SIMULTANEOUS MEASUREMENTS OF PROTON MOTIVE FORCE, ΔpH, MEMBRANE POTENTIAL, AND H^+/O RATIOS IN INTACT *ESCHERICHIA COLI*

ORUGANTI H. SETTY, RICHARD W. HENDLER, AND RICHARD I. SHRAGER
Section on Membrane Enzymology, Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, and Laboratory of Applied Studies, Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD 20205

ABSTRACT An instrument is described that enables the simultaneous monitoring of proton motive force (PMF), membrane potential ($\Delta\Psi$), the ΔpH across a membrane, oxidase activity, proton movements, and H^+/O ratios. We have studied the relationship existing among these parameters of energy transduction as a critical condition is changed during an experiment. The major findings are: (*a*) In the pH range of 4.5 to 7.5, increasing the external pH causes an increase in $\Delta\Psi$, internal pH, and oxidase activity, a decrease in H^+/O ratio, and a peak-plateau in PMF from pH 5.5 to 6.6 where ΔpH is converted to $\Delta\Psi$. (*b*) An increase in $[K^+]$ from 1 to 100 mM, in the presence of 0.5 μM valinomycin, causes the conversion of $\Delta\Psi$ to ΔpH, a gradual decline in PMF and an increase in H^+/O ratio, internal pH, and oxidase activity. (*c*) Increasing valinomycin concentration from 0 to 2.5 μM, in the presence of 50 mM $[K^+]$, causes a decline in $\Delta\Psi$ from 125 to 0 mV, and an increase in ΔpH from 35 to 70 mV. From 2.5 to 10 μM, the ΔpH and the PMF (which it solely represents), stay constant, H^+/O ratio increases mainly from 0 to 0.5 μM and much more slowly from 2.5 to 10 μM. (*d*) Oxygen at only 10% of its concentration in air-saturated buffer can support the generation of 90% or more of the $\Delta\Psi$, ΔpH, and PMF generated in an air-saturated solution. (*e*) The return of extruded protons to the cell (referred to here as "suck-back") represents a complicated process driven by $\Delta\Psi$ and influenced by a variety of factors. (*f*) H^+/O ratios measured by the kinetic technique used here are much higher than those measured by standard oxygen pulse techniques.

INTRODUCTION

It is now known that an electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$) represents an important stage in energy transducing phenomena in all photosynthetic and respiring cells. The energy is stored in this gradient in two forms: an electrical component (membrane potential, $\Delta\Psi$) and a chemical component (ΔpH) such that $\Delta\bar{\mu}_{H^+} = \Delta\Psi - Z\Delta pH$ where $Z = 2.303\ (RT/F)$ or 59.15 at 25°. The relative distribution of energy between the two components is markedly influenced by environmental conditions and cellular requirements.

For example, when *Escherichia coli* cells are suspended in an acidic medium the ΔpH component increases, maintaining the internal pH closer to neutrality (Padan et al., 1976; Booth et al., 1978, 1979; Zilberstein et al., 1979). This pH homeostasis is energy dependent as shown by studies with inhibitors of various proton pumps in both whole cells (Padan et al., 1976) and membrane vesicles (Ramos et al., 1976; Ramos and Kaback, 1977). The magnitudes and relative distribution of energy between the two components are also influenced by the presence of ionophores and permeant ionic species in the medium and by uncouplers which can dissipate energy by discharging the pH gradient.

The origin of the $\Delta\bar{\mu}_{H^+}$ is the unidirectional translocation of protons during respiration. A complete understanding of energy transduction requires the elucidation of the mechanism of proton translocation which, according to Mitchell's hypothesis, has a fixed stoichiometry of one proton per electron per energy transducing site (Mitchell, 1979). This same hypothesis, which depicts proton-carrying components of the respiratory chain as the translocators of the protons, does not allow for proton translocation during the transfer of electrons from cytochrome *c* to oxygen. Many groups disagree with both the stoichiometry and restriction of the Mitchell model (see Wikström and Krab, 1980, for review).

The movement of protons out of a cell or vesicle is retarded by the $\Delta\bar{\mu}_{H^+}$ that forms in response to their leaving. It is necessary, therefore, to obtain the earliest possible measurement of proton movement or dH^+/dt. This objective is obtainable only with instantly responding pH probes or by correcting relatively fast probes (relaxation constant ~1 s) for their response-time error.

The system that we have recently developed, which uses

ion-selective electrodes and a microcomputer, enables rapid kinetic values to be obtained for all of the important and interdependent parameters of energy transduction during an experiment, namely $\Delta\bar{\mu}_{H^+}$, $\Delta\Psi$, ΔpH, dO/dt, dH^+/dt, and H^+/O ratio. This paper describes the results of a study of these components in *E. coli* cells during periods of respiration under a variety of different conditions. It is also shown that the kinetic values for H^+/O ratios obtained with the new system are considerably higher than those obtained by the usual oxygen pulse methods. An additional finding of the current studies is the complexity of the proton suck-back phenomenon that accompanies, and follows, proton extrusion. The influence on this process of $\Delta\Psi$, ΔpH, availability of compensating charges, age of cells, and quantity of oxygen in the pulse is shown.

MATERIALS AND METHODS

Materials

The following chemicals were obtained from the indicated sources: Carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (FCCP) was a gift from Boehringer Mannheim Gmb H, West Germany; sodium tetraphenyl boran, polyvinyl chloride, dioctylphtalate from Aldrich Chemical Co., Inc., Milwaukee, WI; tetraheptyl ammonium iodide, Eastman Kodak Co., Rochester, NY; tetrahydrofuran, Fisher Scientific Co., Fairlawn, NH; TPP^+, Strem Chemicals, Inc., Newburyport, MA; valinomycin, and salicylic acid, Sigma Chemical Co., St. Louis, MO; [^{3}H]-TPP^+, New England Nuclear, Boston, MA (specific activity 4.3 mCi/mmol); salicylic acid (carboxyl ^{14}C), ICN Chemicals and Radioisotope Division, Irvine, CA (specific activity 60 mCi/mM); [^{3}H]-H_2O (1 mCi/g) and [carboxyl ^{14}C]-inulin carboxyl (2 mCi/g), New England Nuclear Corp., Boston, MA; other chemicals used were of analytical grade and obtained from standard sources.

E. coli (W6) was grown in Difco Antibiotic Medium Number 3 (Difco Laboratories, Inc., Detroit, MI) supplemented with 0.03% L-proline at 37° in a 12-liter fermentor with vigorous aeration using a 0.2% (vol/vol) innoculum. Cells were harvested at the mid-logarithmic phase of growth by centrifugation in a continuous Sharples centrifuge (Sharples-Stokes Div., Pennwalt Corp., Warminster, PA). The paste was weighed and then washed four times in a storage medium (9% [wt/vol] glycerol, 1 mM $MgCl_2$, 50 mM Tris-HCl, at pH 7.4). The final suspension was at 41 mg protein/ml in the storage medium which was separated into small aliquots and stored at −61°. These aliquots were thawed as needed and 0.225 ml (9.2 mg protein, 40 μl internal volume) were suspended in a total volume of 6 ml of a medium containing 2 mM potassium phosphate, 25 mM potassium sulphate (KK buffer) adjusted to the desired pH and stored overnight at 4°. This procedure was adopted because cells thawed and placed in KK buffer are initially swollen and slowly shrink to a constant volume over a period of 6 h in the cold. Swelling and shrinking of cells when suspended in KK buffer did not affect any of the measurements because experiments were performed when cells showed a constant internal volume over a long period of time. The internal volumes of nonenergized, energized and uncoupled cells were the same. Just before the experiment, the suspension was brought to room temperature and supplemented with 2.5 mM succinate, 10 to 50 μM TPP^+, and 100 μM salicylic acid.

Procedures

Internal volume was determined using [^{3}H]-H_2O and [^{14}C]-inulin (Rottenberg, 1979). Protein was assayed by the Lowry method (Lowry et al., 1951). The *E. coli* suspension described above was placed in the reaction vessel and brought to anaerobiasis by passage of argon over the surface at a rate of 50 ml/min. 15–20 min after the system was completely anaerobic, respiration was initiated by bubbling oxygen gas through a Teflon needle (30 cm long, 0.032 in. OD, 0.013 in. ID) (Hamilton Co., Reno, NV) into the solution for periods of time up to 10 s. The oxygen electrode was calibrated with NADH according to the method of Chappell (1964) and found to be linear from 0 to 100% of the air-saturated level of oxygen. The concentration in air-saturated buffer was taken to be 475 ngA (nanogram atoms) oxygen/ml at 25°C. With low amounts of oxygen, initial injection levels could not be determined directly from the oxygen electrode because of the combination of succinoxidase activity and slow response of the Clark electrode. Therefore, for the low levels of oxygen, a special calibration procedure was used to relate electrode reading to initial amount of injected oxygen. Known amounts of oxygen in buffer were injected and the actual response of the Clark electrode noted under the same conditions. A calibration curve was constructed relating the delayed electrode recording (in the presence of cells) to the amount of injected oxygen. This procedure was used for the experiments shown in Fig. 1 *c* and *f*, and Fig. 6.

Measurements of $\Delta\Psi$, ΔpH, $[H^+]$, and $[O_2]$. The measurements of $\Delta\Psi$ and ΔpH were based on the distributions of TPP^+ and salicylic acid respectively as detected by ion-selective electrodes. Electrode readings were converted to $\Delta\Psi$ and ΔpH during the experiment in progress by a dedicated microcomputer (an Intel 8080 single-board computer, Intel Corp., Santa Clara, CA). Full details of the experimental system will be published separately. Essential information is provided here. The electrode for measuring TPP^+ concentration was a polyvinyl chloride (PVC) membrane electrode as described by Kamo et al. (1979). The modifications we introduced were the insertion of a very small agar KCl/AgCl salt bridge directly into the electrode body and the use of a micro-Ag/AgCl wire fitted with a gold-plated copper connector pin (Hendler et al., 1977). A PVC membrane electrode selective for salicylate was constructed along similar lines. The selective membrane was cast from a solution containing 0.5 g polyvinyl chloride, 1.5 ml dioctylphthalate and either 100 mg tetraheptyl ammonium iodide (Haynes and Wagenknecht, 1971) for the salicylate electrode or 10.3 mg tetraphenyl boran (Kamo et al., 1979) for the TPP^+ electrode, dissolved in 13.0 ml tetrahydrofuran. The solution was poured into an acid-washed petri dish (100 × 10 mm) and left to set for ~40 h at room temperature with the cover slightly ajar to allow slow evaporation. This membrane can be stored at room temperature for ~4–5 mo. Pieces of membrane were cut as needed with a cork bore (5 mm diameter), fixed to the end of a length of Tygon tubing (4.5 cm length, 3/32 in. ID, 5/32 in. OD) using 5% polyvinyl chloride in tetrahydrofuran as a glue and left overnight at room temperature to set. The filling solution was 100 μM of either TPP^+ or salicylate. The electrodes were conditioned by soaking in a solution of 100 μM of the respective ion for 15–20 h before use. The TPP^+ electrode gave a linear response of 57 mV per decade change of concentration over the entire range of operation but the salicylate electrode demonstrated a concentration-dependent slope. The response times of the two electrodes were as fast as the mixing time (~1 s). These electrodes enable one to know the state of $\Delta\Psi$ and ΔpH from the time of mixing after the injection of oxygen as soon as the TPP^+ and salicylate ions are able to adjust their concentrations across the cell membrane. The rates of equilibration of the probes across the membrane are not known, but the rise in measured $\Delta\Psi$ and ΔpH following an oxygen pulse appears to be almost as fast as the rate of acidification of the external medium (Fig. 1). The system also used a combination pH electrode (Beckman 39505, Beckman Instruments, Inc., Fullerton, CA) and a Clark-type oxygen electrode (YS1 4004, Yellow Springs Instrument Co., OH). The electrode readings were passed through an electronic interface to the microcomputer as indicated in the block diagram in Fig. 2. The electronic interface referenced the voltages of the TPP^+ and salicylate electrodes to the reference electrode of the combination pH electrode, provided isolation for the Clark electrode circuit, accomplished noise reduction and provided gains for the differen-

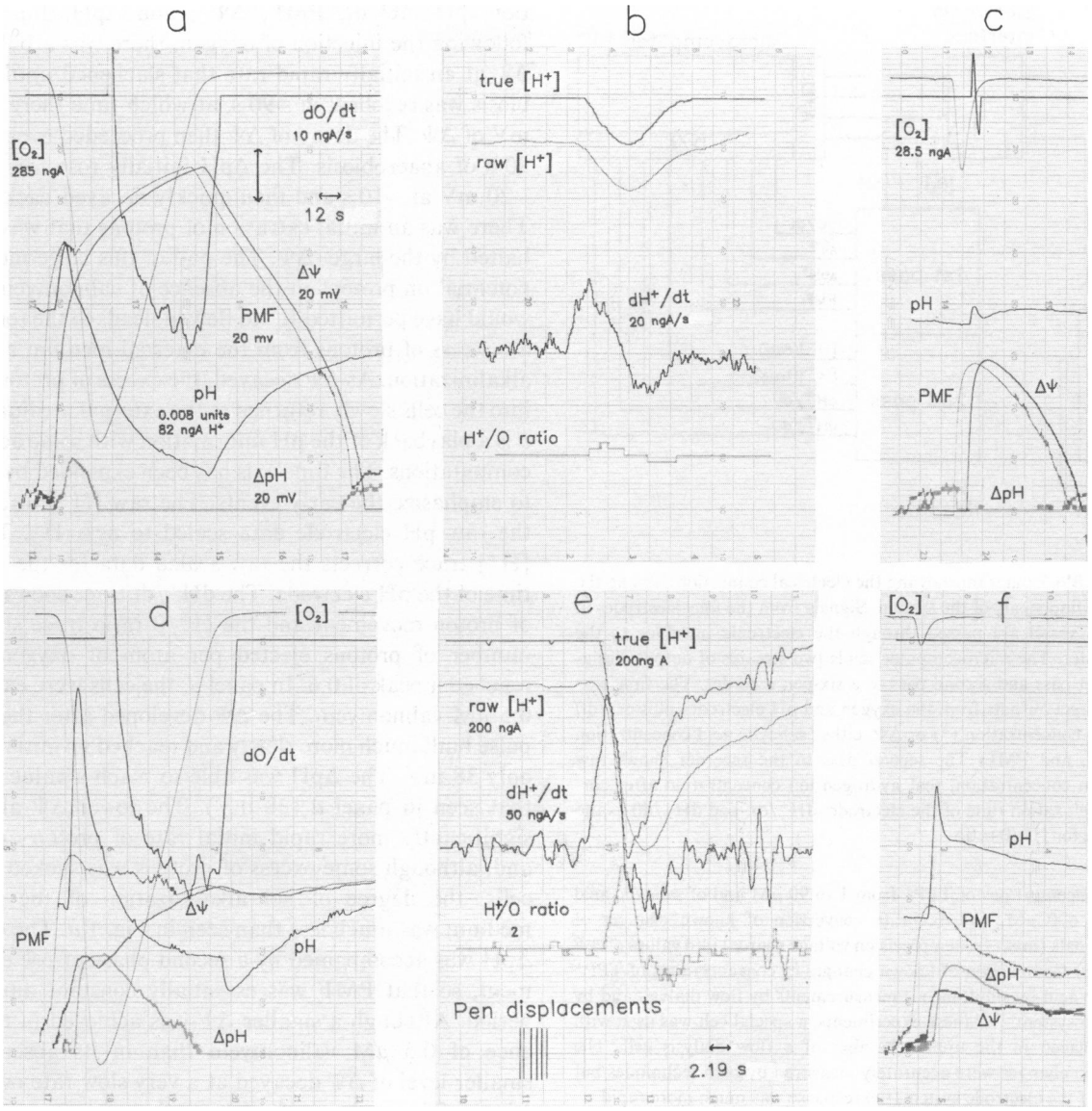

FIGURE 1 Real-time measurements of PMF, $\Delta\Psi$, ΔpH, $[O_2]$, pH, dO/dt, dH^+/dt, and H^+/O ratios during experiments in progress. *E. coli* cells (9.2 mg protein in 225 μl) were suspended in 6.0 ml of 2 mM KH_2PO_4 buffer containing 25 mM K_2SO_4 at pH 7.0 at 4° overnight. The suspension was then brought to room temperature and supplemented with 2.5 mM succinate, 50 μM TPP^+, and 100 μM salicylic acid. The closed assay vessel was flushed with argon for 20 min and then pulsed with gaseous oxygen introduced into the suspension through a Teflon needle. The top three panels were obtained in the absence of valinomycin and the bottom in the presence of 0.5 μM valinomycin. *a*, *c*, *d*, and *f* show first-pass computer outputs for four different experiments. These include PMF, $\Delta\Psi$, ΔpH and dO/dt. Also shown are direct readings of pH and $[O_2]$. Chart speed for these panels was 1 cm/12 s (1 cm shown as a horizontal arrow in *a*). *b* and *e* show the second-pass computer outputs for the experiments shown in *a* and *d*, respectively. These include raw $[H^+]$, true $[H^+]$ corrected for the relaxation time of the pH electrode, dH^+/dt, and H^+/O ratio. The chart speed was 1 cm/2.19 s (1 cm shown as a horizontal arrow in *e*). The quantities shown are for 1/10th full scale, as indicated by the vertical arrow in *a*. Pen displacements are ΔpH, pH, $\Delta\Psi$, dO/dt, PMF and $[O_2]$ from left to right.

tial electrode readings. The voltage representative of the signals from the four electrodes after processing by the electronic interface were digitized by a 12-bit analog-to-digital converter in the microcomputer. The microcomputer was programmed to process incoming signals during the experiment in progress. The processing included digital filtering to increase the signal-to-noise ratios, a correction for the relaxation time of the pH electrode, a concentration-dependent correction for the nonlinearity of the salicylate electrode, conversions of the TPP^+ and salicylate electrode signals to either concentrations of TPP^+ and salicylate or $\Delta\Psi$ and ΔpH, and conversions of the pH electrode and oxygen electrode readings to $[H^+]$, $d[H^+]/dt$, $[O_2]$, $d[O_2]/dt$, and $H^+/0$ ratio. The output of the microcomputer was passed to a six-pen recorder (Linseis Inc., type LS64, Princeton Junction, NJ) in two stages. During the experiment, the recorder showed dO/dt, $\Delta\Psi$, ΔpH, and PMF. In addition, the direct outputs of the oxygen and pH electrodes were displayed. A second stream of outputs from the microcomputer immediately following the experiment showed raw $[H^+]$ scaled in nanograms of H^+ and true $[H^+]$, after correcting for the relaxation time of the pH electrode. The rates of change of $[H^+]$, (dH^+/dt), and $H^+/0$ ratios were also shown. The combined system was checked by the following procedures: (*a*) Accurate reporting

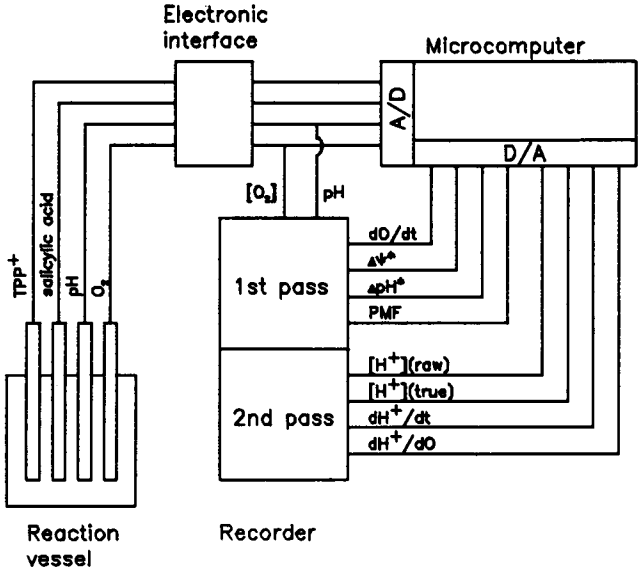

FIGURE 2 Block diagram showing the electrical connections among the four major components of the system. Signals from the four electrodes in the reaction vessel are passed through the electronic interface to the microcomputer. The microcomputer sends two streams of output, designated as first pass and second pass to a six-pen recorder. The first pass shows the direct outputs from the oxygen and pH electrodes plus dO/dt, either TPP^+ concentration (*) or $\Delta\Psi$, either salicylic acid concentration (*) or ΔpH, and PMF. The second pass to the recorder shows raw hydrogen ion concentration, real hydrogen ion concentration after correcting for relaxation time of the electrode, dH^+/dt, and dH^+/dO as the kinetic value for H^+/O ratio.

of known concentrations of TPP^+ from 1 to 90 μM and of salicylic acid from 10 to 600 μM. (*b*) Accurate conversion of known changes of electrode signals (input from a precision voltage source) into values of $\Delta\Psi$ and ΔpH. (*c*) Comparison of known changes of concentrations of TPP^+ and salicylic acid by similtaneous measurements by flow dialysis and by the electrode system. For these experiments, a special cell was used with electrodes placed in the upper chamber of a flow dialysis cell. The concentration changes were accurately measured by both techniques but in the case of the electrode system, the response was much more rapid.

The relaxation time correction for the pH electrode allowed the determination of dH^+/dt rates within the first second after addition of the oxygen pulse. This is important, because as soon as the first protons are translocated and a membrane potential is formed, the further ejection of protons is retarded. In the case of the oxygen electrode such a rapid determination of oxygen consumption rate of the cells is not necessary because once the cytochrome oxidase on the outer surface of the membrane is presented with oxygen at a saturating level, electron transfer at a constant rate should be established and maintained until the level of oxygen is depleted to below saturation of the enzyme. The constant rate of dO/dt becomes evident in the recorder trace following termination of the oxygen pulse period (~10 s) after a delay due to the relaxation time of the electrode. This rate, corrected for the known rate of loss of oxygen (i.e., 0.238% × $[O_2]$) from the solution to the argon gas flowing across the surface, is the oxygen uptake rate of the cell suspension.

RESULTS AND DISCUSSION

Fig. 1 shows direct recorder outputs obtained during several experiments conducted at pH 7.0. The experiments shown in the top row (panels *a*, *b*, and *c*) were performed in the absence of valinomycin. Panel *a* shows the consequences of an oxygen pulse in terms of oxygen concentration, pH, dO/dt, PMF, $\Delta\Psi$, and ΔpH. Immediately following the injection of oxygen, there was a build-up of $\Delta\Psi$ at an initially rapid rate that slackened until anaerobiosis was reached, in ~90 s, at which time there was 130 mV of $\Delta\Psi$. The decay of $\Delta\Psi$ then proceeded over the next 90 s of anaerobiosis. The ΔpH initially rose to a peak of ~20 mV at ~10 s and then quickly decayed back to zero. There was an initial extrusion of protons that was quickly halted by the large $\Delta\Psi$. The pull of this large membrane potential on protons in the absence of valinomycin, which would have permitted K^+ to flow in, leads to the removal of an excess of protons from the external medium and a net alkalinization. As $\Delta\Psi$ decayed, the excess of protons pulled into the cells slowly returned to the external medium. Panel *b* is a playback of the pH information with some additional computations. The time axis has been expanded by 5.5-fold to emphasize the early events. The raw $[H^+]$ trace shows the raw pH electrode data scaled to ngA H^+. The true $[H^+]$ trace corrects the raw scaled data for the response time of the pH electrode. The dH^+/dt trace shows the rate of proton movement and the H^+/O ratio trace shows the number of protons ejected per atom of oxygen, which reached a peak of 0.6. In panel *d*, the cells were exposed to 0.5 μM valinomycin. The $\Delta\Psi$ developed after the oxygen pulse built much more slowly and reached an initial peak of only 38 mV. The ΔpH was able to reach a value of twice that seen in panel *a* (38 mV). The lower $\Delta\Psi$ allowed a significantly more rapid initial rate of proton extrusion, and, although some excess of protons was sucked into the cells, the degree of net alkalinization of the external medium was much less than seen in Fig. 1 *a*. The decay of ΔpH was accompanied by a second phase of $\Delta\Psi$ enhancement, so that PMF was essentially constant during this period. Although a smaller $\Delta\Psi$ was achieved in the presence of 0.5 μM valinomycin than in its absence, this smaller level of $\Delta\Psi$ decayed at a very slow rate over 10 to 15 min. The presence of valinomycin in amounts >2.5 μM prevented any $\Delta\Psi$ from forming (shown below). The playback of the pH information is shown in Fig. 1 *e*, which shows the rapid rate of proton extrusion and the importance of correcting these rapid events for the relaxation time of the electrode (true $[H^+]$). The H^+/O ratio reached a value of 3.2 within 2 s after the injection of oxygen. Panels *c* and *f* show the results obtained with very low levels of oxygen injection. The injection of 40 ngA of oxygen per milliliter (8.5% of air saturation) in the absence of valinomycin (Fig. 1 *c*) led to the rapid build-up (~10 s) of close to 60 mV of $\Delta\Psi$, which then decayed to zero in ~1 min. No ΔpH was formed (there was ~10 mV of noise in the ΔpH channel prior to the injection). A very low level of proton extrusion was seen (~4 ngA H^+/ml). In the presence of 0.5 μM valinomycin Fig. 1 *f*) the injection of 33 ngA of oxygen per ml (7% of air saturation) caused the formation of 50 mV of PMF. Peak values for $\Delta\Psi$ and ΔpH were 24 and 34 mV, respectively. After an initial decay upon anaerobiosis a residual level of both $\Delta\Psi$ and ΔpH

decayed at a slower rate over a period lasting several minutes. The effect of valinomycin on reducing initial $\Delta\Psi$ and increasing the initial rate of proton extrusion is also evident in comparing panels *f* and *c*. It should be noted that, in our hands, valinomycin was effective without the prior treatment of cells with EDTA that Padan et al. (1976) found necessary. Perhaps the strain of cells we used and their history (freezing in glycerol/Tris buffer) are responsible for this difference.

Fig. 3 shows the composite results of a series of experiments conducted at different pH values in the presence and absence of 0.5 μM valinomycin. The data show the relationships for PMF, $\Delta\Psi$, ΔpH, H^+/O ratio, internal pH, and oxygen uptake rate. In both the presence and absence of valinomycin, the ΔpH adjusted so that the internal pH was maintained closer to pH 7 than the pH of the external medium. Therefore, the ΔpH component was higher at the lower pH's, except for pH 4.5 where the oxidase activity was severely limited. The succinoxidase activity as shown in Fig. 3, *a* and *c* (dashed line), generally reflected its pH requirements as described earlier (Hendler and Burgess, 1972).

In the absence of valinomycin, $\Delta\Psi$ is the major component of PMF (panel *b*), and as the external pH rose, the energy lost by diminution of ΔpH was converted to $\Delta\Psi$ so that PMF was essentially constant from pH 5.5 to 6.6. At pH 7.0 and 7.5, the ΔpH was zero when $\Delta\Psi$ was at its maximum. Actually, at pH 7.0 and 7.5, a transient ΔpH of 15–20 mV was formed, but it decayed to zero as $\Delta\Psi$ continued to build to its maximum (see Fig. 1 *a*). In the absence of valinomycin at pH values of 5.5 and higher, H^+/O ratios were 0.5 or lower. However, at pH 5.0 a value of 7.5 was seen, and at pH 4.5 the numbers were much higher but quite variable (15–35) (not shown). This behavior is probably a function of the magnitude of $\Delta\Psi$, which is quite low at extremely acidic pH. The results of the experiment in the presence of valinomycin (panel *d*) were quite different from those obtained in its absence. Here, ΔpH was the major component of PMF until ~pH 6.5. The ΔpH climbed to a higher level than seen in the absence of valinomycin. Although $\Delta\Psi$ did increase with increasing pH, it was always much lower than that seen in the absence of valinomycin. This trend could be continued by increasing the concentration of valinomycin so that above 2.5 μM valinomycin no stable $\Delta\Psi$ could be maintained. Also, the total level of PMF was decreased by opening the cells to potassium ions. The decreased level of $\Delta\Psi$ was reflected by a marked increase in H^+/O ratios. The same trend to higher H^+/O ratios at low pH (panel *d*) and to much higher and more variable values below pH 4.5 was seen (not shown). All of the H^+/O ratios discussed so far were obtained by the kinetic method using the microcomputer. For comparison, H^+/O ratios obtained by standard pulse methods (1.5–2.0) (Lawford and Haddock, 1973) are shown in Fig. 3 *d*. The dashed line (---) with the upright triangles (▲) shows the values obtained by the kinetic technique and the dashed line (---) with upside-down triangles (▼) shows the values obtained by the more usual technique.

Both the probe for $\Delta\Psi$ (TPP^+) and that for ΔpH (salicylic acid) have lipid soluble properties and, therefore, the potential for uncoupling or damaging the system under study. In Fig. 4, effects of increasing concentrations of both probes are shown. With increasing TPP^+, the PMF, $\Delta\Psi$, ΔpH, and respiration were essentially constant in the range of 5–50 μM. This indicates that the metabolic health of the

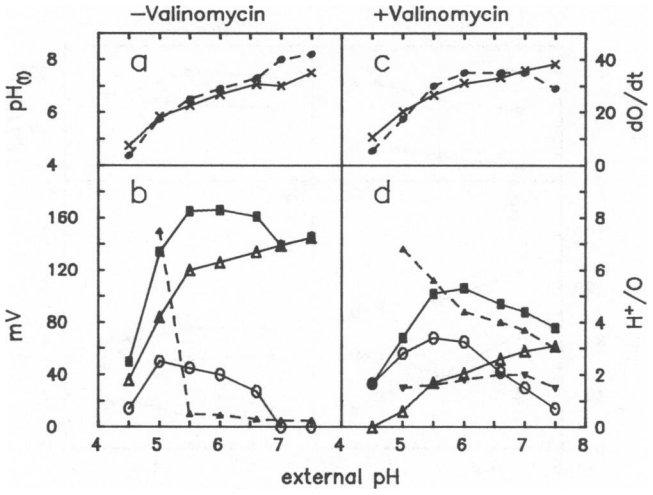

FIGURE 3 Effect of external pH on PMF, $\Delta\Psi$, ΔpH, H^+/O ratio, internal pH and dO/dt in the presence and absence of 0.5 μM valinomycin. Experimental details are the same as described in the legend to Fig. 1 except for the pH, which was varied as indicated. *a* and *b* were obtained in the absence of valinomycin and *c* and *d* in its presence. The ordinates on the left refer to the solid-line curves and those on the right to the dashed-line curves. ■, PMF; Δ, $\Delta\Psi$; ○, ΔpH; ▲, H^+/O by kinetic method; ▼, H^+/O by standard pulse method; X, internal pH; ○, dO/dt.

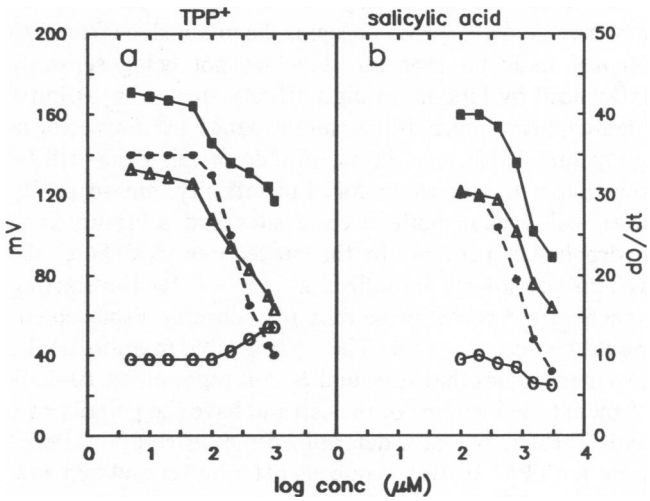

FIGURE 4 Effects of increasing concentrations of TPP^+ and salicylic acid on PMF, $\Delta\Psi$, ΔpH and dO/dt. Experimental details were the same as described in the legend to Fig. 1 except for the pH, which was at 6.0. The left-hand ordinate refers to PMF (■), $\Delta\Psi$ (Δ), and ΔpH (○). The right-hand ordinate refers to dO/dt (●).

cells was not impaired in this range. Above this concentration, PMF, $\Delta\Psi$, and respiration became progressively more inhibited. The fact that ΔpH increased at the expense of $\Delta\Psi$ at high levels of TPP^+ is explained by the ability of the membrane-permeable TPP^+ to neutralize $\Delta\Psi$ just as was seen with potassium in the presence of valinomycin (Fig. 3). With increasing salicylic acid, the PMF, $\Delta\Psi$, ΔpH, and respiration were constant from 100 to 200 μM, indicating that this is a safe range of concentration for this probe. Whereas respiration showed a defect at 400 μM (log = 2.6), the components of PMF were still essentially intact. Higher concentrations of salicylic acid caused precipitous drops in PMF, $\Delta\Psi$, and respiration, and a drop in ΔpH in contrast to the situation seen with higher concentrations of TPP^+. This is reasonable because the weak acid would be expected to show some proton ionophore properties. Our experiments have been conducted with a TPP^+ concentration of 50 μM and salicylic acid at 100 μM.

The presence of binding sites for TPP^+ could introduce inaccuracies in the measurement of $\Delta\Psi$, based on the apparent distribution of the probe. Some of these effects have been discussed, but only in regard to distributions and nonsaturable sites, (Lolkema et al., 1982). Binding could be in the form of saturable sites inside or outside of the cell and in the sequestering of the probe in the apolar interior of the membrane or in internal lipid sites. We have been able to eliminate certain of these potential binding problems. For example, with high affinity sites, either inside or outside the cell, as the TPP^+ concentration was increased from zero and the sites become saturated, the apparent $\Delta\Psi$ rapidly decreased to the true value that remained constant with further increases in TPP^+ concentration. The data in Fig. 4 from 5 to 50 μM TPP^+ concentration can be extrapolated to zero concentration in a linear plot of apparent $\Delta\Psi$ vs. concentration. The value of $\Delta\Psi$ at zero TPP^+ concentration is only ~7 mV higher than the 126 mV indicated at 50 μM. This plus the minor slope from 5 to 50 μM indicates that the data are not being seriously influenced by binding to high affinity sites. Low affinity, high-capacity internal sites can introduce a serious error in the value of $\Delta\Psi$, but the magnitude of this error will be constant with respect to $\Delta\Psi$. Low-affinity, high-capacity sites will include both discrete sites and solubility in a hydrophobic medium. In the procedures used here, the system was always initialized at $\Delta\Psi$ = 0 for the starting nonenergized condition so that any constant displacement would be accounted for. The hydrophobic interiors of the two membranes that surround *E. coli* represented 10–15% of the internal volume of the cell and have the polarity of a hydrocarbon. We have determined the distribution coefficient for TPP^+ between aqueous pH 7 buffer and heptane, and it is ~500 to 1 in favor of the aqueous compartment. Sequestering the probe inside the membrane(s), therefore, does not cause a serious problem. We have not eliminated other kinds of potential binding problems such as low-affinity external sites, moderate-affinity internal or external sites, or sites whose affinity constantly varies with $\Delta\Psi$. A justification for the use of TPP^+ by us and others in spite of these uncertainities is based on the direct demonstration in giant *E. coli* that the value of $\Delta\Psi$ measured directly with microelectrodes and indirectly by TPP^+ distribution is the same (Felle et al. 1980). The same was found to be true in neuroblastoma glioma hybrid cells (Lichtshtein et al., 1979). The considerations discussed in terms of TPP^+ binding also apply to the use of salicylate ion as a probe for ΔpH. Fig. 4 shows that the working concentration for salicylic acid (10^2 μM) is on a plateau for ΔpH, $\Delta\Psi$, PMF, and respiration. The starting nonenergized condition was used to initialize ΔpH = 0, and the distribution coefficient for salicylic acid between pH 7 aqueous buffer and heptane was determined to be 1,000 to 1. Although no independent direct means exists for measuring internal pH, such as an inserted micro pH electrode, salicylic acid and related weak organic acids are widely used for estimation of ΔpH in cell systems. ΔpH determined in *Rhodopseudomonas sphaeroides* with a probe structurally related to salicylic acid (i.e., benzoic acid) was essentially the same as that determined by the completely independent technique of ^{31}P NMR (Nicolay et al., 1981).

The effects of increasing potassium concentration in the presence of 0.5 μM valinomycin at pH 5.5 and 7.0 are shown in Fig. 5. The buffer contained a constant cation concentration of 100 mM made up of different proportions of K^+ and Na^+. The upper panel at pH 5.5 shows that the ability of the cell to maintain an internal pH closer to neutral was enhanced by increasing the potassium concentration. The higher internal pH was reflected by higher succinoxidase activity, as expected from its pH optimum

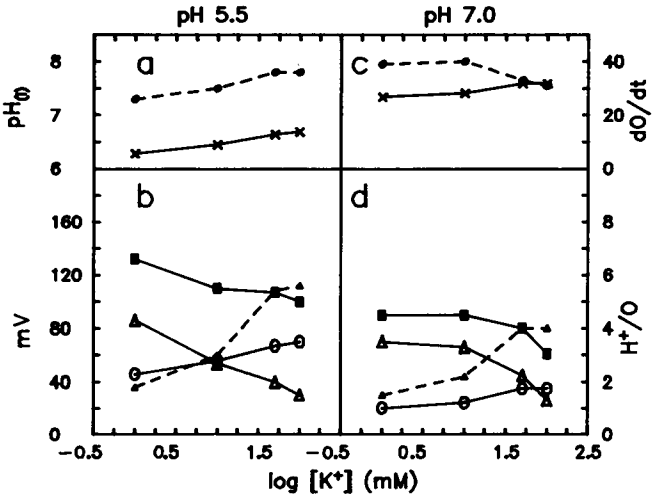

FIGURE 5 Effect of external potassium concentration on PMF, $\Delta\Psi$, ΔpH, H^+/O ratio, dO/d*t*, and internal pH at pH 5.5 and 7.0. Experimental details are the same as described in the legend to Fig. 1 except that K^+ in the buffer was replaced to various extents with Na%+ while keeping the total cation concentration at 100 mM. The experiments shown in *a* and *b* were performed at pH 5.5. The same line and symbol designations as used in Fig. 3 apply here.

which was ~7.2 (Hendler and Burgess, 1972). Fig. 5 *b* shows that increasing potassium progressively neutralized $\Delta\Psi$ with the conversion of some of this energy into ΔpH. A crossover in these two components occurred at 10 mM K^+. The fact that this transfer of energy was not completely conservative is shown by the progressive decline of PMF with higher potassium levels. The H^+/O ratio increased from 1.8 at 1 mM K^+, to 5.5 at 100 mM with most of the rise occurring between 10 and 50 mM K^+. At pH 7.0, the ΔpH is markedly reduced as was already noted in Fig. 3. At this pH, the ΔpH component began to decay while $\Delta\Psi$ was still building. The data presented in panel *d* were obtained at the time when ΔpH was at its maximum and $\Delta\Psi$ was below its maximum value. The qualitative picture in panel *d* is the same as that in panel *b* where PMF and $\Delta\Psi$ decreased and ΔpH and H^+/O ratio increased as potassium was increased, but except for $\Delta\Psi$ the numbers are smaller. Panel *c* shows internal pH was maintained between 7.0 and 7.5 up to 10 mM K^+ and then went above 7.5 at the higher levels of K^+. Succinoxidase activity was at its maximum until the internal pH went above 7.5 and then a small decrease was seen.

Fig. 6 shows the magnitudes of PMF, $\Delta\Psi$, and ΔpH developed in response to different amounts of oxygen used to support respiration. As little as 14 ngA of oxygen per milliliter, which represents 3% of the concentration of oxygen in air-saturated buffer at room temperature, caused the formation of ~50% of the maximum PMF and ~65% of maximal $\Delta\Psi$. Near maximal levels for PMF, $\Delta\Psi$, and ΔpH were attained with 47.5 ng A/ml of oxygen, which still is only 10% of the air-saturated concentration. In fact, increasing the concentration of oxygen to 280 μgA/ml led to very little enhancement in the magnitudes of the components of PMF. This ability of *E. coli* to build and maintain PMF at such low oxygen tension probably represents a mechanism that allows the cells to conserve energy from respiration even under conditions of severe oxygen deprivation.

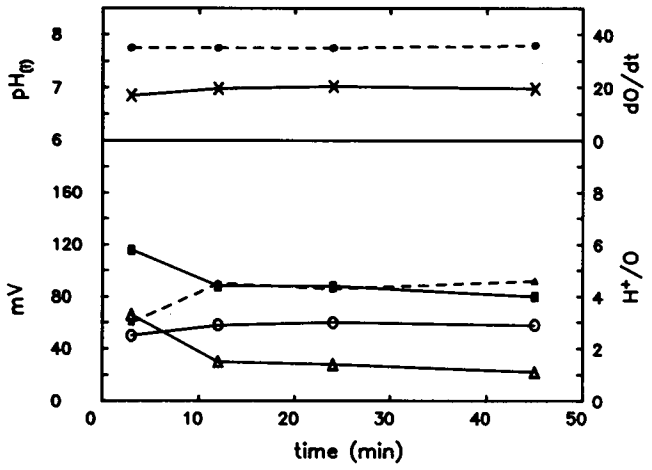

FIGURE 7 The time course of action of 0.5 μM valinomycin on intact *E. coli*. Experimental details are the same as described in the legend to Fig. 1 except for the pH, which was at 6.0. PMF (■), $\Delta\Psi$ (Δ), ΔpH (o), H^+/O ratio (▲), dO/d*t* (●), and interal pH (X).

The time course of action of 0.5 μM valinomycin on intact *E. coli* is shown in Fig. 7. The full effect of the ionophore seems to be exerted within 12 min, as evidenced by the decay of $\Delta\Psi$, and the build-up of ΔpH and the H^+/O ratio (lower panel). The upper panel shows that both the internal pH and succinoxidase activity stayed essentially constant throughout the period of observation. All of the parameters shown in the figure were followed out to 90 min with no further changes seen (not shown).

The effects of increasing concentrations of valinomycin are shown in Fig. 8. At 0.5 μM valinomycin, there was a 67% decrease in $\Delta\Psi$ and an 85% increase in ΔpH. The H^+/O ratio increased from 0.4 with no valinomycin to 5.0

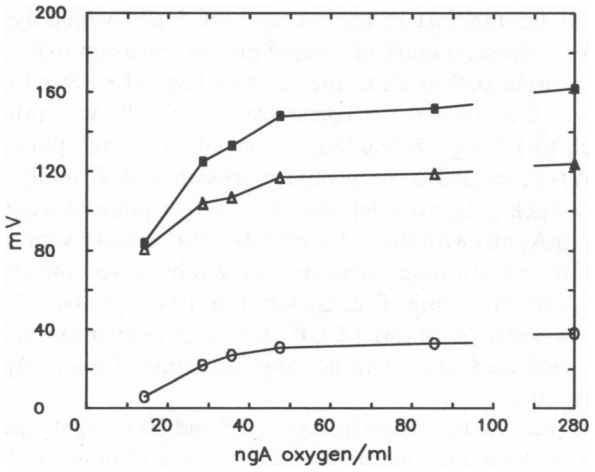

FIGURE 6 Effect of the size of the oxygen pulse on PMF, $\Delta\Psi$, and ΔpH. Experimental details are the same as described in the legend to Fig. 1 except for the pH, which was at 6.0. PMF (■), $\Delta\Psi$ (Δ), and ΔpH (o). The quantities of oxygen were determined as described in the Methods section.

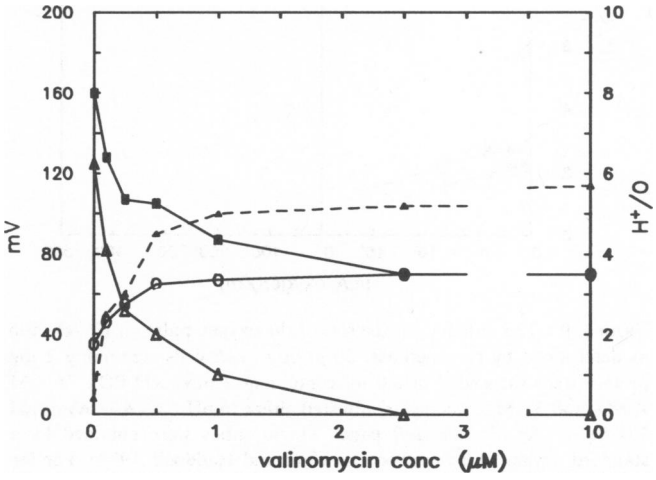

FIGURE 8 Effect of valinomycin concentration on PMF, $\Delta\Psi$, ΔpH, and $H^+/0$ ratio. Experimental details are the same as described in the legend to Fig. 1 except for the pH, which was at 6.0. PMF (■), $\Delta\Psi$ (Δ), ΔpH (o), H^+/O ratio (▲), dO/d*t* (●).

at 0.5 μM valinomycin. Little change in either ΔpH or H^+/O ratio was seen out to 10 μM valinomycin but $\Delta\Psi$ continued to decrease from 40 mV with 0.5 μM valinomycin to zero at 2.5 μM valinomycin. Therefore, with valinomycin concentrations from 2.5 to 10 μM, ΔpH was the sole component of PMF.

The effect of increasing levels of oxygen on the H^+/O ratio was studied by both the kinetic method as used in most of the studies reported here and by the oxygen-pulse method commonly used (Fig. 9). The oxygen-pulse method generally uses quite low levels of oxygen. It is seen that increasing the amounts of oxygen leads to lower measured values for the H^+/O ratio (left panel). When the same cells were used in the kinetic method described in this paper, much higher levels of H^+/O ratio resulted (right panel). Also, increasing the amounts of oxygen up to quite high levels leads to a small enhancement in the level of measured H^+/O ratio.

The Phenomenon of Proton Suck-back

The return of translocated protons to the vesicle interior following anaerobiosis is predicted by thermodynamics and is considered essential in distinguishing proton translocation from a scaler generation of *de novo* protons. This is because at equilibrium the generated proton gradient is dissipated, whereas *de novo* protons can lead to a new equilibrium state of lower pH. The return of protons to their point of origin is driven by the $\Delta\Psi$ generated as a result of their translocation. In our studies, however, we recognize a variety of ways in which extruded protons behave before and after anaerobiosis. The factors that control the kinetics and extent of proton movements are permeability of the membrane as influenced by ionophores or aging, the relative concentrations of external potassium and protons, and the amount of oxygen used in the pulse. As has been previously observed, the experiment depicted in Fig. 10 *a* shows that cells pulsed with oxygen in the absence of valinomycin responded with a small, sluggish ejection of protons. The return of the ejected protons following anaerobiosis was also slow, and the pH did not return to baseline even after 20 min. Aging cells at −61° for 5–6 mo, although the components of PMF were not affected, decreased proton ejection to even lower levels than seen in the fresher cells used in Fig. 10 *a*, and frequently led to the removal of more protons from the external medium than were originally extruded (Fig. 10 *b*). The addition of 0.5 μM valinomycin to either fresh or aged cells led to a high initial rate of proton extrusion (Fig. 10 *c*). The external medium quickly reached a maximum degree of acidification and then there was a loss of protons down to a new level that was maintained until anaerobiosis, at which time a further small drop in external acid was seen. This intermediate level was sometimes above the starting pH of the medium (Fig. 10 *c*, pH 6.0) and sometimes below (Fig. 1 *d*). When valinomycin was present at 5 μM, at which concentration no $\Delta\Psi$ can be formed, there was a large ejection of protons with little or no suck-back, even long after anaerobiosis (not shown). During this period, ΔpH was maintained with only a slow rate of decay. The addition of 1 μM FCCP, however, caused the immediate return of extruded protons and the collapse of the ΔpH. Therefore, although the equilibrium condition required the return of the protons, in the absence of $\Delta\Psi$ there was an insufficient pull to bring the protons back across the membrane. Using a small amount of oxygen in the pulse (4 ngA/ml instead of the usual 330 ngA/ml) and a small amount of cells (0.35 mg protein/ml instead of the usual 1.5 mg/ml) in the presence of 5 μM valinomycin gives a classic picture of a rapid proton extrusion followed by a rapid suck-back to the starting level of external pH (Fig. 10 *d*). When the concentration of cells was raised back to 1.5 mg protein/ml, a very small oxygen pulse, 2 ngA/ml, caused some proton extrusion and virtually no suck-back (Fig. 10 *e*, left trace). A larger pulse of oxygen (32 ngA/ml) with this cell concentration caused a very fast initial rate of proton extrusion followed by or accompanied by a very rapid rate of suck-back (Fig. 10 *e*, right trace). In the presence of 10 μM FCCP, there was neither extrusion nor suck-back of protons nor any formation of $\Delta\Psi$ or ΔpH (Fig. 10 *f*).

If the situations seen in Figs. 10 *d* and 10 *e* (right trace) can be designated as the classic case seen in pulse studies with mitochondria and bacteria where protons are ejected and completely sucked back upon anaerobiosis, we must then recognize the variations that show little or no suck-

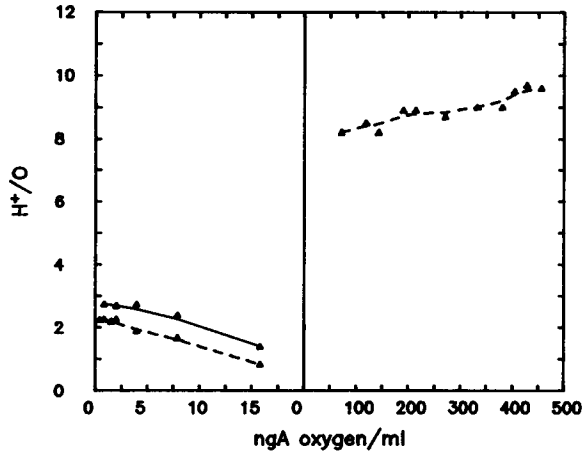

FIGURE 9 The influence of the size of the oxygen pulse on H^+/O ratio as determined by two methods. 50 μl of *E. coli* cells containing 2 mg protein were suspended in 6.0 ml containing 2 mM KH_2PO_4, 75 mM K_2SO_4 and 5 μM valinomycin adjusted either to pH 6.0 (▲----▲) or pH 7.0 (▲——▲). For the *left* panel, H^+/O ratios were obtained by a standard oxygen pulse method (Lawford and Haddock, 1973). For the *right* panel, the kinetic method described here was used. The method of oxygen injection was with oxygenated buffer for the left panel and with the bubbling of oxygen gas into the solution for the right panel as described in the Methods section.

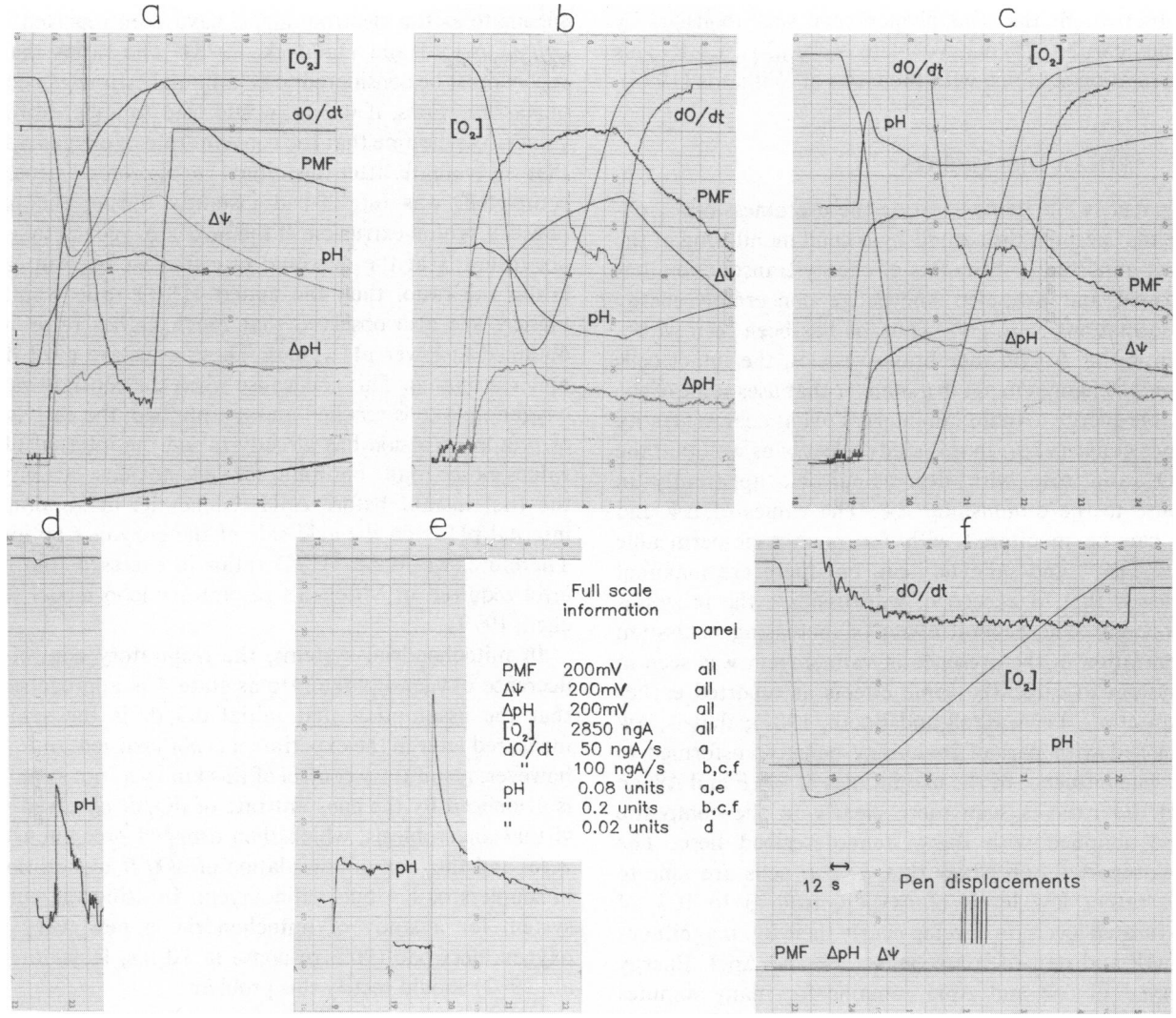

FIGURE 10 Different patterns of proton suck-back in relation to PMF, ΔΨ, and ΔpH. Cell concentration and buffer composition was the same as described in the legend to Fig. 1 except for *d* where 2.0 mg protein of *E. coli* cells were used and except for the pH which was at 6.0. The cells used in *b–f* were stored at −61° in 9% glycerol, 50 mM Tris·Cl, 1 mM $MgCl_2$ at pH 7.4 for 6 mo. The cells used in *a* were stored for 1 mo. Other unique details relating to the experiments shown in the panels are as follows: in *c* the experiment was in the presence of 0.5 μM valinomycin; in *d* and *e* the experiments were in the presence of 5 μM valinomycin and 75 mM K_2SO_4, oxygen was injected in buffer (4 ngA/ml in *d*, 2 ng A/ml in *e*-[*left*], and 32 ngA/ml in *e*-[*right*]) and measurements for PMF, ΔΨ, and ΔpH were not made. The experiment shown in *f* was done in the presence of 0.5 μM valinomycin plus 10 μM FCCP. Pen displacements are ΔpH, pH, ΔΨ, dO/d*t* PMF, and [O_2], from left to right.

back (Fig. 10 *a*), and suck-back to a greater extent than was originally translocated to the external medium (Figs. 1 *a*, 1 *d*, and 10 *b*). The lack of suck-back in Fig. 10 *a* is explained by the extreme proton impermeability of fresh cells. In cases where 5 μM valinomycin was used, with either fresh or aged cells, when there was no ΔΨ to actively pull back the extruded protons there was no suck-back. The cases where more protons were pulled into the cell interior than were initially extruded fall into two categories. In Fig. 1 *a*, where there was no ΔpH, a high ΔΨ, a very low K^+ permeability, and a partial permeability to protons developed upon aging, protons were pulled back by the existing strong membrane potential. Fig. 10 *b*, however, presents a much different situation. Although more protons were pulled into the cell from the external medium than were initially extruded, the ΔpH did not collapse to zero. This same situation, seen to a small extent in Fig. 1 *d*, in the presence of 0.5 μM valinomycin was often encountered at lower external pH's with this level of valinomycin. That is, the external medium showed a net alkalinization while a ΔpH, inside alkaline, still existed. The net alkalinity of both the external and internal aqueous compartments had to be balanced by a supply of protons somewhere else. A possible explanation of this apparent dilemma is that a supply of protons may be sequestered inside the membrane. Consistent with this possibility are

the observations that this phenomenon was abolished by treatment with 0.2% deoxycholate or 10 μM FCCP. This interpretation is in line with the views of Williams (1975).

FINAL DISCUSSION

This paper, while demonstrating the usefulness of a technique that permits the second-by-second monitoring of the various important parameters of energy transduction, has confirmed and extended knowledge concerning energy transformations in *E. coli*. Thus, as has been seen with a suspension of *E. coli* membrane vesicles, the intact cells respond to changes in pH in a manner that uses some of the available energy to maintain internal pH at near neutrality (Ramos et al., 1976). In this study, we saw as well that the H^+/O ratio rose with decreasing pH, apparently in response to the diminishing $\Delta\Psi$. The values of $\Delta\Psi$ and ΔpH can be monitored with the membrane-permeable probes TPP^+, and salicylic acid, but there are maximal safe levels that, if exceeded, will dissipate the processes under study. The general effect of increasing potassium concentration in the presence of valinomycin was seen in our system to cause the same effects as reported earlier (Padan et al., 1976; Kroll and Booth, 1981); that is, $\Delta\Psi$ diminished with part of its energy being transformed to ΔpH. The influence of the magnitudes of PMF and $\Delta\Psi$ on the H^+/O ratio is seen more clearly in the composite picture obtained with the system described here. The studies reported here show that *E. coli* cells are able to utilize rather low levels of oxygen, from 3 to 10% of air-saturated levels, to develop nearly their full magnitudes for PMF and its two components, $\Delta\Psi$ and ΔpH. Energy conserved in $\Delta\Psi$ and ΔpH remains for many minutes beyond the stage of total anaerobiasis. The technique of using a computer to measure H^+/O ratios from the initial rapid rate of extrusion yields considerably higher values than those obtained by the more standard procedure of determining the amount or rate of proton release in response to an injection of a small pulse of oxygen. This is because the most rapid rate was maintained only briefly before the developing $\Delta\bar{\mu}_{H^+}$ retarded further proton release, even in the presence of valinomycin, and because the response (~1 s for 63% of full signal) of even a relatively fast pH electrode leads to an underestimate of initial rates. The magnitude of the H^+/O ratio is important in understanding the mechanism of the conversion of respiratory energy to ATP. According to the concept of Mitchell, the stoichiometry must be two H^+ per energy-transducing site, which would produce a ratio of four for the part of the chain from succinate to oxygen (Mitchell and Moyle, 1967). Values of 6–8 in mitochondria have been reported (Wickström and Krab, 1979; Brand et al., 1976; Vercesi et al., 1978). In *E. coli* only two sites between NADH and oxygen are reported to be coupled to proton translocation with a maximum H^+/O ratio of 4.0 (Lawford and Haddock, 1973; Brookman et al., 1979). Values of <3, using succinate as the electron donor, have been reported in *E. coli* (Lawford and Haddock, 1973). The ratios that we report must be considered to be minimal estimates because our assumptions, if wrong, would lead to underestimated values. We assume that the constant rate of oxygen uptake that we evaluate, after the initial rate of proton extrusion is completed, was established instantly during the initial phase of proton extrusion. If the rate of oxygen uptake was not instantly at the maximal rate that we used in calculating the ratio, then the actual H^+/O ratio would be higher. We also observed that much higher ratios were obtained at lower pH values. These rates are most likely underestimated. This is because when the constant rate of oxygen uptake is reached and maintained, the early stage of proton extrusion has already raised the internal pH to values closer to the optimum for succinoxidase activity. In the first instant before ΔpH reaches its maximum, the internal pH is on the acid side of the enzyme's optimum. Therefore, we found H^+/O ratios in excess of the fixed level required in Mitchell's respiratory loop model (Mitchell, 1979).

In mitochondrial systems, the respiratory control will decrease oxygen uptake rate as state 4 is approached, so that the assumption that initial dO/dt is the same as measured later in the experiment is not justified. In *E. coli*, however, respiratory control of this kind was not seen. This is evidenced by the constant rate of dO/dt observed until virtual anaerobiosis, which then dropped precipitously to zero, and the lack of stimulation of dO/dt by addition of uncouplers or K^+ plus valinomycin. In using the current system for a study of mitochondria, a new, very fast, oxygen electrode (90% response in 10 ms, Reynafarje et al., 1982) should rectify this problem.

Received for publication 1983 and in final form 1983.

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