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Effects of Lipophilic Cations on Motility and Other Physiological Properties of *Bacillus subtilis*

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Lipophilic cations (tetraphenylarsonium, tetraphenylphosphonium, and triphenylmethylphosphonium) caused a number of major changes in the physiology of *Bacillus subtilis*. Macromolecular synthesis was inhibited, adenosine 5'-triphosphate concentration increased, swimming speed was reduced, tumbling was suppressed, and the capacity to take up the cations was greatly enhanced; respiration was not significantly altered. The effects occurred at lipophilic cation concentrations in the range commonly employed for measurement of membrane potential. Neither the enhancement of cation uptake nor the motility inhibition was a consequence of alteration of membrane potential, since both effects were still seen in the presence of valinomycin, with the extent of $^{86}Rb^+$ uptake indicating a constant potential. Because suppression of tumbling accompanied speed reduction, as has also been found when protonmotive force is reduced, it is likely that lipophilic cations are perturbing the process of conversion of proton energy into work, rather than simply causing structural damage.

Many bacterial species are motile and modulate their motility in response to environmental cues such as chemical gradients (14, 22, 24; B. L. Taylor and D. J. Laszlo, *in The Perception of Behavioral Chemicals,* in press). Motility in flagellated species derives from rotation of the helical flagellar filaments (1, 45), and it is controlled by switching of the motors between counterclockwise (CCW) and clockwise (CW) rotation (17). Sustained CCW rotation results in swimming (21), and brief intervals of CW rotation result in tumbling (12, 26). Cells experiencing a "favorable" gradient (e.g., a positive gradient of glucose) suppress tumbling and hence extend their trajectory in a favorable direction (2, 25). Two questions of fundamental importance are the mechanism of rotation and the mechanism of switching between the two senses of rotation.

As a result of studies in a number of laboratories (7, 11, 16, 27-29, 32, 43, 44), it is now evident that the energy for motor rotation is proton electrochemical potential (protonmotive force [PMF]). It is not known how this energy is converted into the mechanical work of rotation, although plausible models of a general sort have been presented whereby the energy is stored in specific hydrogen-bonded arrays between the rotor and stator elements of the motor (7, 19, 23, 33). Such models presume that protons operate in the motor directly, but there is still only negative evidence (the failure to demonstrate an intermediate energy form) in support of the presumption. There are proteins (products of the *motA* and *motB* genes) that do not form part of the motor as presently isolated, but are necessary for its rotation (9, 40); until their function is understood, any model for the conversion of PMF into rotational work must be viewed with caution, and any circumstance in which motor speed is shown to be altered in the absence of a change of PMF becomes particularly significant. Likewise, any circumstance in which the switching probabilities between CCW and CW rotation are perturbed is likely to assist in identification of the elusive "tumble regulator" that is the final output of the process of transduction of sensory information from the environment.

Recent studies (10, 11, 18) have established that any condition which lowers PMF sufficiently to reduce motor speed affects switching probabilities also. CCW → CW events become less probable, CW → CCW events become more probable, and thus, tumbling is suppressed. In other words, PMF is the energy source for rotation of the motor, but it also exerts a regulatory effect on switching properties.

Ordal (35) reported that the lipophilic cation tetraphenylarsonium ($TPAs^+$) caused a complete and permanent suppression of tumbling in *Bacillus subtilis*. de Jong and van der Drift (4) confirmed this finding and noted that swimming speed was reduced also. The effects were

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strongly pH dependent, with much higher cation concentrations being required at pH 5.5 than at neutral pH.

The membrane permeance of cations such as $TPAs^+$ (8) results in a transfer of positive charge into cells. The fact that tumble suppression was accompanied by speed reduction suggested to us that the phenomenon might be another example of perturbation of motor switching as a result of perturbation of PMF, as described above. However, measurements of fluorescence of the cyanine dye diS-$C_3(5)$ had indicated (4) that $TPAs^+$ at concentrations below 100 μM did not cause significant depolarization. Since the proton chemical potential (ΔpH) component of PMF in *B. subtilis* is negligible at pH 7.5 (11, 44), the fluorescence data implied that $TPAs^+$ can reduce motor speed and suppress tumbling without any reduction in the driving force. However, cyanine dye fluorescence as a technique for measuring membrane potential is not fully understood (6a, 47); thus, we decided to examine further the effect of $TPAs^+$ on *B. subtilis*. Answering the question of whether $TPAs^+$ affect membrane potential in *B. subtilis* has not been easy because of major artifacts this compound introduces into the techniques available for potential measurement in small cells; the topic of potential measurement is treated in detail elsewhere (A. Zaritsky, M. Kihara, and R. M. Macnab, J. Membr. Biol., in press).

Here, we describe studies of the effects of lipophilic cations on the motility and bioenergetics of *B. subtilis*. Additionally, we report that these lipophilic cations have profound physiological effects, including inhibition of macromolecular synthesis. The consequent reduction in total energy demand causes an elevation of ATP levels, and indirect evidence suggests that PMF may also be slightly increased under these conditions. The behavioral effects of $TPAs^+$, however, occur even in the presence of valinomycin, with the extent of $^{86}Rb^+$ uptake indicating no change in membrane potential. The reduction in motor speed and the accompanying suppression of tumbling must therefore result from inhibition of the process of conversion of PMF into mechanical work, rather than from an inhibition of PMF generation or from dissipation of PMF by charge transfer across the membrane.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Spores of *B. subtilis* OI1 (*ilvC1, leu-1*) (38), stored in distilled water, were inoculated into Luria broth (20) at 25°C at a low density (10^4 to 10^5 ml^{-1}), and 4 to 5 h later, the culture was diluted 100-fold into glycerol-ammonia medium; this consists of the minimal salt medium of Sargent (41), with glycerol (0.5%, vol/vol) as a carbon source, supplemented with methionine, tryptophan, leucine, isoleucine, and valine (50 μg ml^{-1} each). Cultures were grown aerobically at 25°C with vigorous shaking. Appropriate dilutions with fresh glycerol-ammonia medium were made as necessary to avoid densities higher than about 2×10^8 cells ml^{-1} (optical density at 650 nm [OD_{650}], 0.5). Cells were then centrifuged once (1,600 $\times$ *g* for 10 min at 25°C) and suspended at 25°C in *Bacillus* chemotaxis medium (37), pH 7.5, with glycerol (0.5%, vol/vol) as a carbon source, for behavioral or energetic measurements.

Behavioral observations. Observations of free cells were made at about 2×10^7 cells ml^{-1} under a bridged cover slip, using a Zeiss RA microscope with dark-field illumination. Swimming rates were determined by measuring tracks of single cells, either from video records or from stroboscopic photographs, as described previously (10).

Macromolecular synthesis. Total biosynthesis of macromolecules was estimated by incorporation of label from [^{14}C]glycerol into trichloroacetic acid-insoluble material. Samples (5 ml) of exponentially growing cells (OD_{650}, ca. 0.5) in glycerol-ammonia medium were added to tubes containing 5 ml of the same medium, plus [^{14}C]glycerol (ca. 1 μCi) and various concentrations of $TPAs^+$. Aliquots (1 ml) were taken at various time points and added to equal volumes of an ice-cold trichloroacetic acid solution. After at least 30 min in the cold, each sample was filtered with a membrane filter (0.45-μm pore, 25-mm diameter; Millipore Corp.) and washed six times with 2 ml of cold 5% trichloroacetic acid. The filters were dried (50°C for 2 h), placed in 6 ml of scintillation fluid (Aquasol), and counted.

Determination of PMF. Electrical potential ($\Delta\psi$) measurements, essentially as described previously (10), were made on 1-ml cell suspensions (OD_{650}, ca. 0.4) kept in small vials (12 by 35 mm) to prevent excessive evaporation, stirred vigorously with a magnetic bar, and vigorously aerated via a hypodermic needle. Uptake of ^{3}H-labeled triphenylmethylphosphonium ions ([^{3}H]$TPMP^+$), or of $^{86}Rb^+$ in the presence of 4 μM valinomycin, was determined by filtration assay (42), using Unipore polycarbonate filters (0.6-μm pore, 25-mm diameter; Bio-Rad). A value of 3.2 μl ml^{-1} OD_{650}^{-1} was used for cytoplasmic volume (10). The extent of surface-associated label was estimated with 100 μM carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP); for a critique of various correction procedures, see Zaritsky et al., in press.

Proton chemical potential was measured as described previously (10), by uptake of the weak acid [^{14}C]benzoate, using the technique of flow dialysis (39).

Measurement of ATP level. ATP was assayed by the luciferin-luciferase method of Cole et al. (3), modified as described by Kimmich et al. (13).

Respiration rates. Respiration rates were measured with a Yellow Spring Instrument model 53 oxygen monitor, equipped with a model 5331 oxygen electrode. After measurement of the control rate, $TPAs^+$ was added to the same sample without admitting any further oxygen, and a direct rate comparison was made.

Chemicals. Chemicals were obtained from the following sources: CCCP, luciferin-luciferase extract FLE-50, and valinomycin, Sigma Chemical Co.; diS-$C_3(5)$, A. S. Waggoner, Amherst College, Amherst,

Mass.; $TPAs^+$ chloride, Strem; tetraphenylphosphonium (TPP^+) chloride and $TPMP^+$ bromide, Pfaltz and Bauer; [7-^{14}C]benzoic acid (26 mCi mmol^{-1}), [*U*-^{14}C]-glycerol (135 mCi mmol^{-1}), and ^{86}rubidium chloride (146 mCi mmol^{-1}), New England Nuclear Corp.; [^{3}H]$TPMP^+$ bromide, nonradioactive material tritiated to 6 Ci mmol^{-1} by catalytic exchange, New England Nuclear Corp.

RESULTS

Growth conditions, cell size, and motility in *B. subtilis*. Generally, it is desirable to study cells harvested in exponential growth, when cell parameters have reached steady state (15). However, under exponential growth conditions in rich media, *B. subtilis* forms long filaments (41), and the suppression of cell division is accompanied by a suppression of flagellar assembly; both features have been related to reduced activity of autolytic enzymes (5, 6, 46). We have found that growth-phase dependence of cell size and flagellation can be avoided by cultivation in poorer media, such as the glycerol-ammonia medium of Sargent (41); at the relatively slow growth rate that this medium permits (130-min doubling time at 25°C), the great majority of cells exist as short, highly motile rods at all stages of the cell cycle (Fig. 1, insert).

At the other extreme, we found that the use of a very small inoculum into rich medium resulted in complex, helically-wound cell masses, or "macrofibers" (Fig. 1). These structures have been described in detail by Mendelson (30, 31) and Fein (6) for division-suppressed or lytic-defective mutants, but apparently can occur in wild-type cells, too, provided the initial cell density is low enough. This result suggests that exogenous autolysin concentration may be important in cell division of *B. subtilis*.

The restriction of flagellar synthesis, and hence, tactic responsiveness, to situations in which catabolism is limited (stationary phase or poor media) is a regulatory feature not displayed by gram-negative genera such as *Escherichia* or *Salmonella*.

Behavioral effects of $TPAs^+$ and related

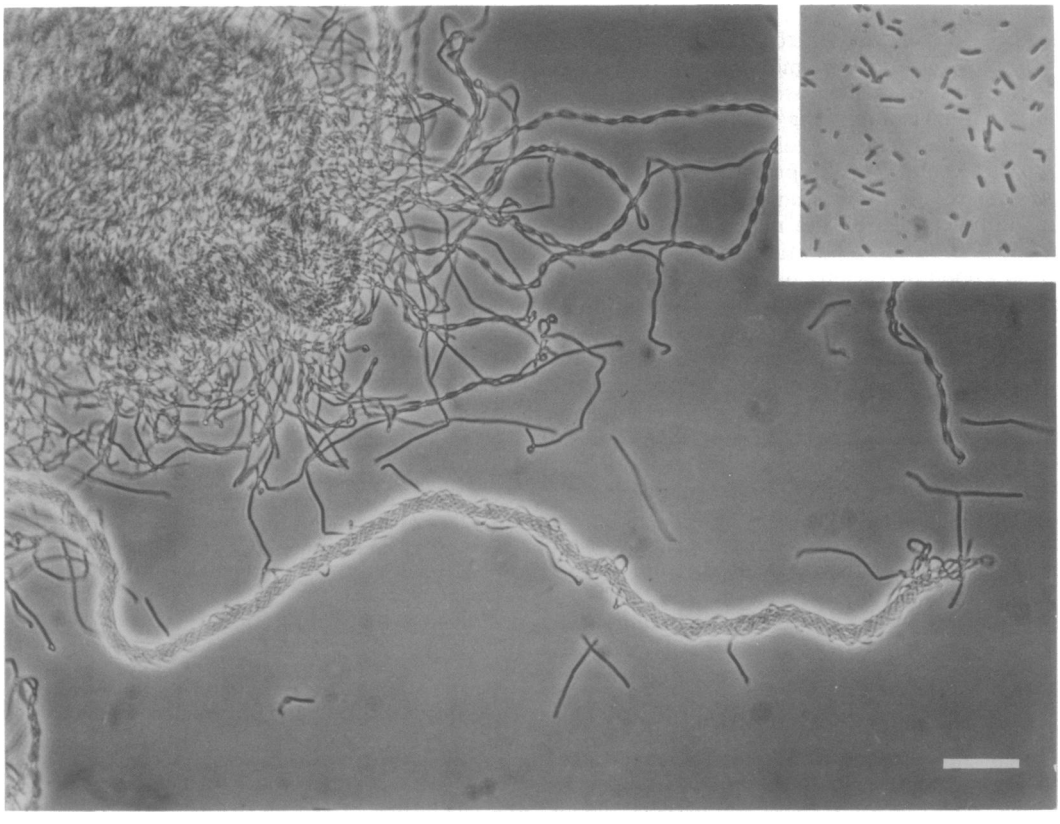


FIG. 1. *Light micrographs of B. subtilis OI1 (wild-type) under different growth conditions. Main figure: Long, helically wound macrofiber structure obtained after exponential growth in tryptone broth at 25°C for at least 20 generations from a very small inoculum. Inset: short, motile rods obtained during exponential growth in glycerol-ammonia medium at 25°C. Bar, 20 μm.*

cations. $TPAs^+$, at concentrations of 2 μM or higher, completely and permanently suppressed tumbling in *B. subtilis* OI1. Swimming speed was not greatly affected at concentrations below 5 μM (Fig. 2), but at higher concentrations it was progressively reduced, and by about 250 μM, most cells were paralyzed. The suppression of tumbling upon addition of the cation was instantaneous; the reduction in speed occurred more gradually but was almost complete by 2 min.

Populations of cells treated with $TPAs^+$ were strikingly homogeneous. All cells swam without tumbling; body wobble or precessional motion was pronounced in some cells, but except at very low speeds, this was clearly distinguishable from tumbling. The standard deviation in speed was small (19% at 50 μM $TPAs^+$; mean of 45 cells). Cells partially deenergized by CCCP or valinomycin were more heterogeneous, ranging from totally immotile to quite vigorous. For example, cells treated with 1 μM valinomycin at 18 mM K^+ had a standard deviation in speed of 36% (mean of 35 cells). Also, whereas the most vigorous cells in valinomycin-treated cultures were aerotactic, accumulating at the edge of the sample, cells treated with 2 μM $TPAs^+$, though vigorous, remained uniformly distributed.

$TPAs^+$ locked the motor strongly in the CCW (swimming) state; a negative attractant gradient (1 → 0.01 mM glutamine), which caused a 30-s tumbling response in untreated cells, had no effect in the presence of 5 μM $TPAs^+$. (A negative attractant gradient was chosen, because all known repellents for *B. subtilis* are themselves membrane-active agents [34, 38]). The effects of $TPAs^+$ on the motor were readily reversible: 20 min after treatment with 50 μM $TPAs^+$, cells were filtered, washed, and suspended in chemotaxis buffer; 3 min later, all cells had resumed tumbling and were swimming at close to normal speed.

Two other lipophilic cations, TPP^+ and $TPMP^+$, had similar effects on motility of *B. subtilis* (Table 1), although in the case of $TPMP^+$, much higher concentrations were needed.

No interaction with magnesium or calcium ion was detected: cells washed twice and suspended in magnesium-free, calcium-free chemotaxis buffer (which normally contains 0.1 mM EDTA and 0.14 mM calcium, but no magnesium) retained vigorous wild-type motility, but became completely smooth swimming upon addition of 5 μM $TPAs^+$, whether or not magnesium (up to 100 mM) or calcium (up to 3 mM) was added.

Inhibition of growth and macromolecular synthesis by $TPAs^+$. Growth was appreciably slowed by $TPAs^+$ at concentrations as low as 1 μM (Fig. 3), when no changes in motility were apparent; at concentrations higher than 3

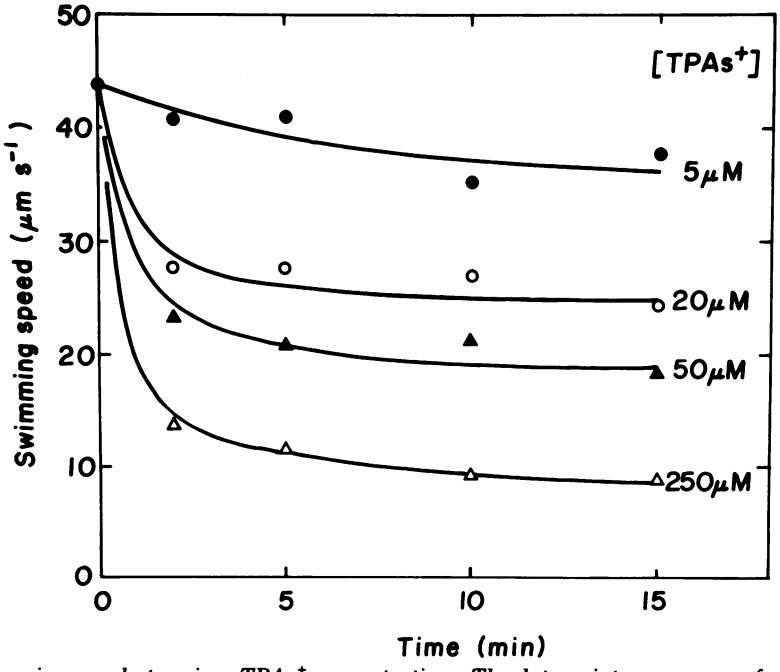


FIG. 2. *Swimming speed at various $TPAs^+$ concentrations. The data points are means of at least 20 cells. The speed at zero concentration was measured on cells swimming smoothly in response to an attractant (alanine, at 10 mM).*

TABLE 1. *Comparison of the effects of lipophilic cations on B. subtilis*

Effect	Concn required (μM)		
	$TPAs^+$	TPP^+	$TPMP^+$
Stimulation of uptake[a]	<3[b]	1	20
Growth inhibition[c]	1	1	20
Macromolecular synthesis[d]	3.2	NT[e]	NT
Tumble suppression	2	2	30
Swimming speed reduction (threshold)	<5	NT	NT
Swimming speed reduction (50%)	35	20	125

[a] The threshold concentration of unlabeled cation for enhancement of uptake of the same radiolabeled cation; based on results in Zaritsky et al., in press.

[b] Testing of lower concentrations was not possible because of low specific activity.

[c] A 50% reduction in growth rate.

[d] K_i for glycerol incorporation into macromolecular material.

[e] NT, Not tested.

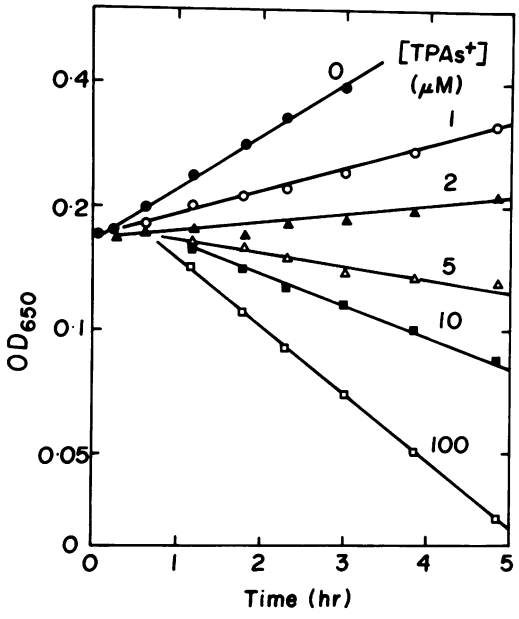


FIG. 3. *OD_{650} of cells in glycerol-ammonia medium at 25°C, after addition (at time zero) of $TPAs^+$ at various concentrations.*

μM, optical density dropped (after a lag period), at least in part because of clumping of cells. TPP^+ and $TPMP^+$ (the latter at a much higher concentration) also arrested growth (Table 1).

The extent of incorporation of [^{14}C]glycerol into macromolecules (defined as trichloroacetic acid-insoluble material), relative to the extent of incorporation in an untreated sample, was reduced by $TPAs^+$, reaching 50% of its final value within 10 min (Fig. 4). The relative extent of incorporation at steady state (2.5 h), as a function of $TPAs^+$ concentration, yielded an inhibition constant of 3.2 μM (estimated from a double-reciprocal plot, not shown).

Bioenergetic effects of $TPAs^+$. When $TPAs^+$ was added at 5 μM (a concentration sufficient to completely suppress tumbling; see above) to a suspension of *B. subtilis* cells, a substantial enhancement of diS-C_3(5) fluorescence was observed. Fluorescence enhancement still occurred in the presence of valinomycin, provided the external potassium concentration was low enough to prevent collapse of the membrane potential by potassium diffusion. We conclude that the $TPAs^+$-induced fluorescence enhancement does not indicate depolarization (Zaritsky et al., in press; see below).

Lipophilic cations such as $TPMP^+$ and TPP^+, chemically rather similar to $TPAs^+$, have been used extensively in radiolabeled form for the measurement of membrane potential. We used [^{3}H]$TPMP^+$ for such measurements, at a concentration (5 μM) considerably lower than is needed for tumble suppression and speed reduction (Table 1). When non-radioactive $TPAs^+$ was added at 5 μM, there was a dramatic increase in the extent of $TPMP^+$ uptake, apparently indicating hyperpolarization. However, since a similar stimulation of $TPMP^+$ uptake occurred in double-label experiments with $^{86}Rb^+$ (in the presence of valinomycin at 5 mM potassium), without any change in the extent of $^{86}Rb^+$ uptake (Fig. 5), we conclude that the stimulated

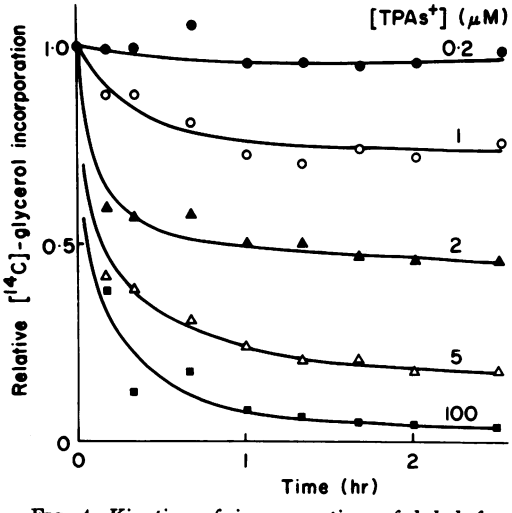


FIG. 4. *Kinetics of incorporation of label from [^{14}C]glycerol into trichloroacetic acid-insoluble material, after addition of $TPAs^+$ at the indicated concentrations. Data are expressed relative to the incorporation in an untreated culture.*

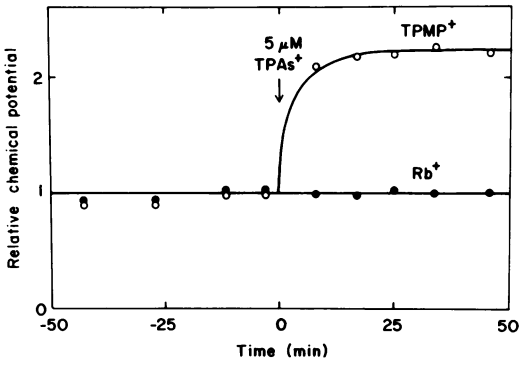


FIG. 5. *Effect of $TPAs^+$ (5 μM) on the uptake of [^{3}H]$TPMP^+$ and $^{86}Rb^+$, in the presence of valinomycin at 5 mM K^+. Uptake is expressed as chemical potential relative to the value immediately before $TPAs^+$ addition, i.e., as (log $conc_{in}/conc_{out})_{after}$/(log $conc_{in}/conc_{out})_{before}$, where conc = concentration.*

uptake of $TPMP^+$ by $TPAs^+$ was not caused by hyperpolarization.

The fact that $^{86}Rb^+$ uptake was not perturbed by addition of 5 μM $TPAs^+$ could have been due to the potential-clamping action of potassium in the presence of valinomycin. Does $TPAs^+$ at this concentration affect membrane potential when valinomycin is absent? This is an important question because these lipophilic cations are used to measure potential. Indirect evidence suggests there may be a slight hyperpolarization: upon addition of 5 μM $TPAs^+$, ATP levels (Fig. 6) increased from 0.8 mM to a final value of 1.5 mM 20 min later, by which time the rate of macromolecular synthesis was severely reduced (Fig. 4). Respiration was only slightly affected under these conditions; 10 μM $TPAs^+$ caused an 11% decrease in rate. By 25 μM, inhibition of respiration was considerable (44%). With a major ATP-consuming process (macromolecular synthesis) inhibited by $TPAs^+$ (Fig. 4), the elevation of ATP pools is evidence that ATP synthesis can still occur. This implies that the membrane-bound ATPase, the principal ATP-producing device under respiratory conditions, is not inhibited by $TPAs^+$. The elevated ATP levels might therefore cause some degree of hyperpolarization.

A second line of evidence involves effects on ΔpH. At pH 5.5, where membrane (electrical) potential (Δψ) is small, and therefore PMF consists almost entirely of the ΔpH component (11, 44), $TPAs^+$ at 100 μM caused a 10% increase in ΔpH (data not shown), suggesting that the energetic state of the cell had been raised.

We attempted to test for hyperpolarization by a "motility rescue" assay. CCCP was added at a concentration (2.5 μM) sufficient to cause a substantial (>50%) impairment of motility. The subsequent addition of 5 μM $TPAs^+$ resulted in full restoration of motility which, however, included tumbling. This result, although providing strong evidence for a restoration of PMF, does not necessarily indicate that $TPAs^+$ per se exerts a hyperpolarizing effect. Being a lipophilic cation, $TPAs^+$ could be forming a neutral complex with CCCP (a lipophilic anion) and thus inactivating it as a proton ionophore; the fact that tumbling was not suppressed by $TPAs^+$ if CCCP was present supports this interpretation.

Motility and membrane potential. Valinomycin did not prevent either the suppression of tumbling or the reduction in swimming speed by $TPAs^+$. Thus, the motility effects are not dependent on any change in membrane potential.

At higher concentrations (>20 μM), $TPAs^+$ caused a gradual depolarization whether valinomycin was absent (depolarization judged by [^{3}H]$TPMP^+$ release) or present (depolarization judged by both [^{3}H]$TPMP^+$ and $^{86}Rb^+$ release). The rate and extent of this depolarization, however, was far too small to explain the rapid deceleration of cells by $TPAs^+$. Even in the absence of valinomycin, swimming speed had dropped almost 50% by 2 min after addition of 50 μM $TPAs^+$ (Fig. 7). The presence of valinomycin at 5 mM K^+ made the deceleration even more rapid (50% after 40 s; data not shown); yet even after 20 min under these conditions, the $^{86}Rb^+$ potential was still at 90% of the prestimulus value (Fig. 7).

DISCUSSION

The three lipophilic cations that we examined ($TPAs^+$, $TPMP^+$, and TPP^+) caused a number

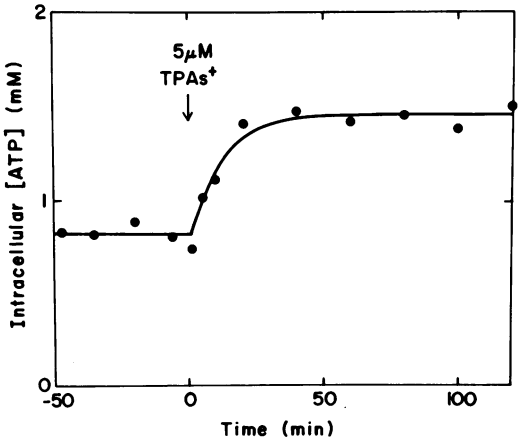


FIG. 6. *Effect of $TPAs^+$ (5 μM) on intracellular ATP concentration.*

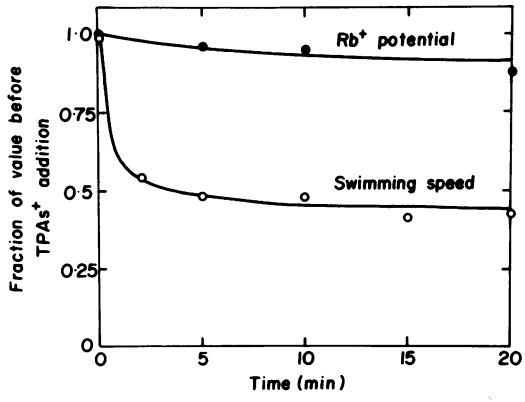


FIG. 7. *Comparison of the effects of TPAs$^+$ (50 μM) on swimming speed and $^{86}Rb^+$ uptake (in the presence of valinomycin at 5 mM K^+), relative to the values before TPAs$^+$ addition. Relative chemical potential as in the legend to Fig. 5.*

of major physiological and behavioral changes in *B. subtilis*.

They also affected two commonly used measures of membrane potential, cyanine dye fluorescence and uptake of [^{3}H]TPMP$^+$. We conclude that neither the enhanced dye fluorescence nor the enhanced uptake of TPMP$^+$ induced by TPAs$^+$ indicates a change in membrane potential. A detailed treatment of the measurement of membrane potential in *B. subtilis*, along with our reasons for rejecting the usual interpretations of the changes in dye fluorescence and TPMP$^+$ uptake, is given elsewhere (Zaritsky et al., in press). Briefly, we conclude that lipophilic cations displace the cyanine dye from a quenching environment, probably the cell envelope, to a nonquenching environment, either the external medium or the cytoplasm. We also suggest that, below a critical concentration, potential-dependent uptake of TPMP$^+$ and related cations occurs only at the cell surface and that, above the critical concentration, potential-dependent uptake occurs into a previously unavailable compartment, which is probably the cytoplasm.

We have confirmed and extended the observations of Ordal (35) and de Jong and van der Drift (4) that tumbling is suppressed and motor speed reduced by lipophilic cations. These effects are seen even in the presence of valinomycin, with the extent of $^{86}Rb^+$ uptake remaining constant, leading to the conclusion that motility changes do not result from a change in membrane potential. This conclusion was reached also by de Jong and van der Drift (4) on the basis of their failure to detect any change in cyanine dye fluorescence upon TPAs$^+$ addition (at the high dye/cell ratio they used, the enhancement artifact is much smaller [Zaritsky et al., in press]).

Although the effects of lipophilic cations on motility are not a consequence of reduction of PMF, they resemble PMF-mediated effects in that reduction in motor speed is accompanied by suppression of tumbling. Previous work has shown that motor speed in *B. subtilis* reaches a limiting value as PMF is increased, and that only below the saturating PMF is tumbling suppressed (11, 44). This suggests that the mechanism of tumble suppression involves the actual protons being used to rotate the motor. Behavioral changes described here are consistent with this notion since TPAs$^+$ simultaneously reduced swimming speed and suppressed tumbling.

The homogeneity of a cell population treated with TPAs$^+$ was striking, particularly since *B. subtilis* cells, if partially deenergized, are broadly distributed from completely immotile to vigorously motile. The heterogeneous motility of partially deenergized cells presumably occurs because cells are in the presaturation range, in which speed is highly dependent upon the magnitude of the PMF (11, 44), which is likely to vary somewhat from cell to cell. However, where the mean PMF of a population is sufficiently high that most cells are above the saturation level, the speed distribution should be rather narrow. For example, in a population of *Salmonella* cells, Macnab and Koshland (Table 1 of reference 25) measured a standard deviation in swimming speed of only 18%. The relative homogeneity of TPAs$^+$-treated *B. subtilis* cells can be explained in the same way if the reduction in speed is a result of inhibition of the use of PMF when the latter is not rate limiting. The homogeneity of TPAs$^+$-treated cells therefore reinforces the conclusion that speed reduction is not a consequence of reduction in PMF.

Lipophilic cations may alter the state of the cell surface (Zaritsky et al., in press). Could this be associated with increased frictional resistance to motor rotation? The fact that tumble suppression and speed reduction occurred concomitantly suggests otherwise, because motor switching probabilities were previously found to be unaffected when speed was reduced by increasing the frictional load (10). Also, the reversal of the effects upon TPAs$^+$ removal argues against a major breakdown of surface architecture. It should be made clear that we are referring here to "external friction," whether between the cell and the external medium or between the motor and its bearings, and not to any activation energy barrier within the actual machinery for transducing proton energy into mechanical work. TPAs$^+$ may very well be affecting a constraint of the latter sort.

Both calcium and magnesium have been reported to regulate tumbling in *B. subtilis* (35, 36). We found that, whether or not calcium or magnesium was present, cells had a normal incidence of tumbling but could be converted to smooth swimming by lipophilic cations.

The effects of lipophilic cations were not confined to motility. In the same concentration range, growth was inhibited (Fig. 3 and 4), the ATP pool was elevated (Fig. 6), and properties of the cell were altered in such a way as to stimulate lipophilic cation uptake (Fig. 5; Zaritsky et al., in press). Indirect evidence suggests that membrane potential may have been slightly increased, provided valinomycin was not present to enable potassium to buffer potential. Respiration rates were not significantly affected. Thus, neither electron transport nor proton translocation coupled to it were inhibited by lipophilic cations; likewise, the elevation of ATP levels in respiring cells indicates that the membrane-bound ATPase was still functioning.

Our results demonstrate that measurement of membrane potential in *B. subtilis* by lipophilic cations is being made on cells in an altered physiological state, since typical concentrations used for membrane potential measurements are above the threshold for the various physiological effects we observed.

Although lipophilic cations affected motility and other processes at quite low concentrations (<5 μM), this does not necessarily indicate high-affinity binding sites because, as a result of the membrane potential (ca. 120 mV) (44; Zaritsky et al., in press), the cations concentrate in the cytoplasm and also the cell surface (Zaritsky et al., in press). The relevant concentration could therefore be >500 μM under these conditions, if the site(s) of action were in the cytoplasm or at the point of lowest electrical potential in the cell surface. The observation (4) that higher external concentrations of $TPAs^+$ are required at lower pH can then readily be explained by the marked decrease in the membrane potential of *B. subtilis* occurring under these conditions (11, 44).

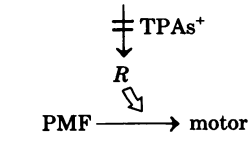
At moderate concentrations, lipophilic cations did not reduce PMF (the energy source for motor rotation) (Fig. 5), and even at high concentrations, the drop in PMF was very slow compared with the behavioral effects (Fig. 7). The cations could therefore be inhibiting the motor directly, by blocking proton entry or inhibiting the energy transduction mechanism:

$$\text{PMF} \xrightarrow{\text{TPAs}^+} \text{motor}$$

Alternatively, they could be inhibiting some process necessary for the operation of the motor but physically remote from it, e.g., the generation of an intermediate energy form X such as an ionic potential or a high-energy compound:

$$\text{PMF} \xrightarrow{\text{TPAs}^+} X \rightarrow \text{motor}$$

It is also possible that the cations are affecting an enabling molecule or process R that constitutes an enabling switch for the motor:



However, the existence of such a molecule or process R seems unlikely, since there is no evidence for a physiological "on-off" control; in energized, uninhibited cells, the motors run incessantly.

Lipophilic cations did not inhibit motility or suppress tumbling in *E. coli* cells (treated with EDTA to increase permeability to the cations), nor did they show anomalous uptake characteristics into *E. coli* cells or vesicles (unpublished data; H. R. Kaback, personal communication). This suggests that the mechanism of inhibition may be specifically related to the properties of the gram-positive cell surface. In vivo identification of binding sites that might be responsible for motility inhibition is likely to be difficult, because of the extent of potential-dependent uptake at the cell surface (Zaritsky et al., in press). Measurement of binding to isolated putative targets, such as the basal body or the *mot* proteins, seems a more promising approach, in spite of the high dissociation constants (0.1 to 1 mM) that are anticipated.

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