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Tetramethyl-*p*-Phenylenediamine Oxidase Reaction in *Azotobacter vinelandii*

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It was possible to quantitate the tetramethyl-*p*-phenylenediamine (TMPD) oxidase reaction in *Azotobacter vinelandii* strain O using turbidimetrically standardized resting cell suspensions. The $Q(O_2)$ value obtained for whole cell oxidation of ascorbate-TMPD appeared to reflect the full measure of the high respiratory oxidative capability usually exhibited by this genera of organisms. The $Q(O_2)$ value for the TMPD oxidase reaction ranged from 1,700 to 2,000, and this value was equivalent to that obtained for the oxidation of the growth substrate, e.g., acetate. The kinetic analyses for TMPD oxidation by whole cells was similar to that obtained for the "particulate" *A. vinelandii* electron transport particle, that fraction which TMPD oxidase activity is exclusively associated with. Under the conditions used, there appeared to be no permeability problems; TMPD (reduced by ascorbate) readily penetrated the cell and oxidized at a rate comparable to that of the growth substrate. This, however, was not true for the oxidation of another electron donor, 2,6-dichloroindophenol, whose whole cell $Q(O_2)$ values, under comparable conditions, were twofold lower. The TMPD oxidase activity in *A. vinelandii* whole cells was found to be affected by the physiological growth conditions, and resting cells obtained from cells grown on sucrose, either under nitrogen-fixing conditions or on nitrate as the combined nitrogen source, exhibited low TMPD oxidase rates. Such low TMPD oxidase rates were also noted for chemically induced pleomorphic *A. vinelandii* cells, which suggests that modified growth conditions can (i) alter the nature of the intracellular terminal oxidase formed (or induced), or (ii) alter surface permeability, depending upon the growth conditions used. Preliminary studies on the quantitative TMPD oxidation reaction in mutant whole cells of both *Azotobacter* and a well-known *Mucor bacilliformis* strain AY1, deficient in cytochrome oxidase activity, showed this assay can be very useful for detecting respiratory deficiencies in the metabolism of whole cells.

The *para*-phenylenediamines (particularly the tetramethyl- and dimethyl-derivatives) are electron donors that have been used in microbiology primarily as qualitative (or partially quantitative) indicators of "oxidase" activity. Paul Ehrlich (3) was the first to use dimethyl-*p*-phenylenediamine and realize its significance in establishing the oxidizing capabilities of various tissues. It was Gordon and McLeod (5), however, who first recognized the usefulness of the *p*-phenylenediamines for measuring oxidizing activities in bacteria. Using a color reaction, they were able to separate bacteria into oxidase-positive or -negative groups. The colonies of oxidase-positive organisms, when exposed to *p*-phenylenediamines, develop a blue color. It was subsequently realized that all oxidase-positive bacteria possessed the "direct oxidase" of Gordon and McLeod which referred to an unidentified enzyme complex that carried out *p*-phenylenediamine oxidations (7). Kovacs (19) standardized the oxidase test using the tetramethyl derivative *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD); later, Steel (22, 23), using Kovacs method, found the test taxonomically useful in classifying bacteria of the family *Neisseriaceae* and *Pseudomonadaceae*. Since then, other modifications have been utilized, such as using compounds like the oxalate derivative of *p*-aminodimethylaniline (dimethyl-*p*-phenylenediamine) (4) or, by indirect methods, analyzing chemically for the presence of intracellular heme or cytochromes with benzidine (2).

Paralleling the use of *p*-phenylenediamine oxidation in microbiological studies was its use for quantitatively estimating cytochrome oxidase activity in tissues and subsequently in

mitochondria (18, 25, 27). The oxidation-reduction potential of TMPD (E_0' = +0.26 V at pH 7.0) indicates that electrons enter the electron transport chain at the level of cytochrome *c* (1, 9), which then allows for TMPD oxidation by the active cytochrome or terminal oxidase complex. In enzyme assays, exogenously added TMPD can be used to replace cytochrome *c*, making it possible to quantitate the terminal oxidase activity particularly in those bacterial electron transport systems where mammalian (horse heart) cytochrome *c* generally serves as a poor electron donor (17, 21).

Yamagutchi (28) first demonstrated that it was possible to measure quantitatively the terminal (or cytochrome) oxidase activity in bacterial whole cells by using *p*-phenylenediamine and dimethyl-*p*-phenylenediamine. A manometric assay system showed that both of these electron donors could be readily oxidized by a wide variety of heterotrophic bacteria. This reaction, in whole cells, was also shown to be cyanide sensitive. In this report we have continued with this type of study and examined TMPD oxidation in *Azotobacter vinelandii* whole cells. We were able to show that in organisms of this genera, which had long been suspected to possess one of the most active bacterial oxidases known, TMPD oxidation can be measured quantitatively with the high rates usually observed for most growth substrate oxidations. Unlike *Neisseria* spp., which exhibit whole cell TMPD oxidase rates that are 10 to 20 times greater than that observed for growth substrate oxidations (15), in *A. vinelandii* the TMPD oxidase rate is equivalent to the rate of oxidation observed for the growth substrate, particularly for resting cell suspensions grown on acetate, under nitrogen fixing conditions, or on nitrate serving as the nitrogen source. In both *A. vinelandii* (12, 14, 17) and *Neisseria catarrhalis* (16), the TMPD oxidase activity concentrates exclusively in the subcellular electron transport particle.

In this report it will be shown that: (i) TMPD oxidase activity can be measured in *A. vinelandii* whole cells without permeability problems; and (ii) TMPD oxidation by whole cell apparently does measure the cytochrome-dependent terminal oxidase reaction. These findings led us to conclude that the TMPD oxidase assay, as first employed by Yamagutchi (28), will probably be very useful in examining the degree to which TMPD oxidation occurs in whole cells of other oxidase-positive bacteria, and that this assay will probably become a useful technique for examining respiratory-deficient mutants in *A. vinelandii* as well as in any other organisms that have previously been shown to lack cytochrome oxidase activity.

MATERIALS AND METHODS

Preparation of resting cells. All organisms used in this study were grown at 30 C in low-form culture flasks placed on a reciprocal shaker (72 cycles per min). Routinely, *A. vinelandii* strain O cells were grown under nitrogen fixing conditions using modified Burk's medium with 1% (wt/vol) sodium acetate, or sucrose, as the sole carbon source (17). On occasion, potassium nitrate (5 mm) or nutrient broth (Difco) was added as the exogenous nitrogen source. Pleomorphic *A. vinelandii* strain O cells were induced by growth on 5% peptone (Difco) as described in detail by Vela and Rosenthal (26). *Mucor bacilliformis* was grown in a 1% peptone broth (Difco) supplemented with 0.5% yeast extract and 5% glucose, pH 5.9 to 6.0 (24).

All cells were harvested by centrifugation after growth to the late logarithmic phase, suspended in cold 0.02 M phosphate buffer, pH 7.5, and allowed to sit overnight at 4 C to reduce the endogenous intracellular reserve by starvation. The following day the cells were washed with freshly prepared, cold phosphate buffer and standardized turbidimetrically so that a 1/100 dilution of a bacterial suspension (in 0.02 M phosphate buffer) gave an optical density reading between 0.70 and 0.80 at 420 nm.

Preparation of Azotobacter electron transport particle (R_3). The *A. vinelandii* electron transport fraction (R_3) was prepared as previously described (12–14, 17).

Chemicals and chemical methods. The sources of the electron donors used in this study are: TMPD; 2,6-dichlorophenolindophenol (DCIP); and L-(+)-ascorbic acid from Eastman Organic Chemicals, Rochester, N.Y. All other chemicals were of reagent grade quality and were obtained from the usual commerical sources (12–17).

A 0.1 M solution of TMPD and a 0.04 M solution of DCIP were prepared in deionized water immediately before use and kept at 4 C in a low actinic glass test tube. A 0.1 M solution of ascorbic acid was always freshly prepared; the pH was adjusted to 6.2 with KOH. Protein concentrations were determined by using the biuret method of Gornall et al. (6).

Assay for TMPD oxidation. The conventional manometric technique, using Warburg vessels, was used to measure TMPD oxidase activity with turbidimetrically standardized resting cell suspensions. All assays for terminal oxidase activity were initiated by the simultaneous addition (from separate side arms) of ascorbate and either TMPD or DCIP. The TMPD oxidase assay was used exactly in the manner previously described (12, 14) except that the reaction was carried out at pH 6.0 at 30 C. DCIP oxidase activity was measured in the identical manner employing 2.6 mM DCIP and 6.7 mM ascorbate. The resting cell concentrations used for estimating TMPD oxidase activity ranged from 0.09 to 0.12 mg dry weight and that for DCIP oxidase

activity ranged from 0.18 to 0.21 mg dry weight.

For both the TMPD and DCIP oxidase assays, suitable controls were always incorporated into the manometric assay system to insure that ascorbate oxidation did not occur with resting cells prior to the addition of TMPD (or DCIP) in the assay, and that no chemical (non-enzymic) autooxidation reaction occurred (12) that would have obscured the terminal oxidase reaction with *A. vinelandii* whole cells.

The manometric assay technique was also employed in measuring the resting cell oxidation rates of the growth substrate, e.g., acetate or sucrose. The conditions used are identical to those previously described for the *Neisseria* studies (15) which were carried out at pH 7.5 employing a final substrate concentration of 16.7 mM. This same technique and conditions were used for measuring the rates of DL-lactate and succinate oxidation by the *A. vinelandii* R_3 electron transport particle (13, 14).

RESULTS

The metabolic patterns for acetate oxidation as well as the two electron donors, TMPD and DCIP, by standardized whole cell suspensions of *A. vinelandii* are shown in Fig. 1. The cells used in this study were grown on acetate as the sole source of carbon under nitrogen-fixing conditions. The microliters of O_2 consumed was plotted as a function of time. The kinetics show that the rate of ascorbate-TMPD oxidation is slightly higher but almost identical to that of acetate oxidation, both exhibiting high and comparable $Q(O_2)$ values (1,764 versus 1,692) that are a common metabolic feature in *Azotobacter* metabolism. This finding suggests that there are no permeability problems; reduced TMPD readily enters the cell and is oxidized at a rate comparable to that of the growth substrate. There was no detectable ascorbate-TMPD (or DCIP) oxidation in *A. vinelandii* cells inactivated by boiling and no detectable ascorbate oxidation in the absence of either TMPD or DCIP. The rate of TMPD oxidation in the absence of ascorbate is decreased [$Q(O_2)$ = 757] to less than one-half the rate as when ascorbate is present in the assay. For the other electron donor system, which is comprised of ascorbate-DCIP, the *Azotobacter* resting cells exhibited $Q(O_2)$ values of about one-half [$Q(O_2)$ = 806] of that noted for the ascorbate-TMPD or acetate oxidations. Therefore it appears that the ascorbate-TMPD oxidation capability, measured in resting cell suspensions of *A. vinelandii* strain O, truly reflects the full oxidative-respiratory potential exhibited by this organism, and there appear to be no problems concerning electron donor permeability relative to that noted for acetate metabolism

The results presented in Fig. 2 show the effects of varying the electron donor concentrations for both the ascorbate-TMPD and ascorbate-DCIP oxidation systems, again employing *A. vinelandii* resting cells as well as the electron transport particle or R_3 fraction (14, 17). The resting cells used in this study also were grown on acetate (under nitrogen-fixing conditions), and the sonic R_3 electron transport fraction was isolated from the same batch of cells. In this study the oxidation rate, expressed as the $Q(O_2)$ value, is plotted as a function of the molar concentrations of the electron donor present in the assay. The ascorbate concentration was kept constant at the 6.7 mM concentration level.

The kinetics of the ascorbate-TMPD oxidation is similar (almost parallel) between the standardized resting cell suspension (dark circles) and the R_3 electron transport fraction (open circles), with the notable exception that

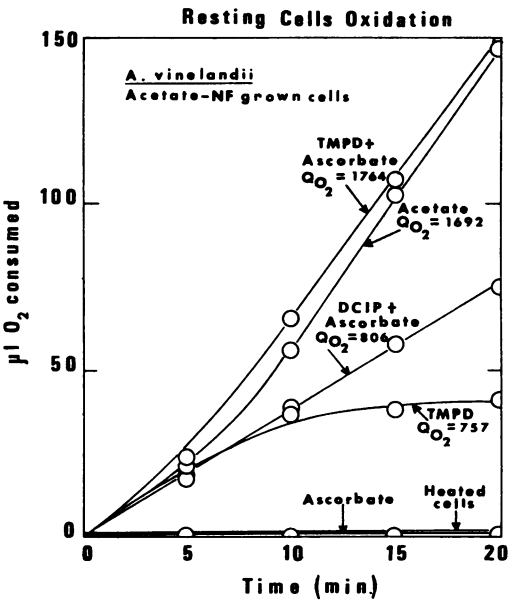

FIG. 1. *The metabolic patterns for oxygen consumption observed during the oxidation of ascorbate-TMPD, acetate, ascorbate-DCIP, and TMPD (in the absence of ascorbate) by turbidimetrically standardized resting cells of A. vinelandii strain O. Resting cells were grown on acetate under N_2-fixing conditions. The oxygen consumed (microliters of O_2 uptake) was plotted as a function of time. The standard manometric assay was carried out as described in the text. The concentration of resting cells used was 0.097 mg dry weight, which was kept constant throughout this study. The specific activities calculated for the oxidation of acetate and the various electron donor systems are shown on this figure and expressed using the conventional $Q(O_2)$ value (microliters of oxygen consumed per hour per milligram dry weight) at 30 C.*

the R_3 fraction exhibited a higher specific activity value for TMPD oxidation. This would be expected since the R_3 fraction represents a partially purified membrane preparation which contains the majority of the electron transport enzymes (12–14, 17). The similarity in the TMPD oxidation patterns that are exhibited by these two curves again suggests that there is no permeability problem regarding reduced TMPD entering the *Azotobacter* cell. The concentration of TMPD used for the standard manometric assay was 3.3 mM, which gives maximal specific activities for both resting cell oxidation as well as the oxidation carried out by the electron transport particle. The kinetics of TMPD oxidation by the *Azotobacter* R_3 fraction has already been described in detail (12).

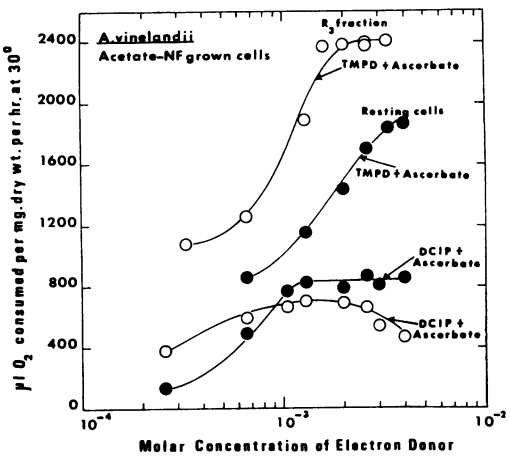

FIG. 2. *Effect of varying concentrations of electron donor on enzymatic oxidation of TMPD and DCIP by standardized suspensions of A. vinelandii whole cells (closed circles) and R_3 electron transport fraction (open circles). The manometric assay was used as described. The concentration of ascorbate was kept constant at the 6.7 mM level. The dry weight concentrations of the Azotobacter R_3 fraction used for measuring TMPD and DCIP oxidations were 0.75 and 1.5 mg/ml, respectively. The dry weight concentrations for the Azotobacter resting cells was 0.12 mg/ml for TMPD oxidation and 0.21 mg/ml for DCIP oxidation.*

The kinetics of ascorbate-DCIP oxidation between resting cells and the R_3 fraction are markedly different than that noted for TMPD oxidation. As shown by the two lower curves in Fig. 2, the DCIP oxidase activities are also substantially lower in comparison to TMPD oxidation for both resting cells (dark circles) and the R_3 electron transport fraction (open circles). At an electron donor concentration of 3 mM, the TMPD oxidation rate for resting cells was two times greater than that noted for DCIP oxidation, whereas TMPD oxidation by the *Azotobacter* R_3 electron transport particle was approximately four times greater than that observed for DCIP oxidation. For the standard manometric assay ascorbate-DCIP oxidase assay the final concentration of DCIP used was 2.6 mM.

Table 1 shows a composite summary comparing *A. vinelandii* resting cell oxidations to that of the R_3 electron transport fraction, using both these electron donors as well as various substrates, two of which (D-lactate and succinate) are known to be oxidized directly by the nonpyridine nucleotide-dependent electron transport system in *A. vinelandii* (13, 14). The *Azotobacter* resting cells and the R_3 fraction were prepared from the same batch of cells for each of the three experiments. All were grown on acetate under nitrogen-fixing conditions. The oxidation rates for the electron donors, TMPD and DCIP, are shown in a comparative relationship to the oxidation rates of some of the substrates known to be directly involved in *A. vinelandii* electron transport reactions. The results also show the extent to which variability can occur during repetitive $Q(O_2)$ estimations for both the resting cells and the R_3 electron transport fraction.

TABLE 1. *Comparative substrate and electron donor oxidation rates for A. vinelandii resting cells and R_3 electron transport fraction*

Substrate	$Q(O_2)$ values[a]					
	Resting cells			R_3 fraction		
	1	2	3	1	2	3
None (endogenous)	10	9	12	0	0	0
Acetate	1,453	2,075	1,764	0	0	0
Sucrose	12	7	11	0	0	0
D,L-Lactate	567	632	499	81	80	137
Succinate	686	729	530	454	383	334
Ascorbate-TMPD	1,851	1,841	1,840	2,582	2,747	2,559
Ascorbate-DCIP			878			669

[a] Expressed in microliters of O_2 consumed per hour per milligram dry weight at 30 C.

Acetate was oxidized readily by the resting cells, but not by the R_3 fraction which lacks necessary soluble cofactors and enzymes. As expected, sucrose was not oxidized by the resting cells or the R_3 fraction. The oxidation of this carbohydrate by whole cells is inducible. D,L-Lactate and succinate, two substrates that can be oxidized directly by the electron transport system of *A. vinelandii*, were oxidized at higher rates by the resting cells than the R_3 fraction, suggesting that the integrated intracellular system of whole cells oxidized these substrates generating other metabolizable products that could be further oxidized by other intracellular enzymes. Of interest, however, is the fact that the ascorbate-TMPD oxidation rate by the *Azotobacter* resting cell preparation was comparable to that of acetate but was approximately three- to fourfold higher than the rate observed for either D,L-lactate or succinate oxidation. As expected, the oxidation of ascorbate-TMPD by the R_3 fraction was higher than the activity noted for the ascorbate-DCIP oxidation by the standardized resting cell preparation (also see Fig. 2). Again, the DCIP oxidation rate was twofold lower than that noted for the ascorbate-TMPD oxidation rate for the *A. vinelandii* resting cells and fourfold lower in the R_3 fraction, but still this DCIP oxidation rate was higher than that noted for D,L-lactate or succinate oxidation by either of the two oxidizing systems (cells and membrane particles). Because of the lower activity rates, and difference in the oxidation kinetics (see Fig. 2), the DCIP oxidase activity was not examined further as a potential electron donor for measuring terminal oxidase activity. This electron donor has been used extensively for studying terminal or cytochrome oxidase reactions by the *Azotobacter* electron transport system (10, 11); in another instance menadione was also used for this purpose (20).

Table 2 shows comparative $Q(O_2)$ value data obtained for both ascorbate-TMPD and growth substrate oxidations by standardized resting cell suspensions of *A. vinelandii* grown under different nutritional conditions. The ascorbate-TMPD oxidation rate levels were high and comparable for resting cells grown on acetate regardless of whether or not nitrate was used as a nitrogen source or if growth had occurred under nitrogen-fixing conditions. However, resting cells prepared from cells grown on sucrose, either in the presence of nitrate or under nitrogen-fixing conditions, showed relatively low TMPD oxidation activities which were depressed when compared to the oxidase rate obtained for sucrose oxidation. This observation strongly suggests that the high sucrose oxidase rates observed for sucrose-grown resting cells (either with NO_3 or N_2 as nitrogen sources) might be mediated by a different type of terminal oxidase, or that the sucrose-grown cells had a new cell surface impermeable to TMPD. Resting cells of *A. vinelandii* grown on nutrient broth, with sucrose present, again exhibited relatively high ascorbate-TMPD oxidase rates comparable to those found for acetate-grown cells. In these cells, the TMPD oxidase rate was twofold higher than the sucrose oxidase rate, the latter also being relatively active [$Q(O_2)$ = 541], but approximately one-half the sucrose oxidase rates noted for the resting cells grown on sucrose with either N_2 or NO_3 [$Q(O_2)$ = 1,159 or 1,341, respectively] serving as the nitrogen source. The study strongly suggests that a major change in TMPD oxidase rates can occur in resting cells grown under different physiological conditions, e.g., using a different carbon source.

In Table 3, $Q(O_2)$ values are presented for ascorbate-TMPD oxidase activities for the parent *Azotobacter* strain O resting cells, and this value is compared to those obtained for some respiratory-deficient mutants. The latter represent *A. vinelandii* strain O strains that were exposed to nitrosoguanidine. Selection was made for slow growing and petite-like colonies (JIG series). Also shown are the TMPD oxidase $Q(O_2)$ values for *A. vinelandii* strain O pleomorphic cells that were chemically induced by growth on 5% peptone (26) as well as a *M. bacilliformis* strain from which a respiratory-deficient mutant was isolated and shown to be lacking the cytochrome oxidase enzyme complex (24).

The results in Table 3 show that strain O respiratory-deficient resting cells, i.e., mutant strains JIG-1 and JIG-2, exhibit lower (approximate fivefold) TMPD oxidation rates when compared to the wild-type strain O cells. This significant decrease in TMPD oxidase activity might possibly indicate a major alteration in the type of terminal oxidase formed. Pleomorphic resting cells of *A. vinelandii* also show a markedly decreased TMPD oxidase rate. Pleomorphic cells obtained after 48 h of growth show an eightfold decrease in TMPD oxidase activity, whereas pleomorphic cells obtained after 72 h of growth show a 28-fold decrease in TMPD (terminal) oxidase activity. An analysis of the electron transport components in *A. vinelandii* pleomorphic cells will be made at a future date.

Table 3 also shows the results in the quantitation of the TMPD oxidase reaction using whole cells suspensions of eukaryotic cells, specifically *M. bacilliformis*. The two *Mucor* strains were analyzed for TMPD oxidase activity

TABLE 2. *The effects of different growth components on the oxidation of ascorbate-TMPD and sucrose by resting cells of A. vinelandii*

Growth components		$Q(O_2)$ values[a]			
		pH 6.0		pH 7.5	
Carbon source	Nitrogen source	Endogenous[b]	Ascorbate-TMPD	Endogenous[b]	Sucrose
Acetate	N_2 (air)	36	1,247	2	4
Acetate	NO_3	20	1,126	8	9
Sucrose	N_2 (air)	8	132	25	1,159[c]
Sucrose	NO_3	7	181	15	1,341[c]
Sucrose	NB[d]	22	1,221	19	541

[a] Microliters of O_2 per hour per milligram dry weight at 30 C.
[b] Endogenous value represents the cellular respiration rate obtained in the absence of any added substrates or electron donor at the pH indicated.
[c] From the data of Zehner (Masters thesis, University of Houston, 1972).
[d] NB, Nutrient broth (Difco).

TABLE 3. *Ascorbate-TMPD oxidase activities for standardized resting cell suspensions of wild-type and respiratory-deficient mutants of Azotobacter vinelandii and Mucor bacilliformis*

Organisms	Growth components		$Q(O_2)$ values[a]	
	Carbon source	Nitrogen source	Endogenous[b]	Ascorbate-TMPD
A. vinelandii strain O				
Wild type	Sucrose	NB[c]	22	1,221
Mutant JIG-1[d]	Sucrose	NB	2	243
Mutant JIG-2[d]	Sucrose	NB	5	293
Pleomorphic cells[e] (48 h)	Peptone (5%)	Peptone (5%)	2	167
Pleomorphic cells[e] (72 h)	Peptone (5%)	Peptone (5%)	1	45
M. bacilliformis				
Wild type	Glucose	Peptone (1%)	8	32
Mutant AY1[f]	Glucose	Peptone (1%)	1	1

[a] Microliters of O_2 per hour per milligram dry weight at 30 C.
[b] Endogenous value represents the cellular respiration rate obtained at pH 6.0 in the absence of any added electron donor.
[c] NB, Nutrient broth (Difco).
[d] Respiratory-deficient mutants of strain O.
[e] Pleomorphism induced by nutritional control (see reference 26).
[f] Respiratory-deficient mutant of *M. bacilliformis* which lacks cytochrome oxidase (see reference 24).

(using yeast-phase cells) and one of these strains is a well-known respiratory-deficient mutant (AY1) which was previously characterized (24) and shown to lack a cytochrome oxidase enzyme complex. As shown by the data, the wild-type *Mucor* strain had a low but measurable TMPD oxidase rate. The respiratory-deficient mutant strain AY1, from *M. bacilliformis*, exhibited a TMPD oxidation rate that was barely measurable and equivalent to the $Q(O_2)$ value obtained for the endogenous respiration. Again, it appears that the TMPD oxidase assay described here will probably serve as a useful tool in quantitating respiratory deficiencies employing standardized resting cell suspensions.

DISCUSSION

It has been shown that a TMPD oxidation assay can be used to quantitatively estimate terminal or cytochrome oxidase activity in *A. vinelandii* whole cells. The whole cell TMPD oxidase $Q(O_2)$ value for *A. vinelandii* was equivalent to that of the growth substrate oxidation when an oxidizable substrate-like acetate was employed as the primary carbon source for growth. This was not the case, however, when resting cells were grown on a fermentable substrate, i.e., sucrose, utilizing either N_2 or NO_3 as the nitrogen source for growth. With the latter type resting cell preparations, there may be another terminal oxidase present, one that

exhibits high turnover rates for sucrose (but not TMPD) oxidase activity or sucrose-grown cells are impermeable to reduced TMPD (see Table 2). This type of TMPD oxidase analysis, which employed turbidimetrically standardized resting cell suspensions, also was successfully used in studies in terminal oxidase activity in various *Neisseria* spp., which are universally known to possess a strongly positive oxidase reaction (15). With the *Neisseria* spp., it was shown that the enzymic whole cell TMPD oxidase activities were 10 to 20 times more active than the growth substrate oxidation rates. This data strongly suggests that electrons (from TMPD) are capable of entering the terminal oxidase site in *Neisseria* whole cells at rates faster than the electrons internally generated by substrate oxidations. This again suggests that the TMPD oxidase assay, even in *Neisseria* sp., can be effectively used to measure terminal oxidase activity in whole cells with negligible permeability problems. This might be expected since the enzymes that would carry out TMPD oxidation would be the membrane-bound terminal oxidases that are localized on the cytoplasmic membrane. The localization of the terminal oxidase complex on the cytoplasmic membrane suggests that reduced TMPD need not penetrate far into the interior of the cell to be enzymically oxidized. This, however, does not appear to be the case for reduced DCIP, whose oxidation by *A. vinelandii* whole cells, under the conditions described and published (10, 11), appears to be restricted (see Fig. 2).

The fact that growth or environmental conditions may alter terminal oxidase activity is a well known fact and is extensively described in two review articles (8, 16a). The effect of oxygen concentration (during growth) is known to markedly alter the biosynthesis (and activity) of terminal oxidase enzymes (8), and the effect of growth substrate, whether it is capable of being oxidized directly by molecular oxygen or is fermentable, would also play a major role in the induction of terminal oxidase activity (16a). This point has already been clearly shown experimentally by the results presented in Table 2 with sucrose-grown resting cells. Finally, the phase of growth at which the cells are harvested has also been shown to be a consideration in the type of terminal oxidase formed (16a), and this difference in terminal oxidase activity might reflect on the age of cells, whose basic oxidative physiology (in the extreme form) might be reflected by data presented for the pleomorphic *Azotobacter* cells (Table 3). Pleomorphic *Azotobacter* cells oxidize TMPD very poorly in relation to the high turnover rates observed for acetate-grown whole cells harvested at the late logarithmic phase.

The usefulness of the TMPD oxidase assay, with resting cells, extends even further. It apparently can be used to analyze and select for respiratory-deficient mutants. By use of this assay it was possible to show with *M. bacilliformis* (yeast phase) whole cells that a known mutant (AY1), deficient in the cytochrome oxidase complex (24), did not have the ability to oxidize TMPD, whereas the wild-type parent readily carried out this oxidation (see Table 3). The usefulness of this test could also be extended to *A. vinelandii* cells, where it appeared that respiratory-deficient mutant resting cells had much lower TMPD oxidase rates. Very little as yet is known on the basic physiology of these respiratory-deficient *A. vinelandii* mutants.

The TMPD oxidase assay as described in this communication can serve as a useful tool to survey large number of organisms and establish the degree to which they may be respiratory deficient (or sufficient) in terminal oxidase activity. This implies, however, that such organisms would have to be oxidase positive and have as one of its major electron transport pathways a terminal oxidase that can be assayed for using the TMPD oxidation reaction described. This would exclude the oxidase-negative organisms, many of which are known to be obligately aerobic.

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