properties of dynein with those derived from observations of flagellar movement.

An alternative to the study of ATPase *in vitro* is to consider the kinetics of muscular contraction, which also involves ATP, and compare activation parameters derived

from this system with those of flagella. For certain muscles the decay of isometric tension or isotonic contraction follows an exponential law of the form

$$A_1 = A_2 e^{-\chi t},$$

where A_1, A_2 are the values of the variable (length or tension) at times separated by an interval t, and χ is the decay constant. The decay constant can be equated with the rate constant of a first-order reaction, and its variation with temperature can be used
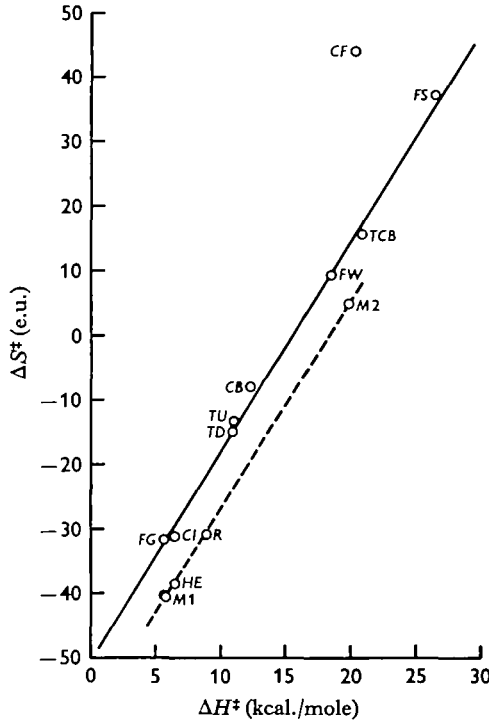
Fig. 4. Comparison of various activation parameters with those of flagella and cilia. The solid line reproduces that of Fig. 2. The broken line indicates a possible correlation between the results for smooth muscles. (For abbreviations see Table 2.)

to determine characteristic values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (Burge & Elliott, 1963). From the data available to us we have calculated activation parameters for a number of muscles; the values we obtained and the original references are given in Table 2 together with published values for ATP. The values are compared with those of flagella in Fig. 4, in which the solid line is the weighted regression line of Fig. 2, derived for the flagella results alone.

There is a striking correlation between the thermodynamic parameters of the striated muscles and those of flagella which leads us to suppose that the rate-limiting reaction of flagellar movement could be identical with the reaction whose rate constant is evident in the exponential form of muscular relaxation. We have already suggested that the former reaction is the breakdown of an ATP-ATPase complex and we will show later how such a reaction may be concerned in muscular relaxation. The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ characteristic of the smooth muscles appear to lie on a different line

in Fig. 4, suggesting that the chemical reaction characterized by the measured rate constants differs in some way from the corresponding reaction in striated muscle.

It is now generally accepted that contraction of striated muscle is produced by the active interdigitation of actin and myosin filaments (e.g. Huxley & Hanson, 1960; Huxley, 1965) and that ATP provides the energy necessary for contraction (Cain et al. 1962) but the precise way in which the interaction of the actomyosin complex

Table 2. *The activation parameters of ATP-ATPase and muscle systems*

	Process	$\Delta H^{\ddagger}$ (kcal./mole)	$\Delta S^{\ddagger}$ (e.u.)	Reference
(CB)	Breakdown of ATP-myosin complex	12·4	−8	Laidler (1958)
(CF)	Formation of ATP-myosin complex	20·4	44	Laidler (1958)
Isometric tension decay in muscles				
(CI)	Cat, intercostal*	6·5	−31·1	Biscoe (1962)
(M1)	*Mytilus*, anterior byssus retractor:†			Bullard (1961)
	in air, 25 V. stimulus of nerve	5·7	−40·3	
	in sea water, 60 V. stimulus of nerve	5·8	−40·5	
(M2)	*Mytilus*, anterior byssus retractor† in sea water, 60 V. stimulus of nerve (second exponential component‡ of the biphasic twitch)	19·5	4·6	Bullard (1961)
(HE)	*Helix*, pharynx retractor†	6·5	−38·5	Abbott & Lowy (1958)
(TCB)	Tortoise, coracobrachialis*	20·9	15 7	Reichel (1960)
(TD)	Toad, semimembranosus*	10·9	−15·0	Abbott & Lowy (1950)
(TU)	Turtle, retractor penis*	11·1	−13·4	Goodall (1957)
Isotonic elongation in muscles				
	Frog, sartorius:*	—	—	Prosser & Brown (1961)
(FS)	Summer frogs	26·6	37·3	—
(FW)	Winter frogs	18 6	9·5	—
(FG)	Frog, gastrocnemius*	5·7	−31·6	Fulton (1926)
(R)	Rabbit, stomach-wall†	8 9	−30·9	Kitisin & Gilson (1954)

* Striated muscle.
† Smooth muscle.
‡ For example see Millman (1963); Lowy & Millman (1959b).
The abbreviations in parentheses refer to points in Fig. 4.

and the ATP induces relative movement of the two types of filament has yet to be elucidated. Of particular importance in the present context are the reactions and conditions necessary for contraction and relaxation in muscle. Contraction appears to be associated with the hydrolysis of ATP bound to myosin or actomyosin in the presence of magnesium and calcium ions, while the removal of the calcium ions from the immediate vicinity of the interacting proteins is essential to the relaxation process (e.g. Needham, 1960; Davies, 1965; Podolsky, 1965; Perry, 1965). The calcium 'pump', which removes the calcium ions and appears to be associated with the sarcoplasmic reticulum, requires a finite time in which to remove the calcium (Hasselbach, 1964; Weber, Herz & Reiss, 1964) so that even in the relaxation phase of muscular activity sufficient calcium may be present to allow a small amount of ATP to be broken down, thus opposing the relaxation.

On the basis of the present evidence there are at least two ways in which the rate of relaxation of muscle could be limited by the dephosphorylation of ATP. If the calcium

is bound (to the ATP molecule or to myosin) while dephosphorylation occurs, and this is postulated in at least one model for muscle contraction (Davies, 1963), then presumably the calcium pump cannot capture the calcium ion associated with a particular ATP molecule until the latter has been hydrolysed. Provided the calcium pump operates quickly enough to remove the calcium before it can participate in another hydrolysis, the decay constant of isometric or isotonic relaxation will be governed by the rate at which the ATP associated with the residual calcium is dephosphorylated, so that, under these conditions, the rate constants derived from the experimental data for striated muscle will relate to the breakdown of an ATP-ATPase complex.

On the above hypothesis each bound calcium ion will be used in the hydrolysis of only one ATP molecule after the calcium pump begins to act. Alternatively, if calcium is removed at a slower rate, thereby allowing a calcium ion to be associated on average with the dephosphorylation of several ATP molecules during relaxation, then the rate at which a muscle relaxes will be determined by the rate at which calcium ions are removed from the activating region and, consequently, by the speed of the calcium pump. This appears to be the more likely situation if a sliding filament model for muscle is accepted since it is reasonable to assume that, during relaxation, links between the actin and myosin will be broken and made several times, particularly during changes in length. Although the mode of action of the calcium pump is obscure it is known to require ATP for its action (Hasselbach & Makinose, 1963). The rate at which the calcium can be removed will depend, in the absence of other limiting factors, on the rate at which energy is supplied to the calcium pump and this in turn is determined by the rate at which ATP is hydrolysed. We are therefore led to the same conclusion as before, namely that the decay of tension or length in muscle is governed by the breakdown of an ATP-ATPase complex, associated in this instance with the calcium pump rather than with the contractile proteins.

With the more explicit elucidation of the chemical reactions which are responsible for the activity of a muscle it is possible that evidence will be found to support a different hypothesis, but the evidence summarized by Fig. 4 makes it probable that the decay constants calculated from observations of muscular relaxation will be associated with the breakdown of an ATP-ATPase complex in the muscle. The arguments used in the previous paragraphs are independent of a particular model for the muscle since they are based on the available chemical evidence which is unlikely to change even if the sliding filament hypothesis is found to require extensive modification. For the same reason, the arguments presented here do not support or conflict with any specific model of muscular contraction, be it a mechanochemical system (Huxley & Hanson, 1954; Huxley & Niedergerke, 1954; Perry, 1965) or an electrostatic mechanism (Davies, 1963; Ingels & Thompson, 1966).

While our results denote a link between the contractile systems of striated muscle and flagella we cannot conclude that the systems are functionally similar except in so much that they both utilize ATP as an energy source, but it may be instructive at this point to compare some of their properties. One apparent difference between the two systems is concerned with the activation of the ATPase. Muscle possesses both a magnesium-activated and a calcium-activated ATPase (although it is not clear that both are concerned directly with the contractile process), whereas the flagellar ATPase

appears to be magnesium-activated (e.g. Perry, 1965; Gibbons, 1965). The point in the contraction–relaxation cycle at which ATP is broken down may also be found to differ in the two systems if model (c) above is shown to represent accurately the activity of flagella. According to this model ATP is broken down in the relaxation stage, whereas for muscle the same reaction occurs during contraction. On the other hand there is evidence from electron microscopy that a sliding filament process may produce bending in certain cilia (Satir, 1965), thus providing a possible point of similarity between the two systems. Theoretical calculations by Rikmenspoel (1965, 1966) suggest similarities between the elastic moduli and tension-developing properties of muscle fibres and those of the nine axonemal fibrils in tails of sea-urchin and bull sperm.

The differences between striated and smooth muscle which emerge from the collected data (see Fig. 4) call for an explanation since the basic chemical reactions would appear to be similar in the two cases although the two types of muscle are obviously struc- turally dissimilar. In *Mytilus* the apparent anomaly may be associated with the protein tropomyosin A, which is found in the paramyosin filaments of molluscan smooth muscle but not in striated muscle and which is believed to be associated with the prolonged maintenance of tension observed in molluscan 'catch' muscles (e.g. the anterior byssus retractor muscle of *Mytilus*). Some authors (Johnson, Kahn & Szent- Györgyi, 1959; Rüegg, 1964) believe that the maintenance of tension during a catch is due to a rigid coupling of the paramyosin filaments while others (Lowy & Millman, 1959a; Lowy, Millman & Hanson, 1964) consider that the holding power of the muscle can be explained in terms of long-lasting cross-links between actin and a protein like myosin in a sliding filament system. Biochemical evidence in favour of the latter proposal is furnished by Minihan & Davies (1965), who found that the amount of ATP broken down during relaxation following the release of a catch in the anterior byssus retractor muscle of *Mytilus* is consistent with the idea that specialized cross- links are responsible for maintaining prolonged tension. It will be necessary to examine the thermal dependence of isometric or isotonic decay in a variety of smooth muscles to ascertain whether the correlation between their parameters which is indicated by Fig. 4 is a valid one, or due merely to a fortuitous relation between the few muscles for which data are available. It is unlikely that experimental errors could account for the observed deviations from the line which is followed by the striated muscles. Results obtained from the extensive biochemical research to which smooth muscle is currently subjected should also prove invaluable in solving this problem.

The muscles for which thermodynamic data are available do not have the rhythmic movement which is so characteristic of flagella, although certain muscles which are non-oscillatory *in vivo* can be induced to oscillate under certain conditions when extracted with ethylene glycol or glycerol (Goodall, 1956; Lorand & Moos, 1956). It appears that the activity of most muscles is regulated by membrane excitation which, directly or indirectly, controls the movement of calcium ions within the muscle (e.g. Birks, 1965). The flight muscles and tymbal muscles of insects have oscillatory movements whose frequencies are limited not by membrane activity, but by the interplay between the elasticity and inertia of the system (Pringle, 1957). Because they are self-oscillatory the insect fibrillar muscles are superficially more like flagella in their action than are the smooth and striated muscles discussed earlier, and it would be interesting to examine the effects of temperature on the activation frequency of

the insect muscles for comparison with the results on flagella. (Temperature measurements on insect flight muscle have been made by Machin, Pringle & Tamasige (1962) but their results are not amenable to our analysis.) Rhythmic oscillation in flagella could not be induced in exactly the same way as in insect muscles, since the inertia of the microscopic system is negligible; possibly the more important viscous forces of flagellar action could replace the inertial properties of the muscle system, thereby providing a viable oscillatory mechanism.

The tentative identification of the breakdown of the ATP-ATPase complex as the rate-limiting process underlying flagellar movement is encouraging but needs confirmation by experiments on various ATP-ATPase systems. The extraction of a flagellar ATPase which can be shown to be implicated in the contraction process, and its subsequent examination, will be of vital importance in this respect. We have mentioned earlier that dynein may be such an ATPase and we eagerly await its further characterization. The connexion between the contractile systems of flagella and muscle remains a tenuous one, but the kind of evidence adduced in the preceding pages adds substance to the hypothesis that, although the two systems may be dissimilar in operation, they rest on a common chemical foundation.

SUMMARY

1. The frequencies of the beat of cilia and flagella from various organisms have been determined at temperatures in the range 5–35° C.

2. Values of the activation enthalpy ($\Delta H^{\ddagger}$, kcal./mole) and activation entropy ($\Delta S^{\ddagger}$, e.u.) derived from the thermal dependence of frequency show a linear correlation of the form, $\Delta S^{\ddagger} = 3 \cdot 25 \, \Delta H^{\ddagger} - 50 \cdot 75$.

3. The corresponding isokinetic activation free energy is 15·6 kcal./mole.

4. The results support a hypothesis that the breakdown of an ATP-ATPase complex could be the common rate-limiting reaction for flagellar activity.

5. Values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the decay of length or tension in striated muscles also fall on the same regression line but some smooth muscles show deviations.

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