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A protonmotive force drives bacterial flagella

(bacterial motility/ionophores/fluorescent dyes)

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ABSTRACT *Streptococcus* strain V4051 is motile in the presence of glucose. The cells move steadily along smooth paths (run), jump about briefly with little net displacement (twiddle), and then run in new directions. They stop swimming when deprived of glucose. These cells become motile when an electrical potential or a pH gradient is imposed across the membrane. Starved cells suspended in a potassium-free medium respond to the addition of valinomycin by a brief period of vigorous twiddling. They also twiddle, although less vigorously, when the external pH is lowered. Valinomycin-induced twiddling occurs in the absence of external alkali or alkaline earth cations and without significant net synthesis of ATP. When a chemoattractant is added to cells swimming in the presence of glucose, twiddles are transiently suppressed, and the cells run for a time. Similarly, when starved cells are suspended in a potassium-free medium containing both valinomycin and an attractant, many cells initially run rather than twiddle. We conclude that the flagella are driven by a protonmotive force.

Chemiosmotic energy coupling, as originally proposed by Mitchell (1–4), is involved in the function of a number of bacterial transport systems, in oxidative phosphorylation, and in pyridine nucleotide transhydrogenation (for reviews see refs. 5 and 6). We undertook the experiments reported here to determine whether bacterial motility is also powered by the proton movements that constitute the primary energy currency of Mitchell's theory. Our results confirm and extend those of Larsen *et al.* (7), who found that the source of energy for motility in *Escherichia coli* is the intermediate in oxidative phosphorylation, not ATP itself (for reviews see refs. 8–10).

We began this work with *E. coli* but were unable to exhaust its endogenous energy reserve without irreversibly impairing motility. This led us to study a motile *Streptococcus* (11). Unlike *E. coli* or *Salmonella typhimurium*, *Streptococcus* strain V4051 lacks an endogenous energy supply and is highly sensitive to ionophores and uncouplers. Thus, it is possible to manipulate either the transmembrane electrical potential or pH gradient and to observe the effects of these manipulations on motility. Similar methods have been used with other *Streptococci* to show that ATP synthesis (12, 13) and the accumulation of amino acids and thiomethylgalactoside (14, 15) are driven by an inward protonmotive force.

MATERIALS AND METHODS

Reagents. All solutions were made from reagent-grade chemicals and glass-distilled water. Ionophores and uncouplers were added in methanol (final methanol concentration 0.1%); ethanol supports motility and ATP synthesis in strain V4051 (our unpublished observations). Valinomycin and firefly lantern extract were purchased from Sigma; sodium nigericin and A23187 (16), from Lilly; gramicidin-D, from Nutritional Biochemicals; trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP), from Du Pont; *N,N'*-dicyclohexylcarbodiimide, from Eastman; choline chloride, α-aminoisobutyric acid, and L-leucine (A grades), from Calbiochem. Monactin was the gift of W. Keller-Shierlein, and 3,3'-dipropyl-2,2'-thiadicarbocyanine iodide [Di-S-C_3(5)] was the gift of Alan Waggoner. The known actions of membrane-active agents used in this work are listed in Table 1 (for reviews see refs. 17 and 18).

Cells. *Streptococcus* strain V4051 (11) was grown at 34° in KTY-glucose medium (19) and harvested in early logarithmic phase at a cell density of 0.15 mg of dry weight per ml. Preparative steps were done at room temperature (22–24°). The cells from 20 ml of culture were trapped on a Millipore nitrocellulose filter (25 mm diameter, 0.45 μm pore size) and washed three times with 5 ml of 100 mM potassium phosphate (pH 7.5). They were resuspended very gently and washed twice by centrifugation in 5 ml of the desired final buffer. A concentrated stock of cells (25–50 mg dry weight per ml) was kept at room temperature and used within 3 hr.

Motility. The motion of the cells was judged by eye or recorded with a Polaroid camera (type 107 film) using a phase contrast microscope (Nikon S-Ke, 30-W tungsten lamp, magnification 200×). When the camera was used, a programmed shutter (Vincent 26XOAOX5) placed between the microscope condenser and stage generated a series of 20-msec exposures. This is a variation of the motility-track method of Macnab and Koshland (20). Cells were diluted 1:1000 from the concentrated stock into the medium in which motility was to be tested, and the mixture was drawn by vacuum into the viewing chamber (21). All observations were made at room temperature.

Protonmotive Force. The protonmotive force, the work per unit charge required to move a proton from the outside to the inside of the cell, is given by

$$\Delta p = \Delta\psi - 2.3(RT/F)\Delta\text{pH}, \qquad [1]$$

in which $\Delta\psi$ is the difference in electrical potential between the inside and outside of the cell, R is the gas constant, T is the absolute temperature, F is the faraday, and ΔpH is the difference in pH between the inside and outside of the cell (2, 3). At 24°, $2.3(RT/F)$ equals 59 mV.

When membrane potentials were induced in starved cells by the addition of valinomycin, $\Delta\psi$ was determined from the ratio of external to internal K^+. A Beckman 39137 glass electrode was used to measure external K^+. Changes in internal K^+ were computed from the initial concentration (0.64 M), the cell density, the sample volume, and changes in external K^+. The potassium diffusion potential was calculated from the Nernst equation, using appropriate K^+ activity coefficients.

In metabolizing cells, $\Delta\psi$ was determined from quenching of the fluorescence of Di-S-C_3(5), a cationic, lipophilic carbocyanine dye (22, 23). Cells were suspended at a density of 0.05 mg of dry weight per ml in a buffer containing Di-S-C_3(5) at

Abbreviations: Di-S-C_3(5), 3,3'-dipropyl-2,2'-thiadicarbocyanine iodide; FCCP, trifluoromethoxycarbonylcyanide phenylhydrazone.

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Table 1. Known actions of membrane-active agents

Compound	Action and relevance to this study
A23187	Exchanges Ca^{2+} or Mg^{2+} for H^+; used in conjunction with EDTA to deplete cells of Ca^{2+} and Mg^{2+}
FCCP	Conducts H^+; rapidly abolishes a protonmotive force
Gramicidin-D	Conducts K^+, Na^+, H^+; abolishes a protonmotive force, rapidly if K^+ or Na^+ is present in the medium
Monactin	Conducts K^+, Na^+; used to deplete cells of K^+ by exchange with external Na^+
Nigericin	Exchanges K^+ for H^+; abolishes a transmembrane pH gradient
Valinomycin	Conducts K^+; generates a diffusion potential if K^+ is present at different concentrations on either side of the membrane

0.5 μM (diluted 1:2000 from a 1 mM stock solution in methanol). These values of cell density and dye concentration gave optimal quenching; they are much lower than those employed previously (22, 24–26). Changes in intensity of fluorescence at 662 nm were followed in a Perkin-Elmer MPF-2A fluorescence spectrophotometer, with excitation at 620 nm. The minimum intensity was determined for each experimental manipulation. A reference intensity was established by adding gramicidin-D at 1 μg/ml (24) or, for cells in Tris buffers, by adding 100 mM NaCl and gramicidin-D. Quenching is defined as the difference between the reference intensity and the minimum intensity, divided by the reference intensity. Calibration curves relating $\Delta\psi$ and quenching were constructed by measuring quenching in cell suspensions containing valinomycin at 1 μg/ml and KCl at eight concentrations ranging from 0.5 mM to 0.1 M; $\Delta\psi$ was calculated from the Nernst equation.

The pH gradient, ΔpH, was computed from the external pH, the initial internal pH, and the buffering capacities of the medium and the cytoplasm. Measurements of pH were made with combination glass electrodes (Beckman 39030 or Radiometer GK2321C). Buffering capacities were determined by titrating cells in weak buffers with 1 mM HCl in the presence and absence of gramicidin-D (2 μg/ml). In the absence of gramicidin-D only the medium and outer cell surfaces are accessible; in the presence of gramicidin-D the cytoplasm also is titrated. The difference in the two titration curves yields the internal buffering capacity. The initial internal pH was determined by lysing cells (3–6 mg of dry weight per ml) at 37° with 10% (vol/vol) 1-butanol; the pH was estimated from the pH change accompanying lysis and from the buffering capacities of the cells and the external medium.

ATP. Measurements of ATP were made with the luciferin/luciferase procedure of Berger and Heppel (27).

Cautions on the Use of Di-S-C_3(5). We had hoped to employ Di-S-C_3(5) more extensively to measure membrane potentials; however, major problems arose that limited our use of this dye. At 0.5 μM the dye abolishes glucose-powered swimming; the cells stop within a few seconds when observed with the phase contrast microscope (see *Motility*). This is a photodynamic effect (28), because it occurs in white or red light (passed by a Schott OG-590 filter) but not in blue light (passed by a Schott BG-12 filter). When motility is induced with valinomycin in the presence of dye, the cells run for a while and then stop, without twiddling; this response does not appear to be photodynamic, because it also occurs in blue light.

When starved cells are equilibrated with the dye in a K^+-free medium and then exposed to valinomycin, the fluorescence intensity drops for 15–30 sec and then remains constant for several minutes. Measurements of K^+ efflux indicate that $\Delta\psi$ decays over this entire period (as in Fig. 3); the rate of this decay is the same whether or not the dye is present. We do not understand why the fluorescence intensity fails to respond to this decay, since a large increase in intensity occurs immediately when $\Delta\psi$ is dissipated by the addition of gramicidin-D or KCl.

RESULTS

Normal Motility Requires an Exogenous Energy Source. Logarithmic cultures of *Streptococcus* V4051 consist largely of peritrichously flagellated diplococci (our unpublished electron micrographs). In the presence of glucose, the diplococci swim in an alternating series of runs and twiddles, like *E. coli* (29), *S. typhimurium* (20), and *Bacillus subtilis* (30). When a chemoattractant is added, twiddles are transiently suppressed (ref. 11; our unpublished observations), as in *E. coli* and *S. typhimurium* (20, 31). The cells stop swimming within a few minutes when transferred to a glucose-free medium but become motile within 10–20 sec when glucose is reintroduced (Fig. 1 *a* and *b*). In contrast, *E. coli* swims for days without an exogenous energy supply (21, 32).

Ionophores Inhibit Motility in Metabolizing Cells. The effects of ionophores (Table 1) on metabolizing cells suggest that motility depends on the protonmotive force and can be supported by either $\Delta\psi$ or ΔpH. The uncoupler FCCP collapses $\Delta\psi$ and ΔpH: at 10 μM it abolishes motility. Valinomycin dissipates $\Delta\psi$ in potassium phosphate (Fig. 2) and enhances it somewhat in sodium phosphate (data not shown). Nigericin increases $\Delta\psi$ slightly in potassium phosphate (Fig. 2) or sodium phosphate. When added separately to cells in either buffer, or together to cells in sodium phosphate, valinomycin and nigericin reduce the number and speed of motile cells, although not dramatically. Their simultaneous addition to cells in potassium phosphate abolishes motility, presumably because both $\Delta\psi$ and ΔpH have been collapsed.

A Potassium Diffusion Potential Induces Transient Motility in Starved Cells. Direct evidence that the protonmotive force powers motility is obtained from experiments in which an artificial $\Delta\psi$ or ΔpH is imposed upon starved cells. These cells twiddle vigorously when diluted into sodium phosphate or Tris chloride buffers containing valinomycin (Fig. 1*c*; Table 2). Under these conditions $\Delta\psi$ is initially about −200 mV. After twiddling subsides (Fig. 1*d*) the cells jiggle (see Table 2, note †) and then stop. When cells are diluted into the same buffers in the absence of valinomycin, they jiggle for at least 4 min (Table 2). This response is not due to a residual energy store; the cells remain nonmotile when diluted into potassium phosphate, the buffer in which they were prepared, whether or not valinomycin is present. Apparently K^+ is able to cross the cytoplasmic membrane more readily than Na^+ or $Tris^+$, even in the absence of ionophores.

When starved cells are diluted into sodium phosphate or Tris chloride containing valinomycin and L-leucine, a potent attractant (11), most of the cells run rather than twiddle (Fig. 1 *e*, and *f*; Table 2). They remain nonmotile in potassium phosphate. Similar results are obtained with α-aminoisobutyric acid, a nonmetabolizable analog of the attractant serine (data not shown).

Smaller valinomycin-induced potentials are generated when the external medium contains potassium chloride. The highest concentration of potassium chloride that consistently allows motility is 20 mM, corresponding to an initial $\Delta\psi$ of −80 mV; some cells run if L-leucine is present, but the runs are slow and short.

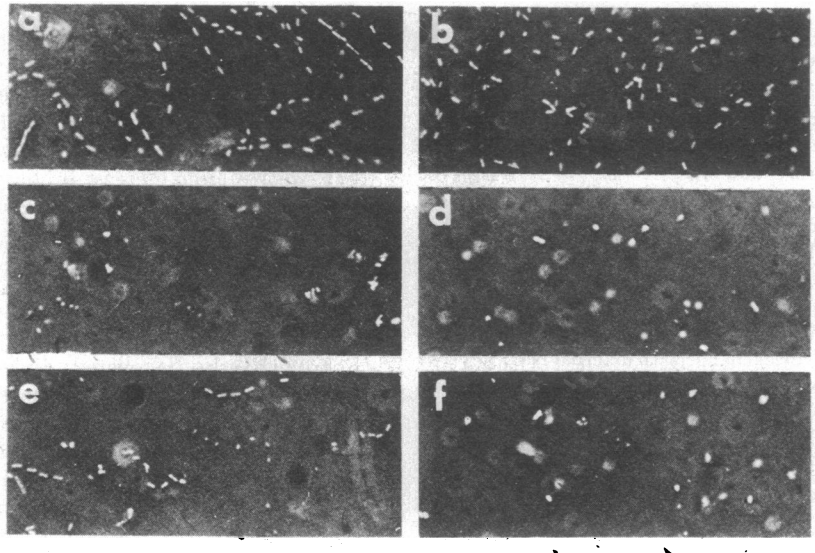

FIG. 1. Motility tracks of *Streptococcus* strain V4051. (×360.) Each print shows four exposures taken at 0.67-sec intervals. Extended tracks of four images indicate runs, clusters of four images indicate twiddles, and single bright images indicate nonmotile cells. Cells (*a*) 20 and (*b*) 80 sec after dilution into 100 mM sodium phosphate (pH 7.5) containing 0.1% glucose. Cells (*c*) 20 and (*d*) 80 sec after dilution into 100 mM sodium phosphate containing 1 μg of valinomycin per ml. Cells (*e*) 20 and (*f*) 80 sec after dilution into 100 mM sodium phosphate containing 1 μg of valinomycin per ml and 1 mM L-leucine.

Gramicidin-D at 1 μg/ml induces motility in cells suspended in Tris chloride; the cells twiddle, or run if L-leucine is present, for about 30 sec. This does not happen in buffers containing Na^+, because Na^+ moves into the cells as K^+ moves out (see Table 1). As expected, gramicidin-D inhibits valinomycin-induced motility in sodium phosphate, but not in Tris chloride. Nigericin at 1 μg/ml and FCCP at 10 μM prevent valinomycin-induced motility in both buffers.

The Valinomycin-Induced Protonmotive Force Declines Rapidly. The short duration of motility induced by valinomycin (Table 2) can be explained by a rapid decay of the protonmotive force. This decay results both from the efflux of K^+, which reduces $\Delta\psi$, and the influx of H^+, which lowers the cytoplasmic pH and thus sets up an outward protonmotive force (15).

The influx of H^+ generates a pH gradient, cell interior acid. If the cell density is sufficiently high and the buffering capacity of the medium sufficiently low, valinomycin-induced changes in external pH and K^+ concentration can be measured simultaneously, and ΔpH, $\Delta\psi$, and Δp computed (Fig. 3). The decay in Δp over the first 60 sec is primarily due to the influx of H^+. The rate of acidification of the cytoplasm is the same in the presence or absence of 1 mM L-leucine (data not shown). The duration of motility is about the same here as in the experiments of Table 2, even with a higher cell density and lower valinomycin-to-cell ratio.

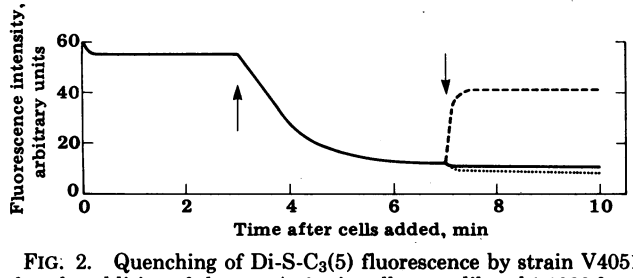

FIG. 2. Quenching of Di-S-C_3(5) fluorescence by strain V4051 after the addition of glucose. At 0 min cells were diluted 1:1000 from a stock (50 mg of dry weight per ml in 100 mM potassium phosphate, pH 7.5) into the same buffer containing 0.5 μM dye. At 3 min (first arrow) glucose was added (final concentration 0.1%). At 7 min (second arrow) ionophores were added in parallel experiments: (- - -) 1 μg of valinomycin per ml; (····) 1 μg of nigericin per ml; (——) 0.1% methanol. The quenching observed at 7 min corresponds to a $\Delta\psi$ of −90 mV. All experiments were done at room temperature.

A pH Gradient Also Induces Motility in Starved Cells. Cells become motile when diluted into acidic buffers. The pH gradient that can be applied in this way is limited, because a low pH inhibits swimming; cells in glucose run and twiddle weakly at pH 5.5. The internal pH of cells prepared at pH 7.5 is 6.8 to 6.9, so a ΔpH of about 1.4 units (−80 mV) is the largest attainable. Cells prepared in potassium phosphate at pH 7.5 jiggle when diluted into potassium phosphate at pH 6.5 or 5.5. They also jiggle when 1 mM L-leucine is present, a result consistent with the finding that chemotaxis to L-leucine drops off sharply below pH 7.0 (11). Attempts to enhance the effect of ΔpH by including valinomycin to dissipate the potential resulting from the entry of H^+ were not successful.

Motility Does Not Require the Synthesis of ATP. ATP levels in starved cells are very low, less than 50 pmol/mg of dry weight. ATP is made rapidly upon the addition of a small amount of glucose and is then used up as the glucose is exhausted (Fig. 4*a*); a short burst of motility coincides with the ATP peak. Because it is known that ATP can be synthesized in response to an artificial protonmotive force (12, 13), we wanted to exclude the possibility that this synthesis might be responsible for the valinomycin-induced motility.

Starved cells do produce a small amount of ATP in response to a potassium diffusion potential at pH 7.5 (Fig. 4*b*). Considerably more ATP is synthesized if the diffusion potential is combined with a pH gradient, as has already been demonstrated for *Streptococcus lactis* (13). The total protonmotive force at pH 7.5 is −160 to −180 mV, whereas at pH 5.5 it is between −280 and −300 mV. However, the synthesis of ATP is not required for motility. Cells preincubated for 30 min in potassium arsenate (pH 7.5) and then resuspended at pH 7.5 in sodium arsenate containing valinomycin make very little ATP, yet they become motile (Fig. 4*b*; Table 2). Even when resuspended in this way at pH 5.5 they make less than 50 pmol of ATP per mg of dry weight. ATP synthesis in phosphate at pH 7.5 is much reduced in the presence of 10 mM KCl, but valinomycin generates motility under these conditions (above). Finally, valinomycin-induced ATP synthesis is abolished at pH 7.5 if the cells are preincubated for 15 min at room temperature

Table 2. Motility induced by a potassium diffusion potential in starved V4051 cells*

Additives	Response observed in a given buffer†		
	Sodium phosphate	Tris chloride	Sodium arsenate
None	Jiggle for at least 240 sec	Jiggle for at least 240 sec	Jiggle for at least 240 sec
L-Leucine	Jiggle and few short runs to 120 sec; jiggle for at least 240 sec	Jiggle and few short runs to 120 sec; jiggle for at least 240 sec	Jiggle and few short runs to 120 sec; jiggle for at least 240 sec
Valinomycin	Twiddle and few short runs to 45 sec; jiggle to 90 sec	Twiddle and few short runs to 45 sec; twiddle to 75 sec; jiggle to 120 sec	Jiggle to 120 sec
Valinomycin + L-leucine	Run to 45 sec; jiggle to 90 sec	Run to 75 sec; jiggle to 120 sec	Run to 45 sec; jiggle to 90 sec

* Cells studied in sodium phosphate or Tris chloride were prepared in potassium phosphate; cells studied in sodium arsenate were prepared in potassium arsenate. All buffers were 100 mM, pH 7.5; the final cell density was 25 μg of dry weight per ml (see *Materials and Methods*). The final buffers contained 0.1% methanol. Other additives are listed in the first column: valinomycin, 1 μg/ml; L-leucine, 1 mM.

† *Run*, to move steadily along a smooth trajectory; *twiddle*, to jump about vigorously with little net displacement; *jiggle*, to move weakly or sporadically more or less in place, yet in a manner clearly distinguishable from Brownian motion.

with 20 μM *N,N'*-dicyclohexylcarbodiimide, a potent inhibitor of membrane-bound, Ca^{2+},Mg^{2+}-dependent adenosinetriphosphatase (33); nevertheless, these cells become motile when suspended in K^+-free media containing valinomycin (data not shown). In all of these experiments the cells run rather than twiddle if L-leucine is present, even when the ATP concentration is less than 50 pmol/mg of dry weight. It remains to be determined whether the cells are motile in the total absence of ATP.

Protons Are the Only External Cations Required for Motility. One might argue that cations other than H^+ respond to a potassium diffusion potential, and that it is their movement into the cell down the electrical gradient that drives the flagella. The most likely candidates are Na^+, Ca^{2+}, and Mg^{2+}. However, a diffusion potential generates motility when these cations are excluded from the external medium, and their addition fails to stimulate such motility. The presence of 1 mM EDTA and/or the ionophore A23187 (see Table 1) at a concentration of 0.5 μg/ml does not impair motility. Thus, it is improbable that external cations other than protons are necessary for normal flagellar function. Intracellular K^+ may not be required for motility either, because cells still swim in the presence of glucose when monactin (Table 1) is used to deplete cells of most of their K^+ (from 0.64 to 0.012 M).

DISCUSSION

Our experiments with the motile *Streptococcus* strain V4051 show that bacterial motility is powered by a proton flux. Rotation of the flagellar motor (9, 10, 34–36) can be driven either by a transmembrane electrical potential ($\Delta\psi$, cell interior negative) or by a transmembrane pH gradient (ΔpH, cell interior alkaline). Neither valinomycin (used with metabolizing cells to dissipate $\Delta\psi$) nor nigericin (used to dissipate ΔpH) strongly inhibits motility; however, a mixture of the two abolishes motility. Starved cells become vigorously motile when a potassium diffusion potential is induced by valinomycin. This response is transient, because an efflux of K^+ reduces $\Delta\psi$ while an influx of H^+ generates a pH gradient, cell interior acid. Starved cells also become motile, although less vigorously, when exposed to a pH gradient, cell interior alkaline. There are two reasons for this lack of vigor: the protonmotive force (Δp) established by the pH gradient is smaller than that established by the diffusion potential, and low pH inhibits motility.

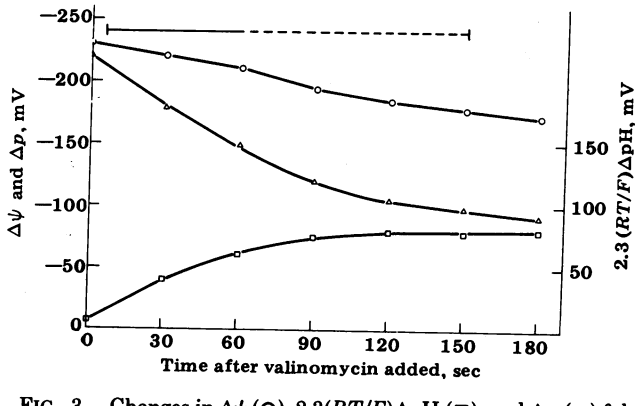

FIG. 3. Changes in $\Delta\psi$ (○), $2.3(RT/F)\Delta pH$ (□), and Δp (△) following the addition of valinomycin to cells in a potassium-free buffer. $\Delta\psi$ and ΔpH were deduced from measurements of the external K^+ concentration and external pH, and Δp was computed from Eq. 1 (see *Materials and Methods*). Cells were suspended at a density of 0.3 mg of dry weight per ml in 0.50 mM Tris chloride (pH 7.0), 100 mM choline chloride, and 0.1 mM EDTA. Valinomycin was added at 1 μg/ml at 0 sec. Parallel measurements of motility are depicted by the horizontal bar: solid for twiddles (or runs with L-leucine), broken for jiggles. The cell density was 12 times higher than that used in the experiments of Table 2. All measurements were made at room temperature.

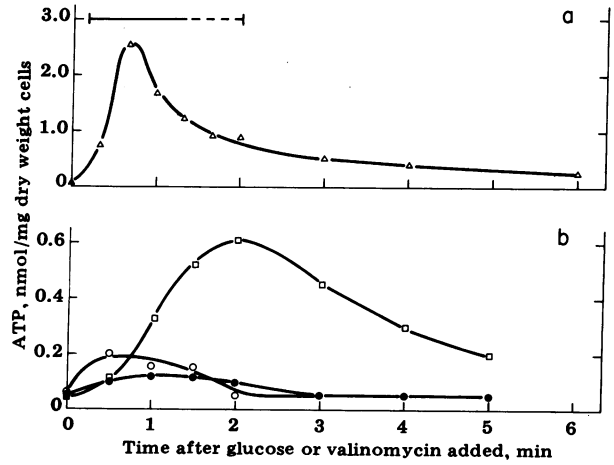

FIG. 4. ATP synthesis induced in starved cells. (*a*) 0.1 mM glucose was added to cells suspended at 3 mg of dry weight per ml in 100 mM potassium phosphate (pH 7.5). Motility was measured every 15 sec on samples diluted 1:100 into the same buffer lacking glucose; the results are depicted by a horizontal bar, as in Fig. 3. (*b*) Valinomycin at 1 μg/ml was added to cells suspended at 1.5 mg of dry weight per ml in 100 mM sodium phosphate at pH 7.5 (○) or 5.5 (□), or in 100 mM sodium arsenate at pH 7.5 or 5.5 (●). ATP levels were determined as described in *Materials and Methods*. All experiments were done at room temperature.

Protons appear to be the only external cations required for the function of the flagellar motor. Other cations that could reasonably be involved, such as Na^+, Ca^{2+}, or Mg^{2+}, are ruled out by our results. External anions should not be driven into the cells in response to a negative $\Delta\psi$. Similarly, internal cations should not be driven out. We have not excluded the possibility that an efflux of internal anions is involved.

The flagellar motor functions at very low levels of ATP, less than 1% of the amount found in cells carrying out glycolysis. Our results agree with the findings of Larsen *et al.* (7) in *E. coli*, and extend their work to include a primarily fermentative organism. Although we have not ruled out the involvement of other biochemical intermediates in the function of the motor, it may be that the torque is generated by proton-dependent changes in the structure of its innermost rings (9, 36).

Clockwise flagellar rotation generates twiddles in *E. coli*, counterclockwise rotation generates runs (37). Preliminary results with tethered cells indicate that the flagella of strain V4051 rotate in the same sense as those of *E. coli* and are therefore left-handed helices (38). An inward protonmotive force turns the flagella of strain V4051 primarily clockwise in the absence of chemoattractants, counter-clockwise in their presence. In *E. coli* and *S. typhimurium*, ATP is required for twiddling (39, 40), presumably because it is a precursor of *S*-adenosyl-L-methionine. Under the conditions we have used to induce motility, cells can run or twiddle when ATP levels are very low; however, small amounts of ATP may be adequate to regenerate adenosylmethionine, or starved cells may retain significant pools of adenosylmethionine.

Treatments that increase (41) or decrease (30, 41, 42) the protonmotive force in metabolizing *B. subtilis* induce twiddling; runs are observed when attractants are added simultaneously (30, 42). We see similar effects when motility is induced in starved *Streptococcus* V4051.

The addition of attractants to *E. coli* causes a transient hyperpolarization of the membrane (43). In our experiments, attractants suppress valinomycin-induced twiddling under conditions in which the potential should be determined by the ratio of external to internal K^+. As Szmelcman and Adler (43) point out, movements of specific ions rather than fluctuations in the membrane potential may be essential to the chemotactic signal.

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