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^{31}P nuclear magnetic resonance studies of bioenergetics and glycolysis in anaerobic *Escherichia coli* cells

(ΔpH/adenosine triphosphatase/dicyclohexylcarbodiimide)

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ABSTRACT **^{31}P nuclear magnetic resonance spectra of glycolyzing, anaerobic *Escherichia coli* cells and their perchloric acid extracts were obtained at 145.7 MHz. Time-dependent intracellular concentrations of nucleoside di- and triphosphates, P_i, and sugar phosphates were measured during glycolysis with 2-min resolution, while intracellular and extracellular pH values were monitored simultaneously. Upon glucose addition, anaerobic *E. coli* cells rapidly produce acids and develop a transmembrane pH gradient (ΔpH). Glycolysis rates were calculated from the changes in the external pH. It was found that glycolysis rates are strongly dependent on internal pH, sharply decreasing when the pH drops below ~7.2. The ATPase inhibitor, dicyclohexylcarbodiimide (DCCD), prevented NTP hydrolysis and inhibited ΔpH formation. The uncoupler, carbonyl cyanide *p*-triflouromethoxyphenyl hydrazone (FCCP), drastically reduced both the ΔpH and the NTP level. When the cells were previously treated with DCCD, FCCP collapsed the ΔpH while the NTP levels remained high. It is concluded that ATP produced by glycolysis is hydrolyzed by the membrane ATPase to generate a ΔpH and that FCCP stimulates ATP hydrolysis by ATPase and collapses the proton gradient.**

High-resolution nuclear magnetic resonance (NMR) of cellular suspensions and tissues can measure intracellular pH from the chemical shifts of metabolites such as inorganic phosphate (P_i), which titrate in the physiological pH range. In addition, resonances from numerous metabolites, including intracellular and extracellular P_i, intermediates of the glycolytic pathway, and nucleoside di- and triphosphates, have been identified and their concentrations *in vivo* followed as a function of stimulation and time (1–5).

In microorganisms a transmembrane proton gradient, ΔpH, and a membrane potential, $\Delta\psi$, are important both for production of ATP during oxidative phosphorylation and for transport of various substrates (6, 7). It has been demonstrated that during respiration, *Escherichia coli* can maintain a large ΔpH and a $\Delta\psi$. The magnitude and the direction of the ΔpH is a function of the external pH, effectively maintaining the internal pH constant at about 7.5 (8). During respiration, the source of the proton electrochemical gradient is proton translocation, which is coupled to substrate oxidation. Under *anaerobic* conditions, dimethyloxazolidinadione measurements on *Streptococcus faecalis* (9) and, more recently, ^{31}P NMR measurements on *E. coli* (4) have shown that glycolyzing cells also maintain a ΔpH. Generation of the ΔpH was inhibited by the membrane-bound ATPase inhibitor dicyclohexylcarbodiimide (DCCD), indicating ATPase-catalyzed hydrolysis of ATP as the source of the proton gradient.

In this study, we extended the earlier ^{31}P NMR studies to measure with 2-min resolution the time course of ΔpH and simultaneously the levels of metabolic intermediates during anaerobic glycolysis in suspensions of *E. coli*. Furthermore, the effects of internal pH, external pH, the ATPase inhibitor DCCD, and the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) on these parameters were investigated. The sensitivity and the chemical shift resolution in these spectra, accumulated for 2 min, is sufficient to allow direct observation *in vivo* of several key intermediates, namely, dihydroxyacetone phosphate, fructose 1,6-bisphosphate (Fru-P_2), P_i, and nucleoside tri-, di-, and monophosphates (NTP, NDP, and NMP, respectively). Several other intermediates not resolved in the *in vivo* spectrum were identified and followed in the extract spectrum. Finally, from the ^{31}P NMR measurements the rate of glycolysis was calculated and was found to decrease when the internal pH dropped below 7.2.

EXPERIMENTAL

E. coli, strain MRE 600, was grown aerobically in 2-liter batches at 37°C in M9 minimal medium (10) supplemented with 0.1 mM $CaCl_2$ and 20 mM glucose. At midlogarithmic phase of growth the cultures were removed from the incubator and cooled to 4°C in an ice bath. During the cooling processes aeration was continued by bubbling O_2 through the cultures. Cells were then centrifuged at ~4°C and the pellets were washed twice and resuspended in an equal volume of cold 10 mM KH_2PO_4/10 mM Na_2HPO_4/100 mM 1,4-piperazinediethanesulfonate (Pipes)/50 mM 4-morpholineethanesulfonate (Mes)/85 mM NaCl at pH 7.3. The cell suspension was kept in an ice bath until ~20 min before addition of glucose, at which point the temperature was brought up to 20°C. External buffer capacities for these suspensions were obtained by titrating them with 1 M HCl and NaOH both before and after catabolism of glucose.

^{31}P NMR spectra were obtained in 10-mm sample tubes at 145.7 MHz on a Bruker HX 360 instrument operating in the Fourier transform mode. Free induction decays were accumulated in 2-min blocks with either a repetition rate of 0.34 sec and a 60° pulse or a repetition rate of 0.68 sec and a 90° pulse. During data accumulation, NMR tubes were continuously flushed with N_2 to maintain conditions anaerobic. All chemical shifts are referenced to external 85% phosphoric acid.

The NMR intensities of external and internal P_i and sugar phosphates were corrected for spin-lattice relaxation (T_1) effects using a spectrum taken with 10-sec intervals between consecutive pulses. The nucleotide phosphate intensities were corrected assuming that their T_1s are ~0.2 sec, as had been measured in similar preparations of *E. coli* (11). Intracellular concentrations were calculated assuming that the total intracellular

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Abbreviations: NMR, nuclear magnetic resonance; DCCD, dicyclohexylcarbodiimide; FCCP, carbonal cyanide *p*-trifluoromethoxyphenyl hydrazone; Pipes, 1,4-piperazinediethanesulfonate; Mes, 4-morpholineethanesulfonate; Fru-P_2, fructose 1,6-bisphosphate.

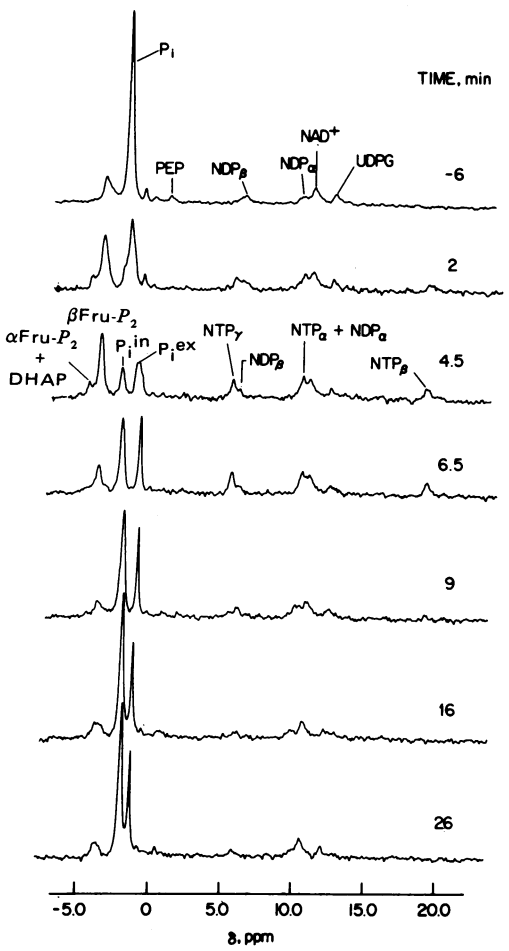

FIG. 1. 145.7 MHz ^{31}P NMR spectra of anaerobic *E. coli* cells in suspension at 20°C. The NMR sample contained ~5 × 10^{11} cells in 1 ml of medium containing 20 mM phosphate/85 mM NaCl/50 mM Mes/100 mM Pipes at pH 7.3. At time 0, glucose was added to a final concentration of 25 mM in the cell suspension. Each spectrum represents a sum of 200 scans obtained with a 90° pulse and a repetition time of 0.6 sec. PEP, phospho*enol*pyruvate; DHAP, dihydroxyacetone phosphate.

volume is half of the pellet volume (8) and consequently one-fourth of the NMR sample volume.

RESULTS

Typical 145.7 MHz ^{31}P NMR spectra of anaerobic *E. coli* cells at different times after glucose addition are shown in Fig. 1. The peaks were identified by their chemical shifts and the pH titration of perchloric acid extracts. The assignments were confirmed by adding to the extracts small quantities of the metabolites purchased from commercial sources in their purified forms. Two extract spectra, one before glucose addition and the other during glycolysis, are shown in Fig. 2. Prior to glucose addition, in the absence of an external carbon source, the intact cell spectrum (Fig. 1 *top*) is characterized by the presence of NDP α- and β-phosphate resonances at 10.0 and 5.6 ppm, respectively, and a single intense P_i peak at −2.52 ppm showing that the intra- and extracellular pHs are the same. The broad phosphomonoester peak observed at −4.2 ppm in the intact cell spectrum before glucose addition is resolved into several distinct resonances in the extract spectrum (Fig. 2). Three of these resonances have been assigned to 3-phosphoglycerate, and 1- and 6-phosphates of β Fru-P_2, and NMP (Fig. 2). The extract spectra further show that the NDP pool is heterogeneous. The two distinct doublets observed for the NDP α-phosphates are assigned to purine and pyrimide nucleoside diphosphates.

Introduction of 25 mM glucose at time 0 led to a rapid build-up of sugar phosphate peaks at −5.2 and −4.4 ppm (at an internal pH of 7.5) concurrent with the formation of NTP α-, β-, and γ-phosphate resonances at 10.0, 18.7, and 5.0 ppm, respectively (Fig. 1). The extract spectra (Fig. 2) show that the NTP peaks are also composite, and include contributions from other nucleotides besides ATP. The single P_i resonance of the intact cell spectrum is broadened 2 min after glucose addition and splits into two distinct peaks at 4.5 min, reflecting the formation of a ΔpH. The cellular NDP pool, as monitored by the partially resolved NDP resonance at 5.6 ppm, is not altered much in the presence of glucose. Note that the total P_i intensity is decreased as sugar phosphates and NTP are synthesized. At 4.5 min the cytoplasmic concentration of P_i is ~9 mM.

In the sugar phosphate region, Fru-P_2, dihydroxyacetone phosphate and glucose 6-P, have been assigned using extracts (Fig. 2). 3-Phosphoglycerate, NMP, and other (unassigned) resonances detectable prior to glucose addition are not observed during the steady state of glycolysis. In the presence of glucose, approximately 90% of the overall sugar phosphate intensity is Fru-P_2 (see Fig. 2). The intracellular concentration of this metabolite at its maximum is ~13 mM, as calculated from the NMR peak intensities of the intact cell. As the sugar phosphate intensity begins to decrease at 0.5 min, the internal P_i intensity increases. The external P_i peak intensity remains essentially unaltered although this resonance gets narrower as the pH stops changing because glucose disappears. After the sugar phosphates disappear, the NTP intensity and the ΔpH diminish with time. Both the cell and the extract spectra obtained (not shown) after the glucose has been consumed are essentially identical to that observed for freshly harvested cells before glucose addition; the only difference is the presence of a residual ΔpH, which collapses later.

A similar sequence of events occurs when, prior to glucose addition, the cells are incubated with DCCD. Although sugar phosphates and NTP are synthesized by the DCCD-treated cells, the ΔpH formed gets significantly smaller with increasing DCCD concentration, as indicated by the smaller separation between the internal and external P_i resonances. In the presence of 2 mM DCCD, the internal P_i resonance appears as a downfield shoulder at 4.5 min and is partially resolved at time ≥7 min (not shown). Similarly, at 4 mM DCCD, a small upfield shoulder in the total P_i resonance appears at 10 min (Fig. 3); the intensity of this partially resolved peak increases when the sugar phosphates are consumed. The data for the experiments with 0, 2, and 4 mm DCCD are plotted as a function of time in Fig. 4.

When FCCP is added to anaerobic *E. coli*, the P_i resonance does not split and, furthermore, the NTP ^{31}P peaks are not detected (Fig. 5). Glucose is metabolized in the presence of FCCP, as shown by the buildup and subsequent disappearance of the sugar phosphates and the continuous upfield shift of the P_i peak as the acidic end-products of glycolysis are formed. Incubation of the cells with 4 mM DCCD before addition of FCCP and glucose, however, removes the effects of FCCP upon metabolism: while ΔpH is still 0, the NTP intensity increases to the levels seen in cells treated with 4 mM DCCD alone. The effects of the DCCD and FCCP on the NTP levels is best shown in Fig. 6, in which signal to noise is enhanced by adding individual 2-min spectra, such as the ones shown in Fig. 1, which displayed the maximum sugar phosphate intensity during the experiment.

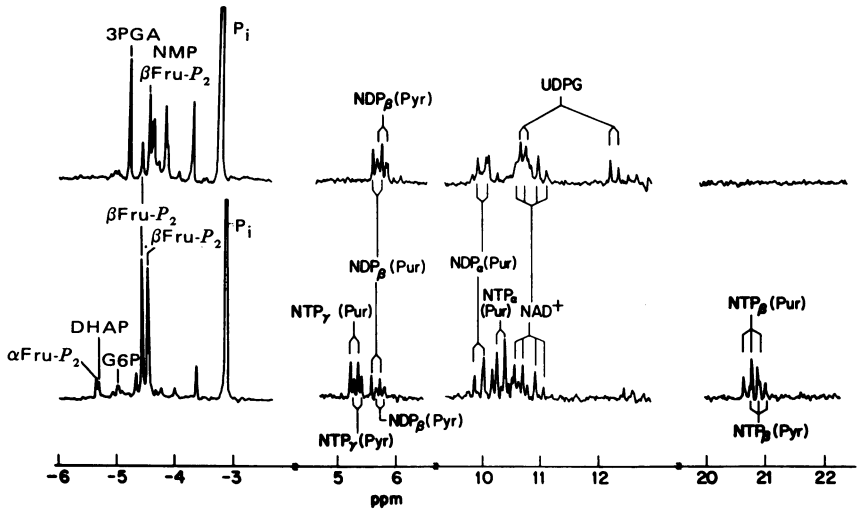

FIG. 2. 145.7 MHz ^{31}P NMR spectra at 20°C of *E. coli* cell extracts before glucose addition (*Upper*) and during glycolysis in the presence of glucose (*Lower*). Experimental conditions for cell growth and suspensions were identical to those of Fig. 1. Extracts were prepared as described in ref. 4. The solution pH was 8.2 and 8 for the upper and lower spectra, respectively. 3 PGA, 3-phosphoglycerate.

DISCUSSION

For reasons given above, it is of interest to follow the connection amongst the ATP levels, the glycolytic pathway, and ΔpH under anaerobic conditions in *E. coli*. Figs. 1 and 4 show that upon the introduction of glucose, *E. coli* establish a substantial ΔpH. Presumably, the ΔpH is generated by the operation of the membrane-bound ATPase which couples ATP hydrolysis to translocating protons out of the cell, thereby creating a proton electrochemical gradient. This is supported by the observation that DCCD, which inhibits the ATPase, also reduces the magnitude of the ΔpH formed (Figs. 1, 3, and 4). Furthermore, consistent with inhibition of the membrane ATPase, during the steady state of glycolysis the NTP/NDP ratio is considerably increased in the presence of DCCD, and after the termination of glycolysis the rate at which this ratio decays (with the concomitant increase in P_i levels) is slower (Fig. 4). In the presence of FCCP, the ΔpH is also reduced, but the NTP/NDP ratio is very low. This indicates that rather than inhibiting the ATPase, FCCP stimulates it by reducing the proton electrochemical

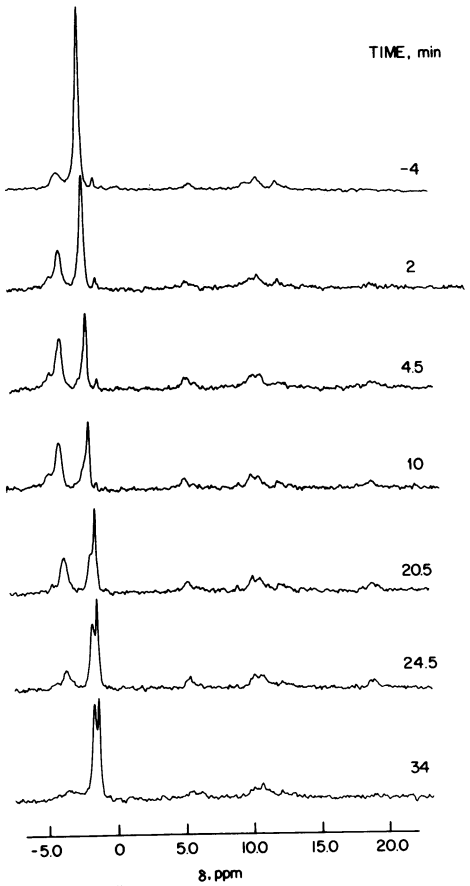

FIG. 3. 145.7 MHz ^{31}P NMR spectra of anaerobic *E. coli* cells treated with 4 mM DCCD. Cells used for this experiment were taken from the same batch used for the experiment shown in Fig. 1. All conditions were identical to those specified for Fig. 1, except that 20 min before glucose addition 4 mM DCCD was added to the NMR sample at 20°C.

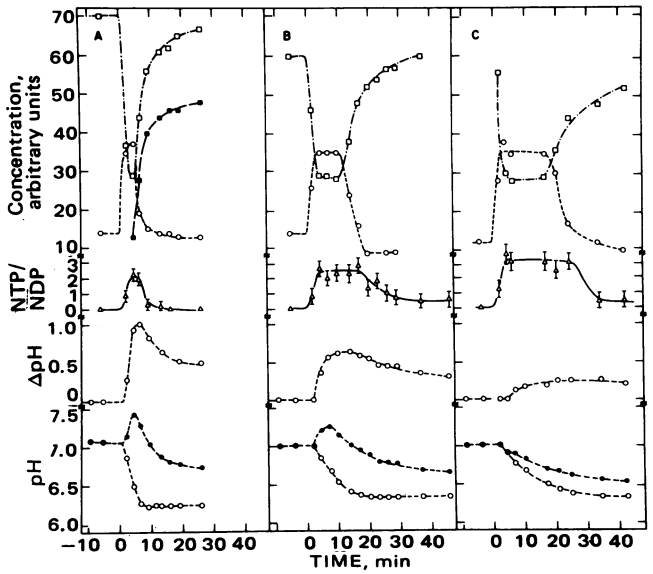

FIG. 4. Time dependence of P_i and total sugar phosphate concentrations, NTP/NDP ratio, internal pH, external pH, and ΔpH in anaerobic *E. coli* cells at 20°C. Data were obtained from the experiments of Figs. 1 and 3 and an experiment performed under identical conditions and with the same batch of cells used for those of Figs. 1 and 3. Each point represents an average of 2 min. (*A*) Control, no DCCD; (*B*) 2 mM DCCD; (*C*) 4 mM DCCD. Top plot: □, total P_i; ■, internal P_i; ○, sugar phosphates.

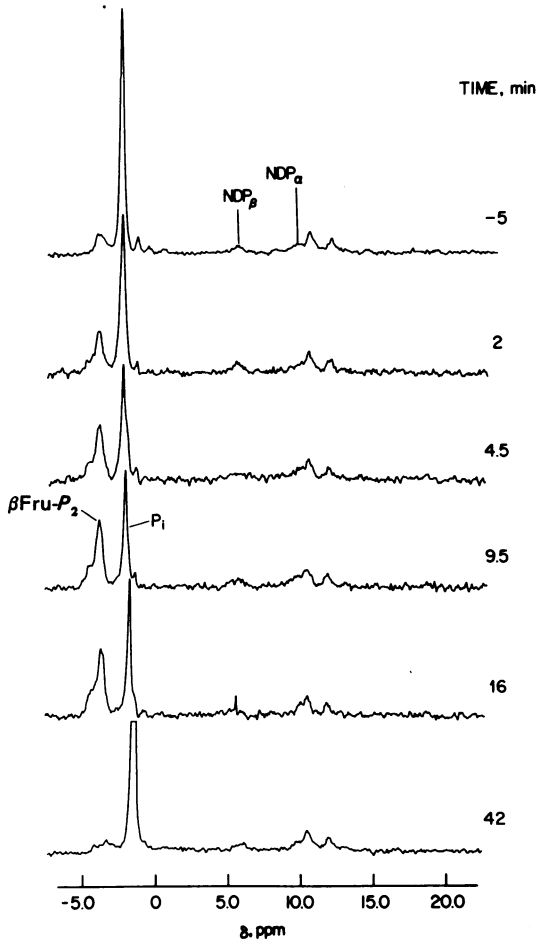

FIG. 5. 145.7 MHz ^{31}P NMR spectra of anaerobic *E. coli* cells in suspension at 20°C in the presence of 75 μM FCCP. Experimental conditions were identical to those given for Fig. 1. FCCP was added 20 min before addition of 25 mM glucose, which was added at time 0.

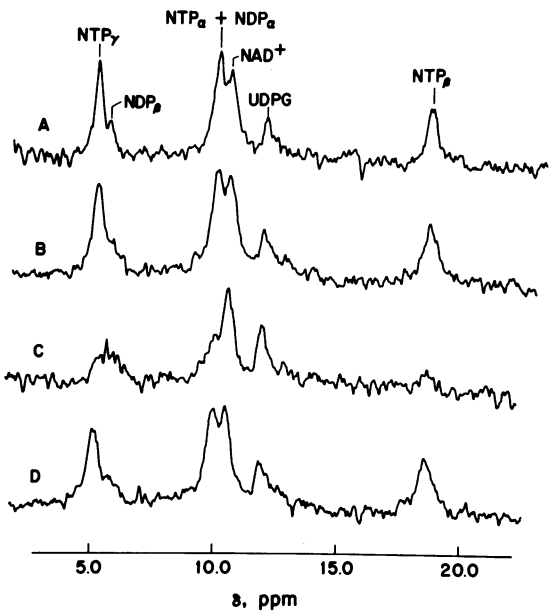

FIG. 6. Upfield region of 145.7 MHz ^{31}P NMR spectra of anaerobic *E. coli* cells. (*A*) Control, 400 scans; (*B*) 4 mM DCCD, 800 scans; (*C*) 75 μM FCCP, 800 scans; (*D*) 75 μM FCCP and 4 mM DCCD, 800 scans. The spectra for *A*, *B*, and *C* were obtained by summing the individual 200-scan spectra of Figs. 1 and 2. *D* was obtained from a different run performed under the conditions of Fig. 1. A control experiment for *D* gave results identical to those of Fig. 1. In each case the sums included spectra that displayed the maximum amount of sugar phosphate intensity observed in that particular series.

potential. Finally, when both DCCD and FCCP are present, no ΔpH is formed while the level of NTP is quite high. This observation clearly shows that the effect of FCCP on the ATP levels must be mediated through the DCCD-sensitive ATPase.

Contrary to the aerobic case, under our conditions the magnitude of the ΔpH maintained by anaerobic *E. coli* is not sufficient to keep its internal pH constant at ~7.5 in the external pH range 6.0–7.5. In our samples, the internal pH reached ~7.5 briefly at the beginning of glycolysis and subsequently decreased simultaneously with external pH.

The cellular ATPase free energy can be calculated from a knowledge of the internal P_i, ADP, and ATP, free Mg^{2+} concentrations, and internal pH (12). Assuming that the ATP/ADP is proportional to the NTP/NDP ratio, and that the cellular free Mg^{2+} concentration is 1 mM (which seems reasonable since all detectable NTP is bound to Mg^{2+}), we calculate the ATPase free energy in our experiments to be ~10 kcal/mol at its maximum.

Glycolysis in anaerobic *E. coli* cells

The main features of the glycolytic process, as observed in Figs. 1 and 3, are that addition of glucose results in about 80% reduction of internal P_i concentration with a simultaneous increase in Fru-P_2 and NTP concentrations. Most of the P_i that disappears is stored in Fru-P_2 during the steady state, with smaller amounts in NTP and other intermediates. At the same time there is a rapid drop of external pH, indicating the formation of acids. When the glucose is depleted the rate of acid production is reduced, together with a rapid drop in the level of Fru-P_2 and the reappearance of P_i. The NTP level, however, remains high even after the level of Fru-P_2 drops, indicating the continuing synthesis of NTP as the pool of phosphorylated intermediates is being exhausted.

A very rough estimate of the rate of glycolysis can be obtained by dividing the amount of added glucose by the duration of the steady state of glycolysis. A better estimate is obtained from following the acidification of the medium, as expressed in the shift of the external P_i resonance. The cell suspensions were titrated, so that from the rate of change of external pH the amount of acid production can be calculated. The rates measured in these two ways agreed very well under many different conditions.

Figs. 1, 3, and 5 show that DCCD and FCCP both drastically reduced the rate of glycolysis. Moreover, the rate of glycolysis decreased during the course of these experiments although the levels of detectable glycolytic intermediates were unaffected. The control of the glycolytic rate is supposed to be mediated mostly by the allosteric enzymes phosphofructokinase and pyruvate kinase (13). Both of these enzymes are affected by the levels of ADP, ATP, Fru-P_2, and phospho*enol*pyruvate; phosphofructokinase in addition is controlled by other metabolites. In our experiments DCCD increased the NTP/NDP ratio and FCCP decreased it, while both reduced the glycolytic rate. In both cases the Fru-P_2 level was not different from the control, and the phospho*enol*pyruvate/ADP ratio is so low that it could not drastically affect phosphofructokinase activity (14). No correlation was found between measurable glycolytic rates and these metabolite levels in our experiments. However, to a first approximation low rates of glycolysis were associated with low internal pH. In order to demonstrate the relationship be-

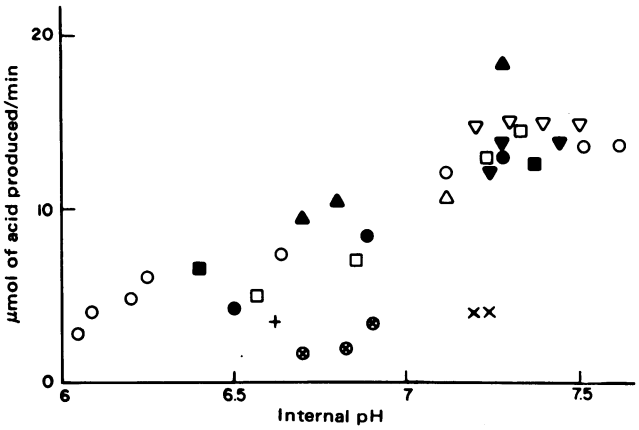

FIG. 7. Rate of acid production in anaerobic *E. coli* cells in the presence of glucose as a function of internal pH at 20°C. This rate was calculated as described in the text from the external medium buffer capacity measured in the presence of the cells and the external pH drift. Rates were obtained only at those points at which the intracellular sugar phosphate concentrations were at their maximum (i.e., during steady state). △, Calculated from the experiment shown in Figs. 1 and 4*A*; experimental conditions are given in the legend of Fig. 1. The cellular suspension medium contained 100 mM Pipes/50 mM Mes/20 mM P_i as buffer. Glucose (25 mM) was added to the NMR sample. ○ and □, Obtained from experiments performed under identical conditions to △ except 50 mM glucose was added to the sample. ▽ and ▼, Calculated from two different experiments in which 50 mM glucose was added to the sample but the external medium buffer capacity was approximately doubled by increasing the Pipes concentration to 200 mM. ▲, ■, and ●, *Initial* rates calculated from three different runs. ▲, Obtained from cells that were divided into three groups and resuspended in media with pH values of 7.4, 6.9, and 6.75. Glucose (25 mM) was added after the NMR sample was allowed to sit at 20°C for ~20 min. ●, Identical to △ except the pH values of the suspension media used were 7.4, 7.0, and 6.6. ■, Identical to ▲ except suspension media used had pH values 7.4 and 6.5. × and ⊗, Rates obtained from cells treated with 2 and 4 mM DCCD, respectively (Fig. 4 *B* and *C*). +, Rate seen in the presence of 75 μM FCCP (shown in Fig. 5). The control for the DCCD- and FCCP-treated cells is △.

tween internal pH and the rate of glycolysis, we have plotted (Fig. 7) both the initial rates of glycolysis as well as the rates measured at subsequent times against internal pH. To obtain initial rates at different internal pH values, we resuspended harvested cells in media with different pH values. When the cells were then allowed to sit at 20°C for ~20 min prior to glucose addition, the internal pH slowly approached that of the external medium. When glycolysis starts at low pH, the rate is initially low, leading to low NTP levels and therefore to smaller ΔpH. The continuous production of acid coupled to the small ΔpH formed leads to even lower internal pH values, further inhibiting the rate of glycolysis in these experiments.

We do not suggest that the rate of glycolysis is solely determined by internal pH. However, our data indicate that under anaerobic conditions internal pH is an important parameter in determining the rate of glycolysis. It is not possible at present to point out the specific reaction that is inhibited by the lowering of internal pH. Most *E. coli* enzymes have high pH optima and thus can be affected. However, it has been shown by *in vitro* experiments that phosphofructokinase from several sources shows a pH rate profile very similar to that shown in Fig. 7 (15, 16). In the presence of DCCD, inhibition of the glycolysis rate appears to be mediated by a factor other than pH because the two points at pH 7.20 and 7.24 coming from the sample with 2 mM DCCD deviate from the remainder of the points in Fig. 7. A similar decrease in the glycolysis rates in the presence of DCCD has been observed in *S. faecalis* (9, 17). Contrary to our results, uncouplers such as FCCP have been known to stimulate glycolysis (9). Unlike our experiments, however, in these experiments (9) the external pH and consequently the internal pH was kept high enough so that glycolysis was probably not inhibited by pH.

These experiments clearly demonstrate that ^{31}P NMR can successfully be used to study aspects of bioenergetics in microorganisms and to follow metabolism. Results presented in this paper generally agree with earlier work based on other techniques and support the critical role assigned to the membrane-bound ATPase in generating transmembrane proton gradients by ATP hydrolysis in the absence of electron transfer. The simultaneous measurements of glycolysis and internal pH revealed that glycolysis slows down for internal pH $\lesssim$7.2.

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