JOURNAL OF BACTERIOLOGY, Aug. 1975, p. 631-636
Vol. 123, No. 2

Printed in U.S.A.

Physiology and Metabolism of Pathogenic *Neisseria*: Partial Characterization of the Respiratory Chain of *Neisseria gonorrhoeae*

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Received for publication 9 April 1975

The cell membrane-associated respiratory electron transport chain of *Neisseria gonorrhoeae* was examined using electron paramagnetic spectroscopy (EPR) at liquid helium temperatures and optical spectroscopy at liquid nitrogen and room temperatures. EPR spectra of dithionite-reduced particles indicated the presence of centers N-1 and N-3 in the site I region of the respiratory chain, whereas reduction with succinate revealed the existence of center S-1 from the succinate cytochrome *c* reductase segment. Free radical(s) resembling that due to flavin semiquinone were observed with both reductants. Low temperature (77 K) optical difference spectra indicated the presence of cytochromes with alpha band maxima at 549, 557, and 562. Bands at 567, 535, and 417 nm, characteristic of the CO compound of cytochrome *o*, were also identified. Cytochromes a_1 and a_3 were not detected; however, a broad but weak absorbance with an alpha band maximum at 600 nm and a Soret shoulder at 440 nm was observed. Hence the respiratory chain of *N. gonorrhoeae* appears to contain several nonheme iron centers, cytochrome *c*, two *b* cytochromes, with cytochrome *o* which probably serves as the terminal oxidase.

Neisseria gonorrhoeae is an obligate aerobe whose only natural habitat is man (18). This organism possesses a terminal oxidase with a high specific activity which catalyzes the oxidation of tetramethyl-*p*-phenylenediamine, a property shared with other members of this genus and most aerobic bacteria. Other than this terminal oxidase reaction (9) little is known about the electron transport components of the organism. Jurtshuk and Milligan (8) have reported difference spectra of the cytochromes observed in *Neisseria catarrhalis* but no information on the site I region of the respiratory chain was presented. Furthermore, *N. catarrhalis* has been shown to be genetically dissimilar from the true *Neisseria* (4) and has subsequently been reclassified *Branhamella catarrhalis* (18). Hence many of the respiratory components of the true *Neisseria* have not been previously reported.

This study reports steady-state data on substrate and dithionite-reducible components associated with the membrane-bound respiratory chain of *N. gonorrhoeae*. The purpose of this study is to describe and characterize some of these respiratory components and to compare them with those of other aerobic organisms as well as other species of *Neisseria*. Furthermore, with the wealth of information concerning the reduced nicotinamide adenine dinucleotide dehydrogenase region of many eucaryotic cells (15, 17), it appeared timely to examine the low temperature (<77 K) electron paramagnetic resonance (EPR) spectra of *N. gonorrhoeae* to establish whether similarities exist between this organism and its host.

MATERIALS AND METHODS

Organism. *N. gonorrhoeae* strain CS-7 was used in this investigation. The properties and maintenance of this strain have been previously described (12, 14) as have the medium and cultural conditions (13).

Preparation of cell membranes. Stationary-phase cultures were harvested by centrifugation (16,000 × *g* for 10 min) in a refrigerated centrifuge, washed twice, and resuspended in 0.4 M tris(hydroxymethyl)-aminomethane-hydrochloride buffer (pH 7.4) containing 0.005 M β-mercaptoethanol. The cell suspension was disrupted by sonic treatment (Biosonik IV; Bronwill, Rochester, N.Y.) for a total of 3 min in 30-s pulses (40% maximum output) with 1-min intervals for cooling. The resulting suspension was centrifuged at low speed (23,000 × *g* for 15 min) to remove cell walls and unbroken cells. The supernatant was centrifuged at 100,000 × *g* for 2 h (type 30 rotor, Spinco model L5-65 ultracentrifuge) and the membrane-rich pellet was resuspended in a small volume of 0.4 M tris(hydroxymethyl)aminomethane-

hydrochloride buffer (pH 7.4) containing 0.005 M β-mercaptoethanol and stored at −30 C until used.

Visible spectroscopy. Room temperature and liquid nitrogen (77 K) spectra of the membrane-rich fraction were recorded on a Cary model 14 spectrophotometer equipped with a model 1462 scattered transmission attachment. The spectrophotometer was calibrated with a holmium oxide standard immediately prior to use. Samples to which reducing agents were added were first made anaerobic by alternately evacuating and flushing with argon. Liquid nitrogen difference spectra were recorded in a 0.2-cm-path-length cuvette attached to a low temperature tee (7). This tee was then partially immersed in liquid nitrogen contained in a glass Dewar fitted with quartz windows.

Carbon monoxide difference spectra were obtained by gently bubbling CO gas through anaerobic dithionite-reduced samples. Samples were periodically withdrawn for spectroscopic analysis.

EPR spectroscopy. Membrane-rich samples (50 mg of protein/ml) were thawed and transferred to anaerobic EPR tubes. Samples were evacuated and flushed with argon at least eight times, reduced with solid sodium dithionite or succinate, and frozen in liquid nitrogen. Liquid helium spectra were recorded with a Varian V-4502 EPR spectrometer. The samples were cooled with a liquid helium transfer system (model LTD-3-110, Air Products and Chemicals, Inc., Allentown, Pa.). Temperatures were monitored with a germanium resistor.

Miscellaneous measurements. Protein was determined by the method of Lowry et al. (11) using bovine serum albumin as a standard.

Chemicals. Carbon monoxide and ultrahigh-purity argon gases were obtained from Matheson Gas Products (Newark, Calif.). Sodium L-malate and L(+)lactic acid were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other reagents were analytical grade or the highest purity available.

RESULTS AND DISCUSSION

EPR spectra. Orme-Johnson et al. (17) and Ohnishi (15) have recently reviewed the iron-sulfur centers arising from the site I region of the respiratory chain in a variety of eucaryotic organisms. These authors have reported g-values for a number of prominent peaks detectable at the temperature of liquid helium and have related these EPR signals to iron-sulfur centers by titrating the signals with reducing equivalents. We have examined the membrane-associated respiratory chain of the aerobic procaryote, *N. gonorrhoeae*, with low-temperature EPR. Figure 1 illustrates the dithionite- and succinate-reduced EPR spectra of the membrane-rich fraction of *N. gonorrhoeae*. The general line shape of the dithionite-reduced spectra is very similar to that of *Candida utilis* and broadly resembles the spectra from bovine heart submitochondrial particles (15). The apparent g-values of several prominent peaks are given in this figure for comparison with site I EPR spectra from other organisms.

It is evident that *N. gonorrhoeae* shows prominent signals at g = 2.02, 1.94, and 1.93 corresponding to center 1 signals according to the designation of Orme-Johnson et al. (17). These investigators give g-values for this center com-

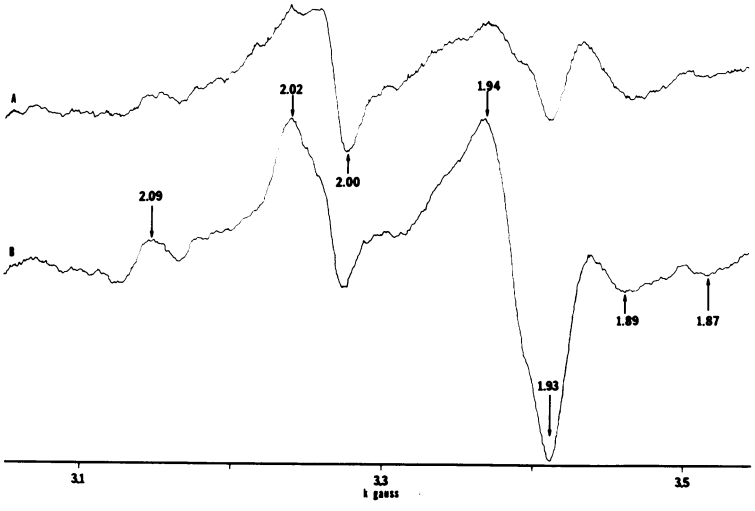

FIG. 1. *Low-temperature EPR spectra of reduced Neisseria gonorrhoeae cell membranes. N. gonorrhoeae membranes (50 mg/ml) dissolved in 0.4 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.005 M mercaptoethanol (pH 7.4) were made anaerobic under argon and reduced with a small amount of solid sodium succinate (A) or dithionite (B). The EPR operating conditions were: modulation amplitude, 9.7 gauss; microwave power, 25 mW; microwave frequency, 9.202 GHz; time constant, 1.0 s; scanning rate, 100 gauss per min; temperature, 18.0 K.*

prised of peaks designated *q*, *r*, and *s* at g = 2.022, 1.938, and 1.923, respectively. Similarly we observed signals at g = 2.09, 1.89, and 1.87 corresponding to peaks *l*, *m*, and *n*, with g-values of 2.099, 1.886, and 1.862, respectively (center 3) (17). These signals, corresponding to centers N-1 and N-3, would be observed upon reduction with dithionite if the site I respiratory chain region of *N. gonorrhoeae* has components similar to those of mammalian mitochondria. We did not observe signals with field positions corresponding to center N-2 at potentials attained with excess succinate or dithionite. Evidence for all the EPR active centers present in *N. gonorrhoeae* membrane will require quantitative redox titration. It should be noted that slightly different g-values for site I centers were reported by Ohnishi (15) for *C. utilis*.

Both the dithionite- and succinate-reduced samples have a g = 2.00 free radical sign, probably arising from a flavin radical as is the case in mitochondria. The succinate-reduced spectrum displays a g = 1.94 central resonance similar to center S-1 observed in succinate dehydrogenase by Beinert and Sands (2). The low potential center S-2 (16) was not observed in gonococcal membrane fractions due to overlap with site I EPR signals. The high potential iron-sulfur protein signals recently observed in the succinate coenzyme Q reductase region (1) were not examined.

Optical difference spectroscopy. Low temperature (77 K)-reduced minus oxidized spectra of *N. gonorrhoeae* cell membrane fragments are shown in Fig. 2. Three alpha bands are resolved in the dithionite-reduced minus ferricyanide-oxidized spectra with absorption maxima at 549 (*c*-type cytochrome) and 557 with a shoulder at 562. The latter two, together with the beta maxima at 528 nm, are indicative of *b*-type cytochromes (10). In addition, there is a broad adsorption band at 600 nm. The increased absorbance observed upon addition of ferricyanide to the reference cuvette is the result of a more complete oxidation of the respiratory chain components, especially those which are not autoxidizable. This was especially apparent in this case since the buffer which the gonococcal membranes were suspended contained β-mercaptoethanol which can donate reducing equivalents to the respiratory chain, necessitating the use of a more effective oxidant. Room temperature difference spectra of dithionite-reduced minus ferricyanide-oxidized (not shown) contain absorbance maxima in the alpha region centered at 553, 560, and 600 nm. These bands were approximately one-fifth as intense at room temperature than at 77 K. The 25 C beta absorbances are centered at 516, 523, and 530. Low-temperature dithionite-reduced minus air-oxidized difference spectra (Fig. 2) gives a Soret maximum at 427 nm. This peak is shifted about 4 nm to lower wave length when the reducing agent is ascorbate. In either case, the Soret band is unresolved but displays a shoulder at 440 nm, typical for α-type cytochromes (10), and a trough at 450 to 452 nm probably due to flavin in various states of reduction.

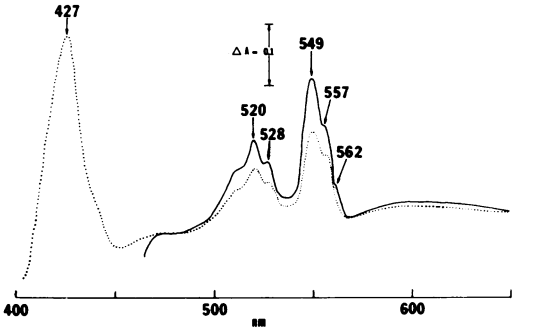

FIG. 2. *Low-temperature optical difference spectra of cell membrane fragments from N. gonorrhoeae CS-7. Dithionite-reduced minus ferricyanide-oxidized (solid line) and dithionite-reduced minus air-oxidized (dotted line) spectra of N. gonorrhoeae cell membrane fragments [10 mg of protein/ml in 0.08 M tris(hydroxymethyl)aminomethane-hydrochloride, 10^{-3} M β-mercaptoethanol, pH 7.4]. Optical difference spectra were recorded as described in Materials and Methods. Samples were reduced under anaerobic conditions with solid sodium dithionite. Ferricyanide (100 μM) was added to the reference sample.*

The observation of a broad absorption band centered around 600 nm with a 440-nm Soret shoulder may indicate the presence of an *a*-type cytochrome in this organism. However, the 600-nm peak does not display the normal sharpness for an alpha band absorbance of an *a*-type cytochrome. Furthermore, the low 440-nm extinction would indicate a very small concentration of this component. On the basis of a similar broad 600-nm absorption, Jurtshuk and Milligan (8) have suggested *B. catarrhalis* contained cytochrome a_1. Even though our CO difference spectra did not indicate the presence of cytochromes a_1 or a_3 (see below), we were unable to eliminate the possibility that an *a*-type exists in the membrane of *N. gonorrhoeae*.

Approximate concentrations of the various cytochromes computed from low-temperature dithionite minus ferricyanide difference spectra by the method of Dietrich and Biggins (6) are presented in Table 1. An estimated millimolar extinction coefficient of $\epsilon = 20\ mM^{-1}cm^{-1}$ (6) was used together with a low temperature intensification factor of five (7) to compute

these concentrations. No correction was made for band overlap. Cytochrome *o* was computed from the dithionite +CO minus dithionite spectrum using the estimation procedure of Daniel (5) (i.e., peak minus trough ϵ = 170 $mM^{-1}cm^{-1}$). The concentrations of cytochromes *c* and *b* calculated in the above manner are higher in *N. gonorrhoeae* than those reported by Smith (19) for Keilin-Hartree heart muscle and other selected bacterial membranes (6, 20), whereas cytochromes *o*- and *a*-type are slightly lower in the organism described here.

Ascorbate, succinate, and L-lactate, but not L-malate, were found by optical criteria to reduce membrane-bound respiratory chain components of *N. gonorrhoeae*. Difference spectra of ascorbate- and succinate-reduced particles (Fig. 3) contain a broad alpha region absorption centered at 551 nm and a broad weak 600-nm band. Particles reduced with succinate display a shoulder at 558 to 560 nm. Since ascorbate does not normally reduce *b*-type cytochromes, the 558 to 560-nm shoulder is not observed when this reductant is employed. The breadth of the absorption at 551 nm suggests that it is composed of more than one band. Reduction with L-lactate diminishes the lower wave length component to a slightly greater extent, splitting the band into 549- and 553-nm peaks.

TABLE 1. *Cytochrome concentration in membrane fragments from N. gonorrhoeae CS-7*

Cytochrome[a]	Concn (nmol/mg of protein)
Cytochrome *c*	1.15
Cytochrome b_{557}	0.75
Cytochrome b_{562}	0.13
Cytochrome *o*	0.18

[a] The designation cytochrome is used here for convenience and is based only upon absorbance peak positions in comparison with established absorbance maxima observed in other bacterial and mitochondrial fragments. No information as to the structure of the heme prosthetic group is intended by this designation.

Figure 4 illustrates that *N. gonorrhoeae* also contains at least one CO binding pigment. Room temperature dithionite plus CO minus dithionite difference spectra shows maxima at 567, 535, and 417 nm and troughs at 553 and 438 nm. These peak and trough positions which have been attributed to cytochrome *o* are within the range of CO binding chromophores found in other microorganisms (10). Broberg and Smith (3) demonstrated a technique for detecting both cytochrome a_3 and cytochrome *o* in bacterial cell membranes. These authors found that initial bubbling with CO produced a small peak at 590 and a trough at 445 nm with a peak plus shoulder at 413 and 430 nm attributed to

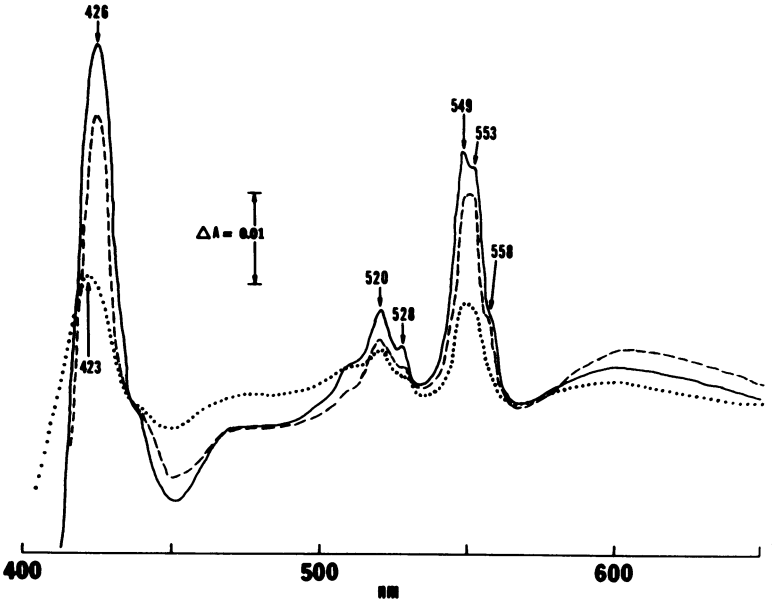

FIG. 3. *Low-temperature optical difference spectra of succinate-, L-lactate-, and ascorbate-reduced N. gonorrhoeae cell membranes. Solid sodium succinate (dashed line), sodium L-lactate (solid line), and sodium L-ascorbate (dotted line) were added to oxygen-free N. gonorrhoeae membrane fragments (10 mg of protein/ml). Optical difference spectra were recorded as described in Materials and Methods.*

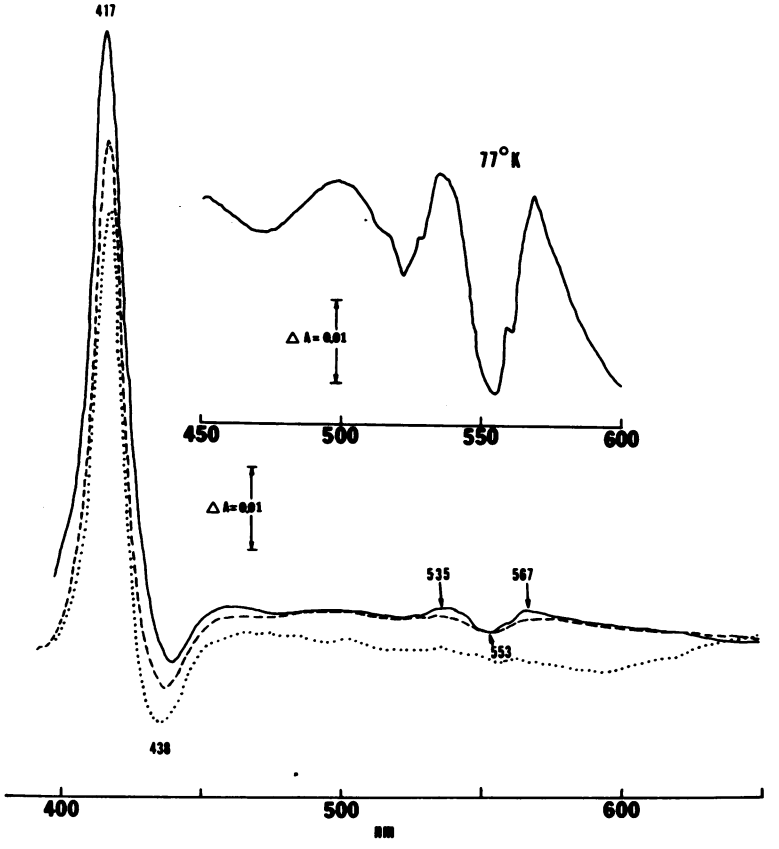

FIG. 4. *Room temperature carbon monoxide difference spectra of N. gonorrhoeae. Dithionite-reduced N. gonorrhoeae cell-membrane particles (12.6 mg of protein/ml) were bubbled with CO for 90 s (dotted line), 5 min (dashed line), and 15 min (solid line), and optical difference spectra were recorded. Inset is the 77 K spectra of the particles bubbled with CO for 15 min.*

cytochrome a_3. Further reaction with CO produced cytochrome *o* absorbances similar to those described above. These differences have been attributed to different CO binding rates of these cytochromes (10). We were unable to detect absorbances due to cytochrome a_3 or cytochrome a_1 (10) by the above procedure. However, a cytochrome *o* absorption spectrum was detected upon bubbling dithionite-reduced *N. gonorrhoeae* membranes for 90 s with CO. Full development of this spectrum took 15 min, at which time a concentration of 0.18 nmol of cytochrome *o* per mg of cell membrane protein was computed from peak 417 minus trough 438-nm absorbances. Jurtshuk and Milligan (8) have presented a CO difference spectra from *N. flava* in which a typical cytochrome *o* spectra was also observed. However, even though the organism studied here is from the same genus, the peak and trough maxima reported by the above authors (8) are shifted 3 to 5 nm to a lower wave length. Discrepancies in peak position of this magnitude are not likely to be due to spectrophotometer calibration differences, hence the equal peak position within a homogeneous genus rule (20) may not apply here.

The carbon monoxide difference spectra suggests the presence of an autoxidizable cytochrome (cytochrome *o*) associated with the membrane of *N. gonorrhoeae*. This cytochrome may be responsible for the high tetramethyl-p-phenylenediamine oxidase activity associated with members of the genus *Neisseria*. Even though cytochrome a_3 or a_1 could not be detected by CO difference spectra by the procedures employed here, the question of the presence of another autoxidizable cytochrome in membranes of *N. gonorrhoeae* is still open since its spectra may be masked by the CO compound of cytochrome *o*.

ACKNOWLEDGMENTS

We wish to thank Howard S. Mason for his suggestions and review of the manuscript.

This research was supported in part by contract no. DAMD17-74-C-4139 from the U. S. Army Medical Research and Development Command and Public Health Service grant AM0718 from the National Institute of Arthritis and Metabolic Diseases.

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