Location and Consequences of 1,1,1-Trichloro-2,2-bis(p-Chlorophenyl) Ethane Uptake by Bacillus megaterium

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No detrimental effects of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) were observed when cells of Bacillus megaterium were grown from small inocula in nutrient media containing up to 100 μg of DDT/ml. However, when the ratio of DDT to biomass of resting cells was held constant, levels of DDT as low as 1 μg/ml (0.5 μg/mg of cell dry weight) enhanced the rate of death in the population. The lethal action of DDT was both time- and dose-dependent so that higher doses required less time to effect the same killing than did lower doses. Intact cells bound a maximum of about 1.7 μg of DDT/mg of cell dry weight, of which about 75% was localized in the protoplast membrane. Much of the bound DDT was subsequently lost to the suspending medium and the aqueous stability of the returned DDT was enhanced, possibly by association with solubilized cell materials. A small quantity of bound DDT was converted to 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane, which was released from cells somewhat faster than DDT. Apparently the lethal action of DDT was related to its binding in the membrane, but respiration was not inhibited. The atypical macroscopic appearance of membranes isolated from treated cells suggested that cell death may result from altered membrane chemistry.

1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) continues to be a heavily used organochlorine insecticide worldwide (21), though recently its use in the United States has been restricted. Its buildup and persistence in the biosphere has aroused considerable interest in the effects of this pesticide on soil microorganisms. When spread on agar containing surface films of various organochlorine insecticides, gram-positive bacteria are generally more susceptible to growth inhibition than are gram-negative bacteria (16). Other effects reported in microorganisms range from increased growth of some species to complete inhibition of others (3, 9, 18). In addition to these gross effects, more detailed mechanisms of action have been suggested. Winemly and San Clemente (18) demonstrated inhibition of respiratory activity in Nitrobacter agilis by 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane (DDD; a common degradation product of DDT) and other pesticides. DDT also may inhibit certain specific enzymes (7).

DDT is known to accumulate in the lipid-containing tissues of many higher organisms, particularly those high in food chains (6), but soil microorganisms also accumulate it. Chacko and Lockwood (2) reported that bacteria centrifuged from an aqueous suspension containing 1 μg of DDT/ml retained between 38% (Bacillus subtilis) and 100% (Agrobacterium tumefaciens) of the DDT to which they were exposed. Selected chlorinated hydrocarbons were taken up from both water and soil by fungal and actinomycete myelia (2, 8). Cells of Euglena gracilis also have been reported to take up DDT from their environment (4). These studies were concerned with uptake by intact cells, and little is known of the distribution of DDT within the cells.

In this paper, we report on experiments designed to relate the gross lethal effects of DDT to the site of localization within cells of B. megaterium, a representative gram-positive soil bacillus. Even with high ambient concentrations of DDT, no effect could be elicited when the ratio of DDT to biomass was allowed to vary. When the ratio of DDT to biomass was held constant, cells of B. megaterium were killed by concentrations of DDT as low as 0.5
µg/mg of cell dry weight. Cells bound a considerable amount of the DDT to which they were exposed, and most of the bound pesticide was located in the limiting membrane. However, inhibition of membrane-bound or other respiratory enzymes was not detected.

MATERIALS AND METHODS

Organism and growth conditions. Cells of the asporogenous KM strain of B. megaterium were grown in aerated 2% (wt/vol) Oxoid peptone (Flow Laboratories, Rockville, Md.) broth (pH 7.0) at 30 ± 1°C. Growth was monitored by following absorbance at 700 nm in a Spectronic-20 spectrophotometer (Bausch & Lomb, Inc.) in 1.1-cm light path cuvettes with distilled water as a blank. Optical densities were correlated with viable cells per milliliter and with cell dry weight per milliliter. Viable counts were made by serially diluting the cultures in 1.2% (wt/vol) Bacto-peptone (Difco) and spreading in triplicate on Trypticase soy agar (BBL). Colonies were counted after approximately 30 h of incubation at room temperature.

Exposure of cells to DDT in growth experiments. Approximately 10⁷ cells were spread on Trypticase soy agar containing 1, 10, or 100 µg of DDT/ml. The appropriate amount of pesticide in 0.1 ml of acetone was added to 100 ml of the warm agar prior to pouring plates. In other experiments, paper disks were wet with acetone solutions of DDT containing 1, 10, or 100 µg of DDT/ml, allowed to dry, and placed on Trypticase soy agar plates previously spread with about 10⁷ bacteria. Bacterial growth was determined visually.

About 3 × 10⁶ cells were also inoculated into each of several flasks of Oxoid peptone broth which contained, variously, 0.1, 1, 10, 50, or 100 µg of DDT/ml, acetone alone, or broth alone. DDT solutions were added by means of a pipette rather than a syringe. The final concentration of acetone was 0.0136 M, or 0.1%. Optical densities at 700 nm were recorded before the addition of cells and at various times through the culture cycle until exponential growth was completed. Viable counts and microscopic observation were also used to assess possible pesticide effects.

Exposure of cells to DDT in survival experiments. Cells were harvested from peptone broth by centrifugation when the optical density at 700 nm reached from 2.20 to 2.40, or about 2.2 ± 0.1 mg of cell dry weight/ml, and were washed twice by resuspension in sterile 0.1 M potassium phosphate buffer, pH 7.00 ± 0.05 (hereafter called buffer). These and all succeeding centrifugations were carried out at 4°C. Cells at this optical density were in the late exponential phase of growth. They were then introduced to 99 ml of buffer-DDT-acetone mixtures to bring the final concentrations to 2.0 mg of cell dry weight/ml and the final volume to 100 ml. Controls consisted of cells in buffer alone and cells in buffer plus 0.0136 M acetone (initial concentration). The flasks containing these mixtures were agitated for 24 h. Periodically, 1.0-ml samples were taken, and the number of survivors was determined by the viable counting procedure. Similarly, the effects of 0.136 or 0.00136 M acetone on these cells was determined.

Respiratory measurements. Oxygen uptake at 30°C was measured by standard Warburg techniques (17). Cells were suspended to a concentration of 2.0 mg of cell dry weight/ml in buffer containing 1, 10, 50, or 100 µg of DDT/ml. Oxygen uptake was measured periodically for various time periods. When desired, 0.1 ml of 2% (wt/wt) glucose was added from the side arm. For oxygen measurements after long-term exposure to DDT, 100 ml of a mixture of cells and DDT in buffer was incubated with aeration at 30°C prior to being placed in the Warburg vessels.

DDT extraction and gas chromatography. Each sample to be analyzed for pesticide content was extracted initially with a mixture of n-hexane and isopropanol (3:1) followed by two more extractions with hexane alone. The hexane fractions were pooled and washed three times with equal volumes of distilled water. If a sample contained a high concentration of sucrose, three volumes of distilled water were added to the sample before the extraction procedure was begun. Any necessary evaporations of the pesticide-hexane solutions were performed at room temperature and were of limited duration. In any case, only a small percentage of the total volume was evaporated. Under these conditions, losses of DDT due to evaporation were negligible. All implements and chemicals were extracted with the hexane-isopropanol solution to test for interfering substances. Glassware was washed with acid and with hexane prior to use. Polypropylene or cellulose nitrate materials were washed with hexane alone.

A Beckman gas chromatograph, model GC-5, equipped with an electron capture detector and a 1.83-m glass column (3-mm inner diameter) was used for pesticide analysis. Three different packings were used on 60 to 80 mesh Glass Chrom Q: (i) 2% DC 11, (ii) 1.5% OV-17/1.95% QF-1, or (iii) 4% SE 30/6% QF-1. Column temperatures were 190, 205, and 205°C, respectively. Inlet temperatures in all cases were 220°C. The carrier gas (helium) had a flow rate of 25 ml/min. Stable base lines were observed in all experiments reported.

Separation of suspended DDT and cells. Discontinuous gradients comprised of six layers, successively 4 ml each of 70, 60, 50, 40, 30, and 29% (wt/wt) sucrose solutions, were employed to separate cells from sedimentible DDT. Cells, suspended at 2.0 mg/kg of cell dry weight/ml, centrifuged (10,400 × g, 10 min), washed with 100 ml of buffer, and resedimented. This procedure eliminated virtually all acetone and non-sedimentible pesticide. The pellet was thoroughly washed in 0.8 ± 0.1 ml of buffer in a centrifuge on a swinging-bucket rotor (Sorvall, HS-4), which was allowed to coast to a stop. The pellets formed a band above the 50% sucrose solution, whereas most DDT was forced into the 70% layer (see Fig. 2). The cell band and other fractions were separated by use of a Cornwall syringe, and each fraction was quantitatively assayed for pesticide.
Other experiments were undertaken to test the efficiency of this separation procedure. An identical preparation of cells, not exposed to DDT, was treated in the same fashion, and the resultant gradient fractions were analyzed for protein by the method of Lowry et al. (11). To facilitate a reasonably accurate protein analysis, each fraction was washed twice (16,300 × g, 10 min) with 100 ml of buffer to reduce the sucrose content (5). Similarly, a 1.0-ml preparation containing approximately 500 μg of DDT and virtually no acetonite was sedimented through an identical gradient. Acetone was removed by heating the suspension at 80 C for 2 h. The gradient was then fractionated and analyzed for DDT.

**DDT uptake by whole cells.** A 500-ml sample of buffer containing 10 μg of DDT/ml was inoculated with 1 g (dry weight) of cells harvested from the exponential phase of the culture cycle when the culture optical density at 700 nm was between 2.4 and 2.6. These optical densities are equivalent to from 2.3 to 2.4 mg of cell dry weight per ml. At periodic intervals over 30 h, 50-ml samples of this cell suspension were prepared for gradient centrifugation and were centrifuged (4,470 × g, 4 min). On removal of the cells from the gradient, 50 ml of buffer was added to dilute the sucrose. The cells were sedimented (10,400 × g, 10 min), resuspended in 10 ml of hexane-isopropanol (3:1), and stored in the cold until the pesticide extraction procedure could be completed. Larger relative centrifugal forces (a speed of 55,000 rpm in an A-321 rotor, representing from 100,000 to 280,000 × g, 1 h) were employed, with the use of preparative ultracentrifuge (IEC model B-60) in an attempt to sediment additional material from the supernatant fluids.

**DDT uptake by limiting membranes.** The procedure was identical to the one for whole cells up to the addition of the hexane-isopropanol mixture, except that the 50-ml samples were prepared and layered on each of two gradients. The cells from the gradients were pooled before sedimentation from the diluted sucrose solution. The cells were then subjected to lysozyme treatment (0.5 mg of lysozyme/20 mg of cell dry weight) for about 2 h at 30 C. After lysis was completed, about 5 mg of deoxyribonuclease was added to the mixture to reduce viscosity. After 1 h of incubation with deoxyribonuclease, the mixture was centrifuged (3,300 × g, 6 min) to remove whole cells, cell debris, and most poly-β-hydroxybutyrate granules. The supernatant fluid was centrifuged (19,600 × g, 20 min) to sediment the membranes. The membrane pellet was washed in 100 ml of buffer and resedimented under identical conditions. The pellet was then suspended in 20.0 ml of buffer and divided into two 10.0-ml portions. One was mixed with the hexane-isopropanol solution and the other was used for dry weight analysis. The supernatant fluids of several successive buffer washes from a membrane preparation not exposed to DDT had approximately equal 260 to 280 nm absorbance ratios. Hence, one wash was deemed adequate to remove most soluble material from the membrane preparation. A Beckman model D poly-0-hydroxybutyrate gradient ultracentrifuge (model D-6) was used for these determinations. Observations of KαMnO₄-stained membrane preparations made with a Philips 300 electron microscope evidenced little, if any, contaminating material.

**Chemicals.** The composition of 99 + % p,p'-DDT (City Chemical Corp., New York, N.Y.) was verified by gas-liquid chromatography. Nanograde n-hexane and Nanograde isopropanol were obtained from Mallinkrodt Chemical Co. (St. Louis, Mo.), lysozyme and deoxyribonuclease from the Sigma Chemical Co. (St. Louis Mo.), and column packing materials from Applied Science Laboratories (State College, Pa.).

**RESULTS**

**Action of DDT on resting cells.** Resting cells of *B. megaterium* were killed by exposure to all concentrations of DDT tested (Fig. 1). After 15 h of exposure, the number of viable organisms in the flask containing 10 μg of DDT/ml was reduced about 10-fold as compared with the number of viable cells in the control flasks. Initially, the cell count per milliliter in the mixtures without DDT, with acetone alone, or with 1 μg of DDT/ml appeared to increase. Microscopic examination of the preparations suggested that these rises in count were due to fragmentation of chains of organisms. Such fragmentation may have resulted from the agitation which the cells suf-

![Fig. 1. Effect of DDT on the viability of *B. megaterium*. Cells (2.0 mg of dry weight/ml) were agitated in buffer containing 1 μg of DDT/ml (○), 10 μg of DDT/ml (△), 50 μg of DDT/ml (□), 100 μg of DDT/ml (◇), acetone alone (▲), or no addition (●). Values are representative of six experiments.](image-url)
fered during the exposure period. Alternatively, endogenous energy and carbon sources may have been able to provide for a limited number of cell divisions. Microscopic examinations dis-
counted the possibility that plate counts were reduced by the aggregation of cells around fine crystals of DDT or other substances. In other experiments, acetone at initial concentrations up to 0.136 M was found to have a negligible effect on cell viability.

Action of DDT on growing cells. Cells grown in peptone broth culture containing DDT were not measurably affected by concentrations up to 100 μg of DDT/ml. No significant differences in culture optical densities or viable counts were found among the controls and pesticide-containing cultures. Optical densities at 700 nm of the broth media were not affected by the addition of DDT or acetone or both. Moreover, growth was not inhibited on plates containing up to 100 μg of DDT/ml of agar nor on agar surfaces with filter-paper disks saturated with DDT solutions of 1, 10, or 100 μg/ml.

Respiratory measurements. No significant differences in the rates of oxygen utilization (measured with and without the addition of glucose) were observed among the various concentrations of pesticide and controls (Table 1). The rates of oxygen uptake dropped evenly over a 24-h period.

Separation of suspended DDT from cells. It soon became apparent that routine centrifugation procedures could not provide adequate separation of cells and fine particles of DDT. Consequently, the separation procedure based on density gradient centrifugation in sucrose solution was developed. In a typical experiment (Fig. 2), the cell pellet from a mixture exposed to 10 μg of DDT/ml (or 5 μg of DDT/mg of cell dry weight) for 4 h was layered

on the gradient and centrifuged. The bulk of the DDT went to the 70% sucrose layer, whereas the DDT in the 40% layer was associated with the cells that banded there (Fig. 2B). The distributions of cells alone as assessed by protein determination (Fig. 2A) or DDT alone (Fig. 2C) confirmed this observation. Most DDT settled into the 70% sucrose, whereas cell protein remained mostly in the 40% layer. When cells not previously exposed to DDT were mixed with 0.5 mg of crystalline DDT just prior to centrifugation, results virtually identical to those shown in Fig. 2C were obtained.

DDT uptake by whole cells or isolated membranes. The amount of pesticide detected in the cells initially increased and then decreased as time progressed (Fig. 3). Decreases were accompanied by corresponding increases in the amounts of pesticide in cell supernatant fluids and cell washes. Thus, the total recovered pesticide, including both DDT and the only detectable degradation product (DDD; see below), remained essentially stable over 24 h. About 48 and 50% of the pesticide could be sedimented from the exposure medium supernatant fluids by ultracentrifugation at 0 and 1.5 h, respectively, compared with 34 and 26% at 8 and 24 h, respectively. The initial amount of DDT in each 50-ml sample was approximately 500 μg. Hence, the maximal uptake of the pesticide by 100 mg (dry weight) of whole cells was about 175 μg or about 35% by weight of the DDT.

Exposure to DDT did not markedly interfere with cell wall dissolution by lysozyme or with subsequent protoplast lysis. However, membranes isolated from treated cells were of a whitish color, whereas those from untreated cells were a pale yellow-brown. Membranes

| Table 1. Effect of exposure to DDT on oxygen uptake by intact cells of B. megaterium* |
|-----------------|-----------------|-----------------|-----------------|
| Exposure time (h) | Control | Acetone | Control | Acetone |
| 0 | 38 | 35 | 38 | 39 | 36 | 110 | 118 | 105 | 107 |
| 4 | 15 | 17 | 15 | 14 | 18 | 16 | 117 | 112 | 110 | 112 | 100 | 105 |
| 8 | 11 | 12 | 9 | 12 | 9 | 79 | 77 | 81 | 81 | 82 |
| 16 | 7 | 5 | 6 | 6 | 5 | 6 | 80 | 80 | 73 | 80 | 80 |
| 24 | 6 | 6 | 3 | 7 | 3 | 55 | 62 | 60 | 59 | 59 |

*Cells were treated with 0.0136 M acetone, 0.0136 M acetone containing DDT, or untreated for the indicated number of hours. Then samples (2.8 ml) were placed in the main compartments of the Warburg flasks. After 20 min of equilibration at 30 C, 0.1 ml of 2% (wt/wt) glucose solution or 0.1 M phosphate buffer was tipped in to start the determination. Center wells contained 0.1 ml of 20% (wt/wt) KOH.
isolated from cells treated with 0.5 μg of DDT/mg of cell dry weight showed an uptake pattern qualitatively similar to that of whole cells (Fig. 3). For the first 1.5 h of exposure, the pesticide content of the membranes increased to a maximum of about 120 μg/100 mg of cell dry weight, and then decreased at a rate similar to the decrease seen with whole cells.

The reported values for the pesticide content of the membranes were calculated on the assumption that the membrane comprised 6.5% of the cell dry weight as given by Yudkin (22). Membrane yields from untreated cells were actually about 6.1%, but those from exposed cells were only about 2.5 to 3.3% of the initial dry weight. The maximal amount of pesticide in the membranes isolated under these conditions represented about 4% (wt/wt) of the recovered membrane dry weight.

The recorded times in Fig. 3 do not reflect the preparation time (approximately 40 min) between the removal of the sample from the exposure medium and the gradient separation of the suspended pesticide and cells. Factors such as lowering of temperature (through centrifugation) and sequential reduction of acetone concentration (through the washing of cells) may have affected the rate of uptake during these intervals.

**Conversion of DDT to DDD.** DDD was produced almost immediately after the addition of the cells to the buffer containing DDT (Fig. 4). However, after about 2 h of exposure, DDD was more easily eluted from the cells than was DDT. Analysis of pesticide in suspension in buffer or in sucrose solution or mixed with heat-killed cells in buffer indicated that DDD production took place only in the presence of viable cells. To exclude the possibility that DDD was being produced after the addition of hexane-isopropanol to the cell or membrane suspensions, DDT was added to unexposed cells previously layered with hexane-isopropanol solution. Under these conditions, no DDD was detected. The solvent also eliminated any viable organisms as determined by plate counting. Periodic checks of known DDT solutions suggested that photoconversion to DDD did not take place.

**DISCUSSION**

The discrepancy between the accelerated reduction in viability observed with resting cells (Fig. 1) and the apparent lack of effect of DDT on growing cells may be due to a number
of factors. The most prominent of these concerns the rather drastic change in the ratio of DDT to biomass in a growing culture. This ratio is halved after each doubling of the population. Under the described growth conditions, the generation time of *B. megaterium* is approximately 80 min. At all DDT levels tested, significant numbers of cells survived after only 80 min of exposure. Hence, a small initial reduction of living cells in broth cultures containing DDT would have little measurable effect on the culture cycle. Differences between the environment of the growing cells and that of the resting cells (peptone-water as opposed to buffer) may have altered the osmotic responses or metabolic pathways of the cells. The extent of uptake of DDT may be influenced by environmental conditions which alter the solubility of DDT or by interactions of DDT with various medium components. The lack of inhibition observed on the agar plates containing DDT might be accounted for in a similar way. Recent results (16) indicated that *B. megaterium* is totally inhibited on agar containing a surface film of 0.5 mg of DDT/cm², a much higher concentration than any used here.

The conditions under which the resting cells were exposed would probably more closely approximate the physiological state of bacteria in soils than would bacteria grown in rich media. Much of the time, undomesticated bacteria are in a condition of relative starvation (1). However, the temptation to draw direct parallels to a soil ecosystem should be avoided.

Soils vary greatly in biological and chemical complexity, and fluctuating parameters, such as water content, affect the degree of adsorption of DDT to soil particles (14). Thus, pesticides may be less readily available to microorganisms in soil than they were in the experiments here.

The solubility of DDT in water, as defined by gravitational stability, has been reported to be about 5 ng/ml (14). The precise solubility of DDT in buffer was neither investigated nor defined, but can be considered to be less than 1 µg/ml as evidenced by the fine suspensions that resulted when DDT in acetone was introduced into buffer. As the DDT suspensions were shaken over a period of hours, slightly larger aggregates of DDT or DDT complexes appeared. These paracrystalline aggregates probably resulted from a reduction in acetone concentration by evaporation. When distilled water was substituted for the buffer, virtually identical results were obtained.

Clearly, the cells took up DDT rather slowly (Fig. 3), but ultimately a maximal concentration of 1.7 µg/mg of cell dry weight was attained. This represented about a 190-fold increase over the environmental concentration of DDT. Thus, the cells act as a DDT sink. The rate at which the cells accumulate DDT appears to be a function of the low water solubility of DDT and seemingly is governed by the rate at which DDT can be pumped from the microcrystal reservoirs through the aqueous medium into the cell sink. A certain amount of the observed uptake may have resulted from direct interactions between cells and solid-phase DDT. Thus, the rate of uptake would have been influenced by the rate at which such transfer occurred. However, it seems that most of the DDT taken up must first pass through a solubilization process because, as previously pointed out, when cell pellets were mixed with crystalline DDT in short-term experiments, there was no detectable uptake. In this context, it has been suggested that various aromatic hydrocarbons are taken up by cells from aqueous solution rather than from the solid phase (19, 20).

Our findings that the cells accumulated a maximum of about 35% of the DDT after 1.5 h of exposure, which dropped to 9% at 24 h, are in apparent quantitative disagreement with previous results. Chacko and Lockwood (2) reported that various soil microorganisms took up between 38 and 100% of the DDT to which they were exposed. However, differences in organism and pesticide concentration, different

![Graph](image-url)
techniques for separating suspended pesticide from cells, and possible differences in cell concentration and viability all preclude direct comparison.

The progressive loss of DDT from the membrane fraction (Fig. 3) was reflected in a concomitant loss by intact cells. Thus, the decrease in DDT in the membrane did not result from partitioning into other subcellular fractions such as the cytoplasm. Rather the DDT returned to the suspending medium. That this DDT may have partitioned into solid-phase DDT is possible but unlikely, since less DDT could be sedimented by ultracentrifugation of the exposure medium after long exposure periods than after short ones. Moreover, the pesticide did not crystallize as it left the cells because pesticide crystals would have been detected by the gradient technique. The increased aqueous stability of the released DDT may have been caused by the association of the DDT with cell materials, perhaps membrane lipids, lost to the medium as the cells gradually deteriorated.

Many of the enzymes of terminal oxidative respiration are bound to the protoplast membrane in B. megaterium (15). Thus, it would not have been surprising had respiratory inhibition resulted from the extensive accumulation of DDT detected in the membrane fraction (Fig. 3). However, there are a number of other important processes that are intimately associated with the limiting membrane, including those involved in cell division, chromosome replication and segregation, and protein synthesis (12). Of particular significance in this regard is the qualitative finding that membranes from treated cells apparently have a slightly different color and probably a different chemistry than those from untreated cells. Several other compounds are known to inhibit selectively the synthesis of certain membrane components, e.g., diphenylamine (13) or to solubilize certain membrane components selectively, e.g., alkylamine HCl (10). Thus, this observation may represent a clue to the way DDT kills bacterial cells.

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