

Kinetic Study of a Change in Intracellular ATP Level Associated with Aerobic Catabolism of Ethanol by *Streptococcus mutans*

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Streptococcus mutans, a group of lactic acid bacteria and a normal inhabitant of the human oral cavity, generates ATP by substrate-level phosphorylation coupled to oxidation of ethanol (an end product of fermentation of sugars) into acetate in the presence of oxygen (K. Fukui, K. Kato, Kodama, H. Ohta, T. Shimamoto, and T. Shimono, Proc. Jpn. Acad. 64B:13-16, 1988). Kinetic measurements were made of the cellular responses of *S. mutans* FA-1 to ethanol in comparison with those to glucose. In contrast to oxygen-independent acid production from glucose, oxygen was absolutely required for acid production from ethanol. Ethanol elicited a marked increase in the intracellular ATP concentration (ATP_i) from a starved level to a steady level which was held constant as long as oxygen was present in the medium. Once oxygen was exhausted, ATP_i returned to the starved level without delay. On the contrary, ATP_i changes induced by glucose, which were independent of oxygen, followed a rather complicated time course before a steady level was established. Both the steady ATP_i and the rate of accompanying oxygen consumption were functions of the ethanol concentration. These two parameters were linearly correlated, indicating that the unimolecular ATP turnover rate, which is independent of the rate of ATP generation in the steady state, can be calculated for cells energized by ethanol. The estimated turnover rate was 1.5 s⁻¹ at 37°C, which is comparable to that for other bacteria energized by glucose under nongrowing conditions.

In lactic acid fermentation by streptococci and related bacteria, sugars are catabolized via pyruvate, depending on conditions, into either lactate or a mixture of formate, acetate, and ethanol through the pathways shown in Fig. 1 (2, 9, 18). The conversion of pyruvate into these fermentation end products functions to oxidize NADH back to NAD⁺ under anaerobic conditions. Hence, the fermenting bacteria per se cannot metabolize these potentially energy-rich end products under anaerobic conditions. Under aerobic conditions, however, these bacteria have another option to regenerate NAD⁺, since they are equipped with a flavin enzyme(s) which catalyzes oxidation of NADH by oxygen (14, 15) (collectively called the NADH oxidase system in this paper). When incubated with glucose or other sugars, therefore, they consume oxygen at an appreciable rate in spite of lacking the respiratory chain (see, for example, reference 12). Furthermore, it is possible, in theory, that steps utilizing NADH in fermentation may be reversed if oxygen is present. Of particular interest is the ethanol branch of pyruvate metabolism, because ATP should be generated by substrate-level phosphorylation associated with oxidation of ethanol to acetate via acetyl coenzyme A (acetyl-CoA) (Fig. 1). Our recent study (4) has shown that this is indeed the case. Thus, concomitant with a rapid oxygen uptake, the intracellular ATP level (ATP_i) increases in several strains of oral streptococci when incubated with ethanol or *n*-propanol under aerobic conditions.

This paper provides a more detailed account of this interesting phenomenon. Our attention was mainly focused on the kinetics of ethanol-induced change in ATP_i of *Streptococcus mutans*. Several characteristic features revealed in the present work indicate that the *S. mutans* ethanol-oxygen

system will be of potential use for studying energy coupling in intact bacterial cells.

MATERIALS AND METHODS

Bacterial cells. *S. mutans* FA-1 (serotype b; alias *S. rattus* ATCC 19645 [3]) was used throughout, since this strain showed the maximum ability to increase ATP_i in response to ethanol among the strains so far tested (4). Cultures were grown anaerobically at 37°C in screw-cap 100-ml bottles as described previously (12). The medium contained (in grams per liter): KH₂PO₄, 1.0; K₂HPO₄, 4.0; MgSO₄, 0.25; peptone (BBL Microbiology Systems), 20; yeast extract (Difco Laboratories), 5; and glucose, 2.0. Growth was followed by measuring the optical density (OD) of cultures at 660 nm (1 OD unit corresponds to a dry weight of 0.42 mg/ml of cell suspension). After cessation of growth at an OD of ~0.90 due to glucose exhaustion, cultures were incubated for another 3 h (Fig. 2). The cells were then collected by centrifugation at 5,000 × *g* for 25 min, washed twice with ice-chilled 10 mM Tris hydrochloride (pH 7.5) containing 5 mM MgCl₂, and finally suspended in the same buffer at a concentration of about 40 mg (dry weight) per ml. The cell suspensions were kept on ice under an N₂ atmosphere until use.

Oxygen consumption and acid production. Ethanol- and glucose-dependent oxygen consumption was measured at 37°C in a reaction vessel (volume, 4.0 ml) fitted with a Clark-type oxygen electrode which was calibrated by assuming that the dissolved oxygen concentration in air-saturated water was 217 μM at 37°C (11). The cell suspension was diluted in the reaction vessel to a concentration of 0.1 to 0.2 mg (dry weight) per ml in a buffer containing 50 mM Tris hydrochloride (pH 7.5), 5 mM KCl, and 5 mM MgSO₄. When acid production was measured simultaneously with a Field Effect Transistor pH sensor (Kuraray, Okayama, Japan)

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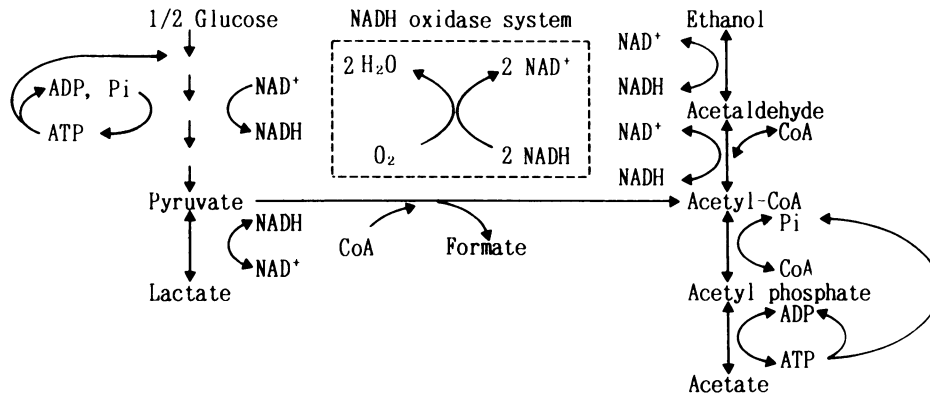


FIG. 1. Fermentation pathway of glucose in streptococci.

inserted into the oxygen electrode vessel, the concentration of Tris was reduced to 1 or 5 mM, but that of KCl was increased to 50 mM. The pH change generated by the addition of a known quantity of 10 mM HCl to the reaction medium was used as a standard for the conversion of pH decrease into moles of acid produced.

ATP determination. A portion (usually 50 μ l) of cultures or the cell suspensions incubated with ethanol or glucose was pipetted at intervals into 1 ml of distilled water in a test tube placed in a boiling-water bath. After 1 min, the tube was transferred onto ice, and the contents were assayed for ATP at 25°C by using a firefly luciferin-luciferase mixture (Laboscience, Tokyo, Japan) in a luminometer (BLR-102; Aloka Co., Tokyo). The instrument was calibrated with an ATP solution (1 pmol) freshly prepared in sterilized distilled water. A value of intracellular water space of 1.45 μ l/mg (dry weight) estimated for *Streptococcus lactis* (10) was assumed to hold also for *S. mutans* to calculate ATP_i.

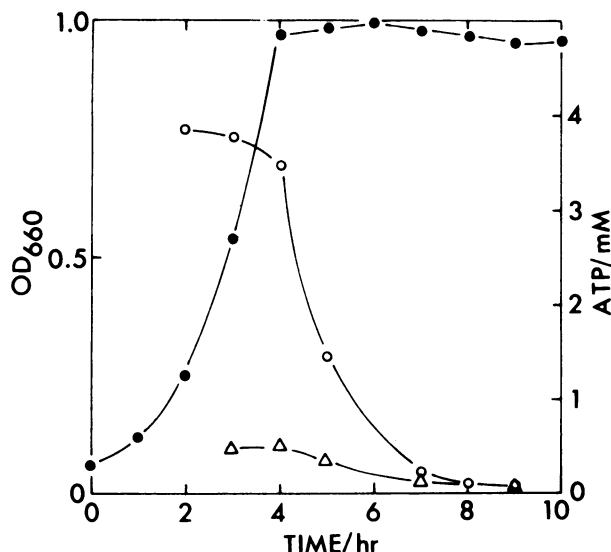


FIG. 2. Growth of *S. mutans* and the accompanying change in the ATP_i level. At intervals, a small sample (10 to 20 ml) of the anaerobic culture was withdrawn, a small portion (50 μ l) was processed directly for the ATP assay (unwashed cells), and the remainder was centrifuged to prepare washed cells for examining the increase in the ATP level in response to glucose and ethanol as described in the text. Symbols: ●, growth; ○, ATP level in unwashed cells; Δ, ATP level in washed cells.

RESULTS

The preparation of cell suspensions is critical in the present work. It was not difficult to deplete the cells of ATP by washing with buffer when glucose-limited cultures (12) had entered the stationary phase of growth (Fig. 2). However, results were variable for the increase in ATP_i and other activities in response to glucose or ethanol if the cells used were harvested within 2 h after cessation of growth. When the postgrowth incubation period was prolonged to 3 or 4 h, the ATP_i of the cells in the culture fell below 0.2 mM. The cells starved to that extent were washed with buffer and used in the experiments described below, so that consistent results were obtained with different cell preparations.

Oxygen involvement in ethanol oxidation. To illustrate the characteristic features of ethanol oxidation by *S. mutans*, the cellular responses to glucose and ethanol were compared (Fig. 3 and 4). When the cell suspension was incubated with glucose, rapid oxygen consumption and acid production took place concomitantly (Fig. 3A). The oxygen consumption can be ascribed to the activity of the putative NADH oxidase system (Fig. 1), which oxidizes the coenzyme reduced in the process of glucose breakdown into pyruvate (14, 15). Acid production continued after the exhaustion of oxygen, since glucose catabolism by *S. mutans* is fundamentally an oxygen-independent process in which reduction of pyruvate to lactate or reduction of acetyl-CoA to ethanol is the coupled reaction to oxidize NADH (Fig. 1).

As shown in Fig. 3B, concomitant acid production and oxygen consumption were also induced by ethanol, as described previously (4). The ethanol-induced acid production, however, ceased completely on exhaustion of oxygen, in contrast to that induced by glucose. When an oxygen pulse was given, acid production resumed and continued as long as the oxygen was present. Formation of acetic acid was confirmed by gas chromatographic analysis of the cell suspension incubated with ethanol. The inset of Fig. 3B is a differential plot of oxygen consumption versus acid production, which gives an oxygen/acid stoichiometric ratio of 0.90. This is in reasonable agreement with the theoretical value of 1 expected for the process shown in Fig. 1, where 1 mol of oxygen is absolutely required for oxidation of 2 mol of NADH generated when 1 mol of acetate is formed from 1 mol of ethanol (4).

The rate of oxygen consumption with glucose was half that with ethanol, whereas the accompanying acid production was much faster than that with ethanol (Fig. 3). The slow oxygen consumption with glucose relative to acid production

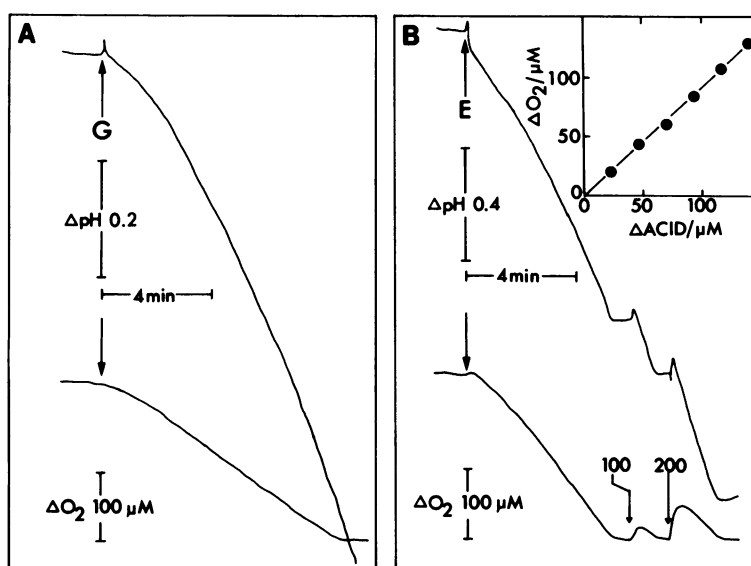


FIG. 3. Simultaneous recording of oxygen consumption (lower trace) and acid production (upper trace) by *S. mutans* incubated with glucose (A) and ethanol (B). Washed cells were incubated at a concentration of 0.25 mg (dry weight) per ml with 10 mM glucose or 520 mM (3%, vol/vol) ethanol in 50 mM KCl-5 mM MgCl₂. The Tris hydrochloride (pH 7.5) concentration was 5 mM (A) or 1 mM (B). Small arrows (B) indicate additions of distilled water (microliters) saturated with pure oxygen at 1 atm. The inset shows the stoichiometric relationship between acid production and oxygen consumption.

can primarily be ascribed to the low theoretical NADH/acid stoichiometry (1:1 for glucose catabolism up to pyruvate, but 1:2 if pyruvate is further metabolized to formate and acetate). Competition of NADH between the NADH oxidase system and dehydrogenases may also be responsible for the observed low oxygen/acid ratio, <0.4, which is unlikely in ethanol oxidation, when NADH should be oxidized exclusively by the oxidase system.

Energization by glucose. Figure 4A shows oxygen consumption and ATP_i change on addition of glucose. As can be seen, there was a rapid rise of ATP_i, which was followed by a sharp drop and then by a slow increase approaching a steady level. Because the glucose catabolism itself took place steadily, as indicated by rapid acid production (Fig. 3A), it is most likely that this complex change of ATP_i after addition of glucose is a reflection of the distribution of the acquired energy into the metabolites (see Discussion). Note that there was no change in the steady ATP_i after exhaustion of oxygen, which is consistent with oxygen-independent acid production (Fig. 3A).

Energization by ethanol. Figure 4B shows a typical result of simultaneous measurement of ethanol-induced change in ATP_i and oxygen consumption. It is obvious that the ATP_i change followed a less complicated time course than that observed with glucose. Ethanol also elicited a rapid rise of ATP_i, which was simply followed by a decrease leading to a steady level (energized state). The initial overshoot of ATP_i was much higher than that attained with glucose (the magnitude of the overshoot was variable for different preparations of cell suspension, but in general it was larger for less-starved cell preparations), whereas the steady level was much the same. ATP_i was held constant until the O₂ concentration reached 5 μM, below which oxygen consumption slowed down apparently; ATP_i then decreased very rapidly to the starved level on exhaustion of oxygen. When an oxygen pulse was given to the cell suspension, the ATP_i of the energized state was rapidly restored without an overshoot. It returned to the starved level again when the oxygen

was exhausted. This oxygen pulse-induced rise of ATP_i could be repeated (not shown), as in the case of acid production shown in Fig. 3B.

Effect of ethanol concentration. The oxygen consumption rate (J_{O_2}) and ATP_i in the steady state were measured simultaneously with a series of different concentrations of ethanol (Fig. 5). Although both quantities increased with the increase in ethanol concentration, ATP_i appeared to level off gradually beyond 1 mM (at ethanol concentrations higher than 50 mM), while J_{O_2} still increased. By double-reciprocal plotting, it was found that an ethanol concentration of 5 to 10 mM gave half-maximum values.

Figure 6 shows ATP_i plotted as a function of J_{O_2} , from which it is possible to estimate the turnover rate of intracellular ATP. The basic assumption is that J_{O_2} is a measure of the rate of ATP generation, since the ATP/oxygen stoichiometry should be 1 in the oxidation of ethanol through the pathway shown in Fig. 1 (see above). In the steady state, where ATP_i is held constant, the rates of ATP generation (V_g) and breakdown (V_b) are balanced, i.e., $V_g = V_b$. As shown in Fig. 6, ATP_i ($[T]$) increased linearly with J_{O_2} or V_g for $[T] < 1$ mM, as described by the equation $[T] = kV_g + [T]_0$, where k is a constant and $[T]_0$ is the ATP_i at the starved state. By neglecting $[T]_0$, as seems plausible, the equation is simplified to $V_g = 1/k[T]$. V_g can, of course, be replaced by V_b . Thus, $1/k$ is the unimolecular ATP turnover rate. The physiological implication of this equation is that the unimolecular ATP turnover is independent of the rate of ATP generation or its breakdown in the steady state. The value which can thus be obtained from Fig. 6 is 1.5 s^{-1} .

DISCUSSION

The discussion is concentrated here on two aspects of ATP synthesis in aerobic catabolism of ethanol by *S. mutans*: (i) differences in the effects of glucose and ethanol on ATP_i and (ii) potential use for studying the energy-coupling mechanisms in intact bacterial cells.

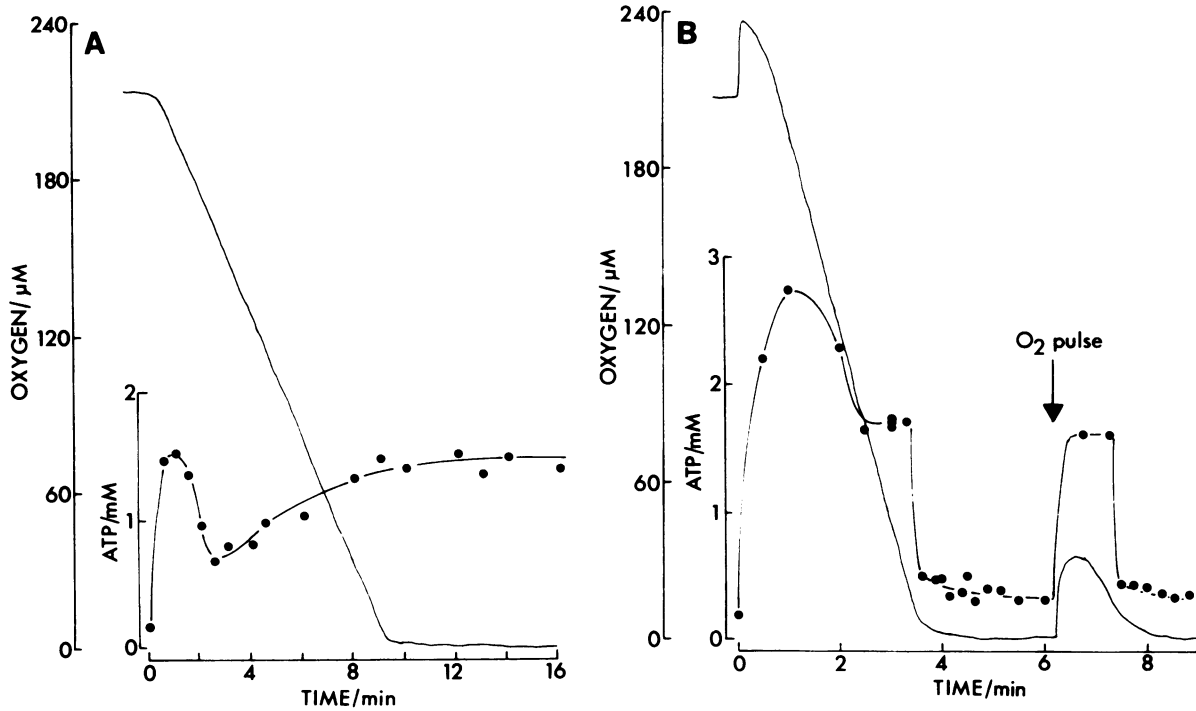


FIG. 4. Oxygen consumption and change in ATP_i level of *S. mutans* incubated with glucose (A) and ethanol (B). The concentrations of glucose and ethanol were 10 mM and 520 mM, respectively. A large upward deflection of the oxygen electrode trace on addition of ethanol was caused by the high solubility of oxygen in ethanol. The arrow indicates the addition of 1 ml of buffer saturated with air. Note the difference in time scales between panels.

The relative simplicity of the ethanol-induced energy charging of the cell (Fig. 4B) seems to be reasonable, because acetate is the only net product of ethanol oxidation. Acetate is poorly metabolized and released as an end prod-

uct of fermentation by streptococci as well as other lactic acid bacteria (2, 16–18). Thus, it is very unlikely that ethanol induces a buildup of a large metabolite pool, except for a possible increase in acetyl phosphate. This view is consistent with the observation of rapid approach of ATP_i to the steady level after addition of ethanol and its rapid fall on oxygen exhaustion. The decrease of ATP_i which follows the initial rise may be ascribed at least in part to phosphoryl transfer from ATP to other nucleoside mono- and diphosphates, i.e., formation of nucleoside triphosphates (NTPs). This is an immediate consequence of ATP formation.

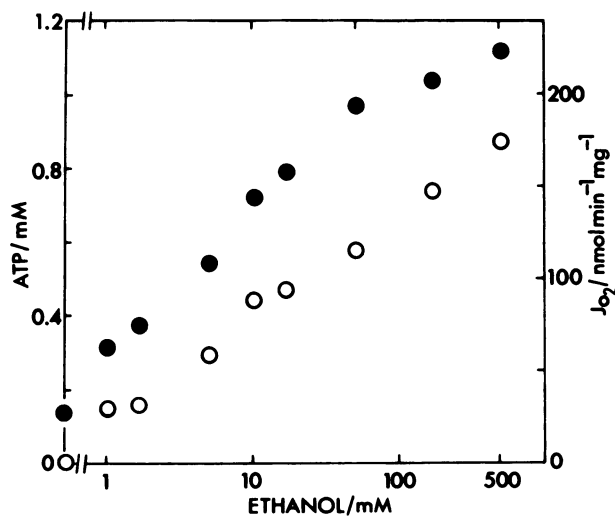


FIG. 5. Effect of ethanol concentration on rate of oxygen consumption and ATP_i level in the steady state. Incubation conditions were essentially the same as in Fig. 4, but a different preparation of cells was used. At each concentration of ethanol, the time course of the change in ATP level was followed and the steady ATP levels (between 3 and 6 min after addition of ethanol) were averaged in the plot. Oxygen consumption was zero order with respect to oxygen concentration in all cases. Symbols: ●, ATP level; ○, oxygen consumption.

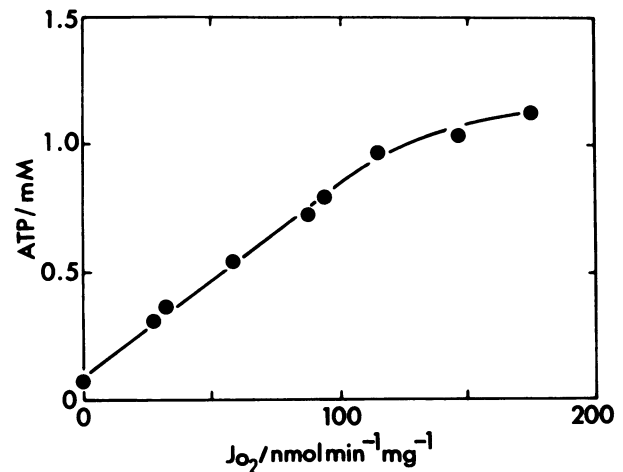


FIG. 6. ATP_i level as a function of oxygen consumption rate. A straight line was fitted up to 1 mM ATP_i by linear regression analysis. The data are the same as in Fig. 5.

By contrast, glucose would induce a rather complicated metabolic change when catabolized. Apart from the formation of NTPs, the glucose breakdown itself would bring a buildup of the phosphorylated metabolite pool, which may also contribute to an apparent decrease in ATP_i following its initial rapid rise (Fig. 4A). Further expansion of the metabolite pool may underlie the slow-increase phase approaching the steady level. Such a difference between the ethanol- and glucose-induced energy chargings is relevant to the second topic of discussion here.

Experimental manipulation of the ATP_i or energy-charging level of bacterial cells has been an important but difficult technique for many years. The use of different carbon (energy) sources, inhibitors (13), or mutants (6) can cause unwanted perturbation of cell metabolism by such manipulations. Glucose is the most conventional energy source. Experiments are usually performed under conditions with glucose (energized) or without glucose (nonenergized) or with glucose and an inhibitor (partially energized or energized but impaired). As described above, bacterial cells respond to glucose not only by increasing ATP_i but also by building up a large phosphorylated metabolite pool which serves as an energy store. It is this latter response that makes it difficult to control ATP_i experimentally when glucose is used.

When ethanol is used, on the other hand, a steady ATP_i can be set at any point between the completely starved and fully energized levels simply by changing its concentration from just below 1 mM to 500 mM, as shown in Fig. 5. The theoretical maximum must, of course, be determined by the total concentration of overall adenine nucleotides, as is reflected in the leveling off of ATP_i above 1 mM (Fig. 5 and 6). The duration of the steady state can be prolonged if a sufficient oxygen supply is ensured for rapid recycling of NAD⁺. In addition, from the linear relationship between ATP_i and the rate of ATP synthesis (Fig. 6), the unimolecular ATP turnover rate can be estimated in the steady state. This estimation is rather straightforward. With other energy sources or other bacteria, although not impossible, it is not an easy task to measure the ATP turnover rate because of difficulty in estimating the rate of ATP synthesis.

The ATP turnover rate (1.5 s⁻¹) in *S. mutans* energized by ethanol is less than half of that (~4 s⁻¹) calculated for the same organism growing on glucose with a doubling time of 50 min (Kodama and Fukui, unpublished). A similar contrast in the ATP turnover rate between nongrowing and growing cells is inferred for other bacteria (1, 5, 7, 8). Even energized by glucose, for example, the rate is as low as ~0.6 s⁻¹ in nongrowing *Escherichia coli* (5), whereas it is much higher, ranging from 4 to 8 s⁻¹, in growing cells (7, 8). Hence, the low ATP turnover rate of *S. mutans* estimated in the present work is probably characteristic of a nongrowing state but not of the state energized by ethanol.

In summary, the ethanol-induced energy charging of *S. mutans* can serve as an interesting experimental system for studying energy coupling in bacteria. Studies are now in progress in this laboratory to ascertain factors affecting the ATP turnover rate.

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