INFECTION AND IMMUNITY, Aug. 1978, p. 462-473
0019-9567/78/0021-0462$02.00/0

Vol. 21, No. 2
Printed in U.S.A.

Respiration and Oxidative Phosphorylation in *Treponema pallidum*

PAUL G. LYSKO AND C. D. COX*
Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003

Received for publication 22 March 1978

Exogenous and endogenously generated reduced pyridine nucleotides caused marked stimulation of O_2 uptake when added to treponemal cell-free extracts, which indicated that terminal electron transport was coupled to the consumption of O_2. Oxidation of reduced nicotinamide adenine dinucleotide (NADH) was shown to correlate stoichiometrically with O_2 reduction, suggesting that NADH was being oxidized through a mainstream respiratory chain dehydrogenase. Oxygen evolution in treponemal extracts was observed after the completion of O_2 uptake which was stimulated by exogenous NADH and endogenously generated reduced NAD phosphate. Oxygen evolution was inhibited by both cyanide and pyruvate, which was consistent with O_2 release from H_2O_2 by catalase. The addition of exogenous H_2O_2 to treponemal extracts caused rapid O_2 evolution characteristic of a catalase reaction. A spectrophotometric assay was used to measure ATP formation in *T. pallidum* cell-free extracts that were stimulated with NADH. P/O ratios from 0.5 to 1.1 were calculated from the amounts of ATP formed versus NADH oxidized. Phosphorylating activity was dependent on P_i concentration and was sensitive to cyanide, *N,N'*-dicyclohexylcarbodiimide, and carbonyl cyanide *m*-chlorophenyl hydrazone. Adenine nucleotide pools of *T. pallidum* were measured by the firefly luciferin-luciferase assay. Shifts in adenine nucleotide levels upon the addition of NADH to cell-free extracts were impossible to evaluate due to the presence of NAD^+ nucleosidase. However, when whole cells, previously incubated under an atmosphere of 95% N_2–5% CO_2, were sparged with air, ATP and ADP levels increased, while AMP levels decreased. The shift was attributed to both oxidative phosphorylation and to the presence of an adenylate kinase activity. *T. pallidum* was also found to possess an Mg^{2+}- and Ca^{2+}-stimulated ATPase activity which was sensitive to *N,N'*-dicyclohexylcarbodiimide. These data indicated a capability for oxidative phosphorylation by *T. pallidum*.

The anaerobic nature of *Treponema pallidum* was recently challenged by a report from this laboratory (16) that *T. pallidum* consumed O_2 at a rate comparable to that of the known aerobe *Leptospira*. This treponeme also possesses enzymes of the Embden-Meyerhof-Parnas and hexose monophosphate pathways, which could provide reducing power and substrate level phosphorylation (7, 35, 41). We have previously reported the presence of cytochromes of the *b* and *c* types as well as large amounts of flavoprotein (29). Difference spectra of the CO-binding pigment identified cytochrome *o* as the terminal oxidase. The terminal electron transport chain was physiologically functional, indicating that *T. pallidum* was capable of aerobic respiration. The purpose of this investigation was to study the nature of O_2 uptake by *T. pallidum* and to determine whether or not terminal electron transport was coupled to oxidative phosphorylation.

MATERIALS AND METHODS

Bacteria. Extraction of *T. pallidum* from infected rabbits was performed as described previously (29). Briefly, treponemes were separated from tissue cells by low-speed centrifugation followed by filtration. Soluble tissue components were removed from the treponemes by differential centrifugation and washing. Washed, pelleted treponemes were disrupted by sonic oscillation to form a cell-free extract (CFE) for use in enzyme assays and in O_2 uptake experiments. Treponemal membranes were obtained for the ATPase assay by centrifuging the CFE for 20 min at 32,000 × *g*. The membrane pellet was washed twice with 1 mM $MgSO_4$ and suspended in 0.1 M tris(hydroxymethyl)aminomethane (Tris), pH 7.5. When whole-cell suspensions were monitored for O_2 uptake, the extraction medium consisted of 10 mM morpholinopropane sulfonic acid, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES), 15 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid, 2 mM NaH_2PO_4, 16 mM $NaHCO_3$, 50 mM NaCl, and 0.1% glucose. The pH was adjusted to 7.4 with NaOH. The organic buffer extraction medium did not consume O_2.

Treponemes for the sparging experiments were extracted from the tissue, washed, and resuspended in this organic extraction medium.

Chemicals. The following enzymes were obtained from Sigma Chemical Co.: glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49) type XV, hexokinase (EC 2.7.1.1) Type C-300, pyruvate kinase (EC 2.7.1.40) type III, adenylate kinase (EC 2.7.4.3) type III, lactate dehydrogenase (EC 1.1.1.27) type III, alkaline phosphatase (EC 3.1.3.1) type III-S, adenylic acid deaminase (EC 3.5.4.6) type IV, and phosphoglycerate kinase (EC 2.7.2.3), and glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12) from the ATP diagnostic kit 366-UV. Nicotinamide adenine dinucleotide (NAD), NADH, NADP, and NADPH were of the highest grade available from Sigma. Firefly extract was obtained from either Worthington Biochemicals or from Sigma. *N,N'*-dicyclodihexylcarbodiimide (DCCD) was obtained from Eastman Kodak Co. and was dissolved in dimethyl sulfoxide (DMSO) grade I from Sigma. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was obtained from Nutritional Biochemicals Corp., and was dissolved in DMSO. All other chemicals, enzyme substrates, and inhibitors were reagent grade or the equivalent.

Determination of oxygen uptake. O_2 uptake was determined as previously described (16). Whole-cell suspensions and CFE of *T. pallidum*, in 4.0-ml volumes, were sparged with air to achieve air saturation before assay. Dissolved oxygen concentration was continuously recorded as percent air saturation versus time. The absolute concentrations of O_2 in the media were determined by the method of Robinson and Cooper (39), which used NADH to quantitatively reduce O_2 through the mediation of *N*-methylphenazonium methosulfate.

Assay for oxidative phosphorylation. The spectrophotometric assay of Pinchot (36) was used for measuring oxidative phosphorylation in *T. pallidum* CFE. Oxidative phosphorylation was measured continuously by coupling ATP formation to the formation of NADPH via the following reactions: $NADH^+ + H^+$

$$+ ADP + P_i + ½ O_2 \xrightarrow{CFE} NAD^+ + ATP + H_2O;$$

$$ATP + glucose \xrightarrow{hexokinase,\ Mg^{2+}} glucose\text{-}6\text{-}phos\text{-}$$

phate + ADP; and glucose-6-phosphate + $NADP^+$ $\xrightarrow{G6PD}$ NADPH + 6-phosphogluconate. The oxidation of NADH to NAD, and the reduction of NADP to NADPH were monitored at 340 nm with a Gilford model 240 spectrophotometer. The control cuvette, which measured only NADH oxidation, contained (micromoles): Tris (pH 7.5), 120; P_i, 2.5; $MgCl_2$, 3.0; glucose, 12.5; and ADP, 0.025. The experimental cuvette, which measured both NADH oxidation and NADP reduction, contained, in addition to the above, 0.1 μmol of NADP, 0.125 U of G6PD, and 0.5 U of hexokinase. Both cuvettes received 50 μg of protein CFE as determined by the method of Lowry et al. (28) and were allowed to equilibrate for a few minutes. To start the oxidative phosphorylation reaction, 0.125 μmol of NADH was added to each cuvette in a volume of 50 μl, which brought the total volume in each cuvette to 0.5 ml. The cuvettes were mixed by inversion, and the change in absorbance at 340 nm was monitored for 5 min.

P_i determination. Assays for P_i were performed essentially by the colorimetric method of Fiske and SubbaRow (20) as described by Klemme and Gest (26). Protein was precipitated with one-half volume of 10% trichloroacetic acid to prepare sample solutions for the phosphate assay. After low-speed centrifugation, the supernatant fluid was assayed. Reagents were added to 10 μl of supernatant fluid in the following order: 100 μl of 1% ammonium molybdate in 2 N H_2SO_4, 50 μl of 2% *p*-methyl-1-aminophenol sulfate in 6% $NaHSO_3$, and 150 μl of distilled water. The solution was mixed, allowed to react for 5 min, and the absorbance at 660 nm was determined. Concentrations of P_i were determined from a standard curve.

Enzyme assays. Adenylate kinase was assayed by the procedure of Colowick (15) which used 5'-adenylic acid deaminase to detect AMP formation from ADP. $NAD(P)^+$ transhydrogenase (EC 1.6.1.1), which catalyzes the conversion of NADPH + NAD^+ to $NADP^+$ + NADH, was assayed by the method of Kaplan (25), except that G6PD was used to generate NADPH from glucose-6-phosphate and NADP, instead of using isocitrate dehydrogenase to generate NADPH from isocitrate and NADP. NAD^+ nucleosidase (EC 3.2.2.5), which cleaves NAD into nicotinamide and ADP-ribose moieties, was assayed by the method of Kaplan (24), which measured the absorbance of an NAD-cyanide complex. ATPase (EC 3.6.1.3) activity was measured by the procedure of Abrams et al. (1), by monitoring the reaction mixture for the release of P_i from ATP. Units of enzyme activity were expressed in terms of micromoles of product formed or lost per minute. Specific activities were reported as units per milligram of protein.

Extraction of adenine nucleotides. Adenine nucleotides were extracted essentially by the chloroform extraction procedure of Dhople and Hanks (17). This procedure was especially suitable for small samples, because the nucleotides were not diluted as in a perchloric acid extraction. Chloroform (0.12 ml) was added to 0.4 ml of either a treponemal suspension or CFE in an acid-cleaned 20-mm glass tube to give a final concentration of 23% (vol/vol) chloroform. This mixture was blended in a Vortex mixer for 15 s, and the tube was placed into a boiling-water bath for 1 min and shaken slightly to prevent bumping. A vacuum of 600 mm of mercury was then applied with a hand pump, and with more shaking the sample was reduced to dryness in approximately 60 s. Samples were rehydrated immediately with 0.4 ml of a buffer solution consisting of 4 mM $MgSO_4$ and 16 mM potassium TES (Mg-TES) pH 7.4, blended in a Vortex mixer for 1 min, and assayed immediately. This procedure effectively extracted 98 to 100% of the adenine nucleotides, as determined by re-extraction. Furthermore, there was no loss of adenine nucleotides when a known amount of mixed standards was extracted by this procedure.

Spectrophotometric assays of adenine nucleotide standards. Concentrations of adenine nucleotide solutions were determined spectrophotometrically, and the solutions were diluted for use as standards in the more sensitive luciferin-luciferase assay. ATP was standardized by use of an ATP diagnostic kit which measured the phosphorylation of 3-phosphoglycerate by phosphoglycerate phosphokinase and ATP. The reaction product, 1,3-diphosphoglycerate, was simultaneously converted to glyceraldehyde-3-phosphate by the glyceraldehyde phosphate dehydrogenase reaction which is dependent on NADH. The decrease in absorbance at 340 nm that resulted from the oxidation of NADH was a measure of the amount of ATP present. ADP and AMP were standardized by a modification of the method of Adam (2). ADP was converted to ATP in the presence of excess phosphoenolpyruvate and pyruvate kinase, forming stoichiometric amounts of pyruvate. Conversion of pyruvate to lactate in the presence of excess lactate dehydrogenase and NADH was measured by monitoring the disappearance of NADH spectrophotometrically at 340 nm. Adenylate kinase was used to convert AMP to ADP. The reaction mixture of 3.0 ml contained (micromoles): Tris (pH 7.5), 480; $MgSO_4$, 75; KCl, 75; tricyclohexylammonium phosphoenolpyruvate, 0.5; ATP, 0.5; NADH, 0.3, and 3.3 U of pyruvate kinase, 7.4 U of lactate dehydrogenase, and approximately 0.025 μmol of AMP. The reaction to determine the ADP concentration was started with the addition of approximately 0.050 μmol of ADP, and the subsequent decrease in absorbance at 340 nm was monitored. When the reaction to determine ADP was completed (about 10 min), 9.6 U of adenylate kinase were added to determine the exact AMP concentration, and the decrease in absorbance at 340 nm was again monitored.

Measurement of adenine nucleotides by the firefly assay. Adenine nucleotide pools of *T. pallidum* were measured by the firefly luciferin-luciferase assay, by the procedures of Robertson and Wolfe (38) and Ball and Atkinson (6), with slight modifications. Freeze-dried firefly extract, which contained 20 mM magnesium sulfate and 50 mM potassium arsenate buffer when reconstituted with 5.0 ml of distilled water, was allowed to stand refrigerated for 2 to 4 h after reconstitution. The suspension was centrifuged at low speed to remove insoluble debris, and the supernatant fluid was used for the assay. ATP was measured by the luciferin-luciferase assay by adding either 75 μl of sample, or portions of a standardized solution of ATP, to an acid-cleaned glass scintillation vial containing 5.0 ml of Mg-TES buffer. The luminescence reaction was initiated by the addition of 25 μl of firefly extract to the scintillation vial which was then swirled to mix contents and introduced within 7 s into the counting chamber of a Packard model 3003 Tri-Carb liquid scintillation spectrometer. The spectrometer was set as for tritium counting, with the base-line discriminator at 50, the upper discriminator at 1,000, and the amplifier gain selector at 100%, with the coincidence circuit off. Each vial was counted for four successive 6-s periods. Samples were run in duplicate, background counts were subtracted, and the second and third 6-s counts were averaged. A standard curve was established for each experiment.

ADP and AMP were measured in the same manner after enzymatic conversion to ATP. For the determination of ADP plus ATP, 100 μl of sample or a solution of mixed standards was added to 400 μl of Mg-TES buffer containing 26.5 U of pyruvate kinase and 0.5 μmol of phosphoenolpyruvate. These amounts were necessary for 100% conversion of ADP to ATP.

For the determination of AMP + ADP + ATP, 100 μl of sample or a solution of mixed standards was added to 400 μl of Mg-TES buffer containing 26.5 U of pyruvate kinase, 0.5 μmol of phosphoenolpyruvate and 48 U of adenylate kinase. ATP was found to be necessary for the total conversion of AMP to ADP by adenylate kinase. Therefore, 50 pmol of ATP was added to the reaction mixtures. The reaction mixtures were incubated at 37°C for 1 h, and portions were removed and assayed for ATP as described above. The picomoles of ATP were determined directly from the standard curve, while ADP and AMP levels were determined by appropriate subtraction.

Sparging experiments. Experiments were performed which involved sparging whole cells with either air or an N_2–CO_2 mixture, to determine the effects upon adenine nucleotide pools. These experiments were performed on treponemes that had been extracted aerobically, as previously described. The treponemal suspension was carefully sparged with a mixture of 95% N_2–5% CO_2 for two 5-min periods, with a 30-min interval between spargings. After an equilibration time of 45 min, the solution was divided in half and sparged for 5 min. One half was sparged with the N_2–CO_2 mixture, while the other half was sparged with air. Adenine nucleotides were then extracted with chloroform and measured by the luciferin-luciferase assay.

RESULTS

Physiological stimulation of O_2 uptake. *T. pallidum* cytochromes have been shown to become physiologically reduced in the presence of various Krebs cycle and glycolytic intermediates plus NAD or NADP (29). The effect of these intermediates on the consumption of O_2 by *T. pallidum* CFE was determined because aerobic electron transport is known to terminate with the reduction of O_2. Oxygen uptake by CFE occurred in the presence of glyceraldehyde-3-phosphate plus NAD, glucose-6-phosphate plus NADP, 6-phosphogluconate plus NADP, malate plus NAD, lactate plus NAD, and isocitrate plus NADP (Fig. 1). Oxygen uptake did not occur in the presence of any substrate when the appropriate pyridine nucleotide was omitted. These data indicated that terminal electron transport was coupled to the consumption of O_2.

O_2 uptake stimulated by NADH. NADH had previously been shown to reduce *T. pallidum* cytochromes to the fullest extent of any physiological reductant used (29). An immediate spike of O_2 consumption was seen when O_2 uptake was monitored after the addition of NADH to CFE (Fig. 2). Lesser amounts of CFE consumed O_2 at a lower rate in the presence of constant amounts of NADH, and O_2 uptake was linear with time whenever NADH was added to the treponemal preparations. When half the amount of NADH was added, half the amount

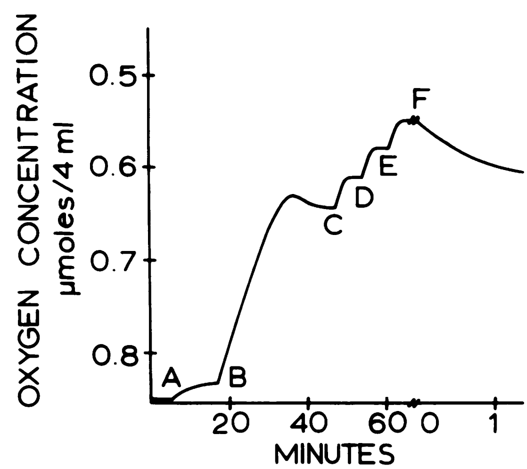

FIG. 1. *Oxygen consumption by T. pallidum CFE. Air-sparged extract initially consumed no oxygen. Endogenous NADH, or NADPH, was generated by the addition of 1.0 μmol each of: (A) glyceraldehyde-3-phosphate plus NAD; (C) malate plus NAD; (D) lactate plus NAD; (B) glucose-6-phosphate plus NADP; and (E) isocitrate plus NADP. Hydrogen peroxide (1.8 μmol) was added (F) to determine catalase activity. Pen deflection occurred immediately after the additions. These experiments were performed with an extract of 1.5 × 10^{10} treponemes in a final volume of 4.0 ml.*

of O_2 was consumed. The micromoles of O_2 consumed were determined to be directly proportional in a 1:2 ratio to the micromoles of NADH added. When calculated from these data, the average activity of O_2 consumption was determined to be 0.1 μmol/min per mg of protein, which was found to correspond to the NADH oxidase activity of 0.2 μmol/min per mg of protein when NADH oxidation was measured spectrophotometrically as a decrease in absorbance at 340 nm. Replicate additions of NADH to resparged CFE continued to cause the consumption of identical amounts of O_2 in the same kinetic fashion, repeatedly without apparent limit. A 2-μmol quantity of NADH is known to be oxidized for every micromole of O_2 reduced during electron transport, and we concluded that all the NADH was being quickly oxidized by *T. pallidum* CFE, with concomitant O_2 reduction.

This "NADH oxidase" activity was found to be partially cyanide insensitive. Oxygen consumption induced by NADH was found to be reduced by half after the addition of NaCN to a concentration of 0.45 μmol/mg of CFE protein, but the rate of O_2 consumption remained the same. Oxygen uptake also occurred with 10 times this concentration of NaCN, but to only 8% of the previous uptake. NADH oxidation was also measured spectrophotometrically at 340 nm. The rate of NADH oxidation in the presence of 100 μmol of NaCN per mg of protein was reduced to 18% of the rate without NaCN. These data indicated that *T. pallidum* CFE contained both cyanide-sensitive and -insensitive NADH oxidases.

Evidence for catalase activity. Oxygen evolution could be repeatedly observed after NADH-stimulated O_2 uptake had ceased due to the depletion of NADH and was thought to be due to O_2 released from H_2O_2 generated by the treponemes during NADH oxidation. After O_2 uptake, the O_2 concentration remained the same for a minute and then increased sharply (Fig. 2). This apparent evolution of O_2 gradually declined over a period of time. We had previously observed during the cytochrome studies that bubbles were released from supernatant fluids of sonically disrupted *T. pallidum* to which H_2O_2 had been added. This tentative identification of catalase, which is known to enzymatically degrade $2H_2O_2$ to $2H_2O$ and O_2, was further substantiated by the addition of pyruvate, which is non-enzymatically degraded to acetate and CO_2 in the presence of H_2O_2 (27). Oxygen evolution

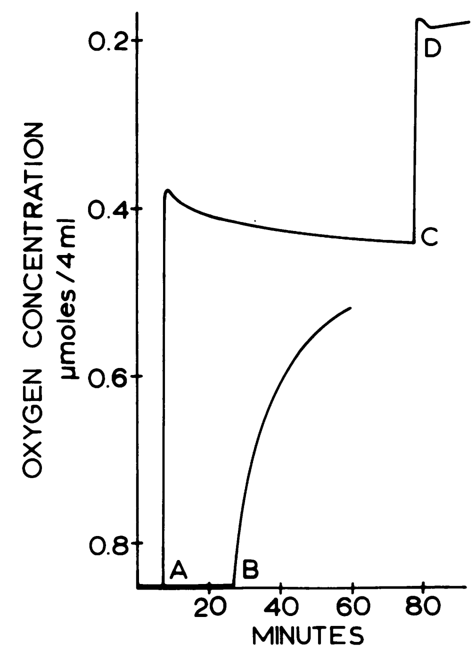

FIG. 2. *Oxygen consumption by T. pallidum CFE. Air-sparged extracts initially consumed no oxygen. NADH (1.0 μmol) was added at time (A) to an extract of 5.4 × 10^{10} treponemes in a final volume of 4.0 ml. NADH (0.5 μmol) was added at (C), and 1.0 μmol of pyruvate was added at (D). NADPH (1.0 μmol) was added at time (B) to another extract of 3.0 × 10^{10} treponemes in a final volume of 4.0 ml. Pen deflection occurred immediately after the additions.*

ceased completely after 1 μmol of pyruvate was added to CFE that was evolving O_2 pursuant to NADH depletion (Fig. 2). Oxygen evolution after NADH addition was also found to be sensitive to 0.1 μmol of NaCN per mg of protein, as would be expected for a heme protein such as catalase. Furthermore, the addition of exogenous H_2O_2 to CFE caused immediate, rapid O_2 evolution that was characteristic of a catalase reaction (40) (Fig. 1). The specific activity of catalase was determined from the linear part of the curve and was expressed as 0.04 μmol of O_2 released per min per mg of protein under these assay conditions.

Oxygen evolution also occurred after O_2 uptake was driven by glucose-6-phosphate plus NADP (Fig. 1) and by 6-phosphogluconate plus NADP. However, the addition of exogenous NADPH caused marked consumption but not evolution of O_2 (Fig. 2). Similar attempts were made to generate endogenous NADH with glyceraldehyde-3-phosphate plus NAD, but O_2 consumption was slight (Fig. 1) as compared with that generated by exogenous NADH (Fig. 2). This may be a reflection of the reported lability of the *T. pallidum* glyceraldehyde-phosphate dehydrogenase (41). When malate plus NAD and lactate plus NAD were used to generate NADH, and when isocitrate plus NADP were used to generate NADPH, O_2 uptake occurred at a fairly high rate, but concomitant O_2 evolution was not seen (Fig. 1). This may be due to the previous finding (29) that only *T. pallidum* cytochrome c_{550} was reduced by these intermediates plus the appropriate pyridine nucleotides. That these intermediates plus pyridine nucleotides were able to consume O_2 in CFE, but not able to reduce the terminal cytochrome *o* oxidase in supernatant fluids of sonically disrupted treponemes may be due to differences of cytochrome association in the two preparations.

Inhibition of O_2 uptake by DCCD and CCCP. The stimulation of O_2 uptake in *T. pallidum* CFE by the tricarboxylic acid cycle and glycolytic intermediates plus pyridine nucleotides, as well as by addition of exogenous NADH and NADPH, strongly suggested that electron transport was coupled to oxidative phosphorylation. Inhibitors of specific components of electron transport and oxidative phosphorylation systems were added to *T. pallidum* CFE to observe the effects on O_2 consumption. Cyanide is known to bind to terminal cytochrome oxidases (10, 21) and has been shown to inhibit O_2 uptake by *T. pallidum* (16). DCCD has been shown to inhibit specifically the ATPase of both mitochondria and bacteria (8, 12, 19, 21, 22) and was found to inhibit O_2 uptake by whole cells of *T. pallidum* (Fig. 3) at a concentration of 90 μmol of DCCD per mg of protein. DCCD was dissolved in DMSO, but the addition of DMSO to CFE had no effect on O_2 uptake. CCCP, which is a well-known uncoupler of mitochondrial respiration, was found to totally inhibit O_2 uptake after 25 min at a concentration of 90 μmol/mg of protein. Inhibition of respiration of whole bacterial cells by CCCP has been reported (10, 46) which suggests that the mode of action of CCCP on bacteria may be different from the mode of action on mitochondria. The above data further suggested that O_2 uptake by *T. pallidum* was due to oxidative phosphorylation and stimulated additional exploration of this subject.

Spectrophotometric assay for oxidative phosphorylation. An example of the measurement of oxidative phosphorylation in *T. pallidum* CFE is given in Table 1. Initial absorbance was calculated by summing the absorbance of the sample before the addition of NADH and the absorbance due to the amount of NADH added. The absorbance after NADH addition was recorded, and the changes in absorbance were determined. Cuvette A lacked the enzymes to measure ATP formation and measured only NADH oxidation, which for the purpose of calculating P/O ratios was presumed to be due solely to oxidative phosphorylation (36). The difference in change between cuvette A and cu-

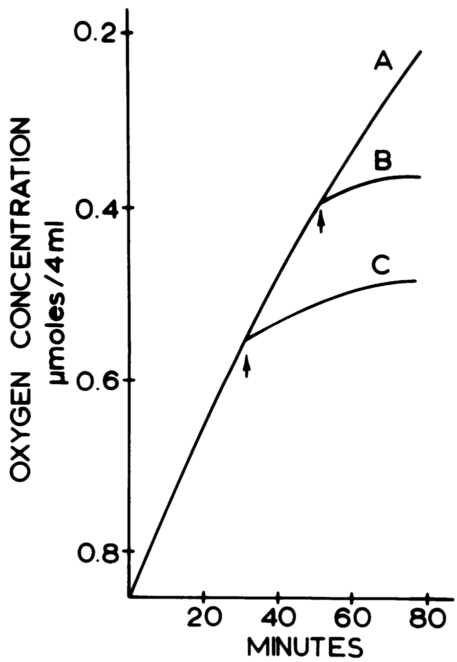

FIG. 3. *Oxygen uptake by 1.1 × 10⁸ T. pallidum per ml in the absence (A) and presence of CCCP (B) and DCCD (C) at final concentrations of 1.0 mM added at the times indicated by the arrows.*

TABLE 1. *Spectrophotometric assay of oxidative phosphorylation in CFE of T. pallidum*

Time (min)	Cuvette A (NADH oxidation)[a]		Cuvette B (ATP formation)[b]		Calculations	
	Absorbance at 340 nm	ΔNADH	Absorbance at 340 nm	Δ(NADH + NADPH)	ΔNADPH	P/O
Before NADH addition						
1.0	0.123		0.167			
2.0	0.122		0.173			
3.0	0.124		0.180			
3.5	0.124		0.185			
NADH addition[c] (Initial absorbance [calculated])	1.337 1.461		1.337 1.522			
After NADH addition						
0.5	1.427	0.034	1.526	+0.004	0.038	1.10
0.75	1.409	0.052	1.521	0.001	0.051	0.99
1.0	1.389	0.072	1.514	0.008	0.064	0.88
1.5	1.360	0.101	1.504	0.018	0.083	0.82
2.0	1.327	0.134	1.491	0.031	0.103	0.77
3.0	1.264	0.197	1.462	0.060	0.137	0.69

[a] Contents were identical to cuvette B except that NADP, hexokinase, and G6PD were omitted.

[b] ATP formation was coupled to NADP reduction by the addition of glucose, hexokinase, NADP, and G6PD. ADP, P_i, and Mg^{2+} were also present.

[c] The absorbance of the NADH solution was previously determined.

vette B was presumed to result from NADPH formation due to ATP production (36) and was reported as ΔNADPH. Table 1 shows that the decrease in absorbance of cuvette A which measured only NADH oxidation, was much greater than that of cuvette B which measured NADPH accumulation as well. During the first 30 s, 0.038 optical density units due to NADPH accumulated in cuvette B while 0.034 optical density units of NADH disappeared from cuvette A, thereby giving a P/O ratio of 1.1. The P/O ratios diminished with time, possibly due to the presence of an NADPH oxidase (36) which had been found in CFE of *T. pallidum* (29). P/O ratios varied among treponemal preparations from 0.5 to 1.1, but were reproducible within a single preparation.

P_i requirement for oxidative phosphorylation. If ATP were indeed generated by oxidative phosphorylation, there should have been a requirement for P_i. Treponemes were harvested as described previously except that the pelleted treponemes were washed with 0.05 M Tris buffer (pH 7.4) and were resuspended in the same buffer for sonic oscillation. P_i was added to cuvette A and B (Table 2) but not to cuvette C. Cuvettes A and B contained 2.9 μmol of P_i, whereas cuvette C contained only 0.11 μmol of P_i. Total depletion of P_i by washing was obviously not feasible. As seen in Table 2, the P/O ratio after 30 s for cuvette B was 0.51, while the P/O ratio for cuvette C, which was P_i deficient, was only 0.12, or 25% of that obtained with the high-P_i level. In other experiments, intermediate concentrations of P_i caused intermediate P/O ratios. These data provided evidence for a phosphate requirement for ATP formation in CFE of *T. pallidum*. The requirement for P_i suggested that oxidative phosphorylation occurred through the action of an ATPase, which would make ATP from ADP and P_i in the presence of an electrochemical gradient (30, 32–34, 45, 47).

Use of inhibitors of oxidative phosphorylation. The validity of the spectrophotometric assay for ATP formation from oxidative phosphorylation was further tested by observing the effects of inhibitors and uncouplers. Cyanide and CCCP are known to inhibit respiration and to uncouple oxidative phosphorylation, respectively, and DCCD is a potent inhibitor of both mitochondrial and bacterial ATPases. A 10 mM final concentration of NaCN completely inhibited the formation of ATP by this system, as did a 100 μM final concentration of DCCD and a 40 μM final concentration of CCCP dissolved in DMSO. The addition of DMSO alone had no effect upon ATP formation.

Detection of adenylate kinase activity. Absorbance in cuvettes B and C increased with time (Tables 1 and 2) during preliminary absorbance readings before the addition of NADH, which indicated that ATP was being formed. There were no changes in the absorbance of the cuvettes which lacked the coupled enzyme systems for the ATP-dependent formation of NADPH. Because ADP was present in the cu-

TABLE 2. *Effect of P_i on oxidative phosphorylation in CFE of T. pallidum*

Time (min)	Cuvette A (NADH oxidation)[a]		Cuvette B (ATP formation)[b]		Cuvette C (ATP formation)[b, c]		Calculations			
							Cuvette B		Cuvette C	
	Absorbance at 340 nm	ΔNADH	Absorbance at 340 nm	Δ(NADH + NADPH)	Absorbance at 340 nm	Δ(NADH + NADPH)	ΔNADPH	P/O	ΔNADPH	P/O
Before NADH addition										
1.0	0.157		0.196		0.221					
2.0	0.158		0.208		0.233					
3.0	0.158		0.220		0.245					
NADH addition[d] (initial absorbance [calculated])	1.394 1.552		1.394 1.614		1.394 1.639					
After NADH addition										
0.5	1.470	0.082	1.574	0.040	1.567	0.072	0.042	0.51	0.010	0.12
0.75	1.445	0.107	1.551	0.063	1.547	0.092	0.044	0.41	0.015	0.14
1.0	1.420	0.132	1.527	0.087	1.520	0.119	0.045	0.34	0.013	0.10
1.5	1.388	0.164	1.502	0.112	1.501	0.138	0.052	0.32	0.026	0.16
2.0	1.356	0.196	1.472	0.142	1.479	0.160	0.054	0.28	0.036	0.18
3.0	1.298	0.254	1.423	0.191	1.432	0.207	0.063	0.25	0.047	0.18

[a] Contents were identical to cuvette B except that NADP, hexokinase, and G6PD were omitted.
[b] ATP formation was coupled to NADP reduction by the addition of glucose, hexokinase, NADP, and G6PD. ADP, P_i, and MG^{2+} were also present.
[c] The phosphate concentration in cuvette C was 4% of the concentration in cuvettes A and B.
[d] The absorbance of the NADH solution was previously determined.

vettes, ATP formation could possibly have occurred via the reaction catalyzed by adenylate kinase, whereby 2 ADP would be converted to an ATP and an AMP. Adenylate kinase was subsequently assayed in *T. pallidum* CFE and found to be present with a specific activity of 0.1 unit/mg of protein.

Inability to detect transhydrogenase activity. The presence of $NAD(P)^+$ transhydrogenase might have caused problems in the interpretation of the spectrophotometric assay for oxidative phosphorylation. However, this enzyme was not detected in CFE of *T. pallidum.* To provide a known positive control for the assay system, an extract of *Pseudomonas fluorescens* was tested and found to contain transhydrogenase.

Determination of ATPase activity. ATPase was assayed to determine that it could be responsible for the phosphorylating activity in the spectrophotometric assay for oxidative phosphorylation. *T. pallidum* membranes were found to contain ATPase activity, and the average specific activity of 0.7 U/mg of protein was comparable to that observed in other bacterial systems (19, 22). These data further substantiated the evidence for ATP generation by oxidative phosphorylation in *T. pallidum.* Magnesium was found to be required for full ATPase activity, because only 28% of the activity occurred in the absence of 5 mM $MgSO_4$. Calcium could be substituted for Mg^{2+} without loss of activity, but no additional activity was detected when both 5 mM $CaCl_2$ and 5 mM $MgSO_4$ were added to the complete reaction mixture. A 10-min pretreatment with DCCD at a concentration of 2 μmol/mg of protein decreased the activity by 50%, which was similar to inhibition seen in other bacterial systems (19, 22). A 5-min pretreatment with 0.25% Triton X-100 did not increase the activity, as has been seen for bacterial membrane vesicles (43, 45). However, detergents have not been shown to have stimulatory effects on ATPases from sonically disrupted extracts prepared in a manner such as that for CFE (43).

Concentrations of adenine nucleotides in *T. pallidum.* Concentrations of the adenine nucleotides present within *T. pallidum* were determined to assess the physiological state of the organism, and to determine whether changes in the adenine nucleotide levels occurred after stimulation of oxidative phosphorylation. Results of a typical extraction of *T. pallidum* adenine nucleotides are shown in Table 3. The adenylate energy charge (AEC) (4) was calculated to be 0.44, which was lower than the values obtained for many other bacteria undergoing active growth (13). A low AEC would normally indicate the possibility that *T. pallidum* was

TABLE 3. *Concentration of adenine nucleotides in chloroform-extracted T. pallidum preparations*

Adenine nucleotide	Concn[a]	% Adenine nucleotide pool
ATP	50.7	8.9
ADP	394.3	69.3
AMP	123.8	21.8 (0.44)[b]

[a] Concentration is expressed as picomoles per 1.1 $\times 10^{10}$ treponemes.
[b] Calculated AEC.

biosynthetically impaired at that time. However, absolute values for nucleotide pools are probably of questionable significance, because ATP concentrations are reported to be lower when cells are damaged by washing or centrifugation (14). Unfortunately, high concentrations of these treponemes can only be obtained by centrifugation and resuspension in smaller volumes.

Attempts were made to stimulate oxidative phosphorylation in CFE by the addition of NADH, which had been shown to physiologically reduce *T. pallidum* cytochromes (29). Changes in adenine nucleotide levels were measured by the luciferin-luciferase assay before and after the addition of 1 μmol of NADH, which was incubated with CFE at 37°C for 1 min before chloroform extraction. Preliminary experiments had indicated that the NADH solution contained substantial amounts of AMP, which was subsequently removed by treatment with alkaline phosphatase (18). However, even after this treatment, all adenine nucleotide (especially ADP and AMP) levels rose after NADH addition. Because ADP levels were expected to fall and ATP levels were expected to rise during oxidative phosphorylation, these results suggested that modifications of the adenine nucleotide levels were being made by other enzymes in the crude CFE.

Detection of NAD nucleosidase activity. NAD^+ nucleosidase, which is known to split NAD into the nicotinamide moiety and the ADP-ribose moiety, could have been responsible for the aberrant nucleotide levels. This enzyme was subsequently assayed and found to be present in CFE with a specific activity of 0.02 U/mg of protein. The presence of this enzyme made it impossible to assess the ability of NADH to effect changes in the adenine nucleotide pools in CFE.

Stimulation of ATP synthesis by increases in O_2 concentration. Because of the inability to assess the effects of NADH on adenine nucleotide pools, attempts were made to stimulate oxidative phosphorylation in *T. pallidum* by sparging whole cell preparations with air. Measurements of the levels of adenine nucleotides in treponemes under low O_2 tensions, and the changes that might occur upon exposure of the treponemes to atmospheric O_2 tensions were also of interest. Therefore, whole-cell preparations were incubated under an atmosphere of 95% N_2–5% CO_2. One half of the cell sample was sparged with air, whereas the other half was resparged with the N_2-CO_2 mixture. Adenine nucleotides were extracted with chloroform, and the levels were determined by the luciferin-luciferase assay. The adenine nucleotide levels shifted when the treponemes were sparged with air (Table 4), resulting in a 13% increase of the ADP level and a 23% decrease of the AMP level. The ATP level increased 33%. The adenine nucleotide levels of the air-sparged cell preparation compared favorably with the levels reported in Table 3 for treponemes that were extracted and prepared totally aerobically. This apparent influence of O_2 was also seen in a comparison of the AEC from each preparation. The treponemal preparation which was incubated under an atmosphere of N_2-CO_2 had an AEC of 0.33 (Table 4), whereas the sample which was prepared entirely aerobically had an AEC of 0.44 (Table 3). The AEC of the preparation that was sparged with air after incubation under an atmosphere of N_2-CO_2 was intermediate at 0.39 (Table 4). These data indicated that ATP synthesis by *T. pallidum* was a function of environmental O_2 tension.

Possibility of tissue contamination affecting interpretation of oxidative phosphorylation data. The possibility of tissue contamination was given serious consideration as it was in the cytochrome studies (29). The pellet obtained from a treponemal preparation that had been further filtered to remove the treponemes was found in those studies to contain only 1% as much protein as the original preparation.

TABLE 4. *Effect of sparging on relative levels of adenine nucleotides in whole-cell preparations of T. pallidum*[a]

Adenine nucleotide	% Adenine nucleotide pool	
	Air sparged[b]	N_2-CO_2 sparged[c]
ATP	8.66	6.48
ADP	60.26	53.25
AMP	31.07 (0.39)[d]	40.27 (0.33)[d]

[a] Cells were extracted from tissue aerobically, then incubated under an atmosphere of 95% N_2–5% CO_2.
[b] Cells were sparged for 5 min with a fine stream of air. Adenine nucleotides were then extracted with chloroform.
[c] Cells were sparged for 5 min with a fine stream of N_2-CO_2. Adenine nucleotides were then extracted with chloroform.
[d] Calculated AEC.

This control convinced us of the purity of our preparations, and was not included in the present study because of the inability to obtain amounts of protein sufficient for enzyme assays. Therefore, testicular extracts from noninfected rabbits were processed as described for testicles from infected rabbits and used as a tissue control. Filtration through 0.8-μm membranes (Nuclepore) removed tissue cells. The filtered tissue membrane fragments were washed, pelleted, and disrupted by sonic oscillation. The resultant extract was used for enzyme assays as described for treponemal preparations. Tissue extracts prepared in this fashion have also been centrifuged at 32,000 $\times$ *g* for 20 min and examined for the presence of cytochromes. No cytochromes have been found, which suggested that if mitochondrial membranes were present in tissue extracts, the membrane cytochrome components were either absent or masked by a much higher concentration of extraneous membrane-bound protein such as that from plasma membranes. Therefore, the likelihood of contamination by mitochondrial membranes in purified treponemal preparations appeared to be slight.

No oxidation of NADH occurred when noninfected tissue control extracts were assayed for oxidative phosphorylation via the spectrophotometric assay. Without NADH oxidation, there could be no oxidative phosphorylation; therefore all phosphorylating activity in the spectrophotometric assay appeared to be of treponemal origin. NAD^+ nucleosidase was detected in noninfected tissue control extracts, but at a specific activity which was only 20% as great as the treponemal activity. These results strongly suggested that tissue NAD^+ nucleosidase was not responsible for significant treponemal activity and that the treponemal activity was due to an intrinsic enzyme. Adenylate kinase activity was also detected in noninfected tissue control extracts, and the specific activity was two to three times higher than the treponemal activity. The two activities could not be distinguished on the basis of a Mg^{2+} requirement or KF sensitivity. Both activities required Mg^{2+} and were inhibited by KF. Nevertheless, as a result of our previous experience that the degree of tissue membrane contamination in treponemal preparations was slight, we felt that some adenylate kinase activity of *T. pallidum* preparations was due to treponemal activity. That half of the protein from our treponemal preparations could be from host tissue was inconceivable. ATPase was also detected in noninfected tissue control extracts and had a specific activity which was twice as high as the activity detected in treponemal extracts. Tissue ATPase was also found to be sensitive to DCCD. Despite the presence of tissue ATPase activity, the lack of an NADH-oxidizing system in tissue vesicles would prevent the tissue ATPase from functioning as an ATP synthetase. Without NADH oxidation and the formation of a proton gradient within the tissue vesicles, the mere presence of an ATPase would not indicate a capacity for oxidative phosphorylation by tissue membrane fragments.

DISCUSSION

The tricarboxylic acid cycle and glycolytic intermediates plus pyridine nucleotides which caused the physiological reduction of *T. pallidum* cytochromes (29), were also shown here to cause O_2 uptake when added to CFE (Fig. 1). These data indicated that the various dehydrogenases which generate reduced pyridine nucleotides are involved in electron transport, probably coupled to oxidative phosphorylation. Malate, lactate, and isocitrate in the presence of pyridine nucleotides have been shown to reduce only cytochrome c_{550} when added to the supernatant fraction of sonically treated *T. pallidum* CFE (29), and have also been shown to cause limited O_2 uptake when added to CFE (Fig. 1). Because O_2 uptake is known to proceed via terminal cytochrome oxidase, cytochrome *o* as well as c_{550} should have been reduced by these intermediates. Sonic oscillation can have the effect of isolating dehydrogenase-cytochrome *c* complexes from the rest of the respiratory components as has been previously mentioned (29). If cytochrome c_{550} were spatially isolated in this manner, perhaps the higher concentrations of membrane components in CFE allow electron transfer from c_{550} to the cytochrome oxidase, resulting in O_2 consumption. Alternatively, cytochrome c_{550} may be an additional cytochrome oxidase, although we have previously demonstrated that CO did not appear to bind to c_{550} (29).

NADH has been shown to cause cytochrome reduction, O_2 uptake, and ATP formation in *T. pallidum* extracts. Some oxidation of NADH appears to be cyanide insensitive and may stimulate O_2 consumption through the action of a flavoprotein oxidase. However, the results of this study indicate that only a small fraction of the NADH oxidase activity is cyanide insensitive, which seems to indicate that aerobic respiration in *T. pallidum* occurs mainly through the action of a NADH dehydrogenase coupled to terminal electron transport and not through the action of a flavoprotein oxidase. The ability of cytochromes to be reduced by NADPH indicated that NADPH oxidation also proceeded through a dehydrogenase coupled to the mainstream electron transport chain.

Endogenous generation of NADH has been shown to result in higher P/O ratios in *Mycobacterium phlei* than when exogenous NADH was added (3, 9, 10). Exogenous NADH was thought to be utilized in that system not only by the particulate respiratory chain leading to the reduction of O_2, but also by the particulate enzymes that exit from the respiratory chain before the terminal oxidase, by soluble flavoprotein oxidases or diaphorases, or by soluble enzymes that reacted either directly with O_2 or re-entered the particulate chain at a higher redox level. Similar phenomena may occur in *T. pallidum*, which might explain the differences in O_2 uptake when CFE was stimulated with exogenous versus endogenously generated pyridine nucleotides. Exogenous NADH caused a spike in O_2 uptake. Endogenous NADH generated from glyceraldehyde-3-phosphate plus NAD may have caused only limited O_2 uptake because of the reported lability of the dehydrogenase (41). The demonstration of limited O_2 uptake with NADH generated by malate and lactate dehydrogenases may be due to a difference in the mediation of electron transport influenced by cytochrome c_{550}, or result from differences in enzyme activity. The addition of exogenous NADPH to CFE caused marked O_2 uptake, although at a lower rate than for exogenous NADH. The rate of O_2 uptake stimulated by exogenous NADPH gradually diminished with time, and exogenous NADPH did not cause O_2 evolution. When stimulated by endogenously generated NADPH, O_2 uptake proceeded at the same rate until presumably all NADPH had been oxidized. At that time, O_2 uptake ceased and O_2 evolution was demonstrated. Oxygen evolution is probably initiated soon after the start of O_2 uptake, but is not evidenced until the cessation of O_2 uptake presumably resulting from substrate depletion.

Catalase activity, which may be expected in an organism of known aerobic capabilities, has been demonstrated in *T. pallidum* extracts by both direct and indirect methods. Catalase activity may be due to an intrinsic treponemal enzyme or due to contaminating activity from tissue extracts which are known to possess catalase activity. Future studies are planned to determine the origin of the catalase activity.

The results of the spectrophotometric assay for oxidative phosphorylation have demonstrated that CFE of *T. pallidum* are capable of oxidative phosphorylation when stimulated with NADH. The P/O ratios that were obtained by this method varied from 0.5 to 1.1. This variability was presumably due to the state of the treponemes before, during, and after extraction from tissue, an event over which we had only limited control. The number of potentially available energy conservation sites in bacteria is known to be subject to change depending upon the growth conditions (44). The variability of the P/O ratios observed in *T. pallidum* may therefore be a reflection of the physiological state of the organism. In any case, P/O ratios should not be considered as absolute values, because a number of studies have shown variability in the phosphorylating capabilities of bacteria. A few studies indicated that bacteria are capable of obtaining a P/O value of 3.0 (5, 23), which is the P/O value for respiring mitochondria. Other studies have indicated that bacteria possess lower phosphorylating capabilities than do mitochondria (10, 21, 42), and advise using caution in the interpretation of data which could lead to falsely high P/O values. Phosphorylating efficiency has been shown to be affected by the state of the bacteria, the manner of preparation, and the type of cytochrome oxidase which may be present (10, 21, 31). In addition, lower P/O values have been obtained when bacterial preparations were stimulated with exogenous NADH, whereas higher values were obtained when NADH was generated endogenously (3, 10).

In addition to the previously mentioned precautions concerning the interpretations of P/O ratios, we should emphasize that our experimental results were obtained with CFE, and not with partially purified electron transport particles. An attempt to isolate electron transport particles from *T. pallidum* and to determine optimal conditions for their coupled phosphorylating activity was unrealistic with the number of treponemes currently available. Therefore, a number of enzymes which have been found in CFE may either add to or subtract from the P/O values that were obtained. Adenylate kinase would be expected to interfere, but even if this activity were subtracted the P/O value for Table 1 would still be 0.97 instead of 1.1. The NADPH oxidase (29) might lower the P/O values and may be responsible for the decrease in P/O values with time by consuming the NADPH which was formed as a result of ATP synthesis. The presence of transhydrogenase activity might be expected to lower P/O values in the same manner, but this activity was not detected in our preparations. The presence of a cyanide-insensitive NADH oxidase could have been responsible for the failure to detect transhydrogenase by oxidizing NADH as soon as transhydrogenase converted NADPH to NADH. *T. pallidum* CFE, in the presence of 1 mM KCN which was routinely included in the reaction mixture, did indeed lower the rate of NADH formation in the *P. fluorescens* transhydrogenase assay. This result could also have been due to the presence of a cyanide-insensitive NADH oxidase, which has been noted in *T. pallidum* CFE. Nevertheless, the P/O values which we obtained were comparable to the values observed by others who have used the spectrophotometric assay for oxidative phosphorylation (11, 36, 37).

The requirement for P_i gave further evidence for the involvement of an ATPase, as did the finding of DCCD inhibition. The activities of NaCN as an inhibitor and CCCP as an uncoupler of oxidative phosphorylation, and the lack of NADH oxidation by tissue controls, further convinced us that *T. pallidum* does indeed contain a membrane-bound ATPase, as do other bacteria that are capable of oxidative phosphorylation (19, 21).

Although absolute values for nucleotide pools are of questionable significance because of required centrifugation, a difference was nevertheless noted in the sparging experiments between whole cells sparged with air or with N_2-CO_2 mixture. The cells that were sparged with N_2-CO_2 had a lower AEC than cells which were prepared totally aerobically. Furthermore, the AEC rose when cells under an atmosphere of N_2-CO_2 were sparged with air. The increase in ATP concentration was thought to be due to oxidative phosphorylation when cells were exposed to higher O_2 tensions as has been seen in other bacteria (5, 23). The shifts in individual adenine nucleotide levels, expressed as percent adenine nucleotide pool in Table 4, may be rationalized as resulting from the combined actions of adenylate kinase and ATPase. The shift in adenine nucleotide levels which occurred in response to increased O_2 tensions resulted in a net increase of 2% ATP and 7% ADP, and a net decrease of 9% AMP (Table 4). The observed shift in adenine nucleotide levels may be represented hypothetically as follows: 11ADP + 11P_i → 11ATP via oxidative phosphorylation, and 9ATP + 9AMP → 18ADP via adenylate kinase. These may be the enzymatic reactions which effected the observed shift. The presence of adenylate kinase may be the reason why a greater increase in ATP was not obtained. When AMP is present in a cell at high levels resulting from biosynthetic reactions, adenylate kinase is known to be the enzyme that returns the mononucleotide to the mainstream of the ATP/ADP cycle by conversion to ADP. This action occurs at the expense of ATP. The action of adenylate kinase by the above hypothesis supports the evidence that this enzyme is an active component of intact, respiring treponemes. The presence of adenylate kinase may reflect the physiological state of the treponemes as being one of active biosynthesis, with ATP being broken down to AMP, which is then recycled. These data seem to indicate that even if the low AEC resulted from centrifugation, the treponemes were still capable of responding in a positive fashion to induced changes in the O_2 tension.

These studies on oxidative phosphorylation complete our investigations of the capability of virulent *T. pallidum* for aerobic respiration. The evidence suggests that *T. pallidum* can derive a large amount of energy from terminal electron transport coupled to oxidative phosphorylation. How well this system functions under the pO_2 of tissue in the host is unknown at this time. We conclude that the inability to cultivate this microorganism is probably not due to intrinsic problems with energy metabolism.

ACKNOWLEDGMENTS

We thank Thomas L. Mason for helpful discussion.

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

LITERATURE CITED

1. **Abrams, A., P. McNamara, and F. B. Johnson.** 1960. Adenosine triphosphatase in isolated bacterial cell membranes. J. Biol. Chem. **235**:3659–3662.
2. **Adam, H.** 1965. Adenosine-5′-diphosphate and adenosine-5′-monophosphate, p. 573–577. *In* Hans-Ulrich Bergmeyer (ed.), Methods of enzymatic analysis. Academic Press, Inc., New York.
3. **Asano, A., and A. F. Brodie.** 1965. The properties of the non-phosphorylative electron transport bypass enzymes of *Mycobacterium phlei*. Biochem. Biophys. Res. Commun. **19**:121–126.
4. **Atkinson, D.E., and G. M. Walton.** 1967. Adenosine triphosphate conservation in metabolic regulation. Rat liver citrate cleavage enzyme. J. Biol. Chem. **242**:3239–3241.
5. **Baak, J. M., and P. W. Postma.** 1971. Oxidative phosphorylation in intact *Azotobacter vinelandii*. FEBS Lett. **19**:189–192.
6. **Ball, W. J., Jr., and D. E. Atkinson.** 1975. Adenylate energy charge in *Saccharomyces cerevisiae* during starvation. J. Bacteriol. **121**:975–982.
7. **Baseman, J. B., J. C. Nichols, and N. S. Hayes.** 1976. Virulent *Treponema pallidum*: aerobe or anaerobe. Infect. Immun. **13**:704–711.
8. **Beechey, R. B.** 1974. Structural aspects of mitochondrial adenosine triphosphatase. Biochem. Soc. Trans. **2**:466–471.
9. **Bogin, E., T. Higashi, and A. F. Brodie.** 1969. Exogenous NADH oxidation and particulate fumarate reductase in *Mycobacterium phlei*. Arch. Biochem. Biophys. **129**:211–220.
10. **Brodie, A. F., and D. L. Gutnick.** 1972. Electron transport and oxidative phosphorylation in microbial systems, p. 599–681. *In* T. E. King and M. Klingenberg (ed.), Electron and coupled energy transfer in biological systems. Marcel Dekker, Inc., New York.
11. **Bryan-Jones, D. G., and R. Whittenbury.** 1969. Hae-

matin-dependent oxidative phosphorylation in *Streptococcus faecalis*. J. Gen. Microbiol. **58**:247-260.
12. **Cattell, K. J., C. R. Lindop, I. G. Knight, and R. B. Beechey.** 1971. The identification of the site of action of N,N'-dicyclohexylcarbodiimide as a proteolipid in mitochondrial membranes. Biochem. J. **125**:169-177.
13. **Chapman, A. G., L. Fall, and D. E. Atkinson.** 1971. Adenylate energy charge in *Escherichia coli* during growth and starvation. J. Bacteriol. **108**:1072-1086.
14. **Cole, H. A., J. W. T. Wimpenny, and D. E. Hughes.** 1967. The ATP pool in *Escherichia coli*. I. Measurement of the pool using a modified luciferase assay. Biochim. Biophys. Acta **143**:445-453.
15. **Colowick, S. P.** 1955. Adenylate kinase (Myokinase, ADP phosphomutase). Methods Enzymol. **2**:598-604.
16. **Cox, C. D., and M. K. Barber.** 1974. Oxygen uptake by *Treponema pallidum*. Infect. Immun. **10**:123-127.
17. **Dhople, A. M., and J. H. Hanks.** 1973. Quantitative extraction of adenine triphosphate from cultivable and host-grown microbes: calculation of adenine triphosphate pools. Appl. Microbiol. **26**:399-403.
18. **Estrabrook, R. W., J. R. Williamson, R. Frenkel, and P. K. Maitra.** 1967. The fluorimetric determination of mitochondrial adenine and pyridine nucleotides. Methods Enzymol. **10**:479-482.
19. **Evans, D. J., Jr.** 1970. Membrane Mg^{2+}-(Ca^{2+})-activated adenosine triphosphatase of *Escherichia coli*: characterization in membrane-bound and solubilized states. J. Bacteriol. **104**:1203-1212.
20. **Fiske, C. H., and Y. SubbaRow.** 1925. The colorimetric determination of phosphorus. J. Biol. Chem. **66**:375-400.
21. **Haddock, B. A., and C. W. Jones.** 1977. Bacterial respiration. Bacteriol. Rev. **41**:47-99.
22. **Harold, F. M., J. R. Baarda, C. Baron, and A. Abrams.** 1969. Inhibition of membrane-bound adenosine triphosphatase and cation transport in *Streptococcus faecalis* by N,N'-dicyclohexylcarbodiimide. J. Biol. Chem. **244**:2261-2268.
23. **Hempfling, W. P.** 1970. Studies of the efficiency of oxidative phosphorylation in intact *Escherichia coli* B. Biochim. Biophys. Acta **205**:169-182.
24. **Kaplan, N. O.** 1955. Animal tissue DPNase (pyridine transglycosidase). Methods Enzymol. **2**:660-663.
25. **Kaplan, N. O.** 1955. Pyridine nucleotide transhydrogenase. Methods Enzymol. **2**:681-687.
26. **Klemme, J. H., and H. Gest.** 1971. Regulatory properties of an inorganic pyrophosphatase from the phostosynthetic bacterium *Rhodospirillum rubrum*. Proc. Natl. Acad. Sci. U.S.A. **68**:721-725.
27. **Lipmann, F.** 1939. An analysis of the pyruvic acid oxidation system. Cold Spring Harbor Symp. Quant. Biol. **7**:248-259.
28. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
29. **Lysko, P. G., and C. D. Cox.** 1977. Terminal electron transport in *Treponema pallidum*. Infect. Immun. **16**:885-890.
30. **Maloney, P. C., E. R. Kashket, and T. H. Wilson.** 1974. A protonmotive force drives ATP synthesis in bacteria. Proc. Natl. Acad. Sci. **71**:3896-3900.
31. **Meyer, D. J., and C. W. Jones.** 1973. Oxidative phosphorylation in bacteria which contain different cytochrome oxidases. Eur. J. Biochem. **36**:144-151.
32. **Mitchell, P.** 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature (London) **191**:144-148.
33. **Mitchell, P.** 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. Cambridge Philos. Soc. **41**:455-502.
34. **Mitchell, P.** 1974. A chemiosmotic molecular mechanism for proton-translocating adenosine triphosphatases. FEBS Lett. **43**:189-194.
35. **Nichols, J. C., and J. B. Baseman.** 1975. Carbon sources utilized by virulent *Treponema pallidum*. Infect. Immun. **12**:1044-1050.
36. **Pinchot, G. B.** 1957. A rapid method for measuring phosphorylation coupled to the oxidation of reduced diphosphopyridine nucleotide. J. Biol. Chem. **229**:11-23.
37. **Ritchey, T. W., and H. W. Seeley, Jr.** 1974. Cytochromes in *Streptococcus faecalis* var. *zymogenes* grown in a haematin-containing medium. J. Gen. Microbiol. **85**:220-228.
38. **Roberton, A. M., and R. S. Wolfe.** 1970. Adenosine triphosphate pools in *Methanobacterium*. J. Bacteriol. **102**:43-51.
39. **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.
40. **Rørth, M., and P. K. Jensen.** 1967. Determination of catalase activity by means of the Clark oxygen electrode. Biochim. Biophys. Acta **139**:171-173.
41. **Schiller, N. L., and C. D. Cox.** 1977. Catabolism of glucose and fatty acids by virulent *Treponema pallidum*. Infect. Immun. **16**:60-68.
42. **van der Beek, E. G., and A. H. Stouthamer.** 1973. Oxidative phosphorylation in intact bacteria. Arch. Mikrobiol. **89**:327-339.
43. **van Thienen, G., and P. W. Postma.** 1973. Coupling between energy conservation and active transport of serine in *Escherichia coli*. Biochim. Biophys. Acta **323**:429-440.
44. **van Verseveld, H. W., and A. H. Stouthamer.** 1976. Oxidative phosphorylation in *Micrococcus denitrificans*. Calculation of the P/O ratio in growing cells. Arch. Microbiol. **107**:241-247.
45. **West, I. C., and P. Mitchell.** 1974. The proton-translocating ATPase of *Escherichia coli*. FEBS Lett. **40**:1-4.
46. **White, R. C., and L. Smith.** 1964. Localization of the enzymes that catalyze hydrogen and electron transport in *Hemophilus parainfluenzae* and the nature of the respiratory chain system. J. Biol. Chem. **239**:3956-3963.
47. **Wilson, D. M., J. F. Alderete, P. C. Maloney, and T. H. Wilson.** 1976. Protonmotive force as the source of energy for adenosine 5'-triphosphate synthesis in *Escherichia coli*. J. Bacteriol. **126**:327-337.