Respiration-induced Changes in the Adenine Nucleotide Pool Composition of Harvested *Beneckea natriegens*

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Changes in adenine nucleotide composition of suspensions of starved *Beneckea natriegens* on aeration and anaerobiosis have been studied using succinate as the respiratory substrate. The anaerobic steady-state adenylate pool is only about 50 to 60% of the aerobic steady-state pool, and the energy charge is also much lower under anaerobic conditions. Aeration of anaerobic suspensions causes the ATP and total adenylate concentrations and the energy charge to rise rapidly with an overshoot in the ATP concentration before the aerobic steady-state is attained. Shifts to anaerobic conditions result in oscillations of the energy charge and adenine nucleotide concentration and the anaerobic steady-state is only attained relatively slowly. Sequential aeration and anaerobiosis stabilizes the adenine nucleotide pool size, permitting estimates of P/O ratios. The P/O ratio, with succinate as the respiratory substrate, has been assayed by pulsing anaerobic suspensions with small amounts of oxygen (generated from H_2O_2 and catalase) and quenching metabolic activity before anaerobiosis had been regained, then analysing the adenine nucleotide composition. P/O ratios of 0.2 decreasing to 0.02 with increasing oxygen pulse size have been observed. Extrapolation to zero pulse size, when little turnover of the adenylates occurs, gives a P/O ratio of about 0.4. This is much lower than the efficiency of energy transduction as indicated from H^+ /O experiments. Therefore, either the observed and extrapolated P/O ratios are unrealistically low and further correction is required, or only a small proportion of the energy transduction is involved in phosphorylation under these conditions.

INTRODUCTION

Only low efficiencies of oxidative phosphorylation are usually found in bacterial cell-free extracts. Other techniques have thus been developed to determine whether the energy transducing systems are labile or inefficient. Studies on growth yields (Stouthamer & Bettenhausen, 1973; Stouthamer, 1976) and proton translocation (Mitchell & Moyle, 1967; Garland, 1977; Jones, 1977) suggest a high level of efficiency of oxidative phosphorylation in bacteria. However, such techniques are indirect and, for example, there have so far been no reports of a correlation between P/O and H^+ /O quotients.

Oxidative phosphorylation can also be estimated by assaying changes in concentration of the adenine nucleotides compared to the initial rate of respiration on aeration of anaerobic suspensions of bacteria. Hempfling (1970*a, b*) and Hempfling & Beeman (1971) measured the initial rate of respiration on aerobiosis of starved *Escherichia coli* by monitoring the decrease in the endogenous NADH:NAD⁺ ratio (it was assumed that no NAD⁺/NADH turnover occurred and that no other donors, such as succinate, contributed to respiratory activity). Baak & Postma (1971) have also used this technique with starved

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Azotobacter vinelandii. However, van der Beek & Stouthamer (1973) and Gadkari & Stolp (1976) have shown that the method leads to incorrect estimates of the efficiency of oxidative phosphorylation. Metabolic activity, including NAD^+/NADH turnover, is presumably stimulated by the increase in energy charge (Atkinson, 1968; Knowles, 1977) as the anhydride-bound phosphate content increases on aeration. The increase in phosphorylation of the adenylates will also cause an increase in their turnover and an underestimate of the $\text{P}/2e^-$ ratio. Provided the true rate of respiration is known, a minimal value of the $\text{P}/2e^-$ ratio can be derived.

Ramirez & Smith (1968) and Knowles & Smith (1970), working with *Rhodospirillum rubrum* and *Azotobacter vinelandii* respectively, compared the rate of ATP synthesis to oxygen uptake on aeration of the organisms. In both cases measurements had to be made at 12 °C to slow the respiratory activity; the maximum ATP concentration was then reached after 15 to 25 s enabling the initial rate of oxygen uptake to be accurately measured. Van der Beek & Stouthamer (1973) mixed anaerobic suspensions of several bacteria with aerated buffer at 30 °C, quenching the reactions after 0 to 30 s. Gadkari & Stolp (1976) carried out similar experiments, quenching activity after 1 to 4 s aeration. However, using a Clark-type or vibrating Pt electrode and chart recorder we have been unable to measure oxygen uptake, following aeration, with a mixing plus response time of less than about 2.5 s (C. J. Knowles & L. Smith, unpublished observations). Adequate control experiments must therefore be carried out before data on oxygen uptake during periods of less than 3 or 4 s after aeration can be considered to be valid.

A different approach was adopted by Baak & Postma (1971) and van der Beek & Stouthamer (1973): anaerobic suspensions of bacteria were mixed with limited amounts of oxygen and the degree of phosphorylation was compared with the oxygen pulse size. It is crucial, when determining ATP/O quotients in experiments of this type, to quench metabolic activity at exactly the moment of oxygen depletion, yet neither group reported how they achieved this.

In this paper, we report our experiments on the effect of changes of aeration on the adenylate pool of harvested *Beneckeia natriegens*, as part of our research into the adenine nucleotide metabolism of this marine bacterium (Niven, Collins & Knowles, 1977*a, b*). To estimate P/O ratios, we have used a modification of the methods of Knowles & Smith (1970) and van der Beek & Stouthamer (1973): small pulses of oxygen were added to anaerobic suspensions and metabolic activity was quenched slightly before anaerobiosis was regained, correcting for the residual oxygen in calculating the P/O quotient.

METHODS

Organism and growth conditions. *Beneckeia natriegens* strain 111 was grown in a 10 mM-succinate/minimal salts medium as described previously (Niven *et al.*, 1977*b*). An E_{680} of 1.0 was equivalent to a density of 0.4 mg dry bacterial wt ml⁻¹ (Niven *et al.*, 1977*a*).

Preparation of bacterial suspensions. Bacteria from a 400 ml culture were harvested by centrifuging at room temperature (23000 g, 10 min), washed with 40 ml of 50 mM-Tris/HCl containing 400 mM-NaCl, 10 mM-KCl, 1.0 mM-MgSO₄, 0.1 mM-K₂HPO₄, pH 7.5, and resuspended to 400 ml in fresh buffered saline solution in a 2 l baffled flask. Such suspensions were starved for 2 h by shaking at 35 °C in a gyrotary shaker (200 rev. min⁻¹), harvested at room temperature as above, washed with 40 ml of 1.0 mM-Tris/HCl containing 380 mM-NaCl, 10 mM-KCl, 1.0 mM-MgSO₄, 10 mM-Na₂HPO₄, pH 7.2, resuspended to about 1.0 ml in fresh buffered saline solution and stored on ice until required (up to a maximum of 1 h). The respiratory activity of bacteria prepared in this way was determined polarographically at 25 °C. In the absence and presence of 10 mM-sodium succinate, the respiratory rates were approximately 30 and 430 ng-atom oxygen min⁻¹ (mg dry wt)⁻¹ respectively.

Adenine nucleotide pool changes during aerobic to anaerobic and anaerobic to aerobic shifts. Bacterial suspension was added to 1.0 mM-Tris/HCl containing 380 mM-NaCl, 10 mM-KCl, 1.0 mM-MgSO₄, 10 mM-Na₂HPO₄, pH 7.2, in a glass vessel (approximately 80 ml capacity) such that the cell density was approximately 1.0 mg dry bacterial wt ml⁻¹ in a final volume of 50 ml. The vessel stopper accommodated air and

nitrogen input tubes, exhaust port, injection/sampling port and oxygen electrode (Yellow Springs Instrument Co., Silver Springs, Ohio, U.S.A.). Oxygen tension was monitored on a W+W Series 1100 recorder (W+W Electronic Inc., Basel, Switzerland). The suspension, which was maintained at 25 °C, was stirred continuously with a Teflon-coated magnetic stirring bar and aerated by passing water-saturated air through it at 2 to 3 l min⁻¹ such that the oxygen tension never fell below that of 50 % air-saturated buffer. Where desired, anaerobiosis was maintained by bubbling water-saturated nitrogen through the suspension instead of air. Prior to initiation of the experiments, suspensions were allowed to equilibrate for 20 min in the presence of either 1 mM or 10 mM-sodium succinate (anaerobic to aerobic and aerobic to anaerobic shifts, respectively).

Estimation of P/O ratios. Bacterial suspension was added to 1.0 mM-Tris/HCl containing 380 mM-NaCl, 10 mM-KCl, 1.0 mM-MgSO₄, 10 mM-Na₂HPO₄, 10 mM-sodium succinate, 0.1 mg catalase ml⁻¹ (EC 1.11.1.6) pH 7.2, to give a cell density of 5 to 7 mg dry bacterial wt ml⁻¹ in a final volume of 14.975 ml. This suspension was contained in an oxygen electrode vessel (Rank Bros, Bottisham, Cambridge) which had been modified such that the vessel capacity was increased to about 19 ml. The vessel was fitted with a no. 53 Suba-Seal stopper (Astell Laboratory Service Co., Catford, London) and an inert atmosphere was maintained above the suspension by passing water-saturated nitrogen (1 l min⁻¹) through a gas line supplying two syringe needles, one passing through the Suba-Seal stopper, the other being open to the atmosphere. Incubation temperature, stirring conditions and monitoring of oxygen tension were as above. The suspension was pulsed five times with 5 µl of anaerobic H₂O₂ (4 µmol) in 400 mM-NaCl, 10 mM-KCl, 1.0 mM-MgSO₄ at 5 min intervals followed by a 15 min anaerobic equilibration period. A sample was then removed for estimation of adenine nucleotide levels. A known amount of anaerobic H₂O₂ (5 to 40 µl of approximately 5 or 10 vol. H₂O₂) in 400 mM-NaCl, 10 mM-KCl, 1.0 mM-MgSO₄ was then quickly injected, and a sample was removed and metabolic activity was quenched with HClO₄ when the oxygen tension, measured polarographically, had returned to that of 10 % air-saturated buffered saline solution. The concentration of H₂O₂ in the anaerobic stock solution was determined spectrophotometrically and by monitoring the oxygen released when small amounts were added to an anaerobic solution of catalase in an oxygen electrode vessel.

Sampling and preparation of extracts. Samples were collected, using a double-stop spring-loaded syringe unit, and extracts were prepared as described previously (Niven *et al.*, 1977a), except that the acidified samples were stored overnight at -22 °C and were thawed, mixed and centrifuged (3000 g, 15 min, 4 °C) prior to neutralization of the supernatant fractions. In a few experiments, samples were removed for estimation of extracellular adenine nucleotide levels. As no adenine nucleotides were detected, this procedure was discontinued.

Determination of adenine nucleotides. Preparation of samples for the luciferase assay, preparation of firefly lantern extract and the luciferase assays were as described previously (Niven *et al.*, 1977a). All luciferase assays were performed in triplicate.

Chemicals. Catalase (C-40, thymol-free) was obtained from Sigma. Sources of other enzymes and biochemicals were as listed previously (Niven *et al.*, 1977a, b). Whenever possible, all other reagents were of analytical grade; glass-distilled water was used throughout.

RESULTS AND DISCUSSION

In the presence of succinate, the steady-state adenine nucleotide content of *B. natriegens* under anaerobic conditions was about 50 to 60 % of the aerobic steady-state level (Fig. 1). The anaerobic energy charge was 0.4 to 0.5 whilst aerobically it was about 0.9.

When bacteria, incubated under anaerobic conditions in the presence of succinate, were aerated, the total adenine nucleotide content rapidly increased then decreased slightly before the aerobic steady-state was attained (Fig. 1). The increase in adenine nucleotide concentration, and the accompanying rise in energy charge from 0.5 to 0.9, was caused by an increase in ATP content, which must be due to either *de novo* synthesis of AMP or increased breakdown of RNA to release AMP, followed by phosphorylation to give the ATP. We have shown previously that in nitrogen-starved non-growing cultures of *B. natriegens*, the adenine nucleotide content is dramatically increased when an energy source (succinate or glucose) is present (Niven *et al.*, 1977a).

When the reverse experiment, the transition from aerobic to anaerobic conditions, was carried out, the energy charge rapidly decreased from 0.9 to about 0.3 then increased to 0.6 to 0.7 and finally fell to about 0.4 (Fig. 2). The cyclical change in energy charge was due to a rapid decrease in ATP content, followed by a smaller increase and a final decrease.

Fig. 1

Fig. 2

Fig. 1. Adenine nucleotide pool changes during an anaerobic to aerobic shift in the presence of succinate: Δ , total adenine nucleotides; $\bullet$, ATP; $\circ$, ADP; $\blacktriangle$, AMP; $\square$, energy charge.

Fig. 2. Adenine nucleotide pool changes during an aerobic to anaerobic shift in the presence of succinate. Symbols as in Fig. 1.

There were converse changes in the concentration of AMP but little change in the ADP pool, presumably due to the action of adenylate kinase. The initial drop and partial recovery of the energy charge (Fig. 2) was also accompanied by a transient fall in total adenine nucleotide content. The initial decrease in adenine nucleotide concentration could have been due to activation of the enzymes of AMP catabolism such that the fall in energy charge was delayed at the expense of the total adenylate pool size. The observed transient recovery of charge may have allowed temporary stabilization of metabolic activity despite the lack of oxygen. The source of energy for the increase in ATP and total adenine nucleotide content during this phase is not known, but it was not from respiration of succinate because of the anaerobic conditions. Some breakdown of glycogen or other endogenous energy source, perhaps accompanied by RNA catabolism to release AMP, could have been stimulated by the preceding fluctuations in energy charge, thus permitting resynthesis of ATP (see Knowles, 1977). Alternatively, AMP could be resynthesized from its catabolic products (IMP, adenosine or inosine; Niven *et al.*, 1977b).

The exact function and mechanism of regulation of the enzymes of AMP catabolism in *B. natriegens* are not known, but the pathway of AMP catabolism in extracts depends on the prevailing ATP concentration or energy charge (Niven *et al.*, 1977b). Similarly, in *A. vinelandii*, aerobic-to-anaerobic transitions cause a decrease in ATP content (Knowles & Smith, 1970) and total adenine nucleotide content (Schramm & Leung, 1973). AMP catabolism by AMP nucleosidase in *A. vinelandii* is activated by decreases in energy charge and inhibited by inorganic orthophosphate; it has been proposed that the AMP nucleosidase acts as a buffering system for maintaining the intracellular energy charge under conditions of fluctuating energy or oxygen supply (Schramm & Leung, 1973; Schramm & Lazoric, 1975).

The changes in total adenine nucleotide concentration that occur on aerobiosis or

Fig. 3

Fig. 4

Fig. 3. Changes in adenine nucleotide pool levels on pulsing a 14.2 ml suspension of anaerobic *B. natriegens* (6.8 mg dry bacterial wt ml⁻¹) containing 10 mM-succinate and 0.1 mg catalase ml⁻¹, with anaerobic H_2O_2 (10 μ l; 8.1 μ mol). Symbols as in Fig. 1.

Fig. 4. Estimation of P/O ratios from pulse aeration of *B. natriegens* using succinate as substrate. The experiment was carried out using various sizes of H_2O_2 pulses in the manner described in Fig. 3. $\circ$ and $\bullet$, P/O ratio; $\square$ and $\blacksquare$, energy charge. Open and closed symbols represent data from two separate experiments using different batches of bacteria.

anaerobiosis of harvested cell suspensions of *B. natriegens* are unusual. In studies of several other bacteria, there have been no reports of such changes in adenylate content (Hempfling, 1970*a, b*; Baak & Postma, 1971; van der Beek & Stouthamer, 1973; Gadkari & Stolp, 1976). However, this could be due to preconditioning of the various bacteria by changes in aeration of the bacteria prior to experimentation (see below).

Attempts to estimate P/O quotients by the increase in anhydride-bound phosphate content of the adenine nucleotide pool on aerobiosis of *B. natriegens* were complicated by the increases in total adenylate content (Fig. 1). However, we observed that 'preconditioning' of suspensions of *B. natriegens* by several small pulses of oxygen (generated by catalase plus H_2O_2) at 5 min intervals gave a high, stable anaerobic content of the adenine nucleotides at a low (0.2 to 0.3) energy charge level. After a further 15 min anaerobic incubation, a small oxygen pulse (8.1 μ g-atom oxygen in Fig. 3) resulted in a rapid increase in ATP and a corresponding decrease in AMP content (with little effect on ADP due to adenylate kinase activity) and an increase in energy charge, but no change in total adenylate concentration. The oxygen, measured polarographically, was 95% consumed after 14 s and totally utilized within 30 to 40 s. The increase in ATP and the decrease in AMP that occurred on addition of oxygen were reversed as anaerobiosis was regained, and the energy charge, after rapidly increasing to 0.8, gradually returned to about 0.2. Only a small decrease in adenine nucleotide content occurred in the next 30 to 60 min.

We therefore measured P/O quotients by pulsing anaerobic suspensions of 'preconditioned' *B. natriegens* with small amounts of oxygen (generated by adding H_2O_2 to catalase-containing suspensions), using succinate as the respiratory substrate. Metabolic activity was quenched using rapid-delivery syringes to remove a portion of the suspension into

HClO_4 (Niven *et al.*, 1977a) when the oxygen tension, measured polarographically, had decreased to 10% of that of air-saturated suspension buffer. Pulses of 3 to 20 μg -atom oxygen in 14.2 ml reaction mixture (i.e. 15 ml initial volume minus 0.8 ml for the sample removed for estimation of the anaerobic adenylate composition) were used and the minimum period before quenching was 8 s, well above the response time of the oxygen electrode. Hence, the oxygen consumed could be accurately determined. Moreover, in this way the changes in concentration of each of the adenine nucleotides could be measured before anaerobiosis was regained and whilst the oxygen tension of the suspension was still well above the K_m values of the oxidases (cf. Weston, Collins & Knowles, 1974).

The observed P/O values varied from about 0.2 using a 5 μg -atom oxygen pulse to 0.02 with a 20 μg -atom oxygen pulse (Fig. 4). When 5 μg -atom oxygen or larger pulses were used, optimum aerobic energy charge values of 0.8 to 0.9 were attained.

In the period immediately after aeration, the ATP content increased dramatically at the expense of AMP, then, as the ATP pool became constant, turnover of ATP occurred, which is not seen as an increase in incorporation of phosphate into the adenylate pool. As metabolic activity is activated by the increase in energy charge, so also the rate of turnover of ATP and total adenine nucleotides will be stimulated. Thus one would expect that the larger the oxygen pulse, the lower the observed P/O quotient, as was shown to occur (Fig. 4).

Ideally, extrapolation to zero oxygen pulse size should give the true P/O quotient. This assumes either that there is a negligibly low anaerobic turnover of the adenylates, which is unlikely even though it will be much reduced, or that such turnover can be estimated and corrections made for it. Extrapolation to zero pulse size gives an accurately reproducible P/O value of 0.38 for succinate oxidation when the data are plotted semi-logarithmically (Fig. 4). It is not clear why a straight line response is obtained on semi-logarithmic plots, but it appears that metabolic activity and ATP turnover must increase logarithmically with the increase in energy charge to give the decrease in the observed P/O ratio as the oxygen pulse size increases.

The P/O ratios (Fig. 4) were calculated as $[2\Delta\text{ATP} + \Delta\text{ADP} (\mu\text{mol})]/\mu\text{g}$ -atom oxygen consumed. Despite the preconditioning procedure, minor changes in the total adenine nucleotide concentration occurred; assuming one ATP molecule is required per AMP molecule synthesized, the P/O ratio can be recalculated as $[3\Delta\text{ATP} + 2\Delta\text{ADP} + \Delta\text{AMP} (\mu\text{mol})]/\mu\text{g}$ -atom oxygen consumed. When the data were recalculated in this manner, the extrapolated P/O value was slightly higher at exactly 0.4.

When pulses of less than 5 μg -atom oxygen were used, the observed P/O ratios were less than the optimum value. At the minimum feasible pulse of 3 μg -atom oxygen, the P/O ratio was about 0.12. With these small pulses, there could have been a switch to a less well coupled pathway of respiration (Weston *et al.*, 1974). Alternatively, the protonmotive force generated by the smaller pulses may have been sufficient only to re-establish, for example, the aerobic osmo-regulatory Na^+ and K^+ gradients, succinate transport, or other non-ATP-requiring but energy-consuming processes. The first possibility seems unlikely since H^+/O ratios have been estimated from oxygen pulse experiments (Mitchell & Moyle, 1967) using pulses giving oxygen tensions of 1% or less of air saturation (D. F. Niven & C. J. Knowles, unpublished observations). With succinate as the substrate, H^+/O quotients measured with separate batches of starved *B. natriegens* were reproducible but varied from 2.45 to 3.6 with different batches. It could be that the efficiency of energy transduction for oxidation of succinate depends on the particular respiratory pathway utilized (Weston *et al.*, 1974) and the variability of H^+/O values depends on the relative flux down the different respiratory branches. Alternatively, the variability and low values could be due to the presence of a Na^+/H^+ antiport operating to different extents in different batches of bacteria (or some other factor).

Assuming an H^+/O quotient of 2 per energy transducing site, the H^+/O values of less

than 4 but greater than 2 indicate that there must be two energy transducing centres, at least, when respiration is via one of the respiratory branches. Since the observed and extrapolated P/O ratios are both much lower than the level of energy transduction, either further correction for turnover of the adenylates is required, or, under these conditions, only a relatively small proportion of energy transduction is directed to phosphorylation. However, as already indicated, the oxygen pulses were much lower in the H^+/O than in the P/O experiments, and the respiratory pathway(s) utilized may be different. Thus, as the oxygen pulse size was increased from 5 to 20 μg -atom oxygen in 14.2 ml in the P/O experiments (Fig. 4), a non-energy-conserving pathway may have been increasingly utilized and the correlation between transduction and phosphorylation could have been closer than observed.

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