Quantitation of the Tetramethyl-p-Phenylenediamine Oxidase Reaction in *Neisseria* Species

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The tetramethyl-p-phenylenediamine oxidase reaction commonly used in the Kovacs oxidase test was quantitatively estimated for various *Neisseria* species employing standardized resting cell suspensions. This genus of microorganisms exhibited very high tetramethyl-p-phenylenediamine oxidase rates comparable to that of *Azotobacter* and *Pseudomonas*, and this reaction was found to be a valid measurement for the respiratory capability possessed by this group of organisms.

The compound tetramethyl-p-phenylenediamine (TMPD) has been used extensively in the Kovacs oxidase reaction as a presumptive test for the identification of pathogenic *Neisseria* species. Because of its simplicity, it has also been employed as a useful reaction for taxonomic purposes (9). Although it was known that different *Neisseria* species have the ability to oxidize TMPD, the extent to which this reaction occurred was never established. Biochemically, TMPD is used almost exclusively as an electron donor to measure bacterial or mitochondrial terminal oxidase reactions in assay systems that are analogous to measuring cytochrome *c* oxidase activity. This activity is associated with membrane-bound enzymes that are tightly attached to the electron transport systems. The availability of a quantitative procedure (6) for measuring TMPD oxidation made it possible to determine whether different *Neisseria* species have (i) high TMPD oxidase rates, and (ii) exhibit disparate oxidative rates when grown under similar conditions.

Resting cell suspensions of various *Neisseria* species were prepared by growing cells in 1-liter quantities at 33°C in Trypticase soy broth (BBL) supplemented with yeast extract (0.5%). Late logarithmic-phase cells were subsequently harvested and homogenized in 0.02 M phosphate buffer, pH 7.5. On occasion, various other carbohydrates (0.25%) were added as supplements to the Trypticase soy broth which already contained glucose. For *Neisseria gonorrhoeae*, the growth procedure differed in that various colony types were heavily streaked on GC base (BBL) plates supplemented with 0.4% glucose, 0.0002% thiamine pyrophosphate, and 0.005% glutamine. After 24 h of incubation at 35°C (in a candle jar), the plates were removed and the organisms were harvested by washing the agar surface with phosphate buffer. These whole cells were then washed in buffer and homogenized, and turbidimetrically standardized testing cells were prepared in the conventional manner.

Manometric assays were used to measure TMPD oxidation using the standardized resting cell suspensions. The TMPD assay measuring terminal oxidation was used exactly in the manner previously described (6), and the resting cell oxidation of carbohydrates (16.7 mM) performed in a similar manner but at pH 7.5. All assays were initiated by addition of substrate (or ascorbate) from the sidearm. Suitable controls were always incorporated into our manometric assay system as, for example, establishing the absence of ascorbate oxidation without TMPD, and all other necessary precautions were taken to insure that no chemical auto-oxidation reactions occurred (6). Values presented in Table 1 and 2 usually represent the average of multiple runs.

Table 1 shows the TMPD-ascorbate oxidation rates obtained from standardized resting cells for the various *Neisseria* species. The data in Table 1 show that the *Neisseria* resting cells exhibit very high TMPD oxidation rates. Most of the organisms exhibited Q(O₂) values ranging from 619 to 1260. Even higher rates were obtained for *N. catarrhalis* (Q(O₂) = 1,825), and *N. lactamica* showed the highest value (Q(O₂) of 2,596) of all the *Neisseriae* examined. The results presented for the studies on the TMPD oxidation suggest that the *Neisseria* possess a very active terminal oxidase and exhibit one of the highest activities for the TMPD oxidation of any of the aerobic organisms examined. Other organisms having very high TMPD oxidation
Table 1. Comparison of TMPD oxidation rates for various Neisseria species employing resting cell preparations

| Neisseria sp.* | Growth substrate | Q(O₂) values* | |
|---------------|------------------|---------------|---|
|               |                  | Endogenous    | Ascorbate-TMPD |
| Saccharolytic |                  |               |               |
| N. sicca (H.H.D.) | Glucose-sucrose | 35.9          | 1,134         |
| N. mucosa (H.H.D.) | Glucose-sucrose | 27.7          | 619           |
| N. flav (14221) | Glucose-fructose | 0.9           | 822           |
| N. lactamica (23970) | Glucose-lactose | 0.5           | 2,596         |
| N. gonorrhoeae (F62) | Glucose |              |               |
| Colony type 1 |                  | 0.0           | 1,077         |
| Colony type 2 |                  | 0.0           | 1,260         |
| Colony type 3 |                  | 0.0           | 934           |
| Colony type 4 |                  | 0.0           | 872           |
| Asaccharolytic |                  |               |               |
| N. elongata (25295) | Glucose | 1.5           | 750           |
| N. catarrhalis (25238) | Glucose | 1.5           | 1,825         |

* H.H.D. represents cultures obtained from the Houston Health Department; five-digit numbers denote ATCC numbers. Strain F62 was obtained from the Center for Disease Control (Atlanta) and was colony typed as described elsewhere (7).

*Expressed in microliters of O₂ uptake per hour per milligram (dry weight) of resting cells at 30 C. Endogenous value represents the cellular respiration rate obtained in the absence of any added substrate, but assayed under the identical physiological conditions used for measuring TMPD oxidation.

*Growth conditions varied from the usual procedure for this organism (see text).

Table 2. A comparative study of carbohydrate oxidation rates [Q(O₂)] for the various Neisseria species

| Neisseria species | Additional growth substrate besides glucose | Carbohydrate or other substrate |
|-------------------|--------------------------------------------|--------------------------------|
|                   |                                            | Endogenous* Glucose | Sucrose | Fructose | Lactose | Lactate |
| Saccharolytic     |                                            |                    |         |          |         |         |
| N. sicca         | Sucrose                                    | 42.0               | 57.0    | 70.0      | -c      | -      |
| N. mucosa        | Sucrose                                    | 31.0               | 33.0    | 44.0      | -      | -      |
| N. flav          | Fructose                                   | 1.7                | 1.2     | -         | 90.2    | -      |
| N. lactamica     | Lactose                                    | 0.7                | 20.0    | -         | -       | 24.0   |
| Asaccharolytic   |                                            |                    |         |          |         |         |
| N. elongata      | None                                       | 1.9                | 9.0     | -         | -       | 49.0   |
| N. catarrhalis   | None                                       | 1.9                | 4.0     | -         | -       | 125.0  |

*Expressed in microliters of O₂ uptake per hour per milligram (dry weight) of resting cells at 30 C.

*Endogenous value represents the cellular respiration rate obtained in the absence of any added substrate but assayed under the identical physiological conditions used for measuring carbohydrate or lactate oxidation.

*Not determined.

rates were Azotobacter, Rhizobium, and Pseudomonas species, whereas gram-negative organisms having low TMPD oxidation rates were primarily the enterobacter species, e.g., Serratia marcescens and Proteus vulgaris (D. N. McQuitty et al., Abstr. Annu. Meet. Amer. Soc. Microbiol., Chicago, p. 170, 1974). Interestingly, N. catarrhalis has been shown to be genetically (1, 3) and biochemically (5, 8) different from other true Neisseriae (2, 3). The results of our study are also consistent with the view of Lambert et al. (8) and Hollis et al. (4), who proposed that N. lactamica be considered a separate species of Neisseria and not a variant of the pathogenic neisseriae.

Table 2 shows the ability of the Neisseriae to oxidize glucose as well as other fermentable carbohydrates and D-lactate. All saccharolytic species, with the exception of N. gonorrhoeae, were grown on a medium which contained glucose as well as an additional carbohydrate, which allowed for the formation of acidic end products. In every case, the ability to oxidize carbohydrates was 10 to 20 times less than the ability to oxidize TMPD, even though the carbohydrate was added as an active growth
substrate allowing for full adaptation. With the asaccharolytic group of Neisseria, it was found that DL-lactate oxidation was high but still not as high as the TMPD oxidation rate. It should be noted, however, that lactate was not added as an additional carbon source in the growth media.

From this study one can conclude that Neisseria species, an oxidase-positive group of organisms, have the capability of oxidizing the electron donor TMPD at a very high catalytic rate using molecular oxygen as a terminal acceptor. This rate of activity is much greater than that obtained for carbohydrate oxidation or for DL-lactate oxidation by the two asaccharolytic strains used in this study.

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