Bacterial and mammalian systems capable of oxidative phosphorylation require the presence of a highly organized particulate structure. Although the methods required for the isolation of particles from bacteria (1) are more vigorous than the procedures used for the isolation of mitochondria from animal tissues, the bacterial particles remain relatively intact and contain the terminal respiratory carriers necessary for coupled oxidative phosphorylation (2). Nevertheless, the particles alone fail to carry out coupled activity unless supplemented with soluble protein factors (2-8). The soluble fraction from Mycobacterium phlei contains both oxidative components and coupling factors (4, 6).

Phosphorylation with a cell-free system from M. phlei was shown to be inextricably associated with oxidation. Thus, an understanding of the mechanisms of oxidative phosphorylation requires detailed knowledge of the respiratory chains. Earlier studies of the electron transport chain (2, 9) suggested broad areas associated with the phosphorylative process; however, more detailed analysis was necessary in order to locate the phosphorylative sites precisely. The respiratory chains of M. phlei appear to be similar to those described for mammalian mitochondria (10). In many respects, this bacterial system resembles the system observed with disrupted mitochondria preparations. Differences between the M. phlei and the mitochondrial system were found in quinone composition (11-14) and in the presence of an additional respiratory chain for malate. In the bacterial system, oxidative phosphorylation with malate can occur by a pathway independent of nicotinamide adenine dinucleotide (15). The flavin adenine dinucleotide-linked malate and NADH-linked chains converge at the naphthoquinone (vitamin K) level and utilize the same terminal respiratory carriers. The succinate chain in M. phlei enters the terminal respiratory chain at the cytochrome b level. The properties of these three respiratory chains in M. phlei will be described in this paper.

**EXPERIMENTAL PROCEDURE**

Vitamin K$_1$ used in these experiments was a gift from Dr. K. Folkers of Merck Sharp and Dohme, Rahway, New Jersey, or was purchased from Mann Research Laboratories. This vitamin was further purified by column chromatography on Permutit resin. The purified vitamin K$_1$ (10 mg) was suspended in 2 ml of 0.05 M Tris, pH 7.4, containing 50 mg of Asolectin with sonic oscillation for 5 minutes. Asolectin (plant phospholipid mixture) was purchased from Associated Concentrates, Woodside, New York. Oligomycin was obtained from the Wisconsin Alumni Foundation, Madison. 2-n-Nonylhydroxyquinoline N-oxide was obtained from Sigma Chemical Company. Yeast hexokinase was obtained from Pabst Laboratories. All of the other chemicals used were commercially available and of reagent grade.

Inhibitors such as 2-n-nonylhydroxyquinoline N-oxide, Dicumarol, carbonyl cyanide m-chlorophenylhydrazone, pentachlorophenol, and sodium Amytal were suspended in 8 ml of water, and 3 N KOH or 1 N HCl was added dropwise with vigorous stirring until a clear solution was obtained. The pH was adjusted to 7.2 or 7.4, and the mixture was brought to a final volume of 10 ml. 2-n-Nonylhydroxyquinoline N-oxide was adjusted to pH 8.5 before dilution. Other relatively insoluble compounds such as rotenone, oligomycin, or thienyltrifluorobutanedione were dissolved in a small amount of acetone (0.1 to 1.0 ml) and then diluted 100 times with distilled water. Many of the solutions, ascorbate, phenazine methosulfate, 2-n-nonylhydroxyquinoline N-oxide, Dicumarol, and carbonyl cyanide m-chlorophenylhydrazone were prepared fresh daily. In addition, the concentration was checked by spectrophotometric means when possible.

**Preparation of Particles and Soluble Components—M. phlei**

ATCC 354 cells were grown and harvested by procedures previously described (16). Sonically disrupted cells were separated into particulate and supernatant fractions by differential centrifugation in the Spinco preparative centrifuge (6). The particles obtained following centrifugation were washed with 0.15 M KCl containing 0.01 M MgCl$_2$ and adjusted to pH 7.4 with Tris buffer (0.01 M). Two types of particles were used in these studies: (a) washed particles freshly prepared and (b) particles stored at 16°, which are referred to as aged particles (2). The supernatant fraction was dialyzed with distilled water for 2 or 3 days with several changes of water. Dialysis was carried out at 4° with constant stirring. The crude supernatant either was used directly or was further fractionated with ammonium sulfate and chromatographed on DEAE-cellulose (17).

**Measurement of Oxidation and Coupled Phosphorylation—Respiration was measured by conventional manometric techniques at 30°.** The main compartment of each vessel contained washed particles (5 to 10 mg of protein), dialyzed supernatant (5 to 15 mg of protein), 15 µmoles of MgCl$_2$, 5 to 12 µmoles of inorganic phosphate, and enzyme preparation. The reaction was started by the addition of substrate. The rate of oxygen uptake was measured at 30°.
phosphate, 3 mg of yeast hexokinase, and 10 μmoles of glucose. The cofactors or inhibitors indicated were added to the main compartment and allowed to incubate with the particulate and supernatant fractions for at least 10 minutes before addition of substrate. The side arm of the vessels contained 25 μmoles of KF, 2.5 μmoles of ADP, and substrate. The total volume was adjusted to 1.5 ml with water. Inorganic phosphate esterification was determined by the method of Fiske and SubbaRow (18). Protein was determined by the turbidimetric method of Stadtman, Novelli, and Lipmann (19) or by the method of Lowry et al. (20).

Measurement of Difference Spectra—The reduced minus oxidized difference spectrum of the particulate preparation was measured following substrate addition with a split beam spectrophotometer (21). A cuvette with a 1- or 0.2-cm light path was used at room temperature; however, at the temperature of liquid nitrogen, a cuvette with a 0.2-cm light path was employed (22). The difference spectrum between a CO-treated reduced preparation and a reduced preparation was measured by the method described by Chance (23) with a Cary model 11 recording spectrophotometer. A gentle stream of carbon monoxide was added to the anaerobic sample for about 1 minute. The inability to change the difference spectrum by the further addition of carbon monoxide was taken to indicate saturation of the binding capacity of this agent.

The double beam spectrophotometer with a vibrating platinum oxygen electrode was used for measurement of the rate of cytochrome reduction. These experiments were carried out in the laboratory of Dr. R. Chance. The dual wave length spectrophotometer (American Instrument Company) was also used to measure the rate of reduction of the terminal respiratory pigments.

Artificial electron acceptors were used to measure malate-vitamin K reductase (15) activity or NADH oxidation. These reactions were followed spectrophotometrically with the Cary model 11 recording spectrophotometer or Gilford automatic recording attachment for the Beckman spectrophotometer.

RESULTS

Respiratory Chain Components—The distribution of respiratory carriers differs in the particulate and supernatant fractions. The particulate fraction contains all of the known respiratory carriers (bound NAD+, flavins, vitamin K₃H, and the cytochromes), whereas the supernatant fraction contains NAD⁺, flavins, and small amounts of vitamin K₃H. The particulate fraction appears to contain sufficient quantities of the respiratory carriers, since oxidative phosphorylation with generated NADH or succinate can be carried out with the particles alone. Stimulation of both oxidation and phosphorylation occurs on the addition of soluble protein components found in the supernatant fraction (6, 17, 24). Oxidation with β-hydroxybutyrate or malate as electron donor requires the addition of cofactors (NAD⁺ or FAD) and the solubilized dehydrogenases for these substrates.

Evidence for participation of the terminal respiratory chain of the particulate fraction was obtained by examining the difference spectrum with various substrates as electron donors. The reduced minus oxidized difference spectrum of the particulate fraction or particulate and supernatant fractions of M. phlei was measured after substrate, or substrate and inhibitor, was added and the system allowed to achieve the anaerobic state.

Following the addition of succinate, absorption bands appear at 528, 562, and 590 μm (Fig. 1). These peaks are characteristic of the α-bands of cytochrome types a, b, and c, respectively. Examination of the Soret region revealed a trough characteristic of flavoprotein (460 μm). The Soret band of cytochrome b was found at 430 μm, while those of cytochromes c and a + a₃ were distinguished as shoulders at 420 and 445 μm, respectively. In the presence of 2-nonylhydroxyquinoline N-oxide the Soret band of cytochrome b was shifted from 430 to 433 μm. The difference spectrum of the particulate fraction with succinate as substrate was independent of the addition of the supernatant fraction; however, stimulation of the rate of reduction of cytochromes b, c₁, and a + a₃ occurred on the addition of the soluble fraction. Reduction of the terminal respiratory pigments with malate or β-hydroxybutyrate, in contrast to succinate, required the addition of supernatant components.

Quantitative differences in reduction of the terminal respiratory pigments were observed with substrates that utilize different pathways of oxidation. The reduced bands of cytochrome b (562 and 430 μm) with succinate or malate as electron donor were less prominent than those observed with β-hydroxybutyrate as substrate. Reduction with sodium dithionite resulted in a difference spectrum which was qualitatively similar to that observed on the addition of substrate and which differed only in that a greater reduction of cytochrome b was observed following chemi...

Fig. 1. Reduced minus oxidized difference spectrum of particles from M. phlei. The system consisted of 30 μmoles of MgCl₂, 107 μmoles of KCl, 3 μmoles of inorganic phosphate, 5.3 μmoles of ADP, 2.5 μmoles of FAD, 6.0 mg of washed particles, 8.6 mg of dialyzed supernatant fluid, 36 μmoles of Tris·HCl buffer (pH 7.4), and water to a final volume of 3.0 ml. The reaction was carried out at room temperature (24°) in a 1-cm cuvette. Curve 1, spectrum obtained following incubation of the system with succinate for 10 minutes (the time necessary to reach the anaerobic state). The reference system was kept in the oxidized state by supplying a stream of oxygen. Curve 2, spectrum obtained following the addition of 2-nonylhydroxyquinoline N-oxide (9.0 μg) and sodium dithionite.

1 Cytochrome c₁ contributes to the absorption in the c region and cannot be distinguished at room temperature; from low temperature studies it would appear that the absorption due to this component at 551 μm is small when compared to that of cytochrome c. Nevertheless, the values reported for the enzymatically reducible cytochrome c represent the absorption of cytochrome c₁ + c. 
The reaction was carried out in a 0.2 cm cuvette at room temperature and contained 2 μmoles of MgCl₂, 32 μmoles of KCl, 4.1 mg of washed particles at the temperature of liquid nitrogen. The system consisted of 15 μmoles of MgCl₂, 23 μmoles of KCl, 1.4 μmoles of NAD+, 11.8 mg of washed particles, 11.1 mg of ammonium sulfate, and water to a final volume of 3.0 ml. Reduction of the cytochrome was accomplished following incubation for 10 minutes with 30 μmoles of succinate and 30 μmoles of p-hydroxybutyrate. The further resolved by low temperature spectroscopy. Under these conditions a cytochrome of the c₁ type appeared as a shoulder at about 554 μm, while cytochrome c was evident at 548 μm. At low temperature the absorption bands in the Soret region revealed the presence of cytochromes a + a₃ (445 μm) and c (423 μm). The band at 438 μm may be due to cytochromes b and a₂.

Carbon monoxide-binding pigments were also observed with the particulate fraction from M. phlei (Fig. 3). A gentle stream of carbon monoxide was added to the enzymatically or chemically reduced (sodium dithionite) sample, and the rapid change in absorption was followed. The reference cuvette contained the preparation that was reduced enzymatically. Chemical reduction with dithionite resulted in an increased reduction of the cytochrome b of the particles; however, the carbon monoxide difference spectrum was identical with that obtained by enzymatic reduction. The carbon monoxide difference spectrum exhibited a trough at 445 μm and a peak at 430 μm. These results are qualitatively similar to those obtained by Chance with mammalian mitochondria, yeast, and Bacillus subtilis (23).

The concentration of the respiratory pigments was determined spectrophotometrically by the method of Chance and Williams (25) and is shown in Table I. The concentration of the respiratory carriers was calculated on the assumption that the cytochromes from this microorganism have molar extinction coefficients similar to those found in mammalian mitochondria. The molar extinction coefficients for pure cytochromes isolated from other microorganisms are in close agreement with those obtained from mitochondria even though the absorption peaks of the cytochromes may differ (28-34). The major absorption peaks of the various cytochromes in M. phlei are similar to those of the homologous cytochromes of mitochondria. The relative concentrations of the respiratory pigments in M. phlei are similar to those found for rat liver (25) and pigeon heart mitochondria determined by a similar method (26). The absolute concentrations of the bacterial respiratory pigments were calculated and expressed as millimicromoles per mg of protein (Table II). The value for cytochrome b in this table was obtained following enzymatic reduction with a combination of succinate, malate, and β-hydroxybutyrate, since addition of a single substrate resulted in only partial reduction of cytochrome b. The further addition of dithionite increased the total amount of cytochrome b reduction by 27%. The amounts of enzymatically or chemically reducible carriers of M. phlei were compared with the amounts of reducible carriers found in different mitochondrial preparations (Table II). The amount of cytochromes b, a + a₃, and a₃ in the bacterial particles was found to be lower than observed in mammalian particles, whereas the amount of cytochrome c was almost the same. The concentration of pyridine nucleotides in M. phlei particles (35) is also substantially lower than the corresponding levels in intact mitochondria.

The reduced steady state level of the terminal respiratory components was studied with substrates that utilize different respiratory pathways (Table III). The individual substrates differed in their capacity to reduce cytochrome b. The highest reduced steady state level of cytochrome b was observed with β-hydroxybutyrate, and the lowest with succinate. The values

![Fig. 2. Reduced minus oxidized difference spectrum of M. phlei particles at the temperature of liquid nitrogen. The system contained 2 μmoles of MgCl₂, 23 μmoles of KCl, 1.4 μmoles of NAD⁺, 11.8 mg of washed particles, 11.1 mg of ammonium sulfate-fractionated supernatant fluid, 100 μmoles of Tris-HCl buffer (pH 7.4), and water to a final volume of 0.6 ml. In addition, one of the cuvettes contained 20 μmoles of succinate and 10 μmole of sodium sulfide. The reaction was carried out in a 0.2 cm cuvette at room temperature and brought to 77° K.](http://www.jbc.org/)

![Fig. 3. Carbon monoxide difference spectrum. The system consisted of 15 μmoles of MgCl₂, 23 μmoles of KCl, 1.4 μmoles of NAD⁺, 11.8 mg of washed particles, 11.1 mg of ammonium sulfate-fractionated supernatant fluid, 100 μmoles of Tris-HCl buffer (pH 7.4), and water to a final volume of 3.0 ml. Reduction of the cytochromes was accomplished following incubation for 10 minutes with 30 μmoles of succinate and 30 μmoles of β-hydroxybutyrate. Curve 1, reduced minus oxidized spectrum; Curve 2, carbon monoxide-treated reduced minus oxidized spectrum; Curve 3, reduced minus oxidized spectrum.](http://www.jbc.org/)
recorded in this table represent the average of eight determinations for each substrate, since the average of enzymatically reducible cytochrome \(b\) varied from preparation to preparation. Although the system from \(M. phlei\) does not exhibit respiratory control, the electron transport sequence was ascertained from studies with respiratory inhibitors and from the reduced steady state level of the carriers following the addition of substrate (Table III). Thus, the electron transfer sequence of the terminal respiratory chain of this microorganism, like the mitochondrial system, flows from cytochrome \(b\) to \(c\) to \(a + a_3\) to oxygen.

Simultaneous determination of oxygen consumption and spectrophotometric changes as described by Chance and Williams (40) revealed that the cytochrome reached a steady state level which was maintained until the oxygen concentration reached about 10 \(\mu\)moles per ml. The rate of oxygen disappearance

Table I
Relative concentration of respiratory carriers in particles from \(M. phlei\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Wave length (\mu)</th>
<th>(E_{	ext{M}}) used (10)</th>
<th>Relative concentration</th>
<th>Mitochondria</th>
<th>Rat liver (a)</th>
<th>Pigeon heart (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome (a + a_3)</td>
<td>558, 635</td>
<td>16</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Cytochrome (b)</td>
<td>562, 574</td>
<td>20</td>
<td>0.88</td>
<td>0.9</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>Cytochrome (c + c_1)</td>
<td>551, 540</td>
<td>9</td>
<td>2.26</td>
<td>1.7</td>
<td>1.28</td>
<td></td>
</tr>
<tr>
<td>Flavoprotein</td>
<td>455, 510</td>
<td>11</td>
<td>2.47</td>
<td>3.6</td>
<td>3.00</td>
<td></td>
</tr>
<tr>
<td>Cytochrome (a_3)</td>
<td>430, 445</td>
<td>91</td>
<td>0.82</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin (K_H)</td>
<td>430, 445</td>
<td>91</td>
<td>43.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(a\) From the data of Chance and Williams (25).
\(b\) From the data of Chance and Hagihara (26).
\(c\) Acid-extractable flavin \(b\).
\(d\) Average of eight determinations.


d Calculated from carbon monoxide difference spectrum. The millimolar extinction coefficient was assumed to be similar to that of heart muscle (28).

e Average of four determinations. Difference of millimolar extinction coefficient of CO compound between 445 and 430 \(\mu\)m was assumed as 82 \(\text{mM}^{-1} \text{cm}^{-1}\) (Chance (28)).

TABLE II
Comparison of enzymatically reducible respiratory carriers of \(M. phlei\) with those of mitochondrial systems

<table>
<thead>
<tr>
<th>Components</th>
<th>Enzymatic reduction</th>
<th>Chemical determinations</th>
<th>Pigeon heart mitochondria</th>
<th>ETP (b)</th>
<th>Beef KHP (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome (a + a_3)</td>
<td>0.27 (d)</td>
<td>0.75</td>
<td>1.62</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>Cytochrome (b)</td>
<td>0.18 (c)</td>
<td>0.65 (f)</td>
<td>0.48</td>
<td>0.85</td>
<td>0.49</td>
</tr>
<tr>
<td>Cytochrome (c + c_1)</td>
<td>0.52</td>
<td>0.37 (g)</td>
<td>0.35</td>
<td>0.63</td>
<td>0.56</td>
</tr>
<tr>
<td>Flavoprotein</td>
<td>0.68</td>
<td>2.2</td>
<td>0.69</td>
<td>0.80</td>
<td>0.39</td>
</tr>
<tr>
<td>Cytochrome (a_3)</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyridine nucleotide</td>
<td>0.33 (h)</td>
<td>8.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinone</td>
<td>12.0 (i)</td>
<td>7.0</td>
<td>4.8</td>
<td>4.26</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) From the data of Chance and Hagihara (20).
\(b\) From the data of Green and Wharton (36).
\(c\) Keilin-Hartree preparation. From the data of King, Nickel, and Jensen (37).
\(d\) Average of eight determinations.
\(e\) Addition of dithionite increased cytochrome \(b\) reduction to 0.25 \(\mu\)mole per mg of protein.
\(f\) Kashket and Brodie (27).
\(g\) Average of four determinations. Difference of millimolar extinction coefficient of CO compound between 445 and 430 \(\mu\)m was assumed as 82 \(\text{mM}^{-1} \text{cm}^{-1}\) (Chance (28)).

The rate of oxygen disappearance was constant over the period measured. Following depletion of oxygen, further reduction of the cytochrome occurred at a rapid rate (Fig. 4). With freshly prepared particles the reduced steady state level was achieved rapidly (Fig. 5), whereas with aged particles reduction of the cytochromes occurred continually and at a slower rate. Thus, it was difficult to determine the reduced steady state level with aged particles.

The turnover number for each of the cytochromes (Table III) was determined from the data as presented in Figs. 4 and 5. The turnover numbers of the cytochromes were of the same magnitude as those described for intact mitochondria. The respiratory activities measured by the conventional manometric technique are in good agreement with those obtained by spectrophotometric methods. The \(Q_{10}\) values obtained with the bacterial particulate preparations are similar to those obtained with intact mitochondria but considerably lower than those obtained with disrupted mitochondria such as \(ETP\) (41) or the Keilin-Hartree heart muscle preparations (42).

The percentage reduction of the cytochromes was determined at the point of transition from the aerobic to anaerobic state as in Fig. 4. The percentage reduction of cytochromes \(a + a_3\), \(b\), and \(c\) is shown in Table IV. Reduction of cytochrome \(b\) at the transition state was lower than that observed for cytochromes \(c\) or \(a + a_3\). In addition, the amount of cytochrome \(b\) reduced at the transition state was lower than the other cytochromes. The properties of the bacterial particles with respect to the rate and transition state behavior of the enzymatically reduced cytochrome \(b\) appear to place the bacterial system between the fully coupled mitochondrial system (40) and the nonphosphorylative Keilin-Hartree preparation (42). The reduction of cytochromes by substrate, in the presence of inhibitors, or on reaching anaerobiosis, follows first order kinetics.
Oxidative Phosphorylation in Fractionated Bacterial Systems. XIV

The Kᵣ value (millimicromoles of O₂ per second per change in optical density) defined by Chance (38) was determined by the method of Estabrook and Mackler (39). The rate of oxygen consumption was calculated by dividing the protein concentration into the Kᵣ value, whereas the Qₒₒ was determined spectrophotometrically and by manometric procedures. The (Kᵣ)ₒ value was obtained from spectrophotometric studies in which the concentration of cytochrome a was determined. The turnover number was calculated by the formula given by Estabrook and Mackler (39), and is equal to Kᵣ × electroequivalents × E × 10⁻⁷, where E represents the millimolar extinction coefficient; the values used in calculating the turnover number for the bacterial system are shown in Table I. The system consisted of 30 μmoles of MgCl₂, 80 μmoles of KCl, washed particles (1.3 to 2.5 mg of protein), ammonium sulfate-fractionated supernatant fluid (2.5 to 3.8 mg of protein), 200 μmoles of Tris-HCl buffer (pH 7.2), and water to a final volume of 3.0 ml. The reaction was started by the addition of 50 pmoles of succinate, dl-malate, or dl-β-hydroxybutyrate. In addition, 0.25 μmole of FAD was added to the reaction mixture when malate was employed as substrate, and 1.5 μmoles of NAD⁺ were used with β-hydroxybutyrate. The reactions were carried out at 23°C in a 1-cm cuvette for the spectrophotometric studies; in the manometric studies the reactions were started by the addition of 50 pmoles of succinate, dl-malate, or dl-β-hydroxybutyrate. In addition, 0.25 μmole of FAD was added to the reaction mixture when malate was employed as substrate, and 1.5 μmoles of NAD⁺ were used with β-hydroxybutyrate. The reactions were carried out at 23°C in a 1-cm cuvette for the spectrophotometric studies; in the manometric studies the reactions were conducted at 30°C for 10 to 15 minutes. The Qₒₒ values calculated from manometric studies represent the average of 10 experiments, whereas the values obtained from spectrophotometric studies represent the average of 4 experiments.

Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ΔO₂a</th>
<th>(Kᵣ)b</th>
<th>Turnover No. of cytochromes</th>
<th>Qₒₒ at 30°C</th>
<th>Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>a₁ b c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>0.73</td>
<td>185</td>
<td>10.2 28.7 5.1</td>
<td>29 39</td>
<td>25 3 12 45</td>
</tr>
<tr>
<td>Malate</td>
<td>0.75</td>
<td>177</td>
<td>12.0 21.5 6.4</td>
<td>30 37</td>
<td>38 4 10 23</td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>1.48</td>
<td>339</td>
<td>22.9 42.6 13.8</td>
<td>60 84</td>
<td>50 6 13 21</td>
</tr>
<tr>
<td>All three substrates</td>
<td>1.67</td>
<td></td>
<td></td>
<td>67</td>
<td>73 10</td>
</tr>
</tbody>
</table>

a Millimicromoles per second per mg of protein (spectrophotometric determination).
b Millimicromoles of O₂ per second per change in optical density at 600 to 623 nm.

c Although the absorption of cytochrome c₁ was found to be relatively low at the wave length used to measure cytochrome c (Fig. 2), the values given above for cytochrome c represent a combination of c + c₁.
d Spectrophotometric.
e Microliters of O₂ per hour per mg of protein.
f The amount of cytochrome b reduced enzymatically was found to be different with the substrates employed. Thus the percentage of the steady state level for this cytochrome was determined with each substrate from the level obtained following complete anaerobiosis (taken as 100%).

Fig. 4. Simultaneous determination of oxygen consumption and cytochrome reduction. The cuvette contained 2.4 μmoles of MgCl₂, 48 μmoles of KCl, 12.0 mg of aged particles, 5.4 mg of ammonium sulfate-fractionated supernatant, 12.0 μmoles of FAD, 2.5 μmoles of dl-malate, 2.5 μmoles of succinate, and 6 μmoles of Tris-HCl buffer, pH 7.4. Total volume, 1.5 ml; optical path, 0.5 cm; temperature, 24°C.

Nature of Respiratory Chains in M. phlei—Three major respiratory pathways have been found in M. phlei which are capable of coupling phosphorylation to oxidation. These pathways can be distinguished by their response to added cofactors, to purified supernatant fractions, and to respiratory inhibitors. The requirement for different cofactors for oxidation of substrates utilizing different pathways in the reconstituted system (washed particles and dialyzed supernatant fluid) is shown in Table V. NAD⁺-linked substrates such as β-hydroxybutyrate or ethanol required the addition of NAD⁺ for activity and were not substantially stimulated by the addition of FAD or FMN. Malate oxidation, however, was stimulated by both FAD and NAD⁺.

Fractionation of the supernatant components on DEAE-cellulose (17) resulted in a resolution of two distinct pathways for malate oxidation. One of the purified enzymes exhibited a requirement for FAD for oxidation of malate when added to the particles. Oxidation by this fraction did not occur when FAD was replaced by NAD⁺. In the absence of particles, this enzyme could be linked to dye (thiazoly1 blue tetrazolium) provided that FAD, vitamin K₁, and phospholipid are added (15). The other purified supernatant fraction exhibited a requirement for FAD⁺ for malate oxidation.

Succinate oxidation by the reconstituted system (particles and supernatant fraction) was not affected by the addition of either FAD or FMN. Some inhibition of succinic oxidase by FAD (3 × 10⁻⁴ M) was observed with particles alone. Inhibition of other flavin enzymes by a high concentration of FAD has been reported by Ernster et al. (45) for mammalian "DJ-diaphorase." Succinate oxidation, however, was stimulated by a high concentration of NAD⁺ when tested with particles and crude supernatant fluid, but not when tested with particles alone (Table V). The particulate fraction has relatively little fumarase activity. The stimulation of succinate oxidation by NAD⁺ exhibited a lag

d The abbreviation used is: FMN, riboflavin phosphate, flavin mononucleotide.
period when tested with particle and supernatant fractions. This lag was presumably due to the time necessary for accumulation of malate, which results in the secondary oxidation via the NAD\(^+\)-linked pathway.

Differences in the three respiratory chains were also shown by

\[
\begin{align*}
(A) & \quad 551^m \rightarrow 540^m \\
(B) & \quad 562^m \rightarrow 574^m \\
(C) & \quad 598^m \rightarrow 623^m
\end{align*}
\]

\[\text{O.D.} \times 0.00087\]

\[\beta HB\]

\[\text{N}_2\text{S}_2\text{O}_4\]

\[60^\circ C\]

**TABLE IV**

Percentage reduction of components of respiratory chain on transition from aerobic to anaerobic state

The system was similar to that described in Table III, except that aged particles (12 mg of protein) were used and the total volume was 1.5 ml. A 0.5-cm cuvette was used, and the reaction was carried out at room temperature (24°C). The amount of cytochrome reduced was measured at the transition point, when the oxygen concentration reached zero. The amount of reduction at the transition point was expressed as the percentage of the amount of cytochrome reduced after complete reduction by substrate.

<table>
<thead>
<tr>
<th>Substrate and fraction</th>
<th>Oxygen consumption with various cofactors</th>
<th>Oxygen consumption with various cofactors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>FAD</td>
</tr>
<tr>
<td>Malate</td>
<td>1.08</td>
<td>6.72</td>
</tr>
<tr>
<td>Particles + supernatant (crude)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particles + supernatant (reductase)</td>
<td>0.96</td>
<td>4.70</td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>1.36</td>
<td>8.75</td>
</tr>
<tr>
<td>Particles + supernatant (crude)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>3.2</td>
<td>1.51</td>
</tr>
<tr>
<td>Particles + supernatant (crude)</td>
<td>3.6</td>
<td>3.1</td>
</tr>
</tbody>
</table>

\(\text{FAD concentration was } 3.3 \times 10^{-4} \text{ M.}\)

**TABLE V**

Effect of cofactors on oxidation by different substrates with various fractions from *M. phlei*

The system consisted of 15 μmoles of MgCl\(_2\), 25 μmoles of KCl, 25 μmoles of ADP, 10 μmoles of glucose, 3 mg of yeast hexokinase, 8 μmoles of inorganic phosphate, washed particles (4.6 to 9.5 mg of protein), ammonium sulfate supernatant ("crude") (6.3 to 8.5 mg of protein) or DEAE-cellulose-fractionated supernatant ("reductase") (3.2 mg of protein), and water to a volume of 1.5 ml. In addition, the vessels indicated contained 40 μmoles of malate, succinate, or β-hydroxybutyrate. The reactions were carried out at 30°C for 15 minutes.

<table>
<thead>
<tr>
<th>Substrate and fraction</th>
<th>Oxygen consumption with various cofactors</th>
<th>Oxygen consumption with various cofactors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>FAD</td>
</tr>
<tr>
<td>Malate</td>
<td>1.08</td>
<td>6.72</td>
</tr>
<tr>
<td>Particles + supernatant (crude)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particles + supernatant (reductase)</td>
<td>0.96</td>
<td>4.70</td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>1.36</td>
<td>8.75</td>
</tr>
<tr>
<td>Particles + supernatant (crude)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>3.2</td>
<td>1.51</td>
</tr>
<tr>
<td>Particles + supernatant (crude)</td>
<td>3.6</td>
<td>3.1</td>
</tr>
</tbody>
</table>

**TABLE VI**

Effect of inhibitors on different respiratory chains of *M. phlei*

The system was similar to that described in Table V. NADH was generated either with yeast alcohol dehydrogenase (0.6 mg) and ethanol (100 μmoles) or with β-hydroxybutyrate (60 μmoles) and the supernatant β-hydroxybutyrate dehydrogenase fraction.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition of oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Succinate</td>
</tr>
<tr>
<td>Amytal, 10^{-2} M</td>
<td>85</td>
</tr>
<tr>
<td>Atebrin, 3 \times 10^{-3} M</td>
<td>85</td>
</tr>
<tr>
<td>Dicumarol, 10^{-4} M</td>
<td>98</td>
</tr>
<tr>
<td>Dicumarol, 10^{-3} M</td>
<td>98</td>
</tr>
<tr>
<td>2-n-Nonylhydroxyquinolne N-oxide, 2 μg per mg of protein</td>
<td>98</td>
</tr>
<tr>
<td>Thionyltrifluorobutanedioine, 10^{-4} M</td>
<td>80</td>
</tr>
<tr>
<td>KCN, 3 \times 10^{-4} M</td>
<td>95</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate, 7 \times 10^{-4} M</td>
<td>88</td>
</tr>
<tr>
<td>Pentachlorophenol, 10^{-4} M</td>
<td>15</td>
</tr>
<tr>
<td>Rotenone, 7 \times 10^{-4} M</td>
<td>5</td>
</tr>
<tr>
<td>Rotenone, 13 \times 10^{-4} M</td>
<td>5</td>
</tr>
</tbody>
</table>

The inhibition of oxidation observed with Amytal occurs only at high concentrations of this agent (10^{-2} M) and is probably nonspecific (44). Rotenone, a specific inhibitor of the NAD\(^+\)-linked chain (45), failed to inhibit this bacterial system. The inhibition by atebrin of malate and succinate oxidation appears to be a reflection of

studies with respiratory inhibitors (Table VI). The inhibition of oxidation observed with Amytal occurs only at high concentrations of this agent (10^{-2} M) and is probably nonspecific (44). Rotenone, a specific inhibitor of the NAD\(^+\)-linked chain (45), failed to inhibit this bacterial system. The inhibition by atebrin of malate and succinate oxidation appears to be a reflection of
the flavin nature of these respiratory chains. The inhibition by 2-n-nonylhydroxyquinoline N-oxide and KCN occurred with all three chains. These observations are in keeping with the findings that all three pathways utilize the terminal respiratory pigments, the succinate chain converging with the malate- and @HB), the flavin nature of these respiratory chains. The inhibition by 2-n-nonylhydroxyquinoline N-oxide and KCN occurred with all three chains. These observations are in keeping with the findings that all three pathways utilize the terminal respiratory pigments, the succinate chain converging with the malate- and NAD4-linked chains at cytochrome b. Differences in inhibition of the various pathways were observed with Dicumarol, penta-chlorophenol, thienyltrifluorobutanedione, and p-chloromercuribenzoate. The insensitivity of the phosphorylative pathway to 

![Difference Spectra](attachment:Difference_Spectra.png)

**FIG. 6.** Requirement for added vitamin K for reduction of endogenous cytochrome b following irradiation. The system consisted of 32 amoles of MgCl2, 107 amoles of KCl, 3 amoles of inorganic phosphate, 4.6 amoles of ADP, washed particles (8.5 mg of protein) irradiated with light at 360 mp for 30 minutes, 25 amoles of FAD, 92 amoles of Tris-HCl buffer (pH 7.2), and water to a final volume of 3.0 ml. NOQNO, 2-n-nonylhydroxyquinoline N-oxide.

![Wave length (mp)](attachment:Wave_length.png)

**Wave length (mp)**

1. [Oxid] - [Oxid] + Base line
2. [Malate + NOQO, Steady state] - [Oxid]
3. [Malate + NOQO + Vit. K1, Steady state] - [Oxid]

**Fig. 7.** The restoration of cytochrome b reduction by vitamin K1 with different substrates. The system was similar to that described in the legend of Fig. 5. The particulate fraction was irradiated with light at 360 mp for the period indicated, whereas the ammonium sulfate-fractionated supernatant fraction was irradiated for 5 hours. The concentration of particles differed: A, 3.2 mg of protein; B, 4.5 mg of protein; C, 4.0 mg of protein. The concentration of the supernatant fraction used was 10.5 mg of protein (A), 5.2 mg of protein (B), and 7.6 mg of protein (C). In addition, NAD4 (30 amoles) was added to the system containing @HB), whereas FAD (25 amoles) was added with malate as substrate.
dye, reactions dependent on the addition of the naphthoquinone, indicated that reduction and oxidation of the added vitamin K were not rate-limiting when optimal concentrations of vitamin K were added. Reduction of dye was found to occur at 2 to 3 times the rate observed for the over-all oxidation of malate by the complete system.

The soluble reductase required the addition of the particulate fraction for oxidation of malate. Reduction of endogenous cytochromes b and c by malate required the addition of the soluble malate-vitamin K reductase (Fig. 9). The requirement for naphthoquinone was not observed with the particles unless the natural quinone was destroyed by irradiation at 360 mp. The malate chain converges with the NAD+-linked chain of the

**TABLE VII**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amytal, 5 x 10⁻⁴ M</td>
<td>% 34 71</td>
</tr>
<tr>
<td>Atebrin, 5 x 10⁻⁴ M</td>
<td>55 75</td>
</tr>
<tr>
<td>Dicumarol, 5 x 10⁻⁴ M</td>
<td>66 92</td>
</tr>
<tr>
<td>2-n-Nonylhydroxyquinoline N-oxide, 1.4 µg per mg of protein</td>
<td>54 56</td>
</tr>
<tr>
<td>KCN, 3 x 10⁻³ M</td>
<td>100 100</td>
</tr>
<tr>
<td>Tween 80, 0.33%</td>
<td>55 100</td>
</tr>
</tbody>
</table>

**TABLE VIII**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Inhibition with reduction of</th>
<th>Cyanide, 1.7 x 10⁻³ M</th>
<th>% 60 60 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Phenanthroline, 5 x 10⁻⁴ M</td>
<td>25 27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α,α′-Dipyridyl, 5 x 10⁻⁴ M</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 9. Effect of the supernatant component on reduction of cytochromes b and c by malate. The system was similar to that described in Fig. 5. In Experiment A, 25 mmoles of FAD, 20 µg of 2-nonylhydroxyquinoline N-oxide (NOQNO), washed particles (P) (4.5 mg of protein), and ammonium sulfate-fractionated supernatant fluid (S) (3.2 mg of protein) were added to the system. The reaction was started by the addition of DL-malate (30 mmoles). Cyanide (5 µmoles) was used as the respiratory inhibitor in Experiment B.

 particles at the naphthoquinone (K₃H) level of oxidation. Reduction of endogenous cytochrome c by malate was inhibited (50%) by 2-nonylhydroxyquinoline N-oxide (1.5 µg per mg of protein).

The site of action of 2-nonylhydroxyquinoline N-oxide in low concentrations with this bacterial system is between cytochromes b and c (48). The addition of 2-nonylhydroxyquinoline N-oxide and oxygen to a succinate-reduced, anaerobic system resulted in the oxidation of cytochromes a and c and in further reduction of cytochrome b. Although the identity of the light-sensitive component on the succinate chain is unknown, metals appear to participate in succinate oxidation (Table VIII). Reduction of thiazolyl blue tetrazolium was found to be inhibited by KCN when succinate was used as an electron donor, whereas no inhibition occurs with malate. In addition, inhibi-
tion of reduction of thiazolyl blue tetrazolium by succinate occurs with o-phenanthroline and α,α'-dipyridyl. Reduction of cytochrome b by succinate was inhibited by cyanide, whereas ferricyanide reduction was not inhibited by this agent.

**DISSECTION of RESPIRATORY CHAINS**—Knowledge of the site of inhibition of several of the inhibitors described in this paper permitted an examination of the sites of interaction of various electron acceptors. The use of exogenous electron acceptors or donors in substrate quantities can afford a means of studying phosphorylating sites in the respiratory chain. A survey of various electron acceptors with the malate and succinate chains is presented in Table IX. Interaction of thiazolyl blue tetrazolium, 2,6-dichlorophenolindophenol, and exogenous cytochrome c with the succinate and malate chains appears to occur in the cytochrome c region. Some interaction of dichlorophenolindophenol occurs before cytochrome b. Addition of NAD+ and malate to the system containing the NAD+-linked malate and succinate chains appears to occur after the cytochrome c region. Some amount of cytochrome c + c1 in the bacterial particles was found to undergo oxidation by two different respiratory pathways. Malate oxidation was found to undergo oxidation by two different respiratory pathways. Malate was found to undergo oxidation by two different respiratory chains in *M. phlei*, one through NAD+ and the other via FAD. The ratio of bound pyridine nucleotide to cytochrome c + c1 in washed particles was 1.2, which is significantly lower than that of the mitochondrial system (usually more than 10 (25, 26)). In addition, the ratio of particle-bound NAD+ to the total NAD+ of the cell was only 0.3%, whereas the ratio of mitochondrial bound NAD+ to the total NAD+ is 7% (35). Most of disrupted mitochondrial systems described do not contain bound pyridine nucleotide and require addition of NAD+ (54–58).

**DISCUSSION**

Systems capable of coupling phosphorylation to oxidation have been obtained from a number of microorganisms (2–8, 16, 50, 51); however, the P:O ratios exhibited by the bacterial systems are consistently lower than those exhibited by the mitochondrial system. Although studies with the bacterial systems have pioneered in the dissection and reconstitution of systems capable of oxidative phosphorylation (3–6), detailed knowledge of the respiratory chains of most of the bacterial system is lacking. Thus, the sites of phosphorylation in most of the microbial systems have not been studied. A detailed examination of the major respiratory pathways of *M. phlei* was undertaken as a first step in obtaining precise knowledge of the respiratory sites which are coupled to phosphorylation.

The respiratory chain of *M. phlei* contains bound pyridine nucleotides (35), flavins (27), a naphthoquinone (vitamin K₃) (11, 12), and cytochromes b, c₁, c, a and a₃. The cytochromes of this microorganism are both qualitatively and quantitatively similar to those found in mammalian mitochondria. On the assumption that the molar extinction coefficients of the cytochromes are the same as those of mitochondrial origin, the amount of cytochrome c + c₁ in the bacterial particles was relatively high compared to cytochromes b, a + a₃, and a₃. Although most of the bacterial cytochromes have extinction coefficients similar to those of mitochondrial origin, the exact concentration of the individual cytochromes from *M. phlei* must await precise determination of these values for this system. Other methods for determining flavins and cytochromes b, c, and c₁ (36, 37) are under investigation. Small amounts of cytochrome b were also found in the soluble fraction; however, this chiefly consisted of the cytochrome c type. The lower value for flavin found on acid extraction than that observed enzymatically may be a reflection of the inability to remove flavin associated with succinic dehydrogenase (52), or may in part be due to absorption contributed by nonheme iron proteins (53). The bacterial dehydrogenases which utilize NAD+ (β-hydroxybutyrate and l-malate) are found in the soluble fraction along with the bulk of the NAD+. The ratio of bound pyridine nucleotide to cytochrome c + a₃ in washed particles was 1.2, which is significantly lower than that of the mitochondrial system (usually more than 10 (25, 26)). In addition, the ratio of particle-bound NAD+ to the total NAD+ of the cell was only 0.3%, whereas the ratio of mitochondrial bound NAD+ to the total NAD+ is 7% (35). Most of disrupted mitochondrial systems described do not contain bound pyridine nucleotide and require addition of NAD+ (54–58).

Kinetic studies of the respiratory chains of *M. phlei* indicated a similarity between it and the mitochondrial system. Although cytochrome b undergoes oxidation and reduction, further study is necessary to define precisely the role of this cytochrome in the respiratory pathways. A major difference between this microorganism and the mammalian mitochondrial system was observed in the pathways utilized in malate oxidation. Malate was found to undergo oxidation by two different respiratory chains in *M. phlei*, one through NAD+ and the other via FAD. The latter pathway corresponds to the α-glycerophosphate pathway of brain, muscle, and liver mitochondria (59–65), or to the choline chain observed in liver mitochondria (44, 66, 67).

The presence of a pyridine nucleotide-independent pathway for malate oxidation in bacteria has been reported for other microorganisms (68, 69). The two malate enzymes may have

### Table IX

**Effects of inhibitors on reduction of different electron acceptors**

The conditions were similar to those described in Table VIII. Reduction of 2,6-dichlorophenolindophenol (0.15 μmole) was measured spectrophotometrically at 600 nm, whereas reduction of horse heart cytochrome c (0.16 μmole) was measured at 550 nm. With malate as substrate, FAD (0.38 μmole) was added.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>2,6-Dichlorophenolindophenol</th>
<th>Cytochrome c</th>
<th>Thiosulphothiazol blue tetrazolium</th>
<th>2,6-Dichlorophenolindophenol</th>
<th>Thiosulphothiazol blue tetrazolium</th>
<th>Ferricyanide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amytal, 10⁻³ M</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Atebrin, 3 × 10⁻⁴ M</td>
<td>35</td>
<td>29</td>
<td>36</td>
<td>69</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Dicumarol, 10⁻⁴ M</td>
<td>67</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irradiation with light at 360 μm</td>
<td>41</td>
<td>81</td>
<td>74</td>
<td>80</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>2-n-Nonylhydroxyquinoline N-oxide</td>
<td>2.5 μg per mg of protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiethyltrifluorobutadine, 5 × 10⁻³ M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The conditions were similar to those described in Table VIII. Reduction of 2,6-dichlorophenolindophenol (0.15 μmole) was measured spectrophotometrically at 600 nm, whereas reduction of horse heart cytochrome c (0.16 μmole) was measured at 550 nm. With malate as substrate, FAD (0.38 μmole) was added.
physiological significance in strictly aerobic microorganisms by providing a means of reoxidizing extraparticulate NADH. Oxaloacetate formed from the bound malate-vitamin K reductase could be reduced to malate by extraparticulate malic dehydrogenase and NADH. This cycle would be analogous to the α-glycerol phosphate cycle of mitochondria suggested by Klingenberg and Bücher (63, 70). The potential of malate/oxaloacetate (Em, -0.102 volt at 38°) (71) is higher than that of NADH/NAD+ (-0.311 at 25°) (72) or other citric acid cycle intermediates with the exception of succinate. The oxidation-reduction potential of malate/oxaloacetate is similar to that reported for other flavin enzymes.

The role of vitamin K in oxidative phosphorylation has been studied extensively (8, 9, 46, 73, 74). This lipid-soluble respiratory cofactor undergoes oxidation and reduction at a rate consistent with the over-all rate of oxidation of malate by the bacterial system (15). The site of action of this cofactor was postulated to be between the flavoprotein and cytochrome b (46). The experiments described in this paper lend further support to the postulated site of interaction of the cofactor. The endogenous naphthoquinone of the particles interacts with both the NAD+ and malate chains. It appears to be the site of entrance of electrons from the solubilized malate-vitamin K reductase. Weber and Rosso (76) have suggested that the naphthoquinone-restored respiration with M. phlei extracts occurs by a bypass of the normal electron transport pathways. Their results are inconsistent with earlier findings (46, 74, 75) as well as with the data presented in this paper. The differences observed by Weber and Rosso may be due to the use of Tween to suspend the naphthoquinone. This detergent in low concentrations (0.13 to 0.33%) was shown earlier to inhibit the main pathways of oxidation in M. phlei and to act as an uncoupling agent in lower concentration (15).

The light-sensitive component (or components) of the succinate chain has been placed between succinic dehydrogenase and cytochrome b. The nature of this component(s) has not been elucidated. Studies with 2-n-nonylhydroxyquinoline N-oxide indicate that succinate converges with the NAD+ chain at the cytochrome b level. Reduction of cytochrome b was lost upon irradiation. A metal also appears to be involved in the succinic oxidase pathway, and its site of interaction appears to be before cytochrome b. Inhibition by cyanide of cytochrome b reduction from succinate with a 2-n-nonylhydroxyquinoline N-oxide-blocked system occurred at the concentration reported to cause a similar inhibition in the Keilin-Hartree preparation (77); however, with the bacterial system prolonged preincubation was not required. The lack of inhibition of the NAD+-linked chain is similar to that reported for other flavin enzymes.

Particles from M. phlei isolated by different procedures exhibit differences in their ability to oxidize various substrates (2), in requirements for supernatant factors and cofactors, and in the intactness of their respiratory chains. Particles prepared from cells 1 to 2 days old differ from those obtained from fresh cells in their response to added supernatant factors. In addition, particles stored at -15° (aged particles) differ from freshly prepared particles in the rate of reduction of their terminal respiratory pigments and in their requirement for soluble components. The reduced steady state level of the cytochromes was difficult to achieve with aged particles.

A schematic representation of the three major coupled respiratory pathways of M. phlei is shown in Fig. 10. Although the bacterial particles are spherical in shape (2), they are shown elongated. The indentations in the particles indicate the probable sites of interaction of the various soluble protein oxidative factors. Since the particles retain some succinic oxidase activity, it was possible to observe the effect of the supernatant factors on the terminal respiratory chain. The amount of reduction of cytochromes c, b, and a + a3 was not influenced by the presence or absence of supernatant fluid; however, with malate the soluble protein components were necessary for reduction of the cytochromes. The malate oxidation factor appears to be malate-vitamin K reductase. In addition, the supernatant contains β-hydroxybutyrate dehydrogenase and another factor necessary.
for reduction of the naphthoquinone. The crude supernatant fraction also contains a number of nonphosphorylative bypass reactions, which will be described in a later publication.

SUMMARY

The respiratory components in *Mycobacterium phlei* extracts have been determined. The particulate fraction contains bound nicotinamide adenine dinucleotide, flavins, a naphthoquinone (vitamin K₃H), and cytochromes b, c₁, c, a, and a₃. The ratios of the enzymatically reducible cytochromes a + a₃, b, c + c₁, flavin, and vitamin K₃H were found to be 1.0, 0.68, 2.3, 2.5, and 43.7, respectively. Carbon monoxide binding of cytochrome a₃ was found. The respiratory activity (Qₒ²) at 30° with succinate, malate, and β-hydroxybutyrate was 39, 37, and 84, respectively. Studies of the cofactor requirements, effect of inhibitors, and requirement for supernatant components revealed the presence of three distinct respiratory chains, namely, succinic oxidase, malate, and NAD⁺-linked chains. The flavin adenine dinucleotide-linked malate pathway was found to be soluble and to converge with the particle-bound NAD⁺-linked pathway at the naphthoquinone (K₃H) level, whereas the succinate chain converges at the cytochrome b level. The sequence of respiratory carriers was studied and is described in detail.

The respiratory chains of *M. phlei* were compared with those of mammalian mitochondria and found to be similar, with the exception of the malate pathway and the presence of a naphthoquinone. The naphthoquinone participates in electron transport between flavoprotein and cytochrome b on the NAD⁺ and malate chains, but not on the succinate chain. An unidentified light-sensitive component on the succinic oxidase pathway functions below cytochrome b. The properties of the *M. phlei* system resemble closely the disrupted mammalian mitochondrial system.

Acknowledgments—The authors would like to express their gratitude to Dr. Britton Chance and his colleagues at the John- 
son Foundation and to Dr. G. Kidder of the Biophysics Department of the Harvard Medical School for their help in some of the spectrophotometric studies, and to Mrs. Jane Ballantine Klubes for her technical assistance.

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Akira Asano and Arnold F. Brodie


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