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# COMPOSITION AND PROPERTIES OF THE MEMBRANE-BOUND RESPIRATORY CHAIN SYSTEM OF *MICROCOCCUS DENITRIFICANS*

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## SUMMARY

1. The respiratory chain system of *Micrococcus denitrificans* closely resembles that of mammalian mitochondria; it includes cytochromes $a + a_3$, two *c*-type and at least one *b*-type cytochrome and DPNH- and succinate dehydrogenases. All of the cytochromes are membrane-bound in preparations from aerobically grown bacteria. Ubiquinone is also localized in the membrane fraction. Although an *o*-type cytochrome may be present, there is no evidence that it is a functional part of the electron transport chain. The DPNH- and succinoxidase activities show sensitivities to inhibitors entirely similar to those of the mammalian system.

2. The DPNH- and succinate dehydrogenases appear to be distinct entities with different sensitivities to the kind and concentrations of ions in the reaction medium and to inhibition by treatment of membranes with detergent. Both dehydrogenases react with the same cytochrome system, and in most circumstances the dehydrogenases are the rate-limiting steps in the oxidation of the substrates.

3. *M. denitrificans* can modify the relative amounts of the different cytochromes present according to the conditions of growth; the content of *b*- and *c*-type cytochromes is higher and the *a*-type lower in cells grown anaerobically with nitrate than in cells grown aerobically. The rates of electron transfer are high in cells grown under all conditions tested. There is no evidence that the respiratory chain system is composed of fixed units with precise stoichiometry.

4. The DPNH- and succinoxidase activities of relatively intact membranes are stimulated markedly by freezing and thawing or exposure to sonic oscillation. Detergents can have both stimulatory and inhibitory effects. The data give evidence of the nature of orientation of the respiratory chain pigments on the membrane.

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## INTRODUCTION

The respiratory chain system on membranes isolated from *Micrococcus denitrificans* resembles that of heart mitochondrial membrane fragments in having the ability to oxidize rapidly DPNH, succinate and mammalian cytochrome $c^{1-3}$. The latter activity has been found in extracts of only a few bacterial species[4]. Further

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Abbreviations: $Q_{10}$, ubiquinone; NQNO, 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide.

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studies reveal additional similarities of the *M. denitrificans* system to the mammalian one: similar complements of cytochromes are present, and the bacterial respiratory chain is sensitive to all of the inhibitors of the mammalian system, including antimycin A and rotenone. Ubiquinone ($Q_{10}$), which is a component of the mitochondrial membranes, can also be extracted from the bacterial membranes.

The respiratory chain system of *M. denitrificans* also resembles that of some other bacterial species in that the absolute and relative amounts of the constituent pigments vary widely with changes in the growth conditions and the period of growth. For example, the bacteria can be grown with oxygen or nitrate as the terminal electron acceptor[5], and the cytochrome complements of the membranes from cells grown with the two different acceptors are qualitatively similar, but the total and relative amounts of the individual cytochromes are quite different. In spite of the relative deficiencies of cytochromes under some conditions of growth, electron transport rates are high in membranes from cells grown under all conditions tested.

*M. denitrificans* are easy to grow in good yield and are lysozyme-sensitive if harvested under proper growth conditions[6]. Relatively intact membranes can be isolated from osmotic lysates of lysozyme-treated cells[6]; this procedure avoids the disruption of the membranes which results from the more drastic procedures often required to break bacterial cells. The DPNH- and succinate oxidase activities of the relatively intact membranes were found to be strongly influenced by the kind and concentration of ions in the suspending medium. In addition, treatment of the membranes with detergents or exposure to sonic oscillation or freezing and thawing can produce marked stimulation of the DPNH- and succinate oxidases. The various data give suggestive evidence about the orientation of the electron transport chains on the membranes.

## METHODS

### *Culture of bacteria and preparation of membrane fractions*

*Micrococcus denitrificans* ATCC 13543 was grown either aerobically without nitrate or anaerobically in the presence of nitrate as described previously[6]. Treatment of the bacteria with lysozyme (EC 3.2.1.17) and isolation of the membrane fraction from osmotic lysates of the lysozyme-treated cells were based on earlier studies[6], employing either bacteria grown aerobically at 30° for 11.5 to 12.5 h (absorbance (turbidity) between 0.35 and 0.5 at 550 mμ measured in a Coleman Junior spectrophotometer) or from anaerobically grown cells harvested after growth for 20 h at 28–30° (absorbance at 550 mμ between 0.5 and 0.7).

Bacteria grown aerobically for 12 h can be completely lysed after treatment with lysozyme and the cytochrome-rich membrane fraction (RR) easily separated after centrifugation of the lysate at 30000 × *g* for 40 min[6]. Not all of the cells of the 20-h anaerobically grown culture are lysed after lysozyme treatment, and the residues collected on centrifugation are contaminated with intact cells. Thus it is usually necessary to wash the membrane fraction RR from anaerobically grown cells two or three times to rid it of whole cells and a hard-packed white residue[6]. The membrane fraction RR was suspended in 0.05 M phosphate buffer, pH 7.0, in concentrations of 5–10 mg protein/ml; small aliquots were used for enzymatic studies as described below.

*Absorption spectra*

Difference spectra[7] were measured in a Cary Model 14 recording spectrophotometer equipped with a more intense light source with the following membrane suspensions:

(a) Anaerobic *minus* aerobic difference spectra were obtained by comparing anaerobic samples containing DPNH or succinate with aerobic suspensions without added substrate in the reference cuvette. The suspensions contain little or no endogenous substrates and the cytochromes are all in the oxidized form, as shown by the absence of change in absorption spectrum on addition of ferricyanide.

(b) Suspensions to which a few grains of solid $Na_2S_2O_4$ were added were compared with either aerobic suspensions or with anaerobic suspensions containing DPNH or succinate.

(c) CO difference spectra were measured either by bubbling a dithionite-reduced sample with CO for 60 sec and comparing this with a similarly reduced sample or by adding CO to an anaerobic sample containing substrate in a Thunberg-type cuvette and comparing this with a similar anaerobic suspension. The Thunberg cuvettes were evacuated and filled with $N_2$ alternately five times before adding the substrate and then filling with CO at atmospheric pressure.

*Measurement of respiration*

$O_2$ uptake was followed polarographically with a Clark oxygen electrode[8] at 25° after addition of DPNH (1 mM) or succinate (10 mM) to suspensions of membranes in phosphate buffer, pH 7.6, $I = 0.2$, except when testing the effect of ionic strength on activity. When potential inhibitors were tested, they were added as small volumes (less than 25 $\mu$l) of strong solutions in water or ethanol. Equivalent volumes of ethanol had no effect on the respiration.

*Isolation and identification of ubiquinone.* Methanol extracts of whole cells or membrane fractions were treated as described by BISHOP, PANDYA AND KING[9] and ubiquinone isolated after alumina chromatography. After taking the extracts up in ethanol, the quinone content was estimated by measuring the change in absorption at 275 m$\mu$ upon addition of sodium borohydride[10]. The nature of the side chain was ascertained by reversed phase chromatography using liquid paraffin as the stationary phase and dimethylformamide as mobile phase[11]. As standard markers $Q_6$ and $Q_{10}$ were obtained from Sigma Chemical Co., and $Q_{10}$, $Q_9$, $Q_8$ and $Q_7$ were isolated from *Rhodospirillum rubrum, Pseudomonas aeruginosa, Escherichia coli* and *Chromatium* respectively, by chromatographing petroleum ether extracts on Eastman Kodak silica gel plates, using 5 % diethyl ether in petroleum ether.

*Membranes were exposed to sonic oscillation* by placing the probe of a Branson sonifier S110 close to the bottom of a 30-ml Pyrex beaker containing 5 ml of a suspension of membranes (7–10 mg protein per ml) and tuning the instrument to maximal output using Step 5. With the beaker in slush ice and with 1-min periods of cooling between each 10 sec of exposure to sonic oscillation, there was very little heating of the suspension.

*Protein content of the membrane fractions* was estimated by the biuret method[12] in the presence of 0.1 % sodium deoxycholate, making a correction for the slight turbidity of the samples by subtracting a blank made with 3 % NaOH in place of the biuret reagent.

### *Chemicals*

Analytical reagents were used where available. The phosphate buffers were prepared from mixtures of $Na_2HPO_4$ and $KH_2PO_4$. Antimycin A, 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide (NQNO) and DPNH, Type III, were obtained from the Sigma Chemical Co.; 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) was a gift from Dr. J.W. LIGHTBOWN; the thenoyltrifluoroacetone was a Fisher product; rotenone was purchased from K and K Laboratories and amobarbital from the Southeastern Biochemical Corp. Sodium succinate was recrystallized from hot water by the addition of ethanol.

## RESULTS

### *The distribution of protein and cytochromes in different fractions of the lysates*

(*a*) *Lysates from bacteria grown aerobically for 12 h.* The supernatant fluids obtained on centrifugation of the osmotic lysates at 30000 × *g* for 45 min contain 75–85 % of the cell protein, and low and variable amounts of cytochromes are present, usually between 0 and 15 % of the cytochrome content of the lysates. The cytochromes of the supernatant fluids can be sedimented by centrifugation at 140000 × *g* for 1 h, indicating that they are bound to membrane fragments of small size (ribosomes are also sedimented at this centrifugal force). The cytochrome pattern of the small fragments is similar to that of the membrane fraction sedimented at 30000 × *g* (Fraction RR), although in some instances it appears to have a relative enrichment of *b*- and *c*-type cytochromes. The heavy white fraction (WR) may also contain small amounts of protein and cytochromes, due to difficulties in separating the two insoluble residues, but the WR fraction can be washed free of protein[6]. Since only occasional intact cells can be found in the lysates, it seems certain that all of the cellular cytochromes are membrane-bound.

(*b*) *Lysates from 20-h anaerobically grown cells.* These lysates can be separated into the same fractions obtained from aerobically grown bacteria, although the membrane fraction must be subjected to more washes to remove the intact bacteria (see METHODS). The supernatant fluid from centrifugation of the lysates of anaerobically grown cells at 140000 × *g* differs from that obtained from aerobically grown bacteria in that it contains several pigments not present in the latter: (1) a *c*-type cytochrome with properties similar to one isolated from this organism by VERNON[1]; (2) a blue protein[13]; (3) a brown pigment resembling a nitrite reductase isolated from *Pseudomonas aeruginosa* by OKUNUKI *et al.*[14]; and (4) a pigment which binds CO to form a compound with an absorption spectrum like the CO compound of cytochrome *o*. These soluble pigments will be described in subsequent papers.

### *The cytochromes of membrane preparations from aerobically and anaerobically grown cells*

Reduced *minus* oxidized difference spectra of membranes from both aerobically and anaerobically grown bacteria show peaks typical of cytochromes $a + a_3$ (peaks around 600–610 and 440 mμ), *b*-type cytochromes (peaks around 560 and 430 mμ) and *c*-type cytochromes (peaks around 550 and 415 mμ), as seen in Fig. 1. The absorption peaks of the *b*- and *c*-type cytochromes fuse in the Soret region of the spectrum (around 420–430 mμ). The data of Table I show that the positions of the peaks in the difference spectra of the two kinds of preparation are at similar wave-

## TABLE I

POSITION OF PEAKS IN REDUCED *minus* OXIDIZED DIFFERENCE SPECTRA OF MEMBRANE FRAGMENTS FROM AEROBICALLY AND ANAEROBICALLY GROWN BACTERIA

Difference spectra were recorded as described in METHODS. Identical anaerobic *minus* aerobic difference spectra were obtained with succinate and with DPNH as substrate. The difference in absorption spectrum between preparations containing dithionite and those made anaerobic with substrate represents cytochromes not reducible *via* substrate. Numbers marked with asterisk refer to shoulders on main peaks.

| | | *Positions of peaks* (*m$\mu$*) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Fragments from cells grown aerobically for 12 h | Anaerobic with substrate *minus* aerobic | 429 | 446 | 524 | 531* | 554 | 561* | | 607 |
| | Dithionite *minus* aerobic | 430 | 445 | 524 | 530* | 553.5 | 558.5 | | 607 |
| | Dithionite *minus* anaerobic with substrate | 432 | | | 530 | | 560 | 566.5 | |
| Fragments from cells grown anaerobically for 20 h | Anaerobic with substrate *minus* aerobic | 426.5 | 443* | 524 | 530* | 552.5 | 560* | | 610 |
| | Dithionite *minus* aerobic | 427 | 443* | 524 | 530* | 552.5 | 560 | | 610 |
| | Dithionite *minus* anaerobic with substrate | 431 | | | 530 | | 560 | 566* | |

## TABLE II

A COMPARISON OF THE CONTENT OF *a*-, *b*- AND *c*-TYPE CYTOCHROMES IN MEMBRANE PREPARATIONS FROM AEROBICALLY AND ANAEROBICALLY GROWN BACTERIA

The content of cytochromes $a + a_3$ is expressed as the difference in absorbance between 607 (or 610) and 630 m$\mu$ in the anaerobic *minus* aerobic difference spectra; that of *c*-type cytochromes as the difference between the absorption peak around 553 and 540 m$\mu$ and that of *b*-type as the difference between the peak around 560 and 575 m$\mu$. The difference between 560 or 565 m$\mu$ and 575 m$\mu$ in the dithionite *minus* anaerobic difference spectrum represents the *b*-type cytochromes reduced by dithionite, but not *via* substrate. The values are calculated for suspensions containing 1 mg of protein per ml for a typical preparation from aerobically grown and one from anaerobically grown bacteria.

| | *Reduced anaerobically with substrate* | | | *Reduced only by dithionite* | |
|---|---|---|---|---|---|
| | $a + a_3$ | *c-type* | *b-type* | *560–575 m$\mu$* | *565–575 m$\mu$* |
| Aerobically grown | 0.0083 | 0.025 | 0.017 | 0.0073 | 0.0066 |
| Anaerobically grown | 0.003 | 0.055 | 0.047 | 0.012 | 0.0081 |
| Anaerobically grown/aerobically grown | 0.36 | 2.2 | 2.7 | 1.6 | 1.2 |

lengths, except for a shift of several mμ in the position of the peaks of cytochromes $a + a_3$. However, the ratio of the *b*- and *c*-type cytochromes to the *a*-type is much greater in the preparation from anaerobically grown cells as is also the content of cytochromes related to membrane protein (Table II). Identical anaerobic *minus* aerobic difference spectra are obtained with DPNH or succinate as substrate with both types of membrane preparation. After the suspensions become anaerobic with either substrate, the peak in the difference spectrum around 560 mμ increases very slowly with time and that in the Soret region shifts slowly from 424 mμ towards 430 mμ; this change takes place rapidly on addition of dithionite. The increased reduction by dithionite, shown in the difference in absorption spectra between dithionite and substrate reduced preparations (Fig. 1), yields absorption peaks at 560 and 565 mμ, giving the appearance of a combination of two *b*-type cytochromes. The presence of two *b*-type and also of two *c*-type cytochromes in these bacteria was previously suggested by isolation experiments of KAMEN AND VERNON[15] and of VERNON[1] and the difference spectra measured by SATO[16]. Two *c*-type cytochromes are also suggested in some difference spectra which show separate peaks at 550 and 553 mμ and by the isolation of a *c*-type cytochrome with an absorption peak at 550 mμ[1,17], several mμ from the peak usually seen in this region of the difference spectra (Table I). Examination of the absorption peaks in the Soret region with a double-beam spectrophotometer also gave evidence for two *c*-type cytochromes*.

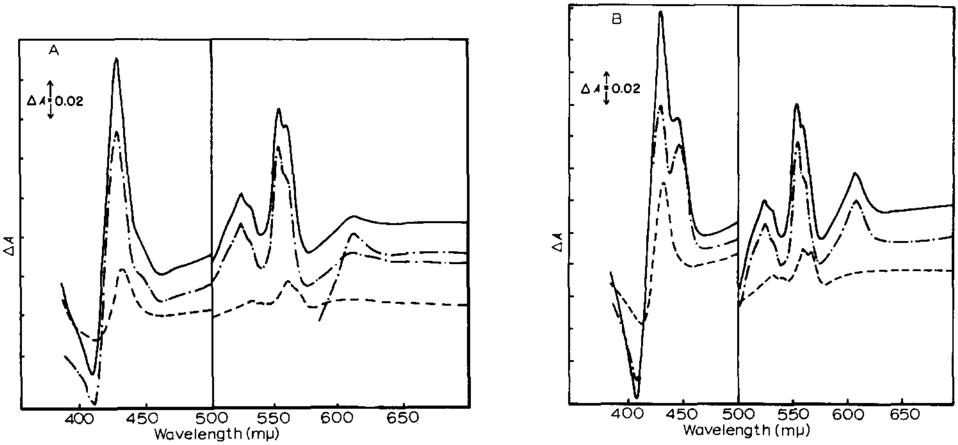

Fig. 1. Reduced *minus* oxidized difference spectra. (A) Preparation from anaerobically grown cells. (—·—·—), (anaerobic *plus* substrate) *minus* aerobic; (————), (reduced with $Na_2S_2O_4$) *minus* aerobic; (-----), (reduced with $Na_2S_2O_4$) *minus* (anaerobic *plus* substrate). The membrane suspension used for the region 350–500 mμ contained 0.33 mg protein/ml, that for the region 500–700 mμ contained 1.11 mg/ml, except for one corresponding to the trace going only from 580–700 mμ, which had 3.33 mg/ml. (B) Preparation from aerobically grown cells. Same as in (A). The preparation used for the region 350–500 mμ contained 1.0 mg protein/ml, that for the region 500–700 mμ had 3.0 mg/ml.

When membrane preparations from cells grown aerobically for 12 h are made anaerobic with either DPNH or succinate, then exposed to CO at atmospheric pressure in a Thunberg cuvette, the resulting difference spectrum with substrate-reduced

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* Some measurements of change in absorbance accompanying the change from aerobic to anaerobic conditions were made with a dual-beam spectrophotometer in the laboratory of Dr. JOHN M. OLSON (Brookhaven National Laboratories), whose kindness is deeply appreciated.

membranes in the reference cuvette is that characteristic of cytochrome $a_3$ (Fig. 2). If the preparation is made from cells grown to the stationary phase, the CO difference spectrum has in addition a shoulder at 415 m$\mu$, similar to that reported for the CO compound of *o*-type cytochromes. The CO difference spectra of anaerobically grown cells reduced with substrate or of dithionite-reduced membranes from cells grown either aerobically or anaerobically at any stage of growth show peaks of the CO compounds of both cytochromes $a_3$ and *o*; the intensity of the absorption peak at 415 m$\mu$ is increased by treating the membranes with Triton X-100 or deoxycholate, as found by PORRA AND LASCELLES[18].

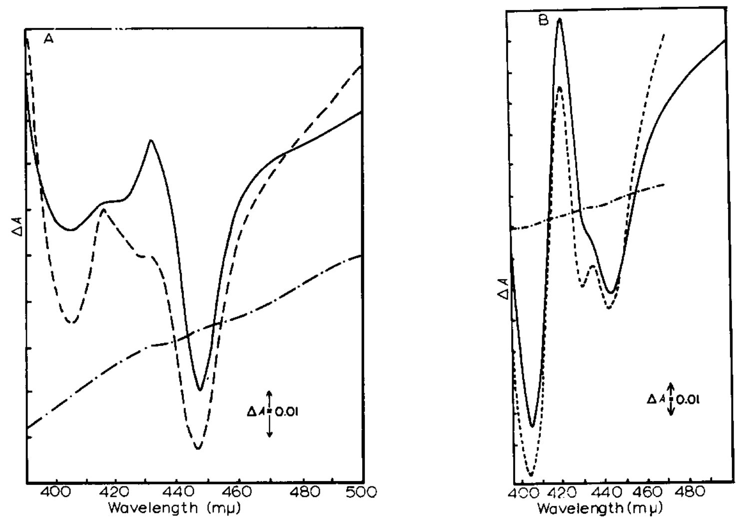

Fig. 2. (Reduced *plus* CO) *minus* reduced difference spectra. (A) Preparation from aerobically grown bacteria. (———), (reduced *plus* substrate *plus* CO) *minus* (reduced *plus* substrate); (-----), (reduced with $Na_2S_2O_4$ + CO) *minus* (reduced with $Na_2S_2O_4$); (–·–·–), baseline (both aerobic, no substrate added). The membrane suspension used contained 1.77 mg protein/ml. (B) Preparation from anaerobically grown bacteria. Same as in (A). The preparation used contained 1.84 mg protein/ml.

### *Identification of ubiquinone*

Ubiquinone ($Q_{10}$) was identified in extracts of whole cells or membranes. The quinone in the cells of a 12-h aerobically grown culture was entirely accounted for in the membrane fraction. The ubiquinone content of *M. denitrificans* (around 0.175 $\mu$mole/g dry weight of whole cells) is low compared to that of some other bacterial species[19].

### *Properties of the membrane-bound DPNH- and succinate oxidase systems*

(*a*) *Effect of pH and ionic strength.* The DPNH- and succinoxidase activities of preparations from both aerobically and anaerobically grown bacteria were optimal at pH 7.6–7.8 in phosphate buffers of constant ionic strength ($I = 0.2$), the succinoxidase of all preparations being more sensitive to changes in pH than the DPNH oxidase. The pH optima were not changed by freezing and thawing the suspensions. Increase in the molarity of phosphate buffer of pH 7.6 from 0.01 to 0.5 M had little effect on the succinoxidase activity of either untreated or frozen preparations from aerobically grown cells, but increased the DPNH oxidase activity as much as 3-fold. The same pattern is seen with preparations from anaerobically grown cells, except that the increase in DPNH oxidase is not so marked.

Replacement of phosphate buffers by Tris–chloride buffers of the same pH and ionic strength decreased both succinoxidase and DPNH oxidase activities, the effect on the former being greater, and the pH optimum for the succinoxidase shifted to 8.0–8.2, while that of the DPNH oxidase remained unchanged. Addition of KCl (or NaCl) to preparations suspended in 0.05 M phosphate buffer showed that the succinoxidase activity, but not the DPNH oxidase, is inhibited by $Cl^-$. Under the conditions used, the DPNH- and succinate oxidase activities were roughly additive.

(*b*) *The effect of physical agents and detergents.* Relatively small increases were sometimes seen in DPNH- and succinate oxidase activities just on storing the suspensions overnight at 1–4°; the activities were then stable on storage at these temperatures for a week. The DPNH- and succinoxidase activities increased around 3-fold after freezing and thawing, which could result from comminution of the relatively intact membranes with an increase in the availability of the substrates to the dehydrogenases. In agreement with this supposition is the increase of both activities by exposure to sonic oscillation. DPNH oxidase was usually increased about 4-fold after 60 sec exposure to sonic oscillation. The increase in succinoxidase was not as great and the level of activity of frozen preparations was never reached, suggesting that the succinoxidase system is both stimulated and adversely affected by this treatment. With some preparations, particularly those from anaerobically grown bacteria, increasing exposure to sonic oscillation resulted in greater discrepancies between the sum of the succinoxidase and DPNH oxidase activities and the respiration with both substrates present. This must indicate a shift of the rate-limiting step from the dehydrogenases toward the terminal oxidase end of the respiratory chain system.

Triton X-100 and other detergents have been used extensively for the purpose of fragmenting membranes. The addition of increasing concentrations of Triton X-100 up to about 0.1 mg/ml produces a pronounced decrease in the DPNH oxidase activity; then the activity decreases more slowly with further increases in Triton concentration (Fig. 3). Succinoxidase activity decreases only slightly with concentrations of Triton up to 0.1 mg/ml, then shows a marked stimulation with further increases until at a level of about 0.15 mg/ml the activity is about twice as great as that with 0.1 mg/ml of Triton. Addition of more Triton results in a rapid decline in activity (Fig. 3). Usually a very slight rise of DPNH oxidase activity is seen at the Triton concentration giving maximal succinoxidase activity.

No increase in succinoxidase activity is seen when Triton is added to frozen or sonicated membrane preparations, although the succinoxidase is more resistant to inhibition than the DPNH oxidase system.

(*c*) *Maximal DPNH- and succinoxidase activities.* DPNH- and succinoxidase rates are usually similar in untreated membrane preparations. A maximal DPNH oxidase activity of 28 mμmoles $O_2$/sec per mg protein (equivalent to a $Q_{O_2}$ of 2280) at 25° was obtained with sonicated preparations from aerobically grown bacteria. The maximal succinoxidase activity was observed with frozen suspensions; this was about 40 % of the maximal DPNH oxidase activity.

(*d*) *The effect of inhibitors.* Table III summarizes the effects of some inhibitors on the membrane-bound DPNH- and succinoxidase systems. Like the mammalian system, the respiration of all preparations is completely inhibited by 1 mM cyanide. Both DPNH- and succinoxidase are inhibited by HQNO, but the inhibition is not complete in all preparations (80–100 % inhibition with 0.016 mM). The DPNH oxidase of membranes from cells grown aerobically for 12 h is inhibited 50 % by 0.27 μg antimycin A per mg protein; similar inhibition is seen in the mammalian system with 0.07 μg per mg protein[20]. Increasing amounts of antimycin A do not always give complete inhibition of the DPNH oxidase (80–100 % inhibition observed). Con-

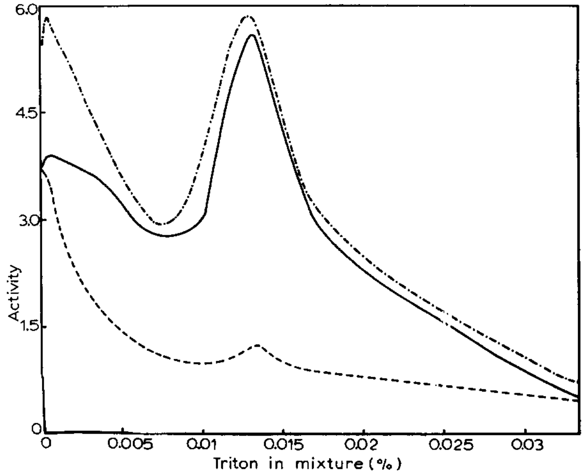

Fig. 3. The effect of Triton X-100 on DPNH- and succinoxidase activities. (-----), DPNH oxidase; (———), succinoxidase; (–·–·–), both substrates present. DPNH and succinoxidase activities are expressed as mμmoles $O_2$ uptake/sec per mg protein. In each assay membrane suspension containing 0.167 mg protein was added to 3 ml of buffer *plus* Triton X-100 as indicated.

TABLE III

THE EFFECT OF INHIBITORS ON THE DPNH- AND SUCCINATE OXIDASE ACTIVITIES OF MEMBRANE FRAGMENTS

All inhibitors except KCN and $KN_3$ were added in small volumes (several μl) of concentrated solution in 95 % ethanol. Similar volumes of ethanol had no effect on the activities tested. The assay system for the preparations from anaerobically grown cells contained 0.25 mg protein; those with preparations from aerobically grown cells contained 0.165 mg protein except for those with Amobarbital, rotenone and thenoyltrifluoroacetone which contained 0.25 mg protein.

| *Inhibitor* | *Final concn. in assay (mM)* | *Per cent inhibition* | | | |
|---|---|---|---|---|---|
| | | *Preparation from aerobically grown cells* | | *Preparation from anaerobically grown cells* | |
| | | *Succinate oxidase* | *DPNH oxidase* | *Succinate oxidase* | *DPNH oxidase* |
| KCN | 1 | 100 | 100 | 100 | 100 |
| $KN_3$ | 10 | 44 | 51 | 32 | 55 |
| Antimycin A | 0.15 (μM) | 84 | 100 | 25 | 88 |
| HQNO | 0.016 | 90 | 79 | 82 | 83 |
| NQNO | 0.032 | 61 | 71 | | |
| Amobarbital | 4.1 | 0 | 78 | 0 | 62 |
| Rotenone | 0.016 | 0 | 100 | 0 | 100 |
| Thenoyltrifluoroacetone | 20 | 100 | 51 | 100 | 37 |

siderable variation is seen in the inhibitory effects of antimycin A on succinoxidase activity. More than 80 % inhibition can be obtained with membranes from 12-h aerobically grown cells, but preparations from older cells are less sensitive. Amytal and rotenone inhibit the DPNH oxidase of all preparations, inhibition being complete with 0.016 mM rotenone. These substances do not inhibit the succinoxidase activity, but it is completely inhibited by 20 mM thenoyltrifluoroacetone.

## DISCUSSION

The cytochromes of aerobically grown cells of *M. denitrificans* are localized entirely on membranes. In osmotic lysates of lysozyme-treated cells, nearly all are associated with membranes large enough to be sedimented at 30000 × *g* for 45 min; electron micrographs show that most of these are relatively intact cytoplasmic membranes[6]. A small fraction of cytochromes is attached to smaller membrane pieces. The nature of fracturing of membranes subjected to extensive stretching is unknown; there is a suggestion that the smaller membrane fragments are enriched in *b*- and *c*-type cytochromes as compared with *a*-type, but the yield of small fragments is so low that it is difficult to be sure.

Cells grown under all conditions tested yield membranes containing cytochromes $a + a_3$, two *c*-type cytochromes and at least one *b*-type cytochrome which are reducible *via* the membrane-bound succinate and DPNH dehydrogenases. The dithionite *minus* substrate reduced difference spectra give evidence of two *b*-type cytochromes reduced only by dithionite. The presence of some cytochrome *b* on the membrane which is not enzymatically reducible is reminiscent of the system on membrane fragments from heart mitochondria, where a cytochrome *b* is reducible only with dithionite[21]. Our data do not tell whether the cytochromes *b* not reducible *via* substrate represent cytochromes displaced from their proper orientation on the membrane, which appears to be the case with the mammalian fragments. One of the *b*-type cytochromes only reducible by dithionite could be the *o*-type cytochrome apparent in dithionite-reduced membranes, since some cytochromes *o* appear to be cytochromes of *b*-type[22,23]. Since the CO spectrum of cytochrome *o* is not seen in membranes of 12-h aerobically grown cells reduced anaerobically with substrate (in Thunberg cuvettes), this pigment appears to have no function in the aerobically grown organism. The observations that some cytochrome *o* is seen in the soluble fraction of anaerobically grown cells (unpublished observations) and that deoxycholate increases the absorption due to the CO compound of cytochrome *o* suggest that the cytochrome *o* may be an altered form of some pigment.

Unlike the respiratory chain system of some other bacteria[24], but like that of heart mitochondrial membrane fragments, the cytochromes of the membranes of *M. denitrificans* are reduced to the same extent when anaerobic in the presence of either DPNH or succinate. In all preparations except for those exposed to sonic oscillation, the DPNH and succinoxidase activities are additive. Apparently the same cytochrome chain is linked to both succinate- and DPNH dehydrogenases, and the reactions of the dehydrogenases represent the rate-limiting steps in all except the sonicated preparations.

The similarity of the *M. denitrificans* respiratory chain system to the mammalian one is striking in many respects. Cytochromes $a + a_3$ are present, and the bacterial oxidase can oxidize mammalian and bacterial ferrocytochromes *c* equally rapidly[25]. Most other bacterial extracts, even some which contain cytochromes $a + a_3$, oxidize the mammalian pigment very slowly, if at all[4]. The membranes of *M. denitrificans* contain ubiquinone, identified as $Q_{10}$, as the only quinone. This is the same quinone found in mammalian mitochondria, and benzoquinones are also found in other Gram negative organisms[9]. However, the content of $Q_{10}$ in *M. denitrificans* is low compared to that of other bacteria[19] (and to the mammalian membranes) and more nearly like that of the cytochromes.

The respiratory chain system of *M. denitrificans* is entirely similar to the mammalian one in sensitivity to inhibitors and different from those of many bacterial species. It is inhibited by antimycin A and rotenone in concentrations (related to protein or cytochrome) similar to those that inhibit the mammalian system. The lack of complete inhibition of the DPNH oxidase by antimycin often seen with the bacterial system is also present in the mammalian one[20]. The greater insensitivity and variability of the succinoxidase system to inhibition by antimycin A is not understood, but again it is apparently not different from the mammalian system in this respect.

In view of the great similarity of the electron transport systems of *M. denitrificans* and mammalian mitochondria, some of the effects of comminution of the membranes are particularly interesting, since complications relating to the presence of the outer mitochondrial membrane are lacking in the bacteria. The addition of DPNH or succinate to spheroplasts stimulates the respiration only slightly. A large proportion of the membranes in the osmotic lysates appears to be empty vesicles somewhat larger than the intact cells[6] which may have "healed" themselves after rupture as other kinds of membranes do[26]. These relatively intact membranes will oxidize both DPNH and succinate to some extent, but the rates of oxidation of both are increased markedly by freezing and thawing or exposure to sonic oscillation. The substrates apparently become accessible to the dehydrogenases only after rupture of the membranes, suggesting that the dehydrogenases are localized on the "inside" of the membranes. This is in agreement with observations with *B. megaterium* (P. BROBERG, unpublished experiments) and *Bacillus subtilis*[27], where DPNH does not increase the respiration of unbroken protoplasts or spheroplasts, but is very rapidly oxidized by fragmented ones. Since soluble ferrocytochrome *c* can be rapidly oxidized by unbroken spheroplasts of *M. denitrificans* and is oxidized at nearly equal rates by untreated and frozen membrane preparations (unpublished observations), the respiratory chain system appears to be oriented across the membrane with the dehydrogenases toward the inside.

Detergents have multiple effects on the membranes. Low concentrations of Triton X-100 can stimulate the succinoxidase activity, probably by disintegration of the membranes, but the DPNH oxidase is inhibited. The DPNH dehydrogenase could be inactivated or removed from the membrane under these conditions. Similar observations with liver mitochondria were made by CHAPPELL[28]. Higher concentrations of the detergent inhibit both DPNH- and succinoxidase and thus appear to further disrupt the membranes with a loss of the integrity of the electron transport chain common to both systems. The lack of stimulatory effect of the detergent on the succinoxidase activity of sonicated preparations is in agreement with these suppositions.

The data show the DPNH- and succinate dehydrogenases to be individual pigments with quite different properties and probably different kinds of linkage to the membranes, since the effect of detergents is very different. The maximal activities that can be obtained with the membrane-bound DPNH- and succinoxidase systems of *M. denitrificans* are high in comparison with those reported for heart mitochondrial membranes or most other bacteria; this must be due to more active dehydrogenases.

The cytochrome system of *M. denitrificans* grown anaerobically with nitrate has a higher content of *b*- and *c*-type cytochromes and a lower content of *a*-type cytochromes than that of bacteria grown aerobically. (PORRA AND LASCELLES[18] also observed a higher content of *b*- and *c*-type cytochromes in bacteria grown anaerobically with nitrate. The near equality of the absorption peaks at 553 and 558 mμ in the dithionite-reduced spectra may had led KAMEN AND VERNON[15] to conclude that the cytochromes of the two types of preparation were similar.) Thus, *M. denitrificans* resemble a number of other kinds of bacteria in possessing the capacity to modify the composition of the cytochrome system according to the conditions of growth[4]. This offers additional evidence against the concept of fixed units of pigments with a definite stoichiometry as the basic elements of the electron transport system. As in other bacteria[29], large discrepancies in the molar ratios of the different cytochromes are compatible with equally rapid electron transport, as shown by the DPNH- and succinoxidase activities and by the involvement of the whole cytochrome system in the oxidation and reduction reactions with substrate and oxygen. There is a small shift of the peaks in the difference spectra of cytochromes $a + a_3$ in preparations grown aerobically and anaerobically. This could result from different ratios of the two *a*-type cytochromes in the two kinds of cells. Unfortunately the data in the Soret region of the spectrum are not good enough to verify this possibility.

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