Antimicrobial Actions of Hexachlorophene: Inhibition of Respiration in Bacillus megaterium

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Hexachlorophene (HCP) inhibits both endogenous and exogenous respiration (oxygen uptake) in Bacillus megaterium, without sparing by any of several substrates. The inhibition is maximal when the cells are treated with 8 μg of HCP per mg of cells (dry weight), which corresponds to the minimal lethal dose. Levels as low as 2 μg/mg are inhibitory but not lethal. HCP also inhibits the respiration of isolated B. megaterium membranes and can act on several components of the electron transport chain in the membranes and on soluble enzymes. Although both forms of nicotinamide adenine dinucleotide, reduced form dehydrogenase and malic dehydrogenase are inhibited by HCP, they are less susceptible than is oxygen uptake. The site of maximal sensitivity is nearer the terminal electron acceptor, but the exact location depends on the cytochrome composition of the membranes. If cytochromes b, a, and a3 are present, but not o, HCP inhibits electron transport on the substrate side of cytochrome b1; if cytochromes b1, a3, and o are present, but not a, the inhibition occurs on the oxygen side of cytochrome b1. Exogenous menadione, an analogue of menaquinone, reverses the inhibition in both circumstances. The primary lethal action of HCP thus appears to be respiratory inhibition at a site within the membrane-bound part of the electron transport chain.

Hexachlorophene [2,2'-methylenebis(3,4,6-trichlorophenol); HCP] has been in use as an antimicrobial agent for about 25 years, yet the primary basis of its lethal action remains uncertain. Four general mechanisms have been suggested: (i) generalized protein denaturation; (ii) membrane disruption; (iii) membrane damage leading to leakage of small cytoplasmic solutes, without membrane disruption; and (iv) respiratory inhibition. The first three of these mechanisms are known consequences of HCP action, but only at doses greater than the minimal amount required to kill cells of a susceptible test bacterium, Bacillus megaterium (8, 16, 21). Consequently, these mechanisms appear secondary.

The inhibition of respiration as a possible primary action was indicated because much of the HCP taken up by intact cells of B. megaterium is localized in the protoplast membrane (21) where many respiratory enzymes are located (20). Furthermore, certain dehydrogenases and cytochromes of B. subtilis, Escherichia coli, and mammalian systems are inhibited by HCP (13, 14). At high concentrations, HCP is an effective inhibitor of respiration in mitochondria isolated from brain or liver and, at low concentrations, apparently uncouples oxidative phosphorylation (7).

Consequently, we undertook study of the effects of HCP on oxygen uptake or electron transport in intact cells, isolated membranes, and solubilized enzymes in the same bacterial system as used previously (8, 16, 21). The results indicated that inhibition in the membrane-bound portion of the electron transport chain accounted for the primary lethal action of the drug for the bacterium.

MATERIALS AND METHODS

Organisms and growth conditions. Cells of the obligately aerobic, asporogenous KM strain of B. megaterium were grown to the late exponential phase in 2% Oxoid peptone broth (Flow Laboratories, Rockville, Md.) at 30 C, as previously described (8). For membrane isolations, 10-liter lots of the broth containing 0.3 g of Dow antifoam per liter were used in a fermentor (model MF-14; New Brunswick Scientific Co.). The fermentor was operated at 30 C with an aeration rate of 10 liter/min and a stirring rate of 250 rpm. Where indicated, the medium was buffered at pH 7.0 with 0.01 M sodium potassium phosphate buffer; otherwise, the pH was periodically adjusted to 7.0 with concentrated HCl during growth.

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Final culture absorbances were 2.60 ± 0.20, or about 2.6 mg of cells (dry weight) per ml of culture medium. Absorbance was determined at 700 nm by means of a Spectronic 20 spectrophotometer (Bausch and Lomb) equipped with a 1.12-cm lightpath cuvettes with distilled water used as a blank.

**Determination of oxygen uptake by intact cells.** Cells were harvested by centrifugation in the cold, and washed by suspension in and sedimentation from a half volume of 0.1 M potassium phosphate buffer (pH 7.0). The sedimented cells were then resuspended in fresh buffer to yield a cell suspension which, contained 27 mg of cells (dry weight) per ml or, in a few experiments, 10 mg/ml. One milliliter of cell suspension was transferred to each main compartment of a requisite number of Warburg flasks. Sidearms contained 0.1 ml of 0.05 M substrate solutions, appropriately concentrated HCP solutions, or buffer. Center wells contained 0.1 ml of 20% (weight/vol) KOH and fluted filter paper. Buffer was added to the main compartment to bring the total aqueous volume in each flask to 3 ml. The flasks were equilibrated in a Warburg bath at 30 C for 10 min with the manometer stopcocks open and for an additional 20 min with the stopcocks closed. Additions were tipped in from the sidearms, and oxygen uptake was measured at 10-min intervals for a minimum of 60 min. Standard practices and nomenclature were used (24).

**Isolation of membranes.** Cells were harvested by means of continuous centrifugation (27,000 × g; 4 C) at a flow rate of 150 ml/min in an RC-2B centrifuge equipped with a Szent-Gyorgi and Blum continuous flow system (Ivan Sorvall, Inc.). Membrane fractions were prepared according to a modification of the method of Broberg and Smith (4). Each gram (wt weight) of washed cells was suspended in 2.5 ml of 0.5 M sucrose solution buffered with 0.01 M sodium potassium phosphate buffer (pH 7.0). Approximately 45 g (wt weight) of cells were used in each isolation. Mucopirnpeptide-N-acetylmutaramyl hydrolyase (EC 3.2.1.17; lysozyme) dissolved in 15 ml of the buffered-sucrose solution was added to the cell suspension to give a final concentration of 10 mg of lysozyme per g of cells (wt weight). The resultant suspension was incubated at 25 C until conversion to single protoplasts was complete (about 60 min.), as judged by microscope examination. The protoplasts were sedimented (25,000 × g; 20 min) at 4 C and then lysed osmotically by suspending the pellet of protoplast residue in 150 ml of 0.01 M phosphate buffer (pH 7.0) containing 0.5 mg of deoxyribonucleic oligonucleotidohydrolyase (EC 3.1.4.5). The mixture was shaken at 25 C for 15 min, at which time lysis of the protoplasts was complete. The resulting membranes were centrifuged at 25,000 × g for 30 min. The buff-colored upper part of the pellet containing the membranes was suspended in 0.1 M N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid-KOH buffer, pH 7.60 (HEPES buffer). This procedure was repeated at least three times, and each time the upper part was scraped off the lower part containing poly-β-hydroxybutyrate granules. The final membrane suspension was stored at −30 C. When needed, an appropriate amount of the frozen membrane preparation was suspended to the desired concentration in HEPES buffer.

**Isolation of soluble dehydrogenases.** The soluble enzymes were contained in the supernatant solution obtained by direct lysis of protoplasts in hypotonic solution. To 88 g of washed cells (wet weight) were added 100 ml of 0.1 M HEPES buffer containing 0.25 g of lysozyme and 0.5 mg of deoxyribonucleate oligonucleotidohydrolyase. This mixture was shaken at 250 rpm in a gyratory shaker at 30 C for 2 h. The protoplasts were further disrupted by alternately freezing and thawing the preparation. Conversion to protoplast ghosts was at least 95% complete as judged by phase microscopy. The membrane fraction was sedimented (25,000 × g for 30 min) and the yellowish supernatant was decanted and stored at 4 C. The buff-colored upper part of the pellet containing the membranes was removed, resuspended in 50 ml of 0.1 M HEPES buffer, and recentrifuged at 25,000 × g for 30 min. The supernatant was again decanted. The pooled supernatants were further clarified by centrifugation at 25,000 rpm with an average centrifugal force of 75,000 × g, for 2 h at 4 C in a preparative ultracentrifuge (International Equipment Co., model B-60) equipped with a swinging bucket rotor (model SB-110). The yellow-green supernatant was removed with a Pasteur pipette and stored at −30 C. When needed, an appropriate amount of the soluble lysate was diluted to the desired concentration in 0.1 M HEPES buffer (pH 7.60). Protein concentrations were estimated by the biuret method (12), with bovine serum albumin as the standard.

**Enzyme assays.** Dehydrogenase activity was determined by the 2,6-dichlorophenolindophenol (DCPIP) reduction assay of Storck and Wachsmann (23), except that HEPES buffer (pH 7.60) was substituted for phosphate buffer (pH 7.0). NaCN was replaced by KCN freshly adjusted to pH 7.6, and the amount of DCPIP was reduced to 0.15 μmol/3.0 ml. HCP was added as indicated. Either reduced dihydrodinitroaniline adenine dinucleotide (NADH) or potassium L-malate as substrate was added to the reference cuvette. The reaction was started by simultaneous injection of 0.25 ml of either the membrane suspension or the soluble lysate, diluted appropriately in 0.1 M HEPES buffer, to both the sample and reference cuvettes. DCPIP reduction was followed at 600 nm in a recording spectrophotometer (Model DB-G, Beckman Instrument Co.) equipped with thermal spacers through which water at 25 C was circulated. Initial velocities were determined in the first 5 to 7 s of assay during which time the rate was linear. The extinction coefficient of DCPIP is 21 liters mmol⁻¹ cm⁻¹ (17).

**Determination of P/O ratios in isolated membranes.** The reaction mixture consisted of 170 μmol of glucose, 50 μmol of MgCl₂, 50 μmol of potassium phosphate, 1 μmol of adenosine 5'-diphosphate, 0.1 mg of ATP; p-hexose 6-phosphotransferase (EC 2.7.1.1; 400 U), 82 mg of membranes (dry weight), and distilled water to a total volume of 2.8 ml. The center well of the Warburg flask contained 0.1
ml 20% KOH. After equilibration for 20 min, the stopcocks were closed and endogenous respiration was followed for an additional 20 min. The reaction was started by tipping in 75 μmol of l-malate and from 1 to 1,000 μmol of HCP in 0.1 ml of 0.1 N NaOH solution from the side wells. Control flasks were immediately removed and the reaction was stopped by pipetting 0.5-ml portions into 9.5 ml of cold 10% trichloroacetic acid. The remaining flasks were incubated for an additional 30 min after which the reaction was stopped in the same manner. Trichloroacetic acid-precipitated protein was removed by centrifugation and a 1.0-ml portion of the supernatant was used for determination of inorganic phosphate (10).

Respiratory inhibition in isolated membranes. Oxygen uptake was followed potentiographically by means of a teflon covered Clark-type oxygen electrode in a standard bath assembly (models YSI 5331 and YSI 5301, respectively; Yellow Springs Instrument Co.) and a modified Beckman oxygen analyzer (model 777, Beckman Instruments, Inc.) equipped with a potentiometric recorder. The temperature was maintained at 25 C by use of a constant temperature circulator. When necessary, 0.05-ml samples of HCP (dissolved in 0.1 N NaOH) or menadione (dissolved in ethanol) or both were injected into the membrane suspension by means of long-tipped micropipettes. The oxygen analyzer was calibrated to read the percentage of O2 in the suspension by setting 0% of O2 with the probe under a stream of argon, 100% O2 with the probe under a stream of O2, and checking atmospheric O2 (20.9%). Irradiation of membranes suspended in 0.1 M HEPES buffer (pH 7.60) was performed with a long wave (365 nm) blacklight (UVSL 13, Ultra Violet Products Inc., San Gabriel, Calif.) at a distance of 1.5 cm for 20 min with slow agitation.

Spectral analyses. Difference spectra were obtained at 25 C by means of a Shimadzu model MPS-501 spectrophotometer (American Instrument Co., Inc., Silver Springs, Md.).

HCP treatment. Stock solutions of HCP in 0.1 N NaOH were prepared fresh daily and diluted as needed to provide the desired amount of HCP in 0.1 ml. The NaOH did not affect the pH of the buffered-cell suspensions. Unless otherwise specified, all doses were based on the dry weight of cells or membranes.

Viable cell counts. The spread-plate technique was used to determine the number of cells surviving various treatments. The diluent was 1% peptone (Difco), and the plating medium was Trypticase soy agar (BBL). Five plates were spread for each plated dilution.

Chemicals. Hexachlorophene was a gift of the Givaudan Corp. (Clifton, N.J.). Enzymes and other biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). Gasses were obtained from Matheson Gas Products (East Rutherford, N.J.). All other chemicals were of reagent grade.

RESULTS

Oxidative metabolism of intact cells. A survey was undertaken to find substrates that could be oxidized by unstarved and starved cells of *B. megaterium* (Table 1). Starvation was accomplished by shaking the cells in phosphate buffer for 2 h, after which time the endogenous rate was reduced by 40%. There was no cell death during the starvation period.

Pyruvate was oxidized at a relatively high rate by both unstarved and starved cells and was therefore selected as the primary exogenous substrate. Among other potential substrates, only l-malate and maltose were oxidized more rapidly than pyruvate. Galactose, lactose, and mannose appeared not to be oxidized. These results are in general accord with the organic acid oxidations of *B. megaterium* (23, 26) and the sugar oxidation pattern of *B. cereus* (3).

Inhibition of respiration in intact cells. At the minimal lethal dose of 8 μg per mg of cells (21), HCP inhibited endogenous respiration and exogenous pyruvate oxidation independently of starvation (Table 2). HCP also inhibited the oxidation of certain other substrates, which were selected because the subcellular distributions of their appropriate dehydrogenase activities in *B. megaterium* had been reported (23, 26). None of these substrates had a significant sparing effect on the action of HCP. For example, the oxidations of pyruvate and of lactate were equally susceptible to HCP, but in *B. megaterium* pyruvic dehydrogenase is a soluble enzyme and lactic dehydrogenase is membrane bound. Thus, the location of the dehydrogenases did not seem to be a factor in the action of HCP.

Concentrations of HCP as low as 2 μg/mg of cells were measurably inhibitory to endogenous respiration and at 8 μg/mg exerted maximal inhibition (Fig. 1). Doses as high as 12 μg/mg were administered but the inhibition was not increased above that of 8 μg/mg.

A dose of 2 μg of HCP per mg is not lethal, whereas a dose of 8 μg/mg kills a treated population within 2 h after exposure (21). To resolve the seeming inconsistency between killing and respiratory inhibition at the lower concentration, the course of inhibition and killing was determined on the same sample of cells. Series of four replicate Warburg flasks were set up at each of three HCP levels. Oxygen uptake was followed and, at 30-min intervals after exposure, one flask from each treatment group was removed and its contents were assayed for viable cells. Levels of 2 μg of HCP per mg of cells and 8 μg of HCP per mg both inhibited oxygen uptake, the latter more so than the former (Fig. 2A). Concomitant with the respiratory inhibition at 8 μg/mg, there was extensive killing (Fig. 2B). At time 0, more than 90% of the population ex-
**RESPIRATORY INHIBITION BY HEXACHLOROPHENE**

**TABLE 1. Oxidative capacity of unstarved and starved cells of Bacillus megaterium**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Unstarved cells</th>
<th>Starved cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of expt.</td>
<td>( Q_0 )</td>
</tr>
<tr>
<td></td>
<td>With substrate</td>
<td>Without</td>
</tr>
<tr>
<td>Endogenous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>30</td>
<td>12.2</td>
</tr>
<tr>
<td>Acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>14</td>
<td>12.5</td>
</tr>
<tr>
<td>Citrate</td>
<td>5</td>
<td>14.2</td>
</tr>
<tr>
<td>Fumarate</td>
<td>8</td>
<td>13.8</td>
</tr>
<tr>
<td>L-lactate</td>
<td>16</td>
<td>13.6</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>9</td>
<td>18.3</td>
</tr>
<tr>
<td>Succinate</td>
<td>17</td>
<td>14.1</td>
</tr>
<tr>
<td>Sugars</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>5</td>
<td>14.3</td>
</tr>
<tr>
<td>Galactose</td>
<td>5</td>
<td>13.1</td>
</tr>
<tr>
<td>Glucose</td>
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<td>13.5</td>
</tr>
<tr>
<td>Lactose</td>
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<td>12.5</td>
</tr>
<tr>
<td>Maltose</td>
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</tr>
<tr>
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<td>12.6</td>
</tr>
<tr>
<td>Mannose</td>
<td>4</td>
<td>12.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>6</td>
<td>14.0</td>
</tr>
</tbody>
</table>

* Cells were prepared as described and used either directly or after 2 h of starvation by shaking in phosphate buffer.

**TABLE 2. Effect of HCP (8 \( \mu g/mg \)) on the metabolism of selected organic acids by Bacillus megaterium**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Substrate</th>
<th>( Q_0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without</td>
<td>With</td>
</tr>
<tr>
<td></td>
<td>HCP</td>
<td>HCP</td>
</tr>
<tr>
<td>Set 1</td>
<td>Unstarved</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endogenous</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>17.4</td>
</tr>
<tr>
<td>Starved</td>
<td>Endogenous</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>18.3</td>
</tr>
<tr>
<td>Set 2</td>
<td>Unstarved</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endogenous</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>L-lactate</td>
<td>14.8</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>13.0</td>
</tr>
</tbody>
</table>

* Cells were prepared as described and used either directly or, where indicated, after 2 h of starvation by shaking in phosphate buffer. Where needed, 0.1 ml of HCP in 0.1 N NaOH was added simultaneously with substrate. Values are means of five determinations (Set 1) or four determinations (Set 2).

Exposed to 8 \( \mu g/mg \) were damaged beyond recovery, whereas cells treated with 2 \( \mu g/mg \) were not so damaged even after 90 min of exposure. The 5-min intervals required for plating were not included in the given exposure times. Even taking these times into account, HCP appeared to kill cells very rapidly, and the time required for the expression of respiratory inhibition was even faster. In fact, in isolated membrane preparations, respiratory inhibition became manifest less than 30 s after exposure to HCP (Fig. 3).
L-malate was used as the substrate rather than pyruvate because malic dehydrogenase occurs in both membrane-bound and soluble forms in B. megaterium, whereas pyruvic dehydrogenase occurs only in the latter form (23). Titration of the HCP inhibition of oxygen uptake by membranes respiring on L-malate revealed that the response was linear from doses of 0.5 μg of HCP per mg of membranes to 24 μg of HCP per mg, with 10 and 53% inhibition, respectively.

The inhibition was partially overcome (Fig. 3, curve B) by addition of 80 μmol of menadione per mg of membranes, i.e., about 20 times the concentration of natural menaquinone reported in protoplast membranes of B. megaterium (18). Irradiation with ultraviolet light inactivates naphthoquinones (9) and irradiated B. megaterium membranes respired very slowly on L-malate. Addition of menadione to these irradiated membranes increased the rate of L-malate oxidation to levels observed with unirradiated membranes (curve C). Menadione-induced L-malate oxidase activity was inhibited by HCP and stimulated by additional exogenous menadione. Exogenously added menadione did not cause oxygen uptake when substrate was absent (curve D) nor did it enhance uptake by unirradiated membranes (curve A).

The foregoing results obtained with membranes from cells grown in buffered peptone were virtually identical with those for mem-

**FIG. 2. Comparison of the inhibitory effect (A) and the lethality (B) of HCP on cells of B. megaterium.** Cells were treated with 0 (O), 2 (Δ), or 8 (□) μg HCP per mg of cells (dry weight). Quadruplicate flasks were prepared each with 27 mg of cells in phosphate buffer. At times 0, 30, 60, or 90 min after the appropriate amount of HCP was tipped in, one flask for each concentration of HCP was removed and the number of survivors was determined by use of the spread plate technique. Uptake values for each flask are plotted as far as uptake was followed. The broken line indicates the amount of killing that occurred in the time interval (about 5 min) between tipping in HCP and completing the plating procedure.

**Fig. 3. Effects of HCP or menadione on oxygen uptake by membranes isolated from cells of B. megaterium grown in buffered peptone.** Oxygen uptake was followed polarographically at 25°C. Each sample chamber contained 0.147 g of membranes (dry weight) in 2.5 ml of HEPES buffer. Curve C represents ultraviolet-irradiated membranes; Curves A, B, and D, unirradiated ones. Symbols: (M) 25 μmol of L-malate, (Q) 11.6 μmol of menadione in 100 μliters of ethanol, (H) 1.0 mg of HCP in 100 μliters 0.1 N NaOH or 6.8 μg of HCP per mg of membranes (dry weight).
branes prepared from cells grown in unbuffered peptone. The P/O ratios of all membrane preparations were zero and were unaffected by sonication and heating procedures designed to restore coupling (2).

**Effects on membrane-associated dehydrogenase activities and their soluble counterparts.** Membrane-bound L-malate: DCPIP oxidoreductase (malic dehydrogenase) showed inhibition kinetics that were typical of a mixed inhibition (Fig. 4A) as defined by Webb (25). In such cases, both the maximum velocity of the enzyme-catalyzed reaction (V_{max}), and the apparent affinity of the enzyme for its substrate (K_m) are reduced. Preincubation with HCP for up to 3 min did not affect the initial velocity. Moreover, the inhibition was of the completely mixed type (Fig. 4B). The inhibition constant (K_i) of HCP for membrane-associated malic dehydrogenase was 23.7 μg of HCP per mg of membranes. Membrane-bound NADH: DCPIP oxidoreductase (EC 1.6.99.3; NADH dehydrogenase) showed inhibition kinetics of the partially and noncompetitive type (Fig. 5). Soluble malic dehydrogenase and soluble NADH dehydrogenase were both inhibited by partially competitive mechanisms. The K_i values for soluble malic dehydrogenase and soluble NADH dehydrogenase were, respectively, 11.04 μg of HCP per mg and 42.47 μg per mg of protein.

Assuming the membrane to comprise about 6% of the cell dry weight (28), the minimal lethal dose of HCP for intact cells (8 μg/mg) is equivalent to about 0.5 μg per mg of membranes. Since the K_i values for membrane-associated malic dehydrogenase and for both the soluble enzymes were greater than this minimal lethal dose, it was apparent that these enzymes are not the primary targets. In the case of membrane-associated NADH dehydrogenase, a 0.5 μg/mg dose elicited only about 5% inhibition. Thus, this enzyme also is not a primary target.

**Action on cytochrome reduction in isolated membranes.** Membranes isolated from cells grown in the phosphate-buffered peptone medium contained a different cytochrome complement than did cells grown in unbuffered peptone medium. Such changes are not unique to *B. megaterium*. Alterations in the pH of the growth medium change the cytochromes in *B. coagulans* (11) and ambient pH or PO_4^3− concentration affects both the composition and the activity of membranous respiratory particles from *Mycobacterium phlei* (19).

For membranes isolated from cells grown in buffered peptone, the reduced minus oxidized spectra contained peaks at 557, 527, and 425 nm (Fig. 6, curve B) that are characteristic of cytochrome b_1 (4, 18). When HCP was added to the L-malate reduced sample, increases in the

![Figure 4](http://aac.asm.org/)  
**Fig. 4.** Effect of HCP on the membrane-associated L-malate: DCPIP oxidoreductase. Velocities are expressed as μmoles of DCPIP reduced per min per 3.0 ml. (A) DCPIP reduction was followed spectrophotometrically at 600 nm. Sample and reference cuvettes contained 0.15 μmol of DCPIP, 15 μmol of MgSO_4, 30 μmol of NaCN, 150 μmol of HEPES buffer, and HCP dissolved in 0.1 N NaOH as indicated. The reaction was started by simultaneous addition of 0.25 ml of the membrane suspension (0.3548 mg of membranes, dry weight) to both cuvettes. All lines were fitted by regression analysis. Symbols: (C) No HCP; (Δ) 14.09 μg of HCP per mg of membranes (dry weight); (○) 28.18 μg of HCP per mg of membranes (dry weight). (B) Reaction conditions as in A except that the concentration of L-malate was held constant at 5.63 μmol per mg of membranes (dry weight).
membranes contained a peak at 425 nm, a shoulder at 419 nm and a trough at 443 to 445 nm (Fig. 7, curve B). The peak at 425 nm and trough at 443 to 445 nm are characteristic of cytochrome a, and the shoulder at 419 nm

cytochrome b, peak heights at 557 and 425 nm were observed (Fig. 6, curve C). The extent of cytochrome b, reduction by L-malate and HCP was greater than the nonenzymatic reduction produced by dithionite. B. megaterium has a fumarate reductase which allows fumarate to accept electrons from cytochrome b, during anaerobiosis (18). However, addition of fumarate to HCP-treated membranes did not result in reoxidation of cytochrome b,. Reduced plus CO minus reduced difference spectra of these

![Graph showing the effect of HCP on the activity of membrane-associated NADH:DCPIP oxidoreductase.](image1)

**Fig. 5.** Effect of HCP on the activity of membrane-associated NADH:DCPIP oxidoreductase. Velocities expressed as in Fig. 4. The reaction was started by simultaneous addition of 0.25 ml of the membrane suspension (0.755 mg of membranes, dry weight) to both cuvettes. Symbols: (A) No HCP; (B) 9.93 μg of HCP per mg of membranes (dry weight). (B) Reaction conditions as in A except the NADH concentration was held constant at 0.0529 M mol per mg of membranes (dry weight).

![Graph comparing HCP and CO on difference spectra of membranes isolated from cells of B. megaterium grown in buffered peptone.](image2)

**Fig. 6.** Effect of HCP on difference spectra of membranes isolated from cells of B. megaterium grown in buffered peptone. Both the sample and reference cuvettes contained 0.442 g of membranes (dry weight) suspended in 2.5 ml HEPPES buffer. Curves: (A) baseline from oxidized minus oxidized difference spectrum; (B) reduced minus oxidized spectrum obtained after the addition of 25 μmol sodium L-malate to the reference cuvette; (C) difference spectrum obtained after injection of 1.0 mg of HCP in 100 μl of 0.1 N NaOH solution to the reduced reference cuvette.

![Graph showing reduced plus CO minus reduced difference spectra of membranes isolated from cells of B. megaterium grown in buffered peptone.](image3)

**Fig. 7.** Reduced plus CO minus reduced difference spectrum of membranes isolated from cells of B. megaterium grown in buffered peptone. Sample and reference cuvettes contained 0.372 g of membranes (dry weight) suspended in 2.5 ml HEPPES buffer. The membranes were reduced with 25 μmol of L-malate. Curves: (A) reduced minus reduced membrane baseline; (B) difference spectrum after bubbling the sample cuvette with CO for 30 s.
probably represents the Soret peak of cytochrome o (4).

For membranes isolated from cells grown in unbuffered peptone, the reduced minus oxidized difference spectra contained the 557-, 527-, and 425-nm peaks of cytochrome b, as well as peaks at 600 nm and at 443 nm (Fig. 8, curve B). Addition of HCP to the L-malate reduced sample caused a decrease in the heights of the cytochrome b, 557-, 535-, and 425-nm peaks, and in the cytochrome a 443-nm Soret peak (Fig. 8, curve C). Reduced plus CO minus reduced difference spectra of these membranes contained a small 425-nm peak and a 445-nm trough characteristic of cytochrome a, (Fig. 9, curve B).

Because the difference spectra obtained by Kröger and Dadák (18) for their strain of B. megaterium were so similar to those obtained here, their equation was used to calculate the effect of HCP on the state of reduction of cytochrome b, (Fig. 10). In the presence of excess L-malate, the extent of reduction of cytochrome b, was greater than the nonenzymatic reduction produced by dithionite and was considered to be the fully reduced state. The aerobic state without substrate was considered to be the fully oxidized state. The redox state of cytochrome b, was calculated from the absorption difference between the fully oxidized state

![Figure 8](http://aac.asm.org/)

**Fig. 8.** Effect of HCP on the difference spectra of membranes isolated from cells of B. megaterium grown in unbuffered peptone. Both the sample and reference cuvettes contained 0.040 g of membrane (dry weight) suspended in 2.5 ml HEPES buffer. Curves: (A) baseline from oxidized minus oxidized difference spectrum; (B) reduced minus oxidized spectrum obtained after addition of 25 μmol of sodium L-malate to the reference cuvette; (C) difference spectrum obtained after injection of 0.6 mg of HCP in 0.1 N NaOH solution to the reduced reference cuvette.

![Figure 9](http://aac.asm.org/)

**Fig. 9.** Reduced plus CO minus reduced difference spectra of membranes isolated from cells of B. megaterium grown in unbuffered peptone. Sample and reference cuvettes contained 0.205 g of membranes (dry weight) suspended in 2.5 ml of HEPES buffer. The membranes were reduced with 25 μmol of L-malate. Curves: (A) reduced minus membrane baseline; (B) difference spectrum after bubbling the sample cuvette with CO for 30 s.

![Figure 10](http://aac.asm.org/)

**Fig. 10.** Effect of HCP on the redox state of the cytochrome b, in membranes isolated from cells of B. megaterium grown in unbuffered peptone. Cytochrome content was calculated from the difference spectra using the difference extinction coefficient given by Kröger and Dadák (18). The completely reduced state was produced by adding 25 μmol of L-malate to the reference cuvette. The indicated amounts of HCP were added to the reference cuvette in 0.1 N NaOH. All lines were fitted by regression analysis. Circles and triangles represent different batches of membranes. Symbols: (O) 0.1082 g of membranes (dry weight) in 2.5 ml of HEPES buffer; (△) 0.2006 g of membranes (dry weight) in 2.5 ml of HEPES buffer.
and the reduced state produced by excess substrate and various amounts of HCP, and was expressed as percent reduction of the fully reduced state. Extrapolation to zero percent reduction indicated that HCP doses between 6.5 and 8.5 μg per mg of membranes (dry weight) were necessary to prevent cytochrome b₁ reduction.

The supernatant solutions of the original protoplast lysates, from which the various membrane preparations were obtained, were examined by means of dithionite-reduced minus oxidized difference spectra to determine whether or not a selective loss of cytochromes from the membranes during preparation could account for the compositional changes. However, this possibility was not supported by the findings. In the case of the supernatant solution associated with membranes that lacked cytochrome a (from cells grown in unbuffered peptone), the spectrum contained only a very broad peak around 435 nm. In the case of the supernatant solution associated with the membranes that contained cytochrome a (from cells grown in buffered peptone), the spectrum showed a trough at 410 nm, a broad flavoprotein peak around 460 nm (5), and peaks at 557 and 425 nm characteristic of cytochrome b₁.

**DISCUSSION**

Treatment of intact cells of *B. megaterium* with the minimal lethal dose of HCP (8 μg/mg of cells) results in maximal inhibition of respiration. Because the degree of inhibition is independent of the nature and presence of exogenous substrate, the electron transport chain, rather than substrate transport systems, is implicated as the target in the primary lethal action of HCP. Moreover, the target must lie among the membrane-bound components of the chain because the soluble components (e.g., dehydrogenases) are less susceptible to HCP than is overall respiration and because HCP interferes with substrate-induced cytochrome reduction in isolated membranes.

HCP killing of *S. aureus* cells is reportedly prevented by Fe²⁺ or Fe³⁺ (1). However, we have not been able to substantiate these findings for *S. aureus* or *B. megaterium* (unpublished data). Thus, the speculation that HCP inhibits respiration and kills cells by binding iron atoms critical to electron transport seems unwarranted. But proteins also bind HCP (13, 15) and these components of the electron transport system seem the more likely sites of interaction with HCP.

We found that the interaction site for HCP depends on the composition of the cytochrome chain in the cells under test. When the membranes contain cytochromes b₁, a, and a₃, but lack detectable cytochrome o (cells grown in unbuffered peptone), HCP treatment of L-malate-reduced membranes causes both cytochrome b₁ and cytochrome a to become oxidized. Thus, HCP inhibits electron transport on the substrate side of cytochrome b₁. Two target sites are immediately apparent. Primary dehydrogenases can be inhibited by HCP but are not the most susceptible targets in this system. For example, electron flow to cytochrome b₁ is totally blocked by a dose of about 7.5 μg of HCP per mg of membranes (dry weight), but the *K*ₘ for malic dehydrogenase is 23.7 μg/mg. Moreover, the flow of electrons from L-malate to DCPIP is completely inhibited only by doses greater than 50 μg/mg. Alternatively, there is in *B. megaterium* a pool of membrane-bound menaquinone which is functionally linked to each dehydrogenase and which is the only mediator of electron flow between the dehydrogenases and the cytochrome chain (18). Menadione, a structural analogue of menaquinone that lacks the 35-carbon side chain, partially restores oxidase activity in HCP-inhibited membranes. HCP may thus interfere with the function of menaquinone either by inactivating the quinone itself or by blocking the association of menaquinone with its oxidoreductase system.

However, when the membranes contain cytochromes b₁, a, and o, but lack detectable cytochrome a (cells grown in buffered peptone), addition of HCP to respiring membranes causes an increase in reduction of cytochrome b₁ alone. Hence, HCP inhibits electron transport on the oxygen side of the cytochrome rather than on the substrate side, although the exact site of inhibition was not elucidated. However, cytochrome oxidase has previously been implicated as the HCP target in mitochondria (6) and in other bacteria (13, 14). In their study of *B. subtilis*, Gould et al. (13) examined HCP inhibition of oxygen uptake induced in whole cells by ascorbate-reduced mammalian cytochrome c. Since Smith (22) later showed that even broken cell preparations of *B. subtilis* have only slight ability to oxidize mammalian cytochrome c, the results of Gould et al. (13) may now be interpreted in other ways. For example, HCP could have reacted with mammalian cytochrome c preventing its reduction by ascorbate.

HCP reportedly is an uncoupler of oxidative phosphorylation in mammalian systems (6, 7). Uncoupling stimulates oxygen uptake and increases cytochrome reduction. But the *B. megaterium* membranes were uncoupled before exposure to HCP and, therefore, uncoupling...
cannot explain the observed increase in cytochrome b1 reduction. Thus, the cytochrome oxidase system is the probable target in cells grown in buffered peptone. It is difficult to reconcile this interpretation with the finding that menadione also reverses HCP inhibition of these cells. However, many bacterial electron transport chains are branched (27) and, although there is no direct evidence for such branching in _B. megaterium_, it may be that exogenous menadione can establish a branch ending with an HCP-unsusceptible sequence.

Our findings clearly point to the electron transport system as the general site of HCP action. They also show that the specific target entity depends on the detailed composition of the respiratory chain. Apparently, there is not one particular enzyme in all cells which is most susceptible to HCP, but rather the target molecule in any given cell may be the one with the largest number of potential HCP-binding sites. Since all of the proteins in the electron transport system probably bind a certain amount of HCP, the one with the greatest number of critical binding sites readily available for interaction will be the first to be inactivated by HCP.

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**LITERATURE CITED**