INFECTION AND IMMUNITY, Mar. 1976, p. 704-711

Vol. 13, No. 3
Printed in U.S.A.

Virulent *Treponema pallidum*: Aerobe or Anaerobe

JOEL B. BASEMAN,* JEAN C. NICHOLS, AND NANCY S. HAYES

Department of Bacteriology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

Received for publication 21 October 1975

Substrate degradation and protein synthesis served as indicators of metabolism in virulent *Treponema pallidum*. Optimal metabolic activity in these spirochetes was observed at 10 to 20% O_2 concentrations, with markedly reduced activity at higher or lower O_2 levels or under anaerobiosis; alternate functioning electron acceptors that might substitute for O_2 were not found. Carbon monoxide and cyanide at concentrations that inactivate cytochrome oxidase were not effective metabolic poisons for *T. pallidum*, although *Micrococcus lutea*, a strict aerobe with cytochrome-dependent respiration, was inhibited under similar experimental conditions. Motility of virulent *T. pallidum* was vigorous in the presence of O_2 and sluggish or inhibited in its absence, reinforcing the role of O_2 in *T. pallidum* metabolism.

Essential nutritional and metabolic data required for the successful cultivation of virulent *Treponema pallidum*, the causative agent of syphilis, are lacking. Since these microorganisms have not been grown in vitro, the majority of literature represents attempts to develop experimental methods that prolong the motility and virulence of *T. pallidum* in artificial media (10, 22, 23). These studies have been performed under anaerobic or chemically reduced conditions because members of the genus *Treponema* are classified as strict anaerobes (7), and reports indicate that anaerobiosis or strong electronegative redox potentials extend survival time of *T. pallidum* in vitro (10, 16, 18, 22, 23).

Until recently, no other techniques for measuring metabolic activity of virulent treponemes have been available. In 1974, Cox and Barber (8) demonstrated cyanide-sensitive oxygen uptake by *T. pallidum* in vitro. They concluded that virulent *T. pallidum* consumed oxygen as a terminal electron acceptor through a cyanide-sensitive cytochrome oxidase. However, numerous difficulties arise when interpreting data based upon the specificity of action and influence of metabolic poisons on complex biological systems (9, 13, 20). Furthermore, it was reported that *T. hyodysenteriae*, a cultivable aerotolerant anaerobic spirochete (11), consumed oxygen at a rate similar to *T. pallidum* (D. L. Harris, R. A. Harris, and J. M. Kinyon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, D4, p. 52). They also indicated that oxygen uptake in both *T. hyodysenteriae* and *T. pallidum* was inhibited by 10 mM cyanide but was insensitive to low cyanide levels, as well as other metabolic inhibitors, in contrast to the aforementioned report by Cox and Barber (8). Therefore, the role of oxygen uptake in virulent treponemes needed further clarification.

Our approach has required the development of techniques that would provide highly sensitive assays for measuring the metabolic capabilities of resting suspensions of virulent treponemes under a variety of experimental conditions. By using radioassays, we recently demonstrated the ability of virulent *T. pallidum* to synthesize protein (3) and to degrade pyruvate and glucose to CO_2 and specific end products (19). Thus, physiological requirements of *T. pallidum*, such as appropriate gas atmospheres, substrate and cofactor dependence, temperature and pH optima, and energy pathways, can be monitored as a function of several physiological indicators. In this study we demonstrate the dependence of *T. pallidum* metabolism on oxygen, which serves as a stimulant of protein synthesis, substrate degradation, and motility.

MATERIALS AND METHODS

Bacteria. Virulent Nichols strain of *T. pallidum* received from the Center for Disease Control, Atlanta, Ga., was maintained by serial passage in rabbits (Pel-Freeze) housed in cubicles kept at 16 to 18 C. Suspensions of virulent treponemes were also stored in liquid nitrogen as described by Nell and Hardy (17) prior to intratesticular inoculation.

Radioisotopes and chemicals. The following radioisotopes were supplied by ICN Radioisotope Division: L-[U-^{14}C]pyruvate, 129 mCi/mmol; L-[U-^{14}C]glucose, 180 mCi/mmol; [1-^{14}C]glucose, 10 mCi/mmol; and [6-^{14}C]glucose, 10 mCi/mmol. [1-^{14}C]pyruvate, 7.2 mCi/mmol, was purchased from

New England Nuclear Corp., and a purified ^3H-labeled amino acid mixture was purchased from Schwarz-Mann. Bovine serum ultrafiltrate was acquired from Microbiological Associates, bovine serum albumin from Armour Pharmaceutical Co., and calf serum from Grand Island Biological Co. Other chemicals used in this study were reagent grade.

High-purity gases. Matheson Gas Products provided cylinders of prepurified N_2 and specific gas mixtures of 50% Ar-35% N_2-10% H_2-5% CO_2 and of 53% CO-30% N_2-10% H_2-5% CO_2-2% O_2. Oxygen (100%) was supplied by Air Products.

Establishment of anaerobic conditions. Anaerobiosis was accomplished by using an anaerobic glove box (Coy Manufacturing, Ann Arbor, Mich.; 21) inflated with Ar, N_2, H_2, and CO_2 and outfitted with necessary equipment, such as a motorized shaker, centrifuge, incubator, and two catalyst trays containing deoxypalladium-coated alumina to remove trace amounts of contaminating O_2. Extraction medium (Table 1), extracting flasks, and glass vaccine assay vials required for the experiment were placed in the glove box for overnight equilibration. Since pyruvate and glutathione are relatively unstable in solution at room temperature, these compounds were weighed out prior to the experiments, placed in the anaerobic hood, dissolved in deoxygenated phosphate-buffered saline (pH 7.4), and allowed to equilibrate for 1 to 2 h before use.

To prepare tissue extract containing treponemes, testes from infected rabbits were removed and immediately placed in the entry lock of the glove box. After two successive evacuations with N_2 and equilibration with Ar, N_2, H_2, and CO_2, the tissue was transferred into the main chamber. Treponemes were then obtained by mincing tissue, extracting in medium for 20 min, and centrifuging twice at 500 × *g* for 5 min to sediment the majority of host cellular components. The low-speed supernatant that contained treponemes and some contaminating animal cells was then added to assay vials (3, 19).

TABLE 1. *Composition of extraction medium*

Component	Concn (mM)
$CaCl_2$	0.9
$MgCl_2$	1.4
NH_4Cl	5.2
KCl	2.7
NaCl	140.0
Na_2HPO_4	4.6
KH_2PO_4	1.5
$NaHCO_3$	60.0
Glucose	30.0
Pyruvate	0.3
Glutathione	2.0
TES[a]	10.0
Bovine serum albumin	0.2%
Bovine serum ultrafiltrate	0.5%
Calf serum	10.0%

[a] *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid. Final pH of the medium was 7.5.

Retention of anaerobiosis in the glove box was monitored by two specific indicators: (i) use of a redox solution containing riboflavin, methylene blue, and disodium ethylenediaminetetraacetate, which detects diffusion of O_2 by the appearance of oxidized dye (15), and (ii) growth or lack of growth of recognized strains of anaerobic and aerobic bacteria.

Assay for substrate degradation by T. pallidum. To determine glucose and pyruvate utilization, the low-speed supernatant from infected testicular extract containing approximately 5×10^7 treponemes/ml was added in a volume of 0.9 ml to 2-ml glass vaccine vials containing a glass insert filled with Hyamine hydroxide to capture released $^{14}CO_2$ (19). For these experiments, testicular tissue was extracted in a medium modified by the omission of glucose or pyruvate when radiolabeling was performed with the respective substrates.

We had previously described procedures for establishing tissue controls from infected testicular extract, which included a high-speed testicular supernatant (18,000 × *g*) lacking both treponemes and animal cells to indicate degradation of ^{14}C-labeled substrate to $^{14}CO_2$ by extracellular enzymes and a control containing high-speed supernatant plus animal cells to measure the contribution of host cells to the $^{14}CO_2$ observed in specific test vials (19). To obtain animal cell controls in the present study, infected testicular extract was centrifuged twice at 500 × *g* to pellet animal cells and tissue debris. The low-speed testicular supernatant was then further clarified by filtering through a 0.22-μm membrane filter (Millipore Corp.), which retained treponemes and remaining host cells. The filtrate was then used to resuspend the low-speed pellet (animal cells) to a cell density equivalent to the number of host cells observed microscopically in the assay vials containing the partially purified treponeme preparation. This method required minimal effort, established proper controls, and confined all manipulations to the anaerobic glove box.

After the assay vials were tightly sealed with rubber stoppers, vials were removed from the hood and various amounts of 100% O_2 were introduced via syringe. The percentage of O_2 in vials was calculated as the ratio of injected O_2 to the final volume of gas in the nonliquid phase of the vials. Other vials receiving an identical volume of the Ar-N_2-H_2-CO_2 gas mixture served as anaerobic controls. To establish O_2 titration curves, various amounts of 100% O_2 were injected into anaerobic vials containing redox indicator (14), and the appearance of oxidized methylene blue was monitored. In addition, similar vials were injected with the anaerobic gas mixture to detect possible O_2 contamination, which might arise during the experimental procedures. Under the conditions described, no O_2 contamination of anaerobic vials was detected.

Methods for analysis of $^{14}CO_2$ release and treponemal end products were previously described (19).

Determination of protein synthesis under aerobic and anaerobic conditions. Measurements of protein synthesis were also performed by using 2-ml glass vaccine vials. To establish base line levels of protein synthesis, erythromycin (5 μg/ml) or cycloheximide

(5 μg/ml) was included in the medium to selectively inhibit protein synthesis in treponemes (3) or animal cells (24), respectively, prior to the addition of ^{3}H-labeled amino acid mixture. Aerobic and anaerobic vials were prepared as described in the preceding sections. General procedures and techniques for determining protein synthesis in *T. pallidum* have been reported (3).

RESULTS

Utilization of ^{14}C-labeled substrate by T. pallidum under aerobic and anaerobic conditions. We had earlier observed the selective degradation of pyruvate and glucose by virulent *T. pallidum* incubated under an air atmosphere (19). At that time, it was not known whether O_2 served as an electron acceptor or whether treponemes preferred anaerobiosis since proper anaerobic controls could not be established. We therefore felt that examination of substrate degradation by *T. pallidum* under controlled aerobic and anaerobic atmospheres would permit the identification of optimal conditions for metabolic and energy-yielding pathways. Increased degradation of [1-^{14}C]glucose and L-[U-^{14}C]pyruvate to $^{14}CO_2$ occurred when *T. pallidum* was exposed to air (Table 2). Little $^{14}CO_2$ was released from [6-^{14}C]glucose under either aerobic (19) or anaerobic conditions.

To identify possible differences in pathways of substrate degradation, end products of metabolism were examined by silicic acid chromatography (19). As seen in Fig. 1A, aerobic oxidation of [1-^{14}C]glucose resulted in the formation of significant amounts of acetate, pyruvate, and lactate; similar results were obtained from utilization of [6-^{14}C]glucose (Fig. 1C). Under anaerobic conditions, glucose was primarily degraded to pyruvate and lactate, with little accumulation of acetate (Fig. 1B and D). No significant level of radioactivity was associated with treponemal cell material during the incubation.

Since we had reported that acetate and lactate were not utilized by virulent treponemes under similar aerobic conditions (19), differences in the ratio of counts per minute from the individual end products to the counts per minute from the total recovered radioactive peaks (Fig. 1) suggested that variations existed in aerobic and anaerobic metabolism. Additional data indicated that accumulation of specific end products continued during longer incubations of treponemes in air, whereas no further increase was apparent after 4 h in an anaerobic atmosphere (unpublished data). These observations suggested that oxygen plays a role in *T. pallidum* metabolism. In Fig. 2, there was a correlation of O_2 concentration to the amount of $^{14}CO_2$ released from L-[U-^{14}C]glucose, with the maximal detection at 20% O_2.

Protein synthesis in virulent T. pallidum under various atmospheric conditions. Although increased levels of O_2 effected a greater CO_2 release from glucose, it was not evident that energy-generating reactions accompanied aerobic or anaerobic metabolism. We therefore examined the ability of virulent treponemes to synthesize protein under similar experimental conditions since continued protein synthesis would require the hydrolysis and regeneration of high-energy bonds. Optimal incorporation of ^{3}H-labeled amino acids into protein occurred at initial concentrations of 10 to 20% O_2, whereas a marked reduction in protein synthesis was observed at high and low O_2 levels and under anaerobic conditions (Table 3). To further characterize the biosynthetic capacity of virulent *T. pallidum* in vitro, the kinetics of protein synthesis were monitored during the incubation of treponemes under anaerobiosis or in the presence of O_2 (Fig. 3). The rate of protein synthesis was almost linear during a 24-h period in 20% O_2 but ceased within 3 to 6 h after anaerobic incubation. If vials containing treponemes were placed in the anaerobic glove chamber 3 h prior

TABLE 2. *Degradation of ^{14}C-labeled substrate to $^{14}CO_2$ under aerobic and anaerobic atmospheres*

Vial	Substrate	Aerobic[a] counts/min[b]	Anaerobic counts/min[b]
Treponemes	[1-^{14}C]glucose	4,161	1,649
Animal cells		671	729
Treponemes	[6-^{14}C]glucose	863	737
Animal cells		703	688
Treponemes	L-[U-^{14}C]pyruvate	60,594	27,397
Animal cells		16,213	18,049

[a] Aerobic conditions were established by exposing vials to air after their removal from the anaerobic glove box.

[b] Average of triplicate values from two separate experiments. ^{14}C substrate (0.5 μCi) was added at zero time, and $^{14}CO_2$ release was determined after 4 h of incubation at 34 C.

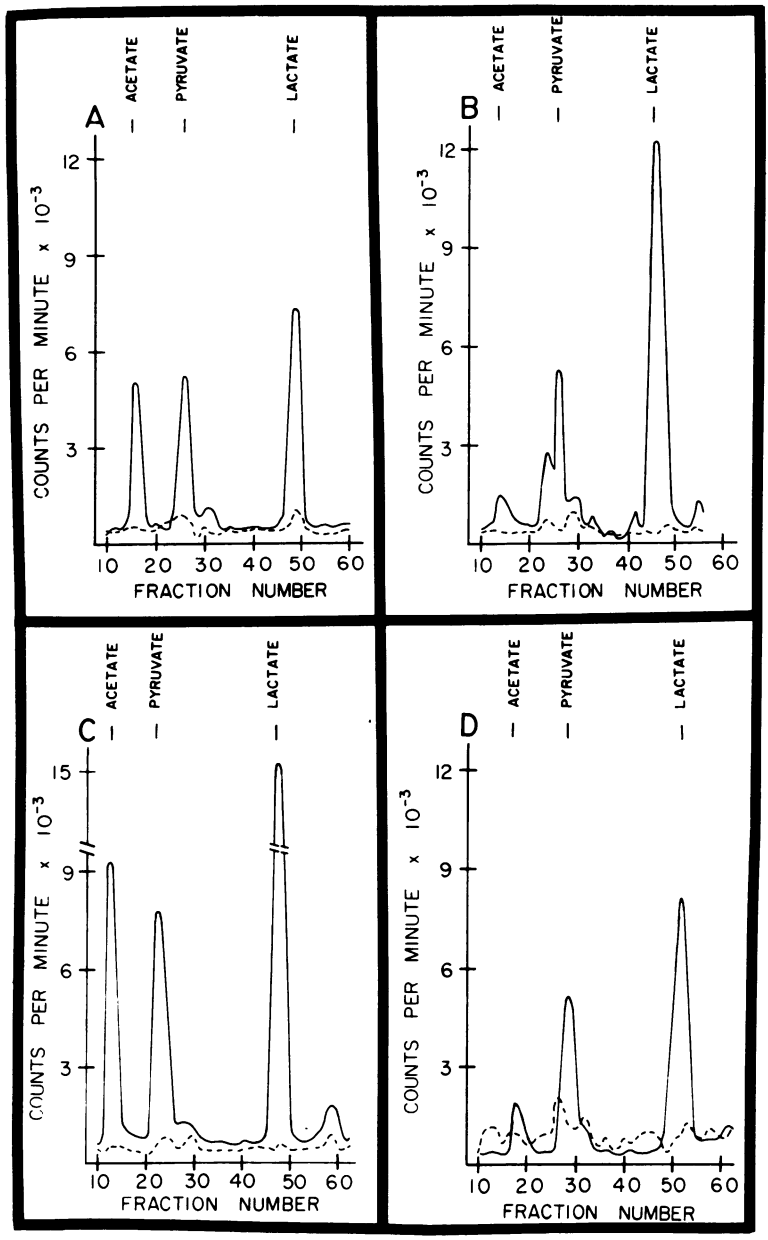

FIG. 1. *End product analysis of glucose degradation by T. pallidum and animal cell controls during aerobic and anaerobic incubation for 4 h at 34 C. (A) [1-^{14}C]glucose in air, (B) [1-^{14}C]glucose anaerobically, (C) [6-^{14}C]glucose in air, (D) [6-^{14}C]glucose anaerobically. Treponemes (solid line); animal cells (broken line).*

to the addition of radioisotope, almost no protein synthesis was detected. These data suggest that early protein synthesis in *T. pallidum* during anaerobic incubation might be attributed to residual O_2 present in the tissue extract or to the metabolism of reserve substrates via substrate level phosphorylation.

Influence of cyanide and carbon monoxide on T. pallidum metabolism. It was suggested from the previous experiments that O_2 served as a functional electron acceptor for *T. pallidum*. Therefore, the effects of cyanide and carbon monoxide on metabolic function in *T. pallidum* were monitored because of their known inhibition of cytochrome oxidase (13, 20). Since CN can be inactivated by forming cyanhydrins

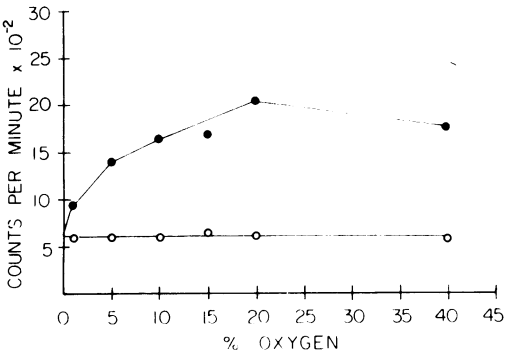

FIG. 2. *Degradation of L-[U-^{14}C]glucose to $^{14}CO_2$ at varying O_2 concentrations. Details as in Table 2. O_2 levels in specific vials and animal cell controls were established as described in the text. Symbols: ●, Treponemes; ○, animal cells.*

TABLE 3. *Effect of different oxygen concentrations on ^{3}H-labeled amino acid incorporation into protein*

Oxygen concn (%)	Counts/min[a]
50	3,989
40	4,743
30	8,474
20	12,518
15	12,381
10	11,087
5	6,085
1	3,468
0	2,092

[a] Average duplicate values from three separate experiments. Varying amounts of O_2 were injected into vials prepared as described in the text. Treponemes were exposed to ^{3}H-labeled amino acid mixture (3 μCi) and incubated in the presence of cycloheximide (5 μg/ml) at 34 C for 24 h.

with keto acids (20), extraction medium lacking pyruvate was prepared, and the influence of CN on substrate utilization and protein synthesis was examined. Only high concentrations of CN reduced *T. pallidum* degradation of pyruvate or glucose to CO_2 (Table 4). Similarly, the sensitivity of protein synthesis to CN occurred only after prolonged incubation in relatively high CN concentrations (Table 5). As a control, *Micrococcus lutea*, a strict aerobe with cytochrome-dependent respiration (7), was grown in Trypticase soy broth, centrifuged, washed twice in phosphate-buffered saline, and resuspended in treponemal extraction medium lacking pyruvate but supplemented with infected tissue filtrate (see Materials and Methods). The latter components constitute a nonpermissive growth medium for *M. lutea*, as determined by viable plate counts, and were included to permit direct comparisons with *T. pallidum* data by avoiding

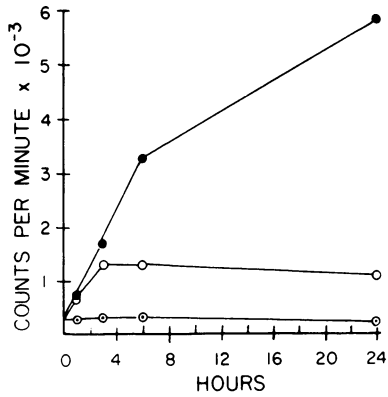

FIG. 3. *Rate of protein synthesis in T. pallidum extracted under anaerobic conditions. Treponemes were incubated with a ^{3}H-labeled amino acid mixture (3 μCi) for 1, 3, 6 or 24 h. Symbols: ●, Treponemes exposed to 20% O_2; ○, treponemes maintained in an anaerobic atmosphere; ⊙, treponemes maintained in 20% O_2 or anaerobic atmospheres in the presence of erythromycin (5 μg/ml).*

experimental variables, such as media and growth differences. Protein synthesis in *M. lutea* was highly sensitive to CN concentrations, with greater than 50% inhibition occurring after 6 h of incubation in levels of CN as low as 0.05 mM (data not shown).

To study the effect of CO, vials were prepared and sealed in the anaerobic hood and then removed and gassed continuously for 2 min with the CO-containing gas mixture. Gassing was accomplished by inserting a 22-gauge needle that was connected to the CO gas cylinder into the rubber stopper of the assay vial. A second needle served as the outlet valve. O_2 was then introduced via syringe to establish a 20% level. As in the case of CN, no reduction in metabolic activity of *T. pallidum* was observed (data not shown). However, pyruvate utilization and protein synthesis in resting cultures of *M. lutea* suspended in treponemal medium containing tissue filtrate were inhibited by greater than 40% within 6 h.

Effect of alternative electron acceptors on T. pallidum metabolism. In an attempt to examine electron flow mechanisms in virulent treponemes, various compounds that might serve as alternate electron acceptors were added to the extraction medium, and metabolic activity of *T. pallidum* was monitored under anaerobiosis and 20% O_2. As seen in Table 6, these compounds were generally ineffective. However, the presence of ferric ammonium citrate in the medium increased degradation of pyruvate to CO_2 while decreasing protein synthesis. Variations in the oxidation-reduction

TABLE 4. *Effect of potassium cyanide on ^{14}C-labeled substrate degradation to $^{14}CO_2$ by virulent T. pallidum and animal cells*[a]

KCN concn (mM)	L-[U-^{14}C]glucose			[1-^{14}C]pyruvate		
	Counts/min[b]		Inhibition (%)[c]	Counts/min[b]		Inhibition (%)[c]
	Treponeme	Animal cell		Treponeme	Animal cell	
	5,980	606		167,960	13,086	
10.0	1,358	535	85	11,116	2,472	96
2.5	3,958	672	39	83,762	3,776	48
1.0	5,978	644	1	130,408	7,043	20
0.25	7,971	650	0	159,242	10,832	4
0.1	7,688	607	0	168,034	12,060	0
0.05	7,570	602	0	188,426	7,120	0

[a] Experimental conditions are described in Table 2, except that incubation was performed in an atmosphere containing 20% O_2. Concentrated solutions of KCN were freshly prepared and adjusted to a pH of 7.5.

[b] Average of duplicate values from two separate experiments.

[c] Determined as (treponeme counts/min − animal cell counts/min) in KCN/(treponeme counts/min − animal cell counts/min), no KCN × 100. This value was then subtracted from 100% to give % inhibition.

TABLE 5. *Protein synthesis by virulent T. pallidum in the presence of varying concentrations of potassium cyanide*[a]

KCN concn (mM)	Counts/min		Inhibition (%)	
	6 h	24 h	6 h	24 h
0	4,238	10,300		
10.0	438	1,772	91	83
1.0	3,892	7,261	8	30
0.25	5,547	6,187	0	40
0.1	3,974	9,210	6	11
0.05	4,928	11,599	0	0

[a] Details as in Table 3, except that treponemes were incubated for 6 and 24 h in an atmosphere containing 20% O_2 prior to harvesting.

potential of the assay medium as a result of adding differing concentrations of potassium ferricyanide to anaerobic vials did not stimulate protein synthesis, thus further implicating the role of O_2 in *T. pallidum* metabolism.

Motility of T. pallidum after aerobic and anaerobic incubation. Dark-field microscopy of media from representative assay vials was performed to determine the degree and percentage of motility in virulent treponemes prior to terminating the experiment. After 24 h of incubation at 34 C, 75 to 90% of *T. pallidum* exposed to O_2 concentrations of 1 to 20% demonstrated vigorous motility. In contrast, 70 to 90% of treponemes incubated under anaerobiosis or high O_2 concentrations (30 to 50% O_2) were nonmotile; the population of motile spirochetes was noticeably sluggish.

DISCUSSION

From prelimary studies it was apparent that any manipulations of infected rabbit testicular tissue, such as mincing or slicing of tissue or centrifugation of crude extract in an air atmosphere (3, 4, 19), permitted considerable diffusion of O_2 into the anaerobic, prereduced extraction medium (Table 1). Attempts to perform these techniques in an open room but under a flow of scrubbed Ar, N_2, H_2, and CO_2 gases still resulted in significant O_2 contamination, as determined by redox indicators. These observations indicated that, in all previous reports that referred to anaerobic culture conditions for *T. pallidum*, significant levels of O_2 must have existed during primary incubations. Therefore, to resolve the role of O_2 in *T. pallidum* metabolism, we employed techniques that permitted all manipulations to occur initially in a glove box under anaerobic conditions (see Materials and Methods).

By using substrate degradation and protein synthesis as separate and distinct parameters of metabolic function, we demonstrated that O_2 significantly influences glucose and pyruvate utilization and the rate and extent of protein synthesis by *T. pallidum*. The latter point is relevant since protein synthesis requires high-energy molecules (adenosine 5'-triphosphate, guanosine 5'-triphosphate), and it is unlikely that their endogenous pool size in *T. pallidum* would permit continued protein synthesis, as indicated in Fig. 3, without replenishment. The role of O_2 is further implicated in *T. pallidum* metabolism since protein synthesis under anaerobic conditions was inhibited after several hours of incubation. In addition, CO_2 release and end-product accumulation that followed the degradation of radiolabeled glucose by virulent treponemes ceased during continued anaerobic incubation, in contrast to aerobic atmospheres. The fact that detectable levels of substrate degradation and protein synthesis

TABLE 6. *Influence of alternate electron acceptors on metabolic activity of T. pallidum under aerobic and anaerobic atmospheres*

Electron acceptor	Concn (%)	[1-^{14}C]pyruvate → $^{14}CO_2$ (counts/min)[a]		^{3}H-labeled amino acids → protein (counts/min)[a]	
		O_2 (20%)	Anaerobic	O_2 (20%)	Anaerobic
None		81,017	26,596	8,604	833
Sulfate	0.05	70,468	39,807	8,624	928
Nitrate	0.05	60,959	30,807	9,038	822
Fumarate	0.1	103,035	25,094	6,431	633
Citrate	0.1	98,752	12,701	10,331	669
Ferric ammonium citrate	0.1	92,547	77,063	3,443	658

[a] These numbers represent the average value of duplicate samples from four separate experiments. Details for pyruvate utilization appear as in the footnotes to Table 2 and for protein synthesis as in Table 3.

(Fig. 2 and 3) were observed during the early hours of anaerobiosis could reflect the presence of limiting amounts of O_2 that are sequestered and remain in testicular tissue during the extraction. Clearly, O_2 must exist in rabbit tissue at the time of sacrifice. It should be noted that ferric ammonium citrate, which stimulated CO_2 release from pyruvate under anaerobic conditions, inhibited protein synthesis under an atmosphere containing 20% O_2. These results suggest that ferric ammonium citrate acted as an artificial electron acceptor, depriving treponemes of a normal, energy-generating electron flow.

Identification of CO_2, acetate, lactate, and pyruvate as metabolic end products during aerobic incubation indicates an incomplete oxidation of glucose by *T. pallidum*. This observation is consistent with our previous demonstration in *T. pallidum* of pyruvate degradation to CO_2, acetate, and lactate (19). However, no significant amount of trichloroacetic acid-precipitable material was associated with *T. pallidum* during incubation for 6 to 12 h in the presence of radioactive glucose or pyruvate (19), although these substrates were clearly degraded. This observation suggests that culture conditions and/or the state of the organisms remain inadequate for growth. It is of interest that *Spirochaeta aurantia*, the only known facultative anaerobic spirochete, accumulates similar end products after aerobic oxidation of glucose (6) but ferments glucose to ethanol, acetate, CO_2, and H_2 (5). Under anaerobic conditions, *T. pallidum* degraded glucose primarily to lactate and pyruvate only during the early hours of incubation.

Little influence of inhibitors of cytochrome oxidase on *T. pallidum* metabolism was apparent. CN was toxic only at high concentrations. This latter observation may be explained by the inactivation of CN by components in the medium (13, 20) which could readily influence relative CN sensitivity of *T. pallidum*, or by limited penetration of CN. However, *T. pallidum* metabolism was also uneffected by CO under similar experimental conditions in which pyruvate utilization and protein synthesis in *M. lutea* were significantly reduced. These data imply that electron flow in virulent *T. pallidum* might terminate at an earlier step in oxidative phosphorylation, such as nicotinamide adenine dinucleotide, flavoprotein, or cytochrome *b* or *c* species, any of which could undergo oxidation in an O_2-containing atmosphere and in the presence of CN and CO. Alternatively, the electron transport pathway in *T. pallidum* may utilize the majority of the respiratory chain but terminate with a cyanide-insensitive oxidase (13). It should be reemphasized, however, that interpretation of data based upon the action of metabolic inhibitors is complicated by numerous experimental variables, thus preventing any definitive statement until specific properties of the electron transport system in *T. pallidum* can be characterized. Furthermore, *T. pallidum* organisms exist as resting cells in vitro, and certain observed reactions may not reflect essential mechanisms required for the growth of these spirochetes. In fact, the possibility still exists that under growth-permissive conditions *T. pallidum* may possess a CN-sensitive respiration as reported by Cox and Barber (8).

In general, active motility of treponemes was observed in a range of initial O_2 concentrations from 1 to 20%, and motility clearly decreased under high O_2 concentrations and anaerobiosis. These data would seem inconsistent with reports in the literature indicating prolonged motility of *T. pallidum* under anaerobic conditions; however, as discussed earlier, experimental procedures of earlier investigations probably did not establish anaerobiosis. The fact that little difference in motility was observed at the low to mid range of O_2 concentra-

tions should caution researchers as to the relevance of motility as an indicator of optimal culture conditions. Protein synthesis and substrate utilization were markedly reduced at low O_2 concentrations (Fig. 2, Table 3) although no change in the motility of *T. pallidum* could be readily detected. This point is further supported by our earlier report of actively motile treponemes after prolonged incubation in erythromycin, which inhibited protein synthesis by greater than 90% (3).

It would appear that electron flow and energy-generating mechanisms in virulent *T. pallidum* are stimulated by O_2. This property separates *T. pallidum* from other species in the genus *Treponema* but not from other members of the *Spirochaetales*. *Leptospira*, a genus of aerobic spirochetes, possesses both a functioning tricarboxylic acid cycle (1) and a multicytochrome system with cytochrome *o* as the terminal oxidase (2). *S. aurantia* has the metabolic capability to grow under both aerobic (6) and anaerobic (5) conditions, and *Borrelia hermsi*, considered to be a strict anaerobe (7), requires small amounts of oxygen for its cultivation (14).

Our data establish a requirement for O_2 in resting suspensions of *T. pallidum*. Unless other functional electron acceptors can be found, initial concentrations of O_2 between 10 to 20% are optimal for metabolic activity of these spirochetes in vitro. Virulent *T. pallidum* may derive energy by oxidation of substrates via an incomplete electron transport chain, involving O_2 as the terminal electron acceptor. However, the possibility that substrate-level phosphorylation serves as a major source of energy in *T. pallidum* metabolism or that different metabolic patterns will be obtained under permissive growth conditions cannot be excluded until the functional role of O_2 is defined. Nonetheless, further attempts to grow *T. pallidum* under anaerobiosis or to use prolonged motility as the critical measure of optimal in vitro growth conditions should be reevaluated at this time.

ACKNOWLEDGMENTS

We thank R. L. Uffen and J. A. Breznak, Michigan State University, and E. C. Hayes, Duke University, for critical review of the manuscript.

This work was supported by Public Health Service grant AI 11283 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Baseman, J. B., and C. D. Cox.** 1969. Intermediate energy metabolism of *Leptospira*. J. Bacteriol. 97:992-1000.
2. **Baseman, J. B., and C. D. Cox.** 1969. Terminal electron transport in *Leptospira*. J. Bacteriol. 97:1001-1004.
3. **Baseman, J. B., and N. S. Hayes.** 1974. Protein synthesis by *Treponema pallidum* extracted from infected rabbit tissue. Infect. Immun. 10:1350-1355.
4. **Baseman, J. B., J. C. Nichols, J. W. Rumpp, and N. S. Hayes.** 1974. Purification of *Treponema pallidum* from infected rabbit tissue: resolution into two treponemal populations. Infect. Immun. 10:1062-1067.
5. **Breznak, J. A., and E. Canale-Parola.** 1972. Metabolism of *Spirochaeta aurantia*. I. Anaerobic energy-yielding pathways. Arch. Microbiol. 83:261-277.
6. **Breznak, J. A., and E. Canale-Parola.** 1972. Metabolism of *Spirochaeta aurantia*. II. Aerobic oxidation of carbohydrates. Arch. Microbiol. 83:278-292.
7. **Buchanan, R. E., and N. E. Gibbons.** 1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
8. **Cox, C. D., and M. K. Barber.** 1974. Oxygen uptake by *Treponema pallidum*. Infect. Immun. 10:123-127.
9. **Gibson, Q. H.** 1963. Inhibitors of gas transport, p. 539-550. *In* R. M. Hochster and J. H. Quastel (ed.), Metabolic inhibitiors, vol. II. Academic Press Inc., New York.
10. **Graves, S. R., P. L. Sandok, H. M. Jenkin, and R. C. Johnson.** 1975. Retention of motility and virulence of *Treponema pallidum* (Nichols strain) in vitro. Infect. Immun. 12:1116-1120.
11. **Harris, D. L., J. M. Kinyon, M. T. Mullin, and R. D. Glock.** 1972. Isolation and propagation of spirochetes from the colon of swine dysentery affected pigs. Can. J. Comp. Med. 36:74-76.
12. **Henry, M. F., and E. J. Nyns.** 1975. Cyanide-insensitive respiration. An alternative mitochondrial pathway. Sub-Cell. Biochem. 4:1-65.
13. **Hewitt, E. J., and D. J. D. Nicholas.** 1963. Cations and anions: inhibitions and interactions in metabolism and in enzyme activity, p. 311-436. *In* R. M. Hochster and J. H. Quastel (ed.), Metabolic inhibitors, vol. II. Academic Press Inc., New York.
14. **Kelly, R.** 1971. Cultivation of *Borrelia hermsi*. Science 173:443-444.
15. **Merkel, J. R., and W. J. Nickerson.** 1954. Riboflavin as a photocatalyst and hydrogen carrier in photochemical reduction. Biochim. Biophys. Acta 14:303-311.
16. **Metzger, M., and W. Smogor.** 1966. Study of the effect of pH and Eh values of the Nelson-Diesendruck medium on the survival of virulent *Treponema pallidum*. Arch. Immunol. Ther. Exp. 14:445-453.
17. **Nell, E. E., and P. H. Hardy.** 1972. The use of freeze-preserved treponemes in the *Treponema pallidum* immobilization test. Cryobiology 9:404-410.
18. **Nelson, R. A., Jr.** 1948. Factors affecting the survival of *Treponema pallidum in vitro*. Am. J. Hyg. 48:120-132.
19. **Nichols, J. C., and J. B. Baseman.** 1975. Carbon sources utilized by virulent *Treponema pallidum*. Infect. Immun. 12:1044-1050.
20. **Slater, E. C.** 1967. Application of inhibitors and uncouplers for a study of oxidative phosphorylation, p. 48-57. *In* R. W. Estabrook and M. E. Pullman (ed.), Methods in enzymology, vol. X. Academic Press Inc., New York.
21. **Uffen, R. L.** 1973. Growth properties of *Rhodospirillum rubrum* mutants and fermentation of pyruvate in anaerobic, dark conditions. J. Bacteriol. 116:874-884.
22. **Weber, M. M.** 1960. Factors influencing the *in vitro* survival of *Treponema pallidum*. Am. J. Hyg. 71:401-417.
23. **Willcox, R. R., and T. Guthe.** 1966. *Treponema pallidum:* a bibliographical review of the morphology, culture and survival of *T. pallidum* and associated organisms. Bull. W.H.O. 35:(Suppl.):1-169.
24. **Willis, M. W., J. B. Baseman, and H. Amos.** 1974. Noncoordinate control of RNA synthesis in eucaryotic cells. Cell 3:179-184.