THE EFFECT OF HEXACHLOROPHENE ON ELECTRON TRANSPORT IN BACILLUS MEGATERIUM

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY

John Joseph Frederick

1974

3 1293 10277 6741

LIBRARY
Michigan State
University

OVERDUE FINES:
25¢ per day per item

RETURNING LIBRARY MATERIALS:
Place in book return to remove charge from circulation records

ABSTRACT

THE EFFECT OF HEXACHLOROPHENE ON ELECTRON TRANSPORT IN *BACILLUS MEGATERIUM*

By

John Joseph Frederick

Hexachlorophene inhibited electron transport in isolated *B. megaterium* KM membranes. One site of inhibition was at the level of soluble and membrane-associated dehydrogenases (substrate:DCPIP oxidoreductases). Kinetic analysis of the effects of HCP on soluble NADH dehydrogenase (with respect to NADH) and soluble L-malate dehydrogenase (with respect to L-malate), indicated for both enzymes an inhibition of the partially competitive type. Whereas the kinetics of hexachlorophene inhibition of membrane-associated L-malate dehydrogenase (with respect to L-malate) were typical of mixed inhibition, hexachlorophene inhibited membrane-associated NADH dehydrogenase (with respect to NADH) in a noncompetitive fashion. However the high K_i's calculated for HCP inhibition of dehydrogenases indicated that this portion of the electron transport system was not the site of maximal HCP sensitivity of isolated membranes.

The effect of HCP on isolated membranes was also investigated by means of difference spectroscopy. The site of maximal HCP sensitivity depended on the cytochrome content of the membranes. When the membranes contained a b_1-type cytochrome, an a-type cytochrome, an a_3-type cytochrome, but lacked o-type cytochrome, about 8 μg HCP/mg membrane dry wt inhibited electron transport on the substrate side of the b_1-type cytochrome,

probably by inhibition of electron transfer from dehydrogenases to menaquinone. Addition of excess exogenous menadione restored oxidase activity to these HCP-inhibited membranes. When the membranes contained a b_1-type cytochrome, an a_3-type cytochrome, an o-type cytochrome, but lacked an a-type cytochrome, as little as 2 μg HCP/mg membrane dry wt inhibited electron transport on the oxygen side of the b_1-type cytochrome, probably at a site between the b_1-type cytochrome and the oxidases via subtle disorganization of the electron carriers in the membrane.

THE EFFECT OF HEXACHLOROPHENE ON ELECTRON TRANSPORT IN *BACILLUS MEGATERIUM*

By

John Joseph Frederick

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1974

GR 134

DEDICATION

Dedicated to my wife, Valerie, whose assistance

encouragement, patience and interest

put the smile on the Cheshire cat

ACKNOWLEDGMENTS

I wish to express my sincere thanks and appreciation to my advisor, Dr. Thomas R. Corner, for his interest and encouragement throughout the course of this work. Thanks also go to the members of my committee, Dr. J. Tiedje, Dr. S. Aust, and Dr. R. Brubaker, for their help and guidance; to Dr. C. San Clemente for the use of his fermenter; and to Dr. T. Akara for the use of his spectrophotometer.

I also thank the Department of Microbiology and Public Health for generous financial assistance.

A special thanks goes to my family for their constant moral and financial support.

Last, but certainly not least, I acknowledge Dr. Rene M. Scherrer whose constant stream of trenchant criticisms helped "... quite much you know."

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

> "...(Alice) had never forgotten that, if you drink much from a bottle marked "poison", it is almost certain to disagree with you, sooner or later."
>
> -Lewis Carroll, 1865

For nearly 25 years, hexachlorophene [2,2'-methylenebis (3,4,6-trichlorophenol); HCP] has been widely used as an antimicrobial compound. Its broad spectrum antimicrobial activity against gram-positive and, to a lesser extent, gram-negative microorganisms, compatibility with soap formulations, stability, lack of odors or color, and economics made it suitable for incorporation into a large number of consumer products (6).

Toxicity for Mammals

Several recent reports indicating high HCP toxicity for mammals (5,7,19,21,24,26,28) have prompted the U.S. Food and Drug Administration to prohibit the nonprescription sale of drugs which contain more than 0.75% HCP and to ban the use of HCP in cosmetics except as a preservative in levels up to 0.1%, and then only when other suitable preservatives are not available (37). The toxicity of HCP for mammals depends on the dose, method of administration, and organism. Repetitive small oral or topical doses (<50 mg/kg) are not immediately toxic whereas large single doses (>100 mg/kg) are generally fatal (16,20,21,25,26,30). HCP has been shown to inhibit dehydrogenases and oxidases of animal

origin and to uncouple oxidative phosphorylation in mammalian systems (5,7,19). The cause of death in acute HCP intoxication of rats may be related to hyperthermia brought about by this uncoupling (32).

Toxicity for Microorganisms

The primary lethal action of HCP in microbes still has not been completely resolved. Some previous suggestions for its mode of action include (i) release and leakage of cytoplasmic materials; (ii) disruption or lysis of the cytoplasmic membranes; (iii) generalized protein denaturation; and (iv) respiratory inhibition. It has been concluded that these first three mechanisms are secondary events to the primary lethal mechanism since they are only observed at concentrations of HCP much higher than the reported minimum lethal concentration of 8 μg HCP/mg cell dry weight for *Bacillus megaterium* (8,23,33). Recently Corner and Gerhardt (unpublished results) have used Warburg respirometry to demonstrate inhibition of oxygen uptake in HCP-treated cells of *B. megaterium*. They found that the rate of oxygen uptake from exogenous or endogenous substrates decreased progressively as the concentration of HCP was increased from 0 to 8 μg HCP/mg cell dry weight. Higher HCP levels did not further depress respiration. Since the concentration of HCP necessary for respiratory inhibition correlated with the value for the minimum lethal concentration, and since inhibition of endogenous respiration ruled out inhibition of transport systems as the lethal event, they reasoned that the respiratory system was the primary target of HCP.

Much earlier Gould *et al.* (18) found that HCP inhibited the activity of selected dehydrogenases (succinic, glucose, lactic) and

cytochrome c oxidase activity in intact cells of both *Escherichia coli* and *B. subtilis*. In their experiments they held the concentration of HCP constant and varied the biomass to achieve a desired reaction rate. Since they did not report the amount of biomass, comparison of their data with other work is precluded. Attempts to enrich for strains of organisms resistant to HCP revealed that anaerobic or facultative organisms were less sensitive to HCP than highly aerobic organisms and that no striking adaptation to the inhibitory effects of HCP took place (20). A lower sensitivity to HCP of the anaerobic or facultative organisms was interpreted to mean that a sensitive oxidase system was bypassed so that some other less sensitive enzyme system became limiting for growth.

Adams and Hobbs have shown that HCP is capable of forming chelates with a variety of metals (1). They found that only Fe^{++} was capable of suppressing the action of HCP against their test organism, *Staphylococcus aureus*, and reasoned that the bactericidal properties of HCP may stem from inhibition of certain iron-containing enzyme systems. However, iron salts neither prevented nor reversed HCP inhibition of bacterial growth when Corner and Gerhardt (unpublished results) repeated the experiments of Adams and Hobbs using *B. megaterium* as the test organism.

Since the primary lethal mechanism of HCP appeared to be respiratory inhibition, the purpose of this investigation was to determine the site(s) of this inhibition in *B. megaterium*.

Respiratory System of *B. megaterium*

The respiratory system of *B. megaterium* is localized in the protoplast membrane. The membrane can be isolated by subjecting

protoplasts of *B. megaterium*, prepared by controlled lysozyme treatment, to osmotic shock followed by differential centrifugation (35,39). These membranes catalyze oxidation of various substrates (2). The respiratory enzyme content of the membranes depends on the growth conditions of the cells before harvest, but one can draw a generalized scheme for the electron transport system of *B. megaterium* KM (Figure 1).

Figure 1. Sequence of electron carriers in *B. megaterium* KM. Redrawn from (29).

This scheme does not take into account the possible involvement of nonheme iron or of c-type cytochromes which have been detected in other strains of *B. megaterium* (40). The precise relationship between the oxidases, cytochrome a_3 or cytochrome o, to either the b_1-type cytochrome or cytochrome a is not known. *B. megaterium* has several membrane-associated and soluble dehydrogenases that supply electrons to the electron transport chain. L-malate dehydrogenase and NADH dehydrogenase were chosen for study because they are present in relatively large amounts and each exists in both membrane-associated and soluble forms (35).

MATERIALS AND METHODS

Organism

The asporogenous KM strain of *B. megaterium* was chosen for this work because its sensitivity to HCP has been well characterized (8,23,33), and details of its respiratory chain are known (2,34,12,13,29). Stock cultures were maintained on 2% trypticase soy agar (Baltimore Biological Laboratories, Cockeysville, Maryland) plates at 30 C.

Cultivation of Cells

Cells of *B. megaterium* were grown to late exponential phase of growth (15-18 hr) in 2% (wt/vol) Oxoid peptone (Flow Laboratories, Rockville, Maryland) in aerated batch cultures on a gyratory shaker (250 rev/min) at 30 C. These cells were used as inocula for 10 L of medium [2% Oxoid peptone in distilled H_2O or 0.01 M sodium-potassium phosphate buffer (pH 7.0) containing 0.3 g Dow antifoam/L] in a fermentor (model MF-14 New Brunswick Scientific Co., New Brunswick, New Jersey) which was operated at 30 C with an aeration rate of 10 L of air per minute and a stirring rate of 250 rpm. When necessary, the pH of the medium was readjusted to pH 7.0 with concentrated HCl. The cells were harvested during the late exponential phase of growth when the optical density of the culture reached about 2.45. This optical density corresponds to about 2.4 mg cell dry wt/ml. Growth was monitored by means of a Spectronic 20 spectrophometer (Bausch and Lomb, Rochester, New York) at a wavelength of 700 nm, with 1.17 cm light-path cuvettes and distilled water used as a blank. The cells were collected by means of continuous

flow centrifugation (27,000 x g; 4 C) at a flow rate of 150 ml/min. A Sorvall RC-2B refrigerated centrifuge equipped with a Szent-Gyorgi and Blum continuous flow system was used. The wet weight yield of the cells was 4.5 $\pm$ 0.1 g/L. The cells were washed twice in cold distilled water and used immediately.

Isolation of Membranes

The membrane fraction of *B. megaterium* was prepared by treatment with mucopeptide N acetylmuramylhydrolase (E.C. 3.2.1.17; lysozyme) followed by osmotic shock according to a modification of the method of Broberg and Smith (2). Each gram (wet wt) 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 (wet wt) of cells were used in each isolation. Crystalline lysozyme (30,000 units activity/mg) dissolved in 15 ml of the buffered sucrose solution was added to the cell suspension to give a final concentration of 0.01 g lysozyme/g cell wet wt. The resultant suspension was incubated at 25 C until conversion to single protoplasts was complete as judged by viewing with a phase contrast microscope (about 60 min). The protoplasts were sedimented (25,000 x g; 20 min) in a Sorvall RC-2B refrigerated centrifuge 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 deoxyribonucleate oligonucleotidohydrolase (E.C. 3.1.4.5; deoxyribonuclease 1). The mixture was shaken at 25 C for 15 min. Complete lysis of the protoplasts occurred under these conditions as judged by microscopic examination. Protoplast ghost membranes were centrifuged at 25,000 x g for 30 minutes. The buff-colored upper part of the pellet containing the membrane fraction was suspended in 0.1 M

N-2-Hydroxyethlpiperazine-N'-2-ethanesulfonic acid-KOH buffer, pH 7.65 (HEPES buffer). This procedure was repeated at least three times and each time the buff-colored membrane pellet was scraped off the heavier white pellet of poly-β-hydroxybutyrate (PHB) granules. The final membrane suspension, which still contained some PHB, was stored at -30 C. When needed, an appropriate amount of the frozen membrane preparation was suspended to the desired concentration in 0.1 M HEPES buffer (pH 7.60), distributed to 15-ml screw-capped test tubes and immediately refrozen in an ethanol-dry ice bath.

Isolation of Soluble Dehydrogenases

The soluble enzymes were contained in the supernatant obtained by direct lysis of protoplasts in hypotonic solution. To 88 g of washed cells (wet wt) were added 100 ml of 0.1 M HEPES buffer containing 0.250 g lysozyme and 0.05 mg deoxyribonuclease. This mixture was shaken at 250 rpm in a gyratory shaker at 30 C for 2 hr. 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 x g; 30 min) and the yellowish supernatant was decanted and stored at 4 C. The buff-colored part of the pellet containing the membranes was scraped off the heavier white PHB layer, resuspended in 50 ml of 0.1 M HEPES buffer, and recentrifuged at 25,000 x g for 30 min. The supernatant was again decanted and pooled with the previous one. The pooled supernatants were further clarified by centrifugation at 25,000 rev/min with an average centrifugal force of 75,000 x g, for 2 hr 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 suspended to the desired concentration in 0.1 M HEPES buffer, distributed to 15-ml screw-capped test tubes, and quickly frozen in an ethanol-dry ice bath. Protein concentrations were estimated by the buiret method (17) using bovine serum albumin as a protein standard.

Enzyme Assays

Dehydrogenase activity was determined by the method of Storck and Wachsman (35) which involves the reduction of 2,6-dichlorophenol-indolphenol (DCPIP). Each 1.0 cm-lightpath cuvette contained 0.15 μmoles DCPIP, 15 μmoles $MgSO_4$, 30 μmoles NaCN (freshly adjusted to pH 7.60 with HCl), 150 μmoles HEPES buffer, and HCP dissolved in 0.1 N NaOH as indicated (final volume = 3.0 ml). Substrate, either reduced dihydro-nicotinamide adenine dinucleotide (NADH) or potassium L-malate, 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 calculated from slopes of the traces during the first 5 to 7 seconds of the assay during which time the rate is linear. The extinction coefficient of DCPIP was taken to be 21 L $\mu M^{-1} cm^{-1}$ (27).

Determination of P/O Ratios

Oxygen uptake at 30 C was measured by standard Warburg techniques (36). The reaction mixture consisted of 170 μmoles glucose,

50 μmoles $MgCl_2$, 50 μmoles potassium phosphate, 1 μmole ADP, 0.1 mg ATP: D-hexose 6-phosphotransferase (EC 2.7.1.1) (400 units), 82 mg membranes (dry wt), and water to a final volume of 2.8 ml. The center well of the flask contained 0.1 ml 20% w/v KOH. After 20 min of equilibration, the stopcocks were closed and endogenous respiration was followed for 20 min. The reaction was started by tipping in 75 μmoles of L-malate and from 1 to 1,000 μmoles of HCP in 0.1 ml 0.1 M NaOH from the side wells. Control flasks were immediately removed and the reaction stopped by pipetting 0.5 ml aliquots into 9.5 ml cold 10% trichloroacetic acid (TCA). The remaining flasks were incubated for an additional 30 min, and the reaction stopped in the same manner. TCA-precipitated protein was removed by centrifugation and a 1.0 ml aliquot of the supernatant was used for inorganic phosphate determination according to the method of Fiske and SubbaRow (14).

Respiratory Inhibition

Oxygen uptake was followed polographically 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 Sargent model SR recorder. The temperature was maintained at 25 C by use of a constant temperature circulator (model FK, Haake Instruments, Inc.). When necessary, 0.05 ml aliquots of HCP (dissolved in 0.1 M NaOH) or 2 methyl-1,4-naphthaquinone (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 percent O_2 in the suspension by settingng zero percent O_2 with the probe under a stream of argon, 100 percent O_2 with the probe under a

stream of O_2, and checking atmospheric O_2 (20.9%). Irradiation of the membrane preparation with ultraviolet light was performed with a long wave (365 nm) blacklight (UVSL 13, Ultra Violet Products Inc., San Gabriel, California). Membranes suspended in 0.1 M HEPES buffer were placed in a glass Petri dish on a magnetic stirrer and irradiated at a distance of 1.5 cm for 20 min while being slowly stirred by a teflon-coated magnetic stirring bar.

Spectra

Difference spectra were obtained with a Shimadzu model MPS-50L spectrophotometer (American Instrument Co., Inc., Silver Springs, Maryland).

RESULTS

Effect of HCP and Menadione on the Rate of Oxygen Uptake by Both Normal and Ultraviolet-irradiated Membranes

Isolated *B. megaterium* membranes contained an electron transport system that was capable of oxidizing L-malate and transferring these electrons to oxygen (Figure 2A, 2B and Figure 3A, 3B). Hexachlorophene inhibited oxygen uptake by isolated *B. megaterium* membranes. This inhibition was partially overcome by addition of 80 μmoles menadione/mg membrane dry wt to the membrane suspension (Figure 2B and Figure 3B). This amount of exogenous menadione was about 20 times the reported concentration of natural menaquinone in protoplast membranes of *B. megaterium* (29). Irradiation with ultraviolet light has been shown to inactivate *Micrococcus lysodeikticus* naphthaquinones (11,15). *B. megaterium* membranes which had been irradiated for 20 min with ultraviolet light respired very slowly on L-malate. Addition of menadione to these membranes increased the rate of L-malate oxidation to the levels observed with unirradiated membranes (Figure 2C and Figure 3C). Menadione-restored L-malate oxidase activity could be inhibited by HCP and stimulated by additional exogenous menadione (Figure 3C). Exogenously added menadione did not cause oxygen uptake when substrate was absent (Figure 3D).

P/O Ratios of Membranes

The P/O ratios of all membrane preparations were zero. Brief sonication or heating of the membrane suspensions did not increase the P/O ratios.

Figure 2. Effect of hexachlorophene and manadione on the rate of L-malate oxidation by isolated membranes of *B. megaterium* grown in unbuffered peptone. Oxygen uptake was followed pologra phically at 25 C. Each sample chamber contained 0.0679 g (dry wt) of membranes in 2.5 ml HEPES buffer. The membrane suspensions were stirred under a stream of O_2 for 3 min immediately before the teflon-covered oxygen electrode was inserted. Curves A and B represent untreated membranes, curve C represents membranes that were irradiated with ultraviolet light to destroy natural quinones (see Materials and Methods). Symbols: (M) 25 μmoles L-malate; (E) 100 μl ethanol; (H) 1.0 mg hexachlorophene in 100 μl 0.1 N NaOH (14.7 μg HCP/mg membrane dry wt); (Q) 5.6 μmoles manadione in 100 μl ethanol.

Figure 3. Effect of hexachlorophene and manadione on the rate of L-malate oxidation by isolated membranes of *B. megaterium* grown in phosphate-buffered peptone. Oxygen uptake was followed pologra phically at 25 C. Each sample chamber contained 0.1470 g (dry wt) of membranes in 2.5 ml HEPES buffer. The membrane suspensions were stirred under a stream of O_2 for 3 min immediately before the teflon-covered oxygen electrode was inserted. Curves A, B and D represent untreated membranes, curve C represents membranes which were irradiated with ultraviolet light to destroy natural quinones (see Materials and Methods). Symbols: (M) 25 μmoles L-malate; (Q) 11.6 μmoles menadione in 100 μl ethanol; (H) 1.0 mg hexachlorophene in 100 μl 0.1 N NaOH (6.8 μg HCP/mg membrane dry wt); (H/2) 0.5 mg HCP in 50 μl 0.1 N NaOH.

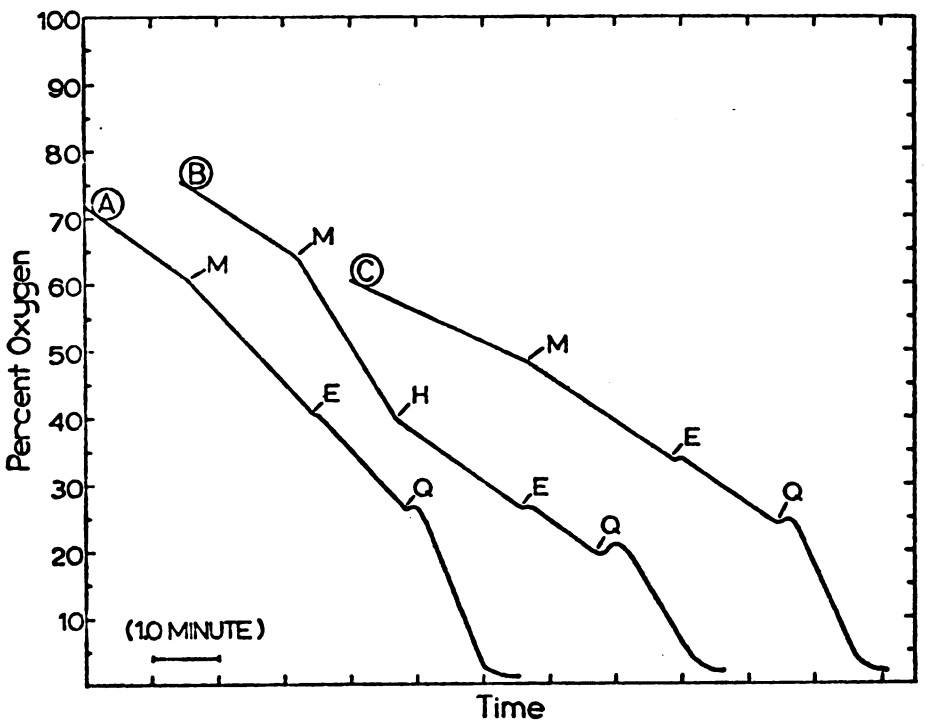

FIGURE 2

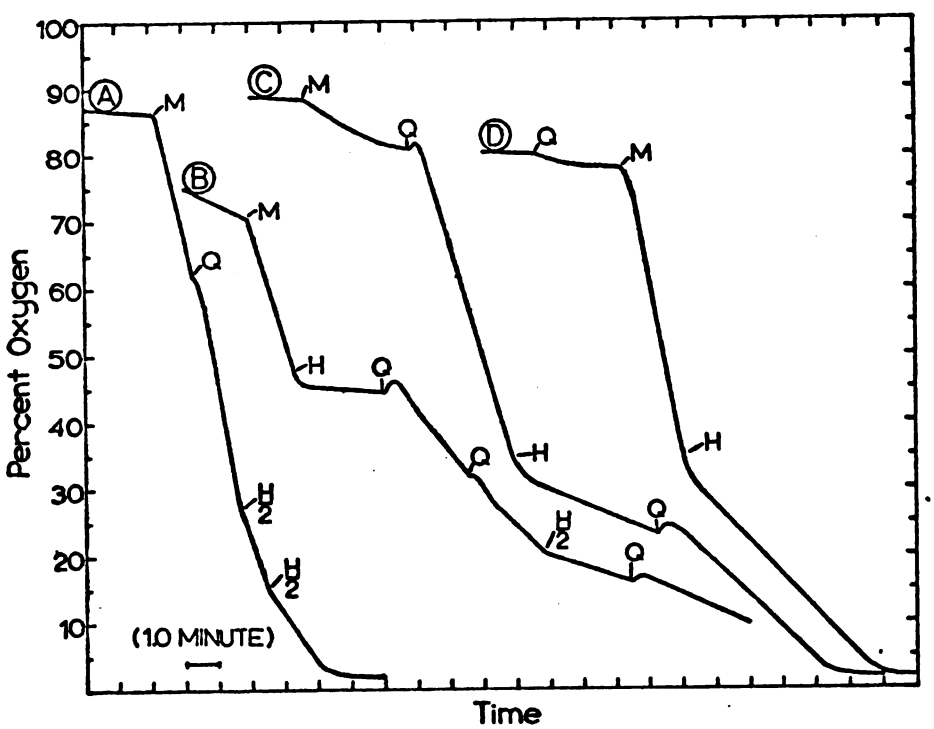

FIGURE 3

Effect of HCP on Membrane-associated L-malate:DCPIP Oxidoreductase

HCP inhibited the membrane-associated form of L-malate:DCPIP oxidoreductase (E.C. 1.1.99.-; L-malate dehydrogenase). When the concentration of DCPIP was held constant, the kinetics of inhibition of L-malate dehydrogenase with respect to L-malate were typical of mixed inhibition as defined by Webb (38) (Figure 4). In such cases both the maximum velocity (V_{max}), and the apparent affinity of the enzyme for its substrates are reduced. Moreover, a plot of velocity vs. HCP concentration indicated that the inhibition was of the completely mixed type since a finite amount of HCP could completely inhibit enzyme activity (Figure 5). The inhibitor constant (K_i) of HCP for the membrane-associated L-malate dehydrogenase was 23.7 µg HCP/mg membrane dry wt (see appendix A for calculations). Preincubation of the membranes with HCP for up to 3 min did not affect the initial velocity.

Effect of HCP on Soluble L-malate:DCPIP Oxidoreductase

Kinetic analysis of the effect of HCP on soluble L-malate dehydrogenase, with respect to L-malate, indicated that the inhibition was of the competitive type (Figure 6). Moreover, a plot of velocity vs. HCP concentration indicated that the inhibition was of the partially competitive type (Figure 7). In such cases inhibition does not increase indefinitely with inhibitor concentration, but increases to a definite limit when all of the susceptible sites on the enzyme are combined with the inhibitor and can then increase no further. The K_i of HCP for soluble L-malate dehydrogenase was 11.04 µg HCP/mg protein (see appendix B for calculations).

FIGURE 4. Effect of HCP on the membrane associated L-malate:DCPIP oxidoreductase (Lineweaver-Burke transformation). DCPIP reduction was followed spectrophotometrically at 600 nm. Sample and reference cuvettes contained 0.15 μmoles DCPIP, 15 μmoles $MgSO_4$, 30 μmoles NaCN, 150 μmoles 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 membrane dry wt) to both cuvettes. All lines shown were fitted by regression analysis. Symbols: (O) No HCP; (Δ) 14.09 μg HCP/mg membrane dry wt; (□) 28.18 μg HCP/mg membrane dry wt.

FIGURE 5. Effect of HCP on the activity of membrane-associated L-malate: DCPIP oxidoreductase. The reaction conditions were as in Figure 4 except that the concentration of L-malate was held constant at 5.63 μmoles/mg membrane dry wt.

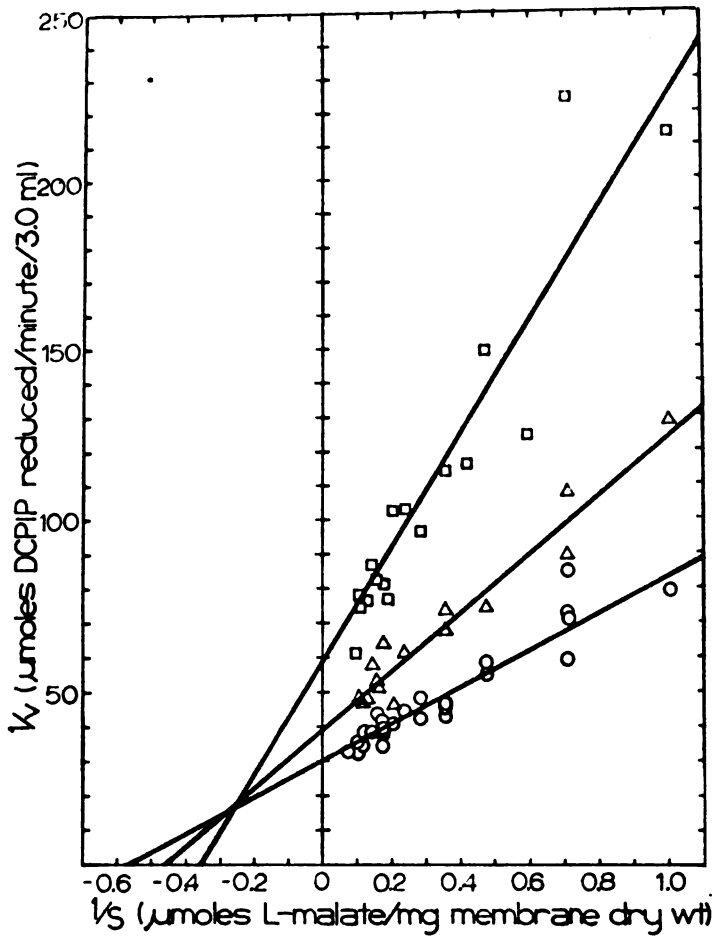

FIGURE 4

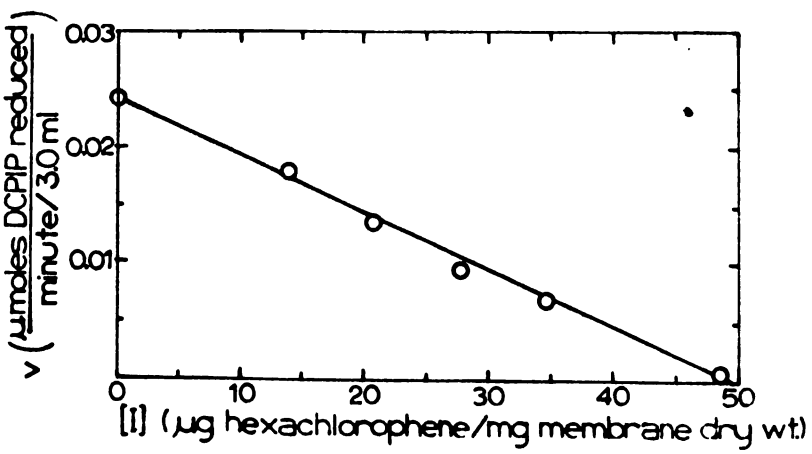

FIGURE 5

FIGURE 6. Effect of HCP on the activity of soluble L-malate:DCPIP oxidoreductase (Lineweaver-Burke transformation). DCPIP reduction was followed spectrophotometrically at 600 nm. Sample and reference cuvettes contained 0.15 µmoles DCPIP, 15 µmoles $MgSO_4$, 30 µmoles NaCN, 150 µmoles 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 soluble lysate containing 2.732 mg protein, to both cuvettes. All lines shown were fitted by regression analysis. Symbols: (O) No HCP; (Δ) 10.98 µg HCP/mg protein; (□) 27.45 µg HCP/mg protein.

FIGURE 7. Effect of HCP on the activity of soluble L-malate:DCPIP oxidoreductase. The reaction conditions were as in Figure 6 except the concentration of L-malate in the sample cuvette was held constant at 0.915 µmoles L-malate/mg protein and the HCP concentration was varied as indicated.

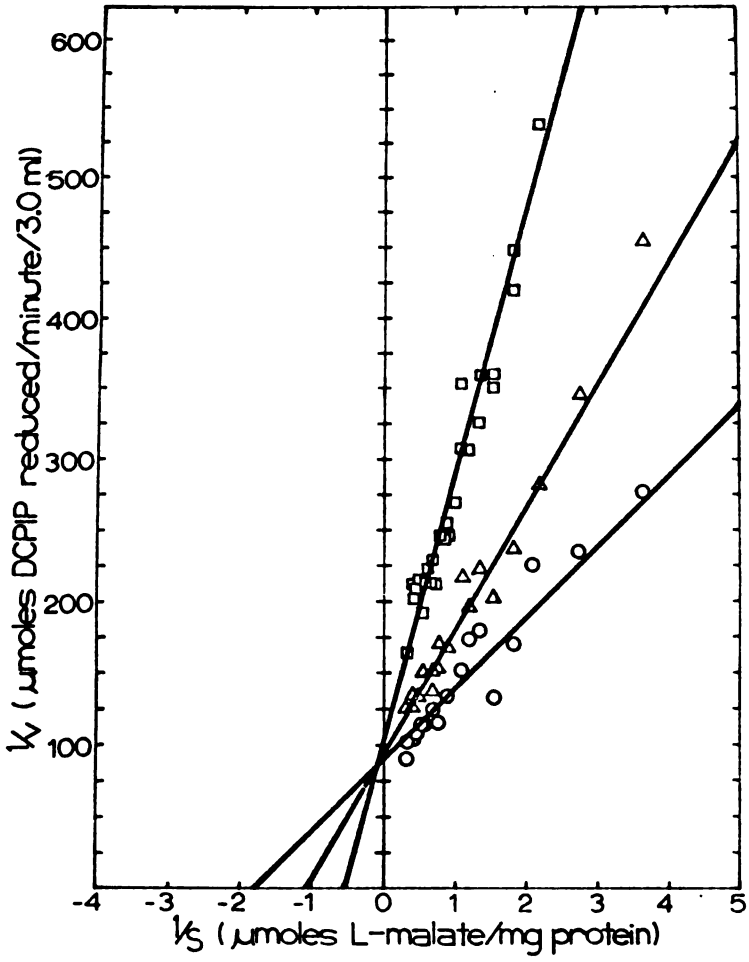

FIGURE 6

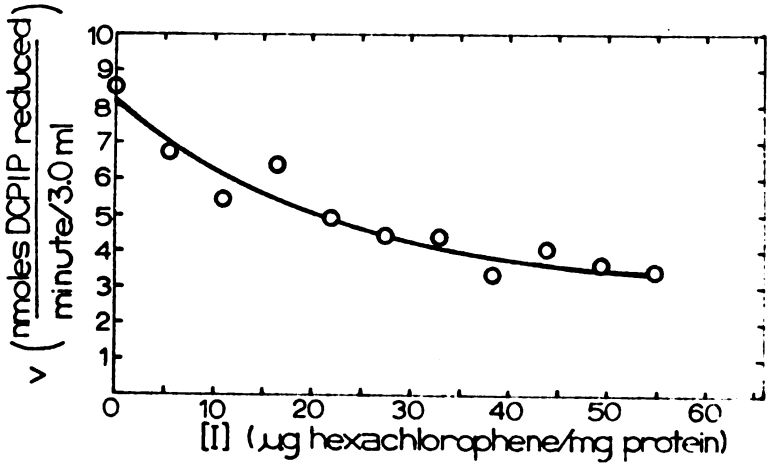

FIGURE 7

Effect of HCP on Membrane-associated NADH:DCPIP Oxidoreductase

Hexachlorophene also inhibited the membrane-associated NADH:DCPIP oxidoreductase (E.C. 1.6.99.3; NADH dehydrogenase) but in a noncompetitive fashion with respect to NADH (Figure 8). A plot of velocity vs. HCP concentration indicated that the inhibition was of the partially noncompetitive type (Figure 9).

Effect of HCP on Soluble NADH:DCPIP Oxidoreductase

Kinetic analysis of the effects of HCP on soluble NADH dehydrogenase, with respect to NADH, indicated that the inhibition was of the partially competitive type (Figure 10 and Figure 11). The K_i of HCP for soluble NADH dehydrogenase was 42.47 µg HCP/mg protein (see appendix B for calculations).

Effect of HCP on Cytochrome Spectra of Membranes Isolated From Cells That Were Grown in Buffered Peptone

B. megaterium membranes isolated from cells grown in very similar conditions were found to differ in their cytochrome content. Reduced minus oxidized difference spectra from cells grown in buffered peptone (0.1 M sodium-potassium phosphate buffer pH 7.0) contained peaks at 557 nm, 527 nm, and 425 nm (Figure 12, curve B). Such peaks are characteristic of cytochrome b_1 (2,29). When HCP was added to the sample cuvette, increases in the b_1-type cytochrome peak heights at 557 nm and at 425 nm were observed (Figure 12, curve C). The extent of reduction of this b_1-type cytochrome by L-malate and HCP was greater than the non-enzymatic reduction produced by dithionite. Kroger and Dadak (29) reported that *B. metgaterium* has a fumarate reductase in equilibrium with cytochrome b_1 which allows fumarate to act as an electron acceptor during the anaerobic state. Addition of fumarate to

FIGURE 8. Effect of HCP on the activity of membrane-associated NADH: DCPIP oxidoreductase (Lineweaver-Burke transformation). DCPIP reduction was followed spectrophotometrically at 600 nm. Sample and reference cuvettes contained 0.15 μmoles DCPIP, 15 μmoles $MgSO_4$, 30 μmoles NaCN, 150 μmoles HEPES, 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 (0.7550 mg membrane dry wt) to both cuvettes. All lines shown were fitted by regression analysis. Symbols: (O) No HCP; (Δ) 9.933 μg HCP/mg membrane dry wt.

FIGURE 9. Effect of HCP on the activity of membrane-associated NADH: DCPIP oxidoreductase. The reaction conditions were as in Figure 8 except the NADH concentration was held constant at 0.0529 μmoles NADH/mg membrane dry wt.

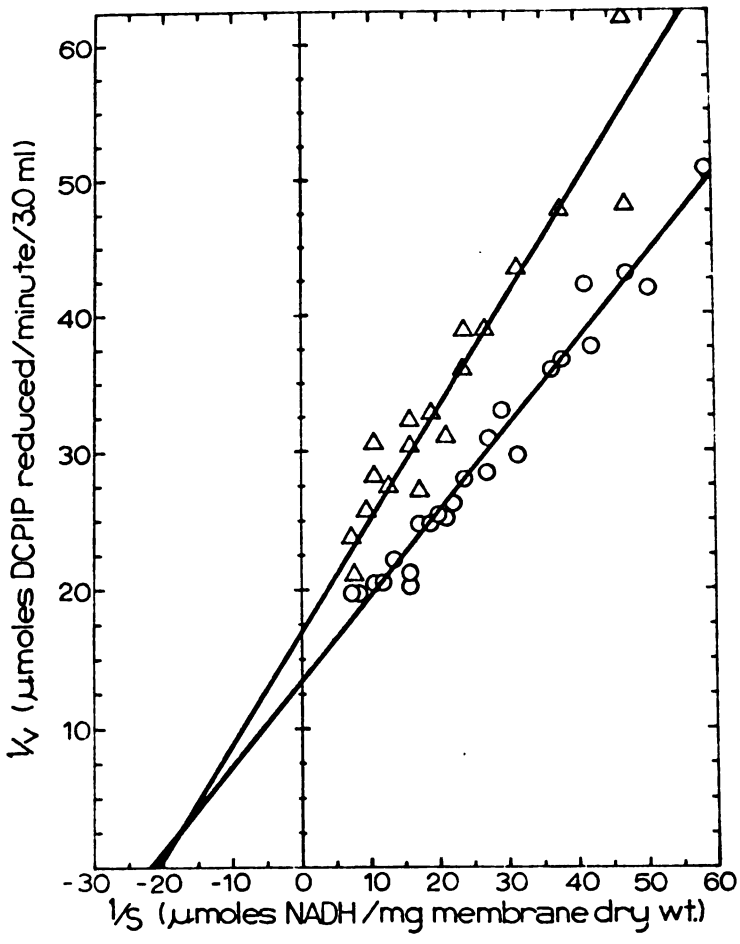

FIGURE 8

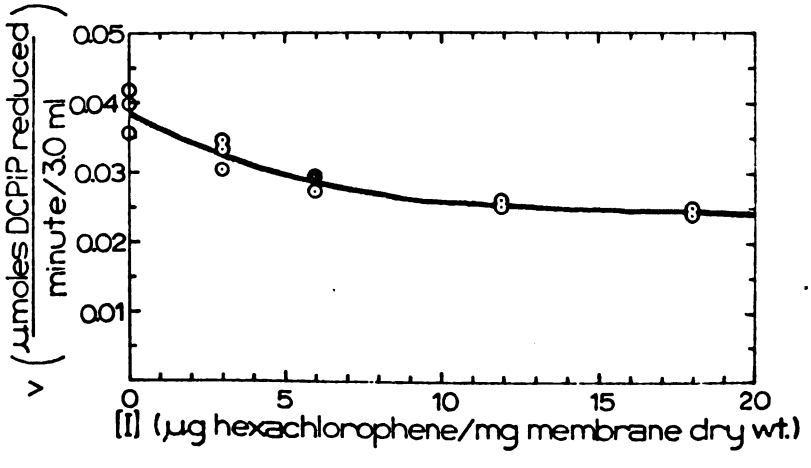

FIGURE 9

FIGURE 10. Effect of HCP on the activity of soluble NADH:DCPIP oxidoreductase (Lineweaver-Burke transformation). DCPIP reduction was followed spectrophotometrically at 600 nm. Sample and reference cuvettes contained 0.15 μmoles DCPIP, 15 μmoles $MgSO_4$, 30 μmoles NaCN, 150 μmoles 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 soluble lysate containing 0.1300 mg protein, to both cuvettes. All lines shown were fitted by regression analysis. Symbols: (O) No HCP; (Δ) 19.23 μg HCP/mg protein; (□) 38.46 μg HCP/mg protein.

FIGURE 11. Effect of HCP on the activity of soluble NADH:DCPIP oxidoreductase. The reaction conditions were as in Figure 10 except the concentration of NADH in the sample cuvette was held constant at 2.461 μmoles NADH/mg protein and the HCP concentration was varied as indicated.

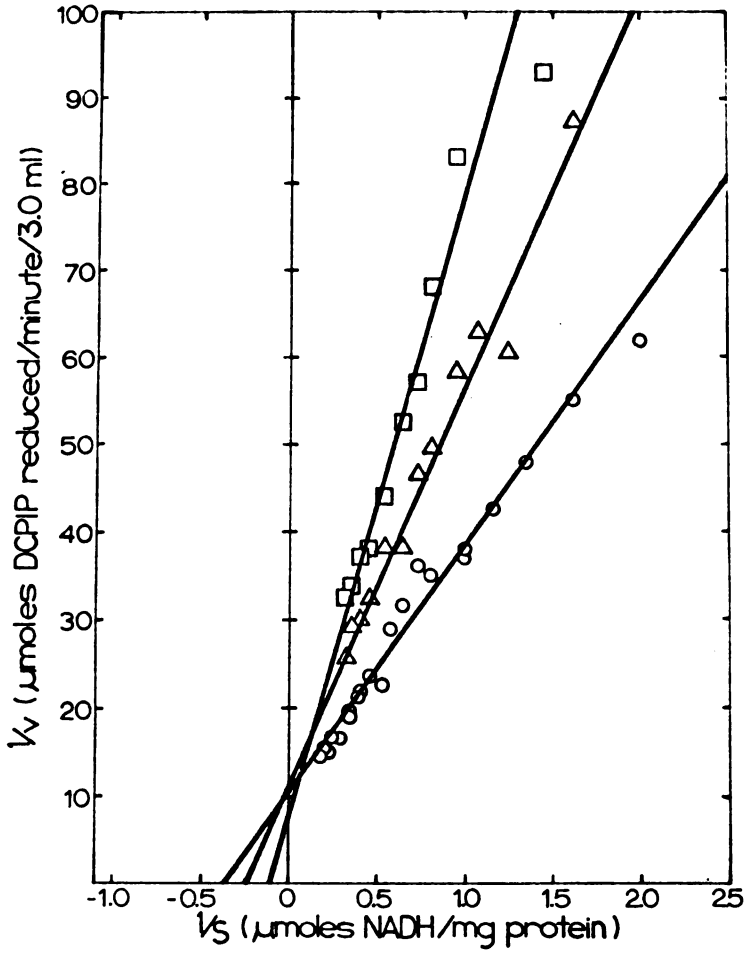

FIGURE 10

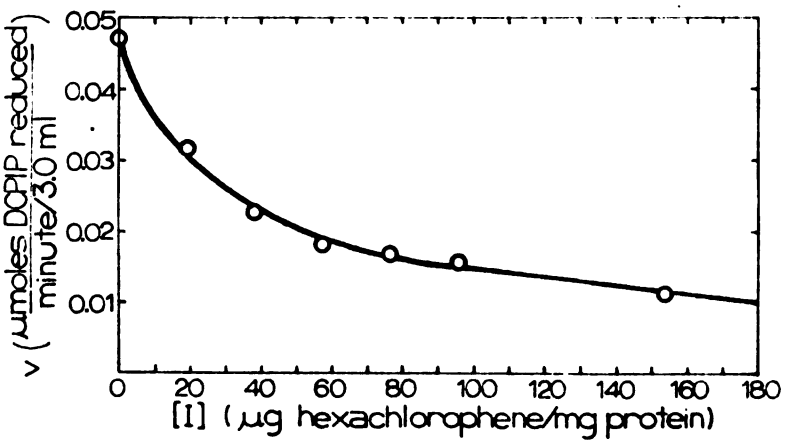

FIGURE 11

FIGURE 12. Effect of hexachlorophene on difference spectra of membranes isolated from cells of *B. megaterium* grown in phosphate buffered peptone. Both the sample and reference cuvettes contained 0.442 g membranes (dry wt) suspended in 2.5 ml HEPES. Symbols: (A) baseline from oxidized minus oxidized difference spectrum; (B) reduced minus oxidized spectrum obtained after the addition of 25 μmoles sodium L-malate to the sample cuvette; (C) difference spectrum obtained after injection of 1.0 mg HCP in 100 μl 0.1 N NaOH to the reduced sample cuvette.

FIGURE 13. Reduced + CO minus reduced difference spectrum of membranes isolated from cells of *B. megaterium* grown in phosphate buffered peptone. Sample and reference cuvettes contained 0.372 g membranes (dry wt) suspended in 2.5 ml HEPES. The membranes were reduced with 25 μmoles L-malate. Symbols: (A) reduced minus reduced membrane baseline; (B) difference spectrum after bubbling the sample cuvette with CO for 30 seconds.

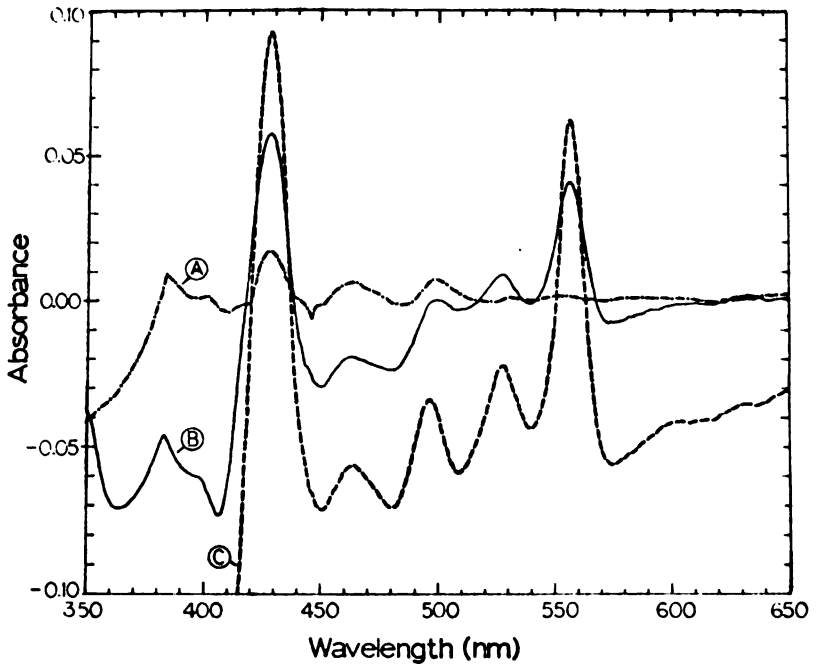

FIGURE 12

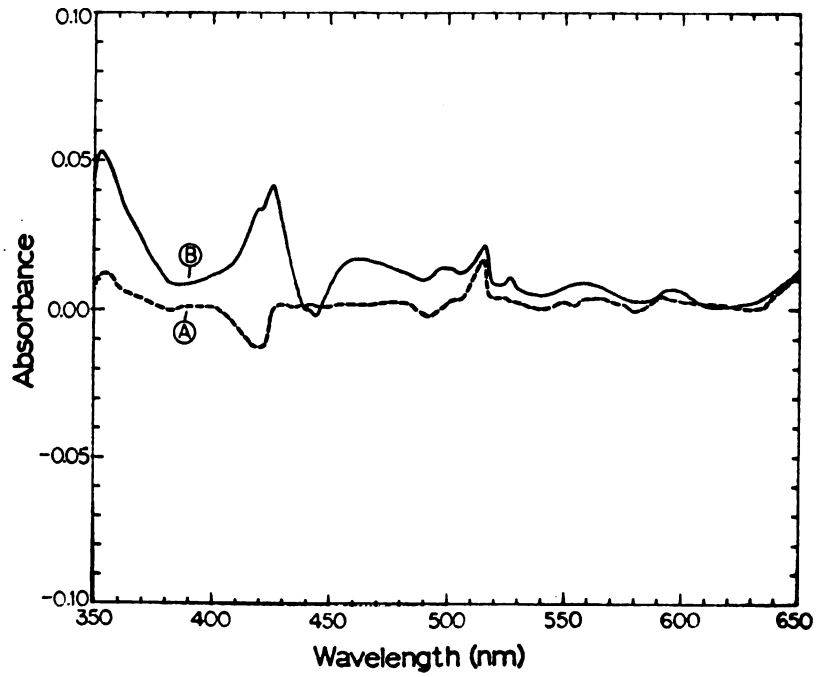

FIGURE 13

HCP-treated membranes did not result in reoxidation of the b_1-type cytochrome. Reduced plus carbon monoxide (CO) minus reduced difference spectra of these membranes contained a peak at 425 nm, a shoulder at 419 nm, and a trough at 443 to 445 nm (Figure 13, curve B). The peak at 425 nm and trough at 443 to 445 nm are characteristic of cytochrome a_3, and the shoulder at 419 nm is probably the Soret peak of cytochrome o (2).

Effect of HCP on Cytochrome Spectra of Membranes Isolated From Cells That Were Grown in Unbuffered Peptone

Reduced minus oxidized difference spectra of membranes isolated from cells grown in unbuffered peptone contained 557 nm, 527 nm, and 425 nm peaks of a b_1-type cytochrome, as well as peaks at 600 nm and at 443 nm (Figure 14, curve B). Addition of HCP to the L-malate reduced sample cuvette caused a decrease in the peak height of the b_1-type cytochrome 557 nm, 535 nm, and 425 nm peaks, and in the a-type cytochrome 443 nm Soret peak (Figure 14, curve C). Reduced + CO minus reduced difference spectra of these membranes contained a small 425 nm peak and a 445 nm trough characteristic of cytochrome a_3 (Figure 15, curve B).

Effect of HCP on the Redox State of the b_1-Type Cytochrome in Membranes Isolated From Cells Grown in Unbuffered Peptone

Kroger and Dadak have calculated the content of *B. megaterium* ATTC No. 14581 b_1-type cytochrome from the absorption difference at the 557.5 nm peak and the 575 trough between the fully oxidized and fully reduced states using the difference extinction coefficient $(\varepsilon_{red}-\varepsilon_{ox})_{557.5nm}-(\varepsilon_{red}-\varepsilon_{ox})_{575nm} = 17.5\ mM^{-1}cm^{-1}$ (2,9). The difference spectra reported for the b_1-type cytochrome in their strain

FIGURE 14. 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 0.0403 g membranes (dry wt) suspended in 2.5 ml HEPES. Symbols: (A) baseline from oxidized minus oxidized difference spectrum; (B) reduced minus oxidized spectrum obtained after addition of 25 μmoles sodium L-malate to the sample cuvette; (C) difference spectrum obtained after injection of 0.6 mg HCP in 0.1 M NaOH to the reduced sample cuvette.

FIGURE 15. Reduced + 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 membranes (dry wt) suspended in 2.5 ml HEPES. The membranes were reduced with 25 μmoles L-malate. Symbols: (A) reduced minus reduced membrane baseline; (B) difference spectrum after bubbling the sample cuvette with CO for 30 seconds.

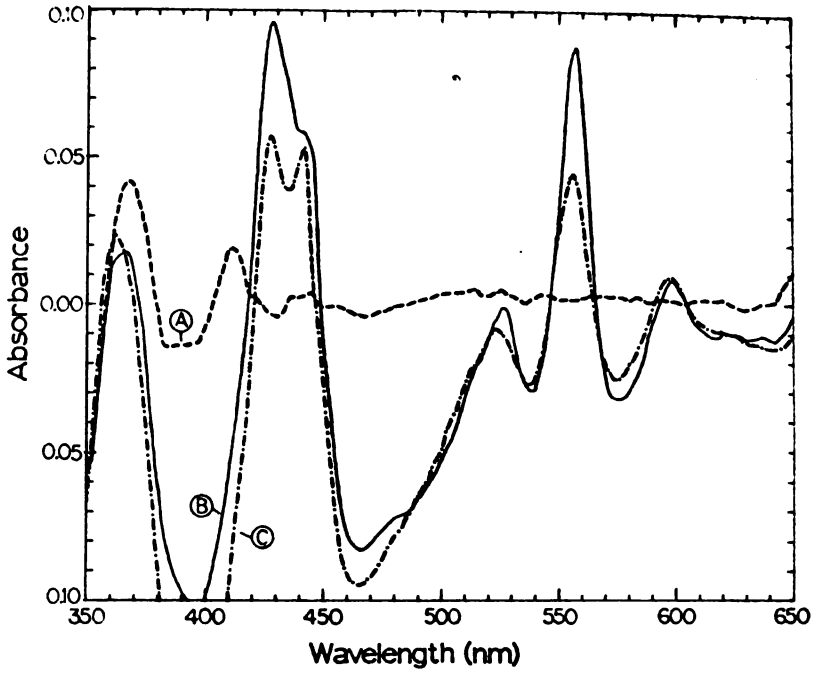

FIGURE 14

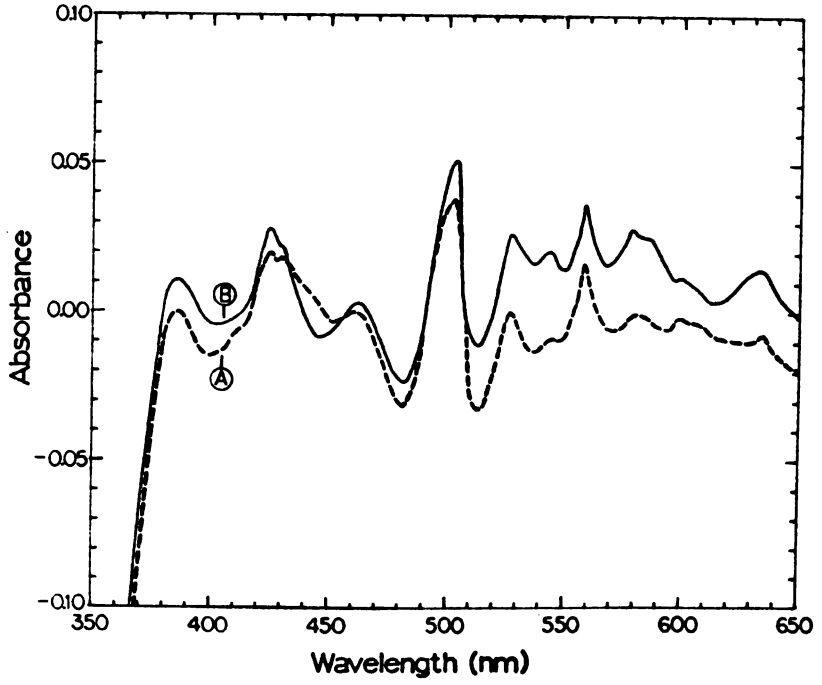

FIGURE 15

of *B. megaterium* was similar to that observed for the KM strain of *B. megaterium* so the above difference extinction coefficient was used to calculate the b-type cytochrome redox state of the KM strain of *B. megaterium*. The extent of reduction of the b-type cytochrome in the presence of excess L-malate was greater than the nonenzymatic reduction produced by dithionite and was considered to be fully reduced state. The aerobic state without substrate was considered to be the fully oxidized state. The redox state of the b_1-type cytochrome was calculated from the absorption difference between the fully oxidized state and the reduced state, produced by excess substrate and various amounts of HCP, and was expressed as percent reduction of the fully reduced state (Figure 16).

Cytochrome Content of Supernatant Solutions

The supernatant solutions of the original protoplast lysates, from which the various membrane preparations were obtained, also were examined by means of dithionite reduced minus oxidized difference spectra. In the case of the supernatant solution associated with the membranes lacking cytochrome a, the spectrum contained only a very broad peak around 435 nm. The spectrum of the supernatant solution associated with the membrane which contained cytochrome a showed a trough at 410 nm, a broad flavoprotein peak around 460 nm (4), and peaks at 557 nm and at 425 nm characteristics of the b_1-type cytochrome.

FIGURE 16. Effect of HCP on the redox state of the b_1 type cytochrome in membranes isolated from cells of *B. megaterium* which were grown in unbuffered peptone. Cytochrome content was calculated from the difference spectra using the difference extinction coefficient $(\varepsilon_{red}-\varepsilon_{ox})_{557.5nm}-(\varepsilon_{red}-\varepsilon_{ox})_{575nm}=$ 17.5 $mM^{-1}cm^{-1}$. The 100 percent reduced state was produced by adding 25 μmoles L-malate to the sample cuvette. The indicated amounts of HCP were added to the sample cuvette in 0.1 NaOH. All lines were fitted by regression analysis. Circles and triangles represent different batches of membranes. Symbols: (O) 0.1082 g membranes (dry wt) in 2.5 ml HEPES; (Δ) 0.2005 g membranes (dry wt) in 2.5 ml HEPES.

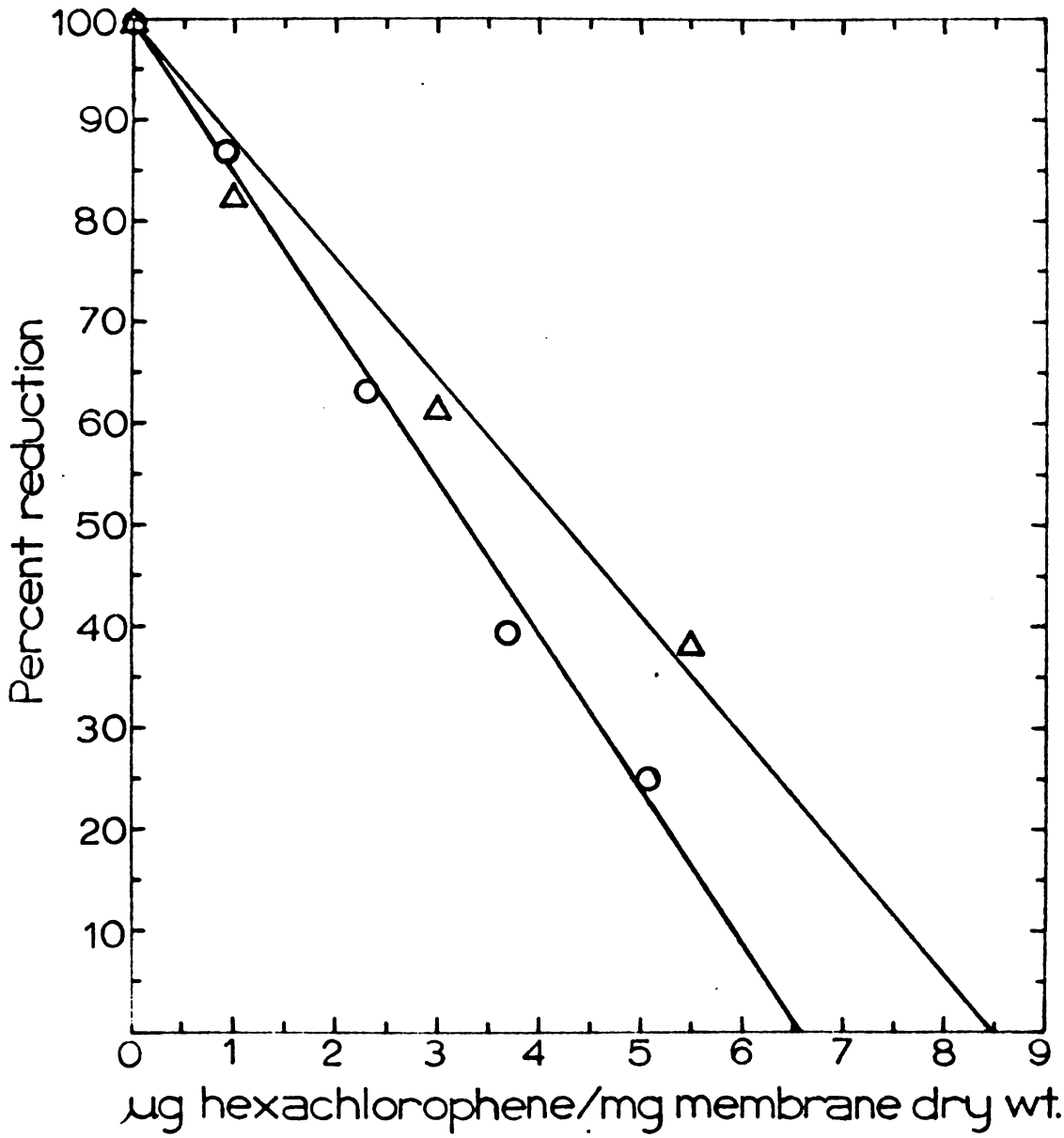
100
90
80
70
60
50
40
30
20
10
Percent reduction
0
1
2
3
4
5
6
7
8
9
µg hexachlorophene/mg membrane dry wt.

FIGURE 16

DISCUSSION

Effect of HCP on Soluble Dehydrogenases

HCP inhibited both membrane-associated and soluble dehydrogenases. Kinetic analysis of the effects of HCP on soluble NADH dehydrogenase (with respect to NADH) and soluble L-malate dehydrogenase (with respect to L-malate), indicated for both enzymes an inhibition of the partially competitive type. The interpretation of these kinetics is that HCP inhibited the association of substrates with the active site of the enzymes but did not affect the rate of transfer of electrons from substrates to DCPIP once the substrate-enzyme-inhibitor-DCPIP complex was formed. The exact nature of the interaction of HCP with the dehydrogenases could not be determined. Analysis of the plots of enzyme activity vs. HCP concentration for the soluble dehydrogenases (Figure 7 and Figure 11) indicated that HCP probably did not act as a substrate analog in reversible equilibrium with the substrate binding sites of the dehydrogenases. Since HCP binds to proteins and synthetic polypeptides (18,22) a working hypothesis could be that HCP inhibited by binding to the soluble dehydrogenases, either altering their tertiary structures or partially blocking their substrate binding sites, the result of such interaction being altered enzymes with lowered substrate affinity. The observation that the K_i for soluble NADH dehydrogenase was fourfold greater than that for soluble L-malate dehydrogenase may reflect the relative concentration of the two enzymes in the cytoplasm rather than different affinities for HCP.

Effect of HCP on Membrane-associated Dehydrogenases

HCP inhibited membrane-associated L-malate dehydrogenase and membrane-associated NADH dehydrogenase. The kinetics of inhibition of membrane-associated L-malate dehydrogenase, with respect to L-malate, were typical of mixed inhibition (Figure 4). The usual interpretation of such inhibition is that HCP inhibited both the association of L-malate with the dehydrogenase and the ability of the dehydrogenase to catalyze the transfer of electrons from L-malate to DCPIP once the substrate-enzyme-inhibitor-DCPIP complex was formed. However, since the dehydrogenase requires two substrates, L-malate and DCPIP, HCP inhibition of association of DCPIP with the dehydrogenase could be the reason for the apparently lower catalytic ability of the membrane-associated L-malate dehydrogenase.

The kinetics of inhibition of membrane-associated NADH dehydrogenase, with respect to NADH, were typical of non-competitive inhibition (Figure 8). The interpretation of these kinetics was that HCP inhibited the ability of the dehydrogenase to catalyze the transfer of electrons from NADH to DCPIP once the substrate-enzyme-inhibitor-DCPIP complex was formed. Again, HCP inhibition of the association of DCPIP with the dehydrogenase could have been the reason for this apparently lower catalytic ability of the membrane-associate NADH dehydrogenase.

Effect of HCP on Electron Transport in Membranes Isolated From Cells of *B. megaterium* Grown in Unbuffered Peptone

HCP appeared to disrupt the flow of electrons from substrate to oxygen in isolated *B. megaterium* membranes. The site of maximum HCP sensitivity appeared to depend on the cytochrome content of the membranes.

When membranes contained a b_1-type cytochrome, an a-type cytochrome, an a_3-type cytochrome, and lacked detectable cytochrome o, HCP caused both the b_1-type cytochrome and the a-type cytochrome to become oxidized (Figure 14). This indicated that HCP inhibited electron transport on the substrate side of the b_1-type cytochrome. Extrapolation of the line relating of percent reduction of the b_1-type cytochrome and HCP concentration to 0% reduction (the fully oxidized state, or the aerobic state without substrate) indicates that at a concentration of HCP within the range of 6 to 9 µg/mg membrane dry wt, HCP completely inhibited the flow of electrons to the b_1-type cytochrome (Figure 16). Since the K_i for the membrane-associated L-malate:DCPIP oxidoreductase is 23.7 µg HCP/mg membrane dry wt, and because the flow of electrons from L-malate to DCPIP is completely inhibited by about 50 µg HCP/mg membrane dry wt (Figure 5), L-malate dehydrogenase may not be the only site of inhibition between substrate and the b_1-type cytochrome. Kroger and Dadak (29) have presented evidence that a pool of membrane bound menaquinone, which is present in sixfold molar excess of the b_1-type cytochrome, is functionally linked to each of the dehydrogenases and is probably the only component which mediates electron flow from the dehydrogenases to the cytochromes. Addition of menadione, which resembles menaquinone but lacks the 35 carbon side chain, partially restored oxidase activity in HCP-inhibited membranes (Figure 2B and Figure 3B). HCP may bind to or inhibit menaquinone and exogenous menadione could then bypass this inhibition.

Alternatively, since the kinetics of inhibition of membrane-associated L-malate dehydrogenase indicated that HCP might inhibit by preventing association of DCPIP with the dehydrogenase, HCP inhibition

of oxidase activity might occur by inhibition of association of the physiological electron acceptor, menaquinone, with the dehydrogenase. The 20-fold excess of soluble menadione may have then overcome this inhibition.

B. megaterium has been reported to have two cytochrome oxidases, cytochrome a_3 and cytochrome o, which can be reduced by substrate and bind CO (2,3). When both cytochromes are present in equal amounts, cytochrome a_3 catalyzes a large fraction of the respiration and is relatively insensitive to CO (4). The relative amounts of these cytochromes vary with the growth stage of the organism (2). Since the reduced + CO minus reduced difference spectra of the membranes grown in unbuffered peptone lacked cytochrome o peaks or troughs, cytochrome a_3 probably catalyzed all of the oxidation and was apparently less sensitive to HCP inhibition than the dehydrogenases and the quinones were.

Effect of HCP on Electron Transport in Membranes Isolated From Cells of *B. megaterium* Grown in Phosphate-buffered Peptone

When the membranes contained a b_1-type cytochrome, an a_3-type cytochrome, and an o-type cytochrome but lacked detectable cytochrome a, addition of HCP to the L-malate-reduced membranes caused an increase in the b_1-type cytochrome reduction (Figure 12).

HCP has been reported to be a potent uncoupler of oxidative phosphorylation in mammalian systems (5,7,31). Uncoupling increases both oxygen uptake and the apparent reduction of the cytochromes. But HCP inhibited oxygen uptake in these membranes and since the P/O ratio of these membranes was zero even without HCP, uncoupling cannot be the reason for the apparent increase in b_1-type cytochrome reduction.

An alternate explanation for the increase in b_1-type cytochrome reduction is that HCP inhibited electron transport on the oxygen side of the cytochrome. The exact site of inhibition could not be determined but previous experimentation has indicated that it could be at cytochrome oxidase since HCP has been shown to inhibit the oxidation of mammalian cytochrome c by rat tissue preparations (5,19) and by intact cells of *Bacillus subtilis* (18). However the experiments of Gould *et al.* with *B. subtilis* involved the inhibition by HCP of ascorbate-reduced mammalian cytochrome c-induced oxygen uptake by intact cells of *B. subtilis*. Since it has been shown that even broken cell preparations of *B. subtilis* have only slight ability to oxidize this cytochrome (34), their results may be interpreted in other ways. For example, the HCP could be reacting with the mammalian cytochrome c to prevent its reduction by ascorbate.

Unless the a_3-type cytochrome in membranes isolated from cells that were grown in buffered peptone is more sensitive to HCP than the same a_3-type cytochrome in the membranes from cells grown in unbuffered peptone, it could presumably catalyze all of the oxidation even if the o-type cytochrome oxidase were totally inhibited by HCP. The site of maximum HCP sensitivity in these membranes isolated from cells grown in unbuffered peptone was probably between the b_1-type cytochrome and the oxidases. Since HCP is a surfactant and has been shown to physically disrupt membranes at high concentrations (>30 μg HCP/mg cell dry wt) (8,23,33), perhaps part of its action derives from disorganization of the cytochromes in the cell membrane. In the membranes which contained an a-type cytochrome between the b_1-type cytochrome and the oxidases, this disorganization apparently did not stop the flow of electrons.

Summary

HCP inhibited both soluble and membrane-associated dehydrogenases. Analysis of the inhibition kinetics indicated that HCP inhibited the association of substrates with the active site of the soluble dehydrogenases but did not affect the rate of transfer of electrons from substrates to DCPIP once the substrate-enzyme-inhibitor-DCPIP complex was formed. In the case of the membrane-associated dehydrogenases, HCP appeared to inhibit both the ability of these dehydrogenases to transfer electrons from substrates to DCPIP once the substrate-enzyme-inhibitor-DCPIP complex was formed, and the association of L-malate with L-malate dehydrogenase. In membranes, the site of HCP inhibition of electron transport appeared to depend on the cytochrome content. When the membranes contained a b_1-type cytochrome, an a-type cytochrome, and an a_3-type cytochrome, about 8 μg HCP/mg membrane dry wt inhibited electron transport on the substrate side of the b_1-type cytochrome, probably by inhibition of electron transfer from dehydrogenases to menaquinone. When the membranes contained a b_1-type cytochrome, an a_3-type cytochrome, an o-type cytochrome, but lacked an a-type cytochrome, as little as 2 μg HCP/mg membrane dry wt inhibited electron transport on the oxygen side of the b_1-type cytochrome, probably at a site between the b_1-type cytochrome and the terminal oxidases. This inhibition may have occurred by means of subtle disorganization of the electron carriers in the membrane.

The concentration of HCP necessary to completely inhibit electron transport in isolated membranes is less than one tenth of the minimum lethal concentration for *B. megaterium* cells. This

difference may be due to a protective effect of bacterial cell walls, or to a degradative effect on the membrane-bound electron transport system during isolation. More information on the effect of HCP on electron transport in cells of *B. megaterium* is now needed.

APPENDIX

APPENDIX A

K_i's have been calculated by the methods described by Webb (38) and by Dixon and Webb (10).

K_i = inhibitor constant. K_m = Michaelis constant. I = inhibitor concentration. $K_p = K_m(1+I/K_m)$. V_m = maximum velocity.

v = velocity. S = substrate concentration

Completely mixed inhibition:

The curve of the double reciprocal plot of 1/v vs. 1/S has a slope of:

$$\frac{K_m}{V_m}\left\{1+\frac{I}{K_i}\right\}$$

For the uninhibited reaction (I = 0), the slope can be simplified to K_m/V_m:

$$\frac{\text{SLOPE}_{\text{inhibited}}}{\text{SLOPE}_{\text{uninhibited}}} = \frac{\frac{K_m}{V_m}\left\{1+\frac{I}{K_i}\right\}}{\frac{K_m}{V_m}} = 1+\frac{I}{K_i} \qquad \text{(AA-1)}$$

In the case of the membrane-associated L-malate:DCPIP oxidoreductase (Figure 4), when I = 0 the slope of the least squares regression line is 53.196. When I = 14.098 µg HCP/mg membrane dry wt, the slope of the regression line is 84.724. Substituting these values into equation AA-1 and solving for K_i:

$$\frac{84.724}{53.196} = 1+\frac{14.098}{K_i}$$

K_i = 23.7 µg HCP/mg membrane dry wt.

APPENDIX B

Partially competitive inhibition:

The K_i for partially competitive inhibition can be calculated from the expression:

$$K_i = \frac{I}{\frac{K_p}{K_m} - 1} \left\{ 1 - \frac{K_p}{K'_m} \right\} \qquad \text{(AB-1)}$$

Where K'_m is the Michaelis constant in the presence of excess inhibitor. This quantity can be obtained by plotting K_p against 1/I and extrapolating the curve to infinite inhibitor concentration.

For soluble L-malate:DCPIP oxidoreductase (Figure 6), $K_m = 0.5472$, $I = 10.980$, $K'_m = 2.07$, and K_p when $I = 10.980$ is 0.9473. Substituting these values into equation AB-1:

$$K_i = \frac{10.980}{\left\{\frac{.9473}{.5472}\right\} - 1} \left\{ 1 - \frac{.5473}{2.070} \right\} = 11.049 \text{ µg HCP/mg protein.}$$

For soluble NADH:DCPIP oxidoreductase (Figure 10), $K_m = 2.799$, $I = 19.230$, $K'_m = 14.900$, and K_p when $I = 19.230$ is 3.6754. Substituting these values into equation AB-1:

$$K_i = \frac{19.230}{\left\{\frac{3.675}{2.799}\right\} - 1} \left\{ 1 - \frac{3.6754}{14.900} \right\} = 42.473 \text{ µg HCP/mg protein.}$$

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Adams, J. B. 1958. The mode of action of chloronated bisphenol antibacterials. Part I. Metal chelates of hexachlorophene and thiobisdichlorophenol. J. Pharm. Pharmacol. 10:507-515.

2. Broberg, P. L., and L. Smith. 1966. The cytochrome system of *Bacillus megaterium* KM. The presence and some properties of two CO-binding cytochromes. Biochim. Biophys. Acta 131:479-489.

3. Broberg, P. L., and L. Smith. 1968. A comparison of the properties of two CO-binding pigments in *Bacillus megaterium* KM, p. 182-187. In M. Kamen and I. Sekuzu (eds.) Structure and Function of Cytochromes. University Park Press, Baltimore, Maryland.

4. Broberg, P. L., M. Welsch, and L. Smith. 1969. Glucose dehydrogenase of *Bacillus megaterium* KM. Coupling of the cytoplasmic enzyme with membrane bound cytochromes. Biochim. Biophys. Acta 172:205-215.

5. Caldwell, R. S., H. S. Nakaue, and D. R. Buhler. 1972. Biochemical lesion in rat liver mitochondria induced by hexachlorophene. Biochem. Pharmacol. 21:2425-2441.

6. Cade, A. R., and W. S. Gump. 1957. The bis-phenols. p. 319-354. In G. F. Reddish (ed.) Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization. Lea and Febiger, Pennsylvania.

7. Cammer, W., and C. L. Moore. 1972. The effect of hexachlorophene on respiration of brain and liver mitochondria. Biochem. Biophys. Res. Comm. 46:1887-1893.

8. Corner, T. R., H. L. Joswick, J. N. Silvernale, and P. Gerhardt. 1971. Antimicrobial actions of hexachlorophene: lysis and fixation of bacterial protoplasts. J. Bacteriol. 108:501-507.

9. Deeb, S. S., and L. P. Hager. 1964. Crystalline cytochrome c from *Escherichia coli*. J. Biol. Chem. 239:1024-1031.

10. Dixon, M., and E. C. Webb. 1964. Enzymes, Academic Press Inc., New York.

11. Eisenberg, R. C. 1972. Reconstitution of *Micrococcus lysodeikticus* reduced nicotinamide adenine dinucleotide and malate dehydrogenase with dehydrogenase-depleted membrane residues: a basis for restoration of oxidase activities. J. Bacteriol. 112:445-452.

12. Eisenberg, R. C., L. Yu, and M. J. Wolin. 1969. Masking of *Bacillus megaterium* KM membrane reduced nicotinamide adenine dinucleotide oxidase and solubilization studies. J. Bacteriol. 102:161-171.

13. Eisenberg, R. C., L. Yu, and M. J. Wolin. 1970. Divalent cation activation of deoxycholate-solubilized and inactivated membrane reduced nicotinamide adenine dinucleotide oxidase of *Bacillus megaterium* KM. J. Bacteriol. 102:172-177.

14. Fiske, C. H., and Y. SubbaRow. 1925. The colorimetric determination of phosphate. J. Biol. Chem. 66:375-400.

15. Fujita, M. S., and N. Shimazono. 1966. Respiratory chain and phosphorylation site of the sonicated membrane fragments of *Micrococcus lysodeikticus*. J. Biochem. 59:104-114.

16. Galey, F. R. 1972. Hexachlorophene influence on ultrastructure of the CNS. p. 338-339. In C. J. Arceneaux (ed.), Proceedings 13th Annual Meeting of Electron Microscopy Society of America. Clailor's Publishing Division, Baton Rouge.

17. Gornall, A. G., C. S. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the buiret reaction. J. Biol. Chem. 177:751-766.

18. Gould, B. S., M. A. Bosinak, S. Niedleman, and S. Gatt. 1953. Effect of hexachlorophene and related bisphenolic compounds on the dehydrogenases and the cytochrome system of *Bacillus subtilis* and *Escherichia coli*. Arch. Biochem. Biophys. 44:284-289.

19. Gould, B. S., N. A. Frigerio, and W. B. Lebowitz. 1955. The action of bisphenolic compounds on succinoxidase, cytochrome c oxidase, and lactic dehydrogenase of animal tissue. Arch. Biochem. Biophys. 56:476-484.

20. Gould, B. S., N. A. Frigero, and J. Hovanesian. 1957. Studies of antibacterial action. The mechanism of adaption of certain bacteria to hexachlorophene (G-11). Antibiotics Chemotherapy. 8:457-454.

21. Gump, W. S. 1969. The toxicological properties of hexachlorophene. J. Soc. Cosmetic Chemists. 20:173-184.

22. Haque, R., and D. R. Buhler. 1972. A proton magnetic resonance study of the interaction of hexachlorophene with amides and polypeptides. J. Am. Chem. Soc. 94:1824-1828.

23. Joswick, H. L., T. R. Corner, J. N. Silvernale, and P. Gerhardt. 1971. Antimicrobial actions of hexachlorophene: release of cytoplasmic materials. J. Bacteriol. 108:492-500.

24. Kimbrough, R. D. 1971. Review of the toxicology of hexachlorophene. Arch. Environ. Health 23:119-122.

25. Kimbrough, R. D. 1973. Review of recent evidence of toxic effects of hexachlorophene. Pediatrics 51S:391-394.

26. Kimbrough, R. D., and T. B. Gaines. 1971. Hexachlorophene effects on the rat brain. Arch. Environ. Health 23:114-118.

27. King, T., and R. L. Howard. 1967. Preparation and properties of soluble NADH dehydrogenase from cardiac muscle. p. 275-276. In R. Estabrook and M. Pullman (eds.) Methods in Enzymology vol. 10. Academic Press, New York.

28. Lockhart, J. D. 1972. How toxic is hexachlorophene? Pediatrics 50:229-235.

29. Kroger, A., and V. Dadak. 1969. On the role of quinones in bacterial electron transport. The respiratory system of *Bacillus megaterium*. Eur. J. Biochem. 11:328-340.

30. Mullick, F. G. 1973. Hexachlorophene toxicity - human experience at the Armed Forces Institute of Pathology. Pediatrics 51S:395-399.

31. Nakue, H. S., R. S. Caldwell, and D. R. Buhler. 1972. Bisphenols-uncouplers of phosphorylating respiration. Biochem. Pharmacol. 21:2273-2277.

32. Nakue, H. S., F. N. Dost, and D. R. Buhler. 1973. Studies on the toxicity of hexachlorophene in the rat. Toxicol. Appl. Pharmacol. 24:239-249.

33. Silvernale, J. N., H. L. Joswick, T. R. Corner, and P. Gerhardt. 1971. Antimicrobial actions of hexachlorophene: cytological manifestations. J. Bacteriol. 108:482-491.

34. Smith, L. 1954. An investigation of cytochrome c oxidase activity in bacteria. Arch. Biochem. Biophys. 50:315-319.

35. Storck, R., and J. T. Wachsman. 1957. Enzyme location in *Bacillus megaterium*. J. Bacteriol. 73:784-790.

36. Umbreit, W. W., R. J. Burris, and J. F. Stauffer. 1964. Manometric Techniques, 4th edition. Burgess Publishing Co., Minneapolis.

37. Wade, N. 1972. Hexachlorophene curbed. Science 177:1175.

38. Webb, J. L. 1963. Enzyme and Metabolic Inhibitors vol. I. Academic Press, New York.

39. Weibull, C. 1953. Characterization of the protoplasmic constituents of *Bacillus megaterium*. J. Bacteriol. 66:696-702.

40. Vernon, L. P., and J. H. Mangum. 1960. Cytochromes of *Bacillus megaterium* and *Bacillus subtilis*. Arch. Biochem. Biophys. 90:103-104.

MICHIGAN STATE UNIV. LIBRARIES
31293102776741