Orientation of the Cell Membrane in Ghosts and Electron Transport Particles of Mycobacterium phlei*

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SUMMARY

The chemical and enzymatic composition and the respiratory components of an intact membrane preparation, ghosts from Mycobacterium phlei, were compared to those of the electron transport particles. The ghosts were found to contain 5 to 7% of some of the soluble enzymes associated with the cytoplasm. In contrast, 97% of the total content of cytochromes b, c, + c, and a + a, was found in the ghost preparation. The content of cytochromes, menaquinone, latent ATPase, and phospholipid was similar in both ghost and electron transport particle preparations. The oxidative activities of the ghosts were similar to or slightly lower than those of the electron transport particles with all substrates tested. Coupled phosphorylation, however, was cryptic with the ghost preparations. Phosphorylation was demonstrable following brief sonication or preincubation of the ghosts with substrates and other components of the phosphate acceptor system. The synthesis of ATP was found to occur within the ghosts. Evidence is presented which suggests that the cell membrane of the ghost is oriented as in the intact cell, whereas the orientation of the membrane in the electron transport particles appears to be inside out.

Studies of oxidative phosphorylation in most bacterial systems have employed disrupted membrane fragments (1-6). These disrupted preparations have been useful for fractionation and reconstitution of coupled phosphorylation (1-5, 7-10). One of the difficulties encountered with these preparations is their low phosphorylation efficiency. Although P:O ratios greater than 1.0 can be obtained with the fragmented systems (ETP)1 from Mycobacterium phlei (1, 7), and these values can be increased by heating at 50° for 10 min (11), these preparations lack respiratory control. On the assumption that respiratory control might be observed in bacterial systems if the cells were not so harshly disrupted, various investigators have used protoplasts (4) or membrane preparations obtained by relatively mild treatment (12). Nevertheless, the P:O ratios of such preparations were still low, especially when compared to sonicated preparations2 (4, 13, 14). It was difficult to understand why milder treatment should result in lower P:O ratios. In some cases, relatively high P:O ratios were reported with a protoplast ghost preparation (12). This discrepancy as well as the increased use of these ghost preparations for active transport (14-16) prompted us to study the energy metabolism of this type of membrane vesicle.

The present investigations were designed to determine the properties of the ghosts of M. phlei and to compare them to the electron transport particles (ETP) of the same organism. Evidence for “masked” phosphorylation in the ghosts will be presented as well as information concerning the vectorial orientation of the cell membrane in the two preparations. A preliminary account of this work has appeared elsewhere (17).

MATERIALS AND METHODS

Preparation of Ghosts

M. phlei ATCC 354 was grown as previously described (18). An osmotically sensitive cell preparation was obtained by a modification of the method of Mizuguchi and Tokunaga (19). Two different procedures (A and B) were employed. They are outlined in Fig. 1. Method B was employed for measuring the amount of cell membrane leakage during lysozyme treatment since it yielded a higher concentration of osmotically sensitive cells, thus providing more reliable experimental values. For all other studies Method A was used.

Method A—M. phlei cells were grown to mid-log phase (usually after 10 to 11 hours of incubation), and glycerol and sucrose were added to a final concentration of 1.55% and 0.5 M, respectively. After 3 hours of incubation at 37°, lysozyme (from egg white, Grade 1, Sigma Chemicals) was added to a final concentration of 50 μg per ml. The appearance of osmotically sensitive cells was followed turbidimetrically in a Klett colorimeter with a Number 42 filter. At frequent intervals an aliquot of the cells was diluted with 4 volumes of water. For control readings, an

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1 The abbreviations used are: ETP, electron transport particles; HEPES, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; DDA+, dimethyldibenzylammonium chloride; BCFI, coupling factor.
equal aliquot was diluted with 4 volumes of 0.5 M sucrose. The decrease in turbidity of the water-diluted (lysed) sample compared to the control was used as an index of the degree of protoplast formation. When the difference between the control and the lysed samples remained the same at two sampling intervals, the incubation was terminated. This was usually 45 min after the addition of lysozyme. The cultures were centrifuged at 5,900 x g for 10 min and the pellets were homogenized in 2 mM MgCl₂ (60 ml/10 g of cells, wet weight) in the presence of 2 mg of DNase I (bovine pancreas, B grade, Calbiochem) per 100 ml of suspension. The ghosts were recovered by centrifugation at 5,900 x g for 10 min, washed with water three times, and re-suspended in 10 mM MgCl₂. The supernatant fraction obtained after centrifugation of ghosts will be hereafter referred to as “ghost supernatant.” Another membrane fraction, containing mesosomes and small membrane fragments, was obtained from the ghost supernatant by centrifugation at 39,000 x g for 30 min.

Preparation of Sonicated Membranes

Electron transport particles (ETP) were prepared from intact cells of *M. phlei* as described previously (7). A membrane preparation similar to ETP was also obtained from ghosts. The ghosts were sonicated by placing plastic centrifuge tubes with 1 to 2 ml of the suspension in a Raytheon sonic oscillator vessel containing water to the level of the material in the tubes. Two successive 10-min sonication periods were employed with 5 min of cooling in between. For large quantity preparation of this membrane preparation, 10-ml aliquots were sonicated directly for 10 min.

**Fig. 1. Preparation of ghosts of Mycobacterium phlei**

**Method A**

- Preparation of ghosts of *M. phlei* as described above and the pellet was suspended in 0.5 M sucrose.
- The cultures were centrifuged at 5,900 x g (10 min) and the lysed samples remained the same at two sampling intervals.
- The difference between the control and the addition of lysozyme. The cultures were centrifuged at 39,000 x g (10 min).
- The supernatant fraction obtained after centrifugation of ghosts will be hereafter referred to as “ghost supernatant.” Another membrane fraction, containing mesosomes and small membrane fragments, was obtained from the ghost supernatant by centrifugation at 39,000 x g for 30 min.

**Method B**—Glycine-treated cells were collected by centrifugation as described above and the pellet was suspended in 0.5 M sucrose containing 1.55% glycine (1 g of cells, wet weight, per 10 ml) and incubated with lysozyme (3.7 mg per g of cells, wet weight) at 30°C for approximately 45 min to form protoplasts as described in Method A. The supernatant fraction obtained after centrifugation of the osmotically sensitive cells is referred to as “lysozyme-treated supernatant.” Possible contamination of the ghost preparation by intact *M. phlei* cells was checked by means of centrifugation (17 hours) of the preparation through a discontinuous sucrose density gradient. The gradient was comprised of 6 ml each of 1.30, 1.55, 1.65, and 1.75 M sucrose and 3 ml of 2.0 M sucrose. The main band containing ghosts appeared in the 1.55 and 1.65 M sucrose (depending on the growth phase of the original cells) whereas the density of the intact cells was the same as or greater than that of 1.80 M sucrose. Thirteen ghost preparations were examined in this manner. In no case was more than a trace of the band containing intact cells observed.

**Sucrose Density Gradient Centrifugation of Ghosts**

Possible contamination of the ghost preparation by intact *M. phlei* cells was checked by means of centrifugation (17 hours) of the preparation through a discontinuous sucrose density gradient. The gradient was comprised of 6 ml each of 1.30, 1.55, 1.65, and 1.75 M sucrose and 3 ml of 2.0 M sucrose. The main band containing ghosts appeared in the 1.55 and 1.65 M sucrose (depending on the growth phase of the original cells) whereas the density of the intact cells was the same as or greater than that of 1.80 M sucrose. Thirteen ghost preparations were examined in this manner. In no case was more than a trace of the band containing intact cells observed.

**Assay of ATP and ADP**

The firefly luciferase assay as described by Stanley and Williams (20) was employed for ATP determinations. Luminescence was measured with a Nuclear Chicago Mark I scintillation counter with the tritium channel (in coincidence). ADP was assayed by converting it to ATP with 1.8 μmoles of phosphoenolpyruvate and 50 μg of pyruvate kinase (Sigma Chemicals, type II). The ATP formed after 15 min at 23°C was assayed and the amount of ATP originally present in the sample was subtracted. Standard curves for ATP and ADP were made each time an assay was conducted.

**Centrifugal Filtration for Assay of Distribution of ATP and ADP**

In order to separate the ATP and ADP located inside the ghosts from that located outside, the centrifugal filtration method of Klingenberg and Pfaff (21) was employed. Centrifuge tubes were prepared by layering 2.5 ml of 0.4 M sucrose containing 5 mM MgCl₂ over 1.0 ml of 1.6 M HClO₄. When reactions were terminated, 1.0 ml of the reaction mixture was layered over the sucrose, and immediately centrifuged at 73,000 x g for 5 min. After centrifugation 1-ml aliquots of the top and bottom (HClO₄) layers were removed. The bottom layer, containing the ghosts, was already acidic; the aliquot from the top layer was acidified with 0.5 ml of 1.6 M HClO₄. The protein precipitate was removed from each aliquot by centrifugation and the clear supernatant fractions were neutralized with KOH before being assayed for ATP and ADP as described above.
Measurement of Oxidative Phosphorylation

Oxidation was measured manometrically using a Gilson Differential Respirometer at 30°, and phosphorylation was assayed by measuring the disappearance of inorganic phosphate as described earlier (8) except that KF was omitted from the system unless otherwise stated. When oxygen uptake was measured with an oxygen electrode (Yellow Springs Instrument Co., Oxygen monitor model 53), phosphorylation was assayed either by measuring ATP and ADP produced from AMP by the luciferase assay or by measuring 32P incorporation into the organic phase by the method of Nielsen and Lehninger (22).

Oxidative phosphorylation of ghost preparations was performed after a preincubation with the reaction mixture at 0° for the required time. The reaction mixture consisted of 20 mM of potassium phosphate buffer (pH 7.5), 15 mM of MgCl2, 10 mM of AMP, 9 to 12 mg of protein (ghosts), 200 mM of sodium succinate, and water (all precooled) to a final volume of 1.0 ml in a precooled test tube. The reaction was started by rapidly increasing the temperature to 30° by adding 0.5 ml of the incubated mixture to 2.0 ml of 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) KOH buffer (pH 7.5) at 30°. An aliquot (0.5 ml) of the reaction mixture was mixed with 0.5 ml of 1.6 M HClO4 and used as a zero time control. Oxygen uptake was measured polarographically. The reaction was stopped by the addition of 0.5 ml of the reaction mixture to 0.5 ml of 1.6 M HClO4. ATP and ADP were assayed as described above and total high energy phosphate synthesized (Δ ~ P) was calculated from these values. For the 32P assay, 0.5 to 5 μCi of purified 32P1 was included in the reaction mixture and the reaction was terminated by the addition of 0.5 ml of 15% trichloroacetic acid containing 30 mM KH2PO4. After processing the sample by the method of Nielsen and Lehninger (22), a 0.1- to 0.5-ml aliquot of the water phase was added to 10 ml of Aquasol (New England Nuclear Corp.), and counted with a Nuclear Chicago Mark I scintillation counter.

Assay of Enzyme Activities

Latent ATPase activity was assayed after trypsin treatment as described by Murthy et al. (23). Malate-vitamin K reductase activity was measured as described previously (24). β-Hydroxybutyrate dehydrogenase was assayed by following NAD+ reduction in the presence of 2 mM KCN, and fumarase was assayed by the method of Racker (25). Adenylate kinase activity was measured by assaying ATP production from ADP (26). Enzyme assay and protein determinations with ghosts were performed following sonication of the preparations in order to overcome possible problems of permeability.

Estimation of Cytochrome and Menaquinone Contents

The cytochrome content was estimated from the dithionite reduced minus ferricyanide oxidized difference spectrum with a Cary model 14 recording spectrophotometer (27). Menaquinone content was measured spectrophotometrically following chloroform-methanol (2:1) extraction as previously described (23).

Chemical Estimation

Phospholipid was extracted from lyophilized samples according to the method of Folch (29), and phospholipid phosphorus was determined after wet ashing with HClO4 by King’s method (30). RNA and DNA were separated by the method of Schmidt and Thannhauser (31). RNA was determined by the orcinol method (32) with yeast RNA as a standard. DNA content was determined with diphenylamine (33) with salmon sperm DNA (Calbiochem, A grade) as a standard. Protein was determined by the method of Lowry et al. with sodium hydroxide pretreatment (34).

Electron Microscopy

Samples were negatively stained by mixing equal volumes of the sample and 2% phosphotungstic acid (pH adjusted to 7.5) containing 0.03% sucrose at 0°. The mixture was dried immediately on grids covered with carbon-coated Formvar. For negative staining of ghost preparations, samples were dried on the grid and then 1% phosphotungstic acid (pH 7.5) was applied. This was done to overcome the aggregation of the ghosts which occurred after the addition of phosphotungstic acid to a ghost suspension. Thin sections were made from glutaraldehyde- and osmium tetroxide- prefixed samples (35) embedded in Vestopal. These sections were stained with uranyl acetate and lead citrate (36). Electron micrographs were taken with an RCA electron microscope model EMU3-F.

RESULTS

Morphology of Ghosts—M. phlei ghosts were examined by electron microscopy. As shown in Figs. 2 and 3, negative staining and staining with uranyl acetate and lead citrate of thin sectioned specimens revealed that most, but not all, of the contents of the cells were removed from the ghosts but some cell wall materials were still present in the preparations, thus maintaining the shape of the ghosts almost similar to that of the intact cells. Therefore, these preparations cannot be called spheroplasts as no spherical forms are seen. As described below, most of the membrane-bound components were retained whereas the preparation was devoid of the soluble enzymes of the intact cells. Therefore, we have called our preparation “M. phlei ghosts.” The unit membrane structure of the cytoplasmic membrane is shown in Fig. 3. Contamination by intact cells was checked by centrifugation through layered sucrose as described under “Materials and Methods”; an example is depicted in Fig. 4. Only trace amounts of material which had the same characteristic as intact cells in layered sucrose were found in ghost preparations and usually about 60 to 80% of the osmotically sensitive membrane preparations was recovered in the main ghost band. The negatively stained membrane fraction is shown in Fig. 5. It is similar in appearance to the mesosomal fractions of Bacillus subtilis.

Distribution of Various Enzymatic Activities of Ghosts—The enzyme content of the ghost fraction was compared to that of other fractions obtained during the preparation of ghosts (Table I). The ghosts contained about 60% of the protein of the intact cells. The absolute value may be lower since the amount of protein removed from the cells during lysozyme treatments could not be measured accurately because of the high concentration of lysozyme, and was therefore omitted from the calculations. The ghosts were found to contain most of the electron transport components. The membrane fraction contained cytochromes and NADH oxidase activity and exhibited high P:O ratios (12% higher than those observed with sonicated ghosts) but represented only a few per cent of the total protein and electron transport components.

For the study of the distribution of soluble enzymes, Method R for the preparation of ghosts was employed to obtain higher concentrations of the soluble fraction (Table I). Adenylate kinase, β-hydroxybutyrate dehydrogenase, and fumarase were found both in lysozyme-treated supernatant and ghost supernatant fractions; little remained in the ghosts. The specific
Fig. 2 (upper left). Electron micrograph of negatively stained ghosts. Magnification, × 82,870.

Fig. 3 (upper right). Electron micrograph of a thin section of ghosts stained with uranyl acetate and lead citrate. Magnification, × 145,550.

Fig. 4. Sucrose density centrifugation of the ghost preparation. Sucrose layers consisted of 3 ml of 2.0 M sucrose and 6 ml each of 1.75, 1.65, 1.55, and 1.30 M sucrose. Three milliliters of the samples suspended in water were layered over the sucrose. Centrifugation was performed in SW 25.1 Beckman rotor and centrifuged at 24,000 rpm for 14 hours. The turbid layers are shown as dark areas.
FIG. 5 (top left). Electron micrograph of a negatively stained fraction containing mesosomes. Magnification, × 92,650.

FIG. 6 (right). Electron micrograph of negatively stained sonicated ghosts. Magnification, × 70,750.

FIG. 7 (bottom left). Electron micrograph of a negatively stained membrane fragment from ghosts. Magnification, × 133,906.
activity of latent ATPase in sonicated ghosts (304 nmoles per min per mg of protein) was compared with that of sonicated particles prepared directly from intact cells (222 nmoles per min per mg of protein). The lack of latent ATPase activity in the supernatant of sonicated ghosts indicated that most of the latent ATPase activity was associated with the ghost fraction.

For comparative studies of oxidative phosphorylation in the ghosts and the membrane vesicles prepared directly from intact cells (ETP), the contents of electron transport carriers, phospholipid, and nucleic acids in the two preparations were determined. The ratios of the cytochromes and menaquinone were similar in both preparations (Table II), and were comparable to those of mammalian mitochondria (27). Phospholipid content was in the same range as that reported for inner mitochondrial membrane and microsomes (37). The high RNA content in the bacterial preparations may indicate contamination with ribosomes. These results show a basic similarity in composition of the ghost preparation and the ETP. The only major difference detected was in the distribution of malate-vitamin K reductase; 60 to 89% of the total content of this enzyme was found in the ghosts, whereas only 8% of the malate-vitamin K reductase was found in ETP (24). This may be attributed to a relatively low affinity of the malate-vitamin K reductase for the membrane.

Oxidative Phosphorylation Activities of Ghosts—A comparison of oxidative phosphorylation in the ghosts from M. phlei with that of the ETP revealed that although the oxidative activities of the ghosts with all substrates were similar to or slightly lower than those of the ETP, the ghosts appeared to lack the ability to couple phosphorylation to oxidation (Table III). Lack of oxidation of malate with sonicated particles corresponded to the low level of malate-vitamin K reductase found in ETP (24). This may be attributed to a relatively low affinity of the malate-vitamin K reductase for the membrane.

### Table I

**Distribution of enzymes in fractions obtained for preparation of ghosts**

<table>
<thead>
<tr>
<th>Parent and daughter preparations</th>
<th>Cells</th>
<th>Ghost supernatant fraction (GS)</th>
<th>Membrane fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ghosts</td>
<td>Ghosts</td>
<td>Supernatant</td>
</tr>
<tr>
<td>Method A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>N.D.</td>
<td>61</td>
<td>35</td>
</tr>
<tr>
<td>Cytochrome a</td>
<td>0.61</td>
<td>97</td>
<td>1.08</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>0.84</td>
<td>96</td>
<td>1.05</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>1.33</td>
<td>97</td>
<td>1.08</td>
</tr>
<tr>
<td>Generated NADH oxidation</td>
<td>29</td>
<td>71</td>
<td>4.84</td>
</tr>
<tr>
<td>Method B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Hydroxybutyrate dehydrogenase</td>
<td>29</td>
<td>71</td>
<td>4.84</td>
</tr>
<tr>
<td>Fumarase</td>
<td>21</td>
<td>79</td>
<td>43.8</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>51</td>
<td>120</td>
<td>7850</td>
</tr>
</tbody>
</table>

*Accurate value could not be determined due to the presence of large amounts of lysozyme. Therefore the percentage of protein content of the other fractions may be higher than the actual value, because the protein content in the supernatant fractions obtained following centrifugation of the ghosts was omitted from the calculations.*

Unpublished observations.
from the preincubation medium lowered the P : 0 ratio.

ponents, since omission of any of the components cited in Table V
not be traced to the impermeability of any one of the single com-
ing the reaction was found to increase the P : 0 ratios (Table V).
ponents of the phosphate acceptor system as found in animal
permeable nature of the membrane to nucleotides or other com-
hosphorylation in the ghost preparation might be due to the im-
ghosts when assayed by the conventional method. It has been
obtained that the addition of a soluble protein factor to the ghost
preparation elicited P : 0 ratios comparable to those observed
with ETP (38). It was possible that the apparent lack of phos-
phorylation in the ghost preparation might be due to the im-
permeable nature of the membrane to nucleotides or other com-
ponents of the phosphate acceptor system as found in animal
cells (13, 39, 40) and intact cells of Escherichia coli (40, 41).

In order to overcome the permeability problem of the ghost
membrane, the ghost preparation was preincubated with high
concentrations of the components necessary for oxidative phos-
phorylation. Preincubation for up to 60 min at 0° before start-
ing the reaction was found to increase the P : 0 ratios (Table V).
Longer preincubation failed to further increase the P : 0 ratios.
Omission of any one of the components necessary for oxidative
phosphorylation from the preincubation medium somewhat de-
creased P : 0 ratios even though these components were added in
the final reaction mixture. This latency of phosphorylation can-
not be traced to the impermeability of any one of the single com-
ponents, since omission of any of the components cited in Table V
from the preincubation medium lowered the P : 0 ratio.

The effects of uncoupling agents, especially those related to ion
transport were studied with this system. The uncoupling agents

### Table II

**Contents of electron transport components and other compounds in ghosts**

Chemical reduction was used to determine the total content of the
cytochromes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Ghosts</th>
<th>ETP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/mg protein</td>
<td>nmoles/mg protein</td>
</tr>
<tr>
<td>Cytochrome a</td>
<td>0.61</td>
<td>0.27</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>1.33</td>
<td>0.62</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>0.84</td>
<td>0.65</td>
</tr>
<tr>
<td>Menaquinone</td>
<td>6.74</td>
<td>7.40</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>0.30 mg</td>
<td>0.39 mg</td>
</tr>
<tr>
<td>RNA</td>
<td>0.15 mg</td>
<td>0.21 mg</td>
</tr>
<tr>
<td>DNA</td>
<td>27 µg</td>
<td>50 µg</td>
</tr>
</tbody>
</table>

### Table III

**Oxidative phosphorylation in ghosts and sonicated particles**

The reaction mixture for generated NADH oxidation was simi-
lar to that described in Table I except that 3.1 mg of ghost protein
or 3.4 mg of ETP protein were added and the duration of the
reaction was 15 min. With succinate as the electron donor the
system consisted of 50 µmoles of sodium succinate and 1.2 mg of
ghost protein or 3.4 mg of ETP protein. The reaction was carried
out for 40 min for ghosts and 15 min for the ETP. With L-malate
(60 µmoles) as the electron donor the system contained 0.15 µmole
of FAD.

### Table IV

**Effects of sonication on oxidation and phosphorylation by ghosts**

Sonication of the ghost preparation was carried out as described
under "Materials and Methods." The conditions were similar to
those described in Table I except that 2.18 mg of protein of ghosts
or sonicated ghosts were added. The duration of the reaction
was 20 min. The reaction mixture for the spectrophotometric
assay of NADH oxidation consisted of 50 µmoles of potassium
phosphate buffer (pH 7.5), 15 µmoles of MgCl₂, 0.2 mg of protein
of ghosts or sonicated ghosts, and water to a final volume of 2.0
ml in a cuvette with a 1.0-cm light path. The reaction was started
by the addition of 0.4 µmole of NADH, and followed at 340 nm.

<table>
<thead>
<tr>
<th>Ghosts</th>
<th>Generated NADH oxidation</th>
<th>NADH oxidation (spectrophotometric, 340): ΔNADH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/min/mg protein</td>
<td>nmoles/min/mg protein</td>
</tr>
<tr>
<td>Ghosts</td>
<td>238</td>
<td>38</td>
</tr>
<tr>
<td>Sonicated ghosts</td>
<td>238</td>
<td>190</td>
</tr>
</tbody>
</table>

### Table V

**Oxidative phosphorylation by ghosts: requirement for preincubation**

The preincubation mixture consisted of 50 µmoles of potassium
phosphate buffer (pH 7.5), 15 µmoles of MgCl₂, 10 µmoles of AMP,
200 µmoles of sodium succinate, ghosts (10.3 mg of protein in Ex-
periment I, 8.7 mg of protein in Experiment II, 7.9 mg of protein
in Experiment III), and water to a final volume of 1.0 ml. When
phosphate buffer was omitted from the preincubation mixture, 50
µmoles of HEPES-KOH buffer (pH 7.5) were added. The reac-
tion was started by adding 0.5 ml of the preincubated mixture to
2 ml of 25 mM HEPES-KOH buffer (pH 7.5) which was kept at
30°. The components omitted from the preincubation mixture
were added to the reaction mixture with the HEPES-KOH buffer
at the start of the reaction. In Experiment I, 1.25 × 10⁶ cpm of
purified 32Pₐ₃ was included and 32P incorporation to acid-soluble
organic phosphorus was assayed as described under "Materials and
Methods."²

<table>
<thead>
<tr>
<th>Preincubation (at 0°)</th>
<th>Reaction (at 30°)</th>
<th>ΔO</th>
<th>ΔP</th>
<th>P : O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>nmoles/ml</td>
<td>nmoles/ml</td>
<td></td>
</tr>
<tr>
<td>Experiment I</td>
<td>0</td>
<td>4</td>
<td>279</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4</td>
<td>337</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>4</td>
<td>349</td>
<td>24.0</td>
</tr>
<tr>
<td>Experiment II</td>
<td>30</td>
<td>5.6</td>
<td>196</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>5.6</td>
<td>234</td>
<td>27.2</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>5.6</td>
<td>284</td>
<td>28.8</td>
</tr>
<tr>
<td></td>
<td>60 (−Mg⁺⁺)</td>
<td>5.6</td>
<td>248</td>
<td>18.4</td>
</tr>
<tr>
<td></td>
<td>60 (−AMP)</td>
<td>5.6</td>
<td>278</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>60 (−Pₐ₃)</td>
<td>5.6</td>
<td>238</td>
<td>22.4</td>
</tr>
<tr>
<td></td>
<td>60 (−succinate)</td>
<td>5.6</td>
<td>338</td>
<td>27.9</td>
</tr>
<tr>
<td>Experiment III</td>
<td>60</td>
<td>7.0</td>
<td>480</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>7.0</td>
<td>480</td>
<td>44.9</td>
</tr>
</tbody>
</table>

² A gift from Dr. H. A. Lardy of the University of Wisconsin.
phenylboron (45) had little or no uncoupling effect (Table VI). This may suggest that an inward transport of a valinomycin-potassium complex and DDA\textsuperscript{3+} occurred as has been shown in intact mitochondria (42-44). Oxidative phosphorylation in the ghost preparation was insensitive to antibodies to coupling factor-latent ATPase (Table VI). It had been previously shown that oxidative phosphorylation in ETP was inhibited by these antibodies (38).

**Distribution of ATP and ADP in Ghosts**—The results described above suggested that the ghost membrane acts as a permeability barrier and that phosphorylation was occurring only on the inside of the membrane. Therefore, the distribution of ATP and ADP formed from AMP was measured by the centrifugal filtration method (21). Almost all of the ATP and half of the ADP were present inside the ghosts after phosphorylation occurred (Table VII). Thus, about two-thirds of the high energy phosphates formed were retained in the ghosts. In contrast, similar experiments with ghosts supplemented with a soluble protein factor showed that only 5% of the high energy phosphates were retained in the ghosts (38). This difference in the localization of ATP corresponded well with the latent nature of the coupled oxidation in the unsupplemented ghosts and the overt oxidative phosphorylation in the supplemented system (38).

**Studies on Sonicated Membrane Preparations**—Since a large increase of phosphorylation was observed after sonication of the ghosts (Table IV), sonicated membrane preparations were studied with special reference to indications as to the “sidedness” of the membrane. Negative staining of sonicated ghosts revealed the presence of sphere-like structures attached by a stalk (repeating units) to the surface of the membrane (Fig. 6). Higher magnification of fragmented ghosts found in the ghost preparations (Fig. 7) showed these structures more clearly. They are similar to those found on the inner membrane of mitochondria (46, 47), although the frequency of the appearance of these structures was less in the bacterial system (see below). The average diameter of these spheres was found to be 112 A, and the average distance from the edge of the membrane to the edge of the sphere (stalk) was 29 A.

**Table VI**

**Effects of uncoupling agents and antibody on oxidative phosphorylation by ghosts**

The preincubation and reaction were carried out as described in Table V except that the duration of preincubation was 60 min, unless indicated otherwise, and the uncoupling agents were added to the preincubation mixture. In Experiment II, 15 \( \mu \)moles of KCl were added to the reaction mixture; therefore the concentration of potassium in the reaction mixture was in the range of 50 to 55 mM. In Experiment III, the potassium salts were replaced by sodium salts. Valinomycin and nigericin were added in ethanol solutions to the preincubation mixture. The total amount of ethanol added was 10 \( \mu l \) and the control (no addition) contained the same amount of ethanol. In Experiment IV, 2.5 \( \mu \)moles of sodium phosphate buffer (pH 7.5) containing 6.24 \( \times \) \( 10^5 \) ATPase (Table VI). It had been previously shown that oxidative phosphorylation in ETP was inhibited by these antibodies (38).

**Table VII**

**Distribution of ATP and ADP synthesized from AMP by ghosts**

The preincubation and the reaction were performed as described under “Materials and Methods” and in Table V, except that 10.0 mg of ghost protein were added to the reaction mixture, and the duration of preincubation was 60 min. After 1 min of reaction, the distribution of ATP and ADP was determined by centrifugal filtration (21). Adenine nucleotides found in the top layer after centrifugation are shown above as “outside of ghosts” and those in the bottom layer are referred to as “inside of ghosts.”

**Table VIII**

**Reconstitution of oxidative phosphorylation and binding of coupling factor-latent ATPase**

The depleted ETP and coupling factor-latent ATPase (BCF\(_4\)) were separated and reconstituted as described previously (8). Oxidative phosphorylation was assayed as described under “Materials and Methods” and in Table I using the NADH-generating system except that 3.36 mg of ETP protein, 0.67 mg of depleted ETP protein, and 0.99 mg of the reconstituted fraction were used. The method of Murthy et al. (23) was employed for the assay of latent ATPase.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>( \Delta O )</th>
<th>( \Delta P )</th>
<th>P:O</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2 ( \mu m )</td>
<td>318</td>
<td>8.6</td>
<td>0.03</td>
</tr>
<tr>
<td>CCP+</td>
<td></td>
<td>349</td>
<td>24.2</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Experiment III</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (K\textsuperscript{+} medium)</td>
<td>80 ( \mu m )</td>
<td>206</td>
<td>5.9</td>
<td>0.03</td>
</tr>
<tr>
<td>Tetrathylphilboron (K\textsuperscript{2+} medium)</td>
<td>8 mm</td>
<td>910</td>
<td>4.6</td>
<td>0.09</td>
</tr>
<tr>
<td>DDA\textsuperscript{+} (K\textsuperscript{2+} medium)</td>
<td>40 ( \mu m )</td>
<td>142</td>
<td>0.0</td>
<td>0.00</td>
</tr>
<tr>
<td>Valinomycin (K\textsuperscript{2+} medium)</td>
<td>2.5 ( \mu m )</td>
<td>91</td>
<td>0.8</td>
<td>0.01</td>
</tr>
<tr>
<td>Nigericin (K\textsuperscript{2+} medium)</td>
<td>1.0 ( \mu m )</td>
<td>130</td>
<td>5.0</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Experiment IV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (Na\textsuperscript{+} medium)</td>
<td>263</td>
<td>4.0</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>Valinomycin (Na\textsuperscript{+} medium)</td>
<td>1.25 ( \mu m )</td>
<td>311</td>
<td>4.8</td>
<td>0.015</td>
</tr>
<tr>
<td><strong>Preparation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (preincubation 30 min.)</td>
<td>184</td>
<td>16.6</td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>176</td>
<td>84</td>
<td>0.079</td>
<td></td>
</tr>
<tr>
<td>Antibody to coupling factor-latent ATPase</td>
<td>31.8 mg</td>
<td>176</td>
<td>12.8</td>
<td>0.073</td>
</tr>
<tr>
<td>CCP+</td>
<td>4 ( \mu m )</td>
<td>186</td>
<td>2.0</td>
<td>0.015</td>
</tr>
</tbody>
</table>

\( ^{a} \) CCP, carbonyl cyanide m-chlorophenylhydrazone.
Higashi et al. (8) were able to remove a coupling factor (BCF₆) from ETP by sucrose density gradient centrifugation in the absence of Mg²⁺. This treatment also removed latent ATPase from the membrane (Fig. 8). These two activities behaved similarly on sucrose density gradients, DEAE-cellulose chromatography, and gel filtration on Sephadex G-200. Rebinding of the solubilized BCF₆ and latent ATPase to the depleted particles restored coupled phosphorylation (8) (Table VIII). Similar observations have been made with sonicated ghost preparations. Repeating units similar to those observed on fragmented ghosts were also seen on reconstituted sonicated particles (Fig. 9) but not on coupling factor-depleted membranes (Fig. 10). In contrast to these observations with ETP and sonicated ghost preparations, sucrose density gradient centrifugation of the ghost fraction in the absence of Mg²⁺ did not dissociate the latent ATPase activity from the membrane (Fig. 8), nor were soluble proteins released from the ghosts by this treatment. The ease of removal of latent ATPase and repeating units from ETP and sonicated ghosts may indicate the localization of the latent ATPase on or near the outer surface of the sonicated membrane vesicles. In contrast, the inability to dissociate latent ATPase from the ghost membrane further indicates a difference between these membrane preparations in the vectorial orientation of the membrane. From the molecular weight of the BCF₆ and specific activities of latent ATPase in purified preparations and ETP, Dr. Higashi et al. estimated the molar ratio of BCF₆ to cytochrome oxidase to be approximately one in ETP. Therefore, the less frequent appearance of repeating units in the bacterial membrane as compared to the mitochondrial preparations may be due to a weaker binding of this coupling factor to the membrane.

4 T. Higashi, E. Bogin, and A. F. Brodie, unpublished observations.

![Diagram of linear density gradient centrifugation of ghosts and sonicated particles (ETP).](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Table IX</th>
<th>Effect of uncoupling agents on oxidative phosphorylation of ETP and sonicated ghosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition</td>
<td>Concentration (uncoupling agent)</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Experiment I</td>
<td>ETP</td>
</tr>
<tr>
<td>None (Na⁺ medium)</td>
<td></td>
</tr>
<tr>
<td>Valinomycin + K⁺ (Na⁺ medium)</td>
<td>3.3 μM</td>
</tr>
<tr>
<td>Nigericin + K⁺ (Na⁺ medium)</td>
<td>1.33 μM</td>
</tr>
<tr>
<td>Valinomycin + NH₄⁺ (Na⁺ medium)</td>
<td>3.3 μM</td>
</tr>
<tr>
<td>Valinomycin + nigericin + K⁺ (Na⁺ medium)</td>
<td>3.3 μM</td>
</tr>
<tr>
<td>Experiment II</td>
<td>ETP</td>
</tr>
<tr>
<td>None (K⁺ medium)</td>
<td></td>
</tr>
<tr>
<td>Tetraphenylboron (K⁺ medium)</td>
<td>16.7 μM</td>
</tr>
<tr>
<td>Tetraphenylboron (K⁺ medium)</td>
<td>57 μM</td>
</tr>
<tr>
<td>DDA⁺ (K⁺ medium)</td>
<td>16.7 mM</td>
</tr>
<tr>
<td>DDA⁺ (K⁺ medium)</td>
<td>67 mM</td>
</tr>
<tr>
<td>Experiment III</td>
<td>Sonicated ghosts</td>
</tr>
<tr>
<td>None (Na⁺ medium)</td>
<td></td>
</tr>
<tr>
<td>Valinomycin (Na⁺ medium)</td>
<td>1.33 μM</td>
</tr>
<tr>
<td>Nigericin (Na⁺ medium)</td>
<td>3.3 μM</td>
</tr>
<tr>
<td>Valinomycin + nigericin (Na⁺ medium)</td>
<td>1.33 μM</td>
</tr>
<tr>
<td>None (K⁺ medium)</td>
<td></td>
</tr>
<tr>
<td>Valinomycin (K⁺ medium)</td>
<td>3.3 μM</td>
</tr>
<tr>
<td>Nigericin (K⁺ medium)</td>
<td>1.33 μM</td>
</tr>
<tr>
<td>Valinomycin + nigericin (K⁺ medium)</td>
<td>3.3 μM</td>
</tr>
</tbody>
</table>

5 Fig. 8. Linear density gradient centrifugation of ghosts and sonicated particles (ETP). A linear sucrose density gradient from 1.25 to 1.0 M was made over 2.5 ml of 2.0 M sucrose as a cushion. Three milliliters each of the samples were layered over the density gradient. Centrifugation was for 15 hours at 24,000 rpm with SW 25.1 Beckman rotor. Fractions were collected and assayed as described under "Materials and Methods."
bacterial membrane compared to the binding of coupling factors to mitochondria.

Examination of the effects of uncoupling agents related to transport on oxidative phosphorylation of ETP and sonicated ghosts (Table IX) revealed differences between these membrane vesicles and ghost preparations (see Table VI). As in submitochondrial particles (48-50), uncoupling of oxidative phosphorylation with ETP and sonicated ghosts was not observed with valinomycin plus potassium ions but required the coexistence of either ammonium ions or nigericin plus potassium. Thus, in contrast to the results described above in Table VI for ghosts, these results indicate that the direction of movement of the valinomycin monocation complex is outward in the case of sonicated membrane vesicles and in order to achieve constant energy discharge by means of this transport, a mechanism is required to maintain the inside concentration of such cations, i.e. a medium containing NH₄⁺ (NH₃ freely permeates the membrane), or a nigericin-mediated H⁺-K⁺ exchange. Another similarity to submitochondrial particles was the insensitivity of ETP toward DDA⁺ (50). The difference between ETP and ghosts in response to these uncoupling agents lends further support to the concept that the orientation of the cell membrane in the two preparations is different. The ghosts appear to be oriented “right side out” as in the intact cell whereas the ETP appear to be oriented “inside out.”

DISCUSSION

An important characteristic of the ghosts of M. phlei was the lack of phosphorylation coupled to the oxidation of several substrates. This phenomenon has also been observed with spheroplasts of E. coli (13). Although the protoplast ghost preparations from Micrococcus lysodeikticus (4) and Micrococcus denitrificans exhibit coupled phosphorylation the P:O ratios observed with these preparations are low when compared to membrane fragments obtained by disruption of the ghost preparations from these microorganisms. In some cases, appreciable P:O ratios were reported for protoplast ghost preparations (12); however, their behavior in a centrifugal field indicated that the preparations might have contained fragmented ghosts or mesosomes. A low efficiency of coupled phosphorylation was also observed with cultured tumor cells and spermatozoa (13, 39, 40).

Mild sonication of the ghost preparations from M. phlei increased the level of phosphorylation, thus indicating that ghost preparations were not inactive with regard to the ability to couple phosphorylation to oxidation. Morton and Lardy (13) also reported that several physical modifications increased the observed P:O ratios of animal cells, although the same methods did not activate phosphorylation of E. coli spheroplasts. This cryptic nature of phosphorylation may be due to the impermeability of the ghost membrane to adenine nucleotide, oxidizable substrates, and other components necessary for oxidative phosphorylation. Similar properties of intact E. coli cells have been reported. These cells showed very low P:O ratios with exogenous ³²P, and an increase of oxidation upon the addition of ADP only after treatment of the cells with EDTA (41). The phenomenon of cryptic phosphorylation observed in M. phlei ghosts may also occur in spheroplast preparations of E. coli which have been used extensively for active transport studies (15, 16). In fact, very...
low P:O ratios (14) and an inability of exogenous ATP to support active transport (15, 16) have been reported for E. coli spheroplasts. With the ghost preparations from M. phlei it is possible to study the properties of oxidative phosphorylation of ghosts which have a high level of active transport of amino acid (51) and thus study the energy requirements for active transport.

A similarity between sonicated bacterial preparations and sub-mitochondrial particles has been previously noted (52). Bacterial ghosts, however, must be compared with animal cells as well as with intact mitochondria, especially when permeability properties are considered. Several studies regarding the “ sidedness” of the bacterial membrane have been reported. Outward translocation of protons coupled to respiration and photo-oxidation was reported for photosynthetic bacteria (53) and M. denitrificans (54), whereas the reverse direction of proton movement was found in chromatophores of the photosynthetic bacteria (55). Since the chromatophore is a discrete entity of the photosynthetic bacterial cell this organelle cannot be contrasted to the bacterial membrane fragments obtained by sonication.

Although the direction of proton movements can be taken as an indication of the polarity of the membrane (53, 54), many other criteria are necessary for the determination of the orientation of any particular bacterial membrane vesicle preparation. Obviously this is especially so if, as in ETP, the preparation contains a mixed population of vesicles, differing in membrane polarity (see below) (55). In such a preparation changes in the pH of the medium as a result of proton translocation would not provide an accurate indication of membrane orientation. A more dependable criterion would be the effects of directionally specific uncouplers such as valinomycin on reactions requiring different membrane orientations, i.e. oxidative phosphorylation and active transport of amino acids (56).

Recent studies on proline transport in M. phlei (51, 55, 56) indicate that the ETP consist of at least two types of membrane vesicles. One type of vesicle appears to be oriented right side out as in the cytoplasmic cell membrane and in the ghosts; these vesicles actively transport amino acid with several substrates. However, the bulk of the ETP are oriented inside out and are capable of nonenzytic oxidative phosphorylation.

In the present study, the following results strongly support the assumption that the membrane of the ghosts was oriented as is the cytoplasmic membrane of intact cells and still presented a permeability barrier to several substances: (a) the requirement for preincubation of ghosts with reaction mixture components for the demonstration of phosphorylation, (b) the retention of synthesized high energy phosphate compounds inside the ghosts, (c) the uncoupling of phosphorylation by transport-linked uncoupling agents such as valinomycin in the presence of K+ and DDA+ which are effective uncoupling agents with intact mitochondria (45, 50, 57) but not with submitochondrial particles, (a) the firm attachment of a latent ATPase to the ghosts in contrast to the ease of removal of the enzyme from sonicated particles, (c) the lack of sensitivity of oxidative phosphorylation in the ghosts to an antibody to latent ATPase (38), and (f) the failure to show “sphere-like structures with stalks” on the outer surface of the intact ghosts. Thus it would appear that the membrane of the ghosts has some characteristics similar to those of the inner membrane of mitochondria but has the permeability characteristics of a cytoplasmic membrane.

The activation of phosphorylation in the ghost preparations following mild sonication suggested that the sonicated membrane preparations (ETP and sonicated ghosts) consisted, at least in part, of vesicles in which the orientation of the membrane was reversed from that in the ghosts. A number of experiments were performed which support this hypothesis. For example, structures were observed on the outer surface of the sonicated membrane preparations which were similar to the repeating units found on the inner mitochondrial membrane (46, 47). These structures were easily removed from the sonicated membrane preparations and found to contain coupling factor activity (BCF) (8) and a latent ATPase which is dicyclohexylcarbodi-imide-sensitive only when the enzyme is bound to the membrane (58). Reconstitution of coupled phosphorylation and restoration of the morphological appearance (membrane structure containing repeating units) occur on addition of the solubilized coupling factor-latent ATPase fraction to the depleted vesicles in the presence of magnesium ions (8). In contrast to the ghost preparations, uncoupling of phosphorylation by transport-linked uncoupling agents was found to occur in ETP and sonicated ghosts under conditions similar to those required for uncoupling of phosphorylation in sub-mitochondrial particles. For example, uncoupling of phosphorylation in ETP and sonicated ghosts was observed to require a combination of valinomycin, nigericin, and potassium ions (48-50) or valinomycin plus ammonium ions (49). It has been shown that active transport of amino acid in ETP preparations, however, is inhibited by valinomycin plus potassium or by DDA+, thus indicating that the membrane orientation required for active transport may be different (60).

Further support that the orientation of the ETP membrane differs from that of the ghost preparation was obtained from studies of the effect of antibodies prepared against the coupling factor-latent ATPase fraction (38). Phosphorylation with ETP was found to be sensitive to the antibodies while phosphorylation in ghost preparations was insensitive. It is interesting to note that the phosphorylation of ghosts supplemented with a soluble protein factor, however, could be inhibited by the antibodies. Moreover, in supplemented ghost preparations the newly synthesized ATP was not retained within the ghosts (38). Finally, an increase of fluorescence of aniline naphthalene sulfonate in the energized state has been observed with the ETP (59) and is similar to that observed with submitochondrial particles (60, 61).

The mesosomal contribution to these sonicated preparations is probably not appreciable since mesosomal structures in ghost preparations were rare as determined by electron microscopy of sectioned ghost preparations. Mesosomes were found more frequently in late log phase cells. Moreover, the cytochrome content of a mesosomal preparation was similar to that of the ghosts, and the mesosomal fraction represented only a few per cent of the membrane preparations. Early studies by Ferrandes et al. (62) claimed that the bacterial cytochromes were localized exclusively in the mesosome. However, recent evidence by these workers has indicated that the major role in respiration is played by the cytoplasmic membrane rather than the mesosome (63, 64).

Upon sonication the orientation of the bacterial membrane is changed, as has been postulated for the mitochondrial system (66) and suggested in the case of membrane vesicles prepared from erythrocyte ghosts (66). It has recently been reported that sub-mitochondrial particles may be a mixture of two different kinds of vesicles, with the membranes oppositely oriented in the two types (67). Phospholipid flip-flop and the rate of outward translocation of phospholipid molecules in a phospholipid bilayer has been reported (68). Studies of the mechanisms and conditions which determine orientation of the bacterial membrane are now in progress.
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Orientation of the Cell Membrane in Ghosts and Electron Transport Particles of *Mycobacterium phlei*

Akira Asano, Natalie S. Cohen, Richard F. Baker and Arnold F. Brodie


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