References

Aster, R. H., & Jandl, J. H. (1964) *J. Clin. Invest. 43,* 843.
Baker, P. F., Hodgekin, A. L., & Shaw, T. I. (1962) *J. Physiol. 164,* 355.
Bakker, E. P., Rottenberg, H., & Caplan, S. R. (1976) *Biochim. Biophys. Acta 440,* 557.
Barber, A. J., & Jamieson, G. A. (1970) *J. Biol. Chem. 245,* 6357.
Beck, J. C., & Sacktor, B. (1975) *J. Biol. Chem. 250,* 8674.
Blaustein, M. P., & Goldring, J. M. (1975) *J. Physiol. 247,* 589.
Blaustein, M. P., & King, A. C. (1976) *J. Membr. Biol. 30,* 153.
Crane, R. K. (1965) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 24,* 1000.
Crane, R. K. (1977) *Rev. Physiol. Biochem. Pharmacol. 78,* 99.
Eddy, A. A. (1968) *Biochem. J. 108,* 195.
Eddy, A. A., Indge, K. J., Backen, K., & Nowacki, J. A. (1970) *Biochem. J. 120,* 845.
Grinius, L. L., Jasaitis, A. A., Kadziauskas, Yu. P., Liberman, E. A., Skulachev, V. P., Topali, V. P., Tsofina, L. M., & Vladimirova, M. A. (1970) *Biochim. Biophys. Acta 216,* 1.
Grollman, E. F., Lee, G., Ambesi-Impiombato, F. S., Meldolesi, M. F., Aloj, S. M., Coon, H. G., Kaback, H. R., & Kohn, L. D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 2352.
Hirata, H., Altendorf, K., & Harold, F. M. (1974) *J. Biol. Chem. 249,* 2939.
Lever, J. E. (1977a) *Biochemistry 16,* 4328.
Lever, J. E. (1977b) *J. Biol. Chem. 252,* 1990.
Lingjaerde, O., Jr. (1971) *Acta Physiol. Scand. 81,* 75.
Martin, D. L. (1973) *J. Neurochem. 21,* 345.
Morville, M., Reid, M., & Eddy, A. A. (1973) *Biochem. J. 134,* 11.
Murer, H., & Hopfer, U. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 484.
Paasonen, M. K. (1968) *Ann. Med. Exp. Biol. Fenn. 46,* 416.
Rudnick, G. (1977) *J. Biol. Chem. 252,* 2170.
Schuldiner, S., & Kaback, H. R. (1975) *Biochemistry 14,* 5451.
Seaston, A., Carr, G., & Eddy, A. A. (1976) *Biochem. J. 154,* 669.
Sneddon, J. M. (1969) *Br. J. Pharmacol. 37,* 680.
Sneddon, J. M. (1973) *Prog. Neurobiol. 1,* 151.

Proton Correlation Nuclear Magnetic Resonance Study of Anaerobic Metabolism of *Escherichia coli*†

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ABSTRACT: Proton correlation nuclear magnetic resonance has been used to investigate anaerobic metabolism of glucose in *Escherichia coli* cells. The time course of the concentrations of six metabolites (ethanol, lactate, acetate, pyruvate, succinate, and formate) has been followed at the very early stage of fermentation, and used to discuss dynamical aspects of the mixed-acid fermentation of glucose by *E. coli*.

In a recent communication (Ogino et al., 1978) we have briefly reported that proton correlation NMR can be used to follow the time course of reactions in cellular systems in a nondestructive way from the very early stage of fermentation. ^{13}C and ^{31}P NMR have been shown to be quite useful in investigating the metabolism in living cells, cellular organelles, and intact tissues (Eakin et al., 1972; Moon & Richards, 1973; Kainosho et al., 1977; Dwek et al., 1977). ^{1}H nuclear magnetic resonance (NMR) has a great advantage that the sensitivity is much better than ^{13}C and ^{31}P NMR, and that ^{1}H nuclei exist virtually in all biologically important molecules. In a series of interesting experiments reported by Williams and co-workers (Daniels et al., 1974, 1976; Dwek et al., 1977), ^{1}H NMR spectra of a variety of compounds in dissected organs such as rat adrenal cortex were observed in the pulse Fourier transform (FT) mode. However, in order to detect a smaller quantity of compounds in biological systems where a large amount of water always exist, a dynamic range much wider than that attained in the pulse FT mode is required. ^{1}H correlation NMR has been recognized as one of most promising techniques for this purpose (Dadok & Sprecher, 1974; Gupta et al., 1974; Arata et al., 1976, 1978). In the present paper, we will discuss, on the basis of ^{1}H correlation NMR data, dynamical aspects of the mixed-acid fermentation of glucose by *E. coli*.

Materials and Methods

E. coli K12 was grown in M9 medium with glucose (0.1 M) as sole carbon source, or in nutrient broth. Cells were harvested at the exponential phase at a cell density of about 0.4×10^9/mL, washed once with the M9 buffer when nutrient broth is used, and resuspended at a density of 0.5 to 2.5×10^9/mL in the same buffer. About 0.5 mL of the suspension was placed in a standard 5-mm NMR tube, bubbled with N_2 gas, and the resultant inhomogeneous cell suspension in the tube was incubated anaerobically in an NMR spectrometer at a probe temperature of 30 °C. ^{1}H NMR spectra were measured with a JEOL PS-100 spectrometer operating at 100 MHz in the

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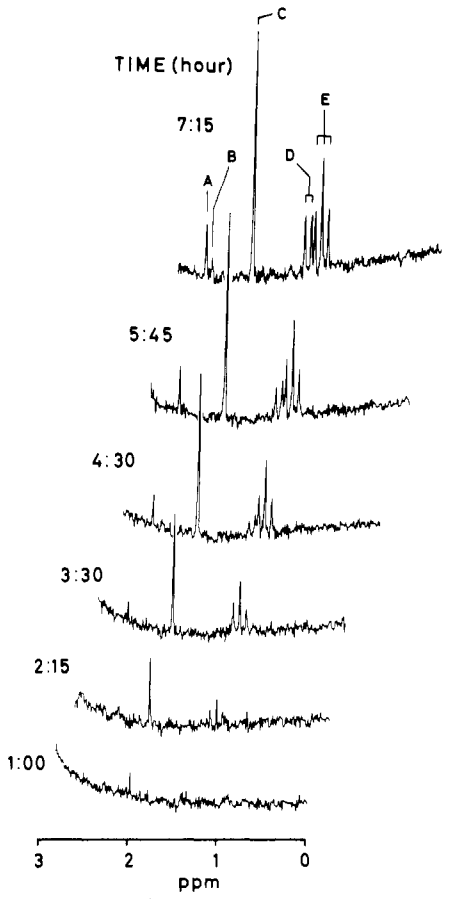

FIGURE 1: The 100-MHz ^{1}H correlation NMR spectra of a suspension of *E. coli* cells grown in an M9 medium with glucose as sole carbon source. *E. coli*, 0.5×10^9/mL; glucose, 0.1 M; pH 7.2, 30 °C. Incubation times after inoculation are given in hours. Each spectrum was accumulated for 5 min in the correlation mode. Chemical shifts are given in ppm from external DSS. Assignments: A, succinate; B, pyruvate; C, acetate; D, lactate; E, ethanol (see text).

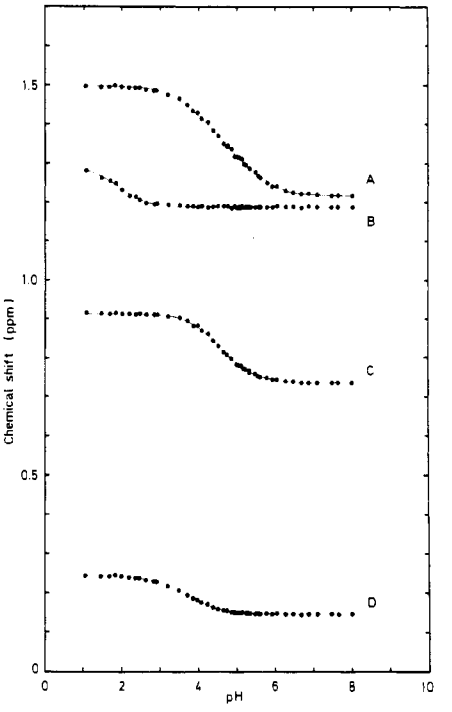

FIGURE 2: Titration curves of metabolites of glucose in *E. coli* cell suspension, which was incubated anaerobically at 30 °C for 48 h in an M9 medium with 0.1 M glucose, where the extracellular pH was changed by adding HCl or NaOH to the cell suspension contained in an NMR tube at 30 °C. Curves A, B, C, and D correspond to succinate, pyruvate, acetate, and lactate, respectively. Chemical shifts are given in ppm from the methyl resonance of ethanol.

correlation mode (Arata et al., 1976, 1978). The concentration of each metabolite was determined by comparing the intensity of a known concentration of an authentic sample in the same buffer.

Results and Discussion

E. coli cells grown to the exponential phase in M9 medium were harvested by low-speed centrifugation, and the pellet obtained was suspended at a cell density of 0.5×10^9/mL in the same buffer containing 0.1 M glucose as sole carbon source. The pH was adjusted to 7.2 at the beginning of the growth. The suspension of *E. coli* cells were grown anaerobically and gave ^{1}H NMR spectra as shown in Figure 1. The pH dependence of these NMR signals was investigated, and the chemical shifts of the metabolite peaks are plotted in Figure 2 as a function of pH. The pH of the medium decreases with the growth of *E. coli* cells. The pH change can be continuously monitored using the ^{1}H chemical shifts of the metabolites. In the above experiment, the pH of the medium initially adjusted to 7.2 decreased to 5.9 after 7.5-h incubation. The results shown in Figure 2 were used along with the chemical shifts of authentic samples dissolved in the same buffer to make assignments of the NMR signals of the metabolites. The metabolites identified in Figure 1 are from low to high field succinate, pyruvate, acetate, lactate, and ethanol. In addition to these metabolites, formate can easily be identified at δ 8.39 ppm. These are well-known metabolites produced in the mixed-acid fermentation by *E. coli* (Metzler, 1977).

As the fermentation proceeds, acetate is the first and lactate the last to be observed. Figure 3 gives the time course of the concentrations of the metabolites observed under the same condition as used to obtain the results shown in Figure 1. It is quite interesting that the amount of lactate produced continues to increase, whereas the concentrations of ethanol, acetate, and succinate clearly level off, even though the supply of glucose was sufficient. It should also be noted that, after these three metabolites reach the plateau, the pyruvate signal becomes clearly observable. It should be emphasized that pyruvate, a key intermediate metabolite in the mixed-acid fermentation of glucose, accumulates in the culture medium. The initiation of pyruvate accumulation suggests that the metabolic pathway has been switched after ethanol, acetate, and succinate level off. This point will be discussed below.

The time course for the anaerobic metabolism in a suspension of *E. coli* cells at a higher cell density of 1.6×10^9/mL is shown in Figure 4. Except that the cells were grown in nutrient broth as the seed culture, other experimental conditions are the same as those used to obtain the results shown in Figure 3. As shown in Figures 3 and 4, ethanol and acetate are almost the same in quantity at an early stage of metabolism. This fact strongly suggests that these two metabolites are derived from the same metabolic intermediate. Enterobacteria, including *E. coli*, are known to convert 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 a half of the acetyl-CoA produced is cleaved phosphorolytically to acetate via acetyl phosphate with 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. In Figure 4, the plateaux for ethanol, acetate, and succinate can be observed more clearly; moreover, it should

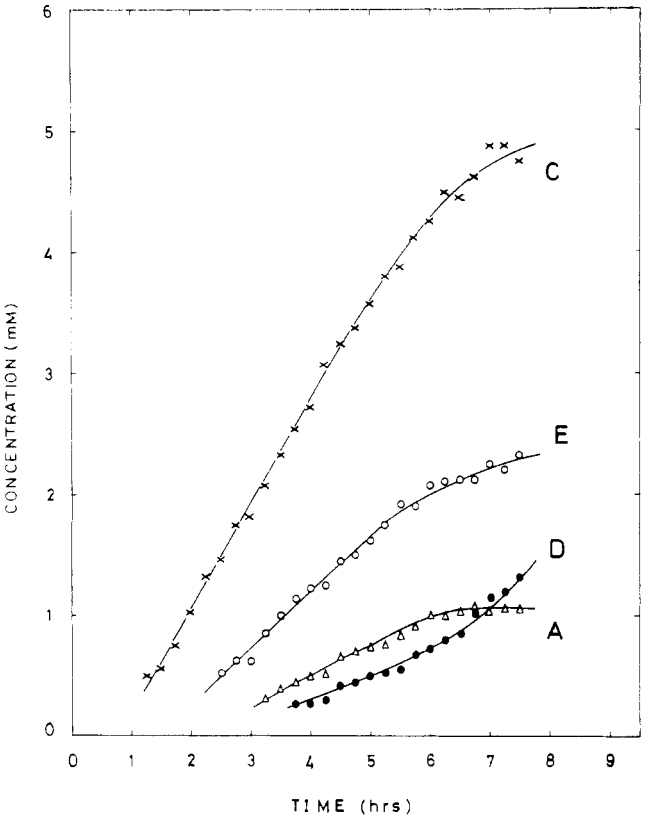

FIGURE 3: The time course for the anaerobic metabolism of glucose in *E. coli* cells. *E. coli*, 0.5 × 10^9/mL; glucose, 0.1 M; pH 7.2, 30 °C. The concentrations of ethanol and lactate plotted in the figure are one-half of actually observed values. Incubation times after inoculation are given in hours. (A) Succinate; (C) acetate; (D) lactate; (E) ethanol.

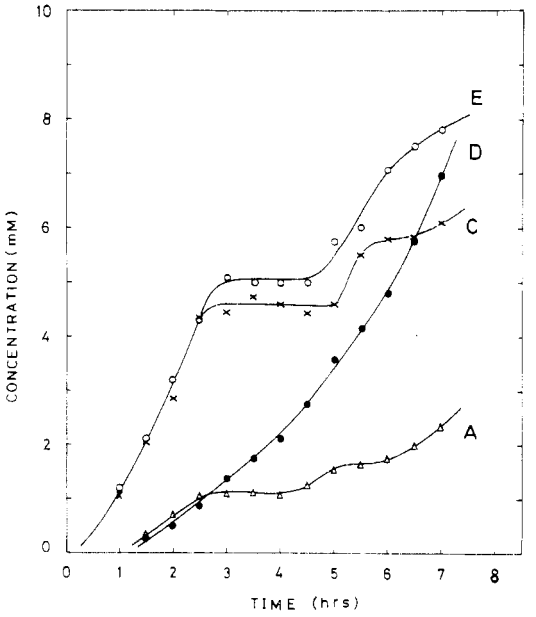

FIGURE 4: The time course for the anaerobic metabolism of glucose in *E. coli* cells at a cell density of 1.6 × 10^9/mL. Other experimental conditions are the same as used to obtain the results shown in Figure 3. The concentrations of lactate plotted in the figure are one-half of actually observed values. Incubation times after inoculation are given in hours. (A) Succinate; (C) acetate; (D) lactate; (E) ethanol.

be noted that, after the plateaux are reached, the rate of formation of acetate becomes much slower than that of ethanol, suggesting that the generation of ATP via acetyl-CoA is also decreased. This result appears to indicate that the physiological

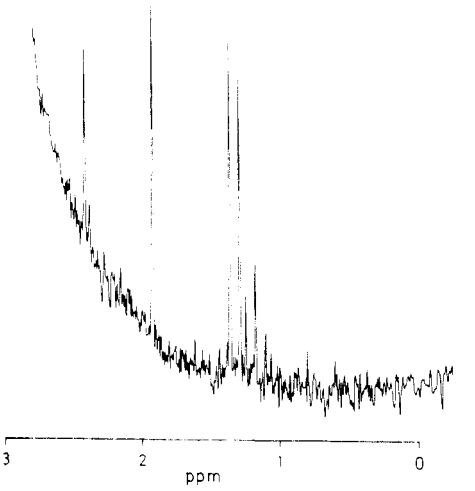

FIGURE 5: The 100-MHz ^{1}H correlation NMR spectrum of a suspension of *E. coli* cells after incubation anaerobically for 7 h in an M9 medium with no nitrogen source. *E. coli*, 2.0 × 10^9/mL; glucose, 0.1 M; pH 7.2, 30 °C. The spectrum was accumulated for 5 min in the correlation mode. Chemical shifts are given in ppm from external DSS.

state of cells has been changed after the concentration of the above three metabolites leveled off, resulting in conversion of the metabolic pathway.

When *E. coli* cells including those in the stationary phase were anaerobically grown in M9 medium at an initial cell density of 0.5–2.5 × 10^9/mL, a similar feature as shown in Figures 3 and 4 was observed. Under the conditions used in the above experiments, the growth of *E. coli* cells was followed using the optical density at 550 nm. It has been confirmed that the growth of *E. coli* cells continues normally, and the decrease in number of *E. coli* cells by autolysis does not occur in the present experiments. In view of this, a possible explanation for the observation of the plateaux for the three metabolites is that the rates of the phosphorolytic cleavage of pyruvate and of the formation of succinate from pyruvate are reduced because of the presence of high concentrations of metabolic substrates such as organic acids, eventually inducing a change in the physiological state of cells to be more adapted for the growth condition. It has been observed that the pH of the medium gradually decreases as the fermentation proceeds. For example, in the experiments shown in Figures 1 and 3, the pH of the medium initially adjusted to 7.2 was observed to have decreased to 5.9 after 7.5-h incubation. On the other hand, the concentration of lactate does not show a plateau and continues to increase. This observation is consistent with a report that the reduction of pyruvate to lactate becomes more active at lower pH (Stokes, 1949).

In the case of resting cells which are grown in a culture medium without any nitrogen source at a cell density of 2.0 × 10^9/mL, the rates of metabolism are an order of magnitude lower than those in the M9 medium. In contrast to the experiments described in Figures 3 and 4, in the case of the resting cells acetate produced is about 50% higher in quantity than ethanol (see Figure 5). This observation suggests that the phosphorolytic cleavage of pyruvate is stimulated in the resting cells promoting ATP synthesis.

In the case of *E. coli*, formate is generally further converted to CO_2 and H_2 by the formic hydrogen-lyase system. However, it should be noted that, under the present condition, formate clearly accumulates in the culture medium. This is probably because pH 7.2 adjusted at the beginning of growth is away from the optimum pH for the enzyme system (Wood, 1952).

In the experiments described above, signals from extracellular metabolites which have diffused through the cell membrane are being observed. Therefore, the rate of formation of each metabolite observed by the present method may also be affected, at least to some extent, by the rate of diffusion of the metabolites through the cell membrane. With a more concentrated cell suspension and/or a large bore NMR tube, it may be possible to observe intracellular metabolites separately, and to determine the intracellular pH by using the chemical shifts of these metabolites (Navon et al., 1977).

It has been noticed that several small signals exist in the low-field NH and aromatic proton region. These signals may also give further information about the anaerobic metabolism of *E. coli* cells under investigation.

References

Arata, Y., & Ozawa, H. (1976) *J. Magn. Reson. 21,* 67.
Arata, Y., Ozawa, H., Ogino, T., & Fujiwara, S. (1978) *Pure Appl. Chem.* (in press).
Dadok, J., & Sprecher, R. F. (1974) *J. Magn. Reson. 13,* 243.
Daniels, A., Korda, A., Tanswell, P., Williams, A., & Williams, R. J. P. (1974) *Proc. R. Soc. London, Ser. B 187,* 353.
Daniels, A., Williams, R. J. P., & Wright, C. F. (1976) *Nature (London) 261,* 321.
Dwek, R. A., Campbell, I. D., Richards, R. E., & Williams, R. J. P., Eds. (1977) *NMR in Biology,* Academic Press, New York, N.Y.
Eakin, R. T., Morgan, L. O., Gregg, C. T., & Matwiyoff, N. A. (1972) *FEBS Lett. 28,* 259.
Gupta, R. K., Ferretti, J. A., & Becker, E. D. (1974) *J. Magn. Reson. 13,* 275.
Kainosho, M., Ajisaka, K., & Nakazawa, H. (1977) *FEBS Lett. 80,* 385.
Metzler, D. E. (1977) *Biochemistry: The Chemical Reactions of Living Cells,* Academic Press, New York, N.Y.
Moon, R. B., & Richards, J. H. (1973) *J. Biol. Chem. 248,* 7276.
Navon, G., Ogawa, S., Shulman, R. G., & Yamane, T. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 888.
Ogino, T., Arata, Y., Fujiwara, S., Shoun, H., & Beppu, T. (1978) *J. Magn. Reson.* (in press).
Stokes, J. L. (1949) *J. Bacteriol. 57,* 147.
Wood, W. A. (1952) *Bacteria 2,* 59.

Three Hydroxylations Incorporating Molecular Oxygen in the Aerobic Biosynthesis of Ubiquinone in *Escherichia coli*†

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ABSTRACT: The biosynthetic origin of the oxygen atoms of ubiquinone 8 from aerobically grown *Escherichia coli* was studied by ^{18}O labeling. An apparatus was developed which allowed the growth of cells under a defined atmosphere. Mass spectral analysis of ubiquinone 8 from cells grown under highly enriched $^{18}O_2$ showed that three oxygen atoms of the quinone are derived from molecular oxygen. It was established that the molecular oxygen is incorporated into the two methoxyl groups (at C-5 and C-6) and one of the carbonyl positions of the ubiquinone molecule by demonstrating that only one of the incorporated oxygens will exchange with water under acidic conditions that specifically catalyze the exchange of carbonyl, but not methoxyl, oxygens. That the C-4 carbonyl oxygen is derived from molecular oxygen was shown by the incorporation of three atoms of $^{18}O_2$ into ubiquinone 8 biosynthesized from added 4-hydroxybenzoic acid. Comparison of ubiquinone 8 and menaquinone 8 from *E. coli* grown under $^{18}O_2$ confirmed that the labeled carbonyl oxygen of the [$^{18}O_2$]ubiquinone 8 is incorporated biosynthetically and not by chemical exchange in the cell. It is concluded that the three hydroxylation reactions involved in the pathway for the aerobic biosynthesis of ubiquinone are all catalyzed by monooxygenases. The implications of this study for the anaerobic biosynthesis of ubiquinone 8 in *E. coli* are discussed.

Ubiquinone is well established as a component of the respiratory chain of mitochondria (Crane & Sun, 1972) and also functions in respiration in many microorganisms (Haddock & Jones, 1977; Jones, 1977). The biosynthetic pathway to ubiquinone (IX) from 4-hydroxybenzoate (I) involves three hydroxylation reactions (Figure 1) (Young et al., 1973). Two of the hydroxyl groups are subsequently methylated and become the C-5 and C-6 ring methoxyl groups of ubiquinone, and the third hydroxyl forms the C-4 carbonyl of the quinone ring. The origin of these three oxygens has received comparatively little attention. It has been shown using ^{18}O labeling that molecular oxygen is incorporated into the two-ring methoxyl groups in the case of the aerobe *Pseudomonas desmolytica* (Uchida & Aida, 1972). However, no labeling of the quinone carbonyl was detected, suggesting that this oxygen might arise from water.

In the case of *E. coli,* which is a facultative anaerobe, the origin of the three hydroxyl groups has not previously been studied. We have recently found that strains of *E. coli* K12 produce up to 70% of the aerobic levels of ubiquinone when grown anaerobically with fumarate as electron acceptor (Alexander & Young, 1978). This finding suggested that *E. coli* is able to synthesize ubiquinone by an anaerobic mechanism and raised the question as to the origin of the three oxygen atoms under aerobic conditions. In the present paper, we show that under aerobic conditions each of the three oxygen atoms is derived from molecular oxygen.

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