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Transmembrane Electrical Potential in *Rickettsia prowazekii* and Its Relationship to Lysine Transport

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The transmembrane electrical potential ($\Delta\Psi$) generated by *Rickettsia prowazekii* metabolizing glutamic acid or ATP was determined by flow dialysis with the lipophilic cation tetraphenylphosphonium and with lysine. At pH 7.0, the rickettsiae generated a $\Delta\Psi$ as measured by tetraphenylphosphonium distribution of 90 mV. Under similar conditions, cells of *R. prowazekii* concentrated lysine to a gradient indicating a $\Delta\Psi$ of 90 mV. Energy-starved cells of *R. prowazekii* were able to utilize exogenously supplied ATP as well as glutamic acid to generate a $\Delta\Psi$ of 110 mV at pH 8.0. Lysine transport was markedly affected by environmental pH, the optimum pH ranging from 8.0 to 8.5. $\Delta\Psi$ as measured with tetraphenylphosphonium was similarly affected in this system, with values ranging from 70 mV at pH 6.0 to 100 mV at pH 8.0. Respiration rates were also affected by the external pH, with a maximum rate of 28 nmol of O_2 consumed per min per mg of rickettsial protein occurring at pH 8.0. The pH effects were readily reversible and with a rapid onset.

The rickettsiae are gram-negative bacteria that require the cytoplasm of a eucaryotic host cell as an environment for multiplying. Although many investigations have attempted to identify a specific metabolic lesion in these bacteria that accounts for their dependence on the host cell, no such lesion has been detected. However, these studies have yielded much information concerning the metabolic capabilities of the rickettsiae (21, 22). Pertinent to the subject of this paper are the observations that rickettsiae synthesize ATP upon the oxidation of glutamate via the tricarboxylic acid cycle by a mechanism sensitive to uncouplers (oxidative phosphorylation) (2, 26, 27, 33) and actively transport at least one amino acid, lysine (18). These processes have been shown (5–7, 9, 13, 24, 28) in other systems to be dependent on both an intact cytoplasmic membrane and a transmembrane proton electrochemical gradient, the protonmotive force (PMF). The fact that both ATP synthesis and lysine transport are inhibited by protonophores and by high concentrations of the lipophilic cation triphenylmethylphosphonium strongly supports the hypothesis that rickettsiae can generate and maintain a PMF (2, 18, 27, 33). Although the integrity of the rickettsial cytoplasmic membrane has been questioned, it appears that this component of the rickettsial cell does provide a barrier to certain compounds, is not generally permeable to small molecules, and contains specific carriers for the transport of some substrates (18, 30, 31). It is not known, however, whether the rickettsial cytoplasmic membrane is permeable to protons or whether a PMF exists in these bacteria. Therefore, we have conducted studies to address this problem.

As defined by Mitchell (11), the PMF is composed of a chemical and an electrical component as described by the equation $PMF = \Delta\tilde{\mu}_{H^+} = \Delta\Psi - (2.3RT/F)\,\Delta pH$. In this report we present data that describe the limiting value of the transmembrane electrical potential ($\Delta\Psi$), one of the components of $\Delta\tilde{\mu}_{H^+}$, in *Rickettsia prowazekii*. The relationship between $\Delta\Psi$ and lysine transport is discussed. A future communication will deal with the proton permeability of the rickettsial cytoplasmic membrane and the magnitude of the total PMF that these bacteria generate.

MATERIALS AND METHODS

Growth and purification of rickettsiae. *R. prowazekii*, Madrid E strain, was cultivated and purified as previously described (31) with the addition of a filtration step through AP-25 filters (Millipore Corp., Bedford, Mass.) (27). When necessary, the rickettsiae were further purified by equilibrium density gradient centrifugation through a linear gradient of 25 to 45% Renografin-76 (E. R. Squibb & Sons, Princeton, N.J.) in a manner similar to that described by Weiss et al. (23). After purification, the rickettsiae were washed twice in 0.218 M sucrose–10 mM potassium phosphate–10 mM glutamic acid (SPG) at the desired pH

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and were suspended in the same buffer. In the buffer containing 10 mM $MgCl_2$, 10 mM morpholinepropanesulfonic acid (MOPS) replaced potassium phosphate (SMG-Mg). The replacement of MOPS for potassium phosphate did not adversely affect the rickettsiae and eliminated the formation of a magnesium phosphate precipitate which formed in SPG containing 10 mM $MgCl_2$ at an alkaline pH.

Transport and $\Delta\Psi$ assays. Lysine transport was assayed at 34°C by previously described filtration methods (18) or, if tetraphenylphosphonium (TPP^+) was involved, at 37°C by flow dialysis (3). The latter method was used for TPP^+ because TPP^+ readily permeates biological membranes, so the filtration method was inappropriate because of a large loss during washing. Flow dialysis was conducted in a controlled atmosphere chamber aerated with 1 atm (101.31 kPa) water-saturated O_2. In flow dialysis experiments, O_2-saturated buffer was pumped through the lower chamber (lower chamber volume, 200 μl) at a rate of 2 ml/min unless stated otherwise. Rickettsiae (20 to 50 mg of protein per ml; total volume, 100 to 150 μl) were added to the upper chamber, which was separated from the lower chamber with a dialysis membrane (exclusion limit, molecular weight of 12,000) that had been previously treated by boiling in 0.5 M EDTA for 1 h and then rinsed with glass-distilled water. Both the upper and lower chambers were stirred with small magnetic stirrers. After allowing 5 min for temperature equilibration, L-[U-^{14}C]lysine (final concentration, 5 μM; 2 μCi/ml) or [*phenyl*-^{3}H]TPP^+Br^- (final concentration, 21.2 μM; 5 μCi/ml) was added to the upper chamber. Fractions of 1 ml of the eluant were collected and assayed for radioactivity. Additions, as indicated, were added directly to the upper chamber as concentrated stock solutions. Corrections for any changes in the volume in the upper chamber owing to these additions were made in the subsequent calculations.

Determination of intracellular water space. Intracellular water was determined essentially by previously described methods (1). Briefly, rickettsiae (approximately 8 mg/ml) were incubated in SPG or SMG-Mg containing ^3H_2O (12.5 μCi/ml) and [^{14}C]sucrose (10 μCi/ml) at room temperature. Samples of 55 μl of this mixture were centrifuged (4°C, 10 min, 10,000 rpm; Beckman Instruments, Inc. [Fullerton, Calif.] Microfuge B) through a layer of dibutyl phthalate (density, 1.0416) into 25 μl of 17% perchloric acid contained in a 250-μl Microfuge tube. After freezing, the tube was cut just above the perchloric acid-dibutyl phthalate interface. The portion of the tube containing the perchloric acid and the pelleted rickettsiae was placed in a vial. Scintillation cocktail was added, and the radioactivity was determined. The total water volume pelleted with the rickettsial cells was calculated from the tritium counts in the perchloric acid layer. Extracellular water was determined by calculating the volume attributable to associated water containing sucrose. The difference of the total water minus the extracellular water was defined as the intracellular space since the rickettsial cytoplasmic membrane is not permeable to sucrose (30). An average value of 3.2 ± 0.06 μl of intracellular water per mg of protein was obtained by this method, and this value was used in the calculations described below.

Preparation of energy-depleted rickettsiae. Purified rickettsiae were washed twice in 20 ml of 0.218 M sucrose–10 mM potassium phosphate (pH 8.0) (SP) at 4°C and were suspended in the same buffer at a density of 19 mg of protein per ml. ADP was added to this preparation at a final concentration of 5 mM to exchange with intracellular ATP (31). After incubation of this suspension at 34°C for 10 min, the rickettsial cells were pelleted by centrifugation at 9,000 × *g* for 10 min, washed twice in 20 ml of SP, and suspended in SP at a final protein concentration of about 20 mg/ml. Rickettsiae prepared this way displayed no hemolytic activity unless an energy source such as glutamic acid was supplied. Reenergization of energy-depleted rickettsiae with glutamic acid resuled in hemolytic activity comparable with that observed with control unstarved cells.

Calculations. The internal concentrations of lysine and TPP^+ were calculated from the difference between the total and extracellular concentrations of the compounds as determined by flow dialysis according to the equation $C_i = (C_tV_t - C_eV_e)/V_i$, in which C_i, C_e, and C_t represent the internal (cytoplasmic), external, and total concentrations of compound, respectively, and V_i, V_e, and V_t similarly designate the internal (cytoplasmic), external, and total volumes, respectively (3). $\Delta\Psi$ was calculated according to the Nernst equation: $\Delta\Psi = (2.3RT/F)\log([\text{cation}]_{in}/[\text{cation}]_{out})$. The calculated value based on the concentration ratios of TPP^+ was taken as the true value of $\Delta\Psi$ since this ion is distributed according to the membrane potential.

Other assays. Protein was determined by a modification (10) of the Lowry method (8) after trichloroacetic acid precipitation of the samples with bovine serum albumin ($A^{1\%}_{1cm}$ at 280 nm, 6.6) as a standard. Oxygen consumption was determined at 34°C with a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio).

Chemicals and radioisotopically labelled compounds. L-[*U*-^{14}C]lysine, ^3H_2O, and [*phenyl*-^{3}H]TPP^+Br^- were purchased from New England Nuclear Corp., Boston, Mass. [^{3}H]TPP^+Br^- was also obtained as a gift from H. R. Kaback. [*U*-^{14}C]sucrose was obtained from ICN Chemical and Radioisotope Division, Irvine, Calif. All other chemicals were the highest available purity obtainable from commercial sources.

RESULTS

Effects of external pH on lysine transport and oxygen uptake. Since the magnitude of a PMF has been shown to vary as a function of pH in other systems (4, 14, 16), we determined the effect of pH on lysine transport in rickettsiae, an easily monitored parameter. The pH of the buffer dramatically affected lysine transport in *R. prowazekii* (Fig. 1). Accumulation of lysine was optimum at pH 8.0 and was virtually absent at pH 5.0. Previously published evidence (18) indicates that lysine transport is dependent, in part, on rickettsial respiratory activity. Therefore, it was of interest to determine the effect of pH on respiration. When oxygen consumption by *R. prowazekii* was monitored at various pH values, it was observed that this variable also affected the respiration rate in these bacteria. Maximal

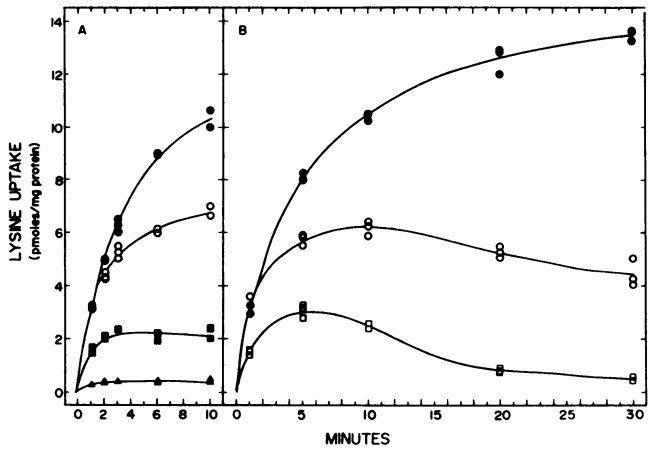

FIG. 1. Effects of pH on lysine uptake by *R. prowazekii*. After purified rickettsiae were incubated for 5 min at 34°C in SMG-Mg at the appropriate pH, L-[*U*-^{14}C]lysine (5 μM, 0.2 μCi/ml) was added (time zero), and uptake of the substrate was monitored for 10 min (A) or 30 min (B) by filtration as described in the text. Symbols represent internalized lysine at pH values of 5.0 (▲), 6.0 (■), 7.0 (○), 8.0 (●), and 9.0 (□).

oxygen consumption occurred at pH 8.0 at a rate of about 28 nnmol of O_2 consumed per min per mg of rickettsial protein.

Correlation between ΔΨ and lysine uptake in *R. prowazekii*. There is strong evidence that lysine is transported in other bacterial systems by a mechanism dependent on ΔΨ (6, 13). It is likely that lysine transport in rickettsiae is also dependent on ΔΨ (18). To test this hypothesis, ΔΨ was measured in *R. prowazekii* with the lipophilic cation TPP^+. The data demonstrate that TPP^+ was accumulated by the rickettsiae (Fig. 2). The addition of the protonophore 2,4-dinitrophenol (DNP) resulted in the release of the accumulated TPP^+ from the rickettsial cells into the surrounding medium. In these experiments, the uptake of lysine was simultaneously monitored, and the data reveal that the rickettsiae interact with the two compounds in a similar manner. Both lysine and TPP^+ were accumulated by the rickettsiae by a mechanism sensitive to DNP (and KCN; data not shown). The time that was necessary for lysine to reach a new steady state after the addition of DNP was longer than that observed for TPP^+. These results may be due to the differences between the net flux by a carrier-mediated transport system (lysine) and by one independent of a carrier (TPP^+). Both compounds were accumulated to a greater extent at pH 8.0 than at pH 7.0. At pH 8.0, TPP^+ was concentrated 49-fold, whereas at pH 7.0 a concentration gradient of 30-fold was achieved. These values reflect ΔΨ values of 103 and 90 mV, respectively. ΔΨ calculated from the steady-state distribution of lysine yielded values of 113 mV at pH 8.0 and 91 mV at pH 7.0. The close correlation between the membrane potential calculated from the steady-state distribution

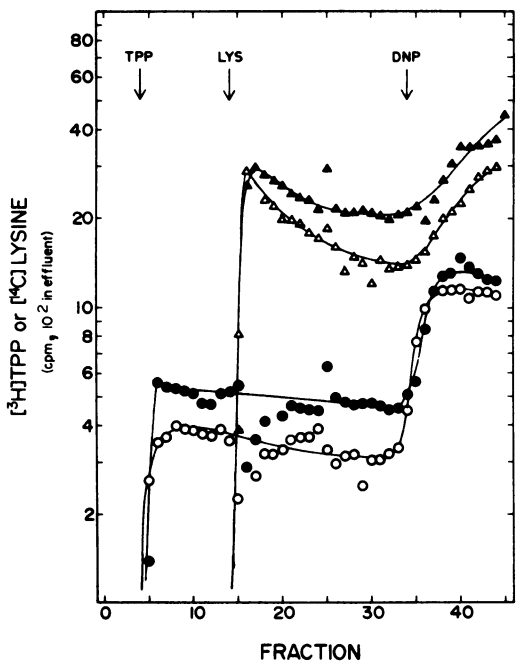

FIG. 2. Flow dialysis analysis of the interaction between *R. prowazekii* and lysine (△,▲) or TPP^+ (○,●) at pH 7.0 (▲,●) and pH 8.0 (△,○). Rickettsiae (150 μl, 20 mg of protein per ml in SPG) were contained in the top chamber of the flow cell (see text). Additions were made at the arrows as follows: [^{3}H]TPP^+, 15 μM, 22 μCi/ml; [^{14}C]lysine (LYS), 5 μM, 2 μCi/ml; and DNP, 1 mM. Values for C_e used to calculate C_i were those observed at fraction 30. C_t values for TPP^+ were taken at fraction 43. Since lysine did not reach a new steady-state value in the time allotted after the addition of DNP, C_t values were determined separately as 9,000 cpm/ml of effluent, and this value was used in the calculations.

TABLE 1. Membrane potential of *R. prowazekii* measured by TPP^+ or lysine distribution as a function of pH

Probe	Expt	Δψ (mV)		
		pH 6	pH 7	pH 8
Lysine	1	76	82	91
	2		81	
	3	50		83
	4	48		78
	5		79	
	6		91	113
TPP^+	1	72	89	106
	6		90	103
	7	61	92	99
	8	69		100
	9		110	
	10		91	106
	11		90	99
	12	73	114	121

of lysine and that calculated from the steady-state distribution of TPP^+ indicates that ΔΨ provides the energy for the active transport of lysine in *R. prowazekii*.

The values of ΔΨ obtained with a number of different preparations of rickettsiae are presented in Table 1. It is clear that the membrane potential generated by *R. prowazekii* increased as the pH of the suspending buffer was increased from pH 6.0 to 8.0. Consistent with other results (Fig. 1), lysine transport was also increased as evidenced by the larger ΔΨ values obtained with this probe as the pH was raised. Also consistent with results presented earlier (Fig. 2), there is a close correlation between the value of ΔΨ obtained by using TPP^+ as a probe and that obtained by using lysine as a probe.

The effect of pH on ΔΨ as evidenced by the steady-state distribution of both TPP^+ (Fig. 3) and lysine (data not shown) was reversible. Cells at an initial pH of 7.8 generated a membrane potential of about 121 mV. When the external pH was quickly changed to pH 5.25, much of the accumulated TPP^+ diffused from the rickettsiae within a very short period of time. Readjusting the external pH to 7.3 resulted in a reaccumulation of TPP^+ by the rickettsiae, indicating a ΔΨ of 114 mV. Many variations of this experiment have been performed with both lysine and TPP^+ as probes, and they all suggest that (i) abrupt environmental pH changes ranging from pH 5.0 to 8.5 appear to do no lasting damage to the ability of rickettsial cells to generate a membrane potential; and (ii) ΔΨ changes rapidly in response to pH changes (apparently faster than the resolution time allowed by the flow-dialysis system employed).

ΔΨ generated by *R. prowazekii* supplied with exogenous ATP. Rickettsiae are unusual in that exogenous ATP can generate the energy required for the active transport of lysine and hemolysis in KCN-poisoned organisms (18, 29) via the ATP transport system and *N,N'*-dicyclohexylcarbodiimide-sensitive ATPase (31, 33). It had been assumed, but not demonstrated, that ATP was restoring the PMF of these poisoned cells. Metabolic poisons may cause nonspecific irreversible effects as well as incomplete inhibition, which would make interpretation of the effects of ATP difficult. Therefore, we evaluated the ability of nonpoisoned rickettsiae, depleted of energy reserves in a complete but reversible manner, to generate a membrane potential by

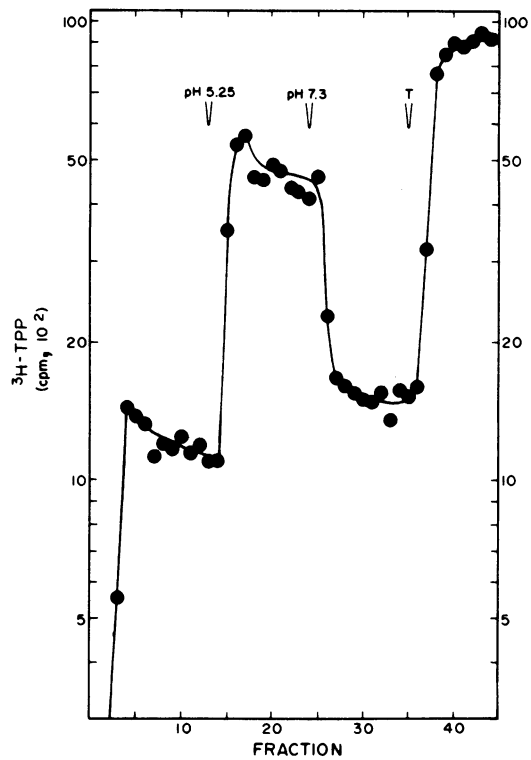

FIG. 3. Effects of rapid pH changes on ΔΨ generated by *R. prowazekii*. Flow dialysis was performed as described in the text. The upper chamber of the flow cell contained 100 μl of rickettsiae (28 mg of protein per ml). [^{3}H]TPP was added at fraction 2. Changes in pH marked on the figure were accomplished by adding 4.8 μl of 0.25 N HCl to the upper chamber at fraction 13 and 4.8 μl of 0.125 M Tris at fraction 24. The rickettsial cells were lysed at fraction 35 (T) by adding 5 μl of 10% Triton X-100 to the upper chamber. Values for C_e used to calculate C_i were those observed at fractions 10 (pH 7.8), 20 (pH 5.25), and 30 (pH 7.3). The value observed at fraction 43 was used for C_t.

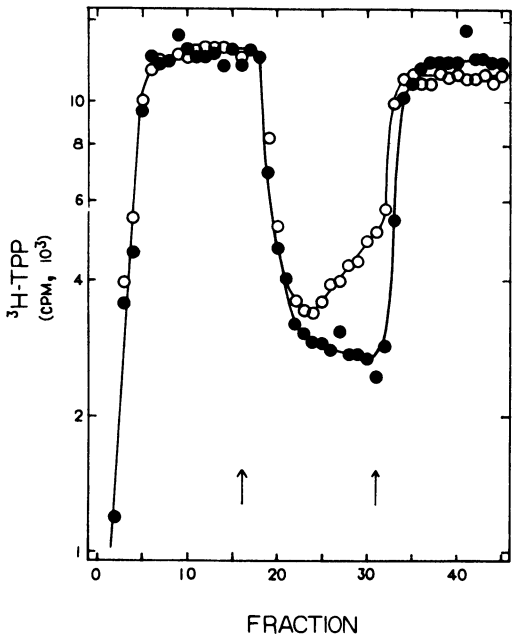

FIG. 4. Generation of ΔΨ by *R. prowazekii* metabolizing ATP or glutamate. Flow dialysis was performed as described in the text. The upper chamber of the flow cell contained 100 μl of energy-depleted rickettsiae (19 mg of protein per ml). [^{3}H]TPP was added at fraction 2. Energization was accomplished by adding either 10 μl of 100 mM ATP (○; final concentration, 8.7 mM) or 10 μl of 100 mM potassium glutamate (●; final concentration, 8.7 mM) at fraction 16 (first arrow). DNP was added at fraction 31 (second arrow). Values for C_e used to calculate C_i were those observed at fractions 12 (before addition of energy source), 24 (after addition of ATP), and 30 (after addition of glutamate). The values observed at fraction 40 were used for C_t.

utilizing exogenous ATP and glutamate. With no exogenous energy source, energy-depleted rickettsiae were unable to concentrate TPP^+, indicating an absence of a membrane potential in these cells (Fig. 4). However, upon addition of either ATP or glutamate to the upper chamber, the probe was rapidly accumulated by the cells. With glutamate as an energy source, a membrane potential of about 110 mV was generated. The steady-state value of the membrane potential generated by hydrolysis of ATP was difficult to determine. Shortly after the addition of ATP to the upper chamber, the TPP^+ was accumulated in a manner similar to that observed when glutamate served as the energy source, achieving a maximum ΔΨ of 108 mV. However, TPP^+ started to diffuse from the rickettsial cells after a short period of time and before a stable steady state was achieved. These results were caused by the rapid utilization of ATP by the high concentration of rickettsiae in the upper chamber since subsequent additions of more ATP to the rickettsial suspension resulted in a reconcentration of TPP^+ which again was retained for a short period of time (data not shown).

DISCUSSION

The definition of the bioenergetic capabilities of the rickettsiae and other obligate intracellular parasites is crucial for understanding why such organisms require host cells as well as for evaluating the magnitude of the energy demand placed on the host cells by these invaders. The number of energy demands imposed by growth and multiplication of the parasite that are met by the metabolic capabilities of the parasite varies with the specific organism. The information available suggests a broad spectrum of bioenergetic interplay between host and intracellular parasite ranging from energy parasites, (bacteria unable to generate their own high energy compounds) to bacteria and protozoa which can apparently meet their own energy needs.

The rickettsiae may not require host-cell-derived high energy compounds to multiply. It has been established (2, 26, 27) that these bacteria possess the metabolic capability through oxidative phosphorylation to generate their own ATP, the main currency of energy in biological systems. However, these bacteria have also evolved ways to optimize the use of the energy-rich environment of the host cell cytoplasm, for example, by developing a transport system for host-generated ATP (31). Other phenomena, such as hemolysis and lysine transport (15, 18, 29), can be energized either by rickettsial electron transport and oxidative phosphorylation or by ATP procured from the environment via the ATP transport system. It is one of our interests to determine whether energy coupling in rickettsiae occurs by the mechanisms elucidated in free-living bacteria or whether different modes of energy transduction and the regulation have evolved because of the particular evolutionary pressures imposed on these bacteria. Accordingly, we have been evaluating the role of rickettsia-derived energy in a number of systems: infection (32), hemolysis (15, 29), lysine transport (18), and ATP synthesis (33). The lysine transport system characterized by Smith and Winkler (18) provides a workable system for manipulating to gain insight into energy coupling in rickettsiae. By the use of the membrane potential probe TPP^+, we have acquired evidence that strongly supports the hypothesis that lysine is transported in *R. prowazekii* by the ΔΨ. This finding is in accordance with the scheme proposed by Niven et al. (13) and Hamilton and Niven (6) that carriers for basic amino acids such as lysine function in response to ΔΨ.

A more general, but very important, point that emerges from this study relates to the fact that a significant membrane potential does indeed exist in the rickettsiae. This observation indicates that the rickettsial membrane cannot be highly permeable to ions in general. A membrane completely permeable to ions such as K^+ or Na^+, as suggested by Myers et al. (12) for extensively purified rickettsiae, would result in the short-circuiting of $\Delta\Psi$ under the conditions we have used in this study (10 mM K^+). This situation would be analogous to that observed with valinomycin-treated *Escherichia coli* in K^+-containing media in which the permeability of the ion allowed by the ionophore resulted in the dissolution of $\Delta\Psi$ (14).

A subtle attribute of *R. prowazekii* cells noticed during these studies is that it was unnecessary to treat the rickettsiae with EDTA to use TPP^+ as a probe for $\Delta\Psi$, as is usually necessary with *E. coli*. This indicates a difference between the two gram-negative bacteria in the structure of their outer membrane. The rickettsial outer membrane has been isolated and examined by Smith and Winkler (19), and differences in the major outer membrane proteins of the two organisms have been demonstrated.

We were able to address the question whether rickettsiae generate a membrane potential when ATP is present as the sole energy source by using energy-depleted *R. prowazekii*. No membrane potential was detected in these cells unless an energy source was added. The addition of ATP resulted in a rapid generation of $\Delta\Psi$ of at least 108 mV. An energy-transducing ATPase has been defined in *R. prowazekii* (33), and it is most likely that it is via this enzyme that these cells generate a $\Delta\Psi$ after transporting exogenously supplied ATP in the cytoplasm.

It is crucial to point out that the values of $\Delta\Psi$ presented here are minimum values. This is due to the fact that the quantity is calculated relative to intracellular water. The preparations of rickettsiae that we obtained unavoidably contained a number of dead and damaged rickettsial cells. Such rickettsial cells would not be expected to have a $\Delta\Psi$ but might contribute to the intracellular water space used in the calculation. The viability varies from preparation to preparation, ranging from about 40 up to 100% (20). This means that the absolute value of $\Delta\Psi$ in these bacteria may be as much as two times the values presented. However, rickettsial viability is usually correlated with the ability of a rickettsial cell either to infect a host cell (25) or to hemolyze an erythrocyte (20). It is possible that an uninfectious or nonhemolytic rickettsial cell could have generated a membrane potential. Thus, we have not corrected for viability in our calculations. In fact, for experiments 9 and 12 (Table 1), the membrane potentials calculated on the basis of TPP^+ distribution at a given pH are quite consistent, suggesting that the preparations used in this study were similar in energy generation.

Although the $\Delta\Psi$ has been observed to vary slightly in *E. coli* as a function of pH, the respiration rate in whole cells of *E. coli* is relatively constant from pH 5.0 to pH 8.5 (14). On the other hand, Repaske and Adler (17) have reported that the membrane potential in *E. coli* is affected by abrupt changes in the external pH. Rapid changes to more alkaline conditions resulted in an increased $\Delta\Psi$ in their experiments. Those authors did not assess the effects that these pH changes had on respiration. We observed that the membrane potential in *R. prowazekii* varied as a function of external pH whether the cells were in contact with a buffer of a given pH for an extended or a very brief period of time (Fig. 2 and 3). The dramatic effect that pH had on respiration, lysine uptake, and $\Delta\Psi$ indicates that this variable should be given serious consideration in evaluating energy-dependent processes in rickettsiae. The sensitivity of these processes in *R. prowazekii* to environmental pH may reflect a lack of development of or a loss of mechanism(s) needed to survive changes in environmental H^+ concentration. Since these bacteria exist primarily in the highly buffered cytoplasm and vascular system of the host, an environment of relatively constant pH, these mechanisms may not be necessary for rickettsiae. It should be mentioned that the hemolytic activity of *R. prowazekii* is also sensitive to the buffer pH but is severely inhibited only at a pH of 5.0 or less (L. Gray and H. H. Winkler, unpublished observations).

We have not attempted to identify the primary process that is affected by pH in *R. prowazekii*. We are determining the intracellular pH, the transmembrane proton gradient, and the permeability of the cytoplasmic membrane to protons in these bacteria. The results of those experiments should provide useful information regarding the sensitivity of energy-coupled processes to pH in rickettsiae.

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