

High-resolution ^{31}P nuclear magnetic resonance studies of metabolism in aerobic *Escherichia coli* cells

(bioenergetics/chemiosmotic hypothesis/intracellular pH/adenosinetriphosphatase/proton gradient)

G. NAVON*, S. OGAWA, R. G. SHULMAN, AND T. YAMANE

Bell Laboratories, Murray Hill, New Jersey 07974

Contributed by R. G. Shulman, December 10, 1976

ABSTRACT ^{31}P nuclear magnetic resonance spectra at 145.7 MHz were obtained of concentrated suspensions of *E. coli* cells. The position of the P_i resonance was used to determine the pH, and in most experiments it was possible to distinguish the intracellular (pH_{in}) and extracellular (pH_{ex}) values. During respiration pH_{in} approached 7.55, while pH_{ex} varied from 6.0 to 8.0. With succinate as a carbon source and in a N_2 environment, $\text{pH}_{\text{in}} = \text{pH}_{\text{ex}}$. Upon addition of glucose, $\text{pH}_{\text{in}} > \text{pH}_{\text{ex}}$. In the presence of an ATPase (adenosinetriphosphatase; ATP phosphohydrolase; EC 3.6.1.3) inhibitor dicyclohexylcarbodiimide, pH_{in} remained equal to pH_{ex} even in the presence of glucose. In other experiments, oxygenation brought pH_{in} above pH_{ex} even in the presence of dicyclohexylcarbodiimide. These experiments are consistent with Mitchell's hypothesis that, first, ΔpH can be created by the reversal of the ATPase reaction and, second, that protons are pumped outward during respiration. In addition to P_i , about 10 more resonances were resolved, several of which were assigned to different phosphate metabolites.

High-resolution ^{31}P nuclear magnetic resonance (NMR) of intact cells has been used to measure intracellular concentrations of phosphate metabolites while simultaneously measuring ΔpH , the difference between intracellular and extracellular pH. In Ehrlich ascites tumor cells, for example (1), well resolved ^{31}P NMR peaks have been assigned to about one dozen metabolites. In other cells, such as intact muscles (2, 3), red blood cells (4, 5), and bakers yeast (6), some of these same metabolites have been identified. These assignments have been made from the spectra of the intact cells supplemented by the resonances observed in perchloric acid digests (1, 3) where the metabolites, *in vitro*, have been identified from their chemical shifts, pH titration, and coupling constants (1). The molecules identified include P_i , AMP, ADP and ATP, fructose 1,6-diphosphate, phosphoenolpyruvate, dihydroxyacetone phosphate, phosphorylcholine, glycerophosphorylcholine, glycerophosphorylethanolamine, NAD^+ , and uridine diphosphoglucose. It has been emphasized that the free concentrations of these molecules in the intact cells are measured by the NMR intensities, while spectra of the acid extracts measure the total concentrations.

Numerous experiments have shown connections between these metabolites and the proton motive force $\Delta\mu_{\text{H}}$ across membranes, composed of ΔpH and an electrostatic contribution $\Delta\psi$ (7). These results generally support the chemiosmotic hypothesis proposed by Mitchell (8, 9).

Recent experiments by Padan *et al.* (10) measured the two separate components of $\Delta\mu_{\text{H}}$ in *Escherichia coli* by chemical means under a variety of conditions. Previously it had been shown that in *E. coli* cells (11) and vesicles (12, 13) specific transport systems are coupled to an energized state of the

membrane which could be energized either from ATP or oxidation-reduction processes. These typical results suggests that high-resolution ^{31}P NMR studies of *E. coli* would be valuable because they could give a simultaneous measure both of ΔpH and the distribution of metabolites. It has been necessary to make measurements rapidly because the life cycle of *E. coli* is less than 1 hr, and often one wants to obtain a well resolved spectrum with adequate signal to noise in a small fraction of a cell cycle. This has been made possible by the resolution and sensitivity of our Bruker HX360 NMR spectrometer. Another technical detail hindering these measurements was the need to measure the NMR spectra under aerobic conditions in the NMR tube. These conditions have been obtained in two ways, one quite simple, as described below.

EXPERIMENTAL

E. coli MRE600 were grown in M9 medium with glucose (18 mM), glycerol (45 mM), or succinate (16 mM) as carbon sources. Cells were harvested in exponential phase at a cell density of about 1×10^9 /ml, washed once with fresh growth medium, and resuspended at a density of 3 to 5×10^{11} /ml in the same buffer. ^{31}P NMR spectra were measured with a Bruker HX360 spectrometer operating in the Fourier Transform mode. Samples were contained in 10-mm diameter tubes and were generally about 1.5 ml in volume. Different methods of bubbling O_2 (or N_2) were used. In one we took advantage of the large change in the lock signal when the oxygen bubble passed through the solution. By synchronizing the pulse trigger to the level of the lock signal, it was possible to pulse the transmitter when the lock signal returned to its original state, indicating that the bubble had passed. In this way the free induction decay was accumulated when the bubble was not present in the solution and the magnetic field homogeneity was preserved. Also, by introducing a variable delay before the transmitter pulse it was possible to synchronize the NMR measurement with respect to the oxygen pulse.

The second method was to bubble oxygen and pulse the transmitter in a nonsynchronous fashion, which gave similar results to the first method except that some line broadening due to magnetic field inhomogeneity was introduced.

RESULTS

Fig. 1 shows the ^{31}P NMR spectra of *E. coli* cells grown in glucose. The top spectrum was summed over a 6-min interval between 7 and 1 min before O_2 was vigorously introduced. The peaks, designated by letters, are tentatively identified by comparison with known standards and with the results obtained more definitely in Ehrlich ascites tumor cells (1).

After the onset of oxygen bubbling, peak A in the phosphoester region disappeared and peaks D, E, F, and G grew. Peak I appeared at a position corresponding to the β phosphate

Abbreviations: NMR, nuclear magnetic resonance; pH_{in} , intracellular pH; pH_{ex} , extracellular pH.

* Present address: Department of Chemistry, Tel Aviv University, Ramat-Aviv, Tel-Aviv, Israel.

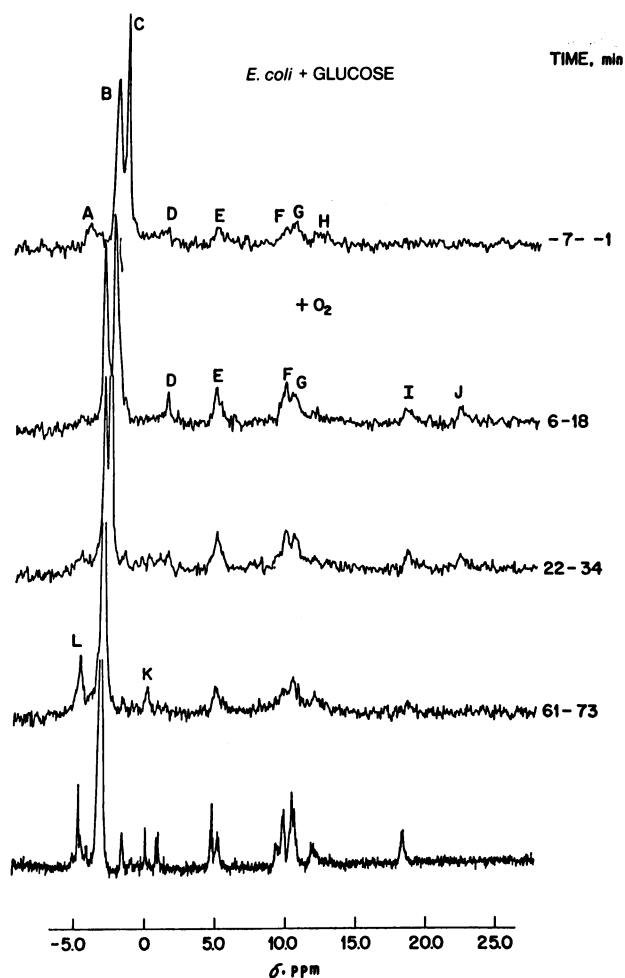


FIG. 1. 145.7 MHz ^{31}P NMR spectra of a suspension of *E. coli* cells grown in M9 medium with glucose as the carbon source. After centrifugation, the pellet (about 1 ml) was suspended in 1 ml of M9 medium with added 3 mM glucose, 50 mM 2-(*N*-morpholino)ethanesulfonate (Mes), 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes), and 20 mM phosphate at pH 6.0. On the basis of chemical shifts and by comparison with acid extracts of ascites cells (1) the tentative assignments are: peaks A and L, phosphomonoesters not identified; peak B, intracellular P_i ; peak C, extracellular P_i ; peak D, not identified; peak E, nucleotide triphosphate, α phosphate; peak F, nucleotide triphosphate, γ phosphate; peaks G, NAD^+ ; peaks H, UDPG; peak I, nucleotide triphosphate, β phosphate; peak J, polyphosphate; and peak K, phosphoenolpyruvate. Spectra measured at 20°.

of ATP and peak J appeared at a position corresponding to polyphosphate. Between 61 and 73 min after the onset of oxygenation peak D disappeared and peaks E, F, and I, assigned to nucleotide triphosphates, became weaker, presumably because the carbon supply was decreasing. Interestingly, at this time peak K, from phosphoenolpyruvate, became quite strong. A similar high concentration of phosphoenolpyruvate in the endogenous state has been reported for yeast (14, 15), while during glycolysis its concentration is an order of magnitude lower. After this spectrum was taken the cells were lysed with perchloric acid and the pH was adjusted to 7.5, which was the intracellular pH in the intact cells as determined from the position of peak B. The NMR spectrum of the extract is shown at the bottom of Fig. 1. In the *E. coli* extracts the nucleotide tri- and diphosphate peaks (Fig. 1E, F, I, and H) are not well resolved, in contrast to the well resolved peaks observed in ascites cell extracts that have been assigned to adenylate nucleotides. We

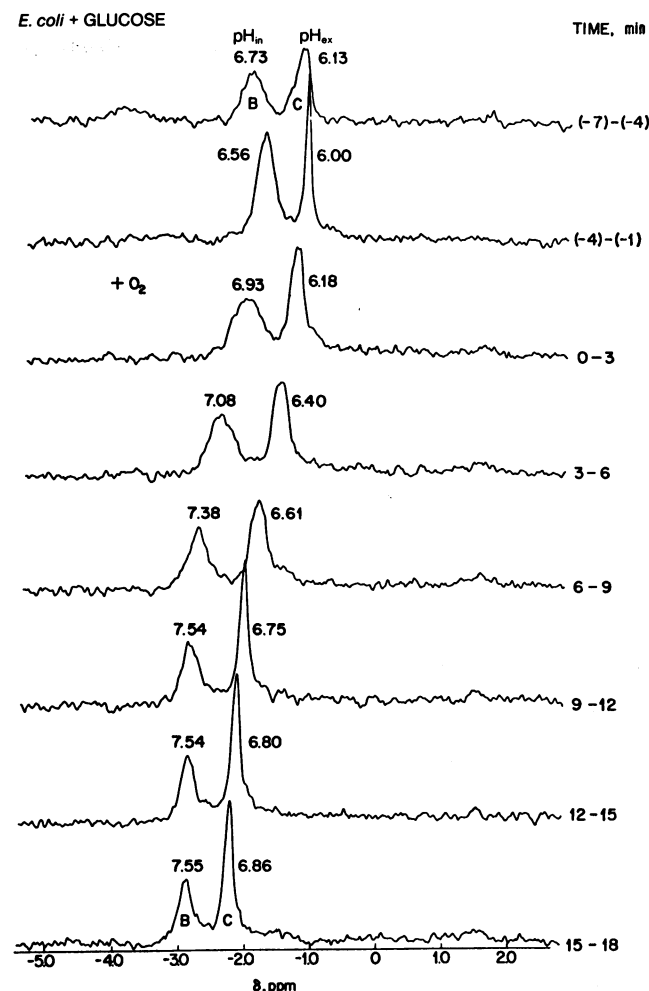


FIG. 2. Same experiment as in Fig. 1 except that spectra accumulated for smaller time intervals are shown.

have shown (unpublished data) that at these high fields peaks from the four nucleotide tri- and diphosphates do not superimpose. Here the *E. coli* spectra show a mixture of tri- and diphosphates, which has been reported for *E. coli* (16) in contrast to the predominance of ATP and ADP reported for ascites cells.

If one is only interested in using the position of the strong P_i peaks to measure the internal (pH_{in}) and external (pH_{ex}) pH, spectra with adequate signal to noise can be obtained in a shorter time than displayed in Fig. 1. Fig. 2 shows spectra of the P_i region accumulated for 3 min each before and after the beginning of oxygen bubbling. Some of these spectra had been added together in Fig. 1. These results, which are typical, show that upon oxygenation the value of pH_{in} becomes constant near 7.55, reaching this value within about 10 min and maintaining it for more than 1 hr, as shown by the position of the P_i peak in Fig. 1. Because pH_{in} depends upon oxygenation, it can be used as an indicator of the adequacy of the oxygen flow. We measured pH_{in} as a function of O_2 bubbling rate and cell concentration. From this, the standard bubbling rate was fixed at 19 ml of O_2 per min. The measured values of pH_{in} compared to pH_{ex} are shown in Fig. 3, where pH_{in} has been measured a few minutes after the onset of O_2 bubbling in fresh samples of *E. coli* cells, with different values of pH_{ex} at the beginning of each experiment.

Fig. 4 shows the values of pH_{in} and pH_{ex} for three different *E. coli* samples, grown on M9 medium plus succinate, centri-

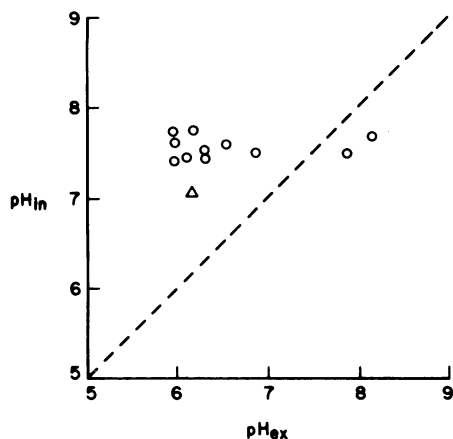


FIG. 3. Values of pH_{in} and pH_{ex} measured at the plateau values of pH_{in} after oxygenation. Cell growth was in glucose- or succinate-enriched M9 medium, and a variety of initial pH values were used. Open circles indicate logarithmic phase cells; triangles indicate stationary phase cells. All experimental points above the dashed line show conditions where $\text{pH}_{\text{in}} > \text{pH}_{\text{ex}}$; points below, where $\text{pH}_{\text{in}} < \text{pH}_{\text{ex}}$.

fused at 15° , washed in medium, recentrifuged, and suspended in an equal volume of medium. The values of pH_{in} and pH_{ex} were determined from the measured positions of the orthophosphate resonances. At time zero, glucose was added to a concentration of 20 mM, as shown in all three parts of Fig. 4. In Fig. 4 (top) the cells were aerobic but contained CN^- , designed to inhibit respiration. It almost completely did so, as

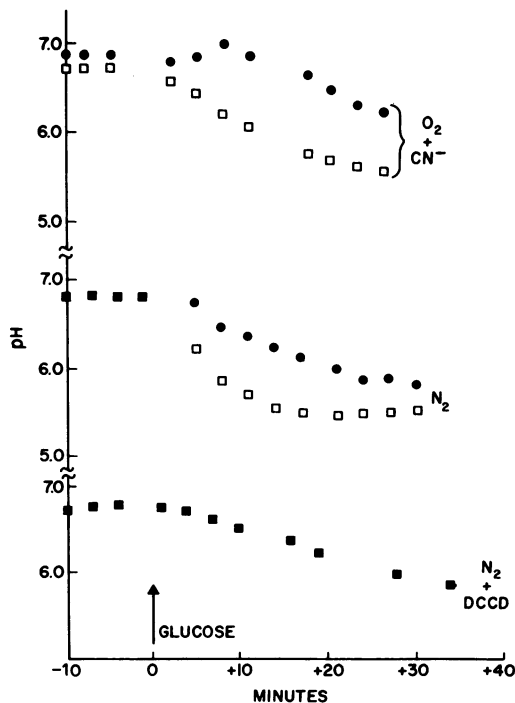


FIG. 4. *E. coli* grown on M9 medium with 16 mM succinate as a carbon source were centrifuged and resuspended in M9 containing 20 mM phosphate and 50 mM Bis-Tris buffer pH 6.8. Glucose was added at time zero to make a concentration of 20 mM. (Top) 1 mM KCN was added before centrifugation and the rate of O_2 bubbling was 18 ml/min. (Bottom) 1 mM dicyclohexylcarbodiimide (DCCD) was added before centrifugation. The rate of N_2 bubbling in the middle and bottom curves was 6.4 ml/min. Circles are pH_{in} , and squares pH_{ex} . Experiments were done at 20° .

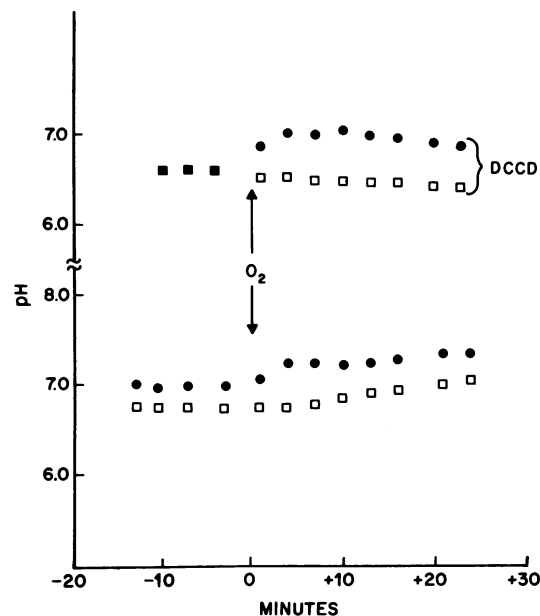


FIG. 5. Same conditions as in Fig. 4 for cells grown in succinate-enriched M9 medium and resuspended in buffer. Oxygen bubbling at 18 ml/min was started at time zero. (●) pH_{in} ; (□) pH_{ex} .

judged from the very small values of ΔpH observed before glucose was introduced. Upon addition of glucose, however, the glycolytic pathway was opened, the cells were energized, and ΔpH increased towards its normal value. Note that the NMR spectra showed that the small value of ΔpH plotted in Fig. 4 (top) before glucose was added actually reflected a homogeneous value of ΔpH and not a spread of ΔpH values, such as would be found if only a fraction of the cells had a large value of ΔpH , while in the rest $\Delta\text{pH} = 0$. Fig. 4 (middle) shows that when N_2 was used to inhibit respiration ΔpH was zero, and while the introduction of glucose lowered both pH_{in} and pH_{ex} it did create a large value of ΔpH . Finally, in Fig. 4 (bottom) dicyclohexylcarbodiimide, an inhibitor of ATPase, was added to the cells, after which glucose had no effect upon the value of ΔpH . Both pH_{in} and pH_{ex} decreased, but the value of ΔpH remained zero.

Fig. 5 (top) shows a complementary experiment in which glycolysis was prohibited by the absence of glucose, while respiration was stimulated by the onset of oxygen bubbling at time zero. Before the onset of respiration the cells were not energized and $\Delta\text{pH} = 0$. Upon oxygenation the value of pH_{in} increased, even in the presence of dicyclohexylcarbodiimide, in accord with the suggestion that protons were being pumped out of the cells during respiration (8). Finally, in Fig. 5 we see that in this batch of *E. coli* cells, the value of pH_{in} did not reach the usual value of 7.5–7.6 but leveled off near pH 7.1, presumably because the cells were harvested closer to their stationary phase.

DISCUSSION

The present experiments illustrate various ways in which ^{31}P NMR measurements can be useful in understanding the functioning of *E. coli* cells. Free concentrations of phosphate metabolites can be followed and observed to vary with changes in the state of the cell, for example, as illustrated in Fig. 1 for phosphoenolpyruvate and nucleotide triphosphates. The P_i resonance is particularly useful and allows us to determine pH_{in} and pH_{ex} separately and accurately. Our pH measurements agree with those reported recently by Paddan *et al.* (10), who

also observed that pH_{in} remains constant, to a first approximation, as pH_{ex} varies from pH 5 to 8. These results are not in agreement with previous reports (17, 18), although it is clear that the cell must be thoroughly energized to reach the present results.

The experiments shown in Figs. 4 and 5 were intended to test two assumptions made by Mitchell in the chemiosmotic hypothesis (8, 9). The first of these is that during respiration protons are pumped out of the cells. The second assumption is that the chemical potential created by the proton translocation is coupled to a vectorial ATPase activity, thereby lowering the free energy needed to form ATP. Hence, by reversing the ATPase reaction one could expect that ATP hydrolysis could be used to form a proton gradient. The proton gradient has been shown to be composed of an electrostatic membrane potential $\Delta\psi$ plus a contribution from the ΔpH . In the present experiments we are measuring only the latter component, so that although the results are not energetically complete, they are qualitative and suggestive.

In Fig. 4 it is shown that ΔpH is very small (top) or zero (middle) before glucose is added, and that it rises to almost one pH unit afterwards. Presumably the ATP formed during glycolysis was hydrolyzed by ATPase and used to create the observed ΔpH . This interpretation was tested by the experiment shown in Fig. 4 (bottom), where dicyclohexylcarbodiimide, an ATPase inhibitor, was added, in which case ΔpH remained zero after the glucose was added. In all three of these experiments respiration was blocked either by CN^- in Fig. 4 (top) or by N_2 (middle and bottom).

In the experiments shown in Fig. 5 respiration in the succinate solution of *E. coli* cells was stimulated by oxygen bubbling which was started at the time indicated. The top experiment containing dicyclohexylcarbodiimide, had no ATPase activity, and before oxygenation had $\Delta\text{pH} = 0$. In contrast, the experiment shown below had its full ATPase activity so that before oxygenation, $\Delta\text{pH} \neq 0$ presumably from the ATPase ac-

tivity. In both cases oxygenation increased the value of ΔpH , which is in accord with Mitchell's assumption that during respiration protons are pumped outward.

It is clear from these experiments that ^{31}P NMR measurements are useful in understanding aspects of bioenergetics in bacteria.

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