OXIDATIVE PHOSPHORYLATION IN BACTERIA

I. OXIDATIVE PHOSPHORYLATION OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

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Oxidative phosphorylation in particulate fractions of eleven bacteria was examined without adding any soluble factor. The bacteria examined which could oxidize both NADH and succinate were divided from their properties of phosphorylation into the following three groups; (i) phosphorylation is scarcely coupled to the oxidation of both substrates, (ii) the P : O ratio in NADH oxidation is nearly equal to that in succinate oxidation, and (iii) the P : O ratio in NADH oxidation is lower than that in succinate oxidation. The bacteria whose P : O ratio in NADH oxidation is higher than that in succinate oxidation were not observed.

In small particles of strains from group (ii) 0.1 mM of cyanide inhibited NADH and succinate oxidation at a similar rate, while in small particles of strains from group (iii) cyanide inhibited succinate oxidation more strongly than NADH oxidation.

These groups agree well with the groups classified by the quinones present. The bacteria in group (ii) contain benzoquinones alone or, at least, mainly, and those in group (iii) contain naphthoquinones alone. From these observations, the role of quinones in oxidative phosphorylation of bacterial particles was discussed.

Studies on oxidative phosphorylation in bacteria have been described by a number of investigators using such strains as Alcaligenes faecalis (1-4), Azotobacter vinelandii (5-7), Mycobacterium phlei (8-10), Micrococcus lyso-deikticus (11-13), Micrococcus denitrificans (14, 15), and so on (16-18).

Properties of oxidative phosphorylation systems in bacteria were found to be different from those of mammalian mitochondrial systems. In general, bacterial systems show lower P : O ratio than mammalian mitochondrial systems and fail to exhibit respiratory control, though few exceptions were reported (14, 19-23). These observations may be partly derived from loose coupling of oxidative phosphorylation to respiration and partly from lack of
phosphorylation sites and occurrence of by-pass reactions in a respiratory chain.

Some bacteria have the same cytochrome pattern as those of yeast and mammalian mitochondria, whereas a number of bacteria lack cytochrome a which is replaced by cytochrome a₁ or by a mixture of cytochrome a₁ and a₂ (24). In addition, bacterial quinones are not always the same as those in the respiratory chain of mammalian mitochondria. Naphthoquinones are found in gram-positive bacteria and sometimes in gram-negative bacteria in place of benzoquinones (25). These lack and replacement of respiratory chain components will result in the lack of phosphorylation sites and non-phosphorylative by-passes.

The object of the present study is to investigate the specific properties of oxidative phosphorylation systems in eleven bacteria which cover various taxonomical groups.

MATERIALS AND METHODS

Bacterial strains. Pseudomonas aeruginosa IAM 1202, Azotobacter vinelandii IAM 1078, Achromobacter cycloclastes IAM 1013, Escherichia coli K 12 IAM 1264, Aerobacter aerogenes IAM 1183, Serratia marcescens IAM 1223, Micrococcus lysodeikticus IAM 1056, Micrococcus luteus IAM 1097, Brevibacterium helvolum IAM 1637, Bacillus megaterium IAM 1166, and Bacillus subtilis were used and stocked on bouillon-agar slants.

Preparation of particulate fractions. The cells cultured to stationary phase in 0.2% succinate-bouillon broth with shaking at 30° were collected by centrifugation, washed twice with 50 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose and 5 mM MgCl₂, and suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 1 M sucrose to the concentration of 1 g wet cells per 20 ml.

Cell-free extract was prepared mainly by the method of Ishikawa and Lehninger (11). To every ml of the cell suspension, 0.1 mg of crystalline lysozyme and 0.5 mg EDTA were added and the mixture was incubated for 30 min at 30°. The cells were recovered by centrifugation at 17,000 × g for 30 min, suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose and 5 mM MgCl₂, and suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 1 M sucrose to the concentration of 1 g wet cells per 20 ml.

Cell-free extract was prepared mainly by the method of Ishikawa and Lehninger (11). To every ml of the cell suspension, 0.1 mg of crystalline lysozyme and 0.5 mg EDTA were added and the mixture was incubated for 30 min at 30°. The cells were recovered by centrifugation at 17,000 × g for 30 min, suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose and 5 mM MgCl₂, and suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 1 M sucrose to the concentration of 1 g wet cells per 20 ml, and incubated for 20 min at 20° to induce osmotic lysis. After disruption of the cells by sonic vibration at 10 kC for 5 min at 2°, the suspension was centrifuged at 8,000 × g for 15 min to remove intact cells and large cell debris. The supernatant fraction obtained was centrifuged at 17,000 × g for 30 min, giving large particles (LP) and supernatant fraction. LP was washed with 50 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose and 5 mM MgCl₂. The supernatant was further fractionated by centrifugation in the Hitachi 55P ultracentrifuge at 105,000 × g for 120 min, giving a red gelatinous sediments of small particles (SP) which were washed with the same buffer. LP and SP
were re-suspended in Tris-HCl buffer (pH 7.4) to a concentration of 25–50 mg protein per ml. These particles were prepared daily.

Protein was estimated by either KALCKAR’s method (26) or LOWRY’s method (27).

Assay of oxidative phosphorylation. Oxygen uptake was measured by amperometry with an oxygen consumption recorder, Yanagimoto PO-100, and calculated as $4O$ (total oxygen consumption minus endogenous oxygen consumption). The amount of phosphate esterification coupled to respiration was measured by HAGIHARA and LARDY’s method (28) and calculated as $4P$ (total esterified P coupled to oxidation of added and endogenous substrates minus esterified P coupled to oxidation of endogenous substrates).

Assay of cytochromes. The absorption spectra of cytochromes in SP were measured with Shimadzu MPS-50L spectrophotometer.

Assay of quinones. Quinones were extracted from lyophilized cells with ethanol-ether (3:1) (29), and purified by BRODIE’s method (30). They were further purified on aluminum oxide column. The purified quinones were identified as naphthoquinone or benzoquinone by measurement with Shimadzu MPS-50L spectrophotometer. Their molecular weights were estimated with Hitachi RMU6L mass spectrometer (chamber voltage, 70 eV; temperature, 230°). The length of side chain was measured by thin-layer chromatography on liquid paraffin-impregnated silica gel, developed with acetone-water (95:5).

Identification of organic phosphorus compounds. $^{32}$P-labeled compounds were identified by BARTLETT’s method (31). Inorganic phosphate and glucose 6-phosphate were separated by HAGIHARA and LARDY’s method (28).

Chemicals. ATP and ADP were obtained from Kyowa Hakko Kogyo Co., Ltd. Yeast hexokinase (Type 1), crystalline lysozyme, and NADH were obtained from Sigma Chemical Co. Other chemicals were of the purest grades commercially available.

RESULTS

Oxidation and phosphorylation by eleven bacterial systems

It is generally said that activity of phosphorylation coupled to oxidation in bacterial cell-free extracts depends on the presence of soluble factors. In most of our preparations, however, phosphorylation coupled to oxidation was observed without addition of any soluble factor, as will be seen in Table 1.

The bacteria examined in the present experiment can be divided from their properties of succinate oxidation in SP systems into two types; (1) the SPs oxidize NADH but not succinate (Ps. aeruginosa, M. luteus, and B. megaterium) and (2) the SPs oxidize both substrates (others). The latter can further be classified from their properties of phosphorylation into three groups; (i) the P : O ratio in oxidation of NADH and succinate is less than 0.1 (Az. vinelandii, and B. subtilis), (ii) the P : O ratio in NADH oxidation is nearly equal to that in succinate oxidation (Ach. cycloclastes, E. coli, A. aerogenes,
Table 1. Oxidation and phosphorylation by

The reaction mixtures consisted of 30 μmoles MgCl₂, 15 μmoles Na₂HPO₄ (containing 600,000 cpm ³²P), 66 μmoles KF, 30 μmoles glucose, 0.5 μmole ATP, 1 μmole ADP, 0.05 mg hexokinase, 50 mM Tris-HCl buffer (pH 7.4), 2 μmoles NADH or 10 μmoles succinate (as substrate), and LP or SP (3–5 mg protein for NADH oxidation

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>SP</th>
<th>NADH</th>
<th>Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔO</td>
<td>ΔP</td>
<td>P/O</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>natom</td>
<td>nmole</td>
<td>0.29</td>
</tr>
<tr>
<td>Az. vinelandii</td>
<td>530</td>
<td>8</td>
<td>0.02</td>
</tr>
<tr>
<td>Ach. cycloclastes</td>
<td>890</td>
<td>384</td>
<td>0.43</td>
</tr>
<tr>
<td>E. coli</td>
<td>910</td>
<td>248</td>
<td>0.27</td>
</tr>
<tr>
<td>A. aerogenes</td>
<td>600</td>
<td>154</td>
<td>0.26</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>550</td>
<td>129</td>
<td>0.23</td>
</tr>
<tr>
<td>M. luteus</td>
<td>870</td>
<td>165</td>
<td>0.19</td>
</tr>
<tr>
<td>M. lysodeikticus</td>
<td>940</td>
<td>300</td>
<td>0.32</td>
</tr>
<tr>
<td>Brev. helvolum</td>
<td>710</td>
<td>177</td>
<td>0.25</td>
</tr>
<tr>
<td>B. megaterium</td>
<td>980</td>
<td>21</td>
<td>0.02</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>630</td>
<td>11</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 2. Effects of inhibitors and uncouplers on

The reaction mixtures were the same as given in Table 1. SP contained 0.1–0.3 mg protein for NADH oxidation and 1.0–3.0 mg protein for succinate oxidation. The reaction was started by adding the substrate after 3 min of incubation with an

<table>
<thead>
<tr>
<th>Inhibitors or uncouplers</th>
<th>M. lysodeikticus</th>
<th>NADH</th>
<th>Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔO</td>
<td>P/O</td>
<td>ΔO</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>(560)</td>
<td>100</td>
</tr>
<tr>
<td>NaCN (0.1 mM)</td>
<td>51</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td>NaN₃ (30 mM)</td>
<td>55</td>
<td>47</td>
<td>10</td>
</tr>
<tr>
<td>Antimycin A (33 mM)</td>
<td>42</td>
<td>8</td>
<td>50</td>
</tr>
<tr>
<td>2,4-Dinitrophenol (1 mM)</td>
<td>89</td>
<td>58</td>
<td>15</td>
</tr>
<tr>
<td>Arsenate (33 mM)</td>
<td>98</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>Oligomycin (50 μg)</td>
<td>89</td>
<td>61</td>
<td>100</td>
</tr>
<tr>
<td>Tributyltinchloride (0.1 mM)</td>
<td>93</td>
<td>32</td>
<td>78</td>
</tr>
</tbody>
</table>
and 30–50 mg protein for succinate oxidation); final volume 3 ml. The reaction was started by adding the substrate after 3 min of pre-incubation, and kept for 5–10 min at 28°.

<table>
<thead>
<tr>
<th>LP</th>
<th>NADH</th>
<th>Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔO</td>
<td>ΔP</td>
</tr>
<tr>
<td>natom</td>
<td>nmole</td>
<td></td>
</tr>
<tr>
<td>460</td>
<td>68</td>
<td>0.15</td>
</tr>
<tr>
<td>540</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>760</td>
<td>296</td>
<td>0.39</td>
</tr>
<tr>
<td>680</td>
<td>287</td>
<td>0.42</td>
</tr>
<tr>
<td>170</td>
<td>68</td>
<td>0.40</td>
</tr>
<tr>
<td>260</td>
<td>30</td>
<td>0.12</td>
</tr>
<tr>
<td>430</td>
<td>167</td>
<td>0.39</td>
</tr>
<tr>
<td>880</td>
<td>650</td>
<td>0.74</td>
</tr>
<tr>
<td>650</td>
<td>216</td>
<td>0.33</td>
</tr>
<tr>
<td>790</td>
<td>23</td>
<td>0.03</td>
</tr>
<tr>
<td>840</td>
<td>15</td>
<td>0.02</td>
</tr>
</tbody>
</table>

oxidation and phosphorylation in small particle.
inhibitor or an uncoupler. The activities of oxidation and phosphorylation were expressed as a relative activity (%). The absolute activity of the control is given in parentheses (ΔO: natom).

<table>
<thead>
<tr>
<th>Brev. helvolum</th>
<th>E. coli K12</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>Succinate</td>
</tr>
<tr>
<td>ΔO</td>
<td>P/O</td>
</tr>
<tr>
<td>100</td>
<td>(970)</td>
</tr>
<tr>
<td>61</td>
<td>44</td>
</tr>
<tr>
<td>94</td>
<td>55</td>
</tr>
<tr>
<td>25</td>
<td>27</td>
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<tr>
<td>80</td>
<td>80</td>
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<td>94</td>
<td>27</td>
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<tr>
<td>75</td>
<td>91</td>
</tr>
<tr>
<td>73</td>
<td>109</td>
</tr>
</tbody>
</table>
and \( S. \) marcescens), and (iii) the \( P:O \) ratio in NADH oxidation is lower than that in succinate oxidation (\( M. \) lysodeikticus and \( Brev. \) helvolum). The bacteria whose \( P:O \) ratio in NADH oxidation is higher than that in succinate oxidation were not observed.

In LP systems, the \( P:O \) ratio in succinate oxidation decreased and became lower than, or, at least, almost equal to that in NADH oxidation (Table 1). It has been reported that some bacteria show different oxidative phosphorylation activities in LP and SP systems (5, 10). Therefore, relationship between particle size and phosphorylative activities was next examined. As shown in Fig. 1, phosphorylative activity was observed in all the preparations, though the \( P:O \) ratio in NADH or succinate oxidation was not constant in these preparations. This agreed with the observation by TISSIERES et al. (5), but not with that by KASHKET and BRODIE (32).

Figure 2 shows the kind of phosphorus compounds found in the reaction

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![Diagram](image_url)

**Fig. 1.** Oxidative and phosphorylative activities of particles obtained at various \( g \) values.

The reaction mixtures were the same as given in Table 1. Particles from \( M. \) lysodeikticus contained 0.1 mg protein for NADH oxidation and 1.0 mg protein for succinate oxidation. Particles from \( B. \) helvolum contained 0.2 mg protein for NADH oxidation and 2.0 mg protein for succinate oxidation.

- •—• \( P:O \) ratio with NADH, ○—○ \( P:O \) ratio with succinate
- •—• \( QO_2 \) with NADH (natom/min/mg protein)
- ○—○ \( QO_2 \) with succinate (natom/min/mg protein)
Fig. 2. Identification of phosphorus compounds in reaction mixture.

The reaction mixtures were the same as given in Table 1. SP from *M. lysodeikticus* contained 0.1 mg protein for NADH oxidation, and 1.0 mg protein for succinate oxidation. They were incubated for 20 min and the reaction was stopped by boiling the mixture for 5 min. Tube numbers 3 to 5 were separated again by hexanol column (28).

\( \times \times \text{G}6\text{P} \) (for succinate as substrate), \( \times \times \text{G}6\text{P} \) (for NADH as substrate), \( \bullet \bullet \) phosphorus compound (for succinate as substrate), \( \bullet \bullet \bullet \) phosphorus compound (for NADH as substrate).

mixture when phosphorylation reaction is over. More than 90% of \(^{32}\text{P}\) esterified was incorporated into the glucose 6-phosphate and ATP fractions which were the end products of oxidative phosphorylation in our systems. Most of the remainder was incorporated into the ADP fraction. Other phosphorus compounds containing \(^{32}\text{P}\) were not found in the reaction mixture. These observations indicate that the data on oxidative phosphorylation in the present work do not include an error from side reactions.

**Effect of inhibitors and uncouplers on oxidation and phosphorylation in small particles**

Three representative strains from the group (ii) and (iii) described above were examined. As shown in Table 2, effect of various inhibitors and uncouplers on oxidation and phosphorylation in the SP of *M. lysodeikticus* is quite similar to that in the SP of *Brev. helvolum* but different from that in the SP of *E. coli*. Cyanide and \(\text{N}_3^-\) inhibited succinate oxidation in the SPs
of the former two bacteria very strongly, while they inhibited succinate oxidation in the SP of the latter one only slightly. Oligomycin and tributyltin chloride lowered P:O ratio with NADH and succinate in M. lysodeikticus slightly while they inhibited phosphorylation in E. coli entirely.

Effect of cyanide was further studied in five strains from group (ii) and (iii). In SPs of strains from group (ii), 0.1 mM of cyanide inhibited NADH and succinate oxidation at a similar rate, while in SPs of strains from group (iii) cyanide inhibited succinate oxidation more strongly than NADH oxidation (Table 3).

**Cytochromes in small particles**

Cytochrome components in SPs of 11 bacteria were qualitatively examined. As shown in Table 4, Ps. aeruginosa, Az. vinelandii, Ach. cycloclastes, M. lysodeikticus, M. luteus, B. megaterium, and B. subtilis showed difference spectra in the α-band region similar to those of yeast and mammalian mitochondria. Ps. aeruginosa, Az. vinelandii, and Ach. cycloclastes lacked the peak of cytochrome a3 in the Soret region. In other words, these three strains contain cytochrome a but not cytochrome a3. E. coli, A. aerogenes, S. mar-

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**Table 3. Effect of cyanide on oxidation and phosphorylation in small particle.**

The reaction mixtures were the same as given in Table 1. SP contained 0.1–0.3 mg protein for NADH oxidation and 1.0–3.0 mg protein for succinate oxidation. The reaction was started by adding the substrate after 3 min of preincubation. The activities of oxidation and phosphorylation are expressed as a relative activity (%).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>CN– added (mm)</th>
<th>NADH</th>
<th>Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ΔO</td>
<td>P/O</td>
</tr>
<tr>
<td><strong>Ach. cycloclastes</strong></td>
<td>0</td>
<td>100</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>14</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>0</td>
<td>100</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>74</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td><strong>A. aerogenes</strong></td>
<td>0</td>
<td>100</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>59</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td><strong>M. lysodeikticus</strong></td>
<td>0</td>
<td>100</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>51</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td><strong>Brev. helvomum</strong></td>
<td>0</td>
<td>100</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>61</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>
Oxidative Phosphorylation in Bacteria

Table 4. Cytochromes in small particles of bacteria.

SPs were suspended in 50 mM Tris-HCl buffer (pH 7.4). They were reduced by sodium hydrosulfitite and were oxidized by hydrogen peroxide. Figures in parentheses indicate indistinct shoulder.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Visible range (nm)</th>
<th>Soret range (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
<td>β</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>607</td>
<td>560</td>
</tr>
<tr>
<td>Az. vinelandii</td>
<td>605</td>
<td>560</td>
</tr>
<tr>
<td>Ach. cycloclastes</td>
<td>605</td>
<td>560</td>
</tr>
<tr>
<td>E. coli</td>
<td>630</td>
<td>600</td>
</tr>
<tr>
<td>A. aerogenes</td>
<td>632</td>
<td>598</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>630</td>
<td>598</td>
</tr>
<tr>
<td>M. luteus</td>
<td>605</td>
<td>560</td>
</tr>
<tr>
<td>M. lysodeikticus</td>
<td>605</td>
<td>565</td>
</tr>
<tr>
<td>Brev. helvolum</td>
<td>633</td>
<td>596</td>
</tr>
<tr>
<td>B. megaterium</td>
<td>603</td>
<td>560</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>608</td>
<td>562</td>
</tr>
</tbody>
</table>

cescens, and Brev. helvolum contained a mixture of cytochrome $a_1$ and $a_2$ in place of cytochrome $a$. The bacteria belonging to Enterobacteriaceae and probably S. marcescens lacked cytochrome $c$.

These bacteria were classified by their cytochrome components into the following 4 groups; (i) a group containing cytochromes $a$, $b$, and $c$, (ii) a group containing cytochromes $a$ and $a_3$, $b$, and $c$, (iii) a group containing cytochromes $a_1$ and $a_2$, and $b$, and (iv) a group containing cytochromes $a_1$ and $a_2$, $b$, and $c$. No relation was found between these 4 groups and the ones classified by properties of oxidative phosphorylation as described above.

Quinones in bacteria

Quinones in the whole cells of the bacteria examined were determined (Table 5). Quinones in SP were qualitatively equal to those in the whole cells.

As has been reported ($25$), only benzoquinones were detected in Ach. cycloclastes, only naphthoquinones in M. lysodeikticus and Brev. helvolum, and both quinones in E. coli and A. aerogenes, though the side-chain length of the quinones in some bacteria was found to differ from those described in the previous reports ($12, 32$). In their mass spectra, main molecular peak was observed at $m/e$ 716 in M. lysodeikticus and E. coli, and at $m/e$ 784 in Brev. helvolum. A lower peak was observed at $m/e$ 784 in the former bacteria and at $m/e$ 852 in the latter bacterium.

According to the results in Table 5, bacterial groups classified by their
quinones seem to agree with the groups classified by the properties of oxidation and phosphorylation, and their sensitivity to cyanide.

**Phosphorylation observed with malate and fumarate oxidation**

As has been reported in the particulate fractions of M. phlei (33), phosphorylation coupled to oxidation of malate was also observed in the SPs of M. lysodeikticus and Brev. helvolum. In the SPs of Ach. cycloclastes and E. coli, malate was not oxidized (Table 6). The P:O ratio with malate was lower than that with succinate, though the former in M. phlei was reported to be equal to the latter (33). Fumarate was not oxidized in SPs of bacteria examined except M. lysodeikticus.

**DISCUSSION**

Oxidative phosphorylation in particulate fractions of eleven bacteria observed without adding any soluble factor is described here. Bacterial particles capable of oxidizing both NADH and succinate were classified into the following three groups; (i) the ones whose phosphorylation is scarcely coupled to the oxidation of both substrates, (ii) the ones whose P:O ratio with both substrates are nearly equal, and (iii) the ones whose P:O ratio with succinate is higher than that with NADH. This classification may not always be applicable to intact cell systems since the P:O ratio of about 3 was reported in intact cell systems of E. coli and A. aerogenes (22, 23), and since the lack of one or two phosphorylation sites by sonic disruption was reported in mitochondrial systems (34), etc.

As shown in Fig. 1, P:O ratio with NADH in M. lysodeikticus system became lower as the particles became smaller in size, while P:O ratio with succinate was almost constant. This might show that the “site I” is very fragile to cell disruption and that the “site II” or the “site II” and “site
III' are relatively stable. However, phosphorylative properties of bacterial particles in group (iii), where P : O ratio in succinate oxidation is higher than that in NADH oxidation, cannot be explained by the fragility of phosphorylation sites. A hypothesis is proposed that NADH is oxidized by nonphosphorylative oxidation systems in particulate fractions similar to those reported in soluble fractions of E. coli and M. phlei systems (32, 35–37). If NADH is oxidized partly through nonphosphorylative by-passes and partly through cytochrome systems, while succinate is mainly oxidized through cytochrome systems, higher P : O ratio with succinate than that with NADH will be observed.

It is of interest that the inhibition of NADH oxidation by cyanide was different in its efficiency from that of succinate oxidation, as shown in Table 3. In SP of Ach. cycloclastes, cyanide equally inhibited NADH oxidation and succinate oxidation and rather higher P : O ratio was observed under the inhibition. In E. coli and A. aerogenes systems, succinate oxidation systems were a little more sensitive to cyanide than NADH oxidation systems, and P : O ratio with NADH decreased by inhibition contrary to a slight increase

### Table 6. Phosphorylation observed with malate and fumarate oxidation.

The reaction mixtures were the same as given in Table 1 except that 10 μmoles malate or 10 μmoles fumarate was used as a substrate. SP contained 0.1–0.3 mg protein for NADH oxidation and 1.0–3.0 mg protein for succinate, fumarate, and malate oxidation.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Substrates</th>
<th>J0 natom</th>
<th>Jp n mole</th>
<th>P/O</th>
<th>Qo2s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ach. cycloclastes</td>
<td>NADH</td>
<td>470</td>
<td>56</td>
<td>0.12</td>
<td>330</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>500</td>
<td>140</td>
<td>0.28</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Fumarate</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>770</td>
<td>104</td>
<td>0.14</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>460</td>
<td>32</td>
<td>0.07</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Fumarate</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>E. coli</td>
<td>NADH</td>
<td>1100</td>
<td>684</td>
<td>0.63</td>
<td>1175</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>750</td>
<td>708</td>
<td>0.97</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Fumarate</td>
<td>765</td>
<td>307</td>
<td>0.41</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>950</td>
<td>535</td>
<td>0.56</td>
<td>638</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>640</td>
<td>147</td>
<td>0.23</td>
<td>1140</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>450</td>
<td>447</td>
<td>0.99</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Fumarate</td>
<td>trace</td>
<td>0</td>
<td>—</td>
<td>trace</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>520</td>
<td>206</td>
<td>0.54</td>
<td>210</td>
</tr>
</tbody>
</table>

* natom, min/mg protein.
in P : O ratio with succinate by the inhibition. In *M. lysodeikticus* and *Brev. helvolum* systems, cyanide inhibited NADH oxidation by about 50\% and P : O ratio was reduced, whereas succinate oxidation was completely inhibited by the same concentration of cyanide. If NADH is oxidized through the same terminal oxidation systems as those of succinate oxidation, cyanide will inhibit the oxidation of both substrates equally. Oxidation and phosphorylation in SP of *Ach. cycloclastes* agree with this supposition. If NADH is oxidized partly through nonphosphorylative by-passes other than cytochrome systems and succinate is oxidized through cytochrome systems, P : O ratio with NADH will be reduced by cyanide while P : O ratio with succinate will be unchanged. Oxidation and phosphorylation in SPs of *E. coli*, *A. aerogenes*, *M. lysodeikticus*, and *Brev. helvolum* agree with this supposition.

Next problem to solve is which respiratory components are related to these oxidative and phosphorylative properties. As shown in Table 4, cytochromes in bacteria were widely varied. The most remarkable differences were found in *a*-type and *c*-type cytochromes. In some bacteria, cytochrome *a* was replaced by cytochrome *a*1 and *a*2, and in some bacteria cytochrome *c* was not detected. However, no correlation was observed between properties of oxidative phosphorylation and cytochrome components contained, except that relatively low P : O ratio was observed in SPs which lacked cytochrome *c*.

**Bishop et al.** and other investigators found benzoquinones alone in gram-negative bacteria, naphthoquinones alone in gram-positive bacteria, and both quinones in *E. coli*, *Proteus vulgaris*, *A. aerogenes*, and *S. marcescens* (38-43). **Kashket** reported that, in *E. coli*, succinate oxidase particles contain ubiquinone alone, whereas NADH oxidase particles contain naphthoquinone alone (44). In our experiments, Tables 3 and 5 suggested that sensitivity of bacterial oxidation system to cyanide is in accord with quinones present in the systems. From these results, we propose the following hypothesis:

NADH is oxidized through benzoquinones and/or naphthoquinones and finally by cytochrome systems. Succinate is oxidized through benzoquinones and then by cytochrome systems or directly through cytochrome systems. From naphthoquinones but not from benzoquinones, electrons flow partly to oxygen directly without coupling to phosphorylation. Cyanide inhibits the cytochrome oxidase but not nonphosphorylative NADH oxidation. Therefore, in the SP of *Ach. cycloclastes* in which electrons from NADH and succinate flow through benzoquinones and then through cytochrome systems, cyanide equally inhibits NADH and succinate oxidation and P : O ratio with both substrates is nearly equal. In the SPs of *M. lysodeikticus* and *Brev. helvolum*, electrons from NADH flow through naphthoquinones and then through cytochrome and/or nonphosphorylative oxidation systems, while electrons from succinate flow through cytochrome systems. Cyanide inhibits succinate oxidation completely, but inhibits NADH oxidation partly. P : O ratio with succinate is higher than that with NADH. In *E. coli* and *A. aerogenes*, which contain both benzoquinones and naphthoquinones, mixed type was observed.
In the present experiments, malate oxidation was detected only in the bacterial systems which contained naphthoquinone alone. It is possible to consider that higher P : O ratio with succinate in these bacteria was from this oxidation system, but such a possibility was denied since the P : O ratio with malate was lower than that with succinate.

REFERENCES