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Terminal Branching of the Respiratory Electron Transport Chain in *Neisseria meningitidis*

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The respiratory components of the envelope membrane preparation of *Neisseria meningitidis* were investigated. Oxidase activities were demonstrated in this fraction in the presence of succinic acid, reduced nicotinamide adenine dinucleotide, and ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylene-diamine (TMPD). Differences in the kinetics of inhibition by terminal oxidase inhibitors on the three oxidase activities indicated that ascorbate-TMPD oxidation involved only an azide-sensitive oxidase, whereas oxidation of the physiological substrates involved two oxidases, one of which was relatively azide resistant. Spectrophotometric studies revealed that ascorbate-TMPD donated its electrons exclusively to cytochrome *o*, whereas the physiological substrates were oxidized via both cytochromes *o* and *a*. The effects of class II inhibitors on the oxidases suggest terminal branching of the electron transport chain at the cytochrome *b* level. A model of the respiratory system in *N. meningitidis* is proposed.

Much work has been reported on the physiology of the genus *Neisseria*, but relatively little attention has been given to the energy requirements of this organism, despite the fact that the genus includes two of the primary pathogens for humans, *Neisseria gonorrhoea* and *Neisseria meningitidis*. In the reports in the past few years on the respiratory system of the genus, there is a suggestion that these organisms possess more than one cytochrome oxidase (11, 17, 20, 30, 33), but there is no definitive evidence to suggest how the cell functions with more than one oxidase. A prior report from this laboratory presented evidence for two cytochrome oxidases, *o* and *a*, in *N. meningitidis* (33), as well as a soluble *c'*-type cytochrome whose function is, as yet, unknown. In this paper we report the findings on studies of the oxygen consumption by *Neisseria meningitidis* in the presence of terminal oxidase inhibitors, as well as cytochrome reduction in the presence of various substrates. The results of this study suggest a terminal branching of the electron transport chain in *N. meningitidis*.

MATERIALS AND METHODS

Organism. The group B *N. meningitidis* (strain SD1C) was obtained from the Neisseria Repository, NAMRU, University of California, Berkeley. Maintenance of both stock and working cultures was described previously (6), as were routine examinations for strain purity. This strain readily dissociates into rough (M3) and smooth (M1) colonial types (9). Only the smooth (M1) strain was used in this study.

Cell growth and fractionation. The growth of cells in aerobic batch culture was described previously (33). Cells were harvested by centrifugation (4°C) at 10,000 × *g* for 10 min and washed once by resuspension in and centrifugation from 0.05 M potassium phosphate buffer, pH 7.5. Cells were disrupted by sonic oscillation (Sonifier Cell Disruptor W-350, Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) by subjecting cells to six treatments for 15-s intervals (tip energy, 250 W) with alternate cooling of probe and sample in an ice bath to avoid loss of enzyme activity due to heat. Cell debris was removed by centrifuging twice (4°C) at 19,000 × *g* for 20 min. The clear supernatant fraction (cell-free extract) was then centrifuged at 345,000 × *g* for 3 h (4°C) to pellet all membranous material. The top one-third of the supernatant fluid was carefully extracted, and the remaining supernatant fraction was discarded. This supernatant fluid was further cleared of residual membrane contamination by centrifuging twice (345,000 × *g*, 3 h) and keeping only the top third portion of each resulting supernatant fraction. The particulate fraction obtained after the initial high-speed ultracentrifugation was resuspended and washed twice. The particulate fraction (referred to as the envelope preparation) was resuspended to form a homogenous suspension in phosphate buffer and used for subsequent oxidase studies. Procedures for the removal and isolation of cell wall blebs (outer membrane) were described by DeVoe and Gilchrist (7). All sample preparations were kept on ice and assayed without subjecting them to freezing and thawing.

Oxidases. Oxidase activities were measured by means of a Rank polarographic oxygen cell (Rank Bros., Bottisham, Cambridge, England). All assays were carried out at 37°C in air-saturated buffer (0.05 M potassium phosphate, pH 7.5; 3 ml). Samples with appropriate dilutions were incubated in buffer to achieve temperature equilibration before the addition of substrates. Inhibitors, when applied, were added 1 min before the addition of substrates. Final concentra-

tions of substrates in a typical run were 3 mM ascorbic acid, 2 mM *N,N,N',N'*-tetramethyl-*p*-phenylene-diamine (TMPD), 30 mM succinic acid, or 2.6 mM NADH. Activity was corrected for endogenous respiration and auto-oxidation. The very small activity due to oxidation of ascorbate was routinely corrected as well. All substrates and inhibitors were prepared just before use and kept on ice. Light sensitive reagents were maintained in the dark. Water-insoluble inhibitors were dissolved in dimethyl formamide or methanol, both of which were shown to have no effect on the assay system during the time course of the assay. Oxidase activities were expressed as nanomoles of O_2 consumed per minute per milligram of protein at 37°C.

Difference spectra. Difference spectra were obtained at room temperature by procedures previously described (33). Substrates (ascorbate-TMPD, succinic acid, and NADH) were all added in saturating quantities (corresponding to concentrations used to achieve V_{max} in oxidase activities) and incubated until maximum reduction occurred. Inhibitors were added in small portions until no further changes were observed in the spectra. CO-binding studies were carried out with Perkin-Elmer UV-Visible Spectrophometer 555.

Miscellaneous assays. Protein was estimated by the method of Lowry et al. (22) with bovine serum albumin as the standard. All samples were pretreated by boiling for 10 min.

Peroxidase was determined by a method described elsewhere (31). Cytochrome *c* peroxidase was assayed by the method of Yonetani (32). Catalase was assayed by the method of Beers and Sizer (3). Succinic dehydrogenase activity was determined by the method of Kasahara and Anraku (19). NADH dehydrogenase activity was assayed by the method of Cheng et al. (5).

Chemicals. All salts, including potassium cyanide, were reagent grade (Fisher Scientific Co., Fairlawn, N.J.). TMPD was obtained from Eastman Organic Chemicals (Rochester, N.Y.). Salicylhydroxamate was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). All other reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Figure 1 shows the variation in oxidase activities for succinate, NADH, and ascorbate-TMPD during the growth cycle of *N. meningitidis*. The specific oxidase activity measured with ascorbate-TMPD increased progressively during the log phase of growth but decreased when cells entered the stationary phase. This finding is consistent with our previous observations (33) on the variation during growth of major membrane-associated cytochromes. Such variations can be reproduced by limiting the oxygen in the environment of the meningococcus (2). The oxidase activities with the two physiological substrates, i.e., NADH and succinate, during growth were the exact opposite of each other. Whereas succinate oxidase specific activity varied directly with that of ascorbate-TMPD during log-phase growth, NADH oxidase activity decreased progressively, reaching a low point as cells entered the stationary phase of growth.

The intracellular distribution of oxidase activity in the cells was investigated after fractionation of the cells (Table 1). Washed whole cells were devoid of detectable endogenous respiration, but were very active on ascorbate-TMPD,

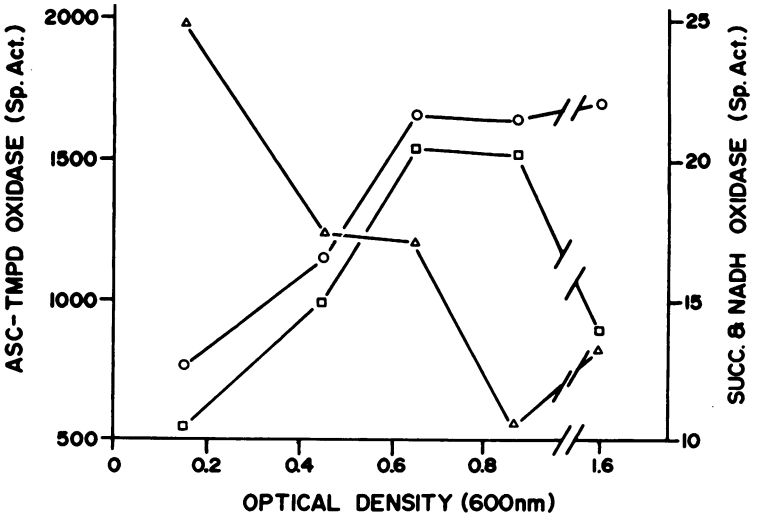

FIG. 1. *Variation in oxidase levels of meningococcal whole cells during aerobic batch culture. Cells grown to an appropriate optical density (optical densities at 600 nm of 0.16, 0.45, and 0.90 for the early-, mid-, and late-logarithmic phase of growth, respectively, and 1.60 for the stationary phase of growth) were harvested and washed, and assayed for oxidase activities. Symbols: □, ascorbate-TMPD oxidase specific activity; ○, succinate oxidase specific activity; △, NADH oxidase specific activity.*

indicating little or no barrier to the premeability of the substrate into the enzyme. The reverse was true for succinate and NADH oxidase activity, i.e., sonic treatment of the cells led to dramatic increases in the oxidase activities for both physiological substrates. As expected, membranous fractions of the cell had high specific activities for all three substrates, whereas the soluble cell fraction was devoid of activity on NADH and succinate but retained a small residual activity on ascorbate-TMPD. The results of enzyme assays on supernatant fractions were uniformly negative for succinate and NADH dehydrogenases as well (data not shown). As previously reported (8) there was also a highly active ascorbate-TMPD oxidase activity associated with isolated blebs of the outer membrane; however, neither succinate nor NADH oxidase activities were detectable in this fraction. The effects of repeated washings of the membrane fraction of the cell increased the specific oxidase activities for all three substrates, as one would expect.

TABLE 1. *Intracellular distribution of oxidases in the meningococcus at the logarithmic phase of growth*

Cell fraction	Sp act		
	Ascorbate-TMPD oxidase	Succinate oxidase	NADH oxidase
Whole cells	1,318.3	23.1	12.0
Cell wall bleb preparation	494.1	<0.2	<0.2
Sonically disrupted cells	1,334.1	99.9	98.1
Cell-free extract	606.9	80.1	72.0
Supernatant fraction, 1st ultracentrifugation	87.9	<0.2	6.0
Supernatant fraction, 2nd ultracentrifugation	60.9	<0.2	1.7
Supernatant fraction, 3rd ultracentrifugation	63.0	<0.2	<0.2
Particulate fraction	1,881.0	150.9	92.0
Particulate fraction, washed once	2,063.1	153.9	148.2
Particulate fraction, washed twice (envelope preparation)	2,222.1	200.1	184.1

To further characterize the oxidase activities in the cell, kinetic studies were carried out in the washed envelope (membrane) preparations. The oxidases on all three substrates obeyed Michaelis-Menton kinetics of reactions. The kinetic constants given in Table 2 were obtained from Eadie-Hofstee plots (v versus $v/[s]$) (10). Oxidase activities on all three substrates exhibited similar sensitivities to inhibition with cyanide and hydroxylamine; however, the ascorbate-TMPD oxidase was markedly more sensitive to inhibition by azide than the other two substrates (Fig. 2). These findings suggest the presence of at

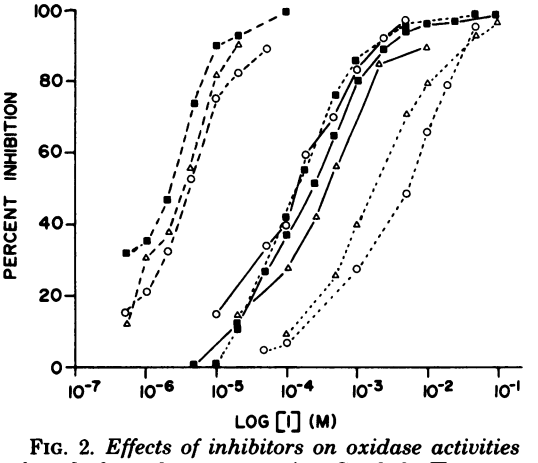

FIG. 2. *Effects of inhibitors on oxidase activities of washed envelope preparation. Symbols:* ■, *ascorbate-TMPD oxidase;* ○, *succinate oxidase;* △, *NADH oxidase;* ----, CN^-; ——, NH_2OH; -----, N_3^-.

TABLE 2. *Effects of terminal oxidase inhibitors on the kinetics of oxidases*

Oxidase	Kinetic constant		Inhibitor	Type of inhibition	Apparent K_i (M)
	V_{max}[a]	K_m (M)			
Ascorbate-TMPD	2,490	5.9×10^{-4}	Cyanide	"Mixed" noncompetitive	3.3×10^{-6}
			Azide	Noncompetitive	2.3×10^{-4}
			Hydroxylamine	Competitive	1.2×10^{-4}
Succinate	221	4.0×10^{-4}	Cyanide	Noncompetitive	6.5×10^{-6}
			Azide	Noncompetitive	5.0×10^{-3}
			Hydroxylamine	Noncompetitive	3.0×10^{-4}
NADH	198	1.2×10^{-4}	Cyanide	"Mixed" noncompetitive	5.5×10^{-6}
			Azide	Noncompetitive	2.7×10^{-3}
			Hydroxylamine	Noncompetitive	5.7×10^{-4}

[a] V_{max} expressed as nanomoles of O_2 consumed per minute per milligram of protein at 37°C.

least two functional terminal oxidases in the envelope of the meningococcus.

A test for this hypothesis was carried out by determining the kinetics of inhibition of terminal oxidase activity. The results (Table 2) revealed differences in the type of inhibition, or the apparent K_i's of the oxidation of the three substrates with selected inhibitors, or both. Assuming the inhibitors were all acting at the terminal oxidase levels and the K_i's represent the dissociation constants for the inhibitor-enzyme or inhibitor-enzyme-substrate complexes, the differences in K_i's observed, particularly those for azide, support the hypothesis that more than one oxidase is operative.

The apparent K_i's for NADH oxidase activity were intermediate between the corresponding K_i's for ascorbate-TMPD and succinate oxidase activities in the presence of azide and cyanide, but not hydroxylamine. An attempt (Fig. 3) was made to determine whether NADH oxidation occurred by sharing electron pathways used for the oxidation of ascorbate-TMPD and succinate, or alternatively, by a third oxidase unique to NADH. A Hill plot slope of $n = 1$ is generally taken to indicate the presence of only one kind of inhibitor binding site, whereas $n < 1$ suggests the existence of multiple components with different affinities for the inhibitor or of independent and different binding sites on a single component (1, 21). The slope generated from the ascorbate-TMPD-azide complex formation was close enough to unity ($n = 0.90$) to justify the conclusion that one component only was involved. The fractional value ($n = 0.75$) for both

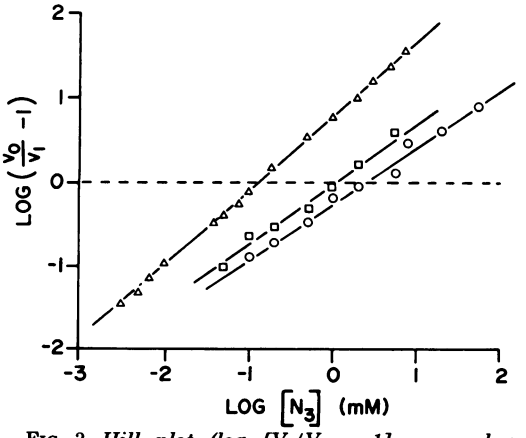

FIG. 3. *Hill plot (log [V_0/V_i − 1] versus log [N_3^-]) of azide titration of oxidase activities in washed envelope preparation. Symbols: △, ascorbate-TMPD oxidase; ○, succinate oxidase; □, NADH oxidase. V_o and V_i are the corresponding oxidase specific activities in the absence and presence of azide, respectively.*

succinate and NADH oxidases, however, suggests multiple or common sites of azide inhibition. The simplest interpretation is that NADH and succinate oxidation may share two pathways differing in azide sensitivity, whereas ascorbate-TMPD oxidation involves a single azide-sensitive pathway. Since the titration curves (Fig. 2) and the double reciprical plots of fractional inhibition versus inhibitor concentration (data not shown) both failed to show any biphasic pattern of inhibition, it seems likely that the more azide-resistant pathway is the preferred one for succinate and NADH oxidation at low inhibitor concentrations. There was no evidence for the distinct switching from one pathway to another with an increase in inhibitor concentration.

An attempt was then made to identify the oxidases which might be involved in branched pathways. Although cytochrome *o* has been proposed as the functional oxidase in neisseriae (17, 20, 23, 30, 33), the presence of an alternate oxidase, although proposed, has not been established. In view of the peroxidase activity in the envelope preparations of the meningococcus (Yu and DeVoe, unpublished data), there existed the possibility that the peroxidase might take electrons from the respiratory chain through cytochrome peroxidation (15, 16). Any interference from the activity of a peroxidase in our membrane preparations could be dismissed, however, in view of the high level of catalase activity in the same fractions when compared with the negligible cytochrome *c* peroxidase activity. The most likely candidate for the alternate oxidase, therefore, appeared to be the *a* cytochrome that we reported earlier in this organism (33). Data from difference spectroscopy support this proposal.

Dithionite-reduced-minus-air-oxidized difference spectra (Fig. 4, curve a), taken as a reference for complete reduction of the entire membrane cytochrome system, revealed in the alpha absorption region a broad band around 600 nm, suggestive of an *a*-type cytochrome, and a sharp peak at 552 nm corresponding to a *c* cytochrome. Marked bleaching due to reduced flavoprotein produced the trough at 450 nm. Positions of shoulders in the *a*-region were readily discernible in expanded scales (Fig. 4B, curve a). These cytochromes were tentatively designated b_{560}, b_{557}, and c_{547}. The shoulder at 563 nm previously reported (33) in dithionite-reduced-minus-ferricyanide-oxidized spectra was not detected in the dithionite-reduced-minus-air-oxidized spectra.

Reduction of the cytochromes by succinate and NADH produced spectra nearly identical to those in which dithionite had been used (Fig. 4,

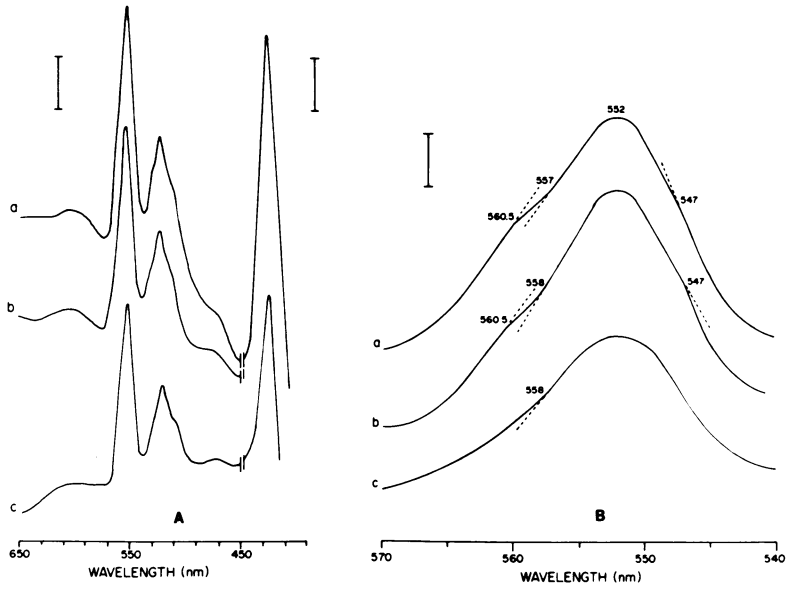

FIG. 4. *Reduced-minus-air-oxidized difference spectra (25°C) of washed envelope preparation. (A) Curve a, dithionite-reduced-minus-air-oxidized; curve b, succinate- or NADH-reduced-minus-air-oxidized; curve c, ascorbate-TMPD-reduced-minus-air-oxidized. Vertical bar represents 0.1 (for Soret, 0.3) absorbance units. Samples contain 20 mg of protein per ml. (B) Corresponding spectra with expanded alpha region.*

curve b) with the exception of the lesser extent of flavoprotein reduction. When ascorbate-TMPD was used as the reductant (Fig. 4, curve c), the 600-nm absorption and the shoulders at 560 nm and 547 nm were absent. Assuming that ascorbate-TMPD enters the electron transport chain at the levels of *c* cytochrome, the results of the spectra (Fig. 4, curve c) suggest that electrons from TMPD go through c_{552} to b_{558}, the cytochrome *o* that we had previously observed in CO-binding studies with this organism (33). Our attempts to isolate the cytochrome by lipase treatment (4, 25) were unsuccessful, although cytochrome c_{552}, but not c_{547}, was solubilized (data not shown). The results, nevertheless, suggest that ascorbate-TMPD oxidation involved only the terminal cytochrome *o*, whereas the physiological substrates were oxidized by both the terminal cytochromes *o* and *a*.

This hypothesis was further substantiated by the data from reduced-plus-CO-minus-reduced spectra (Fig. 5). With dithionite as the reducing agent, a typical cytochrome *a* and *o* spectrum (Soret) was obtained (Fig. 5, curve a) with absorption maxima at 430 and 417 nm, respectively, as we had previously reported (33). Qualitatively similar spectra were obtained with neither NADH nor succinate as the reducing agent (Fig. 5, curves b and c, respectively). However, when the reducing agent was ascorbate-TMPD, the spectrum was markedly different

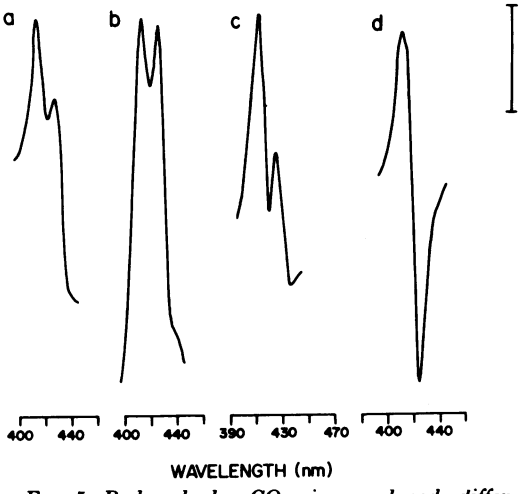

FIG. 5. *Reduced-plus-CO-minus-reduced difference spectra (25°C) of washed envelope preparations. Curve a, dithionite as reductant. Vertical bar represents 0.03 (for Soret, 0.1) absorbance units. Curve b, succinate as reductant. Vertical bar represents 0.01 absorbance units. Curve c, NADH as reductant. Vertical bar represents 0.01 absorbance units. Curve d, ascorbate-TMPD as reductant. Vertical bar represents 0.02 absorbance units. All samples contain 15 mg of protein per ml.*

(Fig. 5, curve d) with absorption due to cytochrome *o* only (a peak at 417 nm and a trough around 430 nm). Considering that cytochrome

oxidases bind CO only when reduced, it is clear that the only oxidase reduced by TMPD was the *o* cytochrome.

The effects of cyanide, azide, or hydroxylamine on the cytochrome spectra were determined in an attempt to define the sites of action of the inhibitors themselves. If an inhibitor should act at a site other than a terminal oxidase, difference spectra in the presence of the inhibitor (at concentrations capable of complete inhibition) should appear qualitatively different from those in the absence of inhibitors. The reduced-plus-inhibitor-minus-air-oxidized and air-oxidized-plus-inhibitor-minus-air-oxidized spectra (data not shown) were essentially the same as those of reduced-minus-air-oxidized spectra (Fig. 4). Such findings provide good evidence that the inhibitors tested were each acting at the terminal oxidase level.

Reduced-plus-inhibitor-minus-reduced spectra were then examined to show the minor changes that might have occurred as a result of inhibitor addition. The results obtained with cyanide (Fig. 6) were similar to the corresponding spectra obtained with azide or hydroxylamine. In the presence of cyanide, succinate or NADH-reduced envelope preparations (Fig. 6, curve a) showed a loss of absorption due to reduced cytochrome *a* (troughs at 600 nm and 440 nm) and to *b*- (or *o*-) and *c*-type cytochromes (troughs at 560 nm and 550 nm, respectively). When ascorbate-TMPD was used as the reductant (Fig. 6, curve b), the absorption due to cytochromes *b* (or *o*) and *c* decreased, but there was no detectable change in *a*-type cytochrome absorption. These results provide further support for the hypothesis that ascorbate-TMPD oxidation occurs exclusively via cytochrome *o*, whereas the oxidation of physiological substrates involved both cytochromes *o* and *a*.

The effects of class I and II inhibitors (refers to those acting at NADH dehydrogenase and between cytochromes *b* and *c*, respectively) on the oxidase activities of envelope preparations (Table 3) suggest that branching of the electron transport chain occurs before the terminal oxidases as well. Oxidation of substrates known to occur via cytochrome *c*, i.e., TMPD, 2,6-dichloroindophenol, and mammalian cytochrome *c* in the presence of excess ascorbate, was not affected significantly by any of the inhibitors listed (Table 3), as one would expect with class I and II inhibitors. Rotenone, shown to be a specific NADH oxidase inhibitor in the mitochondrial system (12), was not effective against oxidases in the envelope preparation (Table 3). Salicylhydroxamate and *o*-phenanthroline, on the other hand, exerted their effects only on NADH oxidase activity, suggesting that if a common pathway exists these inhibitors acted on the NADH oxidase chain before junction with the succinate oxidase pathway.

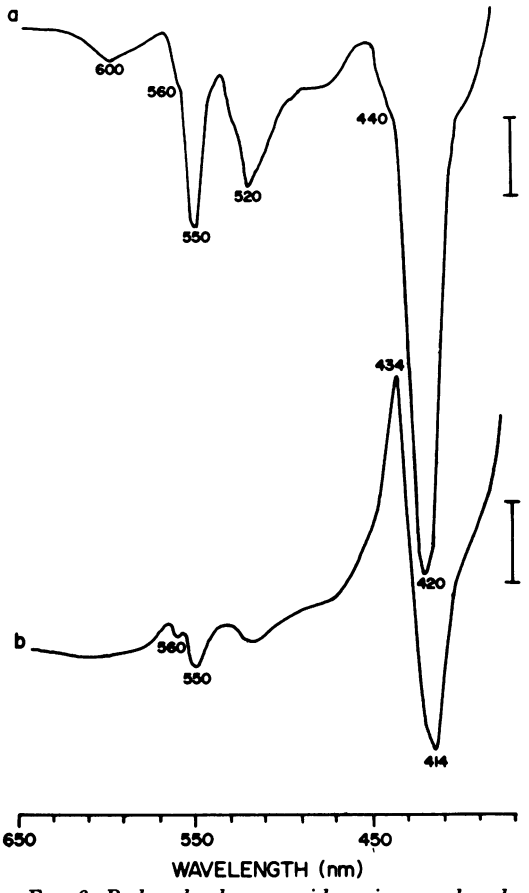

FIG. 6. *Reduced-plus-cyanide-minus-reduced difference spectra (25°C) of washed envelope preparations. Curve a, succinate (or NADH-) reduced-plus-cyanide-minus-succinate- (or NADH-) reduced spectrum. Vertical bar represents 0.01 absorbance units. Curve b, ascorbate-TMPD-reduced-plus-cyanide-minus-ascorbate-TMPD-reduced spectrum. Vertical bar represents 0.03 absorbance units. All samples contain 20 mg of protein per ml.*

The class II inhibitors (antimycin A and 2-*n*-heptyl-4-hydroxylquinoline-*n*-oxide [HOQNO]) were found to be effective against oxidases of physiological substrates only at concentrations much higher than required in the mitochondrial system (27). NADH oxidase activity was more sensitive to the action of both inhibitors, particularly at low inhibitor concentrations (Table 3). Difference spectra (Fig. 7) suggest that both inhibitors act at a level between cytochromes *b* and *c*, since only peaks for cytochromes *o*, *a*, and *c* were detectable. Such inhibitor activities are

TABLE 3. *Effect of inhibitors on oxidase activities in N. meningitidis*

Inhibitor	Final concn (μM)	% Inhibition of oxidase activities				
		Succinate (30 mM)	NADH (2.5 mM)	Ascorbate-TMPD (3-2 mM)	Ascorbate-DCIP[a] (1.5-1 mM)	Ascorbate-cytochrome *c* (1.5-1 mM)
Rotenone	100	21.5	18.4	1.8	13.8	8.5
Salicylhydroxamate	250	<5.0	21.0	<1.0	ND[b]	ND
o-Phenanthroline	500	<5.0	36.0	<1.0	ND	ND
Antimycin A	5	8.4	75.0	ND	ND	ND
	100	65.8	88.3	7.1	5.2	9.1
HOQNO	5	11.6	50.6	ND	ND	ND
	100	43.4	71.4	2.4	8.8	13.6

[a] DCIP, 2,6-Dichloroindophenol.
[b] ND, Not determined.

what one would expect based upon their action on the respiratory chain of mitochondria.

DISCUSSION

There is little definitive information on the respiratory chain of the neisseriae. Although proposals for more than one oxidase in this genus have been put forth (11, 17, 30, 33), the evidence in support of such proposals is cursory. Morse (23) has proposed a linear pathway with cytochrome *o* as the only terminal oxidase in *N. gonorrhoeae*. Recent work with selected inhibitors on gonococcal whole cells and sonically treated cell preparations (20) suggests the existence of a cyanide-sensitive oxidase favored for succinate and malate oxidation and a cyanide-insensitive oxidase involved in NADH oxidation.

In the study here, no cyanide-resistant pathway was found in the envelope preparations of *N. meningitidis*. Our findings with inhibitors, particularly azide, strongly suggest two terminal oxidases. Moreover, the evidence here affirms our earlier suggestion (33) that cytochromes *a* and *o* are the oxidases in this organism.

Ascorbate-TMPD as an electron donor reduced only cytochromes c_{552} and *o*. The pathway for electron flow from TMPD which is consistent with the data is from ascorbate-TMPD via cytochrome c_{552} to cytochrome *o*, as shown schematically in Fig. 8. The inability of ascorbate-TMPD to reduce c_{547} was an unexpected result which suggests that this cytochrome is not accessible to TMPD. As we have reported here, in an unsuccessful attempt to solubilize cytochrome *o*, c_{552} was solubilized; however, c_{547} remained in the membrane. These results indicate that these two *c* cytochromes have a different structural association with the cell membrane. Depending on the positioning of c_{547} in the hydrophobic membrane environment, it could very easily be inaccessible to TMPD, as our data indicate.

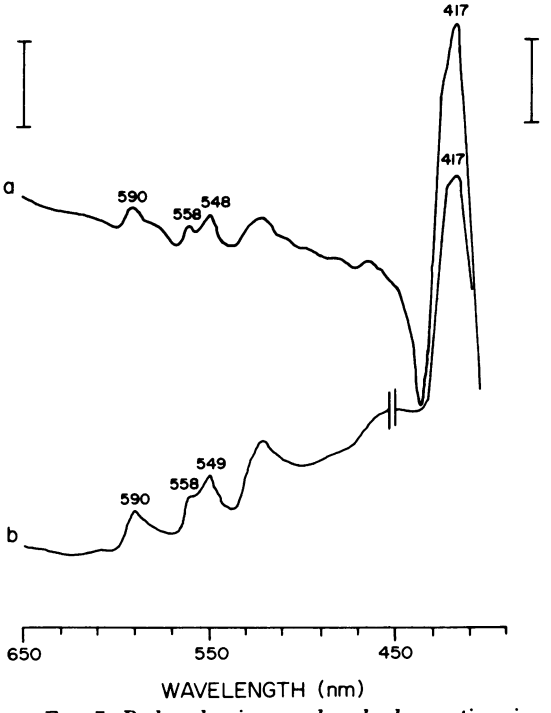

FIG. 7. *Reduced-minus-reduced-plus-antimycin A difference spectra (25°C) of washed envelope preparation. Curve a, NADH-reduced-minus-NADH-reduced-plus-antimycin A spectrum. Vertical bar represents 0.01 absorbance units. Curve b, succinate-reduced-minus-succinate-reduced-plus-antimycin A spectrum. Vertical bar represents 0.01 (for Soret, 0.03) absorbance units. All samples contain 20 mg of protein per ml.*

The oxidation of TMPD was highly sensitive to all the terminal oxidase inhibitors tested, a

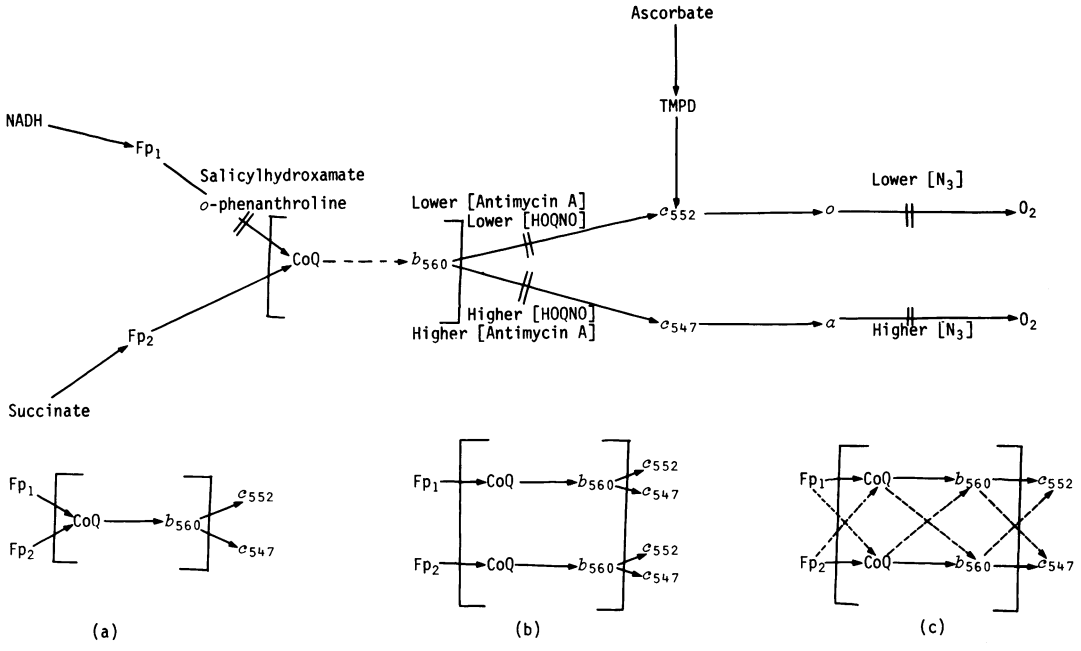

FIG. 8. *Proposed scheme for the respiratory electron transport chain of N. meningitidis. With the data now available, the area set off in the scheme by square brackets could exist as proposed for either (a) a simple terminal branching, (b) totally independent pathways, or (c) parallel pathways with multiple points for crossover of electrons*

finding consistent with reports on other bacteria (14, 16, 18, 24, 28, 29). The two physiological substrates, succinate and NADH, reduced both CO-binding pigments, cytochromes *o* and *a*, and the oxidations of these substrates were more resistant to azide inhibition than the oxidation of ascorbate-TMPD, which is oxidized by means of cytochrome *o*. This suggests that cytochrome *a* is the terminal oxidase of the azide-resistant branch of the electron transport chain, a conclusion in agreement with reports on other microorganisms (26, 28). Although the studies on oxidase inhibition by cyanide and hydroxylamine with the three substrates produced no clues that resolved the oxidases, the kinetic studies with these inhibitors did. There were differences in both the pattern and the kinetics of inhibition which provided further support for a branched electron transport pathway in this organism (Fig. 8).

Additional support was obtained by the action of class II inhibitors on the oxidation of succinate and NADH. If the model is correct, succinate would be oxidized more readily via a pathway relatively insensitive to antimycin A, HOQNO, and azide. However, since complete inhibition could not be achieved, electrons from either substrate could presumably gain access to the alternate branch. Although our spectrophotometric data on the action of the class II inhibitors indicate that they acted classically between cytochromes *b* and *c*, it remains a possibility that the inhibitors acted at more than one site (13, 21) and, therefore, that the model (Fig. 8) as presented would require modification. We now have evidence that the conditions of oxygen limitation stimulate the relative synthesis of the *a* cytochrome and depress the synthesis of cytochrome *o* (Yu and DeVoe, unpublished data). It would appear, therefore, that the substrate preferred and the predominating oxidase are determined by environmental conditions during growth.

Discussions on the preference of electrons from one substrate for a particular oxidase over another, therefore, may be somewhat academic in that the relative amount of a particular oxidase available and the concentration of oxygen in the environment may determine which oxidase is used. Given the information we have at present, we are unable to distinguish between the three alternative schemes for electron flow shown in Fig. 8. The simplest scheme (Fig. 8a) is preferred as the working hypothesis for future work. This scheme is similar in its branching to models proposed for other bacteria (13, 24, 28), which have been shown to have similar responses to inhibitors of electron transport.

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LITERATURE CITED

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