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Changes in Membrane Potential of *Escherichia coli* in Response to Temporal Gradients of Chemicals†

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ABSTRACT: Changes in membrane potential of *Escherichia coli* in response to addition of chemoattractants have been studied by several groups, but their observations and conclusions disagree [e.g., Szmelcman and Adler [Szmelcman, S., & Adler, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4387–4391] and Snyder et al. [Snyder, M. A., Stock, J. B., & Koshland, D. E., Jr. (1981) *J. Mol. Biol. 149*, 241–257]]. This study was undertaken to resolve the differences in these reports. The discrepancies were probably the consequence of differences in the energy level of the bacteria, caused by differences in the availability of oxygen. In the presence of oxygen the relatively small changes in membrane potential that may be correlated with chemotaxis could be masked and compensated for by the changes in membrane potential caused by respiration and the related electrogenic transport processes. In the present study the contribution of the respiratory and related systems was reduced by using electron transport inhibitors such as KCN or amytal or 2-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO). Membrane potential was then monitored by tetraphenylphosphonium distribution in response to the addition of a chemoeffector stimulus. Addition of the chemoattractant galactose caused an increase in the membrane potential only if respiration was inhibited. This hyperpolarization was not caused by ATP hydrolysis since *N*,*N*′-dicyclohexylcarbodiimide (DCCD), an ATPase inhibitor, did not prevent it but rather increased it. The inhibitors used did not abolish the motility or the chemotactic response of the bacteria. Nine other attractants, metabolizable and nonmetabolizable chemicals, were tested under these conditions. All caused hyperpolarization, independent of the receptor identity or the chemotaxis focusing system with which they interact. The significance of these results and of earlier works in light of the present observations is discussed. This study neither proves nor disproves the possible correlation between the observed hyperpolarization and the process of chemotaxis. It describes the conditions that enable the detection of these changes in membrane potential.

Motile bacteria swim toward certain chemicals (attractants) and away from others (repellents), a phenomenon called chemotaxis. In the absence of a stimulating chemical, the motion of bacteria such as *Escherichia coli* is composed of runs in a straight line interrupted by brief tumbles. In an increasing gradient of attractant or a decreasing gradient of repellent, the cells tumble less frequently, with a resultant aggregation toward the attractant or away from the repellent [for recent reviews, see Adler et al. (1979), Koshland (1980a), and Macnab (1980)]. The machinery of bacterial chemotaxis can, in principle, be thought of as the bacterial version of a nervous system. In analogy to eucaryotic nerve cells and protozoa where sensory transduction is mediated by an action potential (Aidley, 1971; Kung et al., 1975), studies have been carried out to determine if membrane potential is involved in the signaling process of bacterial chemotaxis. These studies were of two types: (a) studies of the behavior of bacteria at different levels of membrane potential and (b) measurements of changes in membrane potential in response to addition of chemoattractants.

The investigations in the first category yielded conclusive results: the level of membrane potential affected the behavior of bacteria (de Jong et al., 1976; Manson et al., 1977; Miller & Koshland, 1977, 1980; Khan & Macnab, 1980; Goulbourne & Greenberg, 1981) and consequently a distinct membrane potential signaling protein was postulated (Laszlo & Taylor, 1981).

For the investigations in the second category, the results were in apparent disagreement (Szmelcman & Adler, 1976; Miller & Koshland, 1977; Armitage & Evans, 1979, 1981; Snyder et al., 1981). Even in a single species, *E. coli*, conflicting results were obtained: Szmelcman & Adler (1976) detected changes in membrane potential in response to attractants, but Snyder et al. (1981) and Armitage & Evans (1981) did not find such changes. Snyder et al. reported that a few attractants did cause changes in membrane potential, but these changes were not related to chemotactic sensing. On the other hand, in all of these studies, it was found that some repellents, such as acetate, lead to permanent hyperpolarization[1] in *E. coli* (Szmelcman & Adler, 1976; Snyder et al.,

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1981; Armitage & Evans, 1981). This hyperpolarization was later attributed to the decrease in internal pH caused by the uptake of acetate rather than to chemotaxis (Repaske & Adler, 1981; Kihara & Macnab, 1981).

Indeed, membrane potential per se is not likely to be the signaler for chemotaxis, because so many other processes are affected by membrane potential. However, it is important to determine conclusively whether or not attractants cause changes in membrane potential. Such changes are the reflection of ion fluxes across the cytoplasmic membrane which could well be involved in specific signaling.

Two questions to which conclusive answers have not been given are (1) does binding of chemoattractants to their receptors ultimately result in membrane potential changes and (2) if positive, are these changes in membrane potential correlated to chemotaxis? The purpose of this paper is to answer the first question.

Experimental Procedures

Chemicals. [^{3}H]TPP$^+$Br$^-$ (2.5 Ci/mmol) was a gift from Dr. H. Ronald Kaback (Roche Institute of Molecular Biology, New Jersey). Nonradioactive TPP$^+$Br$^-$ was synthesized according to Dodonow & Medox (1928), yielding 99.96% purity (chemical analysis performed by Spang Microanalytical Laboratory, Michigan) with a sharp melting point at 295.0–295.5 °C. PVC was a gift from B. F. Goodrich Co. Dioctyl phthalate and maltose were purchased from Baker, and L-serine was from ICN. Essentially glucose-free D-galactose, D-fucose, and TPB$^-$Na$^+$ were obtained from Sigma. Maltose was purified by Dr. Arye Tishbee (The Weizmann Institute of Science), using high-performance liquid chromatography with a Lichrosorb-NH_2 (10 μm) 4.6 × 250 mm column, refraction index detector, and mobile phase of 25% (v/v) acetonitryl in water. The purity of the sugars was assayed with paper chromatography (Adler, 1969; Putman, 1957). Purified D-fucose (Adler, 1969) was received as a gift from Dr. Julius Adler (University of Wisconsin at Madison). DCCD was from Fluka, and FCCP was from Boehringer Mannheim. All the rest of the chemicals were of the highest purity commercially available.

Bacteria. The strains used in this study, AW546 (Ordal & Adler, 1974) and RP487 (Toews & Adler, 1979), were obtained from Dr. Julius Adler. They are *E. coli* K-12 derivatives, wild type for chemotaxis.

Preparation of Bacteria. Unless otherwise mentioned, all strains were precultured to the stationary phase (overnight growth) at 35 °C in tryptone broth (Difco) and then diluted 1:50 with H-1 minimal medium of Kaiser & Hogness (1960) containing D-galactose (30 mM) and the required amino acids (1 mM). The bacteria were grown for 6–8 h until they gained vigorous motility (OD_{590} = 0.4–0.8) and then harvested and permeabilized by the Tris–EDTA technique. The procedure of Leive (1965), as modified by Szmelcman & Adler (1976), was used to permeabilize the bacterial cell wall. The efficiency of the permeabilization was determined for each preparation by assaying the loss of motility caused by addition of Triton X-100 (0.1% final concentration). Nonpermeable cells are not affected by Triton X-100 at this concentration. Only batches in which at least 99.9% of the population was permeabilized were used. After permeabilization the bacteria were brought to a concentration of 0.6 mg of protein/mL (unless otherwise specified) in suspending medium [KP_i (10 mM), $MgSO_4$ (5 mM), EDTA (0.1 mM), and L-methionine (0.1 mM) at a final pH of 7.0] and kept on ice until used. The cells remained permeabilized and functional for at least 5 h, if kept on ice in the absence of added energy source.

Heat-treated bacteria, used as a control for TPP$^+$ adsorption, were prepared according to Muratsugu et al. (1979) by incubating permeabilized bacteria at 60 °C for 30 min.

Measurement of Membrane Potential. Since bacteria are too small for the direct measurement of membrane potential by insertion of electrodes, indirect techniques must be used for this purpose. Tetraphenylphosphonium ion (TPP$^+$) is a permeant cation that passes through the cytoplasmic membrane and equilibrates according to the membrane potential. It was shown by Kaback and collaborators, for giant *E. coli* cells (Felle et al., 1980) and for eucaryotic cells (Lichtshtein et al., 1979), that the values of membrane potential obtained from [^{3}H]TPP$^+$ distribution across the membrane are equal to those obtained by direct measurement with microelectrodes. TPP$^+$ was, therefore, used as a probe for membrane potential with two techniques: (a) the filtration technique in which the distribution of TPP$^+$ is determined by the amount of [^{3}H]TPP$^+$ accumulated inside the bacterial cells and (b) continuous measurement with a TPP$^+$-specific electrode, which measures the free TPP$^+$ outside the bacterial cell. The results were comparable with both techniques. In the calculation of membrane potential, the value of 4.4 μL/mg of protein was used for the intracellular water space of the cells (Eisenbach & Adler, 1981).

The filtration technique was used according to Schuldiner & Kaback (1975). The TPP$^+$ electrode was constructed according to Kamo et al. (1979) with slight modifications: the calomel reference electrode was indirectly connected to the measuring vessel through an agar–salt bridge, and a glass tube was used for the electrode to which the PVC membrane was attached by an O ring. A calibration curve for the electrode (the electrode potential vs. the logarithm of TPP$^+$ concentration) at 30 °C yielded a straight line in the region from 10^{-2} to 5 × 10^{-6} M with a slope of −59.7 ± 0.4 mV, as expected from the Nernst equation for this temperature. A TPP$^+$ concentration of 5 μM was found to be most appropriate for the measurements. Although the slope of the curve gradually declined at TPP$^+$ concentrations below 5 μM, the relationship between the electrode potential and TPP$^+$ concentration was linear to the first approximation within the narrow concentration range of a single experiment. Every single experiment was, therefore, calibrated separately for the TPP$^+$ concentration. When not in use, the electrode was immersed in 10 mM TPP$^+$Br$^-$ solution. Under these conditions, a small drift in ΔE was observed at a constant rate of 0.3 mV/h. Changing the electrode immersion solution from 10 mM to 5 μM TPP$^+$ (the concentration used in the measurements) caused an enhanced drift, the rate of which decreased with time for a period of 10 h and only then stabilized on a constant-rate drift of approximately ΔE = 0.6 mV/h. Therefore, before an experiment, the electrode was immersed overnight in the measuring solution [KP_i (10 mM), $MgSO_4$ (5 mM), EDTA (0.1 mM), L-methionine (0.1 mM), and TPP$^+$Br$^-$ (5 μM); pH 7.0]. In agreement with Muratsugu et al. (1979), including

[1] Abbreviations: AIB, α-aminoisobutyrate; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; HOQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; MCP, methyl-accepting chemotaxis protein; α-MeAsp, α-methyl-DL-aspartate; PTS, phosphotransferase system; PVC, poly(vinyl chloride); TPB$^-$, tetraphenylboron; TPP$^+$, tetraphenylphosphonium; ΔE, change in electrode potential; $\Delta\psi$, membrane potential; $\Delta\tilde{\mu}_{H^+}$, proton electrochemical potential (in millivolts). Both $\Delta\psi$ and $\Delta\tilde{\mu}_{H^+}$ are defined to be positive for an outwardly directed potential gradient (inside is positive). Hyperpolarization means, in this study, an increase in the absolute value of $\Delta\psi$ or that $\Delta\psi$ becomes more negative.

0.3 μM TPB^-Na^+ in the reaction mixture did not affect the observations and was not, therefore, used. All measurements were carried out at 30 °C. Membrane potential values from these measurements were calculated according to Muratsugu et al. (1977, 1979) or Grinyus et al. (1980). A correction based on the nonspecific binding of TPP^+ to the cells at $\Delta\psi = 0$ was applied to the TPP^+ accumulation (see, however, the comment in the text concerning membrane potential calculations for Figure 4). Zero membrane potential was accomplished by either one of the following means (with comparable results): heat treatment, anoxia [cf. Skulachev (1980) and Kashket (1981)], or addition of FCCP or gramicidin.

Chemotaxis Assays. Capillary assays, measuring the accumulation of bacteria in attractant-filled capillaries, were performed as described by Adler (1973). Washed cells (or permeabilized cells) were diluted to $OD_{590} = 0.005$ for the capillary assay, unless otherwise noted. Each assay was incubated for 30 min at 30 °C. Chemotaxis was also assayed on tryptone swarm plates and galactose minimal swarm plates (Adler, 1966) or by temporal assays (Macnab & Koshland, 1972) in a phase-contrast microscope carried out as described (Goy et al., 1978).

Results

Different strains of *E. coli* have different properties regarding the use of TPP^+ as a probe for membrane potential: *E. coli* strain B derivatives do not require permeabilization for TPP^+ distribution (Muratsugu et al., 1979), but the K-12 strain AN180 does (Grinyus et al., 1980). For determination of this requirement for the *E. coli* strains used in this study, $[^3H]TPP^+$ uptake was measured with permeabilized and nonpermeabilized cells. Figure 1 shows results in the absence of an externally added energy source, and similar results (with faster kinetics) were observed in the presence of glycerol, which had been the sole energy and carbon source for growth and which had been found to give a much faster rate of respiration than D- or DL-lactate. As shown in Figure 1, only permeabilized cells accumulated $[^3H]TPP^+$. Within 1–2 min essentially full equilibration of TPP^+ was accomplished. The counts found with untreated cells were the result of adsorption (or absorption by the membranes), since the same level of TPP^+ was observed with (a) permeabilized cells in the presence of valinomycin (10 μM) and KCl (0.33 M), under which conditions $\Delta\psi = 0$ (Padan et al., 1976; valinomycin also required permeabilization to be effective), (b) both permeabilized and nonpermeabilized cells in the presence of the uncoupler FCCP (20 μM), and (c) heat-treated cells in the absence of added reagents. Interestingly, the amount of TPP^+ adsorbed to the cells was similar in permeabilized and nonpermeabilized bacteria (2.0 ± 0.2 pmol of TPP^+/mg of protein). This suggests that the adsorption of TPP^+ at zero membrane potential is mainly to the outer membrane [cf. Zaritsky et al. (1981)]. Thus, the *E. coli* K-12 derivatives used in this study do require permeabilization for TPP^+ distribution.

Permeabilization of the cell wall decreased the speed of swimming of the bacteria but did not affect their chemotactic properties. This was concluded on the basis of (a) microscopic observations (temporal assays) with similar response times of both permeabilized and untreated bacteria to the attractant, AIB (see below), and (b) macroscopic measurements (capillary assays), comparing the attraction to L-aspartate (cytoplasmic membrane receptor) and D-fucose (periplasmic receptor) with permeabilized and nonpermeabilized cells.

In published reports, measurements of membrane potential in *E. coli* in response to attractants were carried out in the presence of an energy source for respiration (Szmelcman &

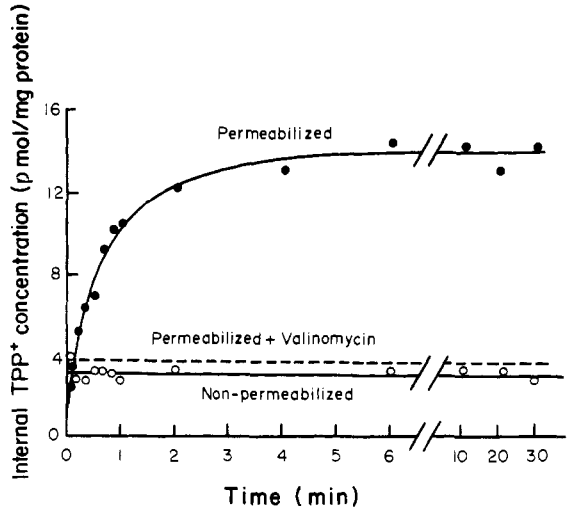

FIGURE 1: TPP^+ uptake by permeabilized and nonpermeabilized *E. coli* cells. Strain RP487, preadapted to growth in Vogel–Bonner minimal medium (Vogel & Bonner, 1956) containing glycerol (50 mM) and 1 mM of each of the required amino acids (L-methionine, L-histidine, L-leucine, L-threonine), was grown fresh in the same medium to $OD_{590} = 0.5$. Half the cells were permeabilized as described under Experimental Procedures, and the second half were washed and resuspended in the same suspending medium as the permeabilized cells: KP_i (10 mM), $MgSO_4$ (5 mM), EDTA (0.1 mM), and L-methionine (0.1 mM) (final pH 7.0). The concentration of the permeabilized (●) and nonpermeabilized (○) cells was 0.106 ± 0.010 and 0.123 ± 0.004 mg of protein/mL, respectively. The dashed line was obtained with permeabilized cells after the addition of KCl (0.33 M final) and valinomycin (10 μM final). The specific activity of the $[^3H]TPP^+$ was 10.6 ± 1.1 cpm/fmol of TPP^+. The final concentration of $[^3H]TPP^+$ in the suspension was 70 nM. Measurement was by the filtration technique described under Experimental Procedures. The value of membrane potential (corrected for TPP^+ adsorption at $\Delta\psi = 0$), calculated from the figure for permeabilized cells, was −111 mV.

Adler, 1976; Snyder et al., 1981). This procedure probably reflected an assumption that energized cells should respond better to stimuli and show larger signals. However, respiration is the major process that forms membrane potential in *E. coli* (Harold, 1977). It may, therefore, mask the attractant-stimulated changes in membrane potential, if the latter changes are relatively small. If respiration is inhibited, these changes in membrane potential may become detectable. We studied the effect of inhibited respiration on the changes in membrane potential stimulated by an attractant. The stimulus chosen was D-galactose, a powerful attractant of *E. coli* (Adler, 1969; Adler et al., 1973) that had been used also by Szmelcman & Adler (1976) and Snyder et al. (1981) in their measurements of membrane potential.

KCN is an efficient respiration inhibitor (Pudek & Bragg, 1974) as shown in Figure 2. Addition of D-galactose to starved cells initiated respiration, which was then inhibited by addition of KCN. Inhibition of respiration by KCN caused a decrease in the membrane potential as shown in Figure 3 (lower trace). (The small transient "hyperpolarization" observed after the addition of KCN was due to dilution, as verified by addition of an identical volume of water to these cells or by addition of KCN to heat-treated bacteria.) In Figure 4 the effect of increasing concentrations of KCN on the membrane potential is shown. The changes in membrane potential in Figure 4 are represented by the amounts of TPP^+ released to the medium, and in Figures 3 and 5 by the electrode potential. Calculated values of membrane potential are not given in these figures because of the uncertainty as to how to correct for the adsorption of TPP^+ to the bacterial membrane (Zaritsky et al.,

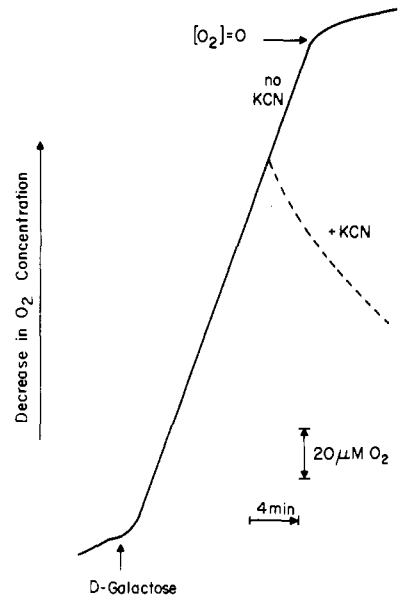

FIGURE 2: Oxygen uptake by *E. coli* cells. Oxygen uptake was measured with an oxygen electrode in a 1-mL (0.6 mg of protein) suspension of permeabilized AW546 cells at 30 °C. The suspending medium was as detailed under Experimental Procedures. Uptake of oxygen was initiated by addition of D-galactose (0.6 mM final concentration). The dashed line was obtained after addition of KCN (5 mM final concentration).

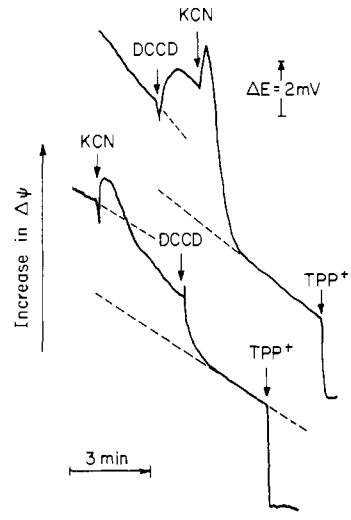

FIGURE 3: Effect of DCCD and KCN on the membrane potential of *E. coli*. The membrane potential was monitored by the TPP^+ electrode as described under Experimental Procedures. The strain used was AW546. Permeabilized bacteria were resuspended in the measuring solution to a concentration of 0.60 mg of protein/mL, and from this suspension, 1.5-mL portions were taken for measurements. The TPP^+ concentration was 5 μM. DCCD and KCN were added when indicated at final concentrations of 20 μM and 5 mM, respectively. At the end of each experiment, TPP^+ (3 μL of 1 mM solution) was added for calibration. The dashed lines are extensions of the base lines.

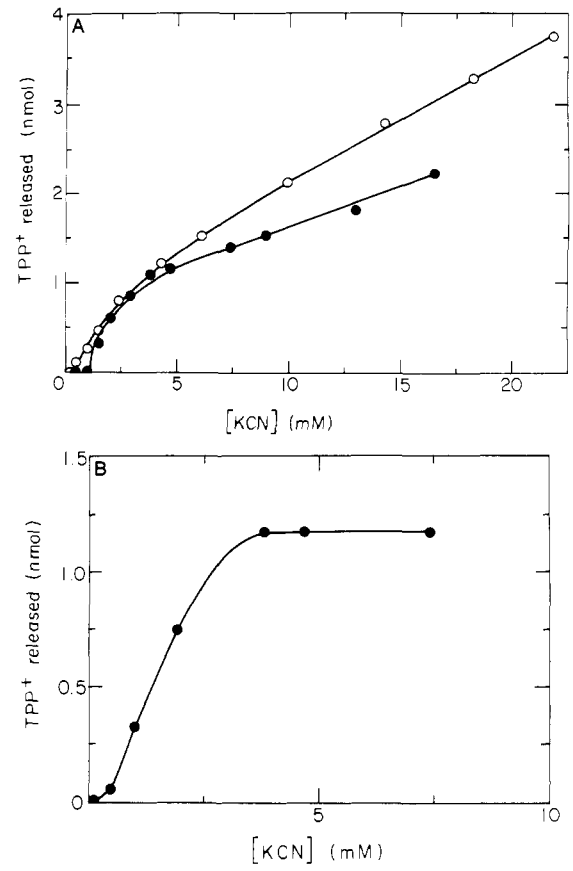

FIGURE 4: Effect of KCN on membrane potential. The change in membrane potential is represented by the amount of TPP^+ released to the medium (see the text). Changes in membrane potential were monitored by the TPP^+ electrode as described under Experimental Procedures. The TPP^+ concentration was 5 μM. The strain used was AW546. Permeabilized bacteria were resuspended in the measuring solution to a concentration of 0.60 mg of protein/mL, and from this suspension, 1.5-mL portions were taken for measurements. Neutralized KCN (0.1 M) was added to the suspension to give the indicated final concentration. (A) KCN was added either to a suspension of cells energized with glycerol (○) or to a suspension of cells without glycerol (●). In either case there was no difference whether the bacteria had been grown on glycerol or on galactose as the sole carbon and energy source. (B) KCN was added to a suspension containing DCCD (20 μM final concentration). At the end of the experiment 3 μL of TPP^+ (1 mM before addition) was added for calibration.

1981; Bakker, 1982; H. Rottenberg, personal communication). It seems that KCN did not abolish the membrane potential even at a concentration that maximally inhibits respiration (5 mM). By correction of the amount of TPP^+ taken up for adsorption at zero membrane potential, the apparent values of membrane potential were −120 and −106 mV (inside negative) for the uninhibited and 5 mM KCN inhibited states, respectively. In the presence of DCCD [an ATPase inhibitor of *E. coli* (Evans, 1970)] the maximal effect of KCN on membrane potential was accomplished already at 3 mM KCN (Figure 4B). In no case did the membrane potential approach zero. This could be the consequence of incomplete inhibition of respiration by the inhibitors (see below) and (but less likely) of electron transport in the fumarate reductase system (Boonstra et al., 1978).

The changes in membrane potential stimulated by galactose are shown in Figure 5. In the absence of KCN, galactose caused mainly a decrease in membrane potential (lower trace), as had been previously observed by Snyder et al. (1981). This slow depolarization was probably the consequence of galactose uptake and the coupled proton uptake (Henderson & Kornberg, 1975; Van Thienen et al., 1977). However, in the presence of KCN, galactose triggered a hyperpolarization (middle trace). A similar hyperpolarization was observed also in the presence of either one of two other respiration inhibitors that were tested, HOQNO (Kaback & Barnes, 1971) and amytal (Chance & Hollunger, 1963; Ernster et al., 1963; Kaback & Barnes, 1971) (not shown). Therefore, the inhibition of respiration, not the inhibitors per se, enables detection of the hyperpolarization. Indeed, recent measurements of membrane potential under anaerobic conditions detected a similar hyperpolarization stimulated by galactose (J. Pierce

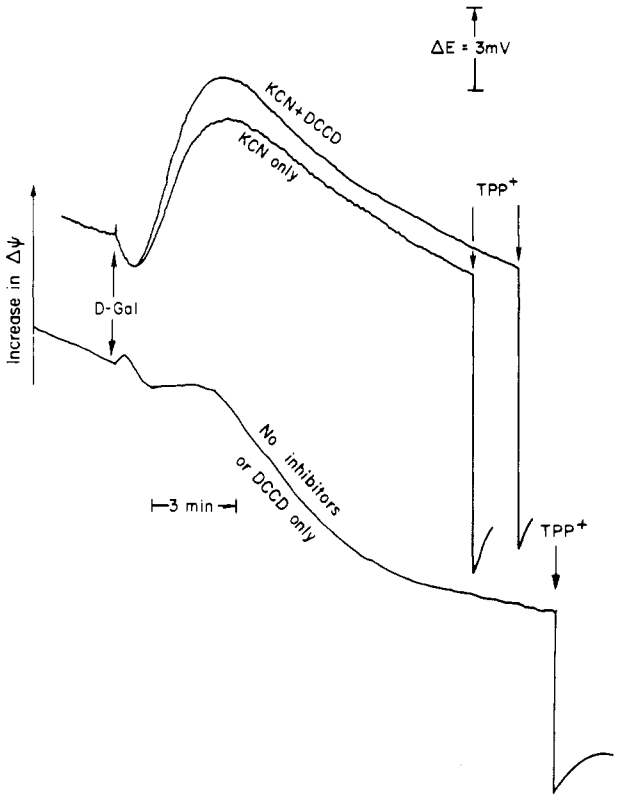

FIGURE 5: Effect of inhibitors on changes in membrane potential stimulated by galactose. Changes in membrane potential were monitored by the TPP^+ electrode as described under Experimental Procedures. The TPP^+ concentration was 5 μM. The strain used was AW546. Permeabilized bacteria were resuspended in the measuring solution to a concentration of 0.60 mg of protein/mL, and from this suspension, 1.5-mL portions were taken for measurements. To one portion of cells, DCCD (20 μM final concentration) and KCN (5 mM final concentration) were added sequentially, and to the others only DCCD or KCN (or no inhibitors) was added. After steady state had been reached, D-galactose (1.1 mM final concentration) was added. At the end of each experiment, TPP^+ (5 μL of 1 mM solution) was added for calibration.

and J. Adler, personal communication). Amytal (3 mM) was only 93% effective in inhibiting oxygen uptake but HOQNO (15 μM) was as effective as 5 mM KCN (cf. Figure 2). Since hyperpolarization was observed even when amytal was used, it seems that complete inhibition of respiration is not necessary for observing the hyperpolarization.

ATPase activity may influence the measured changes in membrane potential; e.g., these changes could have been the reflection of the ATP level of the cells (hydrolysis of ATP via the ATPase system would cause hyperpolarization). To uncouple between the ATP level and the membrane potential, we used DCCD. This inhibitor was effective under our conditions, as is evident from Figure 3: its addition caused hyperpolarization (upper trace), unless it was added after KCN when it caused further depolarization (lower trace). Figure 5 shows that the presence of DCCD, as sole inhibitor in the suspension, did not affect the decrease in the membrane potential stimulated by galactose (lower trace), probably because DCCD could not prevent the galactose:H^+ uptake (Henderson & Kornberg, 1975; Van Thienen et al., 1977). However, in the presence of both KCN and DCCD, galactose caused a larger hyperpolarization than in the presence of KCN alone (upper traces), perhaps because no membrane potential was consumed for phosphorylation in the presence of DCCD. The hyperpolarization by galactose is not, therefore, the reflection of the ATP level in the cells.

The inhibitors used did not abolish the motility or the chemotactic response of the bacteria, based on the following observations. (a) Cells were motile in the presence of any one of the inhibitors for as long as 4.5 h. If, however, a respiration inhibitor and DCCD were present together, a reduction in the swimming speed was observed. The effect of KCN addition was tumbling for 1–2 min [in agreement with similar observations with *Salmonella typhimurium* (Taylor et al., 1979) and *Bacillus subtilis* (Ordal & Goldman, 1976)] and then relaxation into a lower tumbling frequency, which was still higher than the normal unstimulated frequency in the absence of KCN.[2] [Khan & Macnab (1980) found that the effect of KCN on *Salmonella* SL4041 was a decrease in the unstimulated tumbling frequency.] These results agree with the observations that the use of inhibitors does not inhibit respiration as efficiently as oxygen deprivation does: it was reported that anoxia led to complete loss of motility in *S. typhimurium* (Taylor et al., 1979; Khan & Macnab, 1980), *B. subtilis* (Khan & Macnab, 1980), and *E. coli* (Khan & Macnab, 1980; Skulachev, 1980). In this study, however, preventing perturbations of the measurement by respiration are enough, and paralysis of the cells is avoided. (b) Bacterial cells in the presence of KCN (5–10 mM) or amytal (3 mM) or either of these inhibitors with DCCD (100 μM) responded to 100 mM AIB, a nonmetabolizable attractant, in a temporal assay qualitatively the same as did bacteria in the absence of inhibitors: 10–12 min of smooth swimming. This was tested with several bacterial strains, and the results were always the same, whether or not the cells had first been permeabilized. (c) Capillary assays with D-galactose as an attractant gave peak responses at the same galactose concentration with or without KCN (5 mM; both in the capillary and in the pond). The number of bacteria accumulated in the capillary was, however, smaller in the presence of KCN. This was also observed in the L-aspartate control. Attraction into the capillary was observed even with permeabilized cells in the presence of KCN.

D-Galactose was not unique in causing hyperpolarization in the presence of inhibitors. We examined all the attractants that had been tested in previous works (Szmelcman & Adler, 1976; Snyder et al., 1981) and some additional ones. The results are summarized in Table I. All the attractants, metabolizable or not, caused hyperpolarization, indicating that metabolism of the attractant was not the cause of the hyperpolarization. [Maltose, an extremely potent attractant (Koman et al., 1979), caused such a high hyperpolarization that it could be detected even in the absence of inhibitors. The change in the membrane potential was then −14 mV.] No correlation of the magnitude of the responses with the location of the receptors in the cell or with the identity of the focusing system with which they interact was observed.

Discussion

The results presented here demonstrate that under appropriate experimental conditions hyperpolarization by a che-

[2] One phenomenon was always observed when a drop of bacterial suspension already containing KCN was put on the microscope slide (regardless of the period of prior incubation with KCN): the bacteria were nonmotile for 3–6 min and then resumed motility. Although the reason for this phenomenon is not known, it cannot be the result of HCN evaporation from the suspension: when the cells on the slide had resumed their motility, additional KCN (10 mM final) was added to the drop, and the motility remained as vigorous as before. The same phenomenon was observed with amytal (3 mM) but not with HOQNO (15 μM). With the latter inhibitor, the cells were motile from the first moment on the slide.

Table I: Effect of Chemoattractants on *E. coli* Membrane Potential[a]

attractant	concn (M)	receptor location[b]	chemotaxis focusing system[c]	metabolizable[d]	change in $\Delta\psi$ (±SD)[e] (mV)
L-serine	5×10^{-6}	membrane	*tsr* product (MCPI)	+	-5 ± 1
L-serine	5×10^{-4}				-23 ± 2[f]
AIB	1×10^{-2}	membrane	*tsr* product (MCPI)	−	-5 ± 1[f]
L-aspartate	1×10^{-2}	membrane	*tar* product (MCPII)	+	-22 ± 2[g]
α-MeAsp	1×10^{-2}	membrane	*tar* product (MCPII)	−	-24 ± 3[g]
maltose	1×10^{-5}	periplasm	*tar* product (MCPII)	+	-35 ± 4[h,g]
D-ribose	1×10^{-4}	periplasm	*trg* product (MCPIII)	+	-16[i,g]
L-arabinose	1×10^{-4}	periplasm	*trg* product (MCPIII)	+	-19[i,g]
D-galactose	1×10^{-5}	periplasm	*trg* product (MCPIII)	+	-14 ± 2[g]
D-fucose[j]	5×10^{-3}	periplasm	*trg* product (MCPIII)	−	-17[i,g]
D-glucose[j]	1×10^{-5}	membrane and periplasm[k]	PTS system and *trg* product (MCPIII)	+	-12 ± 3[g]

[a] Changes in membrane potential were monitored by the TPP^+ electrode as described under Experimental Procedures. The strain used was AW546, unless otherwise indicated. The sole carbon and energy source for growth was the attractant used unless otherwise mentioned and except for experiments with amino acids as attractants, where glycerol (50 mM) was the carbon and energy source. Permeabilized bacteria were resuspended in the measuring solution to a concentration of 0.50 mg of protein/mL and 1.5-mL portions were taken for measurements. Amytal (2 mM) and DCCD (20 μM) were added at least 10 min before the addition of the attractant. This combination of inhibitors was chosen to allow very slow respiration (amytal) but yet to prevent interferences of the ATPase system (DCCD). This slow respiration due to amytal was advantageous because it finally caused anoxia, allowing easy correction for the nonspecific TPP^+ binding (see Experimental Procedures). Thus, in each experiment the membrane potential was followed until oxygen was totally depleted, followed by TPP^+ addition (3 μL of 1 mM solution) for calibration. [b] According to Mesibov & Adler (1972), Adler et al. (1973), and Koshland (1980b). "Membrane" relates to the cytoplasmic membrane. [c] According to Springer et al. (1979). [d] According to Adler et al. (1973). The plus sign means metabolizable attractant. [e] The minus sign means hyperpolarization. The values given were corrected for the dilution by the attractant (using heat-treated bacteria) and for the nonspecific binding of TPP^+ at $\Delta\psi = 0$, accomplished by anoxia at the end of each experiment. [f] At higher concentrations of attractant the hyperpolarization was followed by depolarization. [g] This hyperpolarization was transient. [h] The value given is for the strain RP487 in the presence of KCN, for which many results have been accumulated. Similar results were obtained for AW546 and for amytal plus DCCD replacing KCN. [i] Based on one determination. [j] The bacteria were grown on minimal medium with D-galactose as the sole carbon and energy source. [k] The periplasmic receptor for glucose is the galactose-binding protein (Zukin et al., 1977).

moattractant is detectable. Under these conditions major processes that cause changes in membrane potential uncorrelated with chemotaxis are at least partially inhibited. Such processes are respiration and uptake of galactose via the galactose–permease system (Rotman et al., 1968). [In this transport system galactose is taken up in a symport mechanism with protons (Henderson & Kornberg, 1975; Van Thienen et al., 1977).] Respiration keeps the membrane potential at a high level, and the electrogenic uptake of galactose may cause partial depolarization. Indeed, galactose added to respiring *E. coli* cells caused slow and continuous depolarization [Figure 5 and Snyder et al. (1981)]. A respiration inhibitor such as KCN also inhibits the $\Delta\tilde{\mu}_{H^+}$-driven uptake of galactose via the galactose permease system (Kerwar et al., 1972; M. Eisenbach, A. Ciobotariu, and T. Raz, unpublished experiments). Thus, on the one hand the inhibitor prevents the partial depolarization caused by galactose uptake, and on the other hand, due to the inhibited respiration, the level of the membrane potential is kept low enough (but unexpectedly still close to normal) to allow detection of the hyperpolarization. This close-to-normal magnitude of membrane potential allows normal motility but is of the correct magnitude to be susceptible to perturbations. Inhibiting processes that utilize membrane potential should further increase the observed hyperpolarization by galactose. An example of this is the increased hyperpolarization observed in the presence of the ATPase inhibitor, DCCD, in Figure 5.

TPP^+ is a probe for measurements of membrane potential (Lichtshtein et al., 1979; Felle et al., 1980; Grinyus et al., 1980). It is generally accepted, however, that a change in the surface potential can be reflected as a change in the membrane potential, measured in the bulk solutions. Is it possible that the galactose-stimulated hyperpolarization is the reflection of a change in the surface potential? [Cf. Armitage & Evans (1981).] This is not likely because such a change would be very short-lived unless supported by a massive ion flow. The potential gradient across the membrane would be quickly compensated for or balanced by ions moving from one side of the membrane to another. For the high and lengthy hyperpolarization seen in this study, a massive flow of ions across the membrane would have to occur. Furthermore, the measured changes in the external TPP^+ concentration were not merely the result of changes in the TPP^+ binding to the cell membrane (Zaritsky et al., 1981). Measurements of membrane potential with $^{86}Rb^+$ in the presence of valinomycin, or with several fluorescent probes, showed a similar hyperpolarization stimulated by galactose as that observed with TPP^+ (J. Friede, H. Rottenberg, and M. Eisenbach, unpublished experiments).

How does the above interpretation on the need to inhibit masking processes explain the previous differences reported in the literature on *E. coli*? It seems that Szmelcman & Adler (1976) worked under anaerobic conditions. They used a very high cell concentration in the presence of an energy source and did not bubble oxygen into the suspension. Under such conditions oxygen is totally depleted from the suspension within 1 min (M. Eisenbach, unpublished experiments), much before an attractant was added to the cell suspension. It seems, therefore, that under the experimental conditions of Szmelcman & Adler the respiration and the electrogenic galactose uptake were inhibited as in this paper, and consequently the hyperpolarization was observed. Under their conditions a depolarization and a second hyperpolarization followed the first hyperpolarization, but this complex pattern could well be the result of oxygen addition to the anaerobic suspension together with the attractant. Indeed, recent experiments based on the results described here and carried out under strict anaerobic conditions confirmed this conclusion (J. Pierce and J. Adler, personal communication). In these experiments galactose was added in an oxygen-free solution and caused a single hyperpolarization, as in the presence of KCN and oxygen (Figure 5).

In the experiments of Snyder et al. (1981), on the other hand, aerated suspensions of bacteria in the presence of an energy source were used. Under such conditions the bacteria

were probably fully energized and the hyperpolarization was, therefore, undetectable. Only the depolarization due to the uninhibited galactose:H^+ uptake could possibly be observed. The above interpretation is consistent for all the attractants that were examined.

This study does not prove or disprove that the hyperpolarization is related to chemotaxis. It describes the conditions that enable the detection of these changes in membrane potential. Studies of the nature and significance of these changes showing correlation to chemotaxis, e.g., by lack of the hyperpolarization in chemotaxis mutants, will be the topic of a subsequent publication (M. Eisenbach, A. Ciobotariu, and T. Raz, unpublished experiments).

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Architecture of *Limulus polyphemus* Hemocyanin†

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ABSTRACT: The architecture of the 48-meric hemocyanin of the horseshoe crab *Limulus polyphemus* has been determined from electron micrographs of whole (48-mer) molecules and half- (24-mer) molecules. The assembly of hexamers of kidney-shaped subunits can produce two dodecameric enantiomorphs, designated as right and left. The assembly of 24-mers can again result in two enantiomorphs. By taking into account the rocking effect described by Van Heel and Frank [Van Heel, M., & Frank, J. (1981) *Ultramicroscopy 6*, 187–194], we deduced that the 24-meric half-molecule is made up of two copies of the left dodecameric enantiomorph. In addition, the two constituent dodecamers of the half-molecule are shifted with respect to a symmetric head-to-tail arrangement, which makes it possible to distinguish two different faces of the 24-mer, termed flip and flop. A model of the whole molecule was built from two copies of the 24-meric half-molecule. This model presents the four distinct views observed in the electron microscope (pentagon, ring, cross, and bowtie). In addition, the model shows the pentagonal view to exist in two varieties: symmetric and asymmetric. An analysis of electron micrographs presenting the pentagonal view by image processing using the statistical technique of correspondence analysis confirmed the existence of two types of pentagonal view, representing projections of a molecule built from two copies of the left 24-meric enantiomorph. In addition, the best fit between the averaged molecule images and the possible models was observed with a flop–flop inter 24-mer contact. The final model is shown in a series of stereo views produced by computer graphical techniques.

The hemocyanin of the horseshoe crab *Limulus polyphemus* is the biggest representative of this class of oxygen carriers in the Arthropod phylum (van Bruggen et al., 1981). It is generally considered to be composed of 48 monomeric subunits assembled in eight groups of six subunits, also called hexamers. The whole molecule is therefore termed an (8 × 6)-meric hemocyanin.

Native hemocyanin can be successively dissociated into 24-mers, 12-mers, and monomers, each form possessing its own domain of stability (Johnson & Yphantis, 1978). The monomers resulting from the dissociation of the native hemocyanin are highly heterogeneous. At least eight different polypeptide chains differing in their chromatographic, electrophoretic, and antigenic properties (Lamy et al., 1979; Brenowitz et al., 1981) and their oxygen affinities (Sullivan et al., 1974) have been found in the dissociation products of the whole molecules.

While the final aim of this work is the determination of the quaternary structure of *L. polyphemus* hemocyanin, we must first determine the architecture of the whole molecule in which the 48 individual subunits are to be localized. A recently developed technique suggested a reasonable approach to this goal. This is the analysis of electron micrographs by computer alignment and the statistical technique of correspondence analysis (Van Heel & Frank, 1981; Frank et al., 1982; Frank & Van Heel, 1982). The application of this technique to the hemocyanin half-molecule (24-mer) of *L. polyphemus* (Van Heel & Frank, 1981) revealed two very interesting structural aspects. The first result was that the molecule in its top view is not a flat structure, as had been generally assumed, but rather a noncoplanar structure resting on three hexamers, with the fourth hexamer suspended above the plane of the support film. Thus, the molecule is able to rock between two stable positions. The second feature discovered in this study was that the molecule in its top view is not perfectly square but rather is slightly rhombic, due to a slight shift of the two dodecameric halves with respect to one another. Therefore, two different top views, called flip and flop, can be distinguished.

The same observations were subsequently made on the 24-meric hemocyanin molecule of the scorpion *Androctonus australis* (Bijholt et al., 1982; Van Heel, 1981). More recently, Sizaret et al. (1982) used the evidence of the rocking effect and the rhombic arrangement to choose between the two possible enantiomorphs of the dodecamer and thus to determine the absolute configuration of the molecule in terms of the positions of all 24 subunits. The aim of this paper is to describe the architecture of the 48-meric native hemocyanin of *L. polyphemus* by the methods successfully employed for

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