# Chemical Modification of *Streptococcus* Flagellar Motors

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Video techniques were used to record changes in motility of cells of *Streptococcus* sp. strain V4051 exposed to a variety of protein modification reagents. Starved cells were tethered to glass by a single flagellum, energized metabolically with glucose, or treated with valinomycin and energized artificially via shifts to media containing low concentrations of potassium ion. Experiments were devised that distinguished reagents that lowered the proton motive force from those that blocked the generation of torque (damaged the flagellar motors). Imidazole reagents blocked the generation of torque. Amino, sulfhydryl, dithiol, and disulfide reagents did not. Some of the imidazole, amino, and sulfhydryl reagents had long-term effects on the direction of flagellar rotation.

A bacterial flagellum is driven at its base by a rotary motor powered by a proton motive force. The morphology, genetics, energetics, and dynamics of this motor have been studied in several species, and models have been presented to explain its function (reviewed in references 6, 17, 20, 24, 39). However, relatively little is known about its biochemistry; the components of interest are buried in the cell wall and cytoplasmic membrane and are not readily purified.

It is possible to use an indirect biochemical approach and treat intact cells with reagents that modify proteins and ask which, if any, reactive groups are essential for function. For example, it has been known for many years that bacterial motility is inhibited by sulfhydryl reagents, e.g., *p*-chloromercuribenzoate (8, 10). Originally, this result was taken as presumptive evidence for the presence of critical sulfhydryl groups in the flagellar filament (10), which was regarded as a contractile organelle. A difficulty with this interpretation, rigid rotation of the filament and absence of cysteine in its protein notwithstanding, is that the experiment fails to distinguish between energy production (generation or maintenance of a proton motive force, $\Delta p$) and energy utilization (chemiosmo-mechanical energy conversion). In brief, it is not known whether the critical sulfhydryl groups are essential for membrane energization or for motor function.

This distinction can be made with the motile *Streptococcus* sp. strain V4051 (44), a primarily fermentative, gram-positive organism that lacks an endogenous energy reserve and is sensitive to ionophores and uncouplers. This organism can be starved, tethered, and artificially energized, either with a potassium diffusion potential or with a pH gradient (6, 20, 27). Cells treated in this way spin for several minutes and can be examined at leisure. We used this method to study the effects on the motor of reagents that modify amino, sulfhydryl, dithiol, disulfide, imidazole, and methionyl residues. Some of these reagents changed the direction of flagellar rotation, an effect studied earlier with *Salmonella typhimurium* (7). Only the imidazole reagents clearly blocked chemiosmo-mechanical energy conversion.

## MATERIALS AND METHODS

**Strains and media.** *Streptococcus* sp. strain V4051 (44) was grown in KTY medium (15) at 35°C in a gyratory incubator.

**Reagents.** Valinomycin, *p*-chloromercuribenzenesulfonic acid, diethylpyrocarbonate, *n*-octane, proflavine hemisulfate, rose bengal (C.I. 45440), and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid were obtained from Sigma Chemical Co.; *N*-ethylmaleimide (NEM), isethionyl acetimidate hydrochloride, and methyl acetimidate hydrochloride, from Pierce Chemical Co.; L-histidine and α-iodoacetamide, from Calbiochem; hydrogen peroxide (30%, wt/wt) and silver nitrate, from Mallinckrodt; sodium arsenite, from Fisher Scientific Co.; 2-mercaptoethanol, from Matheson, Coleman and Bell; eosin Y (C.I. 45380), from Hilton Davis; methylphosphonic acid, from Richmond Organics; silicone oil (high temperature; specific gravity, 1.05 g/cm$^3$), from Aldrich Chemical Co., Inc.; Chelex 100, from Bio-Rad Laboratories; Prosil-28, from PCR Research Chemicals; and $^{86}$RbCl (specific activity, 1.8 mCi/mg) and Aquasol-2, from New England Nuclear.

**Buffers.** Buffers were prepared from reagent-grade chemicals and glass-distilled water. Standard buffer, low-K buffer, and K-free buffer each contained 0.1 M sodium phosphate (pH 7.5, unless otherwise noted) and 10$^{-4}$ M EDTA. Standard buffer contained, in addition, 0.2 M KCl; K-free buffer, 0.19 M NaCl; and low-K buffer, 0.01 M KCl and 0.19 M NaCl. EDTA was omitted from the buffers used with silver nitrate; they were purged of heavy metals by passage through a column of Chelex 100 (1). Reagents that hydrolyze in water, such as methyl acetimidate or diethylpyrocarbonate, were added to the buffers immediately before use; when experiments were replicated, different sets of cells were exposed to solutions of identical age.

**Tethered cells.** Cells were harvested in exponential phase at a density of about 3 × 10$^8$ cells per ml, washed in standard buffer, tethered to glass cover slips, and placed in a small stainless-steel flow cell, as described previously (27); Prosil-28 was used to silanize the cover slips. These and all subsequent manipulations were done at room temperature (~22°C) at the same pH (unless otherwise noted). Experiments were begun once starvation was complete (after about 1 h, as evidenced by total cessation of rotation). Cells to be energized with a potassium diffusion potential were pretreated with valinomycin (1 μg/ml in standard buffer; 1.5 min). Metabolizing cells were given glucose (11 mM). Different solutions were drawn through the chamber at flow rates sufficient to replace 99% of its contents in 5 s (as determined separately by displacement of methylene blue). Experiments were recorded on videotape. Plots were made of the fraction

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of cells spinning as a function of time. Cells that failed to spin or that came off the glass before the end of an experiment were not counted. In some experiments, different cells spun at different times, so that the maximum value of this fraction was <1. Plots also were made of the speeds of a representative set of cells. Speeds in revolutions per second (hertz [Hz]) were measured either by eye and stopwatch or by a system linked to an Apple computer that timed intervals between transits of images over a video cursor. The speeds for each cell were normalized to the maximum observed for that cell during the experiment, and the mean of the normalized speed was computed at each time point. In some experiments, the maximum speeds for different cells occurred at different times, so the maximum value of this function also was <1. The speeds for each metabolizing cell were normalized to the speed obtained for that cell before the addition of reagent. The standard errors in the means of the normalized speeds were typically 0.1 or less.

**Measurements of membrane potential in eosin-treated cells.** Rubidium uptake was used as a measure of the membrane potential generated by potassium diffusion. Cells from a 50-ml culture were harvested in exponential phase at a density of about $7 \times 10^8$ cells per ml, washed twice with 25 ml of standard buffer, and resuspended in 1 ml of the same buffer. The milligrams (dry weight) of cells per milliliter was determined from a measurement of absorbance at 610 nm; with our colorimeter, 1 optical density unit = 0.77 mg (dry weight)/ml. When the protocol called for tethering, the cells were sheared at this point (27). The suspension was diluted to 5 ml with standard buffer, and 20 μl of valinomycin (1 mg/ml in methanol) was added. After about 2 min, the cells were pelleted and resuspended in 0.6 ml of standard buffer. A 0.2-ml aliquot of this suspension was diluted to 0.5 ml with standard buffer, and 2.5 μl of eosin (1 mg/ml in standard buffer) was added. Air was bubbled through this mixture while it was exposed to a collimated beam of light from a 100-W tungsten-halogen lamp (Sylvania FCR, run at about 9 V) focused with a lens from a microscope condenser (Nikon S-Ke). Then the cells were pelleted in an Eppendorf microfuge and resuspended in 0.2 ml of standard buffer. This sample was diluted into 3.8 ml of K-free buffer containing trace amounts of $^{86}$RbCl (about $2.5 \times 10^5$ cpm). The mixture was stirred continuously. At the times indicated, cells in 0.5-ml aliquots were pelleted in the microfuge through a 0.2-ml layer of a 96:4 (vol/vol) mixture of silicone oil–$n$-octane (2). The ratio of the concentrations of rubidium ion in the cell water and the external buffer was determined from the distribution of counts between the pellet and supernatant fractions. Samples were counted in Aquasol-2 in a scintillation spectrometer (Packard 3320). A conversion factor of 1.68 μl/mg (dry weight) was used to relate the volume of cell water to cell dry weight (27). The membrane potential was computed from the Nernst equation (27), assuming similar activity coefficients for rubidium in the intra- and extracellular media. The rubidium uptake experiment, repeated with another 0.2-ml aliquot of the original suspension not exposed to eosin or light, served as a control.

**Measurements of intracellular pH.** Since the concentration of potassium ion is larger inside than outside the cells (free-ion concentration, 0.34 M inside [20] and 0.2 M outside, standard buffer) and the cytoplasmic membrane is slightly permeable to potassium ion, even before treatment with valinomycin (28), starved cells should have a diffusion potential of 13.6 mV (interior negative). If the cells are completely deenergized (if the proton motive force is 0), this potential will be offset by a pH gradient of 0.23 U (interior

acid). We confirmed this expectation by analyzing the $^{31}$P-nuclear magnetic resonance spectra of starved cells, using phosphate as an internal marker and methylphosphonate as an external marker (40); the latter compound was not taken up by our cells. In practice, enough phosphate leaked out of the cells that it could be used as an external marker as well. Cells from a 3-liter culture were harvested at a density of about $5 \times 10^8$ cells per ml and washed twice in standard buffer (400 ml each spin) and twice in a buffer containing 0.01 M $N$-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.5), 0.2 M KCl, and 0.01 M methylphosphonate (200 ml for the first spin, 75 ml for the second). Three samples (2 ml each) were prepared by suspending the cells in the latter buffer at a density of about $3 \times 10^{11}$ cells per ml; one sample was supplemented with sodium phosphate (0.01 M, pH 7.5). The samples were analyzed in a Fourier transform spectrometer (Bruker; $^{31}$P resonance, 202 MHz; field-locked to $D_2O$; sample spun at 20 Hz; average of 256 signals from 35° pulses delivered at 2-s intervals). The spectra were taken about 4 h after the cells were first suspended in standard buffer. The internal pH did not shift significantly 45 min after addition of valinomycin (100 μg per sample). The results from five scans on three samples were $\Delta pH = 0.20 \pm 0.04$ U (interior acid).

## RESULTS

**Rationale.** Reagents that modify proteins were tested for their ability to inhibit the motility of tethered cells energized by a potassium diffusion potential. Starved cells were tethered in standard buffer (0.2 M KCl) and treated with the potassium ionophore valinomycin. Motility was induced by shifting the cells to low-K buffer (0.01 M KCl). A potassium diffusion potential of about 90 mV was generated (cell interior negative; see below). As shown in Fig. 1 (solid curves), this caused the cells to spin; they continued to do so for several minutes. When certain protein modification reagents were added to the low-K buffer (e.g., methyl acetimidate), the number of cells that spun (Fig. 2A, solid curve) and the rate at which they spun (Fig. 2B, solid curve) declined markedly with time. When this happened, we did not know, a priori, (i) whether the reagent made the membranes leaky to protons, which would shunt the motors and increase the rate of acidification of the cytoplasm; (ii) whether the reagent inactivated systems, such as $Na^+/H^+$ antiport, that normally reduce the rate of acidification of the cytoplasm; (iii) whether the reagent made the membranes leaky to other ions, e.g., to $Na^+$ or $Cl^-$, which would reduce the diffusion potential and allow the potassium gradient to run down; or (iv) whether the reagent damaged the motors directly. To find out, we shifted the cells back to standard buffer for an interval of time at least as long as the time of exposure to the low-K buffer and then shifted them back to the low-K buffer once again. When this was done with cells that had not been exposed to reagents, the cells stopped in the standard buffer, as expected, and started up again in the low-K buffer (Fig. 1, dashed curves); these shifts simply turned the potassium diffusion potential off and then on again, albeit to a somewhat lower value. When this was done with cells that had been stopped by some reagents, the cells remained at rest in the standard buffer but started up again in the low-K buffer (Fig. 2, dashed curves). Evidently, the initial intracellular ion concentrations were restored, at least in part, while the cells stood in the standard buffer, and a diffusion potential was generated once again when they were shifted back to the low-K buffer; the motors were still intact and responded to this potential. This is the result expected for reagents that damage the membranes (cases i to iii). The
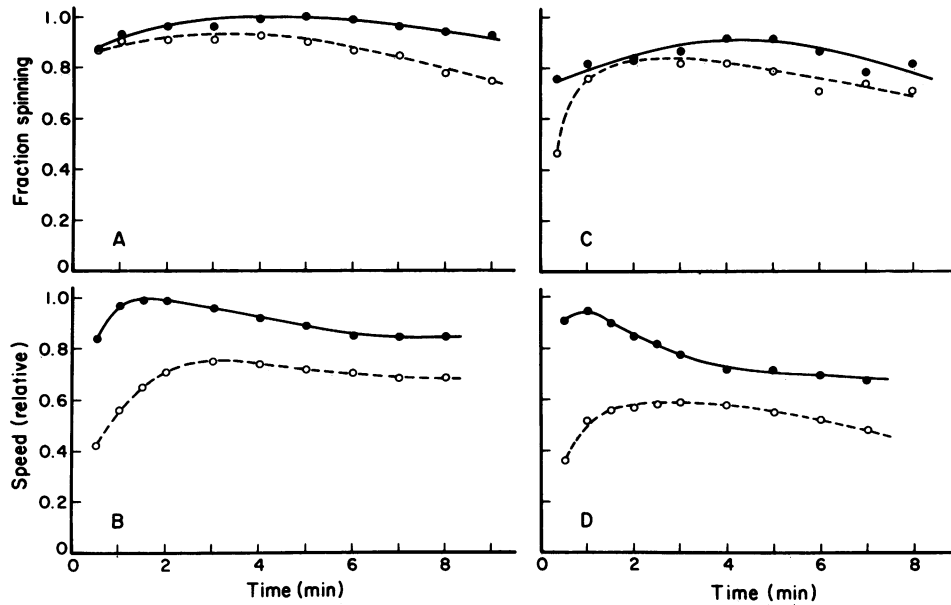
FIG. 1. Rotational behavior of cells energized by a potassium diffusion potential at pH 7.5 (left) or 6.5 (right). (A, C) Number of cells spinning, normalized to the maximum number (A, 68; C, 38). (B, D) Mean speed of six of these cells, each normalized to its maximum speed (mean ± standard deviation of the maximum speeds: B, 4.3 ± 1.3 Hz; D, 2.7 ± 1.1 Hz). Starved cells were treated with valinomycin and shifted from standard buffer to low-K buffer at time zero. Their subsequent behavior is shown by the solid curves (●). At 9 min, the cells were returned to standard buffer, allowed to stand for 12 min (not shown), and then shifted back to low-K buffer (new time zero). Their subsequent behavior is shown by the dashed curves (○). Results obtained at pH 8.0 (not shown) were similar to those obtained at pH 7.5.

alternative possibility, that the motors were damaged by the reagent while the cells were energized and this damage was repaired while the cells were deenergized (even in the presence of fresh reagent), was ruled out in a subsequent experiment by treating the cells with the reagent for a short time before the initial shift to low-K buffer; the motility was inhibited at the start and then declined. When the potassium shift experiments were done with other reagents, the cells failed to spin when shifted to low-K buffer for the second time. When the cells were pretreated with these reagents for a short time the motility was inhibited at the start but did not decline; the membranes remained competent. This is the result expected for reagents that damage the motors directly (case iv). Finally, we studied the effects of both kinds of reagents on metabolizing cells. Some of the reagents of the first type (cases i to iii) failed to inhibit the motility of metabolizing cells, whereas all of the reagents of the second type (case iv) markedly inhibited such motility. Some of the reagents of either type also changed the rotational bias of the flagellar motors. These results are summarized in Table 1 and described in detail in the following sections.

**Amino reagents.** We tested two imidoesters, methyl acetimidate, which is uncharged, and isethionyl acetimidate, which is negatively charged; both react with free amino groups to form the same amidine (45). Since the pK of the amidine is similar to that of the original amino group, these reagents are highly specific; they rarely affect function, unless modifying residues near an active site. Isethionyl acetimidate (50 mM, pH 8.0) did not inhibit motility of either artificially energized cells or metabolizing cells (data not shown). When methyl acetimidate was added to artificially energized cells, the number of cells spinning (Fig. 2A, solid curve) and their speeds (Fig. 2B, solid curve) declined; however, the recovery after exposure to standard buffer (Fig. 2, dashed curves) was substantially larger than would be expected had the reagent acted on the motor directly.

Pretreatment of cells in standard buffer gave surprising results: the cells spun before the shift to low-K buffer; after this shift, however, their speeds declined more rapidly than in the control (data not shown). It is possible that the reagent was hydrolyzed more rapidly in the cytoplasm than in the external medium (liberating ammonia), generating a transient pH gradient (cell interior alkaline). Methyl acetimidate inhibited the motility of metabolizing cells slightly, but most cells continued to spin (data not shown). This reagent shifted the rotational bias of artificially energized cells counterclockwise (CCW; the direction corresponding to runs in swimming cells); these cells normally spin predominantly clockwise (CW; the direction corresponding to tumbles in swimming cells; see reference 20). It also shifted the rotational bias of metabolizing cells CCW; these cells normally spin alternately CW and CCW.

**Sulfhydryl reagents.** We studied the effects of NEM, silver nitrate, p-mercuribenzenesulfonate, and iodoacetamide. With the exception of the latter reagent, which can react slowly with imidazole and methionyl residues, these reagents are highly specific for sulfhydryl groups (at the pH shown, Table 1; see reference 29). Ag$^+$ is positively charged, NEM is uncharged, p-mercuribenzenesulfonate is positively and negatively charged (electrically neutral), and iodoacetamide is uncharged.

The effects of NEM on artificially energized cells are shown in Fig. 3A and B. NEM did not have a marked effect on the number of cells spinning or on their speeds over a time span in which the other reagents were effective (10 min), either with cells energized artificially (Fig. 3A and B) or with metabolizing cells (data not shown). Nor did it change the initial behavior of cells energized artificially when added 10 min before a shift to low-K buffer. Metabolizing cells eventually slowed down, e.g., spinning at about half of their initial speed after 20 min; their rotational bias gradually shifted to CW.
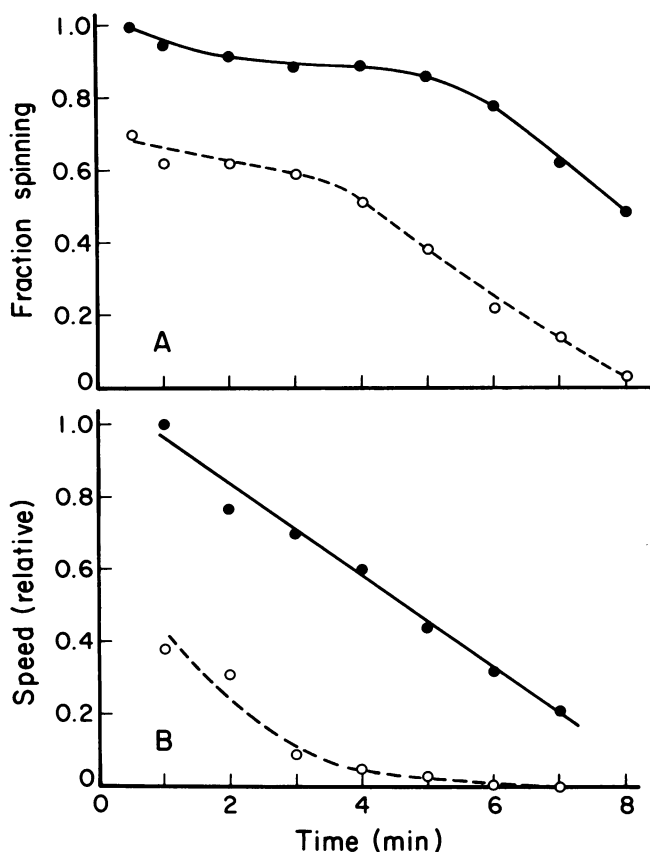
FIG. 2. Rotational behavior of cells energized by a potassium diffusion potential and exposed to methyl acetimidate (50 mM, pH 8.0). (A) Number of cells spinning, normalized to the maximum number, 37. (B) Mean speed of five of these cells, each normalized to its maximum speed (mean ± standard deviation of the maximum speeds, 2.3 ± 0.4 Hz). Starved cells were treated with valinomycin and shifted from standard buffer to low-K buffer (at time zero); the reagent was added 0.5 min later. Their subsequent behavior is shown by the solid curves (●). At 8 min, the cells were returned to standard buffer, allowed to stand for 10 min (not shown), and then shifted back to low-K buffer (new time zero). The subsequent behavior of the cells is shown by the dashed curves (○). Once added, the reagent was present in all of the solutions.

The effects of silver nitrate on artificially energized cells are shown in Fig. 3C and D. Silver nitrate reduced both the number of cells that spun and their speeds, stopping about 90% of the cells in 5 min (cf. Fig. 3C and 1A). However, about 70% of the cells started up again in response to the second shift to low-K buffer, although at a reduced speed. Then the cells stopped a second time, following the earlier time course. Since the reagent was present during the interval between the shifts to low-K buffer, reversibility of the reaction cannot account for this recovery. Similar results were obtained when the reagent was present only during the first exposure to low-K buffer (data not shown). Motility was restored on addition of glucose. When silver nitrate was added to these metabolizing cells, it had little effect. Nor did it shift their rotational bias.

Results with p-mercuribenzenesulfonate were similar to those with silver nitrate, but not as dramatic, because inactivation of motility during the first exposure to low-K buffer was not as rapid (Fig. 4A and B, solid curves). About 50% of the cells started up again in response to the second

shift to low-K buffer, again at a reduced speed (Fig. 4A and B, dashed curves). Now, inactivation was more rapid. Since the reagent was present during the interval between the shifts to low-K buffer, reversibility of the reaction cannot account for this recovery. Pretreatment of cells in standard buffer with the reagent for 11 min before the initial shift to low-K buffer markedly inhibited motility; the cells started up slowly and stopped sooner than before (data not shown). Therefore, inhibition of motility in the experiment shown in Fig. 4A and B would have been virtually complete before the second shift to low-K buffer, were the reagent to have acted on the motor directly. Pretreatment of cells in standard buffer with 2-mercaptoethanol (10 mM, 8 min) before the initial shift to low-K buffer did not change the rate at which p-mercuribenzenesulfonate inhibited motility; the results obtained were similar to those shown in Fig. 4A and B (solid curves). Therefore, critical sulfhydryl residues were not present initially as disulfides (see below).

p-Mercuribenzenesulfonate also inhibited the motility of metabolizing cells (Fig. 4A and B, dotted curves). Essentially all of the cells continued to spin until the mean speed was reduced to about one-tenth of its initial value. By this time, the rotational bias had shifted CW. Once the cells stopped, a substantial number could be started again by a shift to lower pH, as expected if the inhibition were due primarily to reduction of proton motive force.

Iodoacetamide inhibited the motility of both artificially energized cells and metabolizing cells (Fig. 4C and D) but only when used at a relatively high concentration (100 mM). The cells failed to spin when shifted to low-K buffer the second time. This suggests a direct effect on the motor, possibly due to reaction with imidazole or methionyl residues. Iodoacetamide also shifted the rotational bias of metabolizing cells CW; this shift was complete within 1 min.

**Dithiol and disulfide reagents.** We tested sodium arsenite, a reagent highly specific for dithiols (42). Starved cells were treated with valinomycin and shifted from standard buffer to low-K buffer; after 0.5 min, sodium arsenite was added (final concentration, 1 mM). The behavior of the cells was the same as that seen in controls. After 8.5 min, the cells were shifted to standard buffer containing 2-mercaptoethanol (10 mM) and allowed to stand for 12 min. Then they were shifted back to low-K buffer containing sodium arsenite (as before). Again, the reagent had no observable effect. The exposure to 2-mercaptoethanol ruled out the possibility that critical dithiols were present as disulfides. Sodium arsenite did not affect the speeds of metabolizing cells, whether added before or after treatment with 2-mercaptoethanol. The latter reagent had no effect on speeds. Neither reagent had a long-term effect on rotational bias.

**Imidazole reagents.** We studied the effects of photodynamic dyes, which attack imidazole, sulfhydryl, methionyl, and indole residues (29) and are known to affect bacterial motility (31, 32, 41), and diethylpyrocarbonate (ethoxyformic anhydride), which acylates imidazole and amino groups (29, 33). Initially, we tested eosin (31, 32) and proflavine (41), using the light source available in a Nikon Optiphot microscope (a 12-V, 50-W tungsten-halogen lamp run at 9 V, focused by a phase-contrast condenser). Eosin (tetrabromofluorescein) was as effective at 0.008 mM as proflavine was at 0.2 mM, so eosin was chosen for further study. When an orange cutoff filter (Schott OG570) was inserted in the light path, eosin had no effect; this dye absorbs light (maximally at ~516 nm) at wavelengths blocked by the filter (<570 nm). Thus, it was possible to turn the activity of the reagent on or off while continuously monitoring bacterial motion.

TABLE 1. Inhibition of motility by protein modification reagents[a]

| Reagent | Concn (mM) | pH | Artificially energized cells inhibited via: | | Metabolizing cells inhibited | Long-term shift in rotational bias[b] | Reagent charge | Other residues modified at same pH |
|---|---|---|---|---|---|---|---|---|
| | | | Δp | Motor | | | | |
| **Amino** | | | | | | | | |
| Isethionyl acetimidate | 50 | 8.0 | − | − | − | − | − | |
| Methyl acetimidate | 50 | 8.0 | + | − | ? | CCW | 0 | |
| **Sulfhydryl** | | | | | | | | |
| NEM | 5 | 6.5 | − | − | − | CW | 0 | |
| Silver nitrate | 0.01 | 7.5 | + | − | − | − | + | |
| p-Mercuribenzenesulfonate | 2 | 6.5 | + | − | + | CW | + − | |
| Iodoacetamide | 100 | 6.5 | ? | + | + | CW | 0 | Imidazole, methionyl |
| **Dithiol** | | | | | | | | |
| Sodium arsenite[c] | 1 | 7.5 | − | − | − | − | 0[d] | |
| **Disulfide** | | | | | | | | |
| 2-Mercaptoethanol | 10 | 7.5 | − | − | − | − | 0 | |
| **Imidazole** | | | | | | | | |
| Eosin + light | 0.008 | 7.5 | − | + | + | − | − −[e] | Sulfhydryl, Meth- |
| Rose bengal + light | 0.0001 | 7.5 | − | + | + | − | − − | ionyl, Indole |
| Diethylpryocarbonate | 2.5 | 7.5 | − | + | + | CW | 0 | Amino |
| **Methionyl** | | | | | | | | |
| Hydrogen peroxide | 150[f] | 6.5 | ? | ? | + | CW | 0 | Sulfhydryl, Disulfide, Indole, Tyrosyl[g] |

[a] Unless referring to charge: +, yes; ?, maybe; −, no; over a period of ca. 10 min.

[b] CW, Direction corresponding to tumbles in swimming cells; CCW, direction corresponding to runs.

[c] Same results whether cells were untreated, pretreated, or treated concurrently with 10 mM 2-mercaptoethanol.

[d] Arsenious acid, pK 9.2; only 2% ionized at pH 7.5.

[e] Charge on dye; singlet oxygen neutral.

[f] Little effect at 10 mM, except on rotational bias.

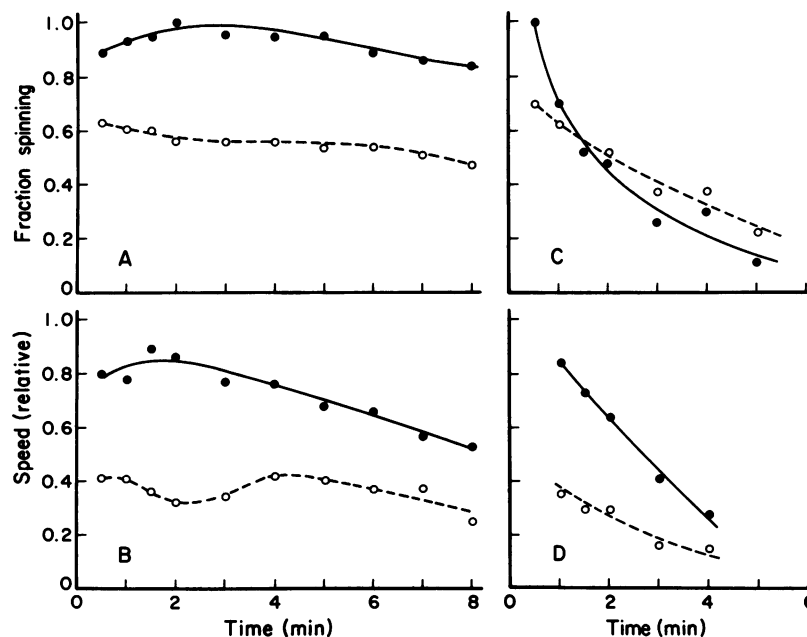[g] Via oxidation of chloride to chlorine.



FIG. 3. Rotational behavior of cells energized by a potassium diffusion potential and exposed to NEM (left; 5 mM, pH 6.5) or to silver nitrate (right; 0.01 mM, pH 7.5). (A, C) Number of cells spinning, normalized to the maximum number (A, 68; C, 26). (B, D) Mean speed of six of these cells, each normalized to its maximum speed (mean ± standard deviation of the maximum speeds: B, 3.1 ± 0.8 Hz; D, 1.4 ± 0.6 Hz). Starved cells were treated with valinomycin and shifted from standard buffer to low-K buffer (at time zero); the reagents were added 0.5 min later. The subsequent behavior of the cells is shown by the solid curves (●). At 8.5 (A) or 5 (C) min, the cells were returned to standard buffer, allowed to stand for 12 (A) or 6 (C) min (not shown), and then shifted back to low-K buffer (new time zero). The subsequent behavior of the cells is shown by the dashed curves (○). Once added, the reagents were present in all of the solutions.
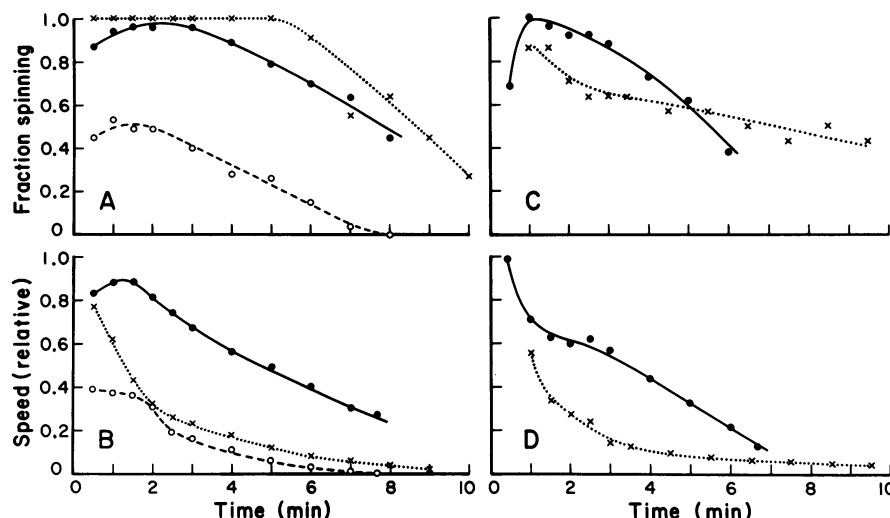
FIG. 4. Rotational behavior of cells exposed to *p*-mercuribenzenesulfonate (left; 2 mM, pH 6.5) or to iodoacetamide (right; 100 mM, pH 6.5). (A, C) Number of cells spinning, normalized to the maximum number. (B, D) Mean speed of six of these cells, each normalized to its maximum speed. Starved cells were treated with valinomycin and shifted from standard buffer to low-K buffer (at time zero); the reagent was added at the same time (A) or 0.5 min later (C). The subsequent behavior of the cells is shown by the solid curves (●; maximum number spinning: A, 47; C, 26; mean ± standard deviation of the maximum speeds: B, 3.3 ± 1.3 Hz; D, 2.2 ± 0.9 Hz). At 8 (A) or 6 (C) min, the cells were returned to standard buffer, allowed to stand for 11 (A) or 10 (C) min (not shown, and then shifted back to low-K buffer (new time zero). Their subsequent behavior is shown by the dashed curves (○); in the experiment with iodoacetamide (right), none of the cells spun. Once added, the reagents were present in all of the solutions. Metabolizing cells also were treated with the reagents, beginning at time zero (A) or at 0.5 min (C). Their behavior is shown by the dotted curves (×; maximum number spinning: A, 11; C, 14: mean ± standard deviation of the initial speeds: B, 9.4 ± 2.2 Hz; D, 7.1 ± 2.1 Hz).

The effect of eosin and light on artificially energized and metabolizing cells is shown in Fig. 5A and B. During the first shift to low-K buffer, there was a substantial decline in the number of cells spinning and in speed (Fig. 5A and B, solid curves). During the second shift, a few cells spun but at very low speed (Fig. 5A and B, dashed curves); some cells continued to spin very slowly for several minutes. The rate of inhibition was similar in metabolizing cells (Fig. 5A and B, dotted curves). The artificially energized cells spun in the usual direction (CW). The metabolizing cells behaved in a more complicated fashion. When the filter was removed there was a transient shift in bias CW; when it was replaced there was a transient shift CCW. When the filter was left off, as in Fig. 5A and B, there was a gradual shift CCW as the cells slowed down. Cells treated for several minutes spun very slowly CCW, but they still gave a CW response when subjected to a repellent stimulus, e.g., removal of glucose. Thus, the photooxidation inhibited motility but did not lock the motor in one or the other rotational state.

Diethylpyrocarbonate also affected the motor directly. It inhibited the motility of both artificially energized and metabolizing cells. When used at a concentration of 2.5 mM (pH 7.5) speeds fell exponentially with a half-time of about 1 min (Fig. 5D). The artificially energized cells failed to recover when returned to standard buffer and shifted back to low-K buffer. The rotational bias of the metabolizing cells rapidly shifted CW.

Similar results were obtained with rose bengal (tetraiodo-tetrachlorofluorescein), a photodynamic dye considered relatively specific for imidazole groups (29). We found it effective in inhibiting motility of both artificially energized and metabolizing cells at 1/100 the concentration required with eosin (using the same light source, stopping the reaction

with a Schott RG-645 filter). Part, but not all, of the increased sensitivity was due to the fact that this dye absorbs light farther in the red (maximally at ~546 nm) where a tungsten-halogen lamp run at half power is relatively more intense. Starved cells energized in low-K buffer and then stopped by exposure to light and rose bengal ($10^{-4}$ mM, pH 7.5) failed to recover when returned to standard buffer and shifted back to low-K buffer (as found with eosin). As these cells slowed to a stop, they continued to spin CW. Metabolizing cells, on the other hand, first spun CW and later CCW (also as found with eosin). The other experiments done with eosin were not repeated.

When cells were pretreated with eosin and light or with diethylpyrocarbonate and then energized artificially, they continued to spin for several minutes at a constant speed (Fig. 6). The longer the pretreatment, the lower the speed. Similar effects were observed with metabolizing cells exposed to eosin when the filter was repeatedly removed and replaced (Fig. 7) or with diethylpyrocarbonate, when the reagent was added and then inactivated with histidine (data not shown). Evidently, the motility was inhibited in some partial, yet stable way. Either the proton motive force was turned down in a stepwise fashion or the torque generated at a given proton motive force was turned down in a stepwise fashion, or both. With artificially energized cells, it is difficult to see how a sizable change in proton motive force could be achieved in a stable way: the proton motive force is reduced when the membrane becomes leaky to protons or other ions (see Rationale above). This leakiness invariably causes the proton motive force to run down; thus, the change is not stable.

To verify this point, we determined the diffusion potential of cells treated with eosin and light from measurements of
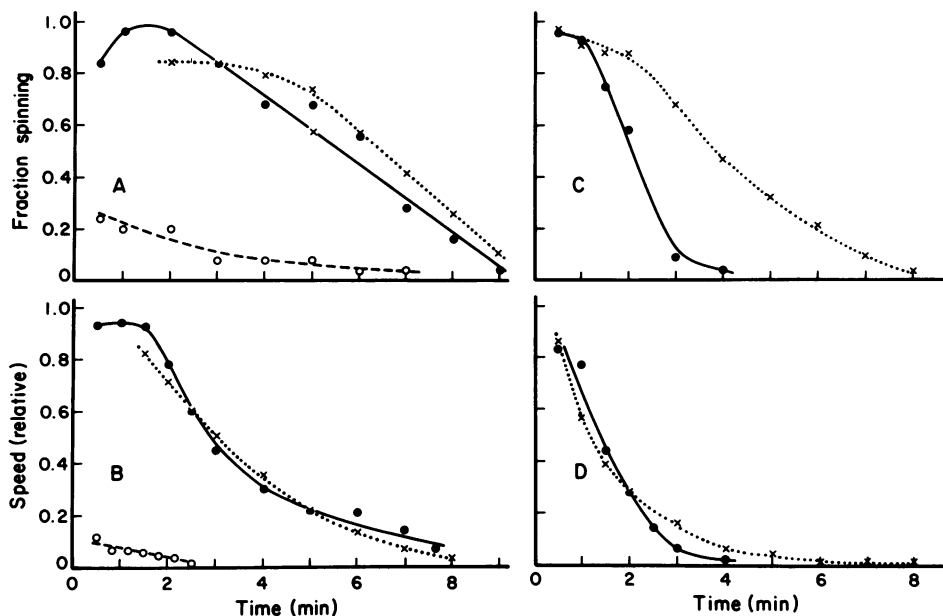
FIG. 5. Rotational behavior of cells exposed to light and eosin (left; 0.008 mM, pH 7.5) or to diethylpyrocarbonate (right; 2.5 mM, pH 7.5). (A, C) Number of cells spinning, normalized to the maximum number. (B, D) Mean speed of six of these cells, each normalized to its maximum speed. In (A) starved cells were treated with valinomycin and shifted from standard buffer to low-K buffer containing eosin (at time zero); the filter blocking light absorbed by eosin was removed 1 min later. In (B) starved cells were treated with valinomycin and shifted from standard buffer to low-K buffer containing diethylpyrocarbonate (at time zero). The behavior of the cells is shown by the solid curves (●; maximum number spinning: A, 25; C, 55; mean ± standard deviation of the maximum speeds: B, 2.8 ± 1.6 Hz; D, 2.3 ± 0.9 Hz). At 8 min (A), the filter was replaced, and the cells were returned to standard buffer, allowed to stand for 12 min (not shown), and then shifted back to low-K buffer (new time zero). Their subsequent behavior is shown by the dashed curves (O). Cells treated with diethylpyrocarbonate (C) failed to recover when returned to standard buffer and shifted back to low-K buffer (with or without reagent). Metabolizing cells also were treated with these reagents (beginning at 1 [A] or 0 [C] min). Their behavior is shown by the dotted curves (×; maximum number of cells spinning: A, 19; C, 34; mean ± standard deviation of the initial speeds: B, 6.0 ± 3.4 Hz; D, 6.4 ± 2.2 Hz).

the equilibrium distribution of tracer amounts of $^{86}Rb^+$ (13, 38). The dye was added to cells in suspension and a sample was placed in a cuvette illuminated from below by light from a 100-W tungsten-halogen lamp, light that was more intense than that available in the microscope. The sample was stirred with a stream of air. Preliminary experiments with swimming cells indicated that an exposure of 2 min was adequate to inhibit motility. Experiments with starved cells, which were tethered and energized artificially, showed that this exposure decreased rotational speeds by a factor of more than 10. The effect of such an exposure on the diffusion potential is shown in Fig. 8, which compares cells exposed to eosin and light (solid curve) with those not treated by the reagent (dotted curve). The potential in cells exposed to eosin and light dropped about 30% in the first 2 min and then leveled off; it was about 75% as large as the control value after 10 min. Thus, whereas some changes in membrane potential did occur, they were far too small to account for the corresponding changes in motility. We concluded that photooxidation inhibited motility primarily through modification of the motor, not through changes in other components of the membrane.

The speed of tethered cells of *Streptococcus* sp. strain V4051 is a linear function of proton motive force to at least −100 mV (20, 27). There is a threshold in the range −8 to −18 mV below which the cells fail to spin or exhibit free rotational Brownian rotation but above which changes in speed are proportional to changes in proton motive force (6). This functional relationship was retained when cells were treated with eosin and light (Fig. 9). The photooxidation

increased the threshold and decreased the constant of proportionality by roughly the same factor (ratio ± standard deviation, 5.6 ± 2.2 and 3.4 ± 0.4, respectively). This is the result expected if the modification reduced the amount of torque generated without altering barriers to rotation, e.g., barriers that arise from friction between the M- and S-rings or between the drive shaft and the cell wall (5). The thresholds (Fig. 9) were lower than expected from earlier work; this might be due to the fact that here the threshold was approached from above rather than from below (S. Khan, personal communication).

It is conceivable that modification of imidazole groups affects the way that the motor responds to a membrane potential differently than the way that it responds to a pH gradient. We looked for such a difference by pretreating cells with diethylpyrocarbonate and then energizing them with either a potassium diffusion potential (as in Fig. 6B) or a pH gradient (by shifting to standard buffer at pH 6.0). The two energy sources remained equivalent.

Two experiments were done to confirm that the effects observed with diethylpyrocarbonate were due to the acylation of histidine side chains. When artificially energized cells were treated with 3.9 mM diethylpyrocarbonate at pH 6.5, 7.0, or 7.5, the half-times for inactivation were 75 ± 3, 54 ± 2, and 37 ± 1 s, respectively (determined from weighted least-squares fits to speed data from 10 cells at each pH). The inactivation rate increased with pH as expected if the critical residue has a pK of 6.8 and is acylated only when unprotonated (16). We also attempted to reverse the action of diethylpyrocarbonate by treatment with hydroxylamine
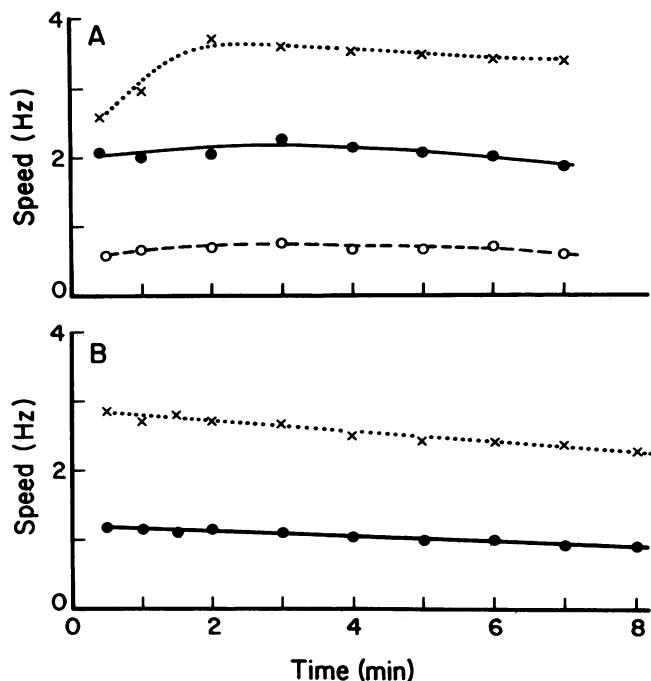
FIG. 6. Rotational speed of cells exposed to light and eosin (A) or to diethylpyrocarbonate (B) and then energized by a potassium diffusion potential. In (A) starved cells were treated with valinomycin and shifted to standard buffer containing eosin (0.008 mM, pH 7.5). Light of short wavelength was blocked by an OG570 filter. The filter was left in place (×) or removed for 1 (●) or 2 (○) min, and then the cells were shifted to low-K buffer (at time zero). In (B) starved cells were treated with valinomycin and shifted to standard buffer containing diethylpyrocarbonate (2.5 mM, pH 7.5). After 1 min, the cells were shifted to standard buffer containing 10 mM histidine (pH 7.5) and allowed to stand for 3 min (to inactivate the reagent). Then they were shifted to low-K buffer (at time zero). The subsequent behavior of these cells is shown by the solid curve (●). The experiment was repeated deleting the step involving diethylpyrocarbonate (×, dashed curve). The mean speed of six cells in each preparation is shown. In (A) the standard errors of the means were about 0.8, 0.4, and 0.3 Hz, respectively; in (B) they were about 0.2 and 0.5 Hz, respectively.
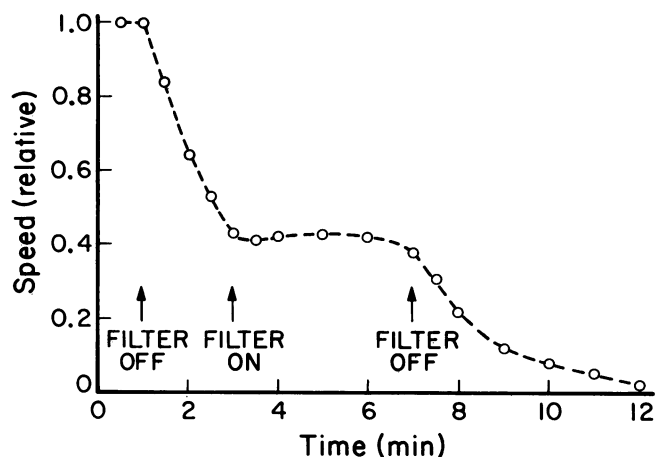


FIG. 7. Rotational speeds of metabolizing cells in standard buffer containing eosin (0.008 mM, pH 7.5). Light of short wavelength was blocked by an OG570 filter. The filter was removed and replaced, as indicated by the arrows. The mean speed of six cells is shown, each normalized to its initial speed (mean ± standard deviation of the initial speeds, 7.1 ± 2.1 Hz).

(29). The difficulty here was that hydroxylamine damaged the cell membranes. Starved cells were treated with valinomycin and shifted to low-K buffer. When hydroxylamine was added (as the hydrochloride, 0.5 M, pH 7.5), the cells stopped within 10 min. However, when the cells were shifted back to standard buffer containing glucose (11 mM), they started up again, beginning to spin after about 1 min and spinning vigorously within 5 min; the bias was predominantly CCW. This experiment was repeated with cells that had been pretreated with diethylpyrocarbonate (2.5 mM, 2 min, followed by 10 mM histidine, 3 min). The cells failed to spin when shifted to low-K buffer or when treated with hydroxylamine (as above). When the cells were shifted back to standard buffer containing glucose, they started up very slowly, beginning to spin after about 5 min, reaching only a moderate speed within 25 min; again, the bias was predominantly CCW. Finally, the experiment was repeated with cells that had been pretreated with diethylpyrocarbonate (as above) and not exposed to hydroxylamine. Again, the cells failed to spin when shifted to low-K buffer. However, they also failed to spin when shifted back to standard buffer containing glucose. Therefore, the effects of diethylpyrocar-

bonate were partly reversed by treatment with hydroxylamine, as expected if the reagent acylates imidazole groups (30, 33).

**Methionyl reagents.** Part of the action of iodoacetamide and the photodynamic dyes might be due to modification of methionyl residues. A possible indication of this was the partial reversal of the inhibition of motility by eosin and light by 2-mercaptoethanol, which reduces methionine sulfoxide to methionine (29). Cells that had been stopped by eosin and light in the experiment shown in Fig. 5A and B were shifted back to standard buffer and allowed to sit for 12 min. Then they were given glucose (11 mM). Cells on regions of the cover slip that had not been exposed to light started up, beginning in about 2 min, but only 1 of the 25 cells that had been photooxidized did so, beginning in about 7 min. Twelve more of these cells started up after treatment with 2-mercaptoethanol (25 mM; four within 2 min, two more within 5 min, five more within 10 min, and one more within 50 min); all of these cells spun very slowly CCW.

Methionyl residues are readily oxidized by hydrogen peroxide, but unfortunately its specificity is low (Table 1). Hydrogen peroxide had relatively little effect on speed when added at a low concentration (10 mM); however, the rotational bias of metabolizing cells shifted CW (within 1 min). This effect was reversed within 1 min when the cells were shifted back to standard buffer and treated with 2-mercaptoethanol (25 mM). Hydrogen peroxide did affect the speeds of both artificially energized and metabolizing cells when used at a higher concentration (150 mM). Whereas most cells in low-K buffer were still spinning after 8 min, only about half started up again when returned to standard buffer and shifted back to low-K buffer; they did so at a relatively slow speed (data not shown). Unfortunately, these effects were not reversed when the cells were shifted back to standard buffer and given 2-mercaptoethanol (25 mM). Thus, although the effects of hydrogen peroxide on speed were substantial, they could not be attributed simply to the oxidation of methionine. Other effects of hydrogen peroxide, e.g., the oxidation of cysteine to cysteic acid or the chlorination of tyrosine after oxidation of chloride to chlorine, would not be reversed by 2-mercaptoethanol.
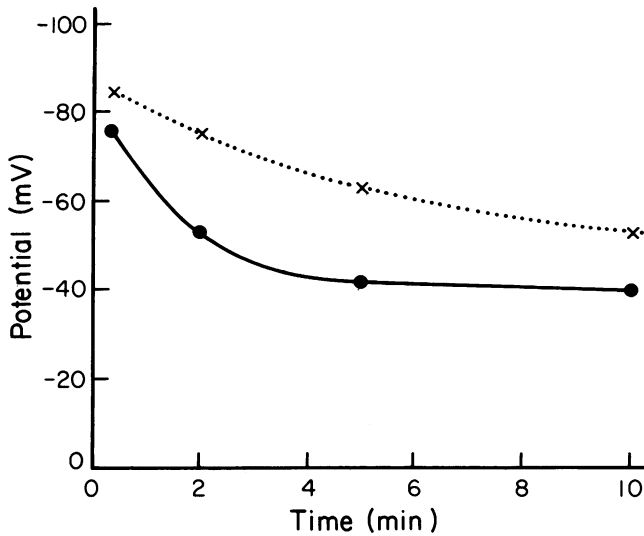
FIG. 8. Potassium diffusion potential determined from $^{86}Rb^+$ uptake. Starved cells were treated with valinomycin and diluted (at time zero) into K-free buffer containing $^{86}Rb^+$ ($\times$, dotted curve) or treated with valinomycin, mixed with eosin (0.008 mM, pH 7.5), exposed to light (see text), and then diluted into K-free buffer containing $^{86}Rb^+$ ($\bullet$, solid curve). The membrane potential was determined at different times from the distribution of $^{86}Rb^+$ between cell water and supernatant fractions, as described in the text. Means obtained from experiments on two different cultures, which gave essentially identical results, are shown.

## DISCUSSION

Our principal findings with tethered cells of *Streptococcus* sp. strain V4051 are summarized in Table 1. We studied the effects on motility of reagents that modify amino, sulfhydryl, dithiol, disulfide, imidazole, and methionyl residues. The reagents were used at concentrations that were large enough to cause substantial changes in speed within about 10 min or were as large or larger than those commonly used to block function in other systems (see below). The pH was chosen to favor the desired reaction or to enhance specificity. Both amino reagents, three of the four sulfhydryl reagents, the dithiol reagent, and the disulfide reagent were highly specific; one of the imidazole reagents was reasonably so; and the methionyl reagent was not. At least one reagent of each class was uncharged (lipid soluble) and, thus, readily able to penetrate the cytoplasmic membrane. The motor was affected directly by reagents that modified imidazole and possibly methionyl residues. The evidence for the involvement of imidazole groups in torque generation was conclusive, but that for the involvement of methionyl groups was relatively weak. Amino, sulfhydryl, dithiol, and disulfide groups did not appear to be involved. Modification of amino and sulfhydryl groups by some of the reagents did, however, interfere with the generation or maintenance of proton motive force. One of the amino reagents generated a long-term CCW shift in rotational bias, whereas some of the sulfhydryl, imidazole, or methionyl reagents generated a CW shift. The shifts generated by iodoacetamide, diethylpyrocarbonate, and hydrogen peroxide were dramatic; they occurred within 1 min after the addition of the reagent.

**Histidine in chemiosmotic coupling.** As noted in the discussions of Fig. 6, 7, and 9, the imidazole reagents reduced the torque in a graded, yet stable way. Evidently, these reagents acted by reducing the number of force generators, the
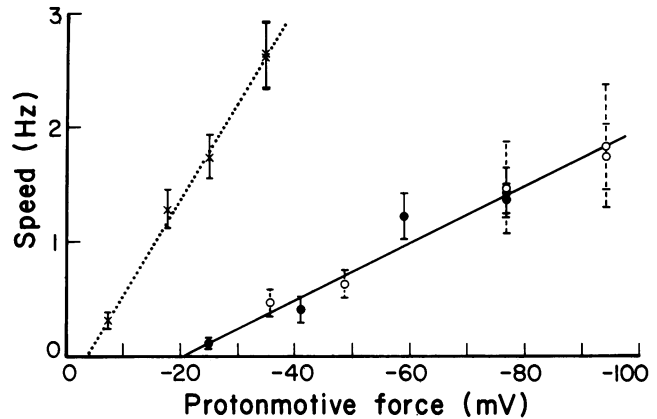


FIG. 9. Functional dependence of speed on proton motive force of untreated cells ($\times$, dotted line) and cells exposed to eosin and light ($\bullet$, $\bigcirc$, solid line). Starved cells were treated with valinomycin, exposed to eosin (0.008 M, pH 7.5) and light (1.5 min, $\bullet$, or 2 min, $\bigcirc$), and then shifted at intervals of 1 to 2 min through a series of solutions of lower potassium concentration (with NaCl added to maintain the ionic strength), starting with the lowest concentration, proceeding upward to the highest concentration, and then returning to the lowest concentration. The experiment also was done with cells from the same culture that had not been exposed to eosin ($\times$). The proton motive force was computed form the Nernst equation (27) assuming that cells in standard buffer were completely deenergized; see text. Cells that had roughly the same radius of gyration were picked for analysis. The error bars are standard errors of the means; the lines are weighted least-squares fits ($\times$—13 cells, slope $\pm$ standard deviation, $0.083 \pm 0.07$, intercept $\pm$ standard deviation, $3.7 \pm 1.3$; $\bullet$ and $\bigcirc$—14 and 12 cells, slope $\pm$ standard deviation, $0.025 \pm 0.002$, intercept $\pm$ standard deviation, $20.8 \pm 3.4$). The reasons why cells exposed to eosin and light for 1.5 min behaved similarly to those exposed for 2 min are not known; the oxygen tension or the light intensity or both might not have been the same in the two experiments.

efficiency at which each generator works, or both. We know from other work that the motor is driven by several independent force generators. First, a starved cell neither spins nor exhibits free rotational diffusion; if the proton motive force is 0, the motor remains rigidly engaged (6, 27; Fig. 9). This implies that there is a barrier to rotation that is large compared with the proton motive force of thermal fluctuation, $kT/e \simeq 25$ mV, where $k$ is Boltzmann's constant, $T$ is the absolute temperature, and $e$ is the proton charge. But the threshold for rotation is quite small, $<25$ mV (6, 27; Fig. 9). It follows that several protons must act in synchrony to overcome the barrier to rotation. The shift in threshold shown in Fig. 9 is consistent with this interpretation; the fewer the number of force generators or the smaller their efficiency, the higher the threshold. Second, fully energized *motB* (paralyzed) mutants of *Escherichia coli* can be tethered, but they fail to spin. When the synthesis of wild-type motB protein is induced in these cells, their rotational speeds increase in a series of equally spaced steps (S. M. Block and H. C. Berg, Nature, in press). Each step signals the addition of another independent force-generating unit comprised, at least in part, of motB protein. The ratio of the final to the initial speeds implies that each motor is driven by about 16 such units.

A model for the flagellar rotary motor has been suggested (5, 20) in which the force generators are transmembrane particles linked elastically to the S-ring (the stator). These particles move from site to site along the periphery of the M-

ring (the rotor). Each particle interacts with two adjacent sites, connecting one to the outside of the cell via a proton well (a proton-specific channel that converts an electric field into a proton gradient; see reference 26) and the other to the inside of the cell via an aqueous pore. A particle (or channel complex) can advance only when the site at the bottom of the proton well is protonated and the site at the bottom of the aqueous pore is unprotonated; it can retreat only when the opposite conditions are met. As a result, its motion is tightly coupled to proton flux. When the complex reaches its equilibrium position, the work done against the elastic constraints is balanced by the energy available when a proton moves down its electrochemical gradient; the elastic restoring force is balanced by the external viscous load.

In the context of this model, the imidazole reagents block channels, uncouple the channel complexes from the M- or S-rings, or destroy the proton-accepting sites. Were channel blockage complete (and the constraints on particle movement absolute), the motor would abruptly stop, because the particles thus affected would no longer be able to move relative to the M-ring. This is not observed. Were proton wells converted to aqueous pores, the motor would no longer be driven with the same efficiency by a transmembrane potential as with a pH gradient (20, 27). This possibility was tested with cells treated with diethylpyrocarbonate. As noted in the discussion of Fig. 6B, the pH gradient and the diffusion potential remained kinetically equivalent. Therefore, the imidazole reagents probably uncouple the channel complexes from the motor or destroy the proton-accepting sites. Indeed, the proton-accepting sites might be imidazole residues, i.e., side chains of histidine.

Imidazole residues have been implicated in chemiosmotic coupling to other membrane devices, notably, the *E. coli* lactose-proton symport system. Diethylpyrocarbonate has been reported to uncouple the carrier protein from the proton electrochemical gradient without affecting the binding of the carrier to lactose, the ability of the carrier to catalyze facilitated diffusion, or the generation and maintenance of the proton motive force (12, 34, 36). Rose bengal behaves similarly, but it also blocks generation of the proton motive force (in membrane vesicles supplied reduced phenazine methosulfate; 12).

**Cysteine and cystine in chemiosmotic coupling.** It has been proposed that dithiol-disulfide interchange may play a general role in membrane-related processes such as transport and energy transduction (22, 37). The present work was inspired, in part, by a scheme of this sort, namely, an earlier model for the motor in which an enzymatically catalyzed redox loop successively breaks and makes disulfide bonds linking the rotor to the stator (6). Unfortunately, none of the reagents that we have tested that are specific for sulfhydryl, dithiol, or disulfide residues appears to affect the motor directly (Table 1; Fig. 3 and Fig. 4A and B).

Sulfhydryl reagents inactivate symport and facilitated diffusion of lactose by reacting at the β-galactoside binding site (e.g., 0.1 mM *p*-chloromercuribenzoate [19]; ~0.1 mM NEM [11]). For example, NEM (0.5 mM, pH 6.6) inhibits lactose transport in membrane vesicles with half-times of 1.3 min when the vesicles are energized and 3.2 min when they are not (9); it does not block the generation of a proton motive force in the presence of reduced phenazine methosulfate (18). In contrast, NEM at 10 times this concentration appears to have little effect on torque generation in the flagellar rotary motor (Table 1; Fig. 3A and B).

Since this work was initiated, models involving redox loops that successively break and make covalent bonds have been ruled out more generally by the absence in chemiosmotic coupling to the motor of both deuterium solvent isotope and thermal effects (20).

**Amino and carboxyl groups in chemiosmotic coupling.** Another model for the motor envisages an electrostatic attraction between a positively charged amino group and a negatively charged carboxyl group followed by proton transfer from one to the other (14). The amino reagents that we have tested did not appear to affect the motor directly (Table 1; Fig. 2). But it could be argued that critical amino groups are not accessible to these reagents (unlikely for methyl acetimidate, which is uncharged ) or that the product, an amidine, having the same pK as the original amino group carries out the same function. *N,N'*-Dicyclohexylcarbodiimide, a reagent that modifies carboxyl groups and can crosslink carboxyls to amines (29), appears to have little effect on motility of *Streptococcus* strain V4051 when applied at concentrations that block the membrane ATPase (0.02 mM; 28). Other reagents that modify carboxyl groups have not been tested. Work with diazoacetates might prove feasible (29).

**Relation to earlier work.** Earlier studies on the effect of protein modification reagents on the mobility of metabolizing cells have demonstrated marked inhibition by reagents that modify amino and guanidino groups (2,3-butanedione, phenyl glyoxal [7]), amino and probably sulfhydryl, imidazole, and tyrosyl groups (glutaraldehyde [7]), sulfhydryl groups (NEM [7]; mercuric chloride or other heavy metals [1, 10]; *p*-chloromercuribenzoate [8, 10]; *p*-chloromercuribenzenesulfonate [3, 7]), sulfhydryl, imidazole, and methionyl groups (iodoacetate [8]; iodoacetamide [7, 10]), or imidazole and other groups subject to photooxidation (eosin, erythrosin, and methylene blue, [31, 32 and earlier references cited therein]; riboflavin and proflavine [41]). As noted at the beginning of this paper, it is impossible to know from these experiments whether the groups modified are essential in energizing the membrane or in driving the motor. This objection does not apply to experiments in which the reagent shifts the rotational bias of the motor without affecting its speed (cf. reference 7).

**Control of direction of rotation.** Control of direction of rotation is the basis for the chemotactic response. For example, cells tend to extend their runs (continue to rotate their flagella CCW) when moving up spatial gradients of chemical attractants or down spatial gradients of chemical repellents (4, 23, 25, 43). The CW rotation of artificially energized *Streptococcus* sp. strain V4051 appears to be due to a chemotactic response to prolonged acidification of the cytoplasm (20). Metabolizing cells spin alternately CW or CCW, with a CCW bias. Many of the reagents that we studied caused these cells to spin almost exclusively CW (Table 1). In the case of iodoacetamide, diethylpyrocarbonate, and hydrogen peroxide, this shift was complete before the reagent had a pronounced effect on rotational speeds. For *p*-chloromercuribenzenesulfonate the shift was fairly rapid; for NEM it was gradual. Only one reagent, methyl acetimidate, shifted the bias CCW. This shift also was gradual. It persisted long after the reagent was removed; therefore, it was not simply due to alkalinization of the cytoplasm. Nor could it be due to a reduction in proton motive force, which has been shown to produce a CCW bias in *E. coli*, *S. typhimurium*, and *Bacillus subtilis* (21). This effect, if it exists in *Streptococcus* strain V4051, requires more stringent deenergization. Artificially energized cells of this strain continue to spin CW until they stop; metabolizing cells have a strong CW bias when spinning very slowly, e.g., as their

supply of glucose is exhausted. We do not know whether the shifts caused by any of these reagents were generated at the level of the flagellar motor. Chemotactic control involves the interaction of a number of different proteins (cf. reference 35), and the modification of any of them might be crucial. Thus, without the use of a variety of chemotactic mutants, experiments of this kind are difficult to interpret.

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