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Estimation of H^+ to Adenosine 5′-Triphosphate Stoichiometry of *Escherichia coli* ATP Synthase Using ^{31}P NMR†

Robert Vink, M. Robin Bendall, Stephen J. Simpson, and Peter J. Rogers*

ABSTRACT: High-field ^{31}P NMR techniques have been used to measure transmembrane ΔpH in wild-type, *unc A*, and *hem A* mutants of *Escherichia coli*. $\Delta\psi$ was measured by distribution methods with radioactive tetraphenylphosphonium bromide and $^{86}Rb^+$ ions as the probes, while intracellular ATP, ADP, and inorganic phosphate concentrations were determined from the ^{31}P NMR spectra. $\Delta G'_p$ and the stoichiometry for ATP synthesis $[\Delta G'_p/(F\Delta p)]$ were then calculated. The stoichiometry of the ATP synthase was found to vary as a function of the cellular metabolic state. In nongrowing, wild-type cells Δp was 192 ± 16 mV with succinate as the substrate and saturating oxygen tension. With limiting oxygen (≃1 μM oxygen), Δp was 125 ± 14 mV. Nucleoside triphosphate synthesis was observed in both cases. The H^+/ATP stoichiometry varied from 2.15 ± 0.35 under aerobic conditions to 3.6 ± 0.8 at low oxygen tension. Δp for *unc A* cells was 140 ± 14 mV with glucose as the substrate (>2.5 μM oxygen) and for *hem A* mutants was 115 ± 10 mV. The bulk phase potentials in oxygen-limited, wild-type cells and in respiratory deficient (*hem A*) cells are comparable, but in the former the ATPase is poised for synthesis while in the latter it generates Δp. The data support a role for localized interactions between the redox and the ATPase sites.

The chemiosmotic theory postulates that the electron-transport chains of bacteria are coupled to ATP synthesis by a protonmotive chemical potential (Δp)[1] across the energy-transducing membrane [for a review, see Mitchell (1979)]. Under aerobic conditions, this protonmotive force can be generated by proton efflux during respiration, and coupling of Δp to ATP synthesis occurs via a membrane-bound ATPase complex. Conversely, during anaerobic growth, or when the respiratory proton pump is impaired, the ATPase complex can act as an ATP-dependent proton pump [for a review, see Harold (1977)].

Studies with artificially generated proton gradients indicate that the ATPase system is poised with Δp potentials of about 200 mV (Maloney, 1982). However, lower values of Δp associated with oxidative growth have been reported [Guffanti et al., 1981; for a review, see Ferguson & Sorgato (1982)]. Combined with the recent evidence that oxidative growth of uncoupler-resistant mutants occurs despite very low values of Δp (Decker & Lang, 1978; Ito & Ohnishi, 1981; Ito et al., 1983), the question has been revived whether localized membrane potentials, in addition to bulk phase potentials, are responsible for activation of the ATP synthase (Williams, 1961; Kell & Morris, 1981; Skulachev, 1982; Westerhoff et al., 1984).

A related question of interest is the H^+/ATP stoichiometry of ATP synthesis catalyzed by the ATPase. According to the chemiosmotic theory, a thermodynamic equilibrium should exist under steady-state conditions (Mitchell, 1979). The stoichiometry (n) of the reaction

$$nH^+_{ext} + ADP + P_i \rightarrow ATP + H_2O + nH^+_{int}$$

is calculated from $\Delta G'_p/(F\Delta p)$, where $\Delta G'_p$, the phosphorylation potential, is the Gibb's free energy of ATP synthesis. $\Delta G'_p$ may be calculated from

$$\Delta G'_p = \Delta G^{\circ\prime} + RT \ln [[ATP]/([ADP][P_i])]$$

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[1] Abbreviations: ATPase, adenosinetriphosphatase (EC 3.6.1.3); CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EDTA, ethylenediaminetetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; *F*, Faraday's constant; FID, free-induction decay; NMR, nuclear magnetic resonance; TPP^+, tetraphenylphosphonium ion; $\Delta G'_p$, phosphorylation potential; $\Delta G^{\circ\prime}$, free energy of ATP hydrolysis; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane proton gradient; Δp, protonmotive force. Subscripts ext and int refer to the external buffer and the cytoplasm, respectively.

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where $\Delta G^{\circ\prime}$ is the standard free energy of ATP hydrolysis. Δp consists of two parameters: the chemical potential (ΔpH) and the transmembrane electrical potential ($\Delta\psi$) as defined by the relationship $\Delta p = \Delta\psi - (RT/F)\Delta$pH. ΔpH and $\Delta\psi$ have usually been measured by distribution of weak acids across the bacterial membrane, while quantitation of P_i and nucleoside phosphates has been based on analyses of cell extracts. High-field ^{31}P NMR allows the simultaneous quantitation of NTP, NDP, and P_i, together with the determination of ΔpH. $\Delta\psi$ must still be measured by distribution methods; however, this value can be obtained on the same set of cells. $\Delta G'_p/(F\Delta p)$ may be evaluated by combining both techniques.

We have estimated n in aerobic and oxygen-limited cells with succinate as the substrate and find that n increases when oxygen becomes limiting. In aerobic cells Δp reached 192 ± 16 mV while at $\simeq$1 μM oxygen Δp was reduced to 125 ± 14 mV, which is comparable to Δp in *unc* mutants lacking F_1F_0-ATPase activity and *hem A* mutants that lack respiratory competence. In the *hem A* mutant, the F_1F_0-ATPase H^+ pump energizes the membrane, whereas, in the oxygen-limited, wild-type cells at a comparable bulk phase membrane potential, redox-dependent ATP synthesis is observed. The data are supportive of the concept of direct energy coupling of respiratory and ATPase sites by local electrochemical gradients.

Materials and Methods

Materials. All chemicals were reagent grade and were obtained commercially, except TPPBr, which was synthesized in our laboratory. Radiochemicals were purchased from Amersham.

Synthesis of TPPBr. A total of 2.62 g of triphenylphosphine, 1.1 mL of bromobenzene, and 2.2 g of nickel bromide was suspended in benzonitrile and refluxed under nitrogen with stirring for 20 h at 190 °C. The reaction mixture was then cooled and steam distilled for 4 h. The residue was extracted 3 times with 20 mL of $CHCl_3$, and the extracts were concentrated by rotary evaporation. The product was purified by recrystallization in ether. Purity was checked by thin-layer chromatography and melting point determination.

Bacterial Strains. Escherichia coli AN704 (*hem A*) and AN721 (*hem A unc A401*) (Brookman et al., 1979; Downie et al., 1979) were obtained from Professor F. Gibson (Australian National University, Canberra). With δ-aminolevulenic acid (ALA) present, normal respiratory competency is developed.

Growth Conditions. Cells were grown at 37 °C in M9 minimal medium (Davis & Mingioli, 1950) supplemented with 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 μM 2,3-dihydroxybenzoic acid, 50 μM thiamine, 20 μg/mL amino acid supplements, and 0.4% w/v glucose or 50 mM succinate.

^{31}P NMR of Intact Cells. Cells were harvested during midlogarithmic phase of growth and washed twice in NMR buffer [100 mM PIPES, 50 mM MES, 5–10 mM orthophosphate ($[K_2HPO_4]/[KH_2PO_4]$ = 1), 100 mM KCl, 5 mM $MgSO_4$, 2 mM EDTA, pH 7.4]. The cells were resuspended in the same buffer (40–50 mg dry wt/mL) and stored on ice for a maximum of 2 h; 5 min prior to use, the cells were warmed to 25 °C.

NMR analyses were carried out in 10 mm diameter tubes with 2 mL of cell suspension in the case of anaerobic studies and 3 mL for aerobic experiments. Aerobic conditions were established with oxygen or air sparging (20–100 mL/min, as specified in the figure legends) from a single capillary exiting below the level of the detection coils. Oxygen levels were recorded in parallel experiments with the same cells under identical conditions by using a galvanic oxygen electrode. Air-saturated medium contained 150 μM oxygen. Electrode response was linear from 0.2 to 100% air in nitrogen. Anaerobic conditions were maintained by gasing with nitrogen in the head space above the sample volume.

^{31}P NMR spectra were obtained with a Bruker CXP-300 spectrometer operating in the Fourier-transform mode at 121.5 MHz. Spectra were collected with a 0.34-s repetition time and a 60° pulse. Previous studies showed that T_1's of $P_{i,int}$ and NTP_γ resonances were 0.4 and 0.2 s for aerobic suspensions of glucose-grown *E. coli* cells (Brown et al., 1977). Ugurbil et al. (1982) reported that only the $P_{i,ext}$ and the NAD resonances were saturated under the rapid pulsing conditions on the basis of the comparability of spectra obtained with 2.5- and 0.34-s repetition times with the same 60° pulse angle. Thus NTP, NDP, and P_i were quantitated by comparison with the resonance strength of authentic standards. Orthophosphoric acid at 85% was used as an external reference.

Sensitivity of P_i Resonance to pH. To relate the chemical shift of P_i to internal pH, *E. coli* cells were resuspended in NMR buffer containing the uncoupler CCCP (50 μM) and titrated with acid or base (Ogawa et al., 1981). The change in chemical shift of P_i in response to acid or base addition was related to pH by measuring the suspension pH before and following spectra accumulation. The levels of K^+, Mg^{2+}, ATP, ADP, and organic acids are known to affect the chemical shift of P_i (Roberts & Jardetzky, 1981). Changes in chemical shift due to small changes in NTP concentration (2–8 mM) are generally low (Ogawa et al., 1981). We have made no corrections for ATP effects upon P_i chemical shifts. Excess Mg^{2+} ions present in the buffer may compensate for changes when the intracellular adenylate charge varies; free intracellular Mg^{2+} concentration in *E. coli* seems to be no greater than approximately 1 mM (Ugurbil et al., 1982). The effects of ionic strength on the pK_a of P_i are less marked when the ionic strength exceeds about 150 mM (Ogawa et al., 1981).

Numerical deconvolution of individual spectra was carried out to determine the corrections to be applied to the observed peak separations when overlap of spectral lines occurred. The large vertical errors arising from instrumental noise (see error bars on the spectra) give rise to an error in this small correction term of about 100%. Generally, the estimated increase in chemical shift separation, after simulations were carried out, was between 10 and 20%.

$\Delta\psi$ Measurements. $\Delta\psi$ was calculated, with the aid of distribution probes, from the Nernst equation (Mitchell & Moyle, 1969). Two probes were used: (a) $^{86}Rb^+$ in the presence of valinomycin and (b) TPP^+Br^-. With $^{86}Rb^+$, the filtration method was used (Schuldiner & Kaback, 1975) and for the latter flow dialysis (Ramos et al., 1979).

(a) The harvested cells were washed twice and resuspended in 20 mM MES, 20 mM PIPES, 10 mM EDTA, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, and 100 mM choline chloride, adjusted to pH 7.4 with NaOH (buffer A). In some experiments the final resuspension buffer was 5 mM Tris, 5 mM MES, and 100 mM choline chloride, pH 7.4. Results from both buffers were similar. The final cell density was between 1 and 5 mg dry wt/mL. Valinomycin was added to a final concentration of 4 μg/mg of bacterial dry wt and the cell suspension incubated at 25 °C for 5 min before ^{86}RbCl was added. Anoxic conditions were established by sparging with nitrogen. Dissolved oxygen concentration was raised by sparging with air or oxygen and varied by manually altering the gas flow rate. Oxygen concentration was measured with a galvanic oxygen electrode. ^{86}RbCl (2 mM, 2 μCi/μmol) was

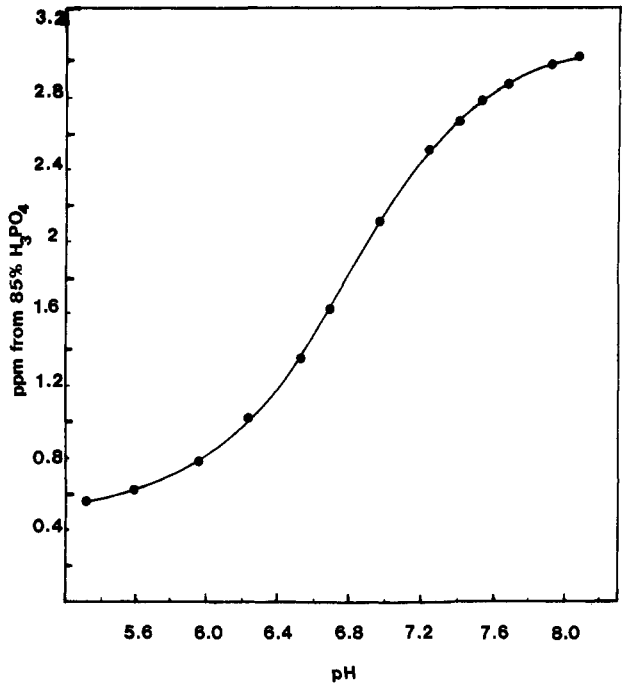

FIGURE 1: Dependence of the chemical shift of P_i on pH. Late logarithmic cells were harvested, washed, and resuspended in 100 mM PIPES, 50 mM MES, 5 mM orthophosphate, 100 mM KCl, 5 mM $MgSO_4$, 2 mM EDTA, and 50 μM CCCP, pH 7.4, at 50 mg dry wt/mL. Acid or base was added, and pH and chemical shift were measured.

added to 0.1 mM final concentration. Glucose or sodium succinate (50 mM) was added 10 min after isotope addition. Aliquots (0.5 mL) were thereafter regularly withdrawn and filtered (0.45 μm, cellulose mixed ester, Nucleopore) and washed twice with 2 mL of 0.2 M LiCl. Filters were counted for radioactivity with Aquasol as the scintillant in a Beckman LS250 counter.

(b) For estimation of $\Delta\psi$ by flow dialysis, with TPP^+ as the probe, a dialysis chamber of the same design as reported by Ramos et al. (1979) was used. Cells were collected and washed in EDTA-containing buffer as described in (a) and finally resuspended in 20 mM MES, 20 mM PIPES, 10 mM EDTA, 5 mM KH_2PO_4, 5 mM K_2HPO_4, and 100 mM KCl, adjusted to pH 7.4 with KOH (buffer B). The final cell density was in the range 1–5 mg dry wt/mL. Dialysis buffer was sparged with air or nitrogen for aerobic or anaerobic conditions, respectively. For measurements under oxygen-limited conditions (<2 μM), the dialysis buffer was sparged with nitrogen until the oxygen level was in the required range. Sparging was then ceased, but a nitrogen atmosphere was maintained above the buffer. The buffer was pumped into the lower chamber at 1 mL/min. TPP^+ (final concentration 10 μM, 116 μCi/μmol) was added to 1.6 mL of buffer B. After 10 min, 0.2 mL of cell suspension (20–40 mg dry wt/mL) was injected; 15 min later, 0.1 mL of substrate (1 M glucose or 1 M sodium succinate) was added. Spent dialyzate was collected in 2-mL fractions and radioactivity measured as before.

Intracellular volume was determined by the method described by Rottenberg (1979a). Values were within the range 1.5–1.7 μL/mg dry wt of cells, for both AN704 and AN721.

Measurement of Respiration and Oxygen K_m. The respiration rate was measured with a Clarke oxygen electrode and a jacketed stirring chamber. Cell suspensions (3 mL) (in buffer A or B, 1–10 mg dry wt/mL) were added with substrate (50 mM succinate), and the rate of oxygen consumption was measured. Respiration rates at low oxygen concentration (<5 μM) were determined at low cell densities, so that the response

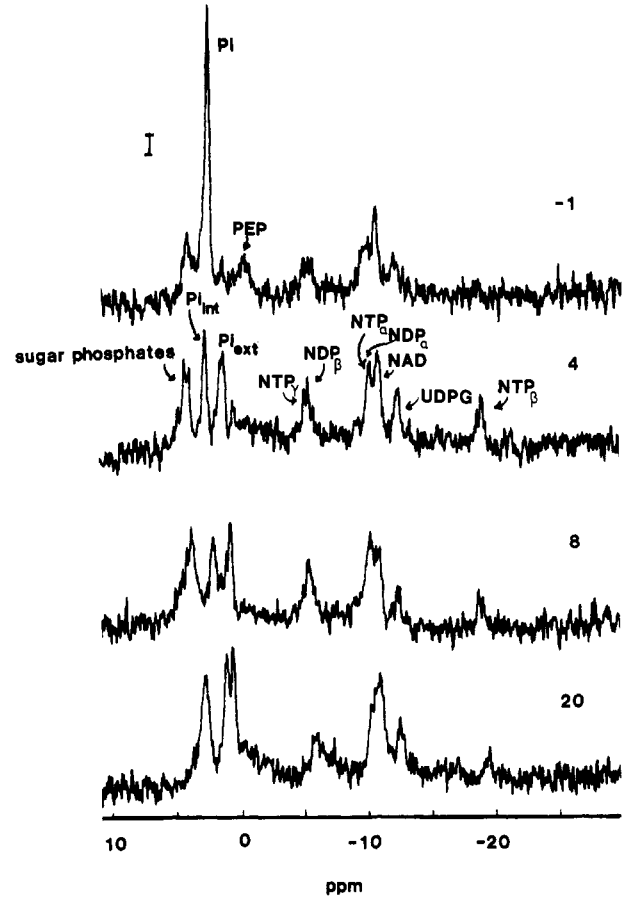

FIGURE 2: The 121.5-MHz ^{31}P NMR spectra of anaerobic, glucose-grown, *E. coli* AN704 (+ALA) cells during glucose metabolism. Each spectrum represents 352 FIDs obtained with a repetition time of 0.34 s and a 60° pulse. Cells were treated and resuspended as detailed in Figure 1 (−CCCP) at 25 °C and a final cell density of 50 mg dry wt/mL. The numbers beside the spectra refer to minutes after glucose addition. Glucose (50 mM) was added at 0 min. Instrumental noise is represented by the error bar.

of the oxygen electrode was not limiting. K_m was 1.5 ± 0.2 μM for late logarithmic phase AN704 cells grown on succinate in minimal medium. The K_m for AN721 (*unc A401*) cells with 50 mM glucose present initially was 1.4 ± 0.2 μM.

Results

Sensitivity of P_i Resonance to pH. Effects of acid or base titration upon the chemical shift of P_i, under conditions approximating the intracellular environment, are shown in Figure 1. The pK_a of the titration curve was 6.90. When the ionic strength was increased 2-fold with KCl, only a slight change in pK_a (6.95) was observed. Since chemical shift measurements were made to within 0.05 ppm, we estimate the error in reported pH values, due to ionic strength fluctuations, to be 0.05 pH unit.

ATPase-Dependent ΔpH Formation in Anaerobic Cells. Figure 2 shows a series of ^{31}P NMR spectra collected prior to and after glucose addition to wild-type cells. Cells were N_2 sparged, and oxygen electrode measurements showed that the dissolved oxygen concentration was <0.1 μM. Prior to glucose addition, only one P_i resonance at 2.65 ppm was observed. Some hexose and triose phosphate peaks were present at 4.2 ppm. The peaks at −10 ppm have been assigned to the α-phosphorus of NDP and also to $NAD(P)^+$, while those at −5.6 and −11.8 ppm have been assigned to the β-phosphorus of NDP and to UDPG, respectively. After glucose addition an NTP_β peak appeared at −19 ppm, and the maximum of the peak previously assigned to NDP_β shifted to −4.8 ppm, indi-

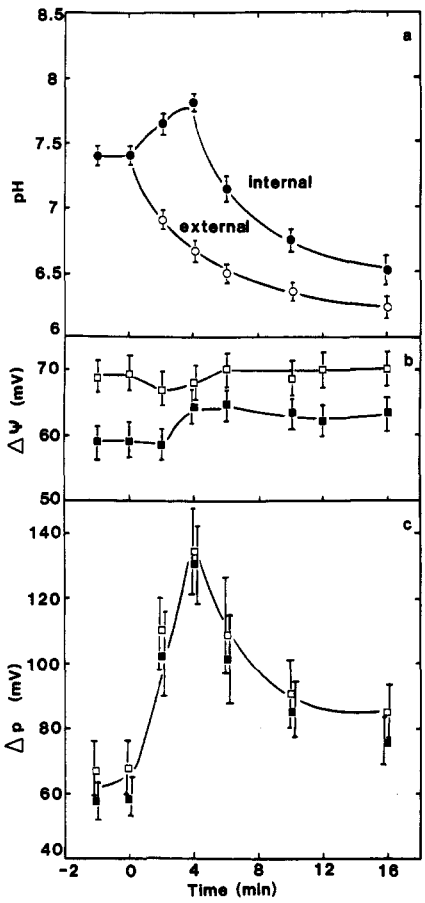

FIGURE 3: ΔpH (a), $\Delta\psi$ (b), and Δp (c) changes accompanying anaerobic, glucose metabolism in *E. coli* AN704 (+ALA) at 25 °C. ΔpH was calculated from the chemical shift data in Figure 2; $\Delta\psi$ was measured by the filtration method with Rb^+ (■) and also by flow dialysis with TPP^+ (□). Confidence limits are included.

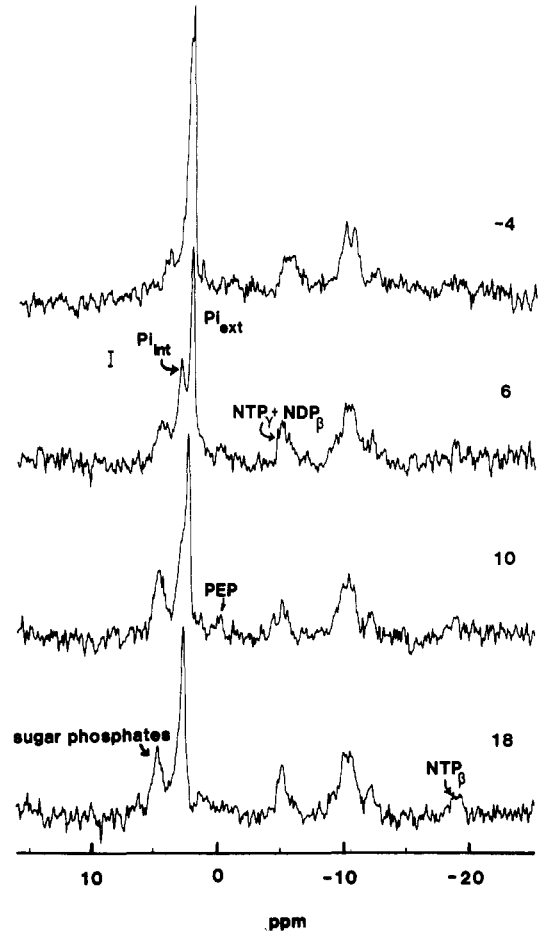

FIGURE 4: The 121.5-MHz ^{31}P NMR spectra of succinate-grown *E. coli* AN704 (+ALA) during aerobic succinate metabolism. Succinate was added to 50 mM at zero time; cell density was 50 mg dry wt/mL. Spectra represent 704 FIDs obtained with a repetition time of 0.34 s and a 60° pulse. The cell suspension was sparged with a single glass capillary at 75 mL/min. Oxygen concentration exceeded 2.5 μM, and the temperature was 25 °C.

cative of NTP synthesis and a decline in NDP concentration (NTP_γ, −5 ppm; NDP_β, −5.6 ppm; Ugurbil et al., 1978). Also, the sugar phosphate signal increased, and the P_i peak split into two; one peak moved upfield indicating a more alkaline environment and the other downfield corresponding to a drop in pH (Figure 3a). Addition of P_i enhanced the latter signal, indicating that it was the external P_i pool. The P_i split was maximal after 4 min. Estimation of pH from the chemical shift of P_i showed that ΔpH exceeded 1.1 units at this time. Thereafter, both the internal pH and the external pH decreased, along with the ΔpH, although pH_{int} was always higher than pH_{ext}. The collapse of ΔpH paralleled the decrease in NTP level—most easily seen from the NTP_β resonance (Figure 2).

The data indicate that the F_1F_0-ATPase is responsible under anaerobic conditions for membrane energization. This is supported by similar studies of the *unc* mutant AN721 (the ATPase catalytic unit F_1 is defective). Anaerobic glucose metabolism in this strain produced no ΔpH during the 30-min incubation period, but relatively high levels of NTP accumulated (spectra not shown). Presumably this happens because the ATPase H^+ pump is inactive. Similar findings were reported by Ugurbil et al. (1982).

ΔpH Formation by Respiring Cells. ΔpH was measured in aerobic cultures of wild-type cells and AN721 with succinate and glucose as the substrates. The *unc A* mutant was studied because limiting membrane energization may be approached in cells that lack ATP synthase activity. ^{31}P NMR spectra of wild-type cells under aerobic conditions (>2.5 μM oxygen) were collected immediately before and following succinate addition (Figure 4). In pre-succinate spectra a large P_i peak, representing extracellular and cytoplasmic pools, was present; very little signal was observed around 4 ppm, indicating the absence of triose and hexose phosphates. The signal centered near −10 ppm may include contributions from NDP_α, NTP_α, and NAD(P). However, since the NTP_β signal is negligible (−19 ppm), the −10 ppm composite is most likely due to NDP_α (−10 ppm) and NAD(P) (−10.7 ppm), on the basis of earlier assignments (Ugurbil et al., 1978). Likewise, as mentioned earlier, the NTP_γ and NDP_β bands have very similar resonances (−5 and −5.6 ppm) (Ugurbil et al., 1978). In pre-succinate cells, there is a peak in the region −5.2 to −6.0 ppm, which probably represents mainly NDP. After succinate was added this peak sharpened, and the peak maximum shifted to −5 ppm, consistent with accumulation of NTP and a decline in NDP concentration. The P_i peak separated into two; the sugar phosphate signal increased (≃4 ppm), and an NTP_β peak was detected. There was also an indication of PEP formation (≃0–1 ppm). Therefore, succinate metabolism produced a transmembrane pH gradient and resulted in NTP and sugar phosphate synthesis. The two P_i resonances on which ΔpH estimation was based showed maximum separation after 6 min although they never completely resolved. To locate the cytoplasmic P_i signal as accurately as possible, the spectra were resolved numerically into two component Lorentzians. In the case of the 6-min spectrum, the simulated peak of the $P_{i,ext}$ component was barely shifted relative to that of the

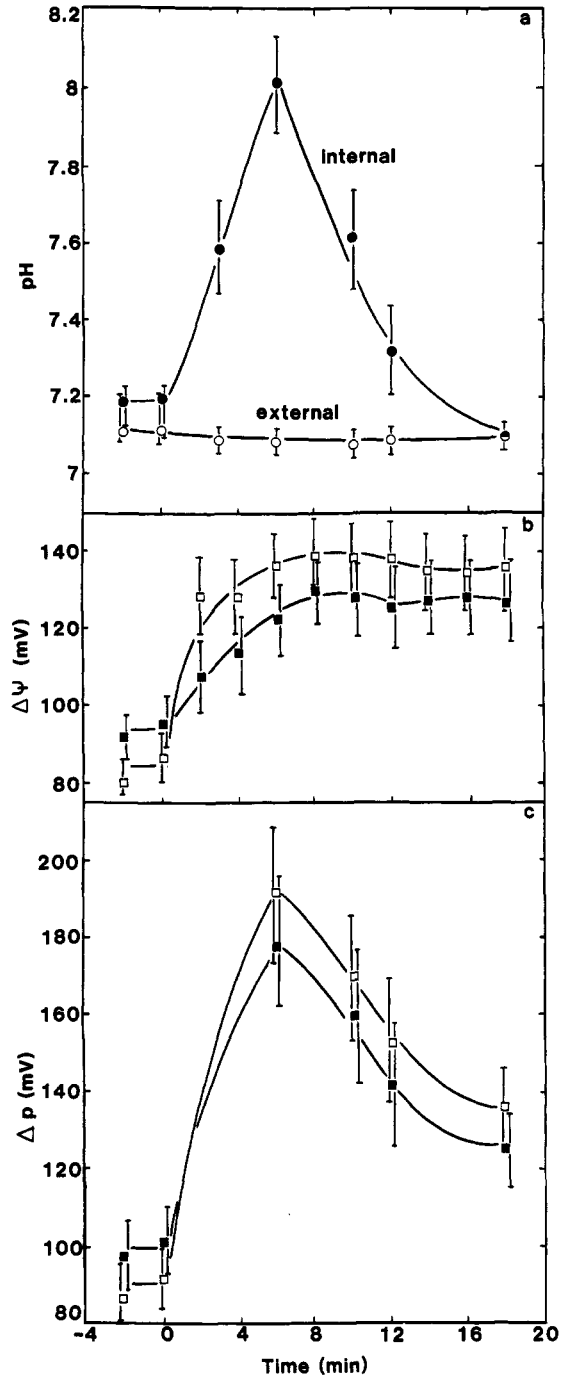

FIGURE 5: ΔpH (a), $\Delta\psi$ (b), and Δp (c) changes accompanying aerobic, succinate metabolism in *E. coli* AN704 (+ALA) at 25 °C. ΔpH was calculated from the chemical shift data in Figure 4; $\Delta\psi$ was measured by the filtration method with the Rb^+ probe (□) and also by flow dialysis with TPP^+ (■). Confidence limits are included.

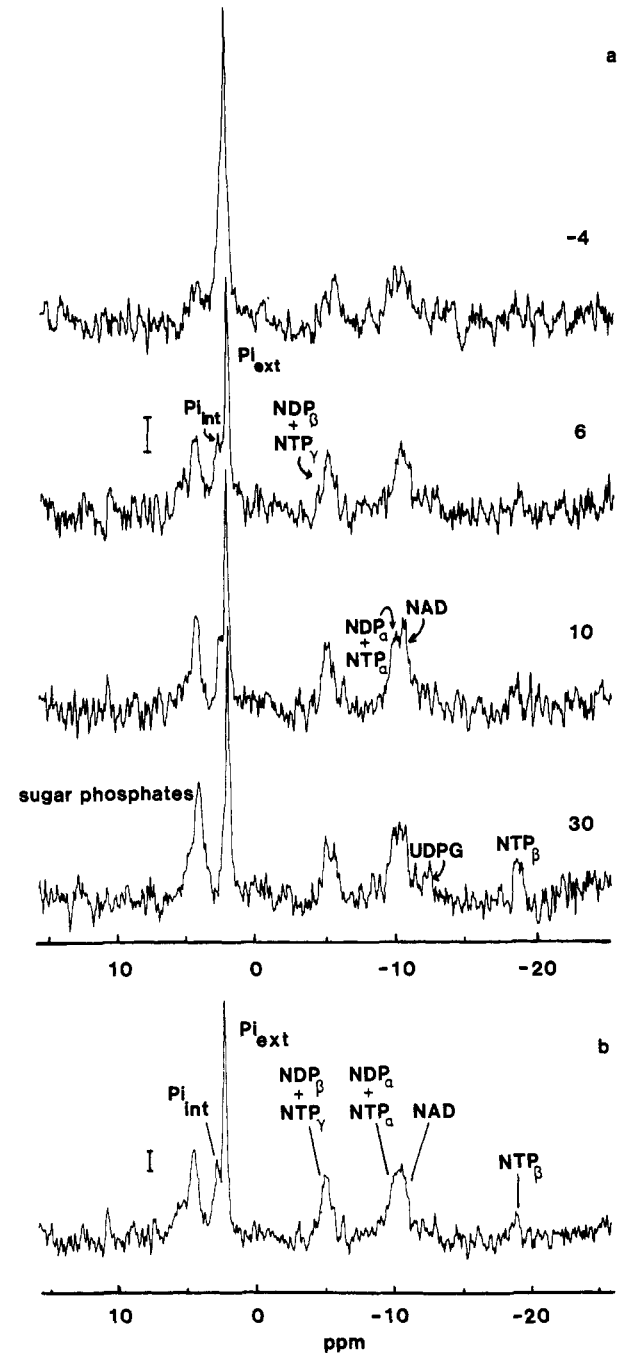

FIGURE 6: The 121.5-MHz ^{31}P NMR spectra of glucose-grown *E. coli* AN721 (+ALA) (resp$^+$ ATPase$^-$ phenotype) during aerobic glucose metabolism. Glucose was added to 50 mM at zero time, and the oxygen concentration exceeded 2.5 μM. Spectra (352 FIDs) were collected with a 0.34-s repetition time and a 60° pulse. Those spectra corresponding to time blocks 2–4, 4–6, and 6–8 min were summed, and the resultant is shown as (b). The initial pH of the cell suspension (50 mg dry wt/mL) was 7.4.

corresponding peak of the observed profile, but the position of the $P_{i,int}$ component was shifted away from that of the $P_{i,ext}$, increasing the separation by almost 15%. The maximum pH separation between cytoplasm and suspension buffer was therefore 0.92 ± 0.16 unit (Figure 5). ΔpH thereafter collapsed quite rapidly and was absent after 12 min (Figure 5). Oxygraph experiments showed that the apparent K_m for respiration was less than 1.5 μM oxygen. Under the oxygenation conditions maintained above, the oxidase system was therefore saturated.

Similar ^{31}P NMR experiments were performed with AN721 (resp$^+$ ATPase$^-$) under aerobic conditions (Figure 6). When glucose was added to thick cell suspensions, the sugar phosphate signal increased as did the NTP_β resonance (−19 ppm); at the same time, the maximum of the NTP_γ + NDP_β composite peak moved from −6 to −5 ppm. The high concentration of sugar phosphates and nucleotides, particularly NTP in the absence of ATPase H^+-pump activity, may account for the reduction in the strength of the cytoplasmic P_i signal. When P_i was omitted from the suspension buffer, there was a rapid decline in the P_i signal strength when glucose was added. Peak position was constant (for 10 min after glucose) and comparable to the position of the shoulder in the 6-min spectrum in Figure 6. However, the latter is within the level of the background noise. By adding the 4-, 6-, and 8-min spectra, it was possible to enhance the signal to noise ratio by $3^{1/2}$

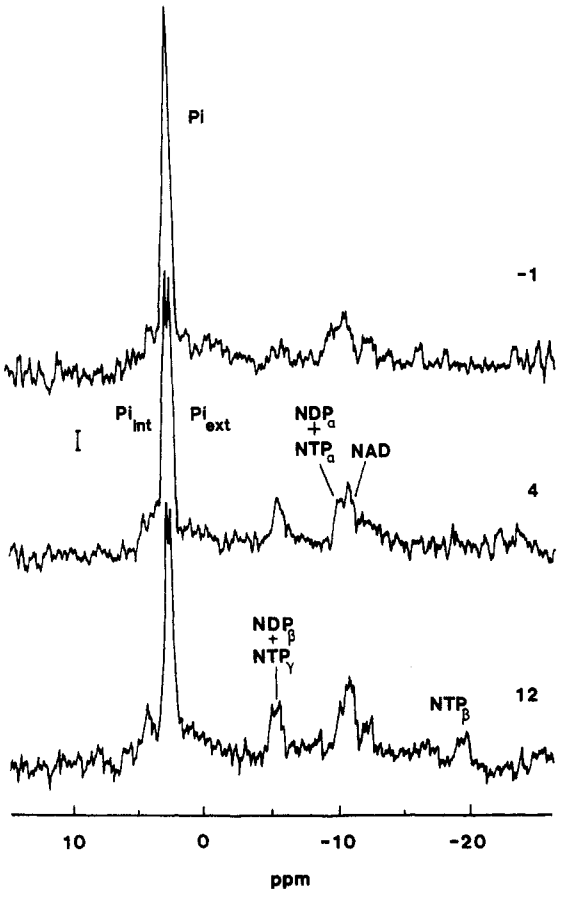

FIGURE 7: The 121.5-MHz ^{31}P NMR spectra of succinate-grown *E. coli* AN704 (+ALA) during succinate metabolism under oxygen limitation. Succinate was added to 50 mM at zero time. The cell suspension was sparged with air (20 mL/min), and the dissolved oxygen level was $\simeq 1$ μM. Spectra (704 FIDs) were collected with a repetition time of 0.34 s and a 60° pulse.

(Figure 6, spectrum b), and deconvolution of the P_i peak and shoulder indicated that the maximum ΔpH was about 0.6 ± 0.2 unit.

ΔpH Formation in Oxygen-Limited Wild-Type Cells. If the oxygen tension was lowered ($\simeq 1$ μM) so the oxidase system was no longer saturated, NTP and a small amount of sugar phosphates were still synthesized by wild-type cells in the presence of succinate. A partial splitting of the P_i resonance occurred (Figure 7), which after deconvolution indicated that the maximum ΔpH formed under these conditions was 0.45 ± 0.17 unit (Figure 8).

ΔpH Formation by Respiratory-Deficient Cells. Comparable studies with respiratory-deficient cells, and the double-mutant phenotype resp$^-$ ATPase$^-$ (AN721 grown in −ALA medium), supported the assumption that the changes in the chemical shift of the P_i peaks reflect pH changes dependent upon the respiratory proton pump or the hydrolytic activity of the F_1F_0-ATPase. ΔpH formation in respiratory-deficient cells (AN704 grown in −ALA medium) in the presence of glucose reached almost 1 unit, irrespective of oxygen tension. On the other hand, the mutants without F_1F_0-ATPase activity and a functional respiratory chain (AN721 grown in −ALA medium) only produced a very small pH gradient (<0.1 unit) toward the end of glycolysis (>40 min, oxygen concentration <2.5 μM). No transient alkalization of the cytoplasm occurred, and the ΔpH formed is probably due to lactic acid efflux (Simpson et al., 1983).

Δψ Measurements. Δψ values were obtained by flow dialysis with TPP^+ as the probe and also by using the filtration method

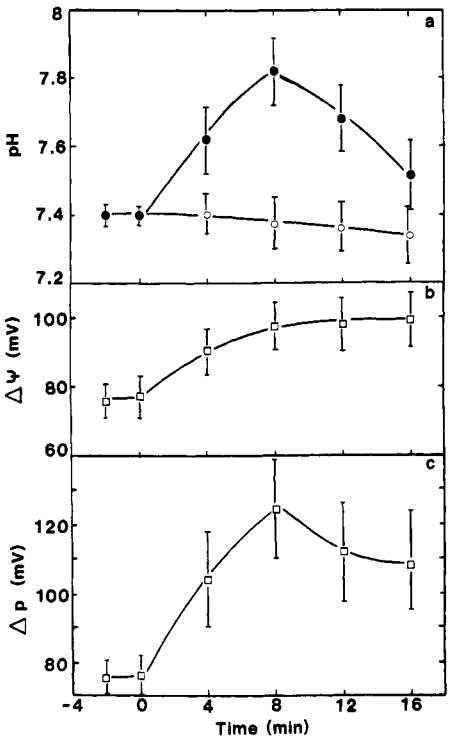

FIGURE 8: ΔpH (a), Δψ (b), and Δp (c) changes during oxygen-limited, succinate metabolism by *E. coli* AN704 (+ALA) cells. ΔpH data were obtained by transforming P_i chemical shifts from typical data (Figure 7). Δψ was estimated by Rb^+ and the filtration method. Confidence limits are shown.

with Rb^+ and valinomycin. Kashket (1981) has reported that high concentrations of EDTA and very small aliquots of cell suspension are necessary with the filtration method to avoid O_2 limitation. She obtained values as high as 164 mV in this way with *E. coli* cells. Using flow dialysis, we found that, with Mg^{2+} omitted from the resuspension buffer and higher EDTA levels (10–20 mM), Δψ values were approximately 20 mV higher for aerobic cells with succinate as the substrate. Variation of the K^+ concentration from 5 to 100 mM had no significant effect on Δψ (<5%), and likewise, Δψ determinations were independent of cell density up to about 5 mg dry wt/mL. At higher cell densities, Δψ decreased. We have assumed that higher values at a given oxygen tension reflect increased accessibility of the probe to the inner membrane surface, and for this reason Δψ measurements were made at low cell density (4 mg dry wt/mL) with high EDTA levels and with Mg^{2+} omitted. With Rb^+ as the probe the K^+ concentration was reduced to <2 mM, and choline was added to replace the omitted K^+ ions.

Under anaerobic conditions, the maximum Δψ value obtained for wild-type cells was 70 ± 4 mV with TPP^+ by flow dialysis and 65 ± 4 mV with Rb^+ by filtration (Figure 3b). Combining the Δψ and the ΔpH data, the maximum Δp was estimated to be 132 ± 14 mV. The maximum Δψ value for AN721 (resp$^-$ ATPase$^-$, −ALA medium) was considerably lower at around 40 mV by similar criteria, while the resp$^-$ ATPase$^+$ phenotype (AN704, −ALA medium) was intermediate in value (50 ± 7 mV; kinetics not presented). Since ΔpH formed during glycolysis by resp$^-$ ATPase$^-$ cells was negligible, these cells have the lowest Δp value of approximately 50 mV. The wild-type and resp$^-$ ATPase$^+$ AN704 cells, which exhibit comparable ΔpH formation ($\simeq 1$ unit) during glycolysis, had similar Δp values.

Δψ values measured under aerobic conditions were much greater in respiratory-competent cells. In the case of wild-type cells (>2.5 μM oxygen) with succinate as the substrate, Δψ

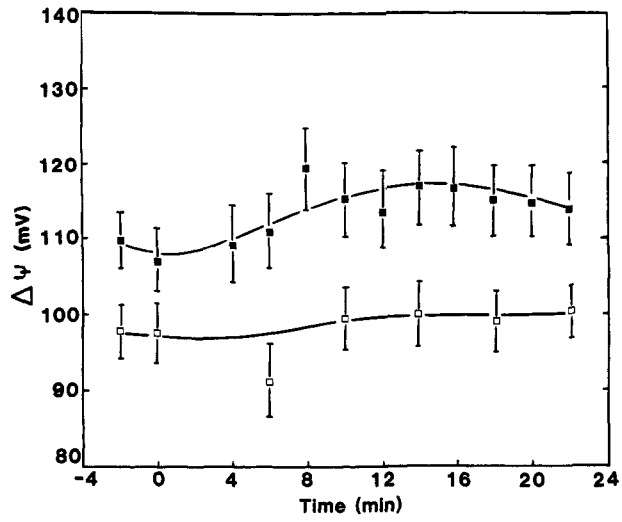

FIGURE 9: Changes in $\Delta\psi$ during aerobic, glucose metabolism in *E. coli* AN721 (+ALA) (resp$^+$ ATPase$^-$ phenotype): (□) Rb^+ and the filtration method; (■) TPP^+ and flow dialysis. The cell density was 4 mg dry wt/mL, and the oxygen concentration was >2.5 μM. Confidence limits are shown.

ranged from 138 ± 10 to 124 ± 9 mV on the basis of the Rb^+ and TPP^+ methods, respectively (Figure 5b). On the basis of the most optimistic values, Δp thus reached 192 ± 18 mV during oxidative metabolism. Under oxygen-limited conditions ($\simeq$1 μM oxygen), $\Delta\psi$ dropped to 100 mV (Figure 8), and Δp was reduced to 125 ± 14 mV. But even under these conditions NTP synthesis was observed.

By comparison the resp$^+$ ATPase$^-$ phenotype exhibited $\Delta\psi$ values of 115 ± 10 mV (TPP^+) when growth aerobically on glucose (Figure 9). $\Delta\psi$ values based on Rb^+ distribution were consistently lower than this and never exceeded 100 mV. Therefore, the highest estimate of Δp in these cells is 140 ± 15 mV.

The respiratory-deficient phenotype (AN704, −ALA medium) reached a ΔpH of 1.1 ± 0.15 during aerobic glycolysis. $\Delta\psi$ remained low (50 ± 6 mV), and Δp reached 116 ± 15 mV. This value is comparable to Δp in wild-type cells during limited oxygenation (125 ± 14 mV) (Figure 8).

$\Delta G'_p$ Calculations. $\Delta G'_p$ can be evaluated according to the relationship $\Delta G'_p = \Delta G^{\circ\prime} + RT \ln [[ATP]/([ADP][P_i])]$. $\Delta G^{\circ\prime}$ is dependent on Mg^{2+} concentration, ionic strength, and pH (Rosing & Slater, 1972; Slater, 1979). We have assumed internal Mg^{2+} concentration in *E. coli* to be approximately 1 mM (Ugurbil et al., 1982). Rosing & Slater (1972) reported that $\Delta G^{\circ\prime}$ for ATP synthesis is 30 kJ/mol at pH 7.4, with 1 mM Mg^{2+} present and the ionic strength in the range 0.1–0.2 M. The [ADP]/[ATP] ratio can be obtained from the ^{31}P NMR data if it is assumed that the nucleotide ratios and ATP/ADP ratios are equivalent and that the NDP and NTP signals represent similar proportions of the total pools (both bound and free).

For wild-type cells oxidizing substrate, the maximum NTP values and corresponding NDP and P_i levels were determined from spectra collected in the 12-min period immediately after succinate addition. The NDP concentration was obtained by subtracting the area of the NTP_β resonance at −19 ppm from the integrated area of the peak at −5 ppm. This was carried out for three separate batches of cells. The internal cytoplasmic volume of wild-type cells from aerobic batch cultures grown on succinate was 1.5–1.7 μL/mg of dry wt. The NTP, NDP, and P_i concentrations in respiring cells were estimated to be 2 ± 1, 6 ± 2, and 4 ± 2 mM, respectively (6-min spectrum). Substitution of these values into $RT \ln [[ATP]/([ADP][P_i])]$ yields 11.1 ± 3.6 kJ/mol and an estimate of $\Delta G'_p$ that ranges from 37 to 45 kJ/mol. The stoichiometry of proton transport coupled to ATP synthesis [$n = \Delta G'_p/(F\Delta p)$] therefore ranges from 1.8 to 2.5.

Quantitation of nucleoside phosphates and P_i by area measurement from NMR spectra is fairly imprecise, first because the resonances are small and noise to signal ratios are high and second because a correction factor must be applied to adjust for saturation effects. Rapid pulsing (0.34 s) during data acquisition is necessary to enhance signal intensity. However, even with allowance for the large confidence limits, the variation in the logarithmic term in the $\Delta G'_p$ expression does not overly affect the values calculated for n (1.8–2.5). On the other hand, the calculations are very sensitive to Δp. At high cell density (>20 mg dry wt/mL) the $\Delta\psi$ of wild-type cells oxidizing succinate (>2.5 μM oxygen) was 90 ± 7 mV as compared to 138 ± 10 mV at lower cell density (<4 mg/mL). Δp could therefore be as low as 145 ± 15 mV; n would then range from 2.4 to 3.6. We said earlier that the higher $\Delta\psi$ values are probably preferable since it can be argued that they reflect greater accessibility of the probe at comparable conditions of substrate and oxygen concentration.

Similar calculations can be made for cells metabolizing succinate under oxygen-limited conditions. In this case, NTP concentration was highest about 10–12 min after substrate addition, which is within the period when maximum Δp was recorded (Figure 8). The NTP concentration was estimated from integrated peak area to be 3 ± 2 mM; that of NDP was 3 ± 2 mM while that of P_i was 6 ± 2 mM. The $RT \ln [[ATP]/([ADP][P_i])]$ term calculated from these measurements varies accordingly from 8 to 17 kJ/mol. Assuming $\Delta G^{\circ\prime}$ is 30 kJ/mol, $\Delta G'_p$ ranges from 38 to 47 kJ/mol. The maximum Δp developed in washed cells under these conditions is 125 ± 14 mV (10 min after succinate addition, Figure 8). On the basis of these figures, n can vary from 2.8 to 4.4; this appears to be significantly greater than the range for cells grown at the higher oxygen tension. If Δp is calculated from $\Delta\psi$ values that were obtained with thick cell suspension ($\Delta\psi$, 85 ± 8 mV; >20 mg dry wt/mL), n is within the range 3–5.

Discussion

$\Delta\psi$ Estimations. $\Delta\psi$ values were obtained by flow dialysis with TPP^+ as the probe and by the filtration method with Rb^+. Using $^{86}Rb^+$ requires addition of valinomycin and low K^+ concentration, while the filtration technique with aerobic cells is limited to low cell densities to prevent anaerobiosis (Kashket, 1981). Thus $\Delta\psi$ (Rb^+) values were not measured simultaneously with the accumulation of ^{31}P NMR spectra but were still obtained on the same batch of cells and under the same conditions of oxygen tension and temperature. Likewise, estimation of $\Delta\psi$ with TPP^+ was carried out with low cell densities. We had hoped that flow dialysis might cope with thick cell densities in the same range as used for the NMR analyses. We found with aerobic cultures that even when oxygen tension was adequately maintained $\Delta\psi$ was significantly decreased at cell densities exceeding about 5 mg dry wt/mL. With anaerobic cultures there was no significant difference between $\Delta\psi$ obtained at 5 and 25 mg dry wt/mL with this method. Increasing the EDTA concentration to 10 mM, and omitting Mg^{2+} from the resuspension medium, resulted in a significant increase in $\Delta\psi$. It is difficult to decide between an intrinsic effect of EDTA on $\Delta\psi$ or a specific effect of EDTA on the membrane permeability of the probe. We have taken the view that higher $\Delta\psi$ values reflect increased accessibility of the cytoplasmic membrane to the probe and are therefore more representative in the case of aerobic cultures

than the lower values obtained at higher cell densities and with no EDTA present. The values of $\Delta\psi$ we have reported are generally in agreement with values already in the literature. Kashket (1981) obtained very low $\Delta\psi$ values ($\simeq$0 mV) for *E. coli* during anaerobic growth in batch culture. For aerobic *E. coli* cells, Padan et al. (1981) obtained values around 125 mV, using Rb^+ as the probe and medium containing little K^+. Kashket (1981) has reported values from 145 to 164 mV, the latter obtained with exponential cells and the filtration method. Ten Brink et al. (1981) showed that binding of TPP^+ is concentration and pH dependent, but they found that $\Delta\psi$ values in aerobic batch cultures of *E. coli* were well below 100 mV when nonspecific binding was corrected for. The values we report, of around 130–140 mV, are in the midrange delineated by these extremes. Distribution of K^+ was not a suitable method for estimating $\Delta\psi$, as active uptake of K^+ occurred during metabolism.

Calculation of n. Previous studies indicate that the H^+/ATP stoichiometry may be considerably greater than 2. Kashket (1982) reported that the H^+/ATP ratio in *E. coli* varied from about 2.5 with external pH 6.25 to 4 at pH greater than 8. In *Paracoccus denitrificans* (McCarthy et al., 1981), *n* was estimated to be 3, and values as high as 7 have been reported for *Bacillus megaterium* (Guffanti et al., 1981). *Streptococcus lactis*, which is a homolactic fermenter with no respiratory system, exhibited a stoichiometry of 2 when an artificial pH and K^+ diffusion potential were applied (Maloney & Saitta, 1982). Steady-state glycolyzing cells on the other hand extruded 3 H^+/ATP, both for *S. lactis* (Maloney, 1982, 1983; Maloney & Saitta, 1982) and for *E. coli* (Kashket, 1983). We found that the H^+/ATP ratio accompanying ATP synthesis was between 1.8 and 2.5, which is in good agreement with the original proposal that the stoichiometry may be 2 (Mitchell, 1979). But the values under oxygen limitation do appear to be higher (2.8–4.4). Studies with isolated mitochondria also suggest that the H^+/ATP stoichiometry may vary depending on metabolic status.

In mitochondria *n* varies with changes in Δp that are caused by ionophore addition (Zoratti et al., 1982). Westerhoff et al. (1981) showed from inhibition of succinate dehydrogenase or addition of ionophores that $\Delta G'_p/(F\Delta p)$ varied from 3.5 at 150 mV to around 10 at 40 mV. Azzone et al. (1978) and Holian & Wilson (1980) also obtained high ratios by treatments that decreased Δp but had a lesser effect on the free energy of ATP synthesis. Ogawa & Lee (1982) have used ^{31}P NMR and K^+ electrode measurements to obtain an apparent *n* value of between 2.2 and 2.4, which showed some dependence upon the metabolic state of the mitochondria. Variable H^+/solute-coupled transport has also been reported for lactate uptake (Ten Brink & Konings, 1980, 1982; Simpson et al., 1983) and for lactose (Ten Brink et al., 1981) and proline uptake (Ramos & Kaback, 1977). It is thus possible that the number of protons transported per ATP synthesized is variable in bacterial systems and depends upon Δp. However, overestimation of the free energy of ATP synthesis, or underestimation of the effective Δp, for instance because of failure to add in localized potential contributions, may give rise to high *n* values.

In the first instance, Ogawa & Lee (1982) proposed that there may be at least two types of ATPase sites: one synthetic that is proximal to the redox centers and thus affected by localized $\Delta\psi$ and ΔpH parameters and one dependent on the bulk phase potential difference. The latter may operate even when ATP synthesis is occurring or when no net ATP synthesis is apparent. Thus the free-energy change for the hydrolytic ATPase would not be zero but positive in sign. If this contribution were reduced from $\Delta G'_p$, all other considerations remaining the same, *n* would decrease.

Westerhoff et al. (1984) have formalized a "mosaic coupling hypothesis" in which the role of Δp, as both the regulator of proton pumps and also as the high-energy substrate for ATP synthesis, has been emphasized. If interactions between the redox and ATPase sites complement the bulk phase differences, then values of H^+/ATP ratios based solely on the latter will be overestimates. This might explain why we obtained very similar Δp values for *E. coli* cells respiring under oxygen-limiting conditions (124 $\pm$ 14 mV) and respiratory-deficient cells utilizing glucose aerobically (116 $\pm$ 15 mV). ΔpH is the major component of Δp in the last case and depends on the ATPase. In the former, redox-dependent ATP synthesis was observed, and ΔpH was less than $\Delta\psi$. Localized potentials could therefore both activate the ATP synthase and also enhance the driving potential.

The idea of a threshold Δp requirement for ATP synthesis has been suggested by Maloney (1982) and Rottenberg (1979b). Studies with isolated ATPase support a role for $\Delta\psi$ (Munoz, 1982). It is now well documented that ATP synthase activity depends upon the membrane attachment of the F_1 catalytic core. Blocking of the proton translocator by oligomycin [for a review, see Racker (1976)] has been attributed to conformational restraints imposed by the altered energetic status of the membrane (Weiss & McCarty, 1977; Ellenson et al., 1978; Sone et al., 1979). There is also a correlation between ATP synthesis, driven by a electrical field imposed across thylakoid membranes, and conformational changes (Rogner et al., 1979). Similar findings have been made by using microelectrodes inserted into the hyphae of *Neurospora* (Hansen et al., 1981).

Acknowledgments

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A Collagen-like Glycoprotein of the Extracellular Matrix Is the Undegraded Form of Type VI Collagen†

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ABSTRACT: The 140 000-dalton collagenous glycoprotein (CGP) from calf aorta and ligament characterized by Gibson & Cleary (1982) [Gibson, M. A., & Cleary, E. G. (1982) *Biochem. Biophys. Res. Commun. 105*, 1288–1295] has been studied. In the electron microscope, rotary-shadowed CGP molecules appear similar to the dimers of type VI collagen (short-chain collagen, intima collagen) described by other authors [Furthmayr, H., Wiedemann, H., Timpl, R., Odermatt, E., & Engel, J. (1983) *Biochem. J. 211*, 303–311] except that they have larger globular domains. As shown by gel electrophoresis, pepsin treatment of CGP at 4 °C either before or after reduction releases polypeptide chains corresponding in size to those of type VI collagen. Electron microscopic examination shows that pepsin digestion of nonreduced CGP removes the outer globular domains, reduces the size of the inner ones, and separates the paired central strands. The residual structures look like type VI collagen dimers. When intact CGP is reduced, monomers with two large globular ends are obtained. Pepsin digestion of monomers removes most or all of both globular domains. In immunoblots, CGP and its pepsin-derived fragments react with antibodies directed against type VI collagen. The results indicate that type VI collagen is an integral component of CGP.

Up until now, short-chain collagen or intima collagen, a component of the extracellular matrix isolated primarily from human or bovine placenta, has been characterized only after pepsin digestion (Furuto & Miller, 1980, 1981; Jander et al., 1981, 1983; Odermatt et al., 1983). As it represents a new and unique collagen, different from other collagen types, the term "type VI collagen" has recently been proposed (Furthmayr et al., 1983; Jander et al., 1983). Amino acid analysis

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