Energy Production During Nitrate Respiration by *Aerobacter aerogenes*

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SUMMARY

The molar growth yield of *Aerobacter aerogenes* growing anaerobically with glucose in a mineral medium was almost doubled when NO_3^- was added as hydrogen acceptor. About half a mole of NO_3^- was reduced to NH_4^+ per mole of glucose. The amount of ATP produced from glucose fermentation calculated from the molar growth yield and the acetate production was about 3 moles ATP/mole glucose. The total amount of ATP produced, deduced from the molar growth yield, was used to calculate that about 3 moles ATP were produced per mole of NO_3^- reduced. Comparable experiments with mannitol as carbon source gave the same results. When nitrite was used as hydrogen acceptor the anaerobic molar growth yield was the same or even smaller than in its absence, although the acetate production increased. The results suggest that during NO_2^- reduction no ATP is produced and that the ATP formed during reduction of NO_3^- to NH_4^+ is completely produced during the reduction of NO_3^- to NO_2^- .

INTRODUCTION

The relation between energy production and growth has been studied and discussed by Bauchop & Elsdén (1960), Gunsalus & Shuster (1961) and Senez (1962). Bauchop & Elsdén found that the growth yield per mole of ATP ($= Y^{\text{ATP}}$) was the same for different micro-organisms. Recently Hadjipetrou, Gerrits, Teulings & Stouthamer (1964) found that the yield of *Aerobacter aerogenes* per mole of ATP produced during glucose fermentation was 10.2 g. By measuring the molar growth yield under aerobic conditions and the oxygen uptake during growth the molar growth yield per atom oxygen taken up ($= Y^0$) has been determined. By dividing Y^0 by Y^{ATP} the number of ATP molecules formed per atom oxygen taken up is found. In this way it was found that the P/O ratio is about three. Many facultative aerobic micro-organisms can utilize NO_3^- as ultimate hydrogen acceptor (see review by Nason, 1962). The influence of nitrate on the formation of fermentation products from glucose with *Aerobacter aerogenes* was studied by Forget & Pichinoty (1964). They concluded that under anaerobic conditions in the presence of NO_3^- the citric acid cycle did not function; that the production of hydrogen, ethanol and formate was decreased and the formation of acetate and CO_2 increased. Until now very little has been known about the production of ATP during nitrate reduction in facultative aerobic organisms. There exists some indirect evidence (increased phosphorus turnover in resting organisms during NO_3^- reduction) which suggests that in *Escherichia coli* and *Pseudomonas denitrificans* phosphorylation coupled to

electron transport from substrate to nitrate takes place (Takahaski, Taniguchi & Egami, 1957; Ohnishi & Mori, 1960). Therefore we decided to study the efficiency of nitrate respiration in *Aerobacter aerogenes* as a continuation of our previous aerobic experiments.

METHODS

The minimal medium and the growth conditions were as described previously (Hadjipetrou *et al.* 1964). NO_3^- was determined as described by Middleton (1959), NO_2^- by the method of Rider & Mellon (1946) and NH_2OH by the method of Czaky (1948). Acetate was determined with a purified preparation of acetatekinase as described by Rose, Grunberg-Manago, Korey & Ochoa (1954). Dry weight was estimated by measuring the extinction at 660 $\text{m}\mu$ in an Unicam SP 600 spectrophotometer. From the extinction the dry weight was calculated with the previously determined relation: $\mu\text{g. dry weight bacteria/ml.} = 380 \times \text{extinction at } 660 \text{ m}\mu$ (Hadjipetrou *et al.* 1964).

RESULTS

Influence of nitrate on the molar growth yield with glucose and mannitol

The molar growth yields of *Aerobacter aerogenes* growing: (1) anaerobically, (2) anaerobically with nitrate as hydrogen acceptor and (3) aerobically with glucose are given in Table 1. It is evident that NO_3^- increased the anaerobic molar growth yield with glucose. However, the growth yield was not as high as when O_2 was the

Table 1. *Molar growth yields and acetate production of Aerobacter aerogenes grown under different conditions with glucose as sole carbon source*

The glucose concentration in all experiments was 3 $\mu\text{moles/ml.}$ The nitrate concentration varied but was larger than 1.33 $\mu\text{moles/ml.}$ (see text); yields of organism are given as equivalent dry weight bacteria obtained turbidimetrically; the numbers in parentheses give the number of determinations.

Growth condition	Molar growth yield (g./mole glucose)	Acetate production ($\mu\text{mole}/\mu\text{mole glucose}$)
Anaerobic	26.1 ± 0.5 (17)	0.84 ± 0.04 (15)
Anaerobic + NO_3^-	45.5 ± 0.3 (10)	1.63 ± 0.08 (4)
Aerobic	72.7 ± 1.0 (21)	—

ultimate hydrogen acceptor. In the presence of NO_3^- about the same amount of fermentation occurred as in its absence (Forget & Pichinoty, 1964): pyruvate was decomposed by a thioclastic reaction to acetyl-CoA and formate, then acetyl-CoA converted to acetate by means of phosphate acetyltransferase and acetatekinase. Thus, above the net gain of 2 moles ATP from the conversion of 1 mole glucose to 2 moles pyruvate, an additional mole of ATP is generated for each mole of acetate formed. Ethanol, which is also formed in this fermentation, is produced by reduction of acetyl-CoA (Dawes & Foster, 1956) by the NADH produced during the formation of pyruvate. During this reduction the energy-rich bond present in acetyl-CoA is lost. The gross fermentation balance for this type of fermentation is: glucose $\rightarrow$ 1 acetate + 1 ethanol + 2 formate. Therefore the net gain of ATP from the fermentation can only be calculated when the amount of acetate is known. The amount of acetate produced at the moment of maximal growth is included in Table 1 for the anaerobic cultures growing in the presence or in the absence of NO_3^- . It is

evident from Table 1 that the production of acetate was much higher in the presence of NO_3^- than in its absence; this confirms the results of Forget & Pichinoty (1964). In contrast to their results, however, no pyruvate accumulation from glucose under an aerobic conditions in the presence of nitrate was detected.

Similar experiments were made with mannitol as sole carbon source. Mannitol is more reduced than glucose and one oxidation step is required to convert mannitol to the hexose oxidation level. Therefore the amount of NADH produced during the conversion of mannitol to pyruvate is larger than the amount produced during the formation of pyruvate from glucose. Thus more of the acetyl-CoA is reduced to ethanol than when glucose is the carbon source. The gross fermentation balance is: 1 mannitol $\rightarrow$ 0.5 acetate + 1.5 ethanol + 2 formate. The results of the growth experiments and of the acetate determinations are given in Table 2. The molar growth yield with mannitol under anaerobic conditions was smaller than with glucose; this is in accordance with the smaller acetate production. In the presence of nitrate as hydrogen acceptor the growth yield with mannitol was larger than with glucose.

Table 2. *Molar growth yield of Aerobacter aerogenes grown with mannitol as sole carbon source*

The mannitol concentration was 3 $\mu\text{moles/ml.}$ and the NO_3^- concentration 6 $\mu\text{moles/ml.}$; other data as for Table 1.

Growth condition	Molar growth yield (g./mole)	Acetate production ($\mu\text{mole}/\mu\text{mole}$ mannitol)
Anaerobic	21.8 ± 0.6 (5)	0.42
Anaerobic + NO_3^-	50.6 (3)	1.07

Products of nitrate reduction

To calculate the amount of ATP produced per mole of NO_3^- we should know how much NO_3^- is reduced per mole of glucose. Therefore experiments were performed in which we studied the growth yield with a fixed amount of glucose (3 $\mu\text{moles/ml.}$) and different amounts of nitrate (from 0 to 35 $\mu\text{moles/ml.}$). The growth yield increased with NO_3^- concentrations between 0 and 1.33 $\mu\text{moles/ml.}$, but did not change when higher nitrate concentrations were used. This suggests that at least $1.33/3 = 0.44$ $\mu\text{mole NO}_3^-$ per μmole glucose was necessary to increase the molar growth yield from the anaerobic value to the maximum value for anaerobic growth in the presence of NO_3^- . Chemical estimation of nitrate revealed that indeed 0.44 μmole nitrate was reduced per μmole glucose at the moment of maximal growth. The amount of nitrite produced from this amount of nitrate was very small: only 0.06 $\mu\text{mole}/\mu\text{mole}$ glucose. In similar experiments with mannitol it was found that 0.93 $\mu\text{mole}/\text{NO}_3^-/\mu\text{mole}$ mannitol were needed to obtain the maximum molar growth yield for anaerobic growth in the presence of NO_3^- . The amount of nitrite produced in this experiment was 0.42 $\mu\text{mole}/\mu\text{mole}$ mannitol.

These results suggest that nitrate was reduced beyond the nitrite stage; this was confirmed by the observation that larger amounts of nitrite were present before maximal growth was obtained. In this aspect our strain differs from the one used by Forget & Pichinoty (1964) because their strain reduced NO_3^- only to NO_2^- .

In the supernatant fluid no hydroxylamine was detected and, as expected, NO_3^- was not reduced to such gaseous products as N_2 or N_2O . That NO_3^- was reduced to

NH_4^+ was confirmed by observing that our *Aerobacter aerogenes* grew in a minimal medium with NO_3^- as sole nitrogen source. The molar growth yields are given in Table 3. Under aerobic conditions the molar growth yield with NO_3^- as sole nitrogen source was much smaller than with NH_4^+ as nitrogen source; a similar observation was made by Pichinoty (1960). Under anaerobic conditions nearly the same yield of organism was obtained in a medium with NH_4^+ as N-source and NO_3^- as hydrogen acceptor, as in the medium with NO_3^- as combined nitrogen source and hydrogen acceptor. These results confirm that NO_3^- is reduced to NH_4^+ .

Table 3. *Molar growth yields of Aerobacter aerogenes grown with NO_3^- as sole nitrogen source and glucose as sole carbon source*

The glucose concentration in these experiments was $1.5 \mu\text{moles/ml.}$; nitrate concentration $35 \mu\text{moles/ml.}$ Other data as for Table 1.

Growth conditions	Molar growth yield (g./mole glucose)
Aerobic	52
Anaerobic	43

Table 4. *Molar growth yield and acetate production of Aerobacter aerogenes grown anaerobically with glucose as sole carbon source and with various concentrations of nitrite as hydrogen acceptor*

The glucose concentration in these experiments was $3 \mu\text{moles/ml.}$ Other data as for Table 1.

Nitrite concentration ($\mu\text{moles/ml.}$)	Molar growth yield (g./mole glucose)	Acetate production $\mu\text{moles}/\mu\text{mole glucose}$
0	26.1	0.84
0.5	27	1.2
1	24.5	1.58
1.5	22.4	1.6
2.6	20.6	Not determined
5	16	Not determined
10	No growth	

The influence of nitrite on the molar growth yield with glucose

The previous results suggested that the production of some ATP by our *Aerobacter aerogenes* was coupled to the reduction of NO_3^- to NH_4^+ . To know to which step in this reduction the ATP production was coupled, experiments were made in which NO_2^- was used as ultimate hydrogen acceptor. NO_2^- was very toxic to the bacteria, especially when there was no other hydrogen acceptor (O_2 , NO_3^-) present. A similar phenomenon was observed for *A. aerogenes* by Lewis & Hinshelwood (1948). The growth yield and the acetate production with different amounts of nitrite are given in Table 4; acetate production was increased in presence of NO_2^- indicating that it indeed acted as hydrogen acceptor. About $0.8 \mu\text{mole NO}_2^-$ were reduced per $\mu\text{mole glucose}$, independent of the initial NO_2^- concentration. Although the acetate production increased, the molar growth yields were similar to or smaller than those obtained in the absence of NO_2^- and decreased as the NO_2^- concentration was increased. This effect was undoubtedly due to growth inhibition by NO_2^- . In experiments in which NO_2^- was added to a complete medium with NH_4^+ as N-

source and the organisms were grown under aerobic conditions, we also found a lower growth yield. A similar decrease in growth yield was obtained when, under anaerobic conditions, a mixture of 35 $\mu\text{moles NO}_3^-$ and 0.5 $\mu\text{mole NO}_2^-$ was used as hydrogen acceptor. Our strain could also utilize NO_2^- as sole N-source. The yields of organisms are given in Table 5; they are much smaller than when NO_3^- was used as sole N-source (compare Table 3). In all these experiments the growth rate was decreased by the presence of NO_2^- . The results indicate that the growth yield was decreased at lower growth rates.

Table 5. *Molar growth yield of Aerobacter aerogenes grown with NO_2^- as sole nitrogen source and glucose as sole carbon source*

The NO_2^- concentration in both experiments was 5 $\mu\text{moles/ml.}$; glucose concentration 3 $\mu\text{moles/ml.}$

Growth condition	Molar growth yield (g./mole glucose)
Aerobic	39
Anaerobic	16

DISCUSSION

Y^{ATP} was calculated from the results of Table 1 (Hadjipetrou *et al.* 1964) to be $Y^{\text{ATP}}_{\text{glucose}} = 10.2$. This value was used to calculate the ATP yield from growth yields under anaerobic conditions in the presence of nitrate, by dividing it into the molar growth yield with nitrate. In this way we find that $45.5/10.2 = 4.46$ moles ATP were produced. Hadjipetrou *et al.* (1964) showed that the carbon content of the *Aerobacter aerogenes* is 40 %, hence 45.5 g. glucose were converted to cell material and $180 - 45.5 = 134.5$ g. were fermented. As the net gain of ATP/mole glucose transformed to pyruvate is 2 moles and as one extra mole is formed per mole of acetate, the amount of ATP formed during fermentation of one mole of glucose was $2(134.5/180) + 1.63 = 3.12$ moles of ATP. From the growth yield we find that the total production of ATP was 4.46 moles, therefore $4.46 - 3.12 = 1.34$ moles of ATP must have been produced by phosphorylation coupled to nitrate reduction. The amount of nitrate used as hydrogen acceptor during the fermentation of glucose was 0.44 $\mu\text{mole}/\mu\text{mole glucose}$. Thus the reduction of 1 mole of NO_3^- gave $(1/0.44)1.34 = 3.0$ moles of ATP.

From the growth and acetate yields after anaerobic growth with mannitol, we can calculate that $Y^{\text{ATP}}_{\text{mannitol}} = 10.0$. This is in good agreement with $Y^{\text{ATP}}_{\text{glucose}} (10.2)$ and $Y^{\text{ATP}}_{\text{fructose}} (10.6)$ determined by Hadjipetrou *et al.* (1964). The molar growth yield for anaerobic growth in the presence of NO_3^- was larger with mannitol than with glucose. In the same way as for glucose we can calculate that during anaerobic growth with mannitol 2.46 moles of ATP must have been produced by phosphorylation coupled to nitrate reduction, which is much more than when glucose is the growth substrate. As mannitol is more reduced than glucose more NO_3^- is needed per mole of mannitol (0.93 mole $\text{NO}_3^-/\text{mole mannitol}$). From the mannitol results we find that $2.46/0.93 = 2.6$ moles of ATP are produced per mole of NO_3^- , which is in good agreement with the value of 3.0 found for glucose.

The growth with NO_3^- as sole nitrogen source shows that NO_3^- is reduced to NH_4^+ . It is likely that ATP production is not coupled to the reduction of NO_2^- to NH_4^+ , since no increased growth yield was obtained with NO_2^- as a hydrogen

acceptor (Table 5). That NO_2^- acts as a hydrogen acceptor follows from the increase in acetate production. This increase implies that an increase in growth yield might have been expected. Instead, the molar growth yields were similar to or even smaller than those obtained in the absence of NO_2^- . The explanation for this phenomenon is that in the presence of nitrite the growth rate is decreased by the growth inhibitory properties of NO_2^- . When the growth rate is decreased part of the ATP may be dissipated, which explains the lowered molar growth yields.

Thus it is most likely that the formation of three ATP molecules is coupled to the reduction of NO_3^- to NO_2^- , indicating that the hydrogen from NADH produced during fermentation is transferred to nitrate in several steps and that cytochromes are involved. Several schemes in which cytochromes are involved in nitrate reduction were given by Nason (1962). Nitrite and the other intermediates of nitrate reduction were most probably reduced directly by NADH. Several such enzymes have been reported present in a number of micro-organisms (Nason, 1962).

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