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MEASUREMENTS OF ATP LEVELS OF INTACT *AZOTOBACTER VINELANDII* UNDER DIFFERENT CONDITIONS

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SUMMARY

1. The ATP level of intact cells of *Azotobacter vinelandii* grown on a nitrogen-free, minimal salts medium with mannitol as carbon source has been examined using the luciferin–luciferase method. Harvested, washed and starved cells had aerobic ATP levels similar to cells taken directly from the culture medium.

2. The aerobic ATP level was always high and about the same with endogenous substrates or with added substrates giving a 10-fold variation of respiratory rates. On anaerobiosis, the ATP level fell to about 1/4 of the aerobic level.

3. Under anaerobic conditions the rate of utilization of ATP was 0.4–0.6 and 1.6–2.3 nmoles/min per mg dry wt. at 12° with endogenous substrate and with added β-hydroxybutyrate or lactate, respectively. The rate of formation on addition of O_2 to anaerobic cells in the presence of β-hydroxybutyrate was 12 nmoles/min per mg dry wt. When the latter was compared with the rate of O_2 uptake with the same samples, P:O ratios of 2 were obtained (corrected for the rates of utilization).

4. The aerobic ATP level was low in early log phase cells, then increased during growth to a maximum of 4.8–6.2 nmoles/mg dry wt. at the end of log phase growth.

5. Aeration of anaerobic cells gave maximal ATP levels within about 30 sec at 12°, but linear O_2 uptake continued for several minutes, until exhaustion of the O_2 in the suspending medium.

6. Oxidative phosphorylation in intact cells of *Azotobacter vinelandii* is an efficient process, but phosphorylation does not appear to be tightly coupled to O_2 uptake.

INTRODUCTION

Measurements of ATP formation linked to substrate oxidation have been made with broken-cell suspensions of numerous different species of bacteria. Phosphorylation of added ADP can only be made with broken-cell extracts, since ADP is not transported into intact bacteria. In general the efficiency of ATP formation (as expressed by the P:O ratio[1]) in disrupted cells is found to be low compared with that of the system in mitochondria of eucaryotic cells[1]. However a few experiments with intact bacteria have suggested efficiencies similar to those of intact mitochondria[2–4].

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The availability of the very sensitive luciferin–luciferase method for measuring ATP concentration has made possible studies of steady-state levels and rates of change of ATP level in relatively dilute suspensions of intact bacteria[3,5–7]. Such studies can show whether the lower efficiency in bacterial extracts results from disruption of the phosphorylating system on cell rupture. They also shed some light on the nature of the phosphorylating pathways, on the lack of respiratory control found in nearly all suspensions of broken bacteria and on relative rates of ATP synthesis and utilization under different conditions. The present work describes such studies with the obligately aerobic *Azotobacter vinelandii*, which contain a very active respiratory chain system and have no fermentative ATP synthesis[8]. Extensive studies have been made on the respiratory chain system and oxidative phosphorylation in cell-free extracts of this species[8,9,10–12].

METHODS

Growth of bacteria

A. vinelandii (NCIB Strain 8660) was grown on the minimal salts medium described by JONES AND REDFEARN[8], which contains no combined nitrogen; the carbon source was mannitol (15 g/l). The bacteria were grown in 700 ml of medium in 2-l flasks on a gyrotory shaker at 30° from a 1% inoculum of log phase cells. They were usually harvested after approx. 35 h, near the stationary growth phase, by centrifugation at 10000 × *g* for 15 min in the cold, then washed once with 50 mM Na_2HPO_4–KH_2PO_4 buffer (pH 7.4) and resuspended in the same buffer to a concentration around 5 mg dry wt./ml. The bacteria were then "starved" of endogenous substrate by aeration at 30° for 90 min, then washed once more and suspended in the same phosphate buffer.

ATP assay

The ATP content of the intact bacteria was measured by rapidly collecting 1-ml samples containing between 5 and 20 mg dry weight into $HClO_4$ and measuring the ATP content of the neutralized $HClO_4$ extracts with the luciferin–luciferase method as described by WELSCH AND SMITH[7]. Calibration curves with pure ATP were made with each experiment.

ATP steady-state levels

The ATP content of cells in the anaerobic state was measured by removing samples from a suspension of cells stirred under a stream of N_2. The aerobic steady-state level of ATP was measured either (a) by removing samples of bacteria at 30-sec intervals after mixing an anaerobic suspension with air in a closed chamber containing an O_2 electrode and a magnetic stirrer[3] or (b) by collecting samples after different intervals of continuous aeration. The aerobic steady-state level was reached in less than 1 min after the addition of O_2 to anaerobic cells at either 10–12° or at 23–25°, and the same level was maintained for periods up to 3 h in the presence of O_2.

Measurement of rates of ATP formation and utilization

The rate of ATP formation was measured following addition of O_2 to an anaerobic suspension of starved cells which had been further incubated for at least 30

min at 0° to insure that the ATP level was low. To do this the bacterial suspension and oxygenated buffer were mixed rapidly, then the mixture injected into cold $HClO_4$ after varying time intervals using the device described by WELSCH AND SMITH[7]. These measurements were made at 10–12°.

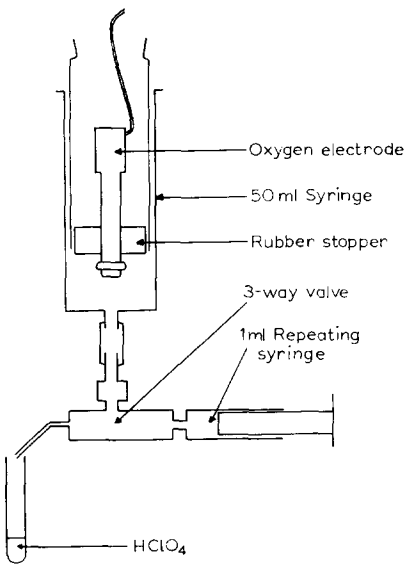

Fig. 1. Diagram of apparatus used to measure the anaerobic rate of ATP utilization. O_2 uptake of aerated bacteria in the 50-ml syringe was followed by means of the inserted O_2 electrode. Immediately before and after anaerobiosis 1-ml samples were taken at 20- or 30-sec intervals, by withdrawing them into the repeating syringe and, *via* the 3-way valve, injecting into 0.2 ml 35% (w/v) $HClO_4$ to stop the reaction. ATP was then assayed as described in METHODS.

The rate of ATP utilization after exhaustion of O_2 was measured in the apparatus shown diagrammatically in Fig. 1. This consisted of a 50-ml syringe with a free-sliding plunger connected by a short piece of rubber tubing to a 1-ml automatic syringe (Scientific Industries, Inc., Springfield, Mass.) and a 3-way automatic valve (B–D Co., Rutherford, N.J.). Both ends of the syringe plunger were removed and a Clark-type O_2 electrode was fitted inside and held securely in a rubber stopper. The syringe was clamped at an angle of 45° so that when samples were removed by the automatic syringe the plunger could slide down the barrell. 20 ml of 50 mM phosphate buffer (pH 7.4) and 12 ml of bacteria (15–20 mg dry wt./ml) were drawn into the syringe and mixed by means of a magnetic stirrer and small stirring bar. When the anaerobic state was approached, as shown by the electrode tracing, 1-ml samples were shot into 0.2 ml of cold 35% $HClO_4$ *via* the 3-way valve at 20- or 30-sec intervals. Fig. 2 shows an O_2 electrode trace from a typical experiment, indicating the times of removal of samples. The rates of utilization were measured at 10–12°.

Measurement of O_2 uptake

O_2 uptake was measured polarographically with a Clark O_2 electrode (Yellow Springs Instrument Co.) either in the apparatus described in the previous paragraph or in a closed chamber as described previously[3].

Chemicals

Chemicals were of the highest grade available in each case. Desiccated firefly tails and ATP were obtained from the Sigma Chemical Co.

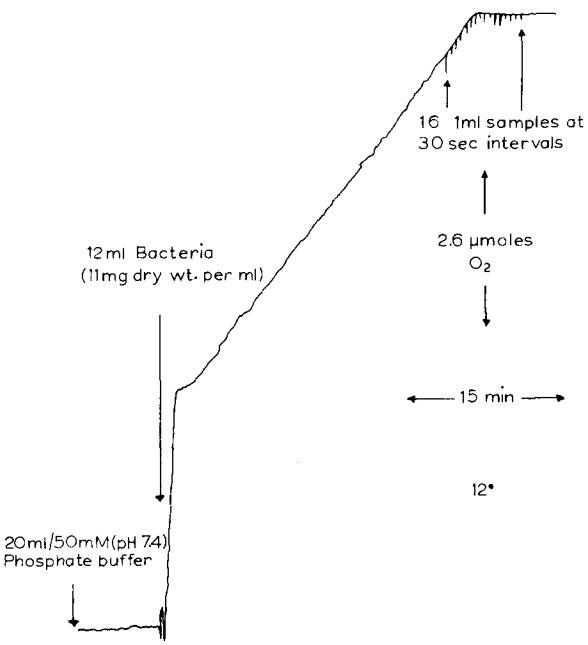

Fig. 2. Trace of O_2 uptake showing collection of samples for assay of the anaerobic rate of ATP utilization. In the apparatus shown in Fig. 1, 20 ml 50 mM phosphate buffer (pH 7.4) were introduced into the 50-ml syringe, followed by 12 ml bacteria (11 mg dry wt./ml). At the end of the aerobic phase 1-ml samples were taken at 30-sec intervals and assayed for ATP content. Experimental temperature was 12°.

RESULTS

COLE *et al.*[6] reported that the aerobic ATP levels of harvested *Escherichia coli* were considerably less than those found in cells taken directly from the growth medium. Thus the effect of harvesting, washing, starving, and rewashing on the aerobic ATP level of *A. vinelandii* was tested. The data of Table I show that these procedures decreased the level of ATP attainable in the aerobic state very little. All of the experiments described below were run with bacteria which had been harvested, washed and starved.

The aerobic ATP levels of washed, starved cells which were harvested at different times during the growth cycle are shown in Fig. 3. The maximal level is reached in cells approaching the stationary phase. In these washed, starved, stationary phase bacteria there is a low constant rate of respiration with endogenous substrate which is maintained during at least 3 h of aeration at room temperature; the aerobic ATP level also remains constant. Values obtained for the latter varied between 4.8 and 6.2 nmoles ATP/mg dry wt. for many batches of bacteria. On anaerobiosis the ATP level rapidly drops to a steady level around 1/4 of that seen in the presence of O_2 (Fig. 4).

Addition of substrates to the starved cells results in increases in the respiration

rate as large as 10-fold but has little effect on the aerobic ATP levels. Table II lists typical values obtained with a few substrates at 24°. At 12° the respiration rates were less than 1/2 of the values at 24°, but again approximately the same aerobic ATP levels were found. As with many other species of bacteria, the O_2 uptake rates with the substrates tested were linear with time until a very low concentration of O_2 was reached (Fig. 2).

Rates of utilization of ATP were estimated by following the decrease in ATP level after the exhaustion of O_2, when no ATP could be formed by oxidative phosphorylation. Aliquots of bacteria were collected into $HClO_4$ at short intervals immediately before and after O_2 was exhausted; the points of collection are designated on the tracing of Fig. 2. The values obtained are plotted in Fig. 5. The usual high level of ATP was observed in the presence of O_2 with either endogenous substrate or with added DL-β-hydroxybutyrate or DL-lactate. On anaerobiosis the ATP level decreases

TABLE I

THE AEROBIC STEADY-STATE LEVEL OF ATP BEFORE AND AFTER HARVESTING

The bacteria were grown to the late log phase and the ATP content measured before and after harvesting, washing and starving. The conditions of harvesting are given in METHODS. 1-ml samples of bacteria were aerated for 2 min at 24° and 0.2 ml 35% (w/v) $HClO_4$ added from a syringe. ATP in the neutralized $HClO_4$ extract was assayed as described in METHODS. ATP concentrations are expressed as nmoles/mg dry wt.

	ATP concn.	% *Initial concn.*
Before harvesting	5.8	100
After harvesting	5.1	88
After washing	5.5	95
After starving	5.3	91

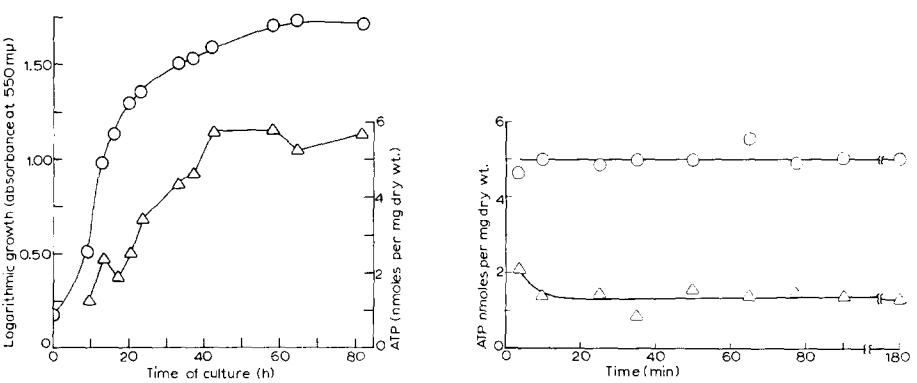

Fig. 3. Variation in the aerobic ATP level during growth. 50- or 25-ml samples were periodically removed, harvested and washed, and resuspended to 5 mg dry wt./ml in 50 mM phosphate buffer (pH 7.4). The aerobic ATP level (—△—) was assayed as described in METHODS, and the growth (—○—) was measured by the absorbance at 550 nm.

Fig. 4. Effect of incubation at 24° on the aerobic and anaerobic ATP pools of harvested, starved and washed cells of *A. vinelandii*. 15 ml bacteria in 50-ml erlenmeyer flasks were stirred aerobically (—○—) and under a N_2 stream (—△—), respectively. 1-ml samples were periodically removed and added to 0.2 ml $HClO_4$, and the ATP content assayed.

TABLE II

VARIATION IN THE AEROBIC STEADY STATE OF ATP WITH ENDOGENOUS AND ADDED SUBSTRATES

ATP concentrations and O_2 uptake were measured at 24° in washed and starved cells as described in METHODS. Added substrates were present at a final concentration of 5 mM. ATP concentrations are expressed as nmoles/mg dry wt. and O_2 uptake as nmoles/min per mg dry wt. Each value is the average of four determinations.

Substrate	*ATP concn.*	*O_2 uptake*
Endogenous	5.1	4.2
DL-Lactate	5.4	40.3
DL-β-Hydroxybutyrate	5.1	10.3
Acetate	5.2	16.4
Pyruvate	5.3	30.6

TABLE III

THE RATE OF ANAEROBIC UTILIZATION OF ATP

The anaerobic utilization of ATP was measured at 12° as described in METHODS. The initial rates of ATP utilization are expressed as nmoles/min per mg dry wt.

Substrate	*ATP utilization*
Endogenous	0.55
DL-Lactate	2.25
DL-β-Hydroxybutyrate	2.05

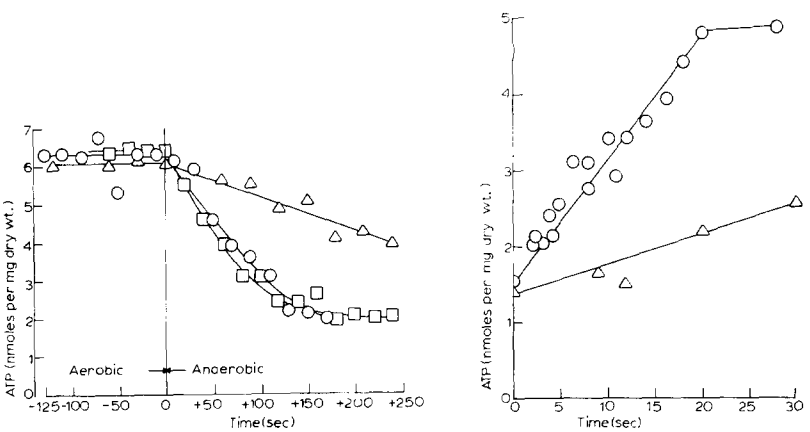

Fig. 5. The anaerobic rate of utilization of ATP at 12°. The ATP pool was assayed at 20- or 30-sec intervals immediately before and after the transition from the aerobic to the anaerobic state as described in the text. (—△—), endogenous substrates; (—○—), 5 mM DL-β-hydroxybutyrate; (—□—), 5 mM DL-lactate.

Fig. 6. Rate of formation of ATP at 12°. 0.5 ml bacteria were mixed with 0.5 ml aerated 50 mM phosphate buffer (pH 7.4) in the apparatus described in METHODS. After various intervals the solutions were injected into 0.2 ml $HClO_4$ and the ATP content measured. (—○—), 5 mM DL-β-hydroxybutyrate; (—△—), endogenous substrates.

slowly in the absence of added substrate, but there is a 4-fold increase in the initial rate of utilization in the presence of either substrate. With a number of different cultures of bacteria the initial rate of ATP utilization was 0.4–0.7, 1.6–2.05 and 1.9–2.3 nmoles/min per mg dry wt. with endogenous substrate, added DL-β-hydroxybutyrate and added DL-lactate, respectively. In one experiment at 23° the rates observed were about 75% higher. The ATP level did not decrease to zero, but levelled off at an ATP level around 2 nmoles/mg dry wt.

The rate of formation of ATP following the addition of O_2 was measured by rapidly mixing aerated 50 mM phosphate buffer (pH 7.4) with an anaerobic suspension of cells that had been starved so that they had a low ATP level. Fig. 6 illustrates the formation of ATP in the presence and absence of DL-β-hydroxybutyrate at 12°. O_2 uptake was measured in a parallel experiment with the same sample of bacteria under the same conditions. It was found that the maximal level of ATP was reached very rapidly in the presence of substrate and that this level was reached well before the O_2 was exhausted. For example, with β-hydroxybutyrate as substrate the maximal ATP level was attained in 15–20 sec at 12°, while the respiration continued for several minutes before the O_2 reached zero. From these data calculations could be made of the corresponding P:O ratios (ratio of the rate of ATP formation:twice the molar rate of O_2 uptake). When the observed rates of respiration in the presence of substrate were corrected by subtraction of the small endogenous respiration rate, the observed values obtained for the P:O ratio in five different experiments were 1.85, 1.65, 1.8, 2.05 and 1.7 with β-hydroxybutyrate as substrate at 12°. If the observed rates of ATP formation were corrected by adding to them the observed corresponding rates of utilization, slightly higher values for the P:O ratios were obtained; the average for the five measurements is 2.0.

DISCUSSION

Our observations with *A. vinelandii* agree with those of HARRISON AND MAITRA[4] on *Klebsiella aerogenes* that a rapid decrease of ATP level follows the exhaustion of O_2 in the suspension; thus the maximal ATP level is only measured when the cells are still aerobic. Keeping this in mind it was found that harvesting, washing and starving *A. vinelandii* had little effect on the aerobic ATP level (Table I). This made it feasible to use the starved bacteria to measure effects following addition of substrates in cells with an apparently unchanged ATP-synthesizing system. *A. vinelandii* seem to differ from *E. coli* in this respect; COLE *et al.*[6] reported that harvested cells had a lower ATP level than those taken directly from the culture medium.

A striking observation with intact *A. vinelandii* is that the ATP level is always high as long as air is present, even in starved cells. Similar observations have been made with *Aerobacter aerogenes*[5]. The aerobic level is increased only slightly (and perhaps not significantly) on addition of substrates to starved cells (Table II). This can be explained by the observation that the rate of synthesis of ATP is so much larger than the rate of utilization, with either endogenous substrate or with added substrate. The ATP level in aerobic growing cultures of *K. aerogenes* was observed to be high and independent of the growth rate and also influenced only little by changes in the rate of respiration[4]. The rate of ATP production in *E. coli* was found to be in balance with the rate of growth under some conditions, and the metabolism of these

bacteria appeared to be geared to the production of an approximately constant ATP pool[6]. Thus the rate of ATP synthesis does not appear to be limiting for growing *K. aerogenes* or *E. coli*. The high aerobic ATP level in a late log phase culture of *A. vinelandii* implies that the same is true for these bacteria in this phase of growth. The lower ATP levels in *A. vinelandii* collected in the early logarithmic phase of growth could result from relatively higher rates of utilization or relatively less total adenine nucleotides. The present data do not distinguish between these two possibilities. Attempts to measure the ADP levels of aerobic bacteria by an enzymatic method[13,14] gave unreliable results, due to the very low levels present. It is interesting that the increases in the aerobic ATP level during growth paralleled increases previously observed in cytochromes and ubiquinone[15].

The similarity of the increased rates of ATP utilization under anaerobic conditions in the presence of added β-hydroxybutyrate or lactate could result from a similar energy requirement for transport of the substrates into the cells. If this is true, then the ATP utilization for transport is an appreciable fraction of the total ATP utilization under these conditions.

The corrected P:O ratios obtained with cells oxidizing β-hydroxybutyrate were near to 2 for several different batches of bacteria. If the rate of ATP utilization is greater aerobically than anaerobically, the P:O ratios would be somewhat higher. Possibly the value of 2 is a reasonable one for these bacteria, since there is evidence of a branched electron transport chain[9], and only one branch may be functional with β-hydroxybutyrate as substrate. The efficiency of oxidative phosphorylation is relatively high in the intact cells and several fold greater than the highest values obtained with broken-cell extracts[12,16]. The lower values obtained with the latter must result from disruption of the phosphorylating system in some way.

On anaerobiosis the ATP level decreases and levels off at around 2 mμmoles/mg dry weight. Some of the cellular ATP could be present in a compartment not rapidly accessible to most of the reactions utilizing ATP. Similar suggestions of compartmentalization have been seen in *Chromatium*[17] and *Rhodospirillum rubrum*[7].

On addition of O_2 to anaerobic cells the ATP level rises rapidly to a maximum, then remains constant while the O_2 uptake continues at a steady rate for several additional minutes until O_2 is exhausted. This seems to imply the absence of tight coupling of phosphorylation to respiration in these bacteria. A continuous production of ADP by the utilization reactions is not a satisfactory explanation, since the ATP could only be regenerated at the same rate as its utilization if there were tight coupling. Under all conditions tested the rate of ATP formation was much greater than its anaerobic rate of utilization. Provided that the aerobic rate of ATP utilization is similar to the measured anaerobic rate, there must be "loose coupling" in these bacteria. The high aerobic ATP levels would seem to rule out appreciable increases in ATP utilization in the presence of air. Other evidences have been cited for a lack of respiratory control in bacteria[4,18].

Measurements with several species of bacteria show a requirement of 1 mole of ATP for the synthesis of 10.5 g dry weight of cells[18,19]. Assuming that a P:O ratio of 2 is appropriate for the oxidation of all substrates by *A. vinelandii*, calculations show that the observed rate of ATP synthesis would be approximately rapid enough to support the measured rate of growth with mannitol as substrate, given the above ATP requirement. An estimate of the rates of ATP synthesis and utilization at differ-

ent times during the growth cycle of *A. vinelandii* would be helpful in understanding the energy balance during growth.

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