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The Oxidation of Glucose by *Acinetobacter calcoaceticus*: Interaction of the Quinoprotein Glucose Dehydrogenase with the Electron Transport Chain

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The coupling of the quinoprotein glucose dehydrogenase to the electron transport chain has been investigated in *Acinetobacter calcoaceticus*. No evidence was obtained to support a previous suggestion that the soluble form of the dehydrogenase and the soluble cytochrome *b* associated with it are involved in the oxidation of glucose. Analysis of cytochrome content, and of reduction of cytochromes in membranes by substrates, and of sensitivity to cyanide indicated that glucose, succinate and NADH are all oxidized by way of the same *b*-type cytochrome(s) and cytochrome oxidases (cytochrome *o* and cytochrome *d*). Mixed inhibition studies [with KCN and hydroxyquinoline *N*-oxide (HQNO)] showed that the *b*-type cytochrome(s) formed a binary complex with the *o*-type oxidase and that there was thus no communication between the electron transport chains at the cytochrome level. Measurements of the reduction of ubiquinone-9 by glucose and NADH, and inhibitor studies using HQNO, indicated that the ubiquinone mediates electron transport from both the glucose and NADH dehydrogenases. In some conditions the quinone pool facilitated communication between the 'glucose oxidase' and 'NADH oxidase' electron transport chains, but in normal conditions these chains were kinetically distinct.

INTRODUCTION

The bacterial oxidation of glucose can occur by routes involving an initial phosphorylation reaction or by a direct oxidative route (Lessie & Phibbs, 1984). In the direct route glucose is oxidized to gluconate by glucose dehydrogenase (GDH), a quinoprotein having pyrroloquinoline-quinone (PQQ) as its prosthetic group (Duine *et al.*, 1979). This enzyme has been described in a variety of bacteria able to grow on glucose; these include, for example, *Pseudomonas* spp. (Ameyama *et al.*, 1981*a*; Matsushita *et al.*, 1980*b*; 1982), *Gluconobacter* spp. (Ameyama *et al.*, 1981*b*) and *Klebsiella aerogenes* (Neijssel *et al.*, 1983). In these bacteria the GDH is always firmly bound to the membrane and it is probably arranged so that the catalytic site is available for periplasmic glucose oxidation (Midgley & Dawes, 1973).

Acinetobacter calcoaceticus is an aerobic, Gram-negative, oxidase-negative organism able to grow on a wide variety of carbon substrates (Baumann *et al.*, 1968; Juni, 1978). Although a characteristic feature of the genus *Acinetobacter* is the inability to utilize glucose as a sole source of carbon and energy, many strains are able to oxidize glucose to gluconic acid by the direct route, which is the only route for glucose oxidation in these bacteria. It has been shown that this single oxidation reaction is able to contribute energy for their growth (De Bont *et al.*, 1984). Although the GDH of *Acinetobacter calcoaceticus* is similar in most properties studied to those from other bacteria, it is unusual in existing in both the membrane-bound form and also in a soluble form (Hauge & Halberg, 1964; Duine *et al.*, 1982).

All quinoproteins catalyse the initial oxidation reactions of energy-transducing electron

Abbreviations: GDH, glucose dehydrogenase; PMS, phenazine methosulphate; DMSI, dimethyl suberimidate; NTB, nitrotetrazolium blue; HQNO, hydroxyquinoline *N*-oxide; TMBZ, 3,3′,5,5′-tetramethylbenzidine.

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transport chains, and their mode of coupling to these chains is clearly of importance in microbial energetics; the present paper describes an investigation into the mode of coupling of the GDH quinoprotein with the electron transport chain of *A. calcoaceticus* LMD 79-39. Dehydrogenases that catalyse the oxidation of organic substrates are usually coupled to electron transport chains by way of membrane-soluble quinones (ubiquinones in Gram-negative bacteria). The two known exceptions to this generalization are the dehydrogenases for methanol and methylamine which, like GDH, are quinoproteins having pyrroloquinoline-quinone as their prosthetic groups. Methanol dehydrogenase interacts with a specific soluble cytochrome *c* (Anthony, 1982; Beardmore-Gray *et al.*, 1983; Beardmore-Gray & Anthony, 1984; Anthony, 1986), whereas a novel blue copper protein called amicyanin is the electron acceptor for methylamine dehydrogenase (Tobari & Harada, 1981; Tobari, 1984; Lawton & Anthony, 1985*a, b*). The two dehydrogenases, the cytochrome *c* and the blue copper protein are soluble, periplasmic proteins that form part of electron transport chains which bypass ubiquinone and mid-potential *b*-type cytochromes.

Acinetobacter spp. do not contain cytochrome *c* but in an early investigation of glucose oxidation in *A. calcoaceticus* (previously called *Bacterium anitratum*) the soluble form of GDH was purified and shown to be closely associated with a soluble, autoxidizable cytochrome *b*. It was suggested that this protein might be the electron acceptor from GDH and that a second, soluble, heat-labile, non-dialysable cofactor may also be involved (Hauge, 1960, 1961). By contrast, in *Gluconobacter suboxydans* (now called *Acetobacter suboxydans*) some indirect evidence suggested that a CO-binding cytochrome *c* may be involved (as in methanol oxidation) whereas other evidence implicated cytochrome *b* (Daniel, 1970; Daniel & Erickson, 1969; Ameyama *et al.*, 1981*b*; Matsushita *et al.*, 1981). In *Pseudomonas aeruginosa* the purified GDH has been shown to react with the artificial electron acceptor ubiquinone, but the rate of reaction with the 'natural' ubiquinone-9 was very slow. Furthermore, the involvement of membrane-associated ubiquinone could not be demonstrated because ubiquinone-depleted, glucose 'oxidase' particles were not stimulated by ubiquinone, whereas NADH and succinate 'oxidases' were stimulated by its addition (Matsushita *et al.*, 1980*a, b*, 1982).

The present paper describes experiments which demonstrate that the atypical soluble GDH of *A. calcoaceticus* does not function in glucose oxidation; that no specific *b*-type cytochromes are involved exclusively in glucose oxidation; that the same cytochromes and cytochrome oxidases are involved in glucose, succinate and NADH oxidation; and that the membrane-bound GDH interacts with the cytochrome chain by way of a ubiquinone pool.

METHODS

Organisms and growth conditions. The strains of *Acinetobacter calcoaceticus* used in this work, and the conditions used for growth, harvesting and preparation of suspensions of whole bacteria were described by Beardmore-Gray & Anthony (1983).

Measurements of respiration rates. These were measured at pH 7·0 in a Rank O_2 electrode at 30 °C using 2 ml reaction volumes. HEPES buffer (25 mM) was used for whole bacteria, and MOPS buffer (20 mM) was used for work with membranes. The concentrations of substrates were as follows: glucose, 50 mM (membranes) or 5 mM (whole bacteria); succinate, 5 mM; and NADH, 1 mM.

Preparation of soluble and membrane fractions, and of spheroplasts. Bacteria were broken either by sonication, and fractionated as described by Beardmore-Gray & Anthony (1983), or by lysozyme treatment of whole bacteria followed by extraction with 1% Triton X-100 as described by Duine *et al.* (1979). Spheroplasts were prepared exactly as described by Jones *et al.* (1982).

Cold shock treatment. The method of Neu & Heppel (1965) was followed. Bacteria (1 g wet weight) were suspended in 80 ml 20% sucrose in 50 mM-Tris/HCl (pH 8·0) at 24 °C; 1 mM-EDTA was added and the suspension gently stirred for 10 min. The cells were then harvested and suspended in 80 ml ice-cold water. After 10 min the bacteria were centrifuged and resuspended in 3 ml 50 mM-Tris/HCl (pH 8·0) and were assayed for the ability to oxidize glucose.

Measurement of phenazine methosulphate (PMS)-linked glucose dehydrogenase activity and of protein. The assay system for glucose dehydrogenase was that described by Duine *et al.* (1979). O_2 consumption was measured at 30 °C by using a Rank O_2 electrode with a 2 ml reaction volume containing 50 mM-Tris/HCl (pH 8·5), 2·8 mM-

PMS and 70 mM-glucose. The K_m for the soluble enzyme in this system was 15 mM and that for the membrane-bound enzyme was 20 mM.

Dimethyl suberimidate (DMSI) treatment. This was done exactly as described by Halpin *et al.* (1981). The experiments were done at two concentrations of DMSI (6 mM or 12 mM). After treatment the bacteria were sonicated; the soluble fraction was concentrated 10-fold using an Amicon B-15 concentration pack and was analysed by isoelectric focusing.

Isoelectric-focusing. The agarose gels used for this were prepared by a method devised by Dr I. Cottingham (University of Southampton, UK). A warm gel solution was poured into casting trays (110 × 100 × 0·4 mm) and overlaid with a piece of LKB Gel-Bond. The gel solution (7 ml) contained 65 mg LKB-agarose Z, 1 g sorbitol, 1% Triton X-100 and 0·4 ml LKB ampholine (pH 3·5–10·0) solution. After 12 h storage at 4 °C the Gel-Bond plus gel was removed; samples (2 μl) were loaded into preformed application wells and electrophoresis was done using an LKB Multiphor system according to the manufacturer's instructions. The gels were run for 15 min at limiting voltage (100 V) followed by 45 min at limiting power (2 W).

After electrophoresis individual lanes were cut out and immersed in appropriate solutions for visualization of the GDH and cytochrome *b*. GDH was stained using a nitrotetrazolium blue (NTB) stain, made up of 150 mg NTB, 15 mg PMS and 2·5 g glucose dissolved in 100 ml 20 mM-phosphate buffer (pH 7·0). The stain for cytochrome *b* consisted of 70 ml 250 mM-acetic acid (pH 5·0) plus 45 mg 3,3′,5,5′-tetramethylbenzidine (TMBZ) dissolved in 30 ml methanol. The gel was immersed in this solution for 10 min in the dark and then 0·34 ml 20% H_2O_2 were added.

Cytochrome measurements. These were all made on a Shimadzu UV-3000 dual wavelength/dual beam spectrophotometer fitted with attachments for stirring, for measurement of O_2 concentration and for measurements at 77 K. Samples were oxidized by air or by addition of a crystal of potassium ferricyanide, or they were reduced by addition of sodium dithionite or of substrate at the same concentration as used for respiration studies. The absorption coefficients and wavelength pairs were those used by Ensley & Finnerty (1980). The cytochrome *o* concentration was estimated from (CO-reduced)-*minus* reduced difference spectra using the wavelength pair 417–490 nm and the absorption coefficient of 80 mM^{-1} cm^{-1} as described by Ensley & Finnerty (1980).

For measurement of the reduction by glucose and NADH of cytochrome *b* in membranes the wavelength pair used was 560–575 nm. The reaction was measured in a 3 ml stirred cuvette containing 0·5–2·0 mg protein ml^{-1}. Substrate concentrations were the same as those used in respiration studies.

Inhibition of respiration. Inhibitors were always incubated with cells or membranes for 5 min before addition of substrate. KCN was dissolved in 10 mM-Tris/HCl (pH 8·0); hydroxyquinoline *N*-oxide (HQNO) was dissolved in ethanol. In each experiment it was confirmed that addition of solvent alone had no effect on results observed. Endogenous respiration was always zero for membrane preparations and less than 10% of the substrate-dependent rate for whole cells. The K_m value for glucose oxidation by membranes was 3 mM and rates of respiration could be limited by varying the glucose concentration around this value. By contrast, the K_m value for NADH was very low (less than 50 μM) and to vary the respiration rate with NADH it was necessary to use a NADH-generating system. This consisted of glucose 6-phosphate (2 mM), NAD^+ (0·1 mM) and glucose phosphate dehydrogenase (0·2–0·6 units). The rate of NADH generation was directly proportional to the dehydrogenase concentration in this system.

Measurement of ubiquinone. Ubiquinones were determined exactly as described by Katayama *et al.* (1980). We are indebted to J. S. Reed of the Biochemistry Department of Southampton University for performing these assays.

RESULTS AND DISCUSSION

The soluble and membrane forms of GDH in A. calcoaceticus LMD 79-39

A. calcoaceticus LMD 79-39 oxidized glucose at similar rates [between 100 and 150 nmol O_2 min^{-1} (mg dry wt)$^{-1}$] when grown on succinate or ethanol in the presence or absence of glucose (20 mM). After ultrasonic disruption, or treatment of whole bacteria with lysozyme followed by Triton X-100, about 60% of PMS-linked GDH was found in the soluble fraction of the bacteria. The specific activity of GDH in the membrane fraction produced by sonication [3·2 μmol O_2 consumed min^{-1} (mg protein)$^{-1}$] was higher than that measured after lysozyme/Triton treatment [2·3 μmol O_2 min^{-1} (mg protein)$^{-1}$]; the specific activity in the soluble fraction was about the same regardless of the disruption method used [1–1·2 μmol O_2 min^{-1} (mg protein)$^{-1}$]. The specific activity of GDH in membranes of *A. calcoaceticus* was similar to that in membranes from other bacteria and was sufficient to account for about 50% of the rate of glucose oxidation measured in whole bacteria.

The rate of respiration of bacteria with glucose was completely unaffected by cold shock treatment which usually diminishes activities which depend upon periplasmic proteins (Neu & Heppel, 1965; Neijssel *et al.*, 1983). Likewise, no effect was observed when respiration was measured in the presence of 200 mM-potassium phosphate (pH 7·0) (±50 mM-EDTA), a treatment that completely inhibits oxidation of methanol by the periplasmic methanol dehydrogenase (Anthony & Zatman, 1964; Anthony, 1975, 1986; Carver *et al.*, 1984).

As shown by Hauge (1961), after ultrasonic disruption the soluble fraction of *A. calcoaceticus* LMD 79-39 contained a *b*-type cytochrome, having peaks at 530 and 558 nm in the reduced-minus-oxidized difference spectrum. Its M_r could not be determined by electrophoresis in sodium or lithium dodecyl sulphate because its haem was rapidly lost. Its isoelectric point (pI) was shown by isoelectric focusing to be 4·5. The concentration of this soluble cytochrome *b* was 25–50 pmol (mg soluble protein)$^{-1}$; this was about 10% of the total cytochrome *b* in these bacteria.

If the soluble GDH is periplasmic and is closely associated with the soluble cytochrome *b* then it might be possible to demonstrate this by treatment with DMSI, a protein cross-linking reagent that is able to penetrate the Gram-negative outer cell wall of bacteria (Halpin *et al.*, 1981). To investigate this possibility whole bacteria, treated with DMSI, were disrupted by sonication, and proteins in the soluble fraction were separated by isoelectric focusing on agarose as described in Methods. Soluble GDH was detected by its enzymic activity and the soluble cytochrome *b* detected by its reaction with TMBZ. The two proteins were very well separated by this method; the pI of GDH was 9·5 and that of cytochrome *b* was 4·5. Neither the amounts of each protein seen on the gels, nor their positions, were altered by prior treatment of bacteria with the cross-linking reagent, suggesting that they are not periplasmic, or are not closely associated or are not accessible for cross-linking by DMSI.

When *A. calcoaceticus* was treated with lysozyme and EDTA by the method of Jones *et al.* (1982), osmotically fragile spheroplasts were produced and a periplasmic fraction was released. This fraction contained less than 10% of the soluble GDH and no detectable cytochrome *b*. When the spheroplasts were subsequently lysed by dilution into dilute suspending medium all the GDH and cytochrome *b* was found to be associated with the membrane fraction, none being detectable in the soluble fraction, indicating that in these mild disruption conditions more than 90% of the GDH is membrane associated.

Membranes of *A. calcoaceticus* LMD 79-39 catalysed a rate of glucose oxidation, measured in the O_2 electrode, similar to that measured with NADH as substrate [250–350 nmol O_2 consumed min^{-1} (mg protein)$^{-1}$]. When a soluble fraction containing GDH (1–3 mg protein) was added to respiring membranes (40 μg protein) the rate of glucose-dependent O_2 consumption remained unchanged. Membranes of *A. calcoaceticus* 8250 lack GDH and so were unable to oxidize glucose, although able to oxidize NADH. When the soluble fraction of strain LMD 79-39 (1–3 mg protein), which contains GDH, was added to the membranes of strain 8250 (250–350 μg protein) no respiration with glucose as substrate was observed. Neither was any reconstituted 'glucose oxidase' activity observed when 25 mM-$MgCl_2$ or potential mediators (duroquinone, ubiquinones-1, 2, 9 or 10; all at 100 μM) were added to the system.

In summary, although none of these results taken separately provide strong evidence, when taken together they do indicate that neither the soluble quinoprotein glucose dehydrogenase of *A. calcoaceticus* nor the soluble cytochrome *b* are periplasmically located, and that these proteins are not likely to play any role in glucose oxidation. It appears probable that, as in other bacteria which have no soluble GDH or cytochrome *b*, the membrane-bound GDH is the only active form in *A. calcoaceticus*, and that the soluble form is removed from the membrane during bacterial disruption and/or is a precursor of the active membrane-bound form, as suggested by Duine *et al.* (1982).

The cytochromes and cytochrome oxidases of A. calcoaceticus LMD 79-39

In order to obtain an indication of whether or not there are any features of electron transport from the membrane-bound GDH that are specific to glucose oxidation in *A. calcoaceticus* it was necessary to characterize the cytochrome chain of that organism. Ensley & Finnerty (1980) have

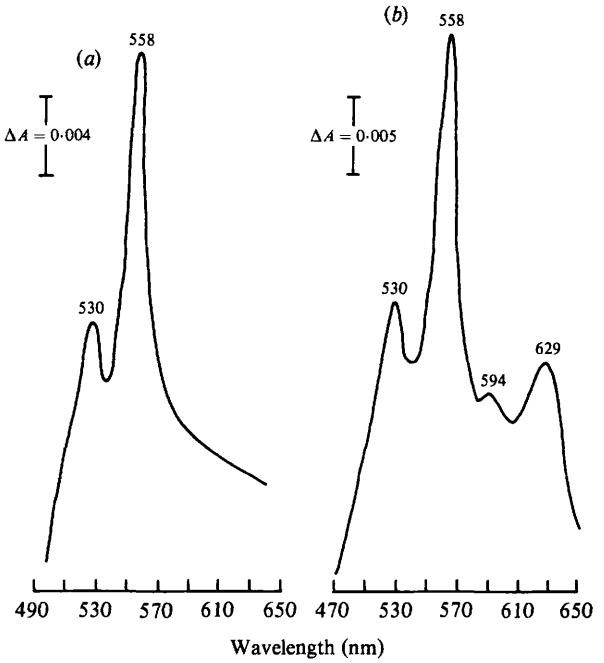


Fig. 1. Effect of growth conditions on cytochrome content of *A. calcoaceticus* LMD 79-39. The spectra, measured at room temperature are reduced-*minus*-oxidized difference spectra of the membrane fractions. The reference cuvette contained untreated membranes which were fully oxidized; the test cuvette contained membranes which had been reduced with either glucose, NADH or sodium dithionite; these all gave the same spectrum. (*a*) Membranes (2·5 mg protein ml^{-1}) from bacteria harvested in the early exponential phase of growth (5 h). (*b*) Membranes (3·5 mg protein ml^{-1}) from bacteria harvested in the late exponential phase (12 h).

shown that the nature of the cytochrome chain of *A. calcoaceticus* strain H01-N was unaffected by the growth substrate (acetate or hexadecane) but was dependent on the availability of O_2 during growth. Under conditions of high aeration cytochrome *b* and an *o*-type oxidase were present. By contrast, under conditions of poor aeration a second oxidase, cytochrome *d*, was also induced together with a trace of cytochrome a_1. As expected, no cytochrome *c* was found under any growth conditions. The results presented in Table 1 and Fig. 1 show that succinate-grown *A. calcoaceticus* LMD 79-39 behaved in a similar fashion to acetate-grown *A. calcoaceticus* strain H01-N and to succinate-grown *A. calcoaceticus* strain 69V (Asperger *et al.*, 1978). During early exponential growth only cytochrome *b* and the *o*-type oxidase were present; as growth proceeded the progressive limitation of O_2 supply led to induction of the alternative oxidase, cytochrome *d*, as found in a number of other bacteria (Poole, 1983). The spectral characteristics of the membrane-bound cytochromes of *A. calcoaceticus* strain LMD 79-39, measured at 298 K, 77 K and after reaction with CO, were very similar to those described by Ensley & Finnerty (1980) in strain H01-N. The low temperature measurements indicated the presence of multiple forms of cytochrome *b*.

Addition of glucose or NADH led to reduction of all the cytochromes present on membranes of strain LMD 79-39 (Fig. 1), thus suggesting that glucose and NADH are oxidized by way of exactly the same cytochromes. That the same oxidases were involved in the oxidation of these substrates (and also succinate) was confirmed by analysis of the effects of respiratory inhibitors. The substrates used were glucose and succinate for whole bacteria, and glucose and NADH for studies of respiration by membrane suspensions. The respiration rates were 150–250 nmol min^{-1} (mg dry weight bacteria)$^{-1}$ for glucose and succinate, and 250–350 nmol min^{-1} (mg membrane protein)$^{-1}$ for both glucose and NADH. These rates were similar in all phases of

Table 1. *Cytochromes of A. calcoaceticus*

Details of growth conditions, membrane preparations, cytochrome measurements and inhibitor studies are given in Methods. The concentration of cytochrome *b* does not include the *b*-type oxidase cytochrome *o*.

Strain/growth substrate	Growth condition	Cytochrome concentrations [pmol (mg membrane protein)$^{-1}$]				Inhibition of respiration by cyanide (I_{50}, μM†)
		Cyt *b*	Cyt *o*	Cyt *d*	Cyt a_1	
LMD 79-39/succinate	Early exponential	170	170	0	0	Monophasic (5)
	Late exponential	200	210	110	T	Biphasic (5, 80)
	Stationary	231	115	227	T	Biphasic (5, 80)
H01-N*/acetate	High aeration	160	180	30	T	Monophasic (36)
	Low aeration	560	170	190	T	Biphasic (36, 300)

T, Trace.
* Data from Ensley & Finnerty (1980).
† Concentration of cyanide giving 50% inhibition of respiration.

Table 2. *Reduction of cytochrome b in membranes by glucose and NADH*

These values are taken from a range of experiments of the sort described in Fig. 6. The reduction of cytochrome *b* was measured in the 'aerobic steady state' part of the reaction sequence in Fig. 6.

Condition of aeration (controlled by stirrer speed)	Percentage of cytochrome *b* reduced by substrate:			[(% reduced by glucose)/(% reduced by glucose + NADH)] × 100	[(% reduced by NADH)/(% reduced by glucose + NADH)] × 100
	glucose	NADH	glucose + NADH		
Anaerobic; stirrer speed zero under N_2 gas phase	97	97	98	99	99
Zero stirrer speed under air	90	90	92	98	98
Low speed	71	71	83	86	86
Medium speed	46	49	68	68	72
High speed	31	34	51	61	67

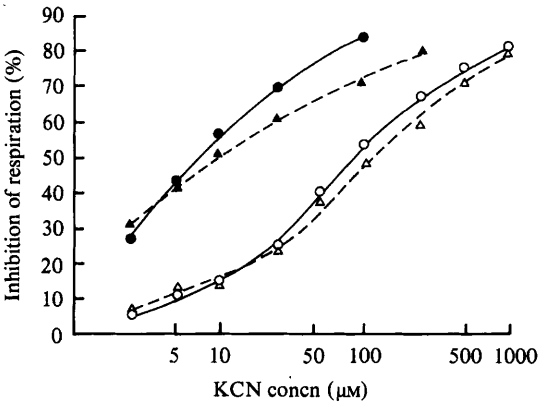


Fig. 2. Inhibition of respiration by cyanide in whole cells and membrane fractions of *A. calcoaceticus* LMD 79-39. ——, Inhibition observed when using whole bacteria harvested after 4·5 h (●) or 8·5 h (○). Identical results were obtained with either glucose or succinate as substrate. – – – –, Inhibition observed when using membrane fractions from bacteria harvested after 5 h (▲) or 15 h (△). Identical results were obtained when using glucose or NADH as substrate. Only one oxidase (cytochrome *o*) was present during the early phase of growth (4·5 or 5 h) but a second oxidase (cytochrome *d*) was also present at the later stages of growth (8·5 and 15 h) (see Table 1 and Fig. 1). The uninhibited respiration rates were in the range 150–250 nmol O_2 min^{-1} (mg dry weight)$^{-1}$ for whole bacteria, and 250–350 nmol O_2 min^{-1} (mg protein)$^{-1}$ for membrane fractions.

growth. Ascorbate plus *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) was not oxidized, as expected for an oxidase-negative organism; this contrasts with the observation of Ensley & Finnerty (1980) who showed that, unexpectedly, strain H01-N was able to oxidize this substrate. Respiration of strain LMD 79-39 was relatively insensitive to azide (1 mM having no effect) and antimycin A; therefore KCN and HQNO were used as inhibitors in this work.

When grown in conditions of high aeration the *o*-type oxidase was the sole oxidase observed in spectra (Fig. 1). In bacteria and membranes having only this oxidase the inhibitor profile for cyanide was monophasic, the concentration giving 50% inhibition (I_{50}) being 5 μM with all substrates (Fig. 2). Using bacteria grown with limiting aeration the second oxidase, cytochrome *d*, was also present (Fig. 1); in these conditions the inhibitor profile for KCN (using glucose, succinate or NADH) was biphasic, the *o*-type oxidase showing the same I_{50}, and the induced cytochrome *d* being relatively cyanide-insensitive with an I_{50} of 80 μM (Fig. 2). These results are similar to those measured for acetate oxidation by whole cells of strain H01-N (Ensley & Finnerty, 1980).

HQNO and antimycin A usually act by preventing reduction of cytochrome *b* by ubiquinones. *A. calcoaceticus* LMD 79-39 was relatively insensitive to antimycin A; the I_{50} for respiration of membranes with either glucose or NADH was about 120 μM. HQNO was therefore used for analysing this part of the electron transport chain. The inhibition profile was identical for glucose and NADH [I_{50}, 2 μM (Fig. 3)]; it was also independent of the nature of the oxidase (cytochromes *o* or *d*). This indicates that glucose and NADH are oxidized by the same route, and that the site of HQNO inhibition in the electron transport chain is before the oxidases. Concentrations of HQNO (20 μM) that almost completely inhibited respiration also prevented the reduction of *b*-type cytochromes by glucose or NADH, confirming that the site of action of HQNO was probably on a *b*-type cytochrome (as for other electron transport chains), acting by preventing its reduction by ubiquinol. These results all suggest that glucose and NADH are oxidized by way of a quinone pool.

Membranes of *A. calcoaceticus* strain LMD 79-39 were shown to contain ubiquinone [3·6 nmol (mg membrane protein)$^{-1}$] and 50% of this could be reduced by addition of either glucose or NADH to an aerobic suspension of membranes. Similar amounts of ubiquinone-9 [3·3 nmol (mg membrane protein)$^{-1}$] were previously shown to be present in *A. calcoaceticus* strain 8250 (Whittaker, 1971).

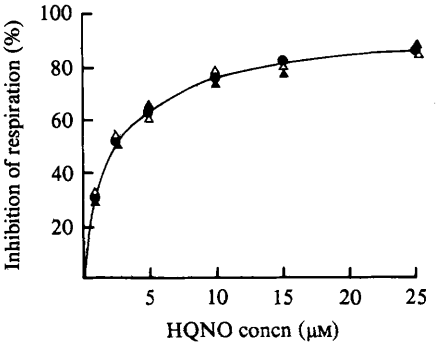


Fig. 3

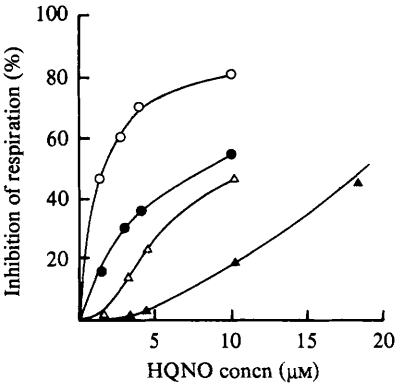


Fig. 4

Fig. 3. Inhibition by HQNO of respiration of glucose and NADH by membrane fractions in oxidase-limited conditions. Inhibition by HQNO was measured either with no KCN present (●), or after sufficient KCN had been added to inhibit the respiration rate by 50% (▲) or 75% (△) (see Fig. 2). Identical results were obtained with either glucose or NADH as substrate. Bacteria were harvested in the early exponential phase of growth when the *o*-type oxidase was the sole oxidase (Table 1, Fig. 1). The initial respiration rate in the absence of inhibitors was 250–350 nmol O_2 min^{-1} (mg protein)$^{-1}$ for both substrates.

Fig. 4. Inhibition by HQNO of respiration of glucose by membranes in substrate-limited conditions. The inhibition of respiration produced by HQNO was measured either under conditions of glucose excess (○) or under conditions in which the uninhibited rate was determined by the concentration of glucose. The glucose concentration was such that the uninhibited rates were 65% (●), 35% (△), or 15% (▲) of the rate with excess glucose. The uninhibited rate in the presence of excess glucose was in the range 250–350 nmol O_2 min^{-1} (mg protein)$^{-1}$. A very similar result was obtained when NADH was used as limiting substrate (see Fig. 5). The method for limiting the respiration rate by substrate concentration is given in Methods.

The interaction between electron transport chains for oxidation of glucose and NADH

If glucose and NADH are oxidized by identical electron transport chains by way of ubiquinones, *b*-type cytochromes and cytochrome oxidases, then communication should be able to occur between these chains by way of any of their mobile components. In the absence of soluble cytochrome *c* such components might be the ubiquinone, or any *b*-type cytochromes mobile in the membrane and independent of the oxidases. The experiments described below were designed to investigate these possibilities; they all used material derived from bacteria harvested from the early phase of growth in which the sole oxidase was the cyanide-sensitive *o*-type oxidase.

The results in Figs 3 and 5 show that the profile (and the I_{50} value) for inhibition by HQNO of respiration of glucose and NADH was not altered when the oxidase was inhibited by cyanide. This confirmed that the HQNO acts at a site before the cyanide-sensitive oxidase and that this site (assumed to be a *b*-type cytochrome) must form part of a binary complex with the oxidase. That is, there is no mobile component between the HQNO-binding cytochrome *b* and the cyanide-sensitive *o*-type oxidase (see Ragan & Cottingham, 1985 and Rich, 1984 for extensive discussion of interpretation of the inhibition kinetics of electron transport systems).

By contrast with the results above, when the rate of oxidation of substrate (glucose or NADH) was determined by the dehydrogenase activity rather than by the oxidase function it was found that the inhibitor profile for HQNO was markedly altered (Figs 4 and 5). Thus, as the rate of respiration was progressively decreased as a result of limitation of substrate availability, the I_{50} values for inhibition of glucose and NADH oxidation by HQNO were progressively increased. This indicates that there is a mobile component between the dehydrogenase and the cytochrome *b*, the obvious candidate for such a mobile component being the ubiquinone. The extent of electron transfer by way of mobile ubiquinone and the 'glucose oxidase' and 'NADH oxidase' respiratory chains should increase when the proportion of the reduced form of the mobile quinone (the quinol) increases. The experiments described in Fig. 6 and summarized in Table 2

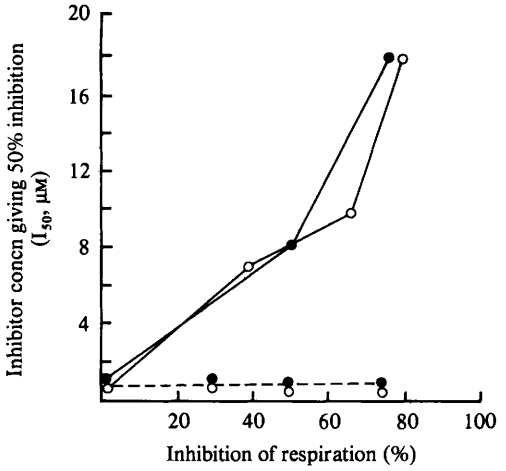


Fig. 5. Dependence of the I_{50} for inhibition by HQNO on the respiration rate. The respiration rate was altered by addition of cyanide (----) or by substrate-limitation (——). The data are taken from Figs 3 and 4. ○, Glucose; ●, NADH.

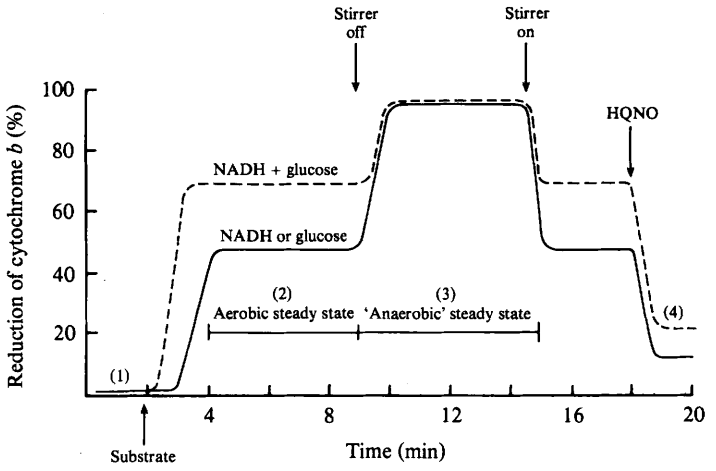


Fig. 6. Reduction of cytochrome *b* in membranes of *A. calcoaceticus* LMD 79-39. The reduction of cytochrome *b* was measured in a 3 ml stirred cuvette in a dual wavelength spectrophotometer using the wavelength pair 560–575 nm; details are given in Methods (the protein concentration was 1·5 mg ml^{-1}). The reduction of cytochrome *b* is expressed as a percentage of the total that it is reducible with sodium dithionite. (1) In the stirred membrane suspension there was no endogenous respiration and all the cytochrome present (cytochrome *b* and cytochrome *o*) was in the oxidized state. (2) Upon addition of substrate, cytochrome reduction was observed after a lag period during which most of the O_2 in the stirred cuvette was consumed, leading to the aerobic steady state; the extent of reduction of cytochrome in this state could be adjusted by modifying the aeration rate or by adding low concentrations of HQNO. (3) The stirrer was turned off thus producing the 'anaerobic' steady state. (4) Addition of HQNO during the aerobic steady state led to an immediate fall in the level of reduced cytochrome *b*. Addition of HQNO in the anaerobic steady state had no effect on the extent of reduction of cytochrome *b*.

show that this did occur. In the anaerobic steady state the cytochrome *b* was almost completely reduced, irrespective of the substrate used (glucose or NADH). The extent of reduction by substrate of cytochrome *b* in the aerobic steady state was dependent on the extent of aeration (governed by the stirrer speed) and was similar with either glucose or NADH as substrate. When

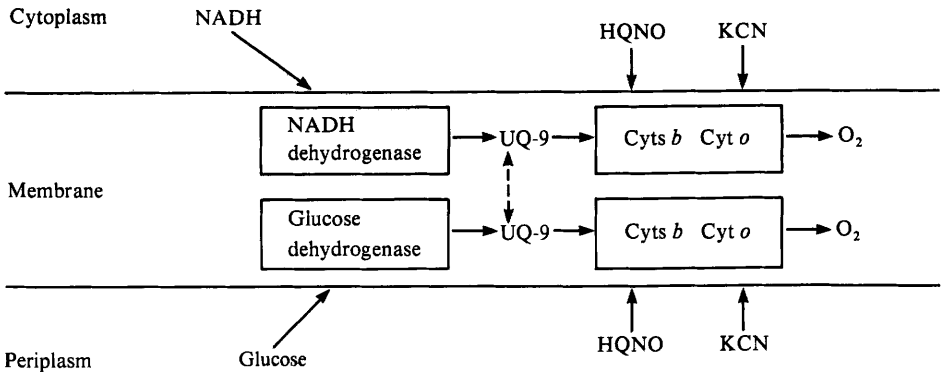


Fig. 7. Oxidation of glucose and NADH in *A. calcoaceticus* LMD 79-39 grown in conditions of oxygen sufficiency. The components in boxes are firmly membrane-bound. There is no mobile intermediate between the *b*-type cytochromes and the *o*-type oxidase. The two dehydrogenases are on opposite sides of the membrane as indicated. The (cyt *b*, cyt *o*) complexes are probably arranged in identical orientations with respect to the membrane, but they differ in their spatial relationships with respect to the two dehydrogenases. The dotted line indicates that in some conditions the ubiquinone pool (UQ-9) mediates interaction between the two kinetically distinct chains for oxidation of NADH and glucose (see text).

a mixture of substrates was used the extent of reduction of cytochrome *b* in the aerobic steady state was always greater than with either substrate alone, and it was inversely proportional to the aeration rate. The results in Fig. 6 show that HQNO prevented the reduction of cytochrome but did not prevent its oxidation.

If, as suggested above, GDH and NADH dehydrogenase are oxidized by identical respiratory chains which are connected by a mobile quinone/quinol pool, then the rates of oxidation of their two substrates should not be perfectly additive in conditions when components other than the initial dehydrogenases are rate-determining. However, the rates of glucose and NADH oxidation remained perfectly additive even when the electron flow to O_2 was inhibited by KCN to only 15% of the maximum capacity. This suggests that although the pathways for oxidation of glucose and NADH have the same components they are, to some extent, kinetically distinct.

General discussion

The results described above all indicate that glucose is oxidized in *A. calcoaceticus* LMD 79-39 by the route shown in Fig. 7. In conditions of O_2 insufficiency the reduced ubiquinone would also be oxidized by the alternative oxidase system containing cytochrome *d*. In bacteria such as *Pseudomonas* spp. that contain *c*-type cytochromes a similar route for glucose oxidation probably operates, except that the *b*-type cytochrome would be oxidized by way of cytochrome *c*. In the scheme shown in Fig. 7 the membrane-bound NADH and glucose dehydrogenases interact with identical but kinetically distinct electron transport chains consisting of ubiquinone-9 and a membrane-bound complex comprising the mid-chain *b*-type cytochromes plus the oxidase cytochrome *o*. In physical terms this means that some of the (cytochrome *b*, cytochrome *o*) complexes are arranged in the membrane sufficiently close to the glucose dehydrogenase (for example) such that the rate of flow of electrons (by way of ubiquinol) from this dehydrogenase to some of the cytochrome *b* is faster than to the other cytochrome *b* complexes which happen to be nearer to the NADH dehydrogenase. In some conditions the quinone pool mediates interaction between the two chains; this occurs when most of the pool is in the reduced form, as occurs in nearly anaerobic conditions.

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