Comparative Study of the Toxic Actions of 2,2-Bis-(p-Chlorophenyl)-1,1,1-Trichloroethane and 2,2-Bis(p-Chlorophenyl)-1,1-Dichloroethylene on the Growth and Respiratory Activity of a Microorganism Used as a Model

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A strain of *Bacillus stearothermophilus* was used as a model for a comparative study of the toxic effects of 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane and 2,2-bis(p-chlorophenyl)-1,1-dichloroethylene. Bacterial growth, the O₂ consumption rate, and respiration-related enzymatic activities provided quantitative data in agreement with results reported for other systems. The use of this bacterium for screening for chemical toxicity is discussed.

Different methods for screening for chemical toxicity have been developed in the last decade. Mitochondria, for example, have provided data that have been correlated with cytotoxicity parameters derived from cell cultures and whole organisms (9, 11, 22). Microorganisms, including yeasts (1, 19, 32, 33) and bacteria (13, 14, 28, 30), have also been used in toxicity tests that have resulted in the establishment of toxicity correlations regarding energy metabolism and cell viability (28–30). Moreover, the use of microorganisms has significantly reduced costly animal experimentation and has alleviated associated ethics issues.

In the present work, the use of a strain of *Bacillus stearothermophilus* as a model to study the toxic effects of membrane-active drugs was motivated by the following reasons: (i) because *B. stearothermophilus* is a gram-positive eubacterium, its cytoplasm is surrounded by a single membrane consisting of a lipid bilayer with phospholipids structurally similar to the eukaryotic counterparts, and pure membrane preparations can be easily isolated; (ii) because this organism is a thermophile, growth occurs at high temperatures, contamination is avoided, and within a couple of hours, a bacterial culture with high final cell density can be easily and economically obtained; and (iii) the growth of this bacterium is very sensitive to environmental agents that interact with the membrane (15, 20, 24), and efficient molecular mechanisms of adaptation mediated by alterations in the membrane lipid composition are induced by adverse growth conditions.

To test our bacterial strain as a tool for examining the toxicological effects of membrane-active xenobiotic compounds, we used the organochlorine insecticide 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane (DDT) and its major metabolite, 2,2-bis(p-chlorophenyl)-1,1-dichloroethylene (DDE), since the mechanisms of action of these compounds have been studied previously in other systems (2–8, 10, 17, 23, 26, 31, 34). Because of the lipophilic character of these compounds, biomembranes are potential target sites for interactions with them (27). Previously, it has been shown that DDT induces significant alterations in the thermotropic behavior of the lipid membranes of our bacterial strain along with impairment of bacterial growth (15). In this work, we extended the growth studies to DDE and attempted to establish a correlation between the effects of these compounds and the growth and respiratory activity of *B. stearothermophilus*.

**Effects of DDT and DDE on growth.** The strain of *B. stearothermophilus* used was grown at 65°C in a complex medium (L broth) to which DDT and DDE from concentrated ethanolic solutions were added to obtain concentrations ranging from 0.5 to 2.5 μM; controls were grown in medium without drugs, but with 10 μl of ethanol (i.e., the maximum amount of solvent used). Increasing concentrations of DDT or DDE induced progressively longer lag periods, lower specific growth rates, and lower bacterial yields (i.e., inhibition of growth). In a typical experiment (Fig. 1), addition of 0.5 μM DDT or 0.5 μM DDE had a negligible effect on growth, but the organism was significantly inhibited by 1 μM DDT or 1 μM DDE, as shown by the increases in the lag time (76% increase in the presence of DDT and 47% increase in the presence of DDE), the decreases in the specific growth rate (45% decrease with DDT and 18% decrease with DDE), and the decreases in the bacterial yield (35% decrease with DTT and 14% decrease with DDE). Growth was completely abolished in cultures containing either compound at a concentration of 2.5 μM.

**Effects of DDT and DDE on O₂ consumption.** O₂ consumption was measured with protoplasts prepared from cells from cultures harvested in the middle of the exponential growth phase by incubation with lysozyme, essentially as described by Wisdom and Welker (35). The protein contents of the protoplasts were determined by the biuret method, calibrated with bovine serum albumin (18). The oxygen consumption by the protoplasts was monitored polarographically with a Clark oxygen electrode connected to a recorder in a thermostat-controlled, water-jacketed, closed chamber (volume, 1 ml) with magnetic stirring at 45°C. DDT and DDE in dimethyl sulfoxide solutions were added to the reaction medium (40 mM HEPES-Tris, 10 mM MgCl₂; pH 7.5) with the protoplasts (protein content, 0.5 mg), and each preparation was incubated for 4 min before the addition of the substrate, either 10 mM NADH or 10 mM ascorbate–600 μM N,N,N’,N’-tetramethyl-p-phenylenediamine (TMPD). These substrates were chosen because they allowed us to study the full respiratory chain (NADH) or only the final oxidase segment (ascorbate-TMPD). Controls were prepared without drugs but with the same volume of...
of dimethyl sulfoxide. When NADH was used as the substrate (Fig. 2A), significant inhibition of the O₂ consumption rate was induced by both compounds, and the inhibition was concentration dependent in the range from 0 to 150 nmol/mg of protein for DDT and from 0 to 400 nmol/mg of protein for DDE. The inhibitory effect of DDT was significantly greater (more than twofold greater) than the inhibitory effect of DDE; at 100 nmol/mg, the respiratory rate was depressed 73 and 35% by DDT and DDE, respectively. When ascorbate-TMPD was used (Fig. 2B), the O₂ consumption rate was considerably less sensitive to inhibition by DDT and DDE. At the maximum concentrations assayed, levels of inhibition of 10 and 20% were observed with DDT (200 nmol/mg) and DDE (400 nmol/mg), respectively, whereas the respiratory rates supported by NADH were inhibited 82 and 63%, respectively. As expected, the O₂ consumption supported by NADH or ascorbate-TMPD was completely impaired by the addition of KCN (1 mM), as a consequence of complete inhibition of cytochrome oxidase. From these data, we concluded that DDT and DDE interact with the respiratory chain of *B. stearothermophilus* at a level(s) preceding the cytochrome oxidase segment.

**Effects of DDT and DDE on the activities of redox complexes.** To assay the activities of the terminal segment (cytochrome oxidase) and a middle segment (succinate-cytochrome c reductase) of the redox chain of *B. stearothermophilus* and the F₁ ATPase activity (reverse of F₀F₁ activity), membranes were isolated from bacterial cells by sonication of cells with a sonicator Vibracell model VC-100 for 10 to 15 min with 6-s pulses until the suspension became translucent. The membranes were collected by centrifugation at 148,000 × g for 90 min at 20°C, and the protein content was determined (18). The cytochrome oxidase activity was measured polarographically; the succinate-cytochrome c reductase activity was measured spectrophotometrically by following the reduction of oxidized cytochrome c as an increase in A₅₅₀. The F₁ ATPase activity (reverse of ATP synthase activity) was determined by monitoring the change in pH associated with ATP hydrolysis with a system composed of a Crison pH meter and a potentiometric recorder calibrated by adding standard acid aliquots at the end of each experiment. All of the enzymatic activities were measured at 45°C. Controls were prepared as in previous experiments by adding volumes of dimethyl sulfoxide corresponding to the maximum drug concentrations used in the enzymatic activity assays.

As expected from the O₂ consumption data, increasing concentrations of DDT (up to 200 nmol/mg of protein) or DDE (up to 400 nmol/mg of protein) induced limited decreases in the cytochrome oxidase activity (Fig. 3). The succinate-cytochrome c reductase activity was considerably inhibited by DDT up to a concentration of 200 nmol per mg of protein (Fig. 3A) and by DDE, although only at concentrations greater than 200 nmol/mg of protein (Fig. 3B). In contrast to the DDT data, the succinate-cytochrome c reductase segment cannot explain the effects of DDE on respiration, since the activity of this enzyme is not sensitive to DDE up to a concentration of 200 nmol/mg of protein, whereas the O₂ consumption supported by NADH was inhibited by increasing concentrations of DDE in the range from 0 to 200 nmol/mg of protein. Since respiration supported by NADH is markedly affected by DDT and DDE without corresponding effects on succinate-cytochrome c reductase, we concluded that NADH oxidase is strongly inhibited by DDT and its metabolite. The F₁ ATPase activity (reverse of F₀F₁ activity) of membranes of *B. stearothermophilus* (Table 1) was not affected by DDT in the concentration range studied (0 to 200 nmol/mg of protein). In the same concentra-

![Figure 1](http://aem.asm.org/) Effects of DDT and DDE on the growth of *B. stearothermophilus* at 65°C in medium (dilute L broth) containing no DDT or DDE (○), 0.5 μM DDT (●), 1.0 μM DDT (▲), 2.5 μM DDT (◇), 0.5 μM DDE (□), 1.0 μM DDE (△), or 2.5 μM DDE (□). Data for growth in the presence of DDT have been reported previously (15) and are included to provide an effective comparison of the effects of DDT and DDE on growth. OD (610 nm), optical density at 610 nm.

![Figure 2](http://aem.asm.org/) Effects of DDT and DDE on the O₂ consumption rate of *B. stearothermophilus* protoplasts when 10 mM NADH (A) or 10 mM ascorbate–600 μM TMPD (B) was used as the substrate. *B. stearothermophilus* was grown at 65°C in dilute L broth under control conditions (without xenobiotic compounds). The graphs show the levels of inhibition of the respiratory rate compared to the control (protoplasts incubated for 4 min without xenobiotic compounds in the presence of a few microliters of dimethyl sulfoxide, the xenobiotic compound solvent).
showed that the O_2 consumption rate supported by NADH was
tase activity, essentially in agreement with our data which
demonstrated in rat liver mitochondria (21), and DDT is more efficient than DDE.
inhibit state 3 and state 4 respiration in cockroach coxal muscle
those obtained with isolated mitochondria which have been
found a reasonable correlation by comparing these data with
nor the F_1 ATPase activity of rat liver mitochondria (17). The
stronger than depression of the succinate-cytochrome
DDE-induced inhibition of state 3 respiration is considerably
ATPase was not affected by the insecticide. On the other hand,
sensitive to DDT, in contrast to succinate-cytochrome c
mitochondrial F_1 ATPase by DDT, whereas the bacterial F_1
to our data, the previous study reported stimulation of the
In contrast to our data, the previous study reported stimulation of the
mitochondrial F_1 ATPase by DDT, whereas the bacterial F_1
ATPase was not affected by the insecticide. On the other hand,
Ferreira et al. (17), using rat liver mitochondria, showed that
DDT-induced inhibition of state 3 respiration is considerably
stronger than depression of the succinate-cytochrome c reductase
activity, essentially in agreement with our data which showed that the O_2 consumption rate supported by NADH
was inhibited by DDE concentrations to which the succinate-cytochrome c reductase activity was not sensitive. Finally, DDE
does not affect the F_1 ATPase activity of B. stearothermophilus
nor the F_1 ATPase activity of rat liver mitochondria (17). The

data obtained with our bacterial model for these membrane-active xenobiotic compounds are consistent with data obtained
with other toxicity tests and have the following advantages: (i) bacterial growth is easy and economic; (ii) our model avoids
ethical issues associated with animal use and is an alternative
in vitro toxicity test; and (iii) our model allows workers to
correlate, in the same model, the toxicity of xenobiotic compounds as assessed in vivo by growth perturbations and the in
vitro toxic effects on cell functions and physical perturbations of membranes.

Conclusions. The greater inhibition of respiratory enzyme
activities by DDT than by DDE is consistent with the greater
inhibitory effect of DDT on bacterial growth. Since very similar
partitionings of DDT and DDE have been estimated to occur
in native and model membranes (7, 34) and in bacterial lipid
membranes (16), the differences in the toxic effects of these
insecticides are not related to effective membrane
central. In other systems, DDE also exhibited lower toxicity than
DDT (10, 12, 21, 25). Therefore, we conclude that growth and
respiration studies performed with our bacterial model, which
reflects the lower toxicity of DDE compared to DDT, may be
potentially useful as an alternative in vitro method for screening
tests for chemical toxicity.

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6. Antunes-Madeira, M. C., and V. M. C. Madeira. 1982. Interaction of insec-

<table>
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<th>Xenobiotic compound</th>
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* Data are means ± standard deviations from three to five independent
experiments.

* Data are expressed as percentages of the control value (without xenobiotic compounds).