BBA 45902

FURTHER STUDIES ON THE NADH OXIDASE OF THE CYTOPLASMIC MEMBRANE OF *MYCOBACTERIUM TUBERCULOSIS*

EDNA B. KEARNEY* AND DEXTER S. GOLDMAN

Tuberculosis Research Laboratory, Veterans Administration Hospital and the Institute for Enzyme Research, University of Wisconsin, Madison, Wisc. 53705 (U.S.A.)

(Received October 13th, 1969)

SUMMARY

The cytoplasmic membrane of the $H_{37}Ra$ strain of *Mycobacterium tuberculosis* has been isolated free of cell wall.

These membrane preparations contain very small quantities of cytochromes *c*, *b* and cytochrome oxidase. The cytochrome *c* is not extracted by any method attempted. The cytochrome *b* is reducible only by dithionite and is believed not to be involved in the direct transfer of electrons during the oxidation of NADH by these preparations. The NADH oxidase activity of the membrane is inhibited by high concentrations of cyanide and also by 2-(*n*-heptyl)-4-hydroxyquinoline-*N*-oxide (HQNO). The cytochrome oxidase of the membrane contains both cytochromes *a* and a_3 and is present in low concentrations relative to cytochrome *c*. The cytochrome a_3 component was identified by characteristic complexes with both CO and cyanide and shows a γ-band absorption maximum at a slightly lower wavelength than the cytochrome oxidase of mammalian mitochondria (442 nm *vs.* 445 nm). The functional activity of the cytochrome oxidase is indicated by the inhibition of reoxidation of reduced cytochromes *c* and *a* in the presence of cyanide.

INTRODUCTION

Previous work from this laboratory[1] has shown that particulate preparations derived from cell-free extracts of the $H_{37}Ra$ strain of *Mycobacterium tuberculosis* catalyze the aerobic oxidation of NADH. The particles contain cytochromes of the *a*, *b* and *c* types in very low concentration; the *a* and *c* type cytochromes were reduced by NADH under anaerobic conditions. The *b* type cytochrome was partially reduced only after a long period of time. These mycobacterial particles differ from respiratory particles obtained from yeast, mammalian tissues, and even from other mycobacteria[2–6] in that NADH is the only substrate used; succinate, malate, β-hydroxybutyrate and lactate are not oxidized by these preparations. No evidence of phosphorylation accompanying NADH oxidation could be found. Finally, the only effective inhibitors of this

Abbreviations: HQNO, 2-(*n*-heptyl)-4-hydroxyquinoline-*N*-oxide; PCMB, *p*-chloromercuribenzoate.

* Present address: Department of Pharmacology, University of California Medical School, San Francisco, Calif., U.S.A.

NADH oxidase were dicumarol and CO; other characteristic respiratory inhibitors such as azide, cyanide and antimycin A were without effect.

While it may be assumed by analogy with other strictly aerobic cells that NADH is oxidized also in the *M. tuberculosis* system *via* the cytochrome system, direct evidence for this pathway of electron transfer has not been reported. Some of the anomalous characteristics referred to above may be more consistent with the operation of an alternative terminal oxidation mechanism. It is of interest to recall at this point that *in vivo* grown virulent mycobacteria show essentially no aerobic metabolism[7]; cultivation through but one *in vitro* passage converted these respiration-inactive bacilli to respiration-active bacilli with no change in pathogenicity. KUSAKA *et al.*[8] later reported that *in vivo* grown mycobacteria lack cytochromes. The possibility therefore remains that *in vitro* grown mycobacteria use a non-cytochrome terminal electron transfer system and the functionality of the cytochrome system must be questioned.

These questions prompted us to re-examine the NADH oxidase of *M. tuberculosis* and we have now found a pattern of inhibition and cytochrome spectral changes which support the presence of a normal cytochrome-mediated oxidation of NADH.

METHODS AND MATERIALS

Growth of bacteria

The procedures for the growth, harvesting and preparation of the cell-free extract of the $H_{37}Ra$ strain of *M. tuberculosis* have been described[9]. The drained, harvested cells may be used immediately or may be stored at —17°.

Preparation of bacterial membrane fragments

The washed cells are disrupted[9] in a nitrogen atmosphere for 30 min in 10 mM phosphate buffer (pH 7.0) containing 1 mM dithiothreitol. The glass beads are removed by decantation and washed with fresh buffer; the washings are added to the cell extract. Cell hulls are then recovered by centrifugation of the extract at 3000 × *g* for 30 min. These cell hulls, shown in Fig. 1, are subjected to another period of grinding to free the cytoplasmic membrane from the cell wall. The second grinding, again in the presence of glass beads, is carried out for 60 min in 10 mM phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$. The glass beads are removed by decantation and intact cells and debris are removed by differential centrifugation at 2000 and at 10000 × *g* for 30 min; the supernatant solutions are recovered. Crude cell wall preparations are next removed by centrifugation at 25000 × *g* for 60 min; the crude cytoplasmic membrane fragments are finally sedimented by centrifugation at 105000 × *g* for 90 min. The residue is suspended in 10 mM phosphate buffer (pH 7.0) – 1 mM $MgCl_2$ and washed through six cycles of differential centrifugation at 30000 × *g* and at 105000 × *g*; each time the 30000 × *g* residue is discarded, the 105000 × *g* residue is recovered. The final washed cytoplasmic membrane preparation (Fig. 2) is free of other cellular elements.

Electron microscopy

We are indebted to Dr. I. Sachs for all electron microscopy of these preparations.

Absorption spectra and assays

Kinetic assays and absorption spectra were measured in a Process and Instruments Co. RS-3 recording spectrophotometer. Estimations of cytochrome content were

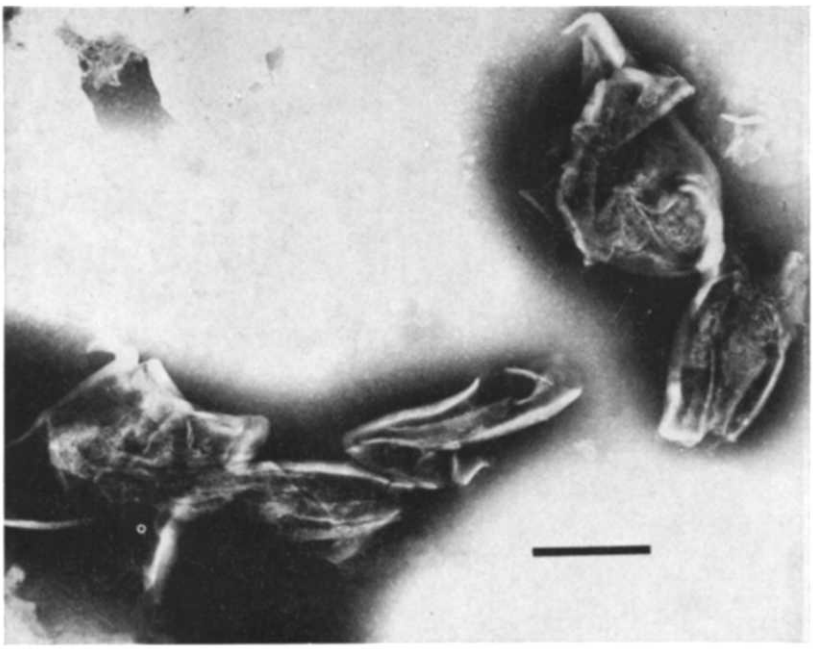

Fig. 1. Electron micrograph of negatively stained cell hulls of *M. tuberculosis*. The indicator mark represents 0.5 μ

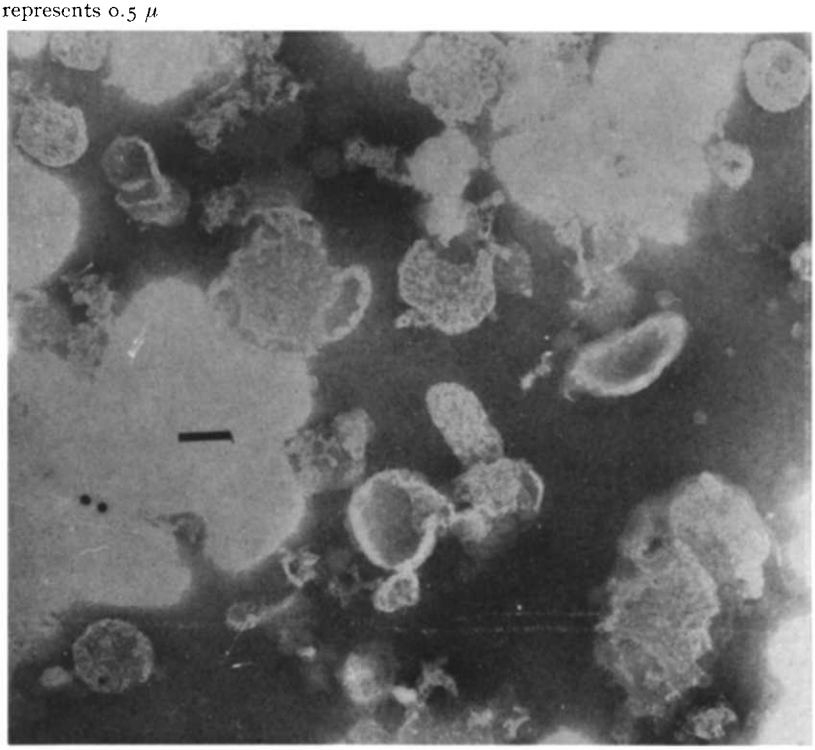

Fig. 2. Electron micrograph of negatively stained cytoplasmic membrane fragments of M. *tuberculosis*. The indicator mark represents 0.1 μ.

essentially according to CHANCE[10] with the wavelength pairs 600–630 nm, 562–540 nm, and 552–540 nm for cyt ($a + a_3$), cyt b and cyt c, respectively.

RESULTS

Effect of respiratory inhibitors on the NADH oxidase system

The data of Table I show that 2-(*n*-heptyl)-4-hydroxyquinoline-*N*-oxide (HQNO) inhibits NADH oxidation by the *M. tuberculosis* NADH oxidase system. HQNO, which has been shown to inhibit the respiratory chain of *Mycobacterium phlei* between cyt b and c_1 (ref. 11), is usually a more effective NADH oxidase inhibitor than antimycin A in bacterial systems[12]. We reported that antimycin A is without effect on the NADH oxidase system of *M. tuberculosis*. A small inhibition is noted with 10 μM HQNO and a 10-fold increase in HQNO concentration caused only a 59 % inhibition. The relative insolubility of the inhibitor prevented the determination of maximum inhibition levels.

In *M. phlei* a partial inhibition of this type could be explained by an NADH oxidation by-pass mechanism[11] which could be inhibited by *p*-chloromercuribenzoate (PCMB)[13,14]. NADH oxidation in our preparations, however, was inhibited by either *N*-ethylmaleimide (1.0 mM) or PCMB (0.5 mM) only to the extent of 13–19 %. Inhibitions by PCMB and HQNO were not additive.

Although we had previously reported[1] that the NADH oxidase of this mycobacterium was insensitive to cyanide, reinvestigation has shown this to be incorrect. The inhibition of cyanide (Fig. 3) was routinely observed; maximal inhibition was about 80 % at 5 mM cyanide.

Effect of NADH and inhibitors on the absorption spectrum of the particulate NADH oxidase

The inhibition of NADH oxidase by HQNO and by cyanide, demonstrated above, along with previous demonstrations of inhibition by CO and by dicumarol, brought the characteristics of the mycobacterial NADH oxidase more into accord with the pro-

TABLE I

INHIBITION BY HQNO OF THE MYCOBACTERIAL NADH OXIDASE

The HQNO* was added to the NADH oxidase reaction mixture which contained 0.4 mg of cytoplasmic membrane protein suspended in 0.1 M Tris buffer (pH 7.5). After temperature equilibration the reaction was started by the addition of 0.15 μmole of NADH. The final volume of the reaction mixture was 1.00 ml; the temperature was 28°.

Final concn. of HQNO (mM)	$\Delta A_{340\,nm}$ *per min*	*Inhibition* (%)
0	0.128	0
0.01	0.107	16
0.03	0.089	31
0.05	0.078	39
0.10	0.052	59

* The HQNO was dissolved at pH 11.5 and then back-titrated to pH

perties expected of a functional cytochrome oxidase system. Direct influence of these inhibitors was then sought through alterations of the absorption spectra. As an indicator of the levels of cytochromes under consideration the cytochrome content of the mycobacterial NADH oxidase is shown in Table II. These data are given as illustrative of the low cytochrome content and should not be taken as wholly accurate. Fig. 4 illustrates the spectra obtained on reduction of the NADH oxidase with NADH and then with dithionite. Under anaerobic conditions NADH reduces only cyt *c* and cyt $(a + a_3)$. Reduced cyt *b* is found only after the addition of dithionite (Fig. 4, insert). In the presence of HQNO the NADH-reduction spectrum is identical to that found in the absence of HQNO. In the presence of dicumarol the normal NADH-reduction spectrum is again found although full reduction of the cyt *c* and *a* was delayed.

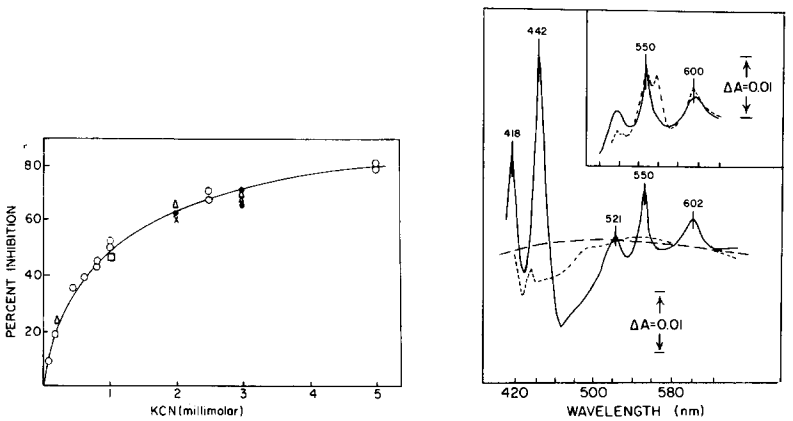

Fig. 3. Inhibition by cyanide of the mycobacterial NADH oxidase. Each reaction mixture contained 0.6 mg of NADH oxidase protein and cyanide as indicated in a final volume of 0.99 ml of 0.1 M Tris buffer (pH 7.5). The assay temperature was 28°. The reaction was started after 3 min of temperature equilibration by the addition of 0.15 μmole of NADH (0.01 ml). The different symbols of the figure represent different NADH oxidase preparations.

Fig. 4. Reduced *minus* oxidized difference spectrum of the mycobacterial cytoplasmic membrane particles (NADH oxidase). Each cuvette (8.0 mm light path) contained 7.4 mg of particle protein in a final volume of 1.00 ml of 0.10 M Tris buffer (pH 7.5) containing 1 % Tween 80.———, baseline; ————, (NADH-reduced) *minus* oxidized; – – – –, reduced *minus* oxidized after aeration. In the insert figure the enzyme (6.0 mg protein per ml) was first reduced with solid NADH (————) and after full reduction had occurred (15 min), further reduced with a few grains of $Na_2S_2O_4$ (– – – –).

TABLE II

CYTOCHROME CONTENT OF MYCOBACTERIAL CYTOPLASMIC MEMBRANE FRAGMENTS

Cytochrome	*Absorption maxima (nm)*	*Prep. No. II-2*	*II-4*	*III*
		(nmole/mg protein)		
$a + a_3$*	442, 600–605	0.04	0.05	0.06
b**	433, 562	0.06	0.06	0.10
c*	417–418, 520–522, 550	0.07	0.10	0.13

* Reduced with NADH.
** Reduced with dithionite. Also measured as the pyridine hemochromogen.

Fig. 5 illustrates the effect of CO on these preparations. The (CO-reduced) *minus* (reduced) difference spectrum is shown at the top of the figure. The shift in absorption maximum from 442 to 430 nm is characteristic of the formation of the CO complex of cyt a_3.

Further evidence for the presence of cyt a_3 in these preparations comes from the effect of cyanide. The spectrum obtained when NADH is added to a cyanide-treated preparation is shown in Fig. 6. Cyt *c* and *a* are clearly reduced by the NADH but cyt a_3 remains oxidized since no absorption peak at 442 nm is found (*cf.* Fig. 4). In contrast to the results illustrated in Fig. 4, aeration of the NADH-reduced, cyanide-treated preparations caused no reoxidation of cyt *c* and *a*. Cyanide also combines with the reduced cytochrome as is shown in Fig. 7. In this experiment the cuvettes were filled completely to eliminate any air space and the NADH oxidase was reduced with a large excess of solid NADH. Cyanide was introduced with as little admission of air as possible. Partial reoxidation of cyt a_3 occurred but the spectrum remained stable. On aeration the 442 nm band of cyt a_3 disappeared immediately while the reduced *c* and *a* peaks remained.

Cytochrome b

Although the cyt *b* of this NADH oxidase is not reduced by substrate it was still of interest to see if its reoxidation is prevented by cyanide. In the experiment illustrated in Fig. 8, dithionite was used to reduce all the cytochromes of the NADH oxidase in the presence of cyanide. The absorption bands of reduced cyt *b* (562, 433 nm) appeared along with those of cyt *c* and *a*. On gentle aeration reduced cyt *b* was partially reoxidized and further aeration resulted in complete reoxidation. Thus reduced cyt *b* is either auto-oxidizable or is oxidized *via* a cyanide-insensitive pathway. In Curve 3 of Fig. 8 the disappearance of the reduced cyt *b* band at 433 nm unexpectedly

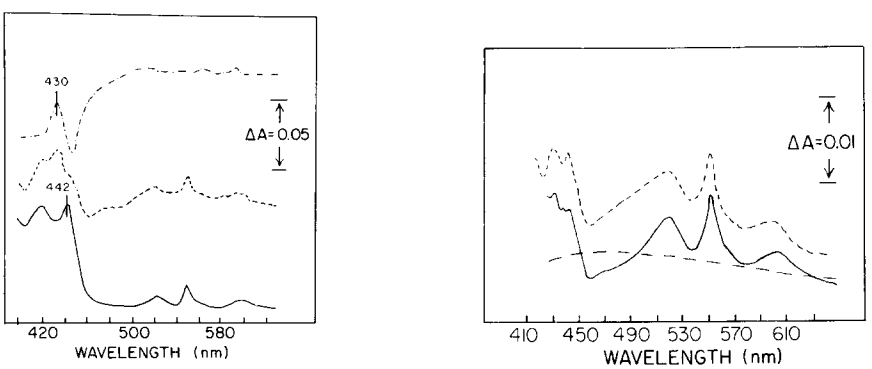

Fig. 5. (Reduced *plus* CO) *minus* reduced difference spectrum. The reaction mixture contained 18 mg of NADH oxidase protein in 0.1 M Tris buffer (pH 7.5)and Tween 80 (1.0 % final concentration). Addition of solid NADH resulted in the reduced *minus* oxidized difference spectrum (————). CO was carefully bubbled through the reduced reaction mixture resulting in the (reduced *plus* CO) *minus* oxidized difference spectrum (– – – –). Subtraction then yielded the (reduced *plus* CO) *minus* reduced difference spectrum (·–·–·–·).

Fig. 6. Reduction of cytochromes *a* and *c* by NADH after treatment of the mycobacterial NADH oxidase with cyanide. The reaction mixture contained 4.6 mg of NADH oxidase protein in 1.00 ml of 0.1 M Tris buffer (pH 7.5) containing 5 mM KCN. – – –, baseline. NADH was added resulting in the (NADH/KCN-reduced) *minus* (KCN, oxidized) difference spectrum (————). The reduced preparation was then gently aerated resulting in the aerated difference spectrum (– – – –) offset in absorbance for the sake of clarity.

revealed a small peak at 442–445 nm; this small peak was lost on further aeration (Curve 4). This may be the result of reduction of the cyanide–a_3 complex by dithionite although cyanide–a_3 complexes are usually difficult to reduce[15].

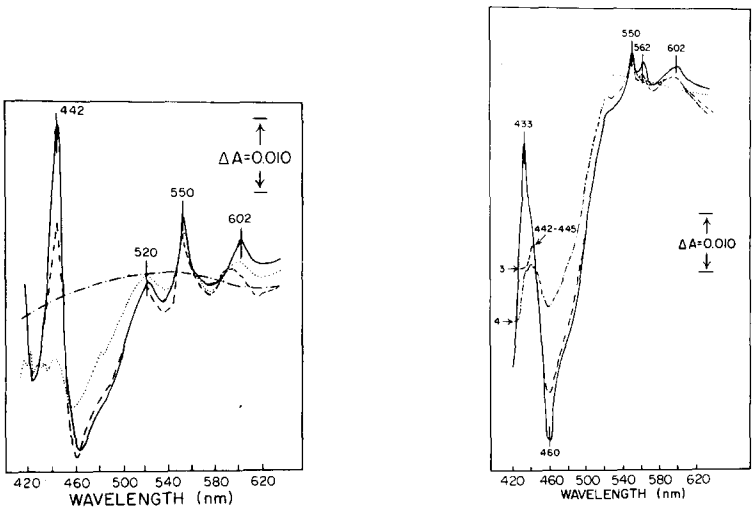

Fig. 7. Reaction of cyanide with reduced NADH oxidase. The reaction mixture contained 7.4 mg of NADH oxidase protein and 10 mg of Tween 80 in each ml of 0.1 M Tris buffer (pH 7.5). This mixture was distributed between two stoppered cuvettes and the baseline (–·–·–·–) was established. The addition of a large excess of solid NADH produced the (NADH-reduced) *minus* oxidized spectrum (——). The addition of KCN to a final concentration of 5 mM occurred with some accidental input of air as shown by the slightly altered (KCN, NADH-reduced) *minus* (KCN, oxidized) spectrum (– – – –). After the reaction mixture had stabilized it was gently aerated resulting in the aerated (KCN, NADH-reduced) *minus* (KCN, oxidized) difference spectrum (·····).

Fig. 8. Cyanide and aeration-induced modifications of the NADH oxidase spectrum. The reaction mixture contained 4.6 mg of NADH oxidase protein and 5 mM (final concn.) cyanide in 1.00 ml of 0.1 M Tris buffer (pH 7.5). The KCN treated enzyme was distributed between two stoppered cuvettes and the baseline (·····) was determined. The addition of a few grains of $Na_2S_2O_4$ to the experimental cuvette yielded the (dithionite-reduced, KCN) *minus* (KCN, oxidized) difference spectrum (——). Partial aeration (Curve 3) yielded the partially oxidized difference spectrum (– – – –); more extensive aeration (Curve 4) yielded a stable oxidized difference spectrum (–·–·–·–).

Cytochrome c

Attempts to assay the cytochrome oxidase of the NADH oxidase directly with ascorbate, with or without mammalian cyt *c*, were unsuccessful. An assay might be possible if mycobacterial cyt *c* could be prepared for use as substrate but the cyt *c* of this mycobacterium could not be extracted by any of the procedures tested (trichloroacetic acid extraction, treatment with *n*-butanol, alkaline extraction of a butanol powder[16]).

DISCUSSION

The experiments described above indicate the presence of both cyt *a* and a_3 in the $H_{37}Ra$ strain of *M. tuberculosis* and provide clear evidence for a functional cytochrome oxidase system in this organism. The levels of cytochromes in the membrane preparations are extremely low but are sufficient for the slow growth of the mycobac-

teria. While our data provide reasonable evidence that terminal electron transfer from NADH to oxygen is mediated by a membrane-bound cytochrome system, we do not fully exclude alternate pathways. The terminal electron system is still anomalous, *viz.* the fact that NADH is the sole substrate oxidized by the membrane system, that succinate dehydrogenase is present in cell-free extracts whereas no succinoxidase can be demonstrated[3,4,9,17] and that phosphorylation coupled to oxidation has not been shown. The non-functional behaviour of cyt *b* and its auto-oxidizability may be relevant to these anomalies. While our consistent lack of success in demonstrating oxidative phosphorylation as well as succinoxidase activity in cell-free extracts of *M. tuberculosis* may have numerous explanations it is reasonable to assume that in this aerobic organism phosphorylation coupled to respiration occurs.

With this assumption in mind it is of interest that KATO AND TANAKA[18] have implicated trehalose-6,6′-dimycolate (cord factor)[19] a toxic glycolipid of mycobacteria, as the factor responsible for the deleterious effects of either cord factor intoxication or the tuberculous infection on the electron transfer process in mouse liver. KATO[20] has extended these findings and has shown an interference with oxidative phosphorylation in liver homogenates prepared from mice treated with cord factor or infected with virulent tubercle bacilli. Although the strain of *M. tuberculosis* used in our experiments does not contain cord factor, it is still possible that the natural content of mycolic acid esters of *M. tuberculosis* is sufficient to interfere with oxidative phosphorylation in cell-free extracts. A corollary of this suggestion would be that in the intact cell these glycolipids are either inactive or, more likely, not available to inhibit this process. Preparations of the cytoplasmic membranes and soluble factors uncontaminated with glycolipids have not yet been obtained. The removal of the cell wall and its adhering glycolipids through the production of viable spheroplasts (Dr. L. Trnka, personal communication) may provide a means for approaching this goal.

While there is little question about the presence and function of the cytochrome system in artificially cultivated $H_{37}Ra$ certain facts make questionable any extension of these data to pathogenic, *i.e.*, *in vivo* grown, mycobacteria. SEGAL AND BLOCH[7] demonstrated that the pathogenic $H_{37}Ra$ strain of *M. tuberculosis* shows essentially no aerobic metabolism when isolated from infected mouse lungs. There is no particular evidence to suggest that the aerobic metabolism of $H_{37}Ra$ changes during primary to late infection stages as the number of organisms and lesions increases. In the absence of evidence to the contrary we must assume that the metabolic capabilities of the organisms in the infected tissues remain the same. These altered oxidative capabilities, taken together with the demonstration (a) that *in vivo* grown Bacille Calmette Guérin (BCG) and *Mycobacterium lepraemurium* have no detectable cytochromes[8] and (b) that pathogenic mycobacteria are sensitive to oxygen[21] again renders questionable the concept that a valid comparison may be made between *in vivo* and *in vitro* mycobacteria.

ACKNOWLEDGEMENTS

This investigation was supported, in part, by Research Grant 02416 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U.S. Public Health Service.

The experiment shown as Fig. 5 was carried out by Dr. A. Worcel to whom we are most grateful for permission to use the data.

REFERENCES

1 D. S. GOLDMAN, M. J. WAGNER, T. ODA AND A. L. SHUG, *Biochim. Biophys. Acta*, 73 (1963) 367.
2 Y. YAMAMURA, M. KUSUNOSE, S. NAGAI AND E. KUSUNOSE, *J. Biochem. Tokio*, 41 (1954) 513.
3 Y. YAMAMURA, M. KUSUNOSE, S. NAGAI, E. KUSUNOSE, Y. YAMAMURA, JR., J. TANI, T. TERRAI AND T. NAGASUGA, *Med. J. Osaka Univ.*, 6 (1955) 489.
4 M. KUSUNOSE, S. NAGAI, E. KUSUNOSE AND Y. YAMAMURA, *J. Bacteriol.*, 72 (1956) 754.
5 A. F. BRODIE AND C. T. GRAY, *J. Biol. Chem.*, 219 (1956) 853.
6 A. F. BRODIE AND J. BALLANTINE, *J. Biol. Chem.*, 235 (1960) 226.
7 W. SEGAL AND H. BLOCH, *J. Bacteriol.*, 72 (1956) 132.
8 T. KUSAKA, R. SATO AND K. SHOJI, *J. Bacteriol.*, 87 (1964) 1383.
9 D. S. GOLDMAN, in H. BIRKHAUSER, H. BLOCH AND G. CANETTI, *Advances in Tuberculosis Research*, Vol 11, Karger, Basel, 1961, p. 1.
10 B. CHANCE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 4, Academic Press, New York, 1957, p. 273.
11 A. ASANO AND A. F. BRODIE, *J. Biol. Chem.*, 239 (1964) 4280.
12 L. SMITH, in T. P. SINGER, *Biological Oxidations*, Interscience, New York, 1968, p. 55.
13 A. ASANO AND A. F. BRODIE, *Biochem. Biophys. Res. Commun.*, 19 (1965) 121.
14 A. ASANO, T. KANESHIRO AND A. F. BRODIE, *J. Biol. Chem.*, 240 (1965) 895.
15 T. YONETANI, in P. D. BOYER, H. A. LARDY AND K. MYRBACK, *The Enzymes*, Vol. 8, Part B, Academic Press, New York, 1963, p. 41.
16 T. YAMAGUCHI, G. TAMURA AND K. ARIMA, *Biochim. Biophys. Acta*, 124 (1966) 413.
17 A. S. YOUMANS, I. MILLMAN AND G. P. YOUMANS, *J. Bacteriol.*, 71 (1956) 565.
18 M. KATO AND A. TANAKA, *Am. Rev. Respirat. Diseases*, 96 (1967) 460.
19 H. NOLL, H. BLOCH, J. ASSELINEAU AND E. LEDERER, *Biochim. Biophys. Acta*, 20 (1956) 299.
20 M. KATO, *Am. Rev. Respirat. Diseases*, 96 (1967) 998.
21 S. F. GOTTLIEB, N. R. ROSE, J. MAURIZI AND E. H. LANPHIER, *J. Bacteriol.*, 87 (1964) 838.