**Influence of Nitrate on Fermentation Pattern,
Molar Growth Yields and Synthesis of Cytochrome *b* in
*Propionibacterium pentosaceum***

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SUMMARY

Under anaerobic conditions, *Propionibacterium pentosaceum* reduces nitrate to nitrite until nitrate is exhausted from the medium, when nitrite is converted into N_2 or N_2O . In the presence of nitrate, fermentation patterns for lactate, glycerol and pyruvate were different from those obtained during anaerobic growth without an inorganic electron acceptor. In the presence of these substrates, a drastic decrease in propionate formation was observed, some pyruvate accumulated during growth with lactate, and acetate was produced from glycerol. Acetate production from lactate and pyruvate was not influenced by the presence of nitrate. Furthermore, CO_2 was produced by citric acid cycle activity. The fermentation pattern during nitrite reduction resembled that of *P. pentosaceum* grown anaerobically without an inorganic electron acceptor. Nitrite has a toxic effect, since bacteria inoculated into a medium with 9 mM-nitrite failed to grow.

The cytochrome spectrum of anaerobically grown *P. pentosaceum* was similar with and without nitrate. In membrane fractions of bacteria grown anaerobically with nitrate, cytochrome *b* functioned in the transfer of electrons from lactate, glycerol 1-phosphate and NADH to nitrate. Molar growth yields were increased in the presence of nitrate, indicating an increased production of ATP. This could be explained by citric acid cycle activity, and by oxidative phosphorylation coupled to nitrate reduction. Assuming that 1 mol ATP is formed in the electron transfer from lactate or glycerol 1-phosphate to nitrate, and that 2 mol ATP are formed in the electron transfer from NADH to nitrate, Y_{ATP} values (g dry wt bacteria/mol ATP) were obtained of between 5.0 and 12.6. The higher Y_{ATP} values were similar to those obtained during anaerobic growth without an inorganic electron acceptor. This supports the assumptions about the efficiency of oxidative phosphorylation for electron transport to nitrate. Low Y_{ATP} values were found when high concentrations of nitrite (15 to 50 mM) accumulated, and were probably due to the toxic effect of nitrite.

INTRODUCTION

Nitrate respiration is a widespread property of bacteria (Payne, 1973; Pichinoty, 1973) and even in anaerobic bacteria such as *Veillonella alcalescens* (Inderlied & Delwiche, 1973; de Vries, van Wijck-Kapteyn & Oosterhuis, 1974), *Selenomonas ruminantium* (de Vries *et al.* 1974) and *Clostridium perfringens* (Ishimoto, Umeyama & Chiba, 1974), nitrate may serve as terminal electron acceptor in anaerobic respiration. In most cases, cytochromes are involved in nitrate reduction (Payne, 1973; Pichinoty, 1973). Cytochromes function in electron transport to fumarate in a number of anaerobic bacteria which form propionate (or succinate) via the succinate pathway. Such bacteria include *Bacteroides* spp. (White,

Bryant & Caldwell, 1962; Rizza *et al.* 1968), *Vibrio succinogenes* (Jacobs & Wolin, 1963), propionic acid bacteria (Chaix & Fromageot, 1942; de Vries, van Wijck-Kapteyn & Stouthamer, 1972; Sone, 1972), *Desulfovibrio gigas* (Hatchikian & Le Gall, 1972), and *V. alcalescens*, *S. ruminantium* and *Anaerovibrio lipolytica* (de Vries *et al.* 1974). Cytochromes are involved in anaerobic electron transport to nitrate in *V. alcalescens* and *S. ruminantium* (de Vries *et al.* 1974), while reduced ferredoxin is the electron donor for nitrate reduction in *C. perfringens* (Chiba & Ishimoto, 1973).

Denitrification is less widespread among bacteria (Payne, 1973; Pichinoty, 1973). The only anaerobe in which it has been observed is *Propionibacterium pentosaceum* (Bergey's *Manual of Determinative Bacteriology*, 1957). de Vries *et al.* (1972) investigated the influence of oxygen on growth, fermentation pattern and cytochrome synthesis in propionic acid bacteria. They found that fermentation does not occur in the presence of air, when these bacteria presumably obtain energy by oxidative phosphorylation with oxygen as electron acceptor although cytochrome synthesis is repressed by oxygen. In the present investigation the influence of denitrification on fermentation pattern and molar growth yields in *P. pentosaceum* was studied in order to determine whether energy is generated by electron transport to nitrate and nitrite. The influence of nitrate on cytochrome synthesis and the possible role of cytochrome *b* in nitrate reduction were also investigated.

METHODS

Organism and growth conditions. *Propionibacterium pentosaceum* (de Vries *et al.* 1972) was used throughout.

Absorption spectra and reduction kinetics of cytochrome *b* were measured on bacteria grown in a complex medium containing (per l deionized water): tryptone, 5 g; yeast extract, 10 g; KH_2PO_4 , 250 mg; MnSO_4 , 5 mg; pH 6.5. Sodium DL-lactate or glycerol were added as required. KNO_3 was added to a concentration of 0.5 or 1 % (w/v).

Growth experiments were performed in a semi-synthetic medium containing (per l deionized water): casein hydrolysate, 5 g; K_2HPO_4 , 3.75 g; KH_2PO_4 , 3.75 g; tryptophan, 100 mg; L-cysteine, 5 mg; sodium thioglycollate, 20 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 325 mg; CaCO_3 , 10 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 22.6 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 7.2 mg; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 5.6 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.2 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1.2 mg; H_3BO_3 , 0.3 mg; adenine, guanine, uracil and xanthine, each 0.5 mg; biotin, 0.14 mg; thiamin, 0.2 mg; riboflavin, 0.2 mg; pyridoxin, 0.4 mg; calcium pantothenate, 0.2 mg; nicotinic acid, 0.2 mg; *p*-aminobenzoic acid, 0.02 mg; pH 6.5, together with growth-limiting concentrations of sodium DL-lactate (up to 25 mM), glycerol (up to 5 mM) or sodium pyruvate (up to 20 mM). Pyruvate was sterilized by filtration. KNO_3 was added as required.

Small volumes of cultures were grown at 30 °C under 95 % N_2 + 5 % CO_2 in stainless steel anaerobic jars manufactured in our laboratory. Larger volumes were grown in a Chemap Vibro-glass fermenter (Chemap AG, Männedorf ZH, Switzerland) at 30 °C under a stream of 95 % N_2 + 5 % CO_2 . In cultures in the complex medium containing glycerol the pH was controlled at 6.5 (de Vries, van Wijck-Kapteyn & Stouthamer, 1973). Under other growth conditions pH control was unnecessary since variations were less than 0.1 unit. Growth was estimated turbidimetrically at 660 nm in 1 cm cuvettes. Bacterial suspensions and membrane fractions were prepared from cultures harvested at an extinction between 0.45 and 1.0. Dry weights were measured by membrane filtration as described by de Vries & Stouthamer (1968).

Determination of lactate, pyruvate, fermentation products and nitrite in supernatant fluids. Total lactate, succinate and propionate were measured by gas-liquid chromatography using

a Hewlett Packard gas chromatograph model 5750G (Hewlett Packard GmbH, Böblingen, Germany) fitted with a flame ionization detector and with N_2 (35 ml/min) as the carrier gas. For measuring lactate and succinate a $6\text{ ft} \times \frac{1}{8}$ in stainless-steel column was used packed with Resoflex LAC-I-R-296 (Burrell Corp., Pittsburg, Pennsylvania, U.S.A.) at a temperature of 130°C . Lactate and succinate were measured as the methyl derivatives, prepared as described in the *Anaerobe Laboratory Manual* (1972). Propionate was measured with the column described above (temperature 135°C) or with a $6\text{ ft} \times \frac{1}{16}$ in glass column (WEBOCO, Zevenaar, Holland) packed with Chromosorb 101 (80/100 mesh; Becker Delft NV, The Netherlands) using a glass injection port and a temperature of 150°C . Propionate was measured by direct injection of culture supernatants into the columns. With the glass column, propionate was completely separated from acetate. The lactate, succinate and propionate contents were calculated from the peak heights and reference to standards. Acetate was determined by the enzymic method of Rose *et al.* (1954), which is based on the colorimetric determination of acetyl phosphate according to Lipmann & Tuttle (1945). L-Lactate was measured enzymically by the method of Hohorst (1970) and D-lactate by the enzymic method of Gahwehn & Bergmeyer (1970). Pyruvate was measured enzymically with lactate dehydrogenase in a reaction mixture containing: tris buffer pH 7.4, 40 mM; $MgCl_2$, 4 mM; NADH, 0.36 mM. Nitrite was measured as described by van 't Riet, Stout-hamer & Planta (1968).

Measurement of carbon dioxide evolution and gas production from nitrate. These were measured by Warburg manometry.

CO_2 evolution was measured in 75 ml capacity Warburg flasks with cultures growing in semi-synthetic medium under N_2/CO_2 at 30°C . Conditions were chosen so that no gas was produced from nitrate (see Results). The pH of the buffered medium remained constant throughout the experiment.

For measuring gas evolution from nitrate, the CO_2 evolved was absorbed by 0.2 ml of a 10% (w/v) KOH solution in the centre well of a 25 ml capacity Warburg flask at 30°C . It was necessary to use pure N_2 instead of the N_2/CO_2 gas mixture for anaerobiosis in these experiments. Since growth under N_2 was very slow in the semi-synthetic medium, a complex medium was used.

During both types of Warburg experiment, samples were taken for the measurement of the extinction and the determination of fermentation products and nitrite.

Preparation of bacterial suspensions and membrane fractions. For the preparation of cell suspensions, bacteria were harvested at 4°C , washed twice with a chilled 0.067 M-phosphate buffer pH 6.8 and resuspended in this buffer to about 15 to 45 mg dry wt bacteria/ml. Membrane fractions were prepared as described by de Vries *et al.* (1972, 1973). Protein was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Measurement of difference spectra and reduction kinetics of cytochrome b. Reduced minus oxidized difference spectra of bacterial suspensions were recorded with an Aminco Chance spectrophotometer (American Instruments Co., Inc.). Cytochrome *b* was estimated from dithionite-reduced minus oxygen-oxidized difference spectra between 400 and 700 nm using $\epsilon = 17500\text{ l/mol/cm}$ (Deeb & Hager, 1964).

The reduction of cytochrome *b* by membrane suspensions was followed with an Aminco Chance spectrophotometer set in the dual-wavelength position using 562 and 578 nm as the sample and reference wavelengths, respectively. To 3 ml (or 1.5 ml) of the membrane suspension (2 to 5 mg protein/ml), oxidized with air, was added 50 μl (or 25 μl) of 0.2 M-NADH, 0.5 M L-lactate, glycerol 1-phosphate or pyruvate, or potassium nitrate.

Fig. 1. Nitrite and gas production in cultures of *P. pentosaceum* growing anaerobically in the presence of 25 mM-lactate and 2.5 mM-KNO₃. Each Warburg flask contained 3 ml of culture and KOH in the centre well. At regular intervals the culture from one flask was harvested for the determination of the nitrite content in the supernatant fluid. Gas evolution was calculated from the mean values of four flasks on the assumption that N₂ was formed; values for NO would be about 2% higher and for N₂O 9% higher.

Chemicals. Tryptone and casein hydrolysate were obtained from Oxoid, and yeast extract from Difco. Sodium DL-lactate solution containing 63% L-lactate and 7% D-lactate was obtained from BDH. Glycerol, sodium pyruvate, potassium nitrate and sodium nitrite were obtained from Merck. NADH, NAD and all the enzymes used were obtained from Boehringer, Mannheim, Germany. Sodium DL-glycerol 1-phosphate and sodium L-lactate were obtained from Serva, Entwicklungslabor, Heidelberg, Germany. 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) was obtained from Sigma.

RESULTS

Strain and medium

One out of three strains of *P. pentosaceum* tested appeared able to reduce nitrate to nitrite and, when nitrate was exhausted, to reduce nitrite further.

In the complex medium, without an additional energy source and without nitrate, bacteria grew anaerobically to an extinction of 0.39 and propionate (6.3 mM) accumulated. In the

Fig. 2. Influence of nitrate reduction on lactate metabolism of *P. pentosaceum*. To a culture growing anaerobically with 25 mM-lactate, KNO_3 was added to a final concentration of 10 mM when the culture density (E_{660}) reached 0.25. x, Growth; +, lactate; ▲, acetate; △, propionate; ●, pyruvate; ○, nitrite.

presence of nitrate (0.5% KNO_3) an extinction of 1.1 was reached, a large amount of nitrite accumulated (24.6 mM), and acetate (4.9 mM) and propionate (0.9 mM) were formed. The complex medium, which was used in an earlier study (de Vries *et al.* 1972), contained fermentable and nitrate-oxidizable organic compounds such as lactate (approx. 6 mM lactate was detected); these disadvantages made it unsuitable for the present study of nitrate reduction in *P. pentosaceum*. In the semi-synthetic medium without added energy source, very low extinctions of 0.05 and 0.12 were reached in the absence and presence of nitrate (0.5% KNO_3) respectively, and furthermore little nitrite (2.5 mM) was produced in the presence of nitrate. Evidently, the semi-synthetic medium contained only a few fermentable and nitrate-oxidizable organic compounds, and it was used for growth experiments. Since growth on this medium with pure N_2 for anaerobiosis was very slow, 95% N_2 + 5% CO_2 was used. In this medium, with lactate as energy source, the specific growth rate was about 0.1 h^{-1} and was not influenced by the presence or absence of nitrate.

Reduction of nitrate to nitrite and further reduction of nitrite

A typical experiment in which a small amount of nitrate was used is shown in Fig. 1. After about 4 h, nitrite production was maximal with 85% of the added nitrate recovered as nitrite. Afterwards, nitrite disappeared and the rate of gas production increased. Since 1 mol gas was formed from 2 mol nitrite, the gas formed was either N_2 or N_2O .

Influence of nitrate on fermentation pattern

Nitrate was added to cultures containing lactate and fermentation products and nitrite, were determined at intervals. The rate of nitrite production was slow initially, but gradually

Table 1. Fermentation balances and molar growth yields of *P. pentosaceum* grown on semi-synthetic medium with excess nitrate

Data line	Substrate	Concn* (mm)	E_{660} at harvesting	$Y_{\text{substrate}}$ (g bacteria/mol)	Carbon recovery† (%)	Products (mol/mol substrate)				Nitrite		Y_{ATP} (g bacteria/mol ATP) for P/NO_3 § of:	
						Acetate	Propionate	Succinate	Pyruvate	mol/mol substrate	mm	0	1 or 2
1	Lactate	†	—	12.9	107	0.33	0.74	—	—	—	—	—	—
2		25 (A)	0.78	13.0	80	0.18	0.09	—	0.53	1.22	29.2	36.1	7.6
3		25 (B)	1.03	17.7	80	0.32	0.10	—	0.38	2.00	47.0	34.1	5.0
4		15 (B)	0.74	22.0	79	0.55	0.15	—	0.09	1.50	21.7	25.9	7.7
5		10 (A)	0.42	22.2	76	0.25	0.11	—	0.40	1.21	11.1	47.2	11.7
6		10 (B)	0.69	27.2	53	0.30	0.10	—	0.13	1.66	18.5	54.4	9.6
7		6 (C)	0.52	36.7	49	0.42	0.07	—	—	1.78	11.7	65.7	11.8
8		6 (C)	0.63	44.5	43	0.34	0.09	—	—	2.72	17.3	85.8	9.0
9		6 (C)	0.75	52.0	33	0.33	—	—	—	3.42	21.5	154.0	8.4
10	Glycerol	†	—	26.3	95	—	0.95	—	—	—	—	—	—
11		5 (C)	0.64	62.0	84	0.58	0.20	0.06	—	3.16	16.8	28.2	8.4
12		5 (C)	0.66	66.2	76	0.46	0.26	0.04	—	3.70	19.3	32.1	7.8
13		3 (C)	0.51	75.0	46	0.40	0.06	—	—	2.70	9.1	48.6	12.6
14		3 (D)	0.53	81.0	38	0.38	—	—	—	3.58	11.7	58.8	10.7
15	Pyruvate	20 (D)	0.94	35.1	43	0.35	0.08	—	—	2.26	46.3	68.9	7.0
16		20 (D)	0.96	35.5	36	0.32	0.04	—	—	2.35	48.0	88.9	7.0
17		6 (C)	0.57	42.0	25	0.17	0.08	—	—	2.24	14.4	127.0	8.7

* For explanation of (A), (B), (C) and (D), see Results.

† Growth was obtained in complex medium in the absence of nitrate (de Vries *et al.* 1973).‡ For the calculation of carbon recoveries the amount of CO_2 formed was estimated from the amount of acetate formed.

§ See Discussion.

Fig. 3. Accumulation and degradation of pyruvate in a culture of *P. pentosaceum* growing with 25 mM-lactate and 1% KNO_3 . For symbols, see Fig. 2.

increased (Fig. 2). Acetate production from lactate was not influenced by the addition of nitrate. Propionate formation diminished and finally ceased while some pyruvate accumulated. No succinate production was detected. At the end of the experiment (10 h after the addition of $10 \mu\text{mol}$ nitrate/ml), 14.7 mM-lactate were converted into 3.4 mM-acetate, 4.2 mM-propionate and 3.3 mM-pyruvate, accompanied by the formation of 10 mM-nitrite. Thus, when high concentrations of nitrate were used, all the nitrate added was converted into nitrite. Assuming that the production of one mole of acetate was accompanied by the formation of one mole of CO_2 , the carbon recovery was 74%.

Since the results showed that fermentation continued for several hours after the addition of nitrate, in subsequent experiments medium containing nitrate was inoculated with cultures grown in the presence of nitrate. To prevent nitrite reduction, an excess of nitrate (final concn 0.5 or 1.0%, w/v, KNO_3) was added.

Fermentation balances and molar growth yields during nitrate reduction

Fermentation balances and molar growth yields for growth of *P. pentosaceum* on lactate, glycerol or pyruvate were determined in the presence of an excess of nitrate (Table 1). For comparison, this Table also contains fermentation balances and molar growth yields for growth with lactate and glycerol in the absence of nitrate (de Vries *et al.* 1973). In cultures growing with lactate (Fig. 3) pyruvate accumulated initially but was degraded when lactate was almost exhausted from the medium, and growth continued at a slower rate. Even when pyruvate had been completely consumed growth continued slowly. In media containing lactate, fermentation balances were determined (see Table 1) at the moment at which lactate was exhausted (A), during growth on the accumulated pyruvate (B) and when pyruvate was exhausted (C). In media containing glycerol and pyruvate, fermentation balances were determined when growth was very slow (C) or had ceased (D). No pyruvate could be

Table 2. *CO₂ production and acetate formation during growth of P. pentosaceum*

Growth (<i>E</i> ₆₈₀)	Acetate (μ mol/ml culture)	CO ₂ (μ mol/ml culture)	Ratio CO ₂ /acetate (mol/mol)	Carbon recovery (%)	
				(A)	(B)
0.085–0.68	4.7	12.7	2.7	69	80
0.74–0.80	1.4	4.6	3.3	55	97

Each Warburg flask contained 20 ml of culture growing with 25 mM-lactate and 1% (w/v) KNO₃. In the first experiment, lactate was used as energy source. In the second experiment, lactate was exhausted from the medium and growth continued on the pyruvate accumulated. Carbon recoveries were calculated (A) without and (B) with the CO₂ produced in excess of acetate.

detected at the moment of harvesting. In all cases, corrections were made for growth on the medium with added nitrate but without added substrate.

From Table 1, it appears that nitrate increased the molar growth yields for both lactate and glycerol. Furthermore, it is evident that the amount of propionate formed per mole of substrate was much less during growth with nitrate. In general, the carbon recovery with lactate and glycerol was about 80% when bacteria were harvested in the early stages of growth. However, when bacteria were harvested later, molar growth yields were higher but the carbon recoveries became less satisfactory. For the calculation of carbon recoveries, it was always assumed that equal quantities of acetate and CO₂ were produced. Furthermore, considering any one substrate concentration, it is apparent that nitrite production (mol/mol substrate) was higher with harvesting later during growth.

One reason for the low carbon recoveries might be the excretion of one or more metabolic intermediates. However, in extensive experiments we could not detect any such metabolic product. Another reason might be CO₂ production by citric acid cycle activity (Delwiche & Carson, 1953; de Vries, unpublished results). In Warburg experiments it was shown that at the end of growth CO₂ production continued at a constant rate whereas acetate production diminished. In growing bacteria CO₂ evolution exceeded acetate production (Table 2), which indicates that CO₂ is indeed produced by citric acid cycle activity. Improved results are achieved by calculating carbon recoveries including the CO₂ produced in excess of acetate. Hence, CO₂ evolution by citric acid cycle activity accounts (at least in part) for the low carbon recoveries in our experiments. The conclusion that CO₂ is produced by citric acid cycle activity is further strengthened by the observation that in general a small decrease in the sum of the amounts of acetate and pyruvate occurred during longer incubation. Furthermore, the nitrite production per mole of substrate converted was higher than the theoretical amount required to convert lactate (2), glycerol (3) and pyruvate (1) into acetate. Therefore, citric acid cycle activity might explain the high molar growth yields and nitrite production when harvesting at the end of growth.

Influence of nitrite reduction on fermentation pattern

With bacteria grown in the presence of limited amounts of nitrate (7 to 12 mM), growth continued after nitrate had been completely converted into nitrite, and nitrite was reduced slowly. Pyruvate, accumulated during nitrate reduction in a medium containing lactate, remained constant and the rate of acetate production did not change. The most important observation during nitrite reduction was that propionate production, which had stopped during nitrate reduction, started again. Thus during nitrite reduction the fermentation

Table 3. *Influence of nitrate on cytochrome b synthesis of P. pentosaceum*

Growth conditions	Substrate	Cytochrome <i>b</i> content ($\mu\text{mol/g}$ dry wt bacteria*)
Anaerobic†	Lactate	0.25
Aerobic†	Lactate	0.05
Anaerobic + 0.5% (w/v) KNO_3	Lactate	0.11–0.19
Anaerobic + 1% (w/v) KNO_3	Glycerol	0.10–0.18

* Dry weights of nitrate-grown cells were calculated from the relationship:

$$\text{dry weight (g/ml)} = 0.43 \times 10^{-3} E_{680}.$$

† de Vries *et al.* (1972).

pattern resembled that of *P. pentosaceum* grown anaerobically without nitrate (de Vries *et al.* 1973).

Influence of nitrate on cytochrome synthesis

After anaerobic growth with excess nitrate, reduced minus oxidized difference spectra of *P. pentosaceum* were essentially the same as those of anaerobically grown *P. freudenreichii* (de Vries *et al.* 1972), with cytochrome *b* (α -peak at 562 nm, β -peak at 532 nm, Soret peak at 429 nm), cytochrome *a* or a_1 (α -peak at 606 nm), flavoproteins (trough at 460 nm) and sometimes cytochrome a_2 (α -peak at 630 nm) present.

The contents of cytochrome *b* were measured in bacteria grown anaerobically with excess nitrate, plus lactate or glycerol (Table 3). The cytochrome *b* content of *P. pentosaceum* after anaerobic growth with lactate was less when nitrate was present than in its absence. However, no drastic decrease of cytochrome *b* content in nitrate-grown bacteria occurred such as was found after aerobic growth by de Vries *et al.* (1972).

Reduction kinetics of cytochrome b in membrane fractions

Dual-wavelength experiments showed that membrane suspensions from *P. pentosaceum* grown with lactate and nitrate reduced cytochrome *b* in the presence of L-lactate and NADH, but not in the presence of pyruvate. After addition of NADH to membrane suspensions oxidized with air (Fig. 4, I), the 'aerobic steady state' (40% reduction of cytochrome *b* as compared with reduction by dithionite) was attained within 40 s. When oxygen was exhausted from the suspension an 'anaerobic steady state' (78% reduction of cytochrome *b*) was reached at about 11 min after addition of NADH. Nitrate oxidized cytochrome *b* to a steady state of 44% reduction. A second steady state (22% reduction) was reached 9 min after addition of nitrate, due to exhaustion of NADH from the suspension. In the presence of HOQNO, an inhibitor of cytochrome *b* function (Cox *et al.* 1970), both the aerobic steady state and the steady state with nitrate were prolonged (Fig. 4, II). L-Lactate reduced cytochrome *b* more slowly than NADH (Fig. 4, III); consequently, the aerobic steady state (31% reduction of cytochrome *b*) lasted longer and more time was needed before the anaerobic steady state (71% reduction of cytochrome *b*) was reached. Nitrate oxidized cytochrome *b* to the same level as during the aerobic steady state.

Membrane suspensions from *P. pentosaceum*, grown with glycerol and nitrate, reduced cytochrome *b* in the presence of glycerol 1-phosphate (Fig. 4, IV) and NADH. Glycerol 1-phosphate reduced cytochrome *b* to 72%, and nitrate oxidized cytochrome *b* to a steady state value of 48% reduction.

Fig. 4. Reduction of cytochrome *b* with L-lactate (III) and NADH in the absence (I) and presence (II) of $2.5 \mu\text{M}$ -HOQNO by a crude membrane fraction (2.1 mg protein/ml) from *P. pentosaceum*, grown with 0.15% (w/v) lactate and 0.5% (w/v) KNO_3 . Reduction of cytochrome *b* with glycerol 1-phosphate (IV) by a crude membrane fraction (3.4 mg protein/ml) from *P. pentosaceum*, grown with 0.17% (w/v) glycerol and 1% (w/v) KNO_3 . Re-oxidation of cytochrome *b* by nitrate.

DISCUSSION

Denitrification has been demonstrated in *P. pentosaceum*. Nitrate interferes, just as does oxygen (de Vries *et al.* 1972), with the normal propionic acid fermentation, and acetate is the main end product during nitrate reduction. In addition, a large amount of CO_2 is formed by citric acid cycle activity. During the reduction of nitrite, however, the propionic acid fermentation occurs normally. Oxygen represses the synthesis of cytochrome *b* in propionibacteria (de Vries *et al.* 1972) and this is the principal reason for the anaerobic character of these bacteria. This effect is not shown by nitrate, although some reduction in the cytochrome *b* content does occur.

The influence of nitrate on molar growth yields has been studied in a number of facultative bacteria. In *Aerobacter aerogenes* (Hadjipetrou & Stouthamer, 1965), *Proteus mirabilis* (Stouthamer & Bettenhausen, 1972) and *Citrobacter freundii* (Kaprálék, 1972) molar growth yields were increased in the presence of nitrate. From these results it was concluded that nitrate reduction is accompanied by oxidative phosphorylation. A similar conclusion can be drawn from this study with *P. pentosaceum*.

Fermentation patterns have been proposed from previous yield studies with propionibacteria growing anaerobically with various substrates in the absence of nitrate (de Vries *et al.* 1973). In these schemes it was postulated that 1 mol ATP is formed in the transfer of an electron pair from lactate and glycerol 1-phosphate to fumarate ($P/2e = 1$, where *P* is ATP, and $2e$ an electron pair) and that 2 mol ATP are formed in the electron transport from NADH to fumarate ($P/2e = 2$). This was based on the finding that in propionibacteria lactate and glycerolphosphate are oxidized by flavoproteins. Consequently in the electron transfer to cytochrome *b*, which is the ultimate electron donor for fumarate reduction, more phosphorylation sites may be present between NADH and

cytochrome *b* than between reduced flavins and cytochrome *b*. These assumptions are supported by the observation that the Y_{ATP} values (g dry wt bacteria/mol ATP) for each organism were similar for growth with different substrates on the same medium. For *P. pentosaceum* grown on semi-synthetic medium, Y_{ATP} values of 11.5 and 12.1 were calculated. Furthermore, unreasonably high Y_{ATP} values were obtained if no allowance was made for oxidative phosphorylation during anaerobic electron transport.

From our experiments Y_{ATP} values were calculated by dividing molar growth yields by ATP yields. ATP yields were calculated from the schemes for lactate and glycerol fermentation and ATP formation postulated by de Vries *et al.* (1973). During nitrate reduction acetate was produced in much larger amounts than propionate (Table 1); therefore sufficient NADH is produced during acetate formation to account for the reduction of fumarate and oxaloacetate in the formation of propionate. Hence 2 mol ATP will be produced during electron transport from NADH to fumarate, and the production of 1 mol propionate or succinate will be accompanied by the production of 2 mol ATP. When ATP production by substrate level phosphorylation and during electron transport to fumarate is taken into account, and oxidative phosphorylation during the electron transport to nitrate is ignored, unreasonably high Y_{ATP} values are found (between 25.9 and 154.0). Forrest & Walker (1971) and Stouthamer (1973) calculated that theoretical maximum Y_{ATP} values lie between 26.7 and 32.1. For some organisms, maximum Y_{ATP} values are found which approach the theoretical values very closely (de Vries *et al.* 1970; Stouthamer & Bettenhausen, 1973). We therefore conclude that nitrate reduction in *P. pentosaceum* is associated with oxidative phosphorylation.

We also propose that 1 mol ATP is formed in the transfer of an electron pair from lactate and glycerol 1-phosphate to nitrate ($P/\text{NO}_3^- = 1$) and 2 mol ATP in the transfer of an electron pair from NADH to nitrate ($P/\text{NO}_3^- = 2$). In our experiments nitrate will be reduced by lactate and glycerol 1-phosphate with a P/NO_3^- value of 1, and by the NADH not used in the reduction of fumarate and oxaloacetate with a P/NO_3^- of 2. With pyruvate as the substrate, all electron transport to nitrate proceeds via NADH ($P/\text{NO}_3^- = 2$).

In our calculations, the extent of oxidative phosphorylation in the electron transport to nitrate was deduced from the amount of nitrite formed. For instance, during growth with lactate (Table 1, data line 2) the nitrite production per mole of lactate is 1.22 mol, 1 mol of which will be produced during the conversion of lactate into pyruvate and the remaining part (0.22 mol) during the further conversion of pyruvate. Thus the ATP yield from lactate may be calculated as follows: 0.18 (acetate formation) $+ 0.09 \times 2$ (propionate formation) $+ 1.0 \times 1$ ($P/\text{NO}_3^- = 1$) $+ 0.22 \times 2$ ($P/\text{NO}_3^- = 2$). The ATP yield from glycerol (Table 1, data line 11) was calculated as follows: 1.0 (Embden-Meyerhof pathway) $+ 0.58$ (acetate formation) $+ 0.20 \times 2$ (propionate formation) $+ 0.06 \times 2$ (succinate formation) $+ 1.0 \times 1$ (glycerolphosphate oxidation with a $P/\text{NO}_3^- = 1$) $+ 2$ (P/NO_3^-) $\times 2.16$ (amount of nitrite formed per mole of glycerol minus one, produced during the oxidation of glycerolphosphate). The ATP yield from pyruvate (Table 1, data line 15) was calculated as follows: 0.35 (acetate formation) $+ 0.08 \times 2$ (propionate formation) $+ 2.26 \times 2$ ($P/\text{NO}_3^- = 2$).

From the ATP yields, Y_{ATP} values between 5.0 and 12.6 were calculated (Table 1). Stouthamer & Bettenhausen (1973) calculated theoretical curves relating Y_{ATP} with the specific growth rate at various values of the maintenance coefficient. In our experiments the specific growth rates varied between 0.06 and 0.12 h⁻¹. Assuming a maintenance coefficient for *P. pentosaceum* of 3 mmol ATP/g dry wt/h (Stouthamer & Bettenhausen, 1973), Y_{ATP} values between 12 and 16 were expected in our experiments. The lower values we obtained might have been caused by the high nitrite concentrations in the medium. This suggestion is

consistent with the results of Hadjipetrou & Stouthamer (1965) who found decreasing Y_{ATP} values in *A. aerogenes* growing in the presence of increasing concentrations of nitrite. Lower Y_{ATP} values were also observed during accumulation of nitrite by *Clostridium perfringens* (Ishimoto *et al.* 1974). With *P. pentosaceum* grown in the presence of nitrate, too, a decrease in Y_{ATP} at increasing nitrite production was observed (Table 1). Furthermore, bacteria grown anaerobically with or without nitrate did not grow when inoculated into a medium containing nitrite (9 mM) but no nitrate. Thus the decrease in Y_{ATP} found during increasing nitrite production from nitrate might be due to uncoupling of growth and energy production. When only small amounts of nitrite were accumulated the expected values for Y_{ATP} were obtained for the various substrates, and we therefore conclude that the results support the basic assumptions made on the efficiency of oxidative phosphorylation for electron transport to nitrate.

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