Proton Correlation Nuclear Magnetic Resonance Study of Metabolic Regulations and Pyruvate Transport in Anaerobic *Escherichia coli* Cells†

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ABSTRACT: Proton correlation nuclear magnetic resonance (NMR) was used to study dynamical aspects of glucose metabolism and pyruvate transport in anaerobic *Escherichia coli* cells. It was shown that, in the anaerobic metabolism of *E. coli* cells, the amount of lactate continues to increase, whereas the concentrations of acetate, ethanol, succinate, and formate level off even when the supply of glucose is sufficient. Metabolisms via the Embden–Meyerhof pathway and the pentose monophosphate pathway were separately quantitated by using glucose-*1*-^{13}C as the sole carbon source. It was concluded that as much as 22% of the glucose is catabolized via the pentose shunt. The ratio of contributions from the two competing pathways remains unchanged even after the rates of metabolism leveled off. It was shown that the rate of glycolysis is drastically reduced at low pH. The decrease in pH of the medium also shifted the fermentation in such a way that the quantity of acetate produced is about twice that of ethanol and that lactate produced is much more abundant than other metabolites observed. These results indicate that under anaerobic conditions the pH of the medium is an important factor in determining the rate of glycolysis and in controlling the metabolic pathways. When glycolysis is inhibited by iodoacetate, an exogenous lactate is taken up by the cell and further metabolized primarily to acetate through pyruvate, resulting in the generation of an additional mole of ATP for each mole of acetate formed. The ^1H NMR data obtained indicate that in the pyruvate metabolism in anaerobic cells the phosphorolytic cleavage of pyruvate to acetate, which is sensitively affected by a small change in environmental conditions, plays a crucial role. It was also shown that a change in pH of the incubation medium results in a rapid efflux of accumulated pyruvate from the cells. The uptake of pyruvate induced by a change in pH was observed to occur against an osmotic gradient of the solute. The uptake was completely inhibited by a proton conductive uncoupler, 2,4-dinitrophenol (Dnp), suggesting that protons play a key role in energy coupling for the active transport of pyruvate. It was also confirmed that a membrane-bound ATPase inhibitor, dicyclohexylcarbodiimide (DCC), does not inhibit pyruvate transport in starving cells. These findings suggest that ATP is not an intermediary in energy coupling under the present conditions. The pyruvate uptake was also observed on changing the physiological state of cells from aerobic to anaerobic conditions. In glycolyzing cells, in contrast to the above experiments on starving cells, the uptake of pyruvate was inhibitied by DCC as well as by Dnp. These observations are consistent with predictions made on the basis of the chemiosmotic hypothesis by Mitchell.

In previous papers we have demonstrated that ^1H correlation NMR[1] is useful in investigating dynamical aspects of metabolism, especially at an early stage, in living cells (Ogino et al., 1978a,b; Arata et al., 1978). The method described is nondestructive, thus enabling one to avoid uncertainties encountered in studying metabolism in living cells by extracting metabolites by conventional chemical methods. It has been shown that ^{13}C and ^{31}P NMR are also quite useful in studying metabolism in various cells (Navon et al., 1977; Ugurbil et al., 1978a), intact tissues (Hoult et al., 1974; Burt et al., 1976), cellular organelles (Casey et al., 1977) and perfused organs of animals (Gadian et al., 1976; Hollis et al., 1977; Dwek et al., 1977). However, in these studies ^{13}C-labeled metabolites originating from ^{13}C-labeled carbon sources or phosphorylated metabolites can only be observed. Natural abundance ^{13}C NMR has also been applied to follow glucose utilization in a suspension of cold acetone-treated yeast cells (Kainosho et al., 1977). However, a long-term signal accumulation is usually required to obtain a ^{13}C signal-to-noise ratio that is sufficient for a detailed and quantitative discussion of the metabolism.

^1H NMR has a great advantage in that its sensitivity is much better than those of ^{13}C and ^{31}P NMR and because ^1H nuclei exist virtually in all biologically important molecules (Daniels et al., 1974, 1976; Dwek et al., 1977; Brown et al., 1977). However, in biological systems where a large amount of water always exists, a dynamic range much wider than that attained in the conventional pulse Fourier transform mode is usually required. ^1H correlation NMR has been recognized as one of most promising techniques for this purpose (Dadok & Sprecher, 1974; Gupta et al., 1974; Arata & Ozawa 1976; Arata et al., 1978).

In the present study, we use ^1H correlation NMR to follow in detail the time course of glucose metabolism in anaerobic *Escherichia coli* cells under various conditions. On the basis of the ^1H correlation NMR data obtained, we will discuss regulatory mechanisms for anaerobic metabolism in *E. coli* cells. Dynamical aspects of metabolism via the Embden–Meyerhof pathway and pentose monophosphate pathway will also be discussed on the basis of experiments which use glucose-*1*-^{13}C as the sole carbon source. We also use ^1H correlation NMR to follow transport processes of pyruvate, a key intermediate of glycolysis, through the membrane. Relations of pyruvate transport to metabolic regulations in anaerobic metabolism in *E. coli* cells will be discussed.

Materials and Methods

Glucose-*1*-^{13}C (90%) was purchased from E. Merck; the extent of ^{13}C isotope enrichment at the C-1 position of glucose was checked by ^1H NMR. All other chemicals used were

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[1] Abbreviations used: ATP, adenosine 5′-triphosphate; DCC, dicyclohexylcarbodiimide; Dnp, 2,4-dinitrophenol; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate; NMR, nuclear magnetic resonance.

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reagent grade and used without further purification.

E. coli K12 (IAM 1264) was grown in an M9 medium with glucose (0.1 M) as the sole carbon source. The composition of the medium was $Na_2HPO_4 \cdot 2H_2O$ (8.8 g), KH_2PO_4 (3.0 g), $MgSO_4 \cdot 7H_2O$ (0.02 g), and NH_4Cl (1.0 g) in H_2O (1000 mL). One milliliter of a solution containing a mixture of trace elements [$Na_2B_4O_7 \cdot 10H_2O$ (88 mg), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (37 mg), $FeCl_3 \cdot 6H_2O$ (470 mg), $ZnSO_4 \cdot 7H_2O$ (880 mg), $CuCl_2 \cdot 2H_2O$ (270 mg), $MnCl_2 \cdot 4H_2O$ (72 mg) in 1000 mL of H_2O] was added to 1000 mL of the medium. Cells were harvested either at an exponential phase or at an early stationary phase of growth, and collected at low-speed centrifugation at 5 °C. The pellet was washed twice with M9 buffer (pH 7.2) and resuspended in the same buffer. Unless otherwise stated, the cells grown to an exponential phase were used. The cell suspension was prepared in a 5-mm NMR tube as described previously (Ogino et al., 1978b) and was incubated anaerobically in an NMR spectrometer at 30 °C.

Membrane protein concentrations were estimated spectrophotometrically by using an optical density of 1.5 at 600 nm, which corresponds to 10 mg of membrane protein/mL (Kaback, 1971). Intracellular concentrations of metabolites were estimated by assuming that the total intracellular volume is 3.4 ± 0.1 mL/g membrane protein (Y. Anraku, private communication). It was confirmed that the intensity of ^1H NMR signals of a cell suspension is the same as that of the supernatant separated by centrifugation of the suspension. It was also confirmed that the cells themselves, immediately after washing with the fresh buffer solution, do not give any signals due to the metabolites; it was observed that the metabolite signals start to appear and increase in intensity within a few minutes after incubation of the same sample in an NMR tube. This point was most carefully checked in the case of pyruvate under different growth conditions. It should be emphasized that this type of experiment can only be performed with the ^1H correlation NMR method, which has great sensitivity and time resolution. These results indicate that signals from *extracellular* metabolites, which have diffused through the cell membrane and accumulated in the medium, are being observed in the present experiments.

^1H NMR spectra were obtained on a JEOL PS-100 NMR spectrometer operating at 100 MHz in the correlation mode (Arata et al., 1978; Arata & Ozawa, 1976). Typically, 128–256 scans were accumulated for 2.5–5 min for a quantitative determination of the metabolites. Chemical shifts are given in parts per million from external DSS. Metabolite peaks were identified from their chemical shifts and pH titration behavior. The assignments were confirmed by adding authentic samples of each of the metabolites to the medium. The concentration of the metabolites was determined by comparing the intensity for each metabolite with that for the authentic sample dissolved at a known concentration in the same M9 buffer.

Results

Figure 1 shows ^1H correlation NMR spectra of a suspension of the resting cells with glucose-*1*-^{13}C used as the sole carbon source. Spectral assignments can be made on the basis of chemical shifts, spin coupling patterns, and ^{13}C–^1H spin coupling constants. The methyl resonances for acetate, lactate, and ethanol clearly show a splitting due to a ^{13}C–^1H coupling of about 145 Hz. By contrast, signals due to ^{13}C-labeled succinate are not observable. All of the above metabolites should be labeled to the same extent because they are known to originate from a common pyruvate or phosphoenolpyruvate pool. As a matter of fact, Ugurbil et al. (1978a) have clearly

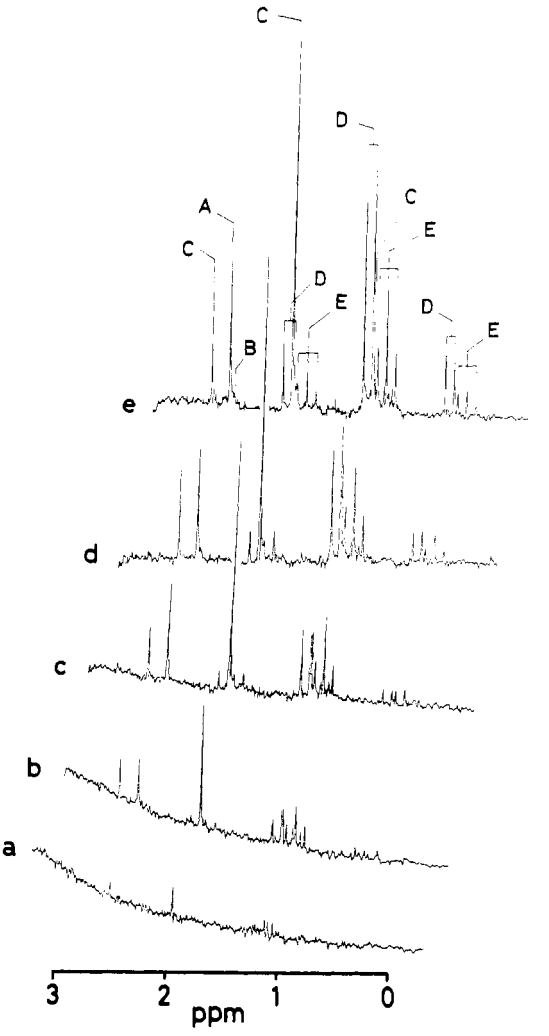

FIGURE 1: The 100-MHz ^1H correlation NMR spectra of a suspension of the resting *E. coli* cells incubated in an M9 buffer with glucose-*1*-^{13}C as the sole carbon source. After centrifugation, the pellet obtained was washed twice with the M9 buffer, which does not contain any nitrogen source, and then resuspended at a cell density of 2×10^9/mL in the same buffer: glucose-*1*-^{13}C, 0.1 M; pH 7.2; 30 °C. Incubation times after inoculation are (a) 0:15, (b) 0:59, (c) 1:59, (d) 2:57, and (e) 4:22 (hours:minutes); (A) succinate; (B) pyruvate; (C) acetate; (D) lactate; (E) ethanol. Chemical shifts are in parts per million from external DSS.

demonstrated this using ^{13}C NMR. Failure to detect the ^{13}C-labeled succinate in the present ^1H study is presumably due to the fact that $^{13}CH_2$–$^{12}CH_2$, which is of the AA'BB'X (X = ^{13}C) type, gives a large number of small signals, which were difficult to observe under the present experimental conditions. It was also observed that the formate peak is a singlet. In Figures 2a and 2b the concentrations of the metabolites that incorporated ^{12}C and ^{13}C from glucose-*1*-^{13}C, respectively, are plotted. These results can be used to separately quantitate contributions from two competing pathways, i.e., the Embden–Meyerhof pathway and the pentose monophosphate pathway, which are of primary importance in glucose metabolism in *E. coli*.

The effect on glycolysis of the pH of the medium was also studied. *E. coli* cells were anaerobically grown in M9 medium with 0.1 M glucose as the sole carbon source at pH 6.5, which is slightly different from the optimum pH. The time course followed by ^1H correlation NMR is plotted in Figure 3.

E. coli cells from the early stationary phase of growth were washed and resuspended in M9 buffer at a cell density of about 10^{11}/mL. Since washed cells of *E. coli* contain a high level

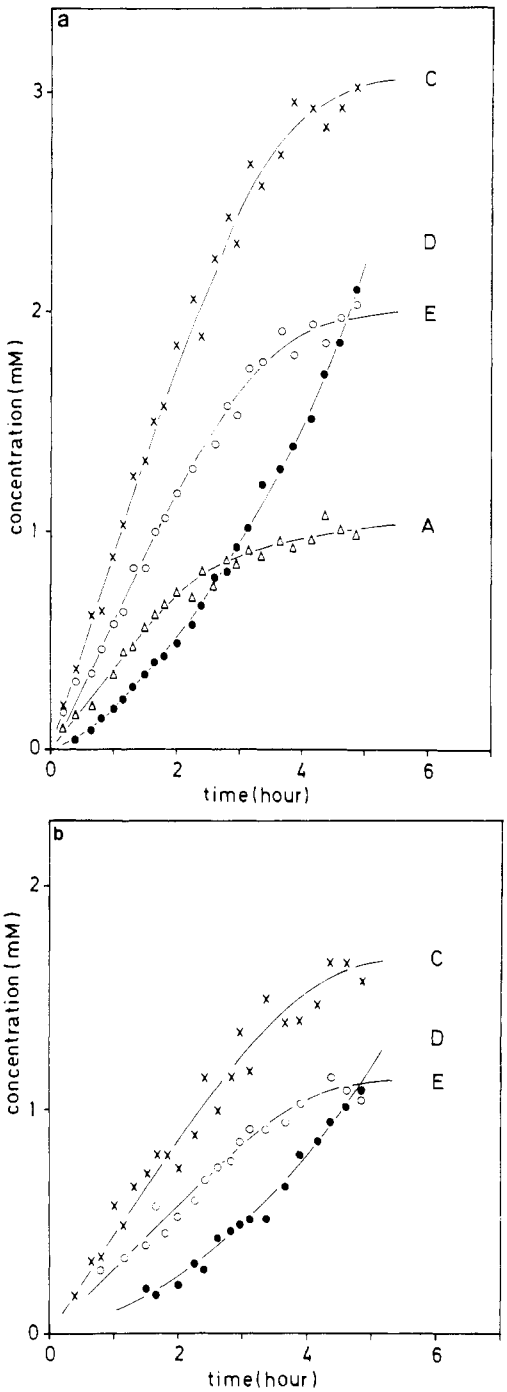

FIGURE 2: The time course of the concentrations of metabolites in anerobic *E. coli* cells that incorporated (a) ^{12}C and (b) ^{13}C from glucose-*1*-^{13}C used as the sole carbon source. The concentrations of lactate plotted in a and b are one-half the actually observed values. In b the concentrations plotted are based on the intensity for one-half of each pair due to ^{13}C–^{1}H coupling of the ^{13}C metabolite signals; therefore, actual concentrations are twice as large as those plotted in the figure: cell density, 2.0×10^9/mL; glucose-*1*-^{13}C, 0.1 M; pH 7.2; 30 °C. Other experimental conditions are the same as used to obtain the results in Figure 1. Incubation times after inoculation are given in hours: (A) succinate; (C) acetate; (D) lactate; (E) ethanol.

of ATP (Maloney et al., 1974), the cells were subjected to a starvation procedure in order to lower the basal level of ATP and eliminate the source of endogenous metabolic energy. For this purpose, the cell suspension was anaerobically preincubated for about 1 h at 30 °C. After the preincubation the pH of the medium was observed to decrease to about 5.0, presumably because of the generation of acid metabolites such as organic acids. The pH of the medium is much lower than

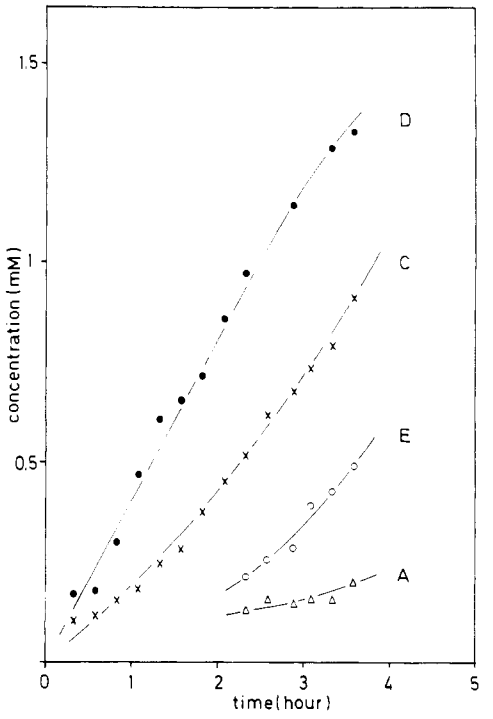

FIGURE 3: The effect of the pH of the medium on anaerboic metabolism in *E. coli* cells. pH was adjusted to 6.5 at the beginning of the growth: cell density, 2.5×10^9/mL; glucose, 0.1 M; 30 °C. Incubation times after inoculation are given in hours: (A) succinate; (C) acetate; (D) lactate; (E) ethanol.

the optimum pH for the growth of the cells; no further developments in the metabolic products were observed in the ^{1}H NMR spectrum.

At this stage the pH of the medium was lowered quickly by adding HCl. Figure 4 shows that the decrease in pH of the medium clearly results in a rapid efflux of accumulated pyruvate from the cells. It should be noted that no change was detectable for the other metabolite peaks. It was confirmed that in the present experiments a decrease in number of the cells did not occur, and normal growth was observed for the cells on an agar plate at 37 °C. Figure 5 plots the extent of the pyruvate efflux as a function of external pH; saturation is clearly observed for the concentration of pyruvate below pH 4.5. The efflux of pyruvate in response to the pH shift was transient. As shown in Figure 6, pyruvate again became undetectable in the ^{1}H NMR spectrum within 5 min after the pH shift, suggesting that pyruvate is taken up by the cells. The pyruvate uptake was actually confirmed by sonicating the cells; after the residue of the sonicated cells was removed by centrifugation, the supernatant gave the ^{1}H NMR spectrum shown in Figure 6c, where the signal of pyruvate accumulated in the cells becomes clearly observable.

Figure 7 shows the effect of an uncoupler, Dnp, on pyruvate transport induced by the pH shift of the incubation medium. It is seen that the uncoupler, which renders bacterial membranes highly permeable to protons, induced a rapid loss of pyruvate from the cells (Figure 7c), which had previously been allowed to accumulate pyruvate, and completely inhibited the transport of pyruvate into the cells. In order to determine whether the pyruvate transport induced by the pH shift requires the activity of the membrane-bound proton translocation ATPase, the effect of an inhibitor, DCC, was also studied. It was confirmed that in the starving cells DCC does not inhibit the accumulation of pyruvate.

Pyruvate uptake was also observed on changing the physiological state of the cells. *E. coli* cells from the early sta-

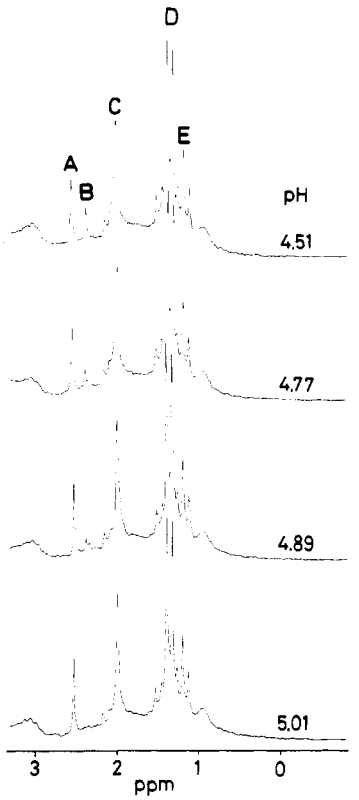

FIGURE 4: The 100-MHz ^1H correlation NMR spectra of a suspension of *E. coli* cells in an M9 medium at pH 7.2. The suspension was anaerobically preincubated at 30 °C for about 1 h to lower the basal level of ATP in the cells; the pH of the medium was then decreased by adding 0.5 M HCl. Each spectrum was accumulated at 30 °C for 2.5 min immediately after lowering the pH. The pH values at which measurements were made are given in the figure: (A) succinate; (B) pyruvate; (C) acetate; (D) lactate; (E) ethanol. Chemical shifts are in parts per million from external DSS.

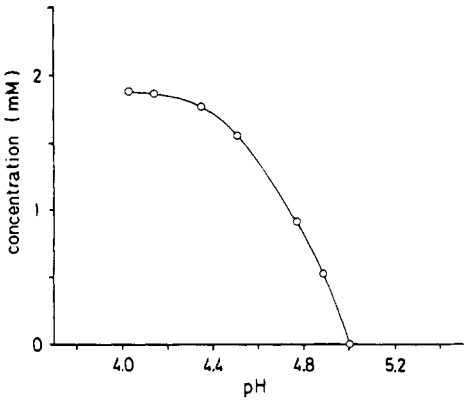

FIGURE 5: The extent of pyruvate efflux at 30 °C as a function of pH. Experimental condtions are as given in Figure 4.

tionary phase of growth were washed and resuspended at a cell density of about 2×10^{10}/mL in the M9 buffer at pH 7.2. After the concentrated cell suspension was *aerobically* preincubated with 0.1 M glucose at 37 °C for ~3 h, the suspension was transferred to an NMR tube and bubbled with N_2 gas. The resultant suspension was anaerobically incubated in an NMR probe at 30 °C and gave the ^1H NMR spectra shown in Figure 8. It is clearly seen that the uptake of pyruvate occurs in response to the change of growth conditions even though the supply of glucose was sufficient. It should also be pointed out that this uptake is specific for pyruvate, which is a key intermediate in glycolysis. In the present case of glycolyzing cells, in contrast to the experiments on starving

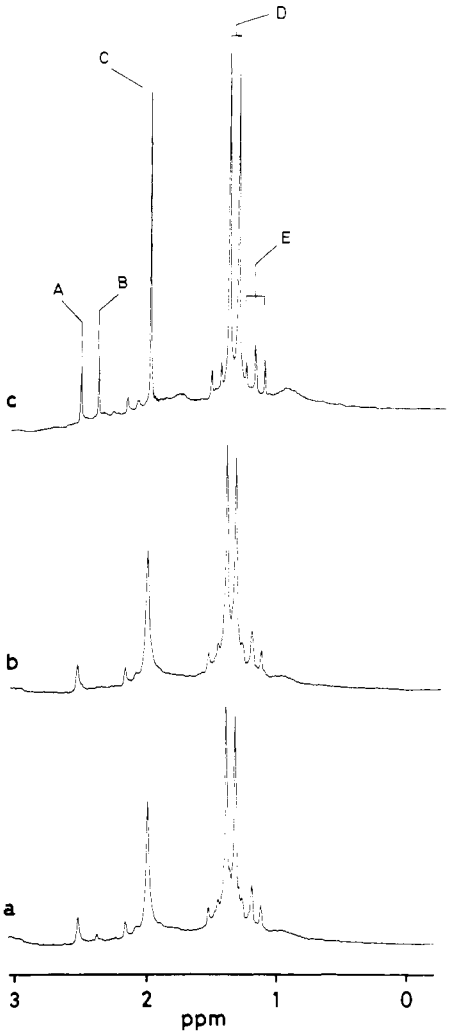

FIGURE 6: The 100-MHz ^1H correlation NMR spectra of a suspension of *E. coli* cells in an M9 medium at 30 °C. The pH of the medium was changed from 5.03 to 4.79 by adding 0.5 M HCl: (a) taken immediately after the pH change; (b) after a 5-min incubation; (c) supernatant of the cells sonicated after the 5-min incubation. Other experimental conditions are the same as in Figure 4: (A) succinate; (B) pyruvate; (C) acetate; (D) lactate; (E) ethanol. Chemical shifts are in parts per million from external DSS.

cells, the uptake of pyruvate was completely inhibited by DCC (1 mM), as well as by Dnp (1 mM).

Discussion

The rate of glycolysis observed in the present ^1H NMR work is almost an order of magnitude lower than that observed by Ugurbil et al. (1978a) in their ^{31}P NMR study. In the ^1H and ^{31}P NMR studies, there is a large difference in glucose concentration per *E. coli* cell; the glucose concentrations are ~2% for 10^9 cells/mL (^1H) and ~0.9% for $3-5 \times 10^{11}$ cells/mL (^{31}P). Glucose concentration is known to influence to a great extent the rate of uptake of glucose by the cells and the rate of metabolism. This appears to be the reason for the observed large difference in the rate of glycolysis in the two experiments.

In a previous paper (Ogino et al., 1978b) we have shown that, in anaerobic metabolism of *E. coli*, the amount of lactate continues to increase, whereas the concentrations of acetate, ethanol, and succinate level off even when the supply of glucose is sufficient. The formation of formate also clearly shows features that are quite similar to those observed for acetate, ethanol, and succinate. It has been confirmed that the growth of *E. coli* cells continues normally, and the decrease in number

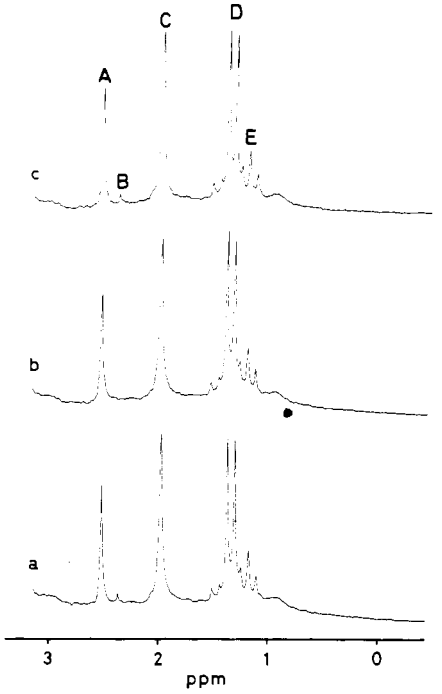

FIGURE 7: The 100-MHz ^1H correlation NMR spectra of a suspension of *E. coli* cells in an M9 medium at 30 °C. The pH of the incubation medium was changed from 5.20 to 4.96 by adding 0.5 M HCl to the medium: (a) taken immediately after the pH change; (b) after a 5-min incubation; (c) Dnp was added at a final concentration of 1 mM to the suspension, which was incubated for an additional 90 min. Other conditions are the same as in Figure 4: (A) succinate; (B) pyruvate; (C) acetate; (D) lactate; (E) ethanol. Chemical shifts are in parts per million from external DSS.

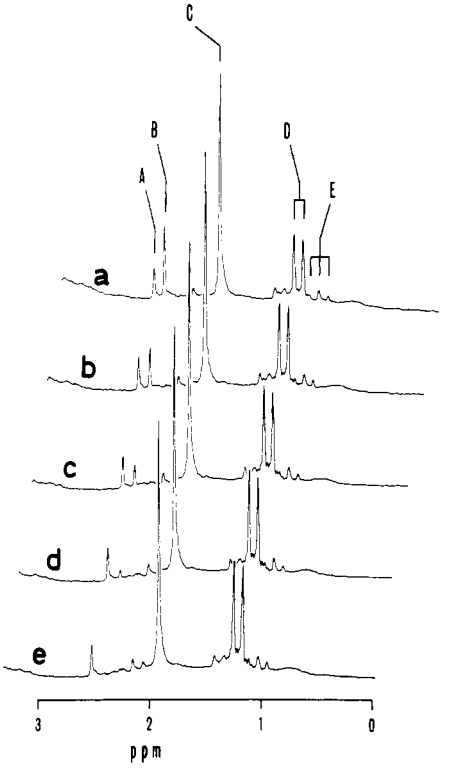

FIGURE 8: The 100-MHz ^1H correlation NMR spectra of a suspension of *E. coli* cells preincubated aerobically at 37 °C for about 3 h with 0.1 M glucose. Each spectrum was taken after the suspension was anaerobically incubated at 30 °C for (a) 0, (b) 20, (c) 35, (d) 50, and (e) 80 min. Chemical shifts are in parts per million from external DSS.

of cells by autolysis did not occur in the present experiments. Before the plateaus are reached for the four metabolites, the amounts of acetate and ethanol are almost identical. However, after the formation of the four metabolites levels off, the concentration of acetate becomes much lower than that of ethanol. At the same time, the pyruvate signal becomes clearly observable (see Figure 1 of our previous paper (Ogino et al., 1978b)). It is of interest that pyruvate, a key intermediate metabolite in the fermentation of glucose, accumulates in the medium. The initiation of pyruvate accumulation and a lower formation rate for acetate than for ethanol indicate that metabolic pathways have been switched in the pyruvate metabolism.

E. coli, a *mixed-acid* bacterium, converts glucose primarily to pyruvate with the formation of two molecules of ATP and of NADH via the Embden–Meyerhof pathway; pyruvate is then cleaved to produce acetyl-CoA and formate. It is also well established that half of the acetyl-CoA produced is cleaved phosphorolytically to acetate via acetylphosphonate with the generation of ATP, while the other half is reduced to ethanol in two steps using two molecules of NADH produced in the initial oxidation of triose phosphate. It has also been shown that lactate and succinate are produced from pyruvate through independent steps.

In the case of resting cells, which are grown in the absence of any nitrogen source, plateaus similar to those observed in M9 medium (with a sufficient amount of nitrogen sources) are observed, although the rate of glycolysis is an order of magnitude lower than that in the M9 medium. In addition, in the resting cells, acetate produced is about 50% larger in quantity than ethanol. Since synthesis of proteins and nucleic acids is inhibited in the resting cells, it is unlikely that the above features observed in the time course of the metabolites are due to the catabolite repression of inducible enzymes.

When glucose-*1*-^{13}C is used as the carbon source, the ^{13}C label is lost as CO_2 in the phosphogluconate dehydrogenase step of the pentose monophosphate shunt, whereas the ^{13}C label is incorporated into the methyl carbon of pyruvate via the Embden–Meyerhof pathway. This means that Figures 2a and 2b can be used to separately quantitate the two fluxes of the competing pathways. It was shown that, in the case of the resting cells, 22% of the glucose utilized by *E. coli* follows the pentose monophosphate shunt. The same result was obtained for anaerobic *E. coli* cells grown in M9 medium. These results are in good agreement with that of Doelle (1975), who reported that in *E. coli* as much as 25% glucose is catabolized via the pentose shunt.

It is observed in Figures 2a and 2b that the ratio for the two competing pathways does not change even after the plateaus are reached for acetate, succinate, ethanol, and formate. These results indicate that pyruvate metabolism is subject to regulation in reactions which are common in the two competing pathways, most likely in the process of a phosphoroclastic split of pyruvate. It should be noted that the cleavage of 1 mol of acetyl-CoA would produce 1 mol each of ATP and acetate. It is seen in Figure 3 that a pH decrease of the incubation medium drastically reduces the rate of glycolysis, indicating that under anaerobic conditions the pH of the medium is an important factor in determining the rate of glycolysis. It has been demonstrated by in vitro experiments that the activity of phosphofructokinase is extremely sensitive to small pH changes in the physiological range (Trivedi & Danforth, 1966; Ui, 1966). It has been suggested that the control of glycolysis by pH as a whole can be attributed primarily to regulation of phosphofructokinase activity (Reinanner & Bruns, 1964; Minakami et al., 1964). As described above, ATP production

is decreased when pyruvate accumulates in the medium. An interesting possibility is that ATP, which is known to be a modifier of phosphofructokinase, controls the rate of glycolysis in anaerobic *E. coli* cells.

It should also be noted that the pH decrease shifts the fermentation in such a way that lactate is in greater quantity than the other metabolites observed and that the yield of acetate is twice that of ethanol. Tikka (1935) has reported that a decrease in pH of the medium shifts the fermentation with an increasing production of lactate. The formation of lactate, however, starts to level off as the fermentation proceeds. This is probably because the lactate production ceases before the pH of the medium falls to a value that is low enough to damage the cells (Chance et al., 1965).

Ugurbil et al. (1978b) used ^{31}P NMR to discuss the relationship between the internal pH of the cell and the rate of glycolysis in anaerobic *E. coli* cells, and suggested that under anaerobic conditions the pH of the medium is an important, but not the sole, factor in determining the rate of glycolysis.

As shown above, the phosphoroclastic split of pyruvate to acetate is stimulated at low pH. This feature was also observed in the resting cells, as shown in Figure 2. These findings suggest that conditions of the media, which are slightly less than optimum for cell growth, drastically reduce glycolysis rates and shift pyruvate metabolism, thus stimulating the phosphoroclastic reaction that generates an additional amount of ATP.

It is of interest that the phosphoroclastic pathway becomes available for lactate when glycolysis is inhibited by iodoacetate; an exogenous lactate is consumed as the carbon source. In this experiment, prior to the addition of glucose (0.1 M) to the medium, the medium was preincubated with 1 mM iodoacetate, an inhibitor of phosphoglyceraldehyde dehydrogenase in glycolysis. Acetate and lactate as the carbon source were then added to the cell suspension at final concentrations of 0.2 and 0.4 mM, respectively. ^1H NMR was observed immediately after the addition of acetate and lactate. It was shown that, after a 28-h incubation, an exogenous lactate was completely depleted. It was concluded from the intensity of pyruvate and acetate produced that the lactate added was converted primarily to acetate via pyruvate as an intermediate. No other metabolites were produced in this experiment. This result clearly shows that, when glycolysis is inhibited, lactate is metabolized as the carbon source. However, the lactate metabolism appears to be limited to the phosphoroclastic split of pyruvate; no other pathways, including that for the production of ethanol, are available. After lactate had been consumed, as long as the cells were kept anaerobic, no further metabolic developments were observed for several hours in the ^1H NMR spectra. It has been shown that, in the anaerobic *E. coli* cells, conversion of pyruvate to acetyl-CoA is inhibited by ATP and that acetyl-CoA activates conversion of phosphoenolpyruvate to oxaloacetate (see Metzler (1977), p 531). It has also been demonstrated that the allosteric enzyme phosphate acetyltransferase, which catalyzes conversion of acetyl-CoA to acetyl phosphate, is activated by pyruvate and is inhibited by $NADH_2^+$ and, more strongly, by ATP (Suzuki, 1969; Suzuki et al., 1969).

In view of the above experimental results, a possible explanation for the selective metabolic regulations observed in the present work is that ATP produced by the cleavage of acetyl-CoA becomes an allosteric inhibitor for feedback inhibition of the phosphoroclastic reaction of pyruvate. It is well known that yeast cells contain an enzyme system that catalyzes the oxidation of acetaldehyde directly to acetate; no associated ATP synthesis has been demonstrated (Metzler, 1977). Using ^1H correlation NMR, we followed the anaerobic metabolism in yeast cells as in the case of *E. coli*. The time course did not show plateaus such as those observed in anaerobic *E. coli* cells. This result supports the above explanation for selective metabolic regulation in anaerobic *E. coli* cells. It should also, in principle, be possible to confirm the above explanation by using a mutant *E. coli* lacking the activity of the phosphoroclastic split of pyruvate. It was shown that succinate is also subject to metabolic regulation. In the anaerobic *E. coli* cells, succinate is known to be produced either from acetyl-CoA in the pyruvate pool or from oxaloacetate in the phosphoenolpyruvate pool. Therefore, the present NMR results suggest that the route of producing succinate from pyruvate is closely related to the phosphoroclastic split of pyruvate and therefore affected by the feedback inhibition by ATP.

Pyruvate Transport in Anaerobic E. coli Cells. As discussed above, it seems quite certain that accumulation of pyruvate in the medium is closely related to metabolic regulation in anaerobic *E. coli* cells. Elucidation of the mechanism of pyruvate transport would therefore be important in understanding in more detail the regulation of pyruvate metabolism. In order to follow the pyruvate metabolism as closely as possible, a varying number of *E. coli* cells per volume was employed in the present work. As described under Materials and Methods, it was confirmed that only extracellular pyruvate is observed in the present experiments.

It is of interest to compare the facilitated passive diffusion of intracellular pyruvate observed in the present experiment with the pyruvate transport in isolated mitochondria (Halepstrap, 1978). It has been demonstrated that the kinetics of pyruvate transport across the mitochondrial membrane is highly dependent on pH and the availability of exchangeable ions such as acetoacetate and lactate; a decrease in the pH gradient across the membrane results in a decrease in the concentration gradient of pyruvate (Papa et al., 1971; Papa & Paradies, 1974; Halepstrap, 1978). Halepstrap (1978) has shown that, in mitochondria prepared at pH 7.5, an increase in pH of the medium (pH_{out}) results in the enhanced efflux of pyruvate. At first sight, this result is contradictory to our results on pyruvate efflux in anaerobic *E.* coli cells. It should, however, be noted in the case of Halepstrap's experiment that inhibitors such as arsenite, oligomycin, rotenone, and antimycin A were added to the medium in order to suppress pyruvate metabolism in mitochondria. The two apparently contradictory experimental results may be explained in terms of a decrease in the pH gradient, ΔpH, across the membrane. In the case of mitochondria, it has been shown that an increase in pH_{out} actually results in a decrease in ΔpH (Papa & Paradies, 1974). This means that the increase in pH_{out} would induce the efflux of pyruvate preloaded in the cells. In the present experiment in anaerobic *E. coli* cells, a decrease in pH_{out} from pH 5 resulted in the efflux of pyruvate. As shown in Figure 4, pyruvate stays in the cells at pH 5.0; i.e., there exists a concentration gradient of pyruvate across the membrane. After preincubation, during which time ATP has presumably been consumed, it is quite likely that a pH gradient is produced across the membrane to maintain the gradient of the pyruvate concentration. This is supported by the observation that in the starving cells pyruvate uptake is inhibited by an uncoupler Dnp, but not by an ATPase inhibitor such as DCC. As Figure 5 shows, pyruvate efflux increases with a decrease in pH; saturation is clearly observed below pH 4.5. These results suggest that the pH-sensitive transport, which is selective to pyruvate, is mediated by a specific carrier protein present in

the cytoplasmic membrane. In view of this, we suggest that in the transport of pyruvate the membrane protein recognizes pyruvate with a high degree of specificity and translocates it across the membrane in a catalytic manner; this protein would interact with pyruvate as a proton donor because pyruvate can be transported across the membrane only in the neutral form (Rosen, 1978). The decrease in pH from 5.0 will first produce a large ΔpH across the membrane. However, in the starving cells where the ATP level is low, it would be difficult to maintain the large ΔpH initially produced; uptake of lactate as an exchangeable ion would partially contribute to the cancellation of ΔpH. Furthermore, the decrease in pH_{out} would increase the concentration of acetate, lactate, and succinate in the undissociated form. This would result in the free diffusion of acetate into the cells, decreasing ΔpH. The apparent pK_a of 4.7 observed in Figure 5 may correspond to the pK_a of 4.75 of acetate. Overall, the decrease in pH_{out} should result in a decrease in ΔpH, inducing the pyruvate efflux. The pyruvate efflux is fast because of the nature of the carrier-mediated facilitated passive diffusion. In summary, in the case of mitochondria as well as of the anaerobic *E. coli* cells, the decrease in ΔpH induces the efflux of pyruvate. At high concentrations of anions, as in the case of lactate and acetate in the present experiment, uptake capability is saturated and, therefore, no change in anion concentration occurs (Palmieri et al., 1970).

In contrast to the experiments that followed the metabolism, as shown in Figure 1, the efflux of pyruvate induced by the pH shift of the medium is transient, and pyruvate is taken up by the cell within several minutes. We suggest that this difference depends on whether a proton gradient across the membrane exists or not. It should be noted that pyruvate accumulated in the cells is at a concentration of about 10–20 mM, which is much higher than that in the culture medium. This result indicates that pyruvate was transported into the cells against an osmotic gradient of pyruvate, suggesting that this uptake is due to active transport, which requires metabolic energy. The same observation has been made by Matin & Konings (1973), who demonstrated using a radiotracer technique that in the presence of ascorbate-phenazine methosulfate, which is a nonphysiological electron donor, active transport of pyruvate occurs in membrane vesicles prepared from *E. coli*, but not in the case of vesicles prepared from *Bacillus subtilis* and *Pseudomonas* species. However, it is quite difficult to use a radiotracer technique to follow, as is performed in the present ^1H NMR experiments, the fate of transported substrates and to discuss the relationship between the transport process of metabolites and the metabolic regulations.

West & Mitchell (1972, 1973) observed in *E. coli* a simultaneous uptake of proton and lactose or its analogues. Hirata et al. (1976) have recently reported that, in membrane vesicles prepared from thermophilic aerobic bacterium PS3, the uptake of alanine is accompanied by a proton flow with a stoichiometric alanine:proton ratio of 1:1. Halepstrap (1975, 1978) has observed pyruvate–proton cotransport in mitochondria. In the present study, the pH change of the incubation medium due to proton uptake can continuously be monitored using the ^1H chemical shifts of the metabolites. However, in the present experiments on intact *E. coli* cells, pyruvate–proton cotransport was not observed. This is probably because the chemical-shift change that arises from the proton uptake was too small to be detected with a limited resolution obtained at 100 MHz. As shown in Figure 8, pyruvate uptake was also observed when the physiological state of the cell was changed from aerobic to anaerobic growth conditions. However, in this case a small change of the peak intensity for other metabolites, especially lactate and acetate, was simultaneously observed. It should also be noted that in glycolyzing cells, in contrast to the experiments using starving cells, the uptake of pyruvate was inhibitied by DCC as well as by Dnp. This difference may be due to the presence in glycolyzing cells of a sufficient amount of ATP, which would establish a proton gradient across the membrane.

Harold et al. (1968; Harold & Papineau, 1972a,b) showed that glycolyzing cells of *Streptococcus faecalis* (*faecium*) establish both a pH gradient (interior alkaline) and membrane potential (interior negative), and that DCC inhibits the formation of each of these components for the proton-motive force. More recently, ^{31}P NMR measurements on *E. coli* (Navon et al., 1977; Ugurbil et al., 1978b) have shown that glycolyzing cells also maintain a pH gradient across the membrane, which is inhibited by DCC. These results suggest that, under anaerobic conditions where the proton cannot be produced by the respiratory chain, the membrane-bound ATPase couples the hydrolysis of ATP to the electrogenic movement of the proton out of the cells for the source of the proton gradient. It appears that, in the present experiments on anaerobic *E. coli* cells, the proton-motive force generated by the pH shift of the medium is utilized by the active transport of pyruvate. These observations are consistent with predictions made by the chemiosmotic hypothesis of Mitchell (1961, 1966; Harold, 1972). It should also be noted that the two distinct effects of DCC on ATPase depend on the availability of metabolic energy and also on the physiological state of the cells, suggesting that the activity of the ATPase also plays an important role in metabolic regulations through the active transport of metabolic substrates.

At present, we cannot conclude that the efflux of pyruvate observed after the level-off of metabolism is entirely controlled by the pH of the medium. However, it is certain from the present data that under anaerobic conditions the pH of the medium is an important factor in causing the efflux of pyruvate from the cells. This explanation may be supported by the observation that the pH of the incubation medium gradually decreases as anaerobic metabolism proceeds. Since the pH of the medium is also an important factor in determining the rate of metabolism and in controlling metabolic pathways, it is quite likely that pyruvate transport and metabolic regulations are closely related to each other.

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