Influences of Growth Substrates and Oxygen on the Electron Transport System in *Acinetobacter* sp. HO1-N

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The electron transport system of *Acinetobacter* sp. HO1-N was studied to determine the specific cytochromes and to measure changes in the composition of the respiratory system due to growth in various concentrations of oxygen or types of growth substrates. Spectrophotometric analysis revealed that the quantity and types of cytochromes changed in response to growth under various concentrations of oxygen. Growth on alkane and nonalkane substrates resulted in only minor differences in cytochrome composition or oxidase activities. Membranes prepared from cells grown under oxygen-limiting conditions contained at least one b-type cytochrome, cytochrome o, cytochrome d, and slight traces of cytochrome a1, whereas membranes prepared from cells grown in the presence of high oxygen concentrations contained only low levels of cytochromes b and o. Polarographic measurements, electron transport inhibitor studies, and photoaction spectrum analyses indicated that cytochromes o, a1, and d were potentially capable of functioning as terminal oxidases in this organism. These experiments also revealed that all three cytochromes may be involved in the oxidation of reduced nicotinamide adenine dinucleotide, succinate, or \( N,N,N',N'\)-tetramethylp-phenylenediamine.

Some bacteria are capable of altering the composition of their respiratory systems in response to changes in the availability of dissolved oxygen. The results of studies with *Proteus vulgaris* (6), *Klebsiella aerogenes* (16), *Haemophilus parainfluenzae* (45), *Escherichia coli* (31), and *Pseudomonas putida* (43) have indicated that cytochrome o is the major terminal oxidase in cells grown in the presence of excess dissolved oxygen, whereas growth under conditions of limited aeration results in the production of cytochrome d (\( \alpha_2 \)) as an additional terminal oxidase.

Analyses of the oxygen uptake kinetics of *E. coli* (37) and *H. parainfluenzae* (45) have supported the proposal that cytochrome d is capable of efficiently reacting with low concentrations of oxygen, permitting continued respiration under conditions of limited oxygen availability. The advantages of this type of respiratory system may be particularly applicable to hydrocarbon-oxidizing bacteria, since the growth of bacteria on hydrocarbons has been associated with a high oxygen demand (26, 34).

*Acinetobacter calcoaceticus*, a bacterium capable of growth on n-alkanes (42), contains respiratory pigments which have been tentatively identified as cytochromes d and o (49). We have measured the influences of limited aeration and alkane growth on the composition of the cytochromes in *Acinetobacter* sp. HO1-N. Cells grown at the expense of acetate or hexadecane contained cytochromes of similar composition. However, growth of the organism under oxygen-limiting conditions resulted in increased levels of cytochromes b, o, and d and synthesis of cytochrome a1. These changes were accompanied by differences in the photoaction spectra and an increased resistance to cyanide-mediated inhibition of respiration.

**MATERIALS AND METHODS**

**Bacterial strain.** *Acinetobacter* sp. HO1-N (5, 20) was used throughout these studies. The bacterium was maintained by serial transfers on agar slants of the following composition (in grams per liter): nutrient broth (Difco), 1.5; yeast extract (Difco), 2.0; peptone (Difco), 5.0; KH2PO4, 3.7; KH2PO4, 1.3; NaCl, 2.0; agar (Difco), 2.0; pH 7.8. Inoculated slants were incubated at room temperature.

**Culture conditions.** The organism was grown in a mineral salts medium described by Scott et al. (39). The minimal medium was supplemented with 0.8% nutrient broth (Difco) plus 0.5% yeast extract (Difco), with a sterilized solution of sodium acetate (Baker) to a final concentration of 0.4%, or with sterilized n-hexadecane (Humphrey Chemical Co.) to a final concentration of 0.5%.

Starter cultures in 100 ml of nutrient broth-yeast extract were inoculated from the agar slants and used to inoculate hexadecane or acetate starter cultures. Cultures were grown at 28°C in 3-liter volumes in a 4-liter Erlenmeyer flask, stirred at 300 rpm with a mag-
nentic stirrer, and either sparged with sterile air at 1 to 2 liters/min for high aeration or not sparged for low aeration. The organism was also grown in 10-liter lots in a Chemapac fermentation vessel (14-liter working volume) with either stirring at 900 rpm without air sparging for low aeration or stirring at 600 rpm with air sparging at 18 liters/min for high aeration. Growth was monitored with a Klett-Summern colorimeter equipped with a 540-nm filter, and the cultures were harvested during the midexponential phase of growth at 28°C. Oxygen concentrations in the fermentation vessel were monitored with a Chemapac oxygen electrode and meter.

Preparation of membranes. Acinetobacter membrane fractions were prepared by lysozyme treatment of washed cells by the method of Scott et al. (39).

Cytoplasmic membranes were enriched from the crude suspension by differential centrifugation (39). The membrane suspension was centrifuged at 65,000 × g for 3 min, and the resulting translucent orange pellet was suspended in 50 mM potassium phosphate buffer, pH 7.8, to a final protein concentration of 5 to 10 mg/ml.

Enriched cytoplasmic membranes were either used immediately or stored at 4°C in the presence of 20 μg of chloramphenicol per ml for not more than 3 days.

Enzyme assays. NADH:cytochrome c reductase (EC 1.6.99.3) and succinate:cytochrome c reductase (EC 1.3.99.1) were assayed by the method of King and Drews (24, 25).

NADH dehydrogenase (EC 1.6.99.3) and succinate: 2,6-dichlorophenolindophenol (DCIP) reductase (EC 1.3.99.1) were assayed by following the rate of reduction of DCIP at 578 nm (24).

Succinate dehydrogenase (EC 1.3.99.1) activity was measured by the phenazine methosulfate-mediated reduction of DCIP as described by Arrigoni and Singer (3), except that a fixed phenazine methosulfate concentration was used.

All activities were measured at 25°C in 3-ml glass cuvettes with a 1-cm light path. Specific activities were calculated by using millimolar extinction coefficients of 20.6 mM−1 cm−1 for DCIP at 578 nm and 21 mM−1 cm−1 for cytochrome c at 546 nm. Control cuvettes contained all components except the substrate, and the low endogenous reduction rates measured were subtracted from the experimental values.

Oxygen uptake measurements. Rates of oxygen utilization by membrane in the presence of various substrates were measured polarographically with a Yellow Springs Instrument model 53 oxygen monitor attached to a Perkin-Elmer model 159 strip chart recorder. All rates were determined in a 3.4-ml jacketed-glass reaction chamber maintained at 30°C with constant stirring. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, and 1 to 2 mg of membrane protein. After endogenous rates of oxygen uptake were determined, the reactions were started by adding one of the following substrates: NADH to 1 mM; sodium succinate to 4 mM; DCIP to 0.3 mM; or N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) (Sigma) to 0.3 mM. The redox dyes DCIP and TMPD were dissolved in water before addition and were kept reduced in the reaction chamber with a twofold molar excess of ascorbic acid (Baker). Succinic and ascorbic acids were dissolved in water and neutralized to pH 7.0 with 0.1 M NaOH. The initial dissolved oxygen concentration in equilibrium with atmospheric oxygen was assumed to be 233 μM.

Inhibition analysis. The effects of various electron transport inhibitors on respiratory activities were determined by polarographic methods as described above. NaN3 (Baker) was dissolved in 50 mM potassium phosphate buffer (pH 7.0), and KCN was dissolved in dilute KOH (pH 11). Antimycin A (Sigma), 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) (Sigma), and rotenone were dissolved in a minimal volume of absolute ethanol. Various concentrations of the inhibitors were incubated with the membrane preparations for 3 min before the reactions were started. The influences of antimycin A, rotenone, and HOQNO were corrected for the inhibition caused by the addition of absolute ethanol.

Optical spectra. Difference absorbance spectra were recorded on an Aminco model DW-2 spectrophotometer. A liquid nitrogen dewar and Pyrex cuvette (path length, 1 mm) were used to record spectra at 77°C. The cytochromes of the respiratory particles were readily oxidized by oxygen; therefore, shaking the particles in air was usually sufficient to produce oxidized samples for the reference cuvette. Samples occasionally were oxidized by the addition of 2 to 12 μl of 50 mM K2Fe(CN)6. Reduced samples of respiratory particles were prepared by the addition of solid sodium dithionite. When carbon monoxide was used, it was bubbled into the reduced sample for 2 min.

Estimates of amounts of cytochromes present were made from room temperature, reduced-minus-oxidized difference spectra by using extinction coefficients and wavelength pairs as follows: cytochrome(s) a, 540 to 575 nm, Σ = 22 mM−1 cm−1 (9); cytochrome d, 625 to 650 nm, Σ = 15 mM−1 cm−1 (17, 47, 48); and cytochrome a1, 585 to 593 nm, Σ = 15 mM−1 cm−1 (38). The cytochrome a concentration was estimated from the wavelength pair 417 to 490 nm with CO-reduced-minus-reduced difference spectra and Σ = 80 mM−1 cm−1 (10, 34).

Photoaction spectra. A photoaction spectrum, the photoinduced reversal of carbon monoxide binding (7, 8), is the classical technique for identification of terminal oxidases in electron transport chains. The utilization of pulsed tunable dye lasers as a source of radiation for photoaction spectra has been reported previously (11, 40). This method has been adapted here for use with a laser producing a continuous beam of light.

Cytoplasmic membranes prepared from acetate cells grown under high and low aerations were mixed with CO-saturated 50 mM phosphate buffer (pH 7.0) to give a final membrane protein concentration of 0.5 to 1 mg/ml in the 3.5-ml reaction volume of a Gilson water-jacketed reaction vessel. The reaction vessel was closed with a ground-glass stopper containing a capillary for addition of reagents. NADH, ascorbate-TPMD, or sodium succinate was added through the capillary to give a final substrate concentration as described for oxygen uptake. Respiration was allowed to proceed until the small amount of residual oxygen in the chamber had been exhausted. After anaerobic
incubation for 3 min, the reaction was started by the addition of 10 to 20 µl of O₂-saturated phosphate buffer, and rates of oxygen uptake at 30°C with constant stirring were monitored polarographically as described previously. Uninhibited membranes were incubated in the presence of N₂-saturated, rather than CO-saturated, phosphate buffer.

Photoaction spectra were measured by comparing oxygen uptake rates in unilluminated experiments with those rates obtained when the reactions were exposed to various wavelengths of laser radiation at a fixed intensity. A Coherent Optics tunable dye laser (model 590) was operated at output wavelengths from 542 to 650 nm, with Rhodamine 6-G dye used to emit from 650 to 750 nm and sodium fluorescein used for wavelengths from 542 to 569 nm. The tunable dye laser was pumped with a Spectra Physics model 164 argon ion laser at an output of 3 W, and the dye laser output was maintained at 60 mW. Wavelength output of the laser was calibrated with an Spex 1401 double monochrometer, and radiation intensity incident on the Gilson cell was measured with a joule meter. Output from the dye laser was reflected from a front-surfaced mirror to a beam splitter which diverted approximately 4% of the beam to a Spectra Physics 1401C power meter photometer for measurements of beam intensity. The majority of the radiation was directed to a convex lens, where the laser beam was spread to a diameter of 1.4 cm at 14 cm. The radiation entered a window in an aluminum foil-covered Gilson reaction vessel and passed through the water jacket to the reaction chamber. A Yellow Springs Instruments high-sensitivity oxygen electrode was used for measurements of oxygen uptake. Operation of the laser for the 2- to 3-min period used in these experiments caused no discernible temperature increase in the reaction chamber.

Oxygen uptake rates were determined for radiation at intervals of 1 to 5 nm for wavelengths varying between 645 and 542 nm. Each experiment measured the effect of one wavelength of radiation, and the results obtained were compared with oxygen uptake rates in the presence of CO with no radiation to determine the degree of inhibition relief at each wavelength. The results were expressed as relief relative to relief at an arbitrary wavelength (550 nm) (19).

Protein estimation. The protein content was estimated by the method of Lowry et al. (27). Bovine serum albumin (Sigma) was used as a protein standard.

RESULTS

Growth on acetate and hexadecane. Acinetobacter sp. H01-N was grown under conditions of high and low aerations in a Chemapac fermentation vessel. It was observed that the rate of growth could be controlled by regulating the flow of air into the fermentation vessel (Fig. 1). Acetate-grown cells exhibited specific growth rates of 0.59 and 0.09 under conditions of high and low aerations, respectively. Hexadecane-grown cells showed specific growth rates of 0.62 and 0.09 under conditions of high and low aera-

![Fig. 1. Growth curves of Acinetobacter sp. H01-N. Symbols: O, growth with high aeration; △, growth with low aeration. Open symbols show growth at the expense of acetate, and closed symbols show growth at the expense of hexadecane.](http://jb.asm.org)
ence spectrum of membranes prepared from acetate-grown cells revealed the presence of at least two b-type cytochromes, with α peaks at 564 and 555 nm, β peaks at 536 and 529 nm, and a broad Soret peak with a maximum at 425 nm (Fig. 3). In addition, the low-temperature spectrum revealed a small peak at 630 nm. Examination of membranes prepared from cells grown at the expense of hexadecane under conditions of high aeration yielded a spectrum which was essentially superimposable on the spectrum obtained from the membranes of acetate-grown cells.

In contrast, growth of *Acinetobacter* sp. HO1-N at the expense of acetate or hexadecane under conditions of limited aeration caused easily detected changes in the cytochrome composition. The absorbance spectrum of membranes prepared from cells grown on acetate or hexadecane under conditions of limited aeration showed distinct absorption maxima at 594 and 630 nm, in addition to the double α and β peaks (Fig. 3). The new peaks were also easily discernible in room temperature spectra. These data suggested that a significant production of new respiratory pigments resulted when cells were exposed to oxygen concentrations low enough to reduce the rate of growth.

Evidence regarding the identity of cytochromes present in cells grown under low oxygen tensions was obtained by carbon monoxide difference spectra. The room temperature difference spectra of membranes prepared from hexadecane-grown cells were obtained for CO-

![Fig. 2. Difference spectra of membranes prepared from *Acinetobacter* sp. HO1-N grown with acetate under high aeration. The dithionite-reduced-minus-oxidized spectrum taken at room temperature (-----) contained a membrane protein concentration of 6.2 mg/ml. The difference spectrum obtained at 77°K (-----) contained membrane protein at a concentration of 3.8 mg/ml.](image)

![Fig. 3. Difference spectra of membranes prepared from *Acinetobacter* sp. HO1-N grown with acetate under low aeration. The spectrum obtained at room temperature (-----) contained a membrane protein concentration of 8.4 mg/ml, whereas the difference spectrum obtained at 77°K (-----) contained a membrane protein concentration of 4.7 mg/ml.](image)

treated and dithionite-reduced membranes versus dithionite-reduced membranes (Fig. 4). Absorbance maxima at 417, 540, and 571 nm, which are characteristic of cytochrome o (6), were obtained. In addition, the spectra of membranes prepared from cells grown under conditions of low aeration showed peaks at 610 and 640 nm, which could represent CO complexes of a; and d, respectively (6). The inference is commonly made that if a reduced cytochrome can form a complex with CO to give a spectrally distinct species, it can also bind with oxygen, although a demonstration of participation in oxygen metabolism requires additional evidence.

The lack of an absorbance maximum in the region of 450 nm indicated that the growth of *Acinetobacter* sp. HO1-N at the expense of hexadecane did not result in the production of detectable levels of cytochrome P-450. A CO-difference spectrum with a pronounced peak at the 450-nm region that appears after treatment with CO and reduction with dithionite represents a diagnostic characteristic for cytochrome P-450 (21, 32, 33).

A comparison of the concentrations of cytochromes in membranes prepared from hexadecane- and acetate-grown cells revealed that growth in hexadecane resulted in a doubling in the concentration of cytochrome o (Table 1). Conversely, cultivation of the organism with growth-limiting concentrations of oxygen caused substantial increases in the levels of membrane-
bound cytochromes. A 3.5-fold increase in cytochrome $b$, a 3-fold increase in cytochrome $o$, and a 6- to 10-fold increase in cytochrome $d$ and synthesis of cytochrome $a_1$ were observed when growth rates were limited to one-sixth the maximum rate by decreased aeration.

Soluble fractions prepared from Acinetobacter sp. HO1-N did not contain detectable levels of hemoprotein, even when the extracts had been concentrated 20-fold by ultrafiltration.

**Photoaction spectra.** Further evidence supporting the identification of cytochromes $o$, $a_1$, and $d$ in Acinetobacter sp. HO1-N was obtained by the use of photochemical techniques. A photoaction spectrum of membrane-bound cytochromes prepared from Acinetobacter sp. HO1-N grown at the expense of acetate with high and low aeration is shown in Fig. 5. With NADH, succinate, or ascorbate-TMPD as electron donors, relief of CO-inhibited oxygen uptake was characterized by three peaks in the region of 540 to 645 nm. The peaks at 570, 595, and 630 nm in the spectrum of membranes prepared from cells grown with limited aeration corresponded to the $a$ bands of photoaction spectra for cytochromes $o$, $a_1$, and $d$, respectively (6). This photochemical evidence established the identity of cytochromes $o$, $a_1$, and $d$ as potential terminal oxidases in Acinetobacter sp. HO1-N. A diminished contribution to respiration by cytochromes $a_1$ and $d$ was observed in the photoaction spectrum of membranes prepared from cells grown under high aeration. Illumination of uninhibited membranes caused no measurable change in the rate of oxygen uptake.

**Oxygen uptake and dehydrogenase activity.** The oxidation of a number of electron donors by membranes prepared from acetate- or hexadecane-grown cells under conditions of high or low aeration is summarized in Table 2. A comparison of the rates of oxidation by membranes prepared from the various sources revealed that neither growth substrate nor aeration rate caused significant changes in $O_2$ uptake. The most rapid oxidation of TMPD and NADH occurred when membranes prepared from $O_2$-limited cells grown on acetate were used, whereas $O_2$-limited growth on hexadecane produced the highest levels of succinate oxidase activity. Reduced cytochrome $c$ and DCIP were not oxidized by membranes prepared from Acinetobacter sp. HO1-N. Soluble fractions contained no oxidase activities, and addition of sol-

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**TABLE 1. Cytochrome composition of membranes**

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Aeration rate</th>
<th>$b$</th>
<th>$o$</th>
<th>$d$</th>
<th>$a_1$</th>
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<tbody>
<tr>
<td>Acetate</td>
<td>High</td>
<td>0.16</td>
<td>0.18</td>
<td>0.03</td>
<td>T</td>
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<tr>
<td></td>
<td>Low</td>
<td>0.56</td>
<td>0.17</td>
<td>0.19</td>
<td>T</td>
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<tr>
<td>Hexadecane</td>
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<td>0.14</td>
<td>0.10</td>
<td>T*</td>
<td>ND*</td>
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<tr>
<td></td>
<td>Low</td>
<td>0.48</td>
<td>0.31</td>
<td>0.17</td>
<td>0.01</td>
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* Aeration rates are as described in the text.
* T, Traces.
* ND, Not detectable.

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**Fig. 4.** Carbon monoxide difference spectrum of membranes prepared from Acinetobacter sp. HO1-N grown on hexadecane under conditions of low aeration. The sample cuvette contained dithionite-reduced, CO-treated membranes, and the reference cuvette contained dithionite-reduced membranes.
A report that growth of *H. parainfluenzae* under oxygen-limited conditions resulted in increased levels of dehydrogenase activities (46) prompted an examination of dehydrogenase activities in *Acinetobacter* (Table 3). Membranes prepared from cells grown with high and low aeration exhibited only small differences in NADH and succinate dehydrogenase activities.

Inhibition analysis. The effects of inhibitors on electron transport can provide data concerning the composition of the electron transport chain (13). Membranes prepared from *Acinetobacter* sp. HO1-N were exposed to various concentrations of selected inhibitors, and the rates of oxidation of NADH, succinate, and TMPD were determined (Table 4). The inhibitor concentrations causing 50% inhibition were extrapolated from plots of percent activity versus inhibitor concentration. Antimycin A, rotenone, and HOQNO were not particularly effective inhibitors of oxygen consumption. One unusual feature of antimycin A inhibition was an effect on the oxidation of TMPD. TMPD oxidation by extracts from *P. putida* and *Azotobacter vinelandii* is not inhibited by similar concentrations of antimycin A (19, 43). Succinate and NADH oxidase activities were inhibited by HOQNO, but TMPD oxidase activity was unaffected by this inhibitor. Only small differences in sensitivity to a particular inhibitor were noted when data concerning membranes prepared from cells grown under high or low aeration were compared.

A significant difference in the inhibitory effect of cyanide was observed with membranes prepared from cells grown under high or low aeration (Fig. 6). Oxidations of NADH, succinate, and TMPD were inhibited to a similar extent in both types of membranes. However, membranes prepared from cells grown with limited aeration were more resistant to inhibition by cyanide. Oxygen uptake was inhibited 50% by 35 to 40 

<table>
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<tr>
<th>Table 2. Oxidase activities of membranes</th>
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<td>Electron donor</td>
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<td>Succinate</td>
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<tr>
<td>NADH</td>
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<tr>
<td>Ascorbate-TMPD</td>
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<tr>
<td>Ascorbate-DCIP</td>
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<td>Ascorbate-cytochrome c</td>
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*Standard deviation of three determinations.


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<th>Table 3. Dehydrogenase activities of membranes</th>
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<td>Electron donor</td>
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<tr>
<td>NADH DCIP</td>
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<tr>
<td>NADH Cytochrome c</td>
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<tr>
<td>Succinate DCIP</td>
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<tr>
<td>Succinate DCIP + phenazine methosulfate</td>
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<td>Succinate Cytochrome c</td>
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<th>Table 4. Effect of inhibitors on oxygen consumption by membranes</th>
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<tr>
<td>Aeration</td>
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<td>High</td>
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<td>Succinate</td>
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<td>Ascorbate-TMPD</td>
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grown with high aeration were examined, whereas greater than 300 µM cyanide was required for 50% inhibition of preparations from cells grown with limited aeration. Concentra-
tions of cyanide as high as 3 mM were necessary for complete inhibition of oxidase activities.

**DISCUSSION**

Absorbance and photoaction spectra of *Acinetobacter* membranes gave evidence for the presence of at least one b-type cytochrome in addition to cytochrome o and small amounts of cytochrome d. When growth was limited by decreasing the dissolved oxygen concentration, increased levels of cytochromes b, o, and d and synthesis of cytochrome a were observed. This response is similar to that found in *H. parainfluenzae* (45), *E. coli* (31), *P. vulgaris* (6), and *K. aerogenes* (16). For each of these species, there is photochemical evidence that both cytochromes d and o can function as terminal oxidases. No significant differences in the cytochrome composition of membranes prepared from cells grown at the expense of acetate or hexadecane could be detected. This finding supports the conclusions of Asperger et al. (4) in their work with *A. calcoaceticus*.

An examination of the data concerning oxidase and dehydrogenase activities of membranes prepared from *Acinetobacter* sp. HO1-N indicated that neither hydrocarbon growth nor limited aeration stimulated increases in enzymatic activity. Dehydrogenase activities were similar in magnitude to those measured in other microorganisms (24, 25), and the specific activities were not significantly altered by growth substrate or aeration rate. Measurements of NADH and succinate dehydrogenase activities from *Paracoccus denitrificans* have also shown that oxygen concentration during growth had no clear effect on the specific activities of these enzymes (36).

Oxidase activities were also unaffected by hydrocarbon growth or low aeration rates. No significant differences were noted for the oxidation rates of NADH, succinate, or TMPD by membranes prepared from *Acinetobacter* grown under the various conditions. The oxidase rates were similar to those measured in aerobically grown *Rhodopseudomonas palustris* (25), *P. putida* (43), and *E. coli* (37).

The lack of an increase in oxidase activities reflecting an increase in cytochrome composition is not peculiar to *Acinetobacter*. Oxidase activities in *E. coli* (32), *P. denitrificans* (38), and *Aerobacter aerogenes* (16) have been reported to be unaffected by increases in the cytochrome composition of the cells. This has been considered an indication that the dehydrogenase reactions are the rate-limiting step in respiration (1). The low dehydrogenase activities measured in membranes prepared from *Acinetobacter* tend to support the conclusion that respiration rates in this organism were also limited by the dehydrogenase.

Data concerning the influence of hydrocarbon growth on the levels of respiratory proteins in *Acinetobacter* should be interpreted with caution. The relative amounts of the respiratory proteins remained constant when measured per unit of membrane protein, but there is strong evidence that hydrocarbon growth causes a sig-
significant increase in the amount of membrane. Previous studies with this organism have documented amounts of lipid phosphorous and membrane protein two to three times greater in cells grown at the expense of hexadecane (22, 28, 39). These experimental findings suggest that the total levels of respiratory proteins in *Acinetobacter*, when calculated on the basis of units per whole cell, are increased at least threefold by growth on hexadecane. This consideration relates further to the induction of intracytoplasmic membranes in *Acinetobacter* by growth on hexadecane (22, 23, 39). If these specialized structures do not contain respiratory enzymes, their contamination of cytoplasmic membrane preparations would dilute the specific activities of respiratory proteins and mask hydrocarbon-induced increases in respiratory capacity.

The flow of electrons through the respiratory chain of *Acinetobacter* was analyzed with electron transport inhibitors. Cyanide was the only inhibitor tested whose effects were significantly influenced by the source of the respiratory particles. Membranes prepared from cells grown with limited aeration displayed less sensitivity to inhibition by cyanide. This effect is common in a number of bacteria (31, 45, 48) and has been attributed to increased synthesis of a cyanide-resistant cytochrome *d* (35). Since NADH and succinate oxidations were inhibited to a similar extent by the same concentration of cyanide, regardless of the source of respiratory particles, electrons abstracted from these substrates appear to follow the same route through the electron transport chain. This inference is supported by data obtained with photorelief of carbon monoxide inhibition. Identical photoaction spectra were measured with both NADH and succinate as electron donors, again indicating a similar pattern of electron flow.

The observation of TMPD oxidase activity in membrane preparations derived from *Acinetobacter* was surprising, since this organism has been characterized as oxidase negative (5). Whole cells would not measurably oxidize TMPD and could not be stimulated to do so by treatment with toluene. However, membrane preparations would oxidize TMPD at one-half to one-third the rate of NADH oxidation, which was significantly greater than the autooxidation rate of ascorbate plus TMPD. Baumann et al. proposed that a positive oxidase test demonstrates that the organism possesses a cytochrome *c* component as part of its electron transport system (5). These workers also showed a lack of spectrally detectable cytochrome *c* in six typically oxidase-negative strains of *Acinetobacter* (5). Our studies also failed to detect the presence of cytochrome *c* in *Acinetobacter* sp. H01-N. Recent studies concerning the functional organization of electron transport chains have placed cytochrome *c* at the outer surface of the cytoplasmic membrane (15, 18). If this model is correct, the requirement for cytochrome *c* in a positive oxidase reaction may be related to the location of this protein near the cell surface.

Further analysis of TMPD oxidation by membranes prepared from *Acinetobacter* revealed a number of differences when compared to data from studies of oxidase-positive organisms. Cyanide inhibited TMPD oxidation by *Acinetobacter* membranes to approximately the same extent as it did NADH and succinate oxidations. In addition, membranes containing elevated levels of cytochrome *d* demonstrated an increased resistance to cyanide inhibition of TMPD oxidation. This is in direct contrast to findings obtained with a number of other organisms. Potassium cyanide at concentrations sufficient to inhibit completely TMPD oxidation by extracts of *P. putida* would only inhibit NADH and succinate oxidations by 30% (43). Membranes prepared from *P. putida* containing high levels of cytochrome *d* exhibited increased resistance to cyanide inhibition of succinate and NADH oxidations, but not TMPD oxidation (18). The oxidation of TMPD is also confined to a cyanide-sensitive branch of respiratory systems in *Beneckea natriegens* (44) and *A. vinelandii* (19). The results obtained in the present study of *Acinetobacter* suggest that electrons from TMPD are not limited to a particular branch of the respiratory pathway. Photoaction spectra of *Acinetobacter* membranes with TMPD as the electron donor showed relief peaks at wavelengths corresponding to all three terminal oxidase cytochromes, again suggesting that TMPD may be oxidized by all of the terminal oxidase in *Acinetobacter*.

Based on results of the present study, the electron transport chain shown in Fig. 7 is proposed for *Acinetobacter* sp. H01-N. NADH and succinate donate electrons via routes presently unknown to the respiratory system. The isolation of a ubiquinone from *Acinetobacter* (28), the ability of crude extracts to reduce menadione in the presence of NADH (Finnerty, unpublished data), and the inhibitory effects of HQNO on the oxidations of NADH and succinate implicate ubiquinone as an essential intermediate in the respiratory system. No order of components in the chain has been demonstrated yet, but their arrangement probably forms the basis of electron flow to the oxidases *o, d*, and possibly *a*.1
The multiple terminal oxidases of the electron transport system proposed in Fig. 7 provide alternative routes for the flow of electrons to oxygen. Little is known at present about factors which might favor respiration through one terminal oxidase over the others. Results from cyanide inhibition studies led Pudek and Bragg to conclude that, when present in E. coli, both cytochrome o and d function simultaneously as oxidases (36) and that the relative flow of electrons through these cytochromes is a function of their pool size in the membrane. However, a recent study on the kinetics of oxygen binding by cytochromes o and d in E. coli indicated that ambient oxygen concentrations can regulate the contribution to respiratory activity by either of these cytochromes (37).

The respiratory function of cytochrome a₁ in Acinetobacter is uncertain. Only trace amounts of this pigment were measured, although concentrations did seem to increase in response to growth of the organism under oxygen-limiting conditions. Photoaction spectra gave evidence that cytochrome a₁ in Acinetobacter is potentially a terminal oxidase, but photoaction spectra of the H. parainfluenzae respiratory chain showed a peak of relief corresponding to cytochrome a₁, even though Smith et al. demonstrated that this cytochrome a₁ is not kinetically competent to support respiration (41). Cytochrome a₁ in E. coli is also not considered to contribute to observed respiration rates (14). However, in certain bacteria such as Acetobacter species, cytochrome a₁ is thought to function as a terminal oxidase (6, 30).

The electron transport system proposed here for Acinetobacter is similar in many respects to the respiratory system in Achromobacter, as reported by Arima and Oka (2). Achromobacter also lacks a c-type cytochrome, and electron flow does not appear to be restricted to a particular branch of the respiratory pathway. In the Achromobacter model, terminal oxidases o, d, and a₁ are all functional, but no photochemical evidence was given to support this contention.

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LITERATURE CITED


