Phosphorus-31 Nuclear Magnetic Resonance Studies of Bioenergetics in Wild-Type and Adenosinetriphosphatase(1−) *Escherichia coli* Cells†

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ABSTRACT: By use of ^{31}P NMR, the transmembrane pH gradient (ΔpH) and the intracellular levels of phosphorylated metabolites were measured in aerobic suspensions of wild-type *Escherichia coli* cells in the presence and absence of the adenosinetriphosphatase (ATPase) inhibitor dicyclohexylcarbodiimide (DCCD); the same parameters were also determined in *E. coli* mutants deficient in ATPase activity under both anaerobic and aerobic conditions. A method is described by which dense suspensions of *E. coli* cells ($\sim 3 \times 10^{11}$ cells/mL) were oxygenated so that steady-state O_2 levels in the suspensions were far greater than the K_m for O_2 consumption. Under these conditions, in wild-type MRE600 cells, the intracellular concentrations of P_i, NTP, and NDP were measured to be 3.0 ± 1.5, 8 ± 1, and 1.2 ± 1 mM, respectively, while the intracellular pH was ~7.5 over the external pH range studied (6 to ~7.0). Upon treatment with DCCD, the intracellular NTP level was drastically reduced and intracellular P_i concentration increased in respiring wild-type cells; in the same cells, however, DCCD did not affect the intracellular pH and the ΔpH. During respiration in the presence of lactate, ATPase$^-$ cells established a ΔpH but failed to synthesize any detectable levels of NTP. Conversely, ATPase$^-$ cells accumulated high levels of NTP but did not generate a ΔpH during glycolysis under *anaerobic* conditions. These results are in complete agreement with the generally accepted chemiosmotic hypothesis. ^{31}P NMR data on intact ATPase$^-$ NR70 cells were in agreement with the previously proposed [Rosen, B. P., Brey, R., & Hasan, S. (1978) *J. Bacteriol. 134*, 1030] existence of a proton leak in this strain which is sealed by DCCD or by spontaneous mutation into strain NR71. However, the NMR data also indicated that other major differences exist between NR71 and NR70 cells.

High-resolution ^{31}P NMR[1] spectroscopy has been used to monitor levels of phosphorylated metabolites such as nucleotides, sugar phosphates, and inorganic phosphate in intact cell suspensions and in tissue (Henderson et al., 1974; Hoult et al., 1974; Salhany et al., 1975; Burt et al., 1976; Navon et al., 1977a,b; Ugurbil et al., 1978, 1979a,b; Shulman et al., 1979). In addition, intracellular pH has been determined from the ^{31}P chemical shifts of ionizable metabolites which titrate in the physiological pH range (Henderson et al., 1974; Salhany et al., 1975; Burt et al., 1976; Navon et al., 1977b; Ogawa et al., 1978; Ugurbil et al., 1978). ^{31}P NMR measurements have shown that *Escherichia coli* cells maintain a transmembrane ΔpH under both anaerobic and aerobic conditions (Navon et al., 1977b). In a more recent ^{31}P NMR study, the magnitude of this ΔpH was measured simultaneously with the levels of P_i, NDP, NTP, and other phosphorylated metabolites in *E. coli* cells during anaerobic glycolysis (Ugurbil et al., 1978); it was demonstrated that a ΔpH was generated concurrently with the accumulation of intracellular NTP. The formation of the ΔpH was selectively blocked by the inhibition of the ATPase, whereas an uncoupler, FCCP, prevented both the ΔpH generation and the accumulation of NTP.

In this study, we report measurements of intracellular pH and intracellular concentrations of P_i, NTP, and NDP in *respiring* wild-type *E. coli* cells and in ATPase$^-$ mutants during both respiration and fermentation. In general, these cells are able to grow under aerobic conditions on fermentative carbon sources like glucose but not on pure electron-donating substrates such as succinate. However, they are capable of oxidizing electron-donating substrates, in some cases faster than their wild-type parental strains (Simoni & Postma, 1975). In the context of the chemiosmotic hypothesis [Mitchell, 1966, 1968; for reviews, see Harold (1972, 1977) and Rottenberg (1975)], these measurements are complementary to the earlier ^{31}P NMR studies on *E. coli* under anaerobic conditions. During *anaerobic* glycolysis, a ΔpH and presumably a $\Delta\psi$ are created by ATPase-mediated hydrolysis of the ATP synthesized by substrate-level phosphorylation; during *respiration*, substrate oxidation is expected to generate a protonmotive force with ΔpH and $\Delta\psi$ components, which in turn should drive ATP synthesis by the ATPase.

We also present detailed measurements of intracellular pH in ATPase$^-$ *E. coli* strains NR70 and NR71. It is thought that in strain NR70, the F_0 portion of the H^+-ATPase acts as an open proton channel which is sealed by DCCD or by spontaneous mutation of NR70 into strain NR71. This conclusion, however, was reached primarily on the basis of experiments with membrane vesicles prepared from NR70 and its nonleaky derivative NR71 (Rosen et al., 1978); consequences of such a H^+ leak on the intracellular pH of the intact cells has not previously been explored.

Experimental Procedures

The strains of *E. coli* used in these experiments were MRE600, AN180, AN120 (unc$^-$; ATPase$^-$), K-12 strain 7, NR70 (unc$^-$, ATPase$^-$), and NR71 (ATPase$^-$). AN120 and NR70 are the unc$^-$ ATPase$^-$ derivatives of AN180 and K-12 strain 7, respectively. NR71 is an ATPase$^-$ strain which arises from NR70 by spontaneous mutation. This strain can slowly

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[1] Abbreviations: NMR, nuclear magnetic resonance; FID, free induction decay; P_i, inorganic orthophosphate; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; ATPase, adenosinetriphosphatase; NAD, nicotinamide adenine dinucleotide; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, *p*-(trifluoromethoxy)phenylhydrazone.

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grow in minimal media with succinate as the carbon source (Rosen et al., 1978); therefore, it does not get the unc$^-$ designation. Strains AN180 and AN120 were supplied by Dr. Robert Fillingame, University of Wisconsin Medical School, Department of Physiological Chemistry, Madison, WI. Strains NR70 and NR71 were obtained from Dr. Barry Rosen, University of Maryland, School of Medicine, Baltimore, MD. Experiments reported on strains NR70 and NR71 were performed within 2 weeks after they were supplied to us by Dr. Rosen. All strains were first grown aerobically at 37 °C in 250 mL of enriched medium (L broth) which was inoculated directly with cells maintained on solid agar plates. These liquid cultures were subsequently used to inoculate 2-L batches of M9 minimal medium (5 g/L NaCl, 3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, and 1 g/L NH_4Cl) supplemented with 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, and 0.1 g/L thiamin; the carbon source for growth was 37 mM succinate for MRE600 and 20 mM glucose for the other strains. In addition, the 2-L cultures for AN180 and AN120 which are arg$^-$ strains contained 0.2 mM arginine. The 2-L cultures were aerated at 37 °C in a rotatory shaker. At mid-logarithmic phase of growth, the cultures were removed from the incubator and cooled to 4 °C in an ice bath. Aeration of the cultures was continued during the cooling process by bubbling O_2 through them. Cells were collected by ~10-min centrifugation at ~4 °C and subsequently washed twice and resuspended in ice-cold buffered media which consisted of 100 mM Pipes, 50 mM Mes, 40 mM NaCl, and 4–20 mM orthophosphate ($[Na_2HPO_4]$:$[KH_2PO_4]$ = 1:1); the P_i concentration was varied according to cell density, and low P_i concentrations were used with suspensions with low cell densities. When the KH_2PO_4 concentration was less than 10 mM, KCl was added to the suspension to retain the K^+ concentration at 10 mM. The pH of this resuspension medium was adjusted to the desired pH values with NaOH. The cells were kept in an ice bath until use which occurred no later than 2 h after final resuspension. The unc$^-$ ATPase$^-$ cells grown for NMR experiments were always checked for reversion to wild type by monitoring their growth at 37 °C on minimal agar plates containing succinate as the carbon source. No growth of NR70 cells was detected even after 7–10 days of incubation; occasional growth was observed for AN120 cells.

NMR spectra were obtained in 10-mm sample tubes at 145.7 MHz on a Bruker HX360 instrument operating in the Fourier transform mode. The spin–lattice relaxation times (T_1's) of ^{31}P resonances observed from intracellular molecules in *E. coli* cells are relatively short; in glucose-grown MRE600 *E. coli* cells, T_1's of the intracellular P_i and NTP_γ resonances were previously measured to be 0.4 and 0.2 s, respectively (Brown et al., 1977). These short T_1's allow the use of rapid pulse rates for optimum signal-to-noise ratios. Consequently, the ^{31}P NMR spectra were obtained by using either 90° pulses and a 0.68-s repetition time or 60° pulses and a 0.34-s repetition time. Saturation of the resonances were checked by comparing these spectra with spectra obtained from the same sample with the same pulse angle and a 2.5-s repetition time. For this comparison, spectra with the slow and rapid pulsing rates were recorded in alternating 1-min time blocks in order to average out equally any changes occurring during the time of accumulation. All chemical shifts are reported relative to external 85% phosphoric acid; they were obtained by using phosphoglycerocholine as an internal standard resonating at −0.49 ppm. The suspensions were oxygenated by continuously bubbling O_2 through the NMR samples either through a single capillary at the rate of ~25–30 mL/min or through two capillaries with a low (~20 mL/min) and a high (~150 mL/min) O_2 flow rate. In the latter method, the capillary with the lower gas flow rate generated bubbles at the bottom of the NMR tube, below the level of the detection coils; the capillary with the higher flow rate generated bubbles at ~5 mm above the detection coil.

^{13}C NMR spectra were obtained at 90.52 MHz on the same spectrometer by using a 0.34-s repetition time and 40° pulses. Experimental conditions were identical with those used in the corresponding ^{31}P NMR measurements except that the unlabeled succinate was replaced with 90% labeled [2-^{13}C]-succinate (Merck Sharp & Dohme). Initially, at the extracellular pH of ~6, a strong resonance from [2-^{13}C]succinate was observed at −33.8 ppm (the chemical shift was measured by using dioxane as an internal standard in one sample and is expressed relative to Me_4Si). A second, ~15-fold weaker resonance assigned to intracellular [2-^{13}C]succinate was also detected at −34.9 ppm. The resolution of the extra- and intracellular components of succinate arises from the transmembrane pH gradient. During the course of the experiment, as the extracellular pH increased, the extracellular succinate resonance shifted downfield and eventually overlapped with the resonance from intracellular succinate.

Cell extracts were prepared by rapidly mixing the cell suspension in the NMR tube with 20% by volume of ice-cold 60% perchloric acid. After the cellular debris was removed by centrifugation, the supernatant was neutralized by K_2CO_3 and centrifuged again to remove the precipitated $KClO_4$. Subsequently, the supernatant was diluted ~20-fold in H_2O and passed through a Chelex-100 (Bio-Rad) column equilibrated to pH 7 with 5 mM Tris buffer. The eluate was lyophilized and redissolved in H_2O equal in volume to the original sample.

Results

Figure 1 illustrates a sequence of 145.7-MHz ^{31}P NMR spectra of succinate-grown MRE600 *E. coli* cells before, during, and after oxygenation in the presence of succinate. Each spectrum was accumulated for ~2 min. Peak assignments are based on chemical shifts (Navon et al., 1977a; Ugurbil et al., 1978) and were checked by the addition of the pure compounds into the cell extracts. As previously reported for glucose-grown *E. coli* (MRE 600) (Ugurbil et al., 1978), the nucleotide phosphate resonances included contributions from both purine and pyrimidine nucleotides which were not resolved in intact cell spectra but were separable in the extract spectra (not shown). The NAD peak may contain contributions from both the reduced and the oxidized forms because the two forms are not resolved in the intact cell spectra. The broad phosphomonoester peak labeled S-P stems from several overlapping resonances which have not been identified.

The intra- and extracellular pH values (pH^{in} and pH^{ex}, respectively) shown in Figure 1 were obtained by using P_i chemical shifts and the pH titration profile of P_i in the growth medium; the titration of P_i in the suspension medium used in the NMR experiments was the same as that observed in M9. The chemical shift of P_i at a given pH value in the pH range of interest (~5 to ~9) depends on ionic strength only through the salt dependence of its pK_a value (Ogawa et al., 1981). However, this ionic strength dependence is not very strong at high salt concentrations (≳50 mM). In our growth medium (which contains 170 mM Na^+, as well as 1 mM Mg^{2+} and 0.1 mM Ca^{2+}) the pK_a of P_i is 6.7 at 20 °C. In 150 mM KCl, it is 6.73 and it decreases by less than 0.1 pH unit if the KCl concentration is doubled (Ogawa et al., 1981). Since *E. coli* cells are thought to accumulate 200 mM K^+, we estimate the systematic error in our intracellular pH measurements due to

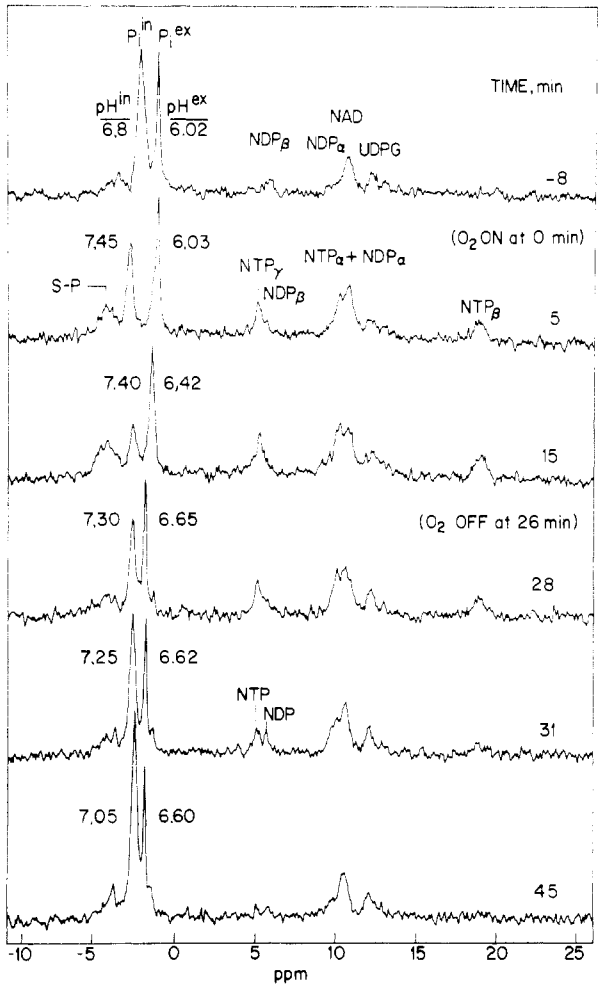

FIGURE 1: 145.7-MHz ^{31}P NMR spectra of succinate-grown *E. coli* MRE600 cells at 20° before, during, and after oxygenation. Each spectrum consists of 200 FID's obtained in ~2 min by using a repetition time of 0.68 s and 90° pulses. Cells were grown and harvested at the mid-log phase of growth as described under Experimental Procedures. The recovered pellets were washed twice and resuspended in an equal volume of 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 40 mM NaCl, 50 mM Mes, and 100 mM Pipes (pH 6.1). This gives a suspension with a cell density of $\sim 5 \times 10^{11}$ cells/mL. The suspensions were kept in an ice bath until ~20 min prior to O_2 bubbling at which point they were warmed up to 20 °C. 75 mM succinate was added prior to oxygenation. Oxygen was introduced by continuous bubbling through a single capillary at the rate of 25 mL/min. The NMR sample was 2 mL in volume.

uncertainties in the pK_a of P_i in the *E. coli* cytoplasm to be $\lesssim$0.1 unit. The chemical shifts are determined with an accuracy of ±0.05 ppm; this corresponds to an error of ±0.08 pH unit at pH 7.4 and to ±0.04 pH unit in the pH region between ~7.1 and ~6.3 where the P_i chemical shift dependence on pH is approximately linear.

The difference between the internal and external pHs observed prior to oxygenation in the top spectrum of Figure 1 is an artifact of sample preparation. When aerobically grown, mid-log phase cells are harvested at ~4 °C and the cultures are maintained aerobic during the cooling process prior to harvesting, the internal pH of recovered cells is usually between 7.3 and 7.5. In the experiment shown in Figure 1, the cells were resuspended in a medium whose pH was 6.1, and after the addition of 75 mM succinate to the cold cells, the external pH was 6.0. Consequently, an artificial transmembrane pH gradient was created. This gradient was stable over periods of 2–3 h in the cold (~2 °C). However, in the absence of oxygen, or a fermentable carbon source, it slowly collapsed at 20 °C (see Figure 4).

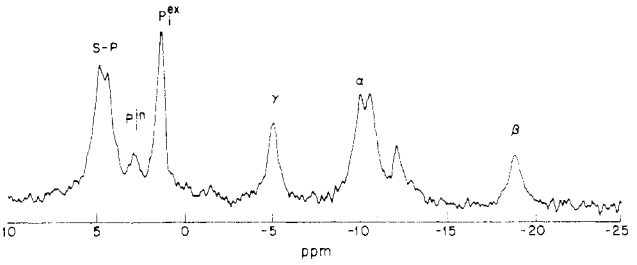

FIGURE 2: 145.7-MHz ^{31}P NMR spectrum of *E. coli* MRE600 cells at 20 °C in the presence of O_2 and 75 mM succinate. Cells were grown and harvested as described and were suspended in the same medium as in Figure 1 except the cell density was 66% of what it was for the experiment of Figure 1. Cells were oxygenated with two bubblers as described under Experimental Procedures. The NMR sample was 3 mL in size. The spectrum is the sum of 1000 FID's obtained with 60° pulses and a 0.34-s repetition time.

After the onset of oxygenation in the presence of succinate, the P_i^{in} peak rapidly shifted downfield and remained at approximately −2.8 ppm during oxygenation, indicating an internal pH of ~7.4. During the same period, P_i^{ex} moved upfield slowly, reflecting a change in pH^{ex} from 6.0 to 6.4 in ~15 min. The intensity of the P_i^{in} resonance decreased in the presence of O_2 as phosphoric esters such as sugar phosphates (S-P) and NTP were synthesized. When O_2 was turned off, however, P_i recovered its initial intensity within ~15 min. Furthermore, after oxygenation was stopped, the P_i^{in} resonance now shifted upfield, indicating a decrease in internal pH.

In the experiment shown in Figure 1, the cells were oxygenated by generating O_2 bubbles at the bottom of the NMR tube at ~20 mL/min. Under these conditions and at these high cell densities ($\sim 5 \times 10^{11}$ cells/mL), previous measurements had shown that the steady-state O_2 levels in the suspension were ~0.15 μM which corresponds to ~20% of the K_m value for O_2 consumption by MRE600 *E. coli* cells (Ogawa et al., 1978). It was, however, also demonstrated that this did not affect the internal pH attained by these cells. In order to fully oxygenate *E. coli* suspensions at high cell densities, we developed a method which uses two capillaries, one generating O_2 bubbles at the bottom of the NMR tube at low flow rates (20–30 mL/min) and the other generating bubbles at higher rates (100–200 mL/min) ~5 mm above the level of the detection cell. This technique allowed vigorous oxygenation and stirring of the suspension without significantly disturbing the magnetic field homogeneity; under these conditions, the steady-state O_2 concentration measured at the level of the detection coil was ~0.2 to ~1 mM in a dense suspension ($\sim 3 \times 10^{11}$ cells/mL) of MRE600 cells respiring on succinate. This O_2 concentration far exceeds the K_m value of 0.7 μM for O_2 usage (Ogawa et al., 1978). The major difference observed in the NMR spectrum with higher O_2 levels is higher levels of sugar phosphates and a lower level of internal P_i (Figure 2). In agreement with earlier results (Ogawa et al., 1978), pH^{in} was not affected by the higher efficiency of oxygenation.

The spectrum shown in Figure 2 was obtained from cells which were washed and resuspended in a medium lacking a nitrogen source. So that the conditions where cell growth occurs could be duplicated, the same experiment was repeated with cells washed and resuspended in the same medium plus 25 mM NH_4Cl. The spectrum observed under these conditions was not different from the one shown in Figure 2.

The absolute intensities of the resonances observed in Figure 2 were calibrated by comparing it to a spectrum obtained with 60° pulses and a 2.5-s repetition time. It was observed that only the P_i^{ex} and the NAD resonances were saturated under the rapid pulsing conditions of Figure 2. This observation,

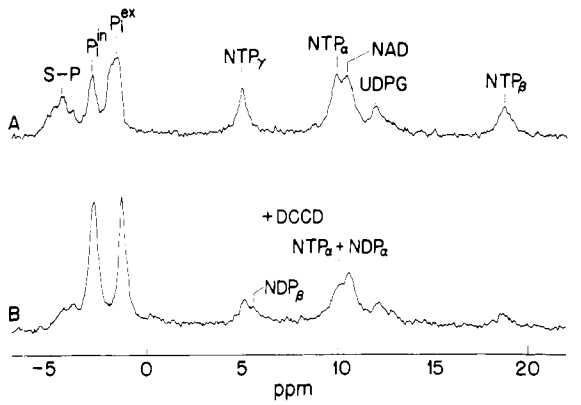

FIGURE 3: Effect of DCCD on the ^{31}P NMR spectra of succinate-grown *E. coli* MRE600 cells respiring on succinate. (A) Untreated cells; (B) cells treated with 3 mM DCCD. All experimental conditions were identical with those given for Figure 1. The recovered cells in suspension were divided into two samples. Both samples were kept in an ice bath until ~20 min prior to O_2 bubbling at which point they were warmed up to 20 °C. 3 mM DCCD was added to one sample immediately after it was warmed up to 20 °C. The O_2 flow rate was 25 mL/min. NMR samples contained 75 mM succinate. The data were obtained in 2-min blocks as in Figure 1. The spectra shown were obtained by adding together eight consecutive 2-min spectra between 4 and 20 min after the onset of O_2 bubbling. These 2-min spectra displayed constant pH (see Figure 4) and constant NTP, P_i, and sugar phosphate levels.

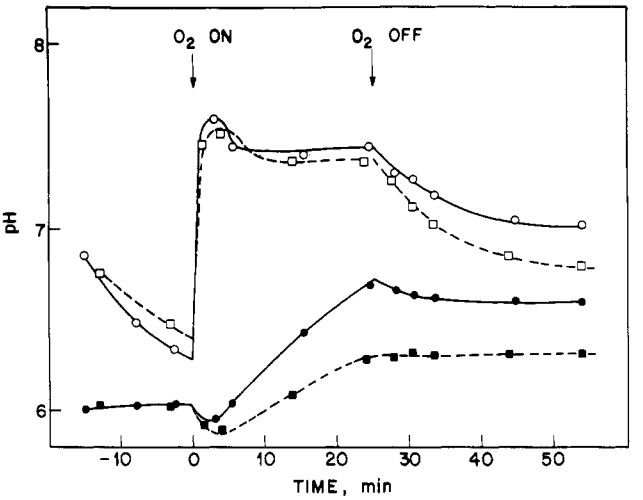

FIGURE 4: Effect of DCCD on the internal and the external pH in succinate-grown MRE600 cells respiring on succinate at 20 °C. Internal pH and external pH are designated by open and closed symbols, respectively. Squares represent the DCCD-treated cells, and circles represent the control. These pH data and the spectra shown in Figure 3 were taken from the same experiment. The intra- and extracellular pH was obtained from the P_i chemical shifts of the individual 2-min spectra in the series (see the legend to Figure 3).

however, does not imply that the other resonances have extremely short T_1's ($\lesssim$0.2 s) because vigorous aeration of the 3-mL samples results in extensive stirring; therefore, the ~0.8-mL volume actually seen by the detection coil is at least partially exchanged with fresh, unsaturated spins between consecutive pulses. The T_1's of P_i^{in} and NTP_γ resonances were previously measured to be 0.4 and 0.2 s, respectively, in aerobic suspensions of glucose-grown *E. coli* cells (Brown et al., 1977).

From the integrated intensities of the P^{in} and the NTP_β resonance in Figure 2, and assuming that the intracellular volume is 50% of the cell pellet volume, we calculate the intracellular P_i and NTP concentrations to be 5 and 9 mM, respectively. The NDP concentration is calculated to be 2 mM from the difference in the integrated intensities of the peak at 5.0 ppm and the NTP_β resonance. The intracellular pH and intensities of P_i, NTP, and NDP resonances measured in MRE600 cells were highly reproducible. From five measurements under the conditions of Figure 2, on five different batches of MRE600 cells grown at different times, the average P_i^{in}, NTP, and NDP concentrations measured were 3 ± 1.5, 8 ± 1, and 1.2 ± 1 mM, where the errors are average deviations from the mean. In the same five experiments, pH^{in} of respiring cells was between 7.40 and 7.60. By use of the mean values obtained for intracellular P_i, NTP, and NDP concentrations, the parameter RT ln [[NTP]/([NDP][P_i])] in the *E. coli* cells is calculated to be 4.5 kcal/mol at 20 °C. If it is assumed that the NTP/NDP ratio is proportional to the ATP/ADP ratio, the Gibbs free energy of ATP synthesis in these cells is therefore 4.5 kcal/mol plus the standard free energy, $\Delta G°$, for this reaction. $\Delta G°$ of ATP hydrolysis has been measured as a function of pH, ionic strength, and free Mg^{2+} concentration (Rosing & Slater, 1972); at pH 7.5 and an ionic strength of 0.2, $-\Delta G°$ was 7.8, 7.25, and 7.33 kcal/mol for a free Mg^{2+} concentration of 0.1, 1, and 10 mM, respectively. Furthermore, the effect of ionic strength on $\Delta G°$ was less than or equal to 0.1 kcal in the 0.1–0.2 ionic strength range. Therefore, if the free Mg^{2+} concentration in *E. coli* cells is approximately 1 mM, and if we assume that the [NTP]/[NDP] ratio is the same as the [ATP]/[ADP] ratio, the phosphorylation potential of ADP to form ATP is approximately 12 kcal/mol.

DCCD Effect. Figure 3 compares the ^{31}P NMR spectra of the control and the DCCD-treated MRE600 cells; for determination of the NTP and NDP levels with greater accuracy than is possible from a single 2-min spectrum, eight consecutive 2-min spectra which displayed constant values of pH^{in} and approximately constant levels of P_i and NTP were summed to obtain the spectra shown in Figure 3. During oxygenation, the control cells maintained high levels of NTP, and treatment with 3 mM DCCD reduced the [NTP]/[NDP] ratio by a factor of ~10 (Figure 3); in the DCCD-treated cells, a contribution from the NDP β-phosphate resonance was clearly observable at 5.5 ppm, slightly upfield of the NTP_γ peak (Figure 3B), whereas none was detectable in the absence of DCCD (Figure 3A). Furthermore, the intracellular P_i level in the DCCD-treated cells was higher and the sugar phosphate level was lower than that observed in the control. The small amount of NTP detectable in the DCCD-treated cells probably came from substrate-level phosphorylation; for example, each turn of the tricarboxylate cycle produces one GTP by substrate-level phosphorylation at the succinyl thiokinase step. The NTP observed in the DCCD-treated cells during oxygenation lasted ~30 min after O_2 was turned off. By contrast, NTP disappeared within ~10 min after oxygenation was stopped in the control cells (Figure 1).

Figure 4 shows that the pH^{in} values attained by the DCCD-inhibited cells were the same as in the control cells and, furthermore, both were constant during oxygenation after the initial burst. In the presence of O_2 and succinate, pH^{ex} increased with time after the initial acidification in suspensions of both the DCCD-treated and the untreated cells (Figure 4). This pH^{ex} increase is associated with succinate consumption because its occurrence required the presence of succinate in the suspension. Therefore, the slower rate at which pH^{ex} increased for the DCCD-treated cells relative to that of the control suggested that succinate consumption may have been inhibited by DCCD. This was shown to be the case by monitoring the usage of [2-^{13}C]succinate with ^{13}C NMR under experimental conditions identical with the ^{31}P experiments. It is seen (Figure 5) that during the initial period between ~3

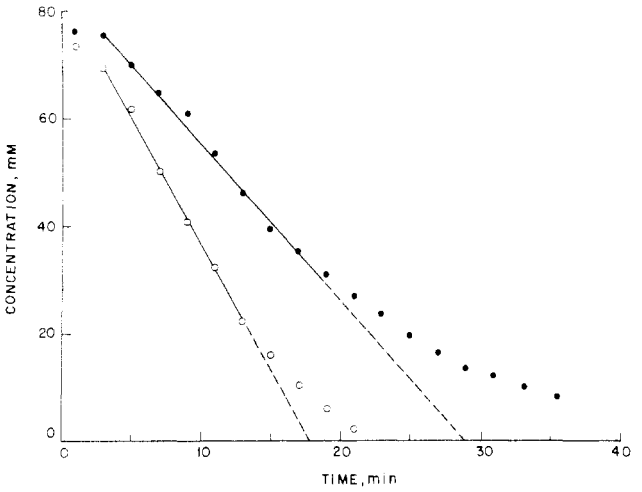

FIGURE 5: Time course of succinate consumption by *E. coli* MRE600 cells treated with 3 mM DCCD (●) and the untreated control (○) at 20 °C. The MRE600 cells were grown, harvested, and resuspended for NMR measurements as described in Figure 1. From the final suspension of cells, two 2-mL NMR samples were taken. The samples, which were kept in an ice bath, were warmed up to 20 °C ~20 min prior to oxygenation. To one NMR sample 3 mM DCCD was added immediately after it was warmed up to 20 °C. 75 mM [2-^{13}C]-succinate was added to both samples prior to oxygenation. Oxygen was introduced by bubbling through a single capillary at the rate of 25 mL/min. ^{13}C NMR spectra were obtained in 1-min blocks (200 FID's) at 90.52 MHz by using a 0.34-s repetition time and 40° pulses. The data show the *extracellular* succinate concentration vs. time; extra- and intracellular [2-^{13}C]succinate resonances were resolved from each other in the NMR spectrum due to the pH^{in} and pH^{ex} difference. Intracellular succinate concentration was constant in the presence of oxygen.

and 15 min, where the succinate consumption was linear in time, the rate of succinate usage was approximately 2 times slower in the DCCD-treated cells; this is exactly what was observed for the rate of alkalinization of pH^{ex} (Figure 4). This observation implies that although the pH^{in} is maintained at ~7.4 in both DCCD-treated and untreated cells, the DCCD-treated cells accomplish this at half the rate of electron transfer.

ATPase⁻ Mutants. ^{31}P NMR results obtained on the ATPase⁻ mutant NR70 and its wild-type parental strain 7 were qualitatively analogous to those observed in strain MRE600 in the presence and absence of DCCD, respectively. In the presence of oxygen and lactate, ATPase⁻ NR70 cells developed a ΔpH as shown by the presence of the two well-resolved P_i peaks (Figure 6B and Table I); however, no net NTP synthesis occurred (Figure 6). On the other hand, under anaerobic conditions, while metabolizing glucose, NR70 cells displayed high levels of NTP but did not generate a ΔpH (Figure 7) as can be inferred from the unsplit P_i peak during the first 37 min of anaerobic glycolysis; during this period, the extra- and intracellular pH decreased from an initial value of ~7.1 to ~6.2 due to lactic acid production. Subsequently, when the suspension was oxygenated, a ΔpH was established as indicated by the two resolved P_i resonances observed in the top spectrum (Figure 7). When the same anaerobic experiment was repeated with the ATPase⁻ AN120 cells, the results were again qualitatively identical: the AN120 cells accumulated NTP but did not generate a ΔpH during glycolysis under anaerobic conditions; subsequent oxygenation, however, resulted in a ΔpH. The experiment described in Figure 6 was not repeated with this strain. It has already been shown by ^{31}P NMR that during anaerobic glycolysis, under similar conditions, wild-type MRE600 cells establish a transmembrane ΔpH and display a high NTP/NDP ratio (Ugurbil et al., 1978).

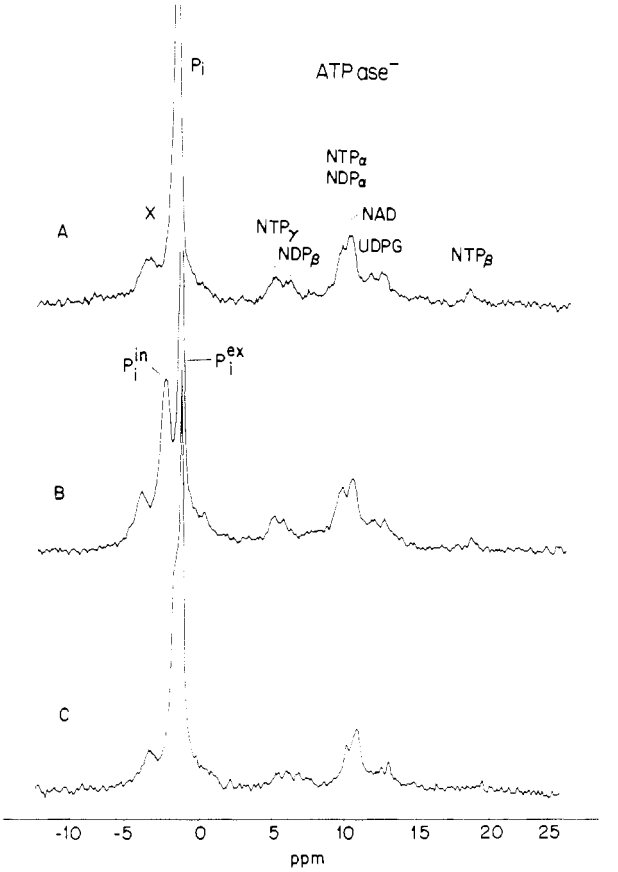

FIGURE 6: ^{31}P NMR spectra of the *E. coli* ATPase⁻ mutant, strain NR70, before (A), during (B), and after (C) oxygenation in the presence of 16 mM DL-lactate at 20 °C. Cells were grown and harvested as described under Experimental Procedures. The pellet collected at the end of harvesting and washing was suspended in 3 times the pellet volume of 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 100 mM Pipes, 50 mM Mes, and 40 mM NaCl, pH 6.5. This density corresponds approximately to 1.3×10^{11} cells/mL. Oxygenation was accomplished by bubbling continuously at the rate of 20 mL/min. The data were collected in consecutive 2-min blocks (400 scans, 0.34-s repetition time, and 45° pulses). The spectra shown represent the sum of four such 2-min spectra.

The parental wild-type strain AN180 and K-12 strain 7 cells behaved very similarly to MRE600 cells; either anaerobically when fed glucose or in the presence of lactate and O_2, they both synthesized high levels of NTP and generated a ΔpH. The pH^{in} values measured in these strains in the presence of O_2 are given in Table I.

Under aerobic conditions, in experiments performed at different external pH values between 6 and 7, all of the coupled strains maintained a constant internal pH irrespective of the external pH (Table I). In NR70 cells, however, pH^{in} was generally lower and not independent of pH^{ex} (Table I). At an external pH of 6.4, two different NR70 suspensions prepared for NMR measurements from different liquid cultures gave a pH^{in} of 7.0 while respiring on lactate; when the cells were suspended in a medium with a lower pH, a pH^{in} of 6.8, 6.8, and 6.9 was measured at pH^{ex} of ~6.2 in three different NR70 suspensions again prepared from separate liquid cultures.

DCCD has been shown to enhance respiration-dependent transport in NR70 membrane vesicles (Tsuchiya & Rosen, 1975; Rosen et al., 1978) and to a lesser extent in intact NR70 cells (Rosen, 1973a,b). Therefore, we have tested the effect of DCCD on the ΔpH generated by the NR70 cells. In several experiments at external pH values between 6.0 and 6.4, treatment with 1–5 mM DCCD of NR70 suspensions prepared

Table I: Intracellular pH of Respiring Wild-Type and ATPase$^-$ *E. coli* Cells at 20 °C[a]

strain	pH^{ex}	pH^{in} [d]
MRE600	6.0–7.0[b]	7.50 ± 0.08
MRE600 (+DCCD)	6.0–6.4[b]	7.46 ± 0.09
AN180	6.2–6.7[b]	7.35 ± 0.08
AN120[e]	6.3–6.6[b]	7.35 ± 1.0
K-12 strain 7	6.2–6.8[b]	7.60 ± 0.07
NR70[e]	6.3/6.4[c]	7.00 ± 0.06
	6.1/6.15[c]	6.84 ± 0.05
NR70[e] (+DCCD)	6.35/6.45[c]	7.18 ± 0.06
	6.0/6.1[c]	7.14 ± 0.08
NR71[e]	6.3/6.45[c]	7.20 ± 0.07
	6.0/6.15[c]	7.19
	5.90/5.95[c]	6.98
NR71[e] (+DCCD)	6.35/6.41[c]	7.18

[a] Cells were harvested and suspended at a density of ~1.3×10^{11} to ~5×10^{11} cells/mL as described under Experimental Procedures. The carbon source used was 75 mM succinate (initial concentration) for MRE600 cells and 16 mM DL-lactate for the other strains. ^{31}P NMR spectra were obtained in 2-min consecutive blocks. pH^{in} was measured over an ~10- to ~40-min period during the steady state reached after the O_2 was turned on; during this period, pH^{ex} changed by ~0.8 pH unit for MRE600 (in the absence of DCCD), ~0.4 pH unit for AN180, K-12 strain 7, and AN120, and ≲0.2 pH unit for NR70 and NR71. The pH^{ex} range reported stems both from this change in individual samples and from starting experiments at different pH^{ex} values. [b] The pH^{ex} range given stems from starting experiments at different pH^{ex} values and from the gradual change in pH^{ex} in individual samples during the observation period. [c] The two pH^{ex} values given represent the change in pH^{ex} in the samples during the measurement of the corresponding pH^{in}. [d] In cases where several measurements were performed on different samples, average deviations from the mean are given. When no error is given, the random error in the measurement stems from inaccuracies in chemical shift measurements; this error is ±0.04 pH unit at pHs between ~6.3 and ~7.1 and ±0.08 at pH 7.4. [e] These strains are all ATPase$^-$ and except for NR71 are all unc$^-$. NR71 does not get an unc$^-$ designation because it can grow very slowly on minimal medium with succinate as the carbon source (Rosen et al., 1978).

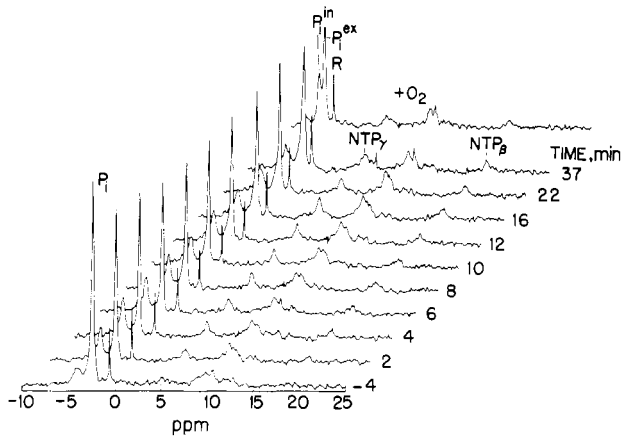

FIGURE 7: ^{31}P NMR spectra of *E. coli* NR70 (unc$^-$; ATPase$^-$) during anaerobic glycolysis on glucose and when oxygenated subsequently (top spectrum). Cells were suspended in the medium specified for Figure 6 except that the medium lacked NaCl and its pH was adjusted to 7.5 with 1 M NaOH. The pH^{ex} in the final cell suspension just prior to the NMR measurements was ~7.1. 25 mM glucose was added at time zero. The suspension was kept anaerobic for 40 min after addition and subsequently oxygenated by bubbling O_2 through it. Each spectrum consists of 400 free induction decays, obtained with 45° pulses and a 0.34-s repetition time at 20 °C. The 37-min spectrum and the spectrum of the oxygenated cells show a spike (due to rf pulse feed through) which, subsequent to application of an exponential filter, appears as a peak overlapping the NAD resonance.

for NMR (containing ~30% cell pellet by volume, or ~0.3 g wet weight/mL) caused an increase of approximately 0.2–0.3

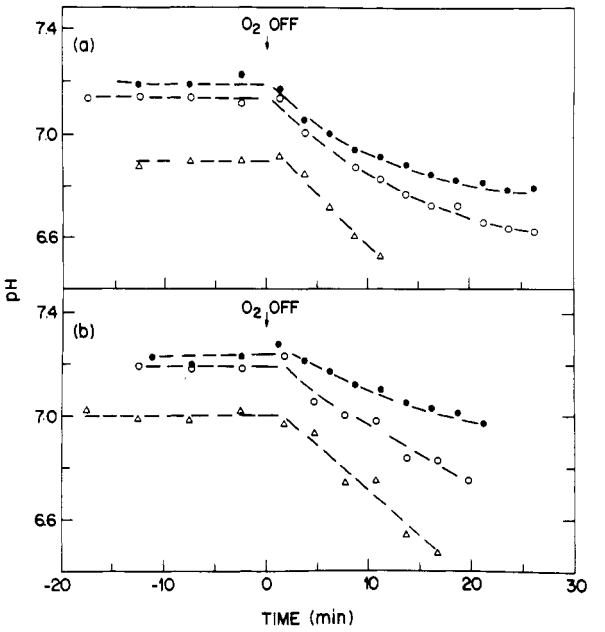

FIGURE 8: Intracellular pH of NR70 (△), DCCD-treated NR70 (○), and NR71 (●) cells at 20 °C during oxygenation and after oxygenation is stopped. (a) External pH 6.4; (b) external pH 6.1. Cells were suspended in 100 mM Pipes, 50 mM Mes, 40 mM NaCl, 8 mM KCl, and 4 mM P_i ($[Na_2HPO_4]$:$[KH_2PO_4]$ = 1:1). Total suspension volume was 4 times the cell pellet volume. The pH^{ex} was ~6.3 and ~6.0 in (a) and (b), respectively, at the time the oxygenation was started (at −40 min); in both cases, pH^{ex} increased by ~0.1 to ~0.15 unit during oxygenation and remained constant after O_2 was turned off.

pH unit in intracellular pH (Table I and Figure 8). In addition to increasing the pH^{in}, DCCD also slowed down by ~40% the rate at which the pH^{in} decreased after oxygenation was ceased (Figure 8); since pH^{ex} was constant after O_2 was turned off, the rate of pH^{in} decrease also represents the rate at which the transmembrane ΔpH collapsed.

NR70 cells spontaneously mutate into a ATPase$^-$ strain identified as NR71 (Rosen et al., 1978). Membrane vesicles prepared from NR71 cells exhibit properties similar to those of DCCD-treated vesicles of NR70: they are not deficient in respiration-dependent transport and this transport rate is not further enhanced by DCCD (Rosen et al., 1978). Our measurements of pH^{in} in suspensions of NR71 cells showed that at external pH values of 6.0–6.4, these cells generated a pH^{in} comparable to that observed in DCCD-treated NR70 cells (Table I and Figure 8). DCCD addition to the NR71 cell suspension did not increase the pH^{in} of these cells (Table I).

During the ^{31}P measurements, a major difference was observed between NR70 and NR71 cells. Generally, when *E. coli* cells are harvested and resuspended at high cell densities in a buffered salt medium lacking an exogenous carbon source as described under Experimental Procedures, the NMR spectra obtained from them show some NDP and little or no NTP (see top spectrum in Figure 1). This is clearly the case in NR70 (Figures 6 and 9a) as well as in MRE600 (Figure 1, top spectrum) cells. In NR71 cells, however, large NTP signals were observed after harvesting and in the *absence* of O_2 or an exogenous energy source (Figure 9b). These NTP signals were stable over 6–7 h at ~5 °C; at 20 °C, they disappeared slowly over a period of 90 min.

In addition, NR71 cells displayed a strong resonance at −1.4 ppm which was absent in NR70 cells. At pH^{ex} >6.5 when the extracellular P_i is shifted to −1.75 ppm or lower, this resonance is also observable in MRE600 cells (Ugurbil et al., 1978, 1979b). It stems from a phosphodiester compound which has not yet been identified.

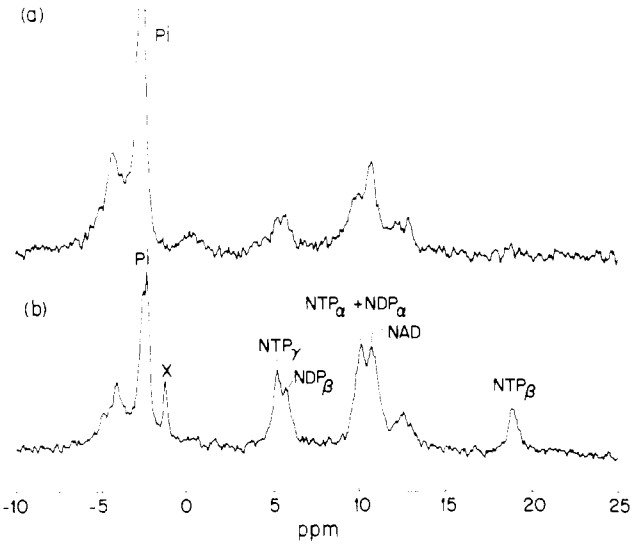

FIGURE 9: ^{31}P NMR spectra of NR70 (a) and NR71 (b) cells in suspension at 145.7 MHz and ~5 °C, in the absence of an exogenous carbon source. The spectra are the sum of 2000 FID's obtained with 45° pulses and a 0.34-s repetition time. Cells were grown and harvested as described under Experimental Procedures and finally suspended in cold 200 mM Pipes, 10 mM KCl, and 8 mM NaH_2PO_4, with the pH adjusted to 7.2 (b) and to 7.5 (a) with NaOH. Spectra were obtained immediately after the final suspension.

Discussion

It is clearly seen from the ^{31}P experiments shown in Figures 1 and 3 that *E. coli* generate a transmembrane pH gradient during respiration in the presence of a purely electron-donating carbon source, such as succinate. In the external pH range examined, the internal pH of respiring cells is regulated and maintained at a constant value between 7.35 and 7.60, depending on the strain (Table I). Consequently, the magnitude of the ΔpH generated in this pH^{ex} range is strictly determined by the pH^{ex}. These results are in agreement with the earlier pH measurements on aerobic *E. coli* cells calculated from the dimethyloxazolidinedione distribution (Padan et al., 1976) and from the ^{31}P NMR shifts (Navon et al., 1977a,b; Ogawa et al., 1978).

The inhibition of the membrane-bound ATPase with DCCD does not affect the generation of ΔpH during respiration; this is expected because during respiration, proton translocation by the electron transfer chain should generate a pH gradient independent of the ATPase activity. In fact, as in the case of control cells, *E. coli* treated with DCCD continue to regulate their internal pH at ~7.5, thus generating a ΔpH whose magnitude is determined by the extracellular pH. DCCD treatment, however, drastically reduces the level of NTP in respiring cells, as expected.

These results are in agreement with the generally accepted chemiosmotic hypothesis (Mitchell, 1966, 1968). According to this hypothesis, a reversible coupling is mediated by the membrane-bound, vectorial ATPase between a transmembrane electrochemical proton gradient which consists of a ΔpH and a $\Delta\psi$ component and intracellular ATP levels. It was shown for *E. coli* under *anaerobic* conditions that ATP generated during glycolysis was hydrolyzed by the ATPase to create a ΔpH (Ugurbil et al., 1978); contrary to what is observed in respiring cells, formation of this ΔpH was inhibited by DCCD (Navon et al., 1977b; Ugurbil et al., 1978). Furthermore, inhibition of the ATPase by DCCD during anaerobic glycolysis resulted in higher NTP levels and higher NTP/NDP ratios, again in direct contract to results obtained on respiring cells (Ugurbil et al., 1978).

The ATPase$^-$ cells are expected to behave like the DCCD treated wild-type cells. This, in fact, is what is observed. ATPase$^-$ NR70 cells are capable of generating a ΔpH but fail to synthesize any NTP during substrate oxidation in the presence of O_2. Conversely, under aerobic conditions, glucose catabolism in both NR70 and AN120 cells results in the accumulation of NTP, but a ΔpH is not generated. The observation that NR70 cells do not synthesize ATP during substrate oxidation is not surprising because this mutant is characterized as being uncoupled, i.e., deficient in oxidative phosphorylation and lacking ATPase activity. However, the inability of both NR70 and AN120 to generate a ΔpH during glucose catabolism under *anaerobic* conditions is not a priori expected outside the context of the chemiosmotic hypothesis.

In addition to being uncoupled, NR70 vesicles and to a lesser extent whole cells have been reported to display a marked defect in respiration-driven transport (Rosen, 1973a,b; Tsuchiya & Rosen, 1975; Rosen et al., 1978). There are extensive data which show that NR70 membrane vesicles do not contain F_1 and that these vesicles are unable to generate a transmembrane ΔpH (Tsuchiya & Rosen, 1975; Rosen et al., 1978). Addition of F_1 prepared from the parental strain 7 or treatment with DCCD allows NR70 vesicles to generate a ΔpH and repair their transport defect. It was, therefore, suggested that in the absence of F_1, the DCCD-sensitive F_0 fraction of the *E. coli* ATPase acts as an "open" proton channel in NR70 vesicles and cells; the DCCD reaction with F_0 seals the proton leak, restoring respiration-coupled transport and the ability to maintain a pH gradient. The membrane vesicles of NR71 cells also lack F_1; however, unlike the vesicles prepared from NR70 cells, NR71 vesicles are capable of establishing a ΔpH and are not deficient in respiration-dependent transport. It was therefore concluded that although F_0–F_1 complexes still do not exist in NR71 vesicles, the spontaneous mutation responsible for the conversion of NR70 to NR71 has sealed the open proton channel in F_0 in an analogous fashion to the DCCD reaction (Rosen et al., 1978).

Our measurements demonstrate that NR70 cells are unable to maintain a pH^{in} and a ΔpH as large as that of the wild-type strains. DCCD increases the pH^{in} and consequently the ΔpH and inhibits the rate at which this transmembrane proton concentration gradient collapses in the absence of respiration. Both observations are consistent with the notion that DCCD seals a H^+ leak in these cells. The pH^{in} and the ΔpH of DCCD-treated NR70 cells are comparable to those observed in NR71 cells; this, again, is consistent with the suggestion that the DCCD treatment and the genetic alterations which transform strain NR70 into NR71 have analogous effects. However, both in the DCCD-treated NR70 and in NR71 cells, pH^{in} values remain ~0.3 unit less than the pH^{in} displayed by their parental strain K-12 strain 7 and other wild-type cells (Table I). The reason for this major difference between these ATPase$^-$ strains and the wild-type cells is not understood.

Unlike the NR70 membrane vesicles (Rosen et al., 1978), respiring NR70 cells do establish a ΔpH, albeit a deficient one (Table I and Figure 6). This difference between the vesicles and the intact cells may simply reflect a much higher rate of respiration-linked H^+ translocation by the electron transport chain in the intact cells compared to that in the membrane vesicles. The H^+ flux through the open proton channel is probably directly proportional to the magnitude of the ΔpH. Therefore, the ΔpH should increase until the leak rate is equal to the H^+ translocation rate of the electron transport chain. Under these circumstances, the intracellular pH is not expected to be regulated at a constant value irrespective of the extra-

cellular pH; this is exactly what is observed in NR70 cells. ^{31}P NMR results demonstrate that in NR71 cells, the rate at which the NTP pool disappears in the absence of an exogenous carbon source is less than that in the NR70 cells (Figure 9); the specific reactions responsible for this are not known. Furthermore, NR71 cells accumulate large amounts of a phosphodiester compound (resonance X in Figure 9a) which has not been identified. Clearly, there exist some unexplained differences between the intermediary metabolism of strains NR70 and NR71; these differences could have arisen as an indirect consequence of the higher pH^{in} levels maintained by the NR71 cells.

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Mechanism of Tubulin Assembly: Guanosine 5′-Triphosphate Hydrolysis Decreases the Rate of Microtubule Depolymerization†

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ABSTRACT: The rate of depolymerization of microtubules upon lowering the temperature was found to depend on the amount of time elapsed since the beginning of the assembly process. In the first minutes following self-assembly at 37 °C, microtubules are more cold sensitive and depolymerize faster than later at the steady state. In the meanwhile, no change occurred in the average length nor in the shape of the distribution of microtubules. On the other hand, the evolution with time of the apparent dissociation rate constant of tubulin from microtubules was in good correlation with the GTP content of microtubules following assembly, showing that GTP hydrolysis modifies the tubulin–tubulin interactions. Microtubule-bound GTP was not exchangeable for GDP, but steady-state GTP hydrolysis was inhibited by GDP. This result indicates that GDP and GTP exhibit different affinities for tubulin in the body and at the ends of microtubules. It is proposed that GTP–tubulin dissociates faster from microtubules than GDP–tubulin. In other words GTP hydrolysis contributes to the stabilization of microtubules.

Since the discovery of a guanyl nucleotide requirement for microtubule polymerization (Weisenberg, 1972), and GTP hydrolysis during polymerization (Berry & Shelanski, 1972), the exact role of nucleotide hydrolysis in the structure and function of microtubules remains unknown.

It is well established that GTP hydrolysis occurs at the "exchangeable" E site of tubulin during polymerization, P_i is released in the medium and GDP remains blocked in the microtubule (Jacobs et al., 1974; Kobayashi, 1975; Weisenberg et al., 1976; David-Pfeuty et al., 1977).

The stoichiometry is one GTP hydrolyzed per molecule of tubulin dimer incorporated in the polymer (McNeal & Purich, 1978).

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