INFECTION AND IMMUNITY, July 1974, p. 123-127

Vol. 10, No. 1
Printed in U.S.A.

Oxygen Uptake by *Treponema pallidum*

C. D. COX AND M. K. BARBER

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01002

Received for publication 15 April 1974

Virulent *Treponema pallidum* has been shown to consume O_2 at a rate similar to that of the known aerobic spirochaete, *Leptospira*. Such O_2 uptake is cyanide sensitive, indicating a functioning cytochrome oxidase. Inhibition of O_2 uptake by azide, chlorpromazine, and amytal further suggests a functioning electron transport system for the oxidation of nicotinamide adenine dinucleotide (reduced) to O_2. Evidence is consistent with the probability that this terminal electron-transport system is coupled to oxidative phosphorylation. The potential significance of these findings is discussed.

Excellent reviews of old and recent literature have substantiated the conclusion that *Treponema pallidum*, the cause of syphilis, has not been cultivated in vitro (18, 24). Most of the information pertinent to the physiology of this microorganism has been obtained from studies on the use of various media for the in vitro survival of virulent *T. pallidum* (13, 23), and from nutritional studies on cultivatable but nonvirulent treponemes (6). Regrettably, these studies have not yielded information which has led to the successful cultivation of virulent *T. pallidum*.

T. pallidum is considered to be an anaerobe (3). Evidence for its anaerobiosis seems to have arisen from observations that viability of virulent *T. pallidum* decreases more rapidly in the presence of air than in atmospheres of nitrogen or hydrogen (13, 24), and from the anaerobic nature of the cultivatable but nonvirulent strains (6). The apparent in vitro deleterious effect of air on virulent *T. pallidum* should not necessarily warrant its being labeled as an anaerobe, especially in the absence of information on terminal electron acceptors and in view of the knowledge that this organism grows in vivo under the pO_2 of tissue (22). This investigation was planned in an attempt to resolve this apparent enigma by making measurements on the possible O_2 consumption by these bacteria and by studying the terminal electron transport process using metabolic inhibitors of O_2 consumption. Techniques were selected for their sensitivity and applicability to working with the small numbers of virulent *T. pallidum* obtainable from tissue.

MATERIALS AND METHODS

Bacteria. The virulent Nichols strain of *T. pallidum* was used throughout the study and was obtained from U. S. Kuhn from the Center for Disease Control, Atlanta, Ga. Procedures for the growth of this strain in rabbit testicles have been previously described (8, 22, 24). Rabbits were screened for absence of syphilitic antibodies by rapid plasma reagin (RPR) and fluorescein treponemal antibody (not adsorbed) (FTA) tests (15, 7). The use of cortisone acetate permitted minimal tissue cell infiltration (8), and those cells which were found in the tissue extracts were easily removed by low-speed centrifugation (8). Testicular extracts were made in a medium consisting of 10% (vol/vol) inactivated pooled rabbit serum (Pel-Freez, Inc., Rogers, Ark.), 15% (vol/vol) glycerin (11) and 0.075 M sodium citrate (8), and the extracts were adjusted for cell density in the same medium. Suspensions were either used fresh or stored in liquid nitrogen in the liquid phase (12). *Leptospira* B_{16} was isolated in this laboratory and was used as a control for a known aerobic spirochaete. This strain was chosen because of our knowledge of its physiology and terminal electron transport process (1). *Leptospira* B_{16} was grown throughout these studies in SM-6 (21). *Spirochaeta stenostrepta* was obtained from E. Canale-Parola of this department. It was used as a control for a known anaerobic spirochaete (9) and grown in a previously described medium containing cysteine (4). All chemicals used in the media and as metabolic inhibitors were reagent grade or the equivalent.

Assay of O_2 uptake. Dissolved O_2 concentrations were determined by the use of the Clark-type electrode in a Yellow Springs Instrument Co. model 53 oxygen monitor with a water bath temperature controlled by a Tamson, TZ 9-100 instrument to $< \pm 0.1$ C. All cell suspensions were sparged before assay in order to achieve air saturation of the medium. Values were continuously recorded as percent air saturation versus time. Absolute concentrations of O_2 in all media were determined by the method of Robinson and Cooper (19), and the recordings were then converted to plots of O_2 uptake versus time. In all cases testicular extracts from noninfected rabbits showed minimal O_2 uptake over a period of 1 to 2 h. Nevertheless, these values were subtracted from the

O_2 uptake figures for similar extracts from infected rabbits in determining O_2 uptake values for *T. pallidum*.

RESULTS

Preliminary evidence indicated that cells of virulent *T. pallidum* were indeed consuming O_2 at a rate similar to that observed with cells of the known aerobic *Leptospira*. It became important to determine whether this O_2 consumption was a function of the cells themselves or some intermediate in the surrounding medium. A suspension of virulent *T. pallidum* was centrifuged at 10,000 × *g* for 30 min. All cells were motile after centrifugation. The top half of the supernatant fluid containing no observable treponemes was compared with the bottom half of the supernatant fluid, in which the pellet of treponemes was suspended at a density of 4.0×10^8 cells per ml. Oxygen consumption was found to be confined to the cell suspension in which O_2 uptake was linear, and 90% of the available O_2 was consumed in 1 h. At the end of this experiment 78% of the cells were still motile. Cultures of *S. stenostrepta* in anaerobic medium also gave appreciable O_2 uptake values in similar studies. However, such activity was equally associated with supernatant fluids as well as cells, and could be removed from cells by repeated washings in medium lacking cysteine without disturbing motility. Such findings could be explained by the auto-oxidation of intermediates, such as sulfide ions, in supernatant fluids and on cell surfaces from which they could be removed by repeated washings. Oxygen uptake by *T. pallidum* was not found to be dependent upon such intermediates.

The rate of O_2 uptake was indeed a function of treponeme concentration and was quite similar to that observed with *Leptospira* (Fig. 1). Previous studies (1) had already demonstrated the existence of a functioning cytochrome system in this *Leptospira* strain. Subsequent studies have shown that values for rates of O_2 uptake (QO_2) could be determined from those portions of the curves which were most linear (between 10 and 50 min), and QO_2 could be expressed as microliters per milliliter per minute per 10^8 treponemes. Expressions per unit of dry weight, or per weight of protein, were not feasible with these bacteria in the extraction medium, and repeated high-speed centrifugations and washings in protein-free media did not permit maintenance of viability. QO_2 values expressed per 10^8 treponemes were quite constant within the same extract at any of the cell densities measured. Thus, calculations of QO_2 values of *T. pallidum* from Fig. 1 shows a value of 0.0181 ± 0.0015 μliters of O_2 per ml per min per 10^8 treponemes, using cell densities varying from 0.6×10^8 to 2.8×10^8 cells per ml from the same lot.

The optimal temperature for O_2 uptake was near 38 C (Table 1). Appreciable differences in metabolic activity, as rates of O_2 uptake, were noticed between different extracted cell preparations, and therefore agreement should not be expected between different lots in Table 1. However, excellent reproducibility has been observed upon repeated determinations within the same lot. Although this optimum should not be considered necessarily as the optimum temperature for growth, it is not inconsistent with such data (22, 24).

Conclusions that O_2 consumption was linked to cytochrome oxidase activity would be indicated by evidence that O_2 uptake was inhibited by cyanide (2, 10, 14, 20) and azide (10, 14, 20). Figures 2 and 3 provide such evidence. Attempts to provide similar evidence for involve-

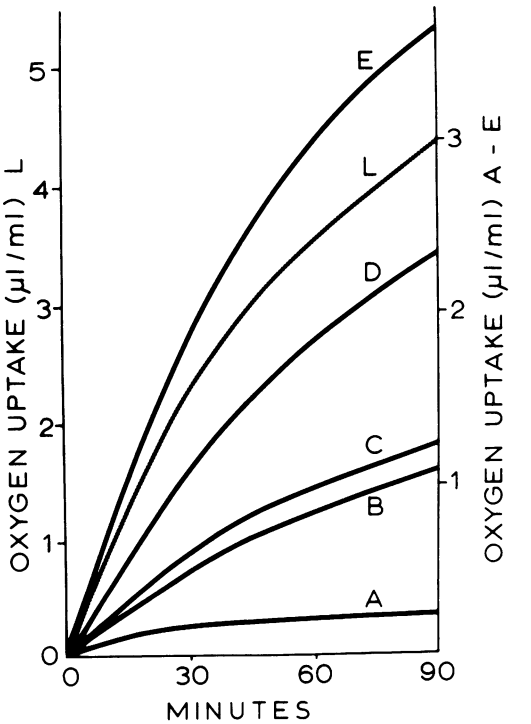

FIG. 1. *Oxygen uptake at different cell densities of virulent T. pallidum compared with a known aerobic spirochaete, Leptospira* B_{16}. *Cell densities for T. pallidum were* 0.6×10^8 *per ml (curve B),* 0.7×10^8 *per ml (curve C),* 1.8×10^8 *per ml (curve D),* 2.8×10^8 *per ml (curve E). Leptospira* B_{16} *was used in a cell density of* 2.0×10^8 *per ml (curve L). Curve A represented tissue extract from an uninoculated rabbit in maintenance medium for T. pallidum.*

TABLE 1. Optimal temperature for O_2 uptake by virulent *T. pallidum*

Temp (C)	QO_2 values for different lots of cell preparations[a]			
	Lot A	Lot B	Lot C	Lot D
42	0.0068[a]			
40	0.0159	0.0239		
38	0.0193	0.0264	0.0253	
37	0.0104		0.0243	
36			0.0226	0.0188
34			0.0192	0.0148
33			0.0139	0.0102
30				0.0094

[a] QO_2 values are expressed as microliters of O_2 per milliliter per minute per 10^8 cells of *T. pallidum*.

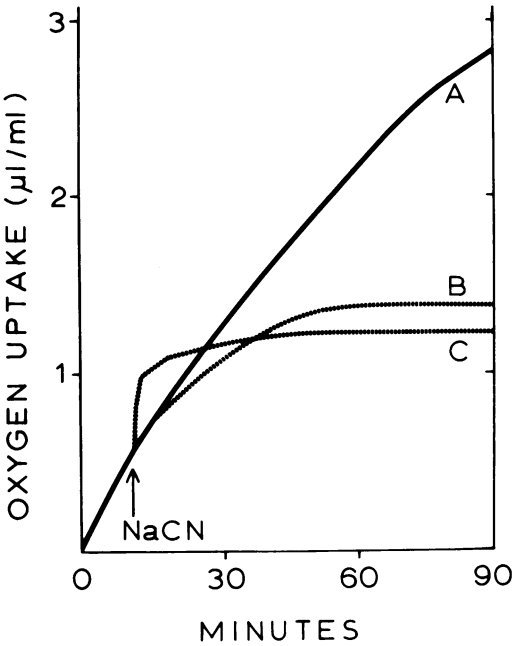

FIG. 2. *Oxygen uptake by virulent T. pallidum (2.0 × 10^8 cells per ml) in the absence (A) and presence of NaCN at final concentrations of 20 µM (B) and 10 mM (C) added at the time indicated by the arrow. Motility at 120 min was 80% for curve A and 0% for B and C.*

ment at the levels of cytochromes *b* and *c*, and flavoprotein using 2-heptyl-4-hydroxy-quinoline-*n*-oxide (HOQNO) and rotenone were not successful. No inhibition of O_2 uptake occurred with concentrations of HOQNO up to 257 µM and concentrations of rotenone up to 86 µM. In contrast, complete inhibition of O_2 uptake by *Leptospira* B_{16} occurred with 57 µM HOQNO and 28 µM rotenone. Recognizing the possibility of permeability problems with these inhibitors by using *T. pallidum*, we sought other inhibitors reported to be active at these sites in the electron transport chain. Chlorpromazine and amytal were selected because of their solubility in water. Amytal has been shown to inhibit O_2 uptake in guinea pig brain cortex slices (17) and rat liver mitochondria (16). The site for action of amytal in blocking electron transport has generally been thought to be in the region of nicotinamide adenine dinucleotide (NAD)-linked flavoprotein (16). Figure 4 shows inhibition of O_2 uptake in *T. pallidum* by amytal. Inhibition of electron transport between NADH and cytochrome *c* in phosphorylating systems has been attributed to chlorpromazine (5). Figure 5 demonstrates inhibition of O_2 uptake with this drug by *T. pallidum*.

Figure 5 shows slight and temporary evidence of uncoupling of oxidative phosphorylation by chlorpromazine. Such evidence was more pronounced by increasing the chlorpromazine level to 22.7 mM. In addition, 10 mM NaCN showed marked evidence of uncoupling (increased rate of O_2 uptake) (Fig. 2). Complete inhibition of O_2 uptake occurred with 93 µM 2,4-dinitrophenol (DNP) but only slight inhibition with 19.6 µM concentration, and uncoupling by DNP was not

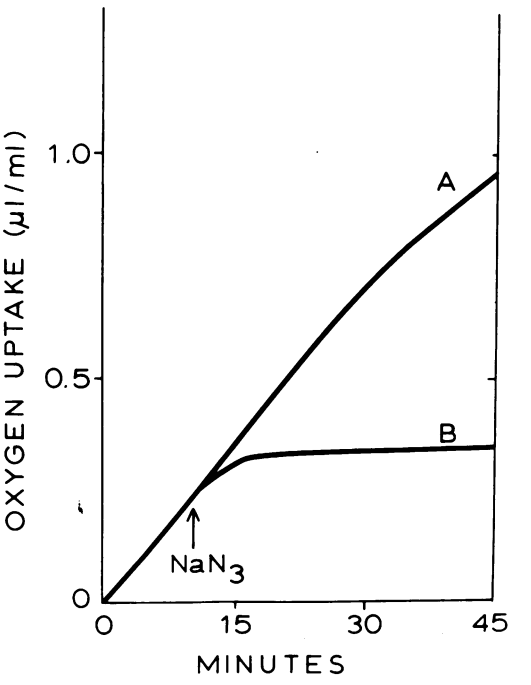

FIG. 3. *Oxygen uptake by virulent T. pallidum (1.0 × 10^8 cells per ml) in the absence (A) and presence (B) of NaN_3 at a final concentration of 28.0 mM added at the time indicated by the arrow. Motility at 120 min was 95% for curve A and 0% for B.*

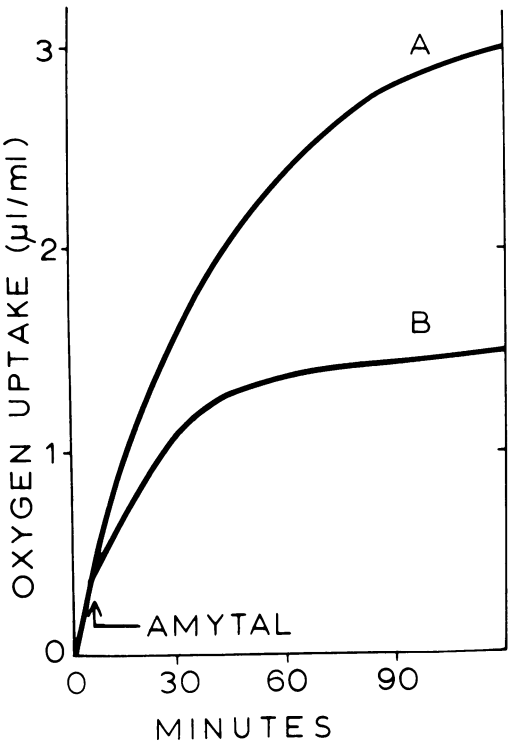

FIG. 4. *Oxygen uptake by virulent T. pallidum (1.35 × 10^8 cells per ml) in the absence (A) and presence (B) of sodium amytal at a final concentration of 1.1 mM added at the time indicated by the arrow. Motility at 130 min was 65% for A and 0% for B.*

observed at these levels. Such findings are consistent with published data indicating that DNP is not as active in uncoupling oxidative phosphorylation in most bacterial systems as it is in mammalian systems (20). These data suggest that oxidative phosphorylation is coupled to the terminal electron transport system.

DISCUSSION

The above data convinces us that virulent *T. pallidum* is an aerobe in the sense that it consumes O_2 as a final electron acceptor through a cyanide-sensitive cytochrome oxidase. Additional data provide evidence for a functioning flavoprotein-cytochrome mediated oxidation of NADH to O_2, and the evidence suggests that this is coupled to oxidative phosphorylation. Such findings do not rule out the possibility of other mechanisms for oxidation of NADH, but one must consider the strong possibility that O_2 is required for reproduction and growth of the organism, and that past findings of toxicity of air could be attributed to the accumulation of oxidized intermediates, such as the superoxide radical or peroxides, rather than O_2 itself. Anaerobic conditions would prevent the accumulation of toxic oxidized intermediates which virulent *T. pallidum* alone could not remove, but the absence of O_2 would prevent growth or reproduction.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-10982 from the National Institute of Allergy and Infectious Diseases.

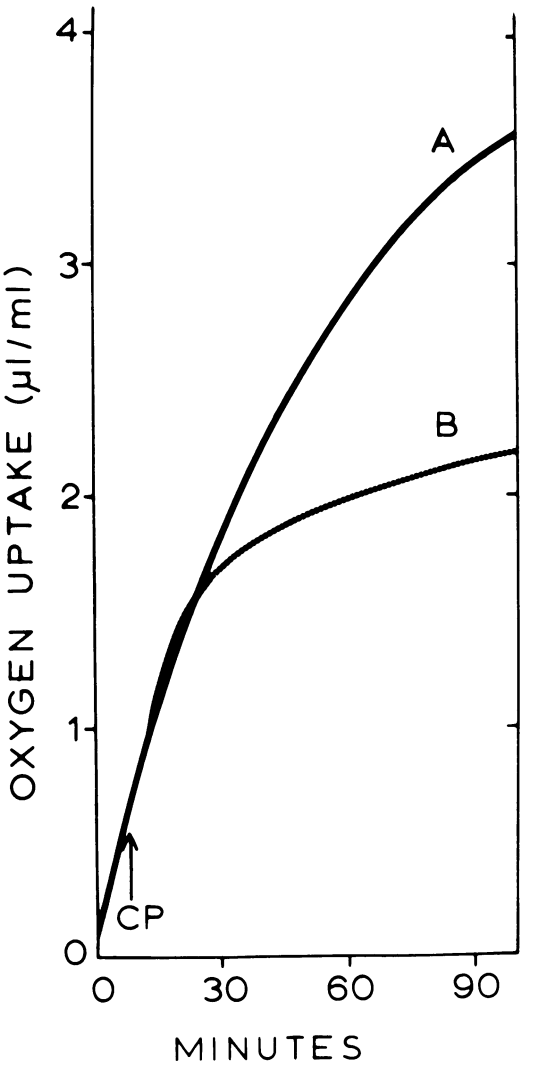

FIG. 5. *Oxygen uptake by virulent T. pallidum (1.35 × 10^8 cells per ml) in the absence (A) and presence (B) of chlorpromazine at a final concentration of 0.227 mM added at the time indicated by the arrow (CP). Motility at 110 min was 88% for curve A and 5% for B.*

Technical assistance of James Embree is gratefully acknowledged.

LITERATURE CITED

1. Baseman, J. B., and C. D. Cox. 1969. Terminal electron transport in *Leptospira*. J. Bacteriol. **97**:1001-1004.
2. Biggins, J., and W. E. Dietrich. 1968. Respiratory mechanisms in the *Flexibacteriaceae*. Arch. Biochem. Biophys. **128**:40-50.
3. Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology, seventh edition. The Williams & Wilkins Co., Baltimore.
4. Canale-Parola, E. 1973. Isolation, growth and maintenance of anaerobic free-living spirochetes, p. 61-73. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 8. Academic Press Inc., New York.
5. Dawkins, M. J. R., J. D. Judah, and K. R. Rees. 1959. The mechanism of action of chlorpromazine. Biochem. **73**:16-23.
6. Eagle, H., and H. G. Steinman. 1948. The nutritional requirements of treponemata. J. Bacteriol. **56**:163-176.
7. Hardy, P. H., and E. E. Nell. 1972. A study of the properties of sorbent, the reagent employed in the fluorescent antibody-absorption test. Amer. J. Epidemiol. **96**:141-152.
8. Hardy, P. H., and E. E. Nell. 1955. Specific agglutination of *Treponema pallidum* by sera from rabbits and human beings with treponemal infections. J. Exp. Med. **101**:367-382.
9. Hespell, R. B., and E. Canale-Parola. 1970. Carbohydrate metabolism in *Spirochaeta stenostrepta*. J. Bacteriol. **103**:216-226.
10. Hewitt, E. J., and D. J. D. Nicholas. 1963. Cations and anions: inhibitors and interactions in metabolism and enzyme activity, p. 311-436. *In* R. M. Hochster and J. H. Quastel (ed.), Metabolic inhibitors, vol. II. Academic Press Inc., New York.
11. Hollander, D. H., and E. E. Nell. 1954. Improved preservation of *Treponema pallidum* and other bacteria by freezing with glycerol. Appl. Microbiol. **2**:164-170.
12. Nell, E. E., and P. H. Hardy. 1972. The use of freeze-preserved treponemes in the *Treponema pallidum* immobilization test. Cryobiology **9**:404-410.
13. Nelson, R. A., and H. G. Steinman, 1948. Factors affecting the survival of *Treponema pallidum in vitro*. Proc. Soc. Exp. Biol. Med. **68**:588.
14. Nicholls, P. 1968. Cytochrome oxidase as an allosteric enzyme, p. 76-86. *In* K. Okunuki, M. D. Kamen, and I. Sekuzu (ed.), Structure and function of cytochromes. University Park Press, Baltimore.
15. Portnoy, J., J. H. Brewer, and A. Harris. 1962. Rapid plasma reagin card test for syphilis and other treponematoses. Publ. Health Rep. **77**:645-652.
16. Pumphrey, A. M., and E. R. Redfearn. 1963. Inhibition of succinate oxidation by barbiturates in tightly coupled mitochondria. Biochim. Biophys. Acta **74**:317-327.
17. Quastel, J. H. 1963. Effects of anesthetics, depressants, and tranquilizers on cerebral metabolism, p. 517-538. *In* R. M. Hochster, and J. H. Quastel (ed.), Metabolic inhibitors, vol. II. Academic Press Inc., New York.
18. Report of a WHO scientific group. 1970. Treponematoses research. World Health Organization Technical Report Series no. 455. Geneva.
19. Robinson, J., and J. M. Cooper. 1970. Method of determining oxygen concentrations in biological media, suitable for calibration of the oxygen electrode. Anal. Biochem. **33**:390-399.
20. Smith, L. 1961. Cytochrome systems in aerobic electron transport, p. 365-396. *In* I. C. Gunsalus, and R. Y. Stanier (ed.), The bacteria, vol. II, metabolism. Academic Press Inc., New York.
21. Staneck, J. L., R. C. Henneberry, and C. D. Cox. 1973. Growth requirements of pathogenic *Leptospira*. Infect. Immunity **7**:886-897.
22. Turner, T. B., and D. H. Hollander. 1957. Biology of the treponematoses. Monograph series no. 35. World Health Organization, Geneva.
23. Weber, M. M. 1960. Factors influencing the *in vitro* survival of *Treponema pallidum*. Amer. J. Hyg. **71**:410-417.
24. Willcox, R. R., and T. Guthe. 1966. *Treponema pallidum*. Supplement to vol. 35 of the Bulletin of the World Health Organization. Geneva.