DISTRIBUTION AND EFFECTS OF DDT AND 2,4,5-T IN BACILLUS MEGATERIUM

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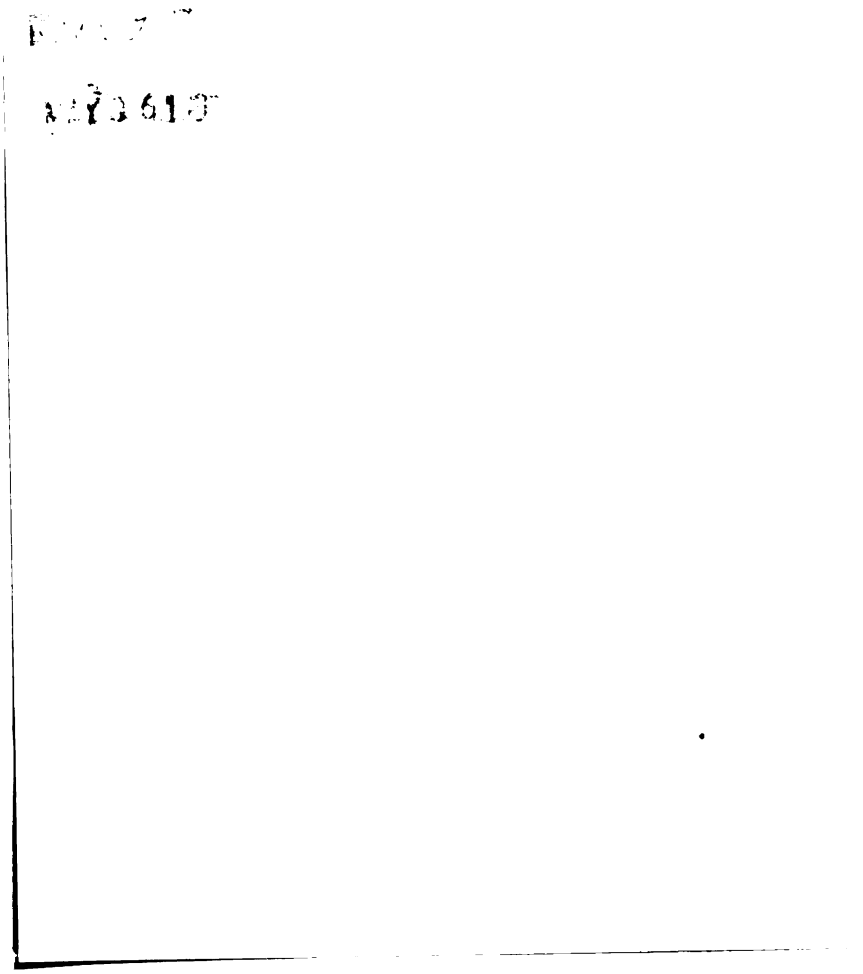

ABSTRACT

DISTRIBUTION AND EFFECTS OF DDT AND 2,4,5-T IN *BACILLUS MEGATERIUM*

By

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DDT, a water insoluble insecticide, and 2,4,5-T, a water soluble herbicide, exerted similar effects on cells of *Bacillus megaterium*. The viability of cells in non-nutritive media was reduced after several h exposure to 10 µg/ml (5 µg/mg cell dry weight) of either pesticide. Respiration was not affected by either pesticide at 100 µg/ml. Growth was affected only at 100 µg 2,4,5-T/ml. A small amount of DDT was degraded to a structurally similar compound, DDD, but no other degradation products were evident.

A sucrose gradient centrifugation procedure that separated particulate DDT from cells in suspension was developed and characterized to allow precise measurement of DDT uptake by cells *in vitro*. Each pesticide partitioned into viable cells which retained 175 µg DDT or 0.5 µg 2,4,5-T/100 mg of cells (dry weight). About 75% of the accumulated DDT or 60% of the accumulated 2,4,5-T

was sequestered by the limiting membrane. The DDT retained in the membrane represented about 2% of the membrane dry weight. Chemical analyses indicated that DDT but not 2,4,5-T replaced membrane lipids. After about 1.5 h exposure, DDT and the generated DDD were slowly eluted from the cells but DDD was eluted at a faster rate. The eluted DDT also became much more gravitationally stable in aqueous media, as measured by centrifugation characteristics, probably due to its association with membranes or other cellular material. These elution patterns offer an attractive explanation of how DDT can enter and move through lower trophic levels. Residual 2,4,5-T in cells was not eluted but remained associated with cells and membranes even after the viability of the cell population was reduced. Thus it appears that the limiting membrane is a major site of interaction of these pesticides with bacteria.

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By

Garland F. Hicks, Jr.

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DEDICATION

This dissertation is dedicated to my wife,
who through her support and infinitely
good humor made my doctoral program possible.

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The author expresses his gratitude to Dr. Thomas R. Corner, whose patient guidance in this investigation and throughout my graduate program was most highly appreciated. I thank guidance committee members Dr. James Tiedje, Dr. Arthur Wolcott, Dr. Clarence Suelter and Dr. Charles San Clemente for their interest and helpful criticisms.

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INTRODUCTION

Insecticide and herbicide use accelerated markedly after World War II with the advent of the scientific discoveries and the technology that led to the production of these pesticides in large amounts at low cost. The primary use of pesticides has been in agriculturally related areas. Scientists initiated investigations to determine the effects of these compounds on both target and non-target organisms which resulted in a voluminous accumulation of data. Many of these data concern the effect of pesticides on basic soil processes or, more directly, on the microbial inhabitants of soils and waters.

Of the hundreds of papers pertaining to pesticide interaction with microflora, four general research goals were apparent: (i) the characteristics of pesticides as they are degraded and move through the biosphere, (ii) the toxic effects on soil and water microflora and the ensuing ecological effects, (iii) the role microorganisms play in concentrating various pesticides and how microorganisms introduce the pesticides to higher organisms through food chains, and (iv) microorganisms as cellular models for other organisms to determine the toxic effects of pesticides.

DDT[1] has been the most heavily and most widely used of all the insecticides, and the relative number of research publications pertaining wholly or in part to DDT reflects this. The reported effects of DDT in higher organisms, notably the more conspicuous birds of prey, has created emotionally charged political issues that may have accelerated this research. Moreover, the effects that DDT may have on any living system are decidedly subtle, may be of many years' duration and, by virtue of the movement of DDT and its metabolites over the entire surface of the earth (Harrison *et al.*, 1970; Woodwell *et al.*, 1971), may be applicable to all organisms.

During the late 1960s many reports appeared in both the scientific and lay press which indicated that DDT could be metabolized or otherwise degraded by microorganisms. This capacity seemed to lessen somewhat the controversy over the widespread use of DDT. At the same time a group of very recalcitrant compounds called the polychlorinated biphenyls were being investigated extensively and found to have vaguely similar toxicological and physical properties as DDT and the slightly modified degradative products of DDT (DDD and DDE). Recently a theoretical connection has been proposed between photodegradation of DDT and the polychlorinated biphenyls, and to some extent between

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[1]See Table 1 and Figure 1 for full chemical names and structures of DDT and degradation products discussed herein.

photodegradation of DDT and several of the DDT degradation products heretofore attributed to microbial breakdown. Thus, the ecological fate of DDT is still uncertain.

As evidenced by many thousands of determinations on higher organisms, DDT can and does become highly concentrated in the upper trophic levels of food chains. However, valid studies on DDT uptake at the bases of most food chains, i.e., in microorganisms, are rare and not illustrative, probably because of the techniques used for evaluation. In addition, no papers report any data concerned with intracellular DDT buildup or distribution. Again due to techniques used as well as divergent experimental conditions, data indicating the altering effects that pesticides of any sort have on microorganisms are so contradictory as to be meaningless.

The overall goal of this research report was to contrast the effects of two pesticides, DDT and 2,4,5-T, a water soluble herbicide, on a model bacterium. These effects include uptake by the whole cell and membrane of the cell, the effects on growth and viability, the intracellular distribution of the pesticides and other effects which the pesticides may have on the whole cell or parts of the cell. Implicit in this was a major program to develop and test techniques critically needed to perform and evaluate this research.

LITERATURE REVIEW

Microbial Degradation of DDT

DDT and its immediate degradative products, DDD and DDE, are extremely recalcitrant in the natural environment (Alexander, 1965). This has led many investigators to look for additional evidence of microbial degradation of these compounds and to look for the physiological and biochemical circumstances associated with the conversion.

Apparently the only previous study ever undertaken to elucidate the involvement of pesticides directly with bacterial membranes was done by French and Hoopingarner (1970). These authors were interested in finding if the conversion of DDT to DDD was a biologically dependent process and subsequently determining the intracellular site of such a process. Membranes or cytoplasm isolated from *Escherichia coli* were associated with some DDD formation but large quantities of DDD were produced by isolated membranes only in the presence of reduced flavine adenine dinucleotide (FAD). Conversely, cytoplasm alone or cytoplasm containing boiled membrane fractions produced little DDD, which indicated that the process may be largely enzymatically mediated. With the site of DDD production

established, it was noted that the absence of Kreb's cycle activity, due to anaerobiosis, was correlated with increased DDD production and that electrons from the cytochrome system did not contribute significantly, if at all, to DDD generation.

Wedemeyer (1966) concluded that reduced cytochrome oxidase (cytochrome a_3) was the enzyme responsible for DDD production in *Aerobacter (Enterobacter) aerogenes*. French and Hoopingarner (1970) disagreed because an active Kreb's cycle should contribute more electrons to the cytochrome system and thus aerobic conditions would facilitate rather than inhibit DDD generation. These results may not be inconsistent; anaerobic conditions, i.e., a block at the cytochrome oxidase level, may result in a more highly reduced cytochrome oxidase than if the cytochrome system were actively passing electrons to oxygen. If this were the case, the electrons necessary to produce DDD might come from sources other than the cytochromes. In any event, French and Hoopingarner suggest that FADH may be the active molecule associated with the enzymatic conversion of DDT to DDD.

Production of DDD under natural conditions may not be limited to functional biological systems. Iron porphyrins are oxidized by alkyl halides (Castro, 1954) and reduced porphyrins can contribute electrons necessary for the conversion (Miskus *et al.*, 1965). Ecobichon and

Saschenbrecker (1967) described the appearance of DDD in frozen blood, where presumably enzyme activity is minimal or nonexistent, when only DDT was added.

The production of DDD from DDT commands more than an isolated academic interest. Chacko *et al.* (1966) were the first to illustrate this reduction by soil microorganisms. Ko and Lockwood (1968b) reported that DDD may be somewhat more resistant to degradation than DDT and DDD also may be more toxic to soil microorganisms as measured by growth inhibition on artificial media. Ko and Lockwood also observed that DDD formation was accelerated under anaerobic conditions and further accelerated on the addition of an organic substrate (alfalfa). Chacko *et al.* (1966) found that, of the organisms tested, two genera of actinomycetes, but no fungi, degraded DDT to DDD in significant quantities. However, in an observation that led to an apparent contradiction of conclusions, maximal DDD production was attained during the active growth phase of aerated cultures.

DDD production from DDT is a phenomenon that occurs in many microbial environments (e.g., Cope, 1966; Pfaender and Alexander, 1972) and may be chacteristic of many bacteria (Chacko *et al.*, 1966; Barker *et al.*, 1965; Braunberg and Beck, 1968; Johnson *et al.*, 1967; Mendel and Walters, 1966; Wedemeyer, 1966, 1967; Kokke, 1968; Malone, 1970), some fungi (Kallman and Andrews, 1963;

Matsumura and Bousch, 1968; Anderson and Lichtenstein, 1971), and one alga (Kiel and Priester, 1969).

The biological conversion of DDT to DDD is probably limited to microorganisms. Barker and Morrison (1964) found DDD only in the alimentary tracts of mice fed DDT, and there only in areas of high microbial activity. DDT injected peritoneally was not significantly degraded, suggesting that the liver had little to do with the "detoxification reaction" (however, little evidence exists to indicate that DDD is any more or less toxic to higher organisms than DDT). Subsequently, Barker *et al.* (1965) isolated from intestines of mice a *Proteus vulgaris* strain that would generate DDD *in vitro*. The authors implied that DDD found in mammalian and avian cadavers may be a function of bacterial processes, as *P. vulgaris* is a primary invader of deceased animals.

Less commonly reported are the microbial conversion of DDT to DDE (e.g., Braunberg and Beck, 1968; Wedemeyer, 1967; Kokke, 1970a,b; Pfaender and Alexander, 1972; Miyazaki and Thorstein, 1972) and the conversion of DDD or DDE to other products by microbes. Wedemeyer (1967) has shown, by means of sequential analysis, that many other degradative products of DDT are generated by cell-free extracts of *E. aerogenes*. A representation of his analysis is included in Figure 1. In this model, DDE is not an intermediate of DDD generation.

Sink *et al.* (1972) found that DDD, DDE, and DDMU were produced in ovine rumen fluid *in vitro*, but DDE was an end product rather than an intermediate. Peterson and Robison (1964) noted that rats contained DDD and DDE upon oral administration of DDT. Wedemeyer speculated that metabolites similar to those produced by *E. aerogenes* may also be produced by the intestinal flora.

Guenzi and Beard (1968) found that DDT was degraded to DDD (and possibly other unidentified products) by anaerobic soil over a period of several weeks, whereas sterile soil facilitated no DDD production. Unidentified alfalfa volatiles tended to accelerate DDD generation. Ko and Lockwood (1968b) reported similar results. Thus DDD generation seemed dependent on viable organisms. Burge (1971) indicated that autoclaved soil may have undergone changes (e.g., free radicals may have been quenched or other physical and chemical properties may have been modified) sufficient to inhibit nonbiological transformations of DDT. To test the conclusions of Ko and Lockwood (1968b) and Guenzi and Beard (1968), Burge added a very small inoculum of natural soil to autoclaved soil containing DDT. The processes that converted DDT to DDD were restored by this procedure, and all DDT eventually disappeared. He too noted that alfalfa volatiles accelerated the disappearance of the DDT, and that 2% oxygen contributed to the stability of the pesticide.

DDD was the only breakdown product detected, and by addition of DDD and DDE to other samples it was found these compounds were somewhat more resistant to degradation than DDT. It was concluded that unknown pathways of DDT breakdown, not involving DDD as an intermediate, may exist.

Stenersen (1965) isolated *Serratia marcescens*, *E. coli* and an unidentified bacterium from excrements of DDT-resistant or -susceptible flies. Each bacterium, when grown with an oxygen deficiency, converted about 90 per cent of the DDT to DDD, and about 5 per cent to DDE. The flies themselves were analyzed for pesticide content and no difference in products was found in the two strains. Stenersen concluded that the ability to degrade DDT, by the flies proper or by associated microflora, was not a factor that determined resistance to DDT. Dinamarca *et al.* (1971) later suggested that the conversion of DDT to DDE was a mechanism of resistance in houseflies.

Although the relative toxicity of DDT, DDE, and DDD for microorganisms is unclear, Kiel and Priester (1969) suggested that DDE, produced *in vitro* by a marine diatom, may be less toxic to water microflora because it is much more water soluble than DDT and thus would more easily be leached from cells. This hypothesis apparently has not received support.

Alexander and colleagues (1970, 1972, 1973) reported that a *Hydrogenomonas* sp. degraded DDM to one or more

products and that cell-free extracts of the bacterium, under anaerobic conditions, could transform DDT to DDD, DDMS, and DBP. PCPA was found when oxygen was added to the extract. An *Arthrobacter* sp. was shown to degrade PCPA to PCPGA. Sewage and fresh water stream samples degraded DDT to large quantities of DBP, DDD and to smaller quantities of DDE, but no PCPA. Focht (1972) isolated a *Fusarium* sp. that could degrade both DDM and PCPA to H_2O, CO_2, and HCl. Thus, a plausible model has been established to account for complete microbial degradation of DDT (Figure 1).

The enzymes involved in the DDT transformation are constitutive in *Hydrogenomonas* and are associated with a cometabolic process (Pfaender and Alexander, 1973), i.e., a degradative pathway in which the organism derives no energy or elemental requirements. The concept of cometabolism attained prominence largely from studies on pesticide degradation (Horvath, 1972) and represents a wide and curious aspect of microbiology.

Several reports claim that some animals converted o,p'-DDT to p,p'-DDT (summarized in Bitman *et al.*, 1971), which prompted others to examine *E. aerogenes* for this ability, but no evidence of the isomerization was found. Bitman *et al.* (1971) refuted the original reports by demonstrating that the original data were a result of poor experimental technique.

Figure 1. Reported pathways for DDT degradation (a composite diagram adapted from many authors' work--see text).

Solid lines indicate proposed pathways of DDT degradation by individual microbial species, cell-free extracts of specific miroorganisms or microbial ecosystems. Broken lines indicate that degradative products were reported but no sequence necessarily was implied in original articles. Dotted lines illustrate proposed pathways for photoconversion of DDT. Specific investigators and organisms or ecosystems studied, if applicable, are as follows:

1. Wedemeyer, 1967; *E. aerogenes* (cell-free extracts and whole cells).

2. Focht and Alexander, 1972; *Fusarium* sp.

3a. Pfaender and Alexander, 1972; microbial communities of sewage and fresh water.

3b. Pfaender and Alexander, 1972; whole cells of *Hydrogenomonas* sp. + O_2.

3c. Pfaender and Alexander, 1972; cell-free extracts of *Hydrogenomonas* sp.

4. Sink *et al.*, 1972; rumen fluid.

5. Focht and Alexander, 1970.

6. Maugh, 1973.

7. Plimmer *et al.*, 1970.

8. Ahmed and Focht, 1973; *Achromobacter* sp.

9. Kaiser and Wong, 1974; unspecified organism(s).

10. Numerous other investigators (see text).

11. Bitman *et al.*, 1971.

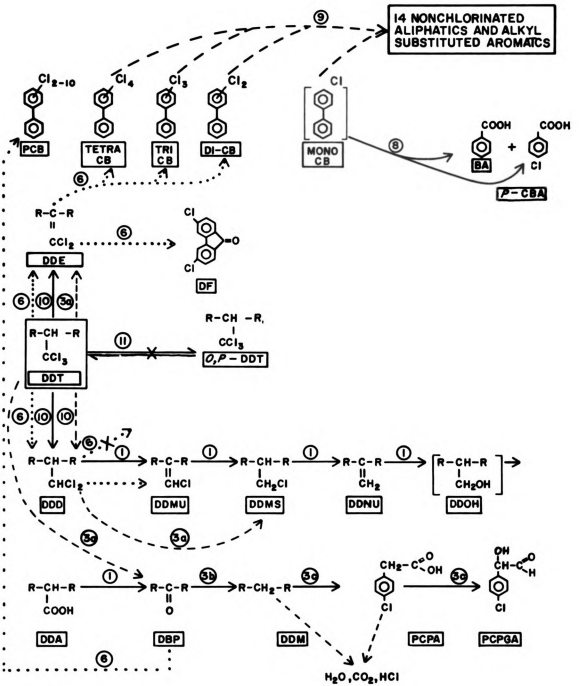
14 NONCHLORINATED ALIPHATICS AND ALKYL SUBSTITUTED AROMATICS
PCB
TETRA CB
TRI CB
DI-CB
MONO CB
BA
P-CBA
DDE
DF
DDT
O,P-DDT
DDD
DDMU
DDMS
DDNU
DDOH
DDA
DBP
DDM
PCPA
PCPGA
H2O,CO2,HCl

Figure 1

Table 1. Chemical names and abbreviations

BA = benzoic acid
p-CBA = p-chlorobenzoic acid
CB = chlorinated biphenyl
DBP = 4,4'-dichlorobenzophenone
DDA = 2,2-bis(p-chlorophenyl)acetate
DDD = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane
DDE = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
DF = 3,6-dichlorofluorenone
DDOH = 2,2-bis(p-chlorophenyl)ethanol
DDM = bis(p-chlorophenyl)methane
DDMS = 1-chloro-2,2-bis(p-chlorophenyl)ethane
DDMU = 1-chloro-2,2-bis(p-chlorophenyl)ethylene
DDNU = UNSYM-bis(p-chlorophenyl)ethylene
DDT = 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
PCB = polychlorinated biphenyl
PCPA = p-chlorophenylacetic acid
PCPGA = p-chlorophenylglycolaldehyde
R = p-chlorophenyl group
R_1 = o-chlorophenyl group

Photooxidation of DDT

Apart from DDT degradation associated with biological materials, a considerable body of data emanating from *in vitro* experiments indicates that DDT can be photooxidized rather extensively. The vapor pressure of DDT is high (Lichtenstein and Schulz, 1970; Spencer and Cliath, 1972) and that of DDE is even higher (Maugh, 1973). Thus the potential photooxidation of the compounds could have important ecological significance.

Plimmer *et al.* (1970) described UV-induced photooxidation of DDT to several products, including polychlorinated biphenyls (PCBs). Maugh (1973) condensed the research of several workers and came to some guarded conclusions which are summarized here. During the last decade the PCBs were discovered to be as ubiquitous as DDT in the biosphere (e.g., Reynolds, 1969; Risebrough, 1968). The PCBs were thought to have originated from industrial processes. However, unlike DDT, PCBs are relatively nonvolatile, and their appearance in the arctic and in newly created artificial lakes was mysterious. In addition, the fate of the large quantities of DDT used in the past three decades was, and remains, unaccounted for in view of its recalcitrance. This led to the finding that radiation found in the lower atmosphere (290 to 310 nm) converted vaporized DDT to various PCBs, with intermediates consisting of DDE, DDMU and DBP. DDD, however,

was formed slowly, as its formation is dependent on a hydrogen donor, and was not further reduced or oxidized.

Maugh's description of DDT photooxidation may help to clarify several longstanding uncertainties about DDT degradation: (i) the ubiquity and quantity of PCBs in the biosphere, (ii) the disappearance of at least some DDT from the earth's surface, (iii) the fate of DDE, which previously was unknown, and (iv) the failure of any researcher to observe *in situ* the DDT degradation schemes in their entirety proposed by Pfaender and Alexander (1973), Wedemeyer (1967), and others; indeed, in no papers reviewed here is there mention of intensively controlled experiments to check specifically for photoconversion of DDT, either acting alone or in synergy with microbial cometabolism. Radiation of wave lengths greater than 286 nm will penetrate Pyrex glass (Su and Zabik, 1972), which may give particular significance to this latter point.

Microbial Degradation of Chlorinated Biphenyls

Only recently has evidence appeared describing microbial degradation of some PCBs. 4-Chlorobiphenyl was degraded by two *Achromobacter* spp. to benzoic acid and parachlorobenzoic acid (Ahmed and Focht, 1973). Although the literature pertaining to DDT degradation does not account for a direct link to monochlorobiphenyl, unidentified bacterial isolates degraded a mixture of

mono-, di-, tri-, and tetrachlorobiphenyls to 14 non-chlorinated aliphatic and alkyl aromatic compounds, but no chlorinated degradation products were found (Kaiser and Wong, 1974).

Uptake of DDT by Microbial Cells: Methods Employed to Measure Uptake

Many investigators have attempted to determine quantitatively the ability of microflora to accumulate DDT from aqueous surroundings. This is difficult to do with a compound that has a solubility in water of about 5 ng/ml, as defined by gravitational stability (Shin *et al.*, 1970) or about 1.2 ng/ml as defined by ultracentrifugation sedimentation characteristics (Bowman *et al.*, 1960). The problem of separating the particulate DDT from suspended cells is critical to the attainment of accurate uptake data; investigators have attacked this problem in various ways.

DeKoning and Mortimer (1971) inoculated a growing culture of *Euglena gracilis* with ^{14}C-DDT and periodically took samples of the culture to determine the extent of DDT uptake by the cells. Their method of removing the DDT from the medium consisted of extracting the aliquots with hexane. They assumed the DDT in the medium or on the cell surfaces would be removed with hexane but DDT within the cell would be unaffected, an assumption not checked experimentally. In any event, the data were

presented in terms of radioactive material within the cells but no cell weights were given. Thus the actual quantities of DDT accumulated by the cells were impossible to determine. One unrelated conclusion given by the authors was that DDT uptake could involve physiological control, since a polychlorinated biphenyl accidentally introduced into a *Euglena* culture was not taken up whereas DDT was.

A more popular way of separating the water-insoluble DDT from suspended cells was simply to wash the cells with water. Chacko and Lockwood (1967) washed several bacteria-pesticide or fungi-pesticide mixtures through a Millipore filter and analyzed the material on the filter for pesticide content. No mention was made of checking the filter for nonfilterable pesticide. The authors felt this was a valid procedure because *Streptomyces griseus* was found to contain 80 per cent of the pesticide to which it was exposed after the mycelia were washed through an 80-mesh wire sieve. The materials on the filter contained from 38 to 100 per cent of the DDT originally added. Similar experiments suggested that uptake was independent of pH or cell viability.

Using a somewhat different procedure, Ko and Lockwood (1968a) mixed fungal mycelia with soil treated with DDT. The mycelia were removed from the soil and washed through a wire sieve. The DDT concentration in the mycelia of *Rhizoctonia solani* increased over 50 h, from about 3 to

60 μg DDT/gram mycelium, and at 24 h had accumulated about 50 μg. Ko and Lockwood concluded that the microorganisms accumulated only about 10 per cent of the pesticide to which they were exposed but under natural soil conditions the uptake could be higher due to the greater surface area of fungi actually growing in the soil. They also speculated that uptake by soil microorganisms could contribute to the persistence of DDT in soils and that pesticide retention "...by microorganisms may also reduce the biological activity of [DDT] in soil."

To circumvent the problem of aqueous insolubility of DDT, Johnson and Kennedy (1973) attempted to determine quantitative uptake of DDT by *E. aerogenes* or *B. subtilis* by exposing each organism to from 1 to 5 ng DDT/ml suspension and subsequently analyzing the DDT content of the cell pellet after centrifugation. The pellets contained between 13 and 17 per cent of the DDT to which the bacteria were exposed. No correction factors were imposed to account for interstitial space or void space within the peptidoglycan. These percentages were reported to represent magnification factors between 2800 and 4200, which were independent of pesticide concentrations in the exposure medium. As the exposure time increased up to 24 h, the uptake of DDT increased only slightly. The aqueous media in which the cells were suspended were composed of, variously, distilled H_2O, 0.05 M phosphate

buffer or water containing salts that approximated those found in pond water. The composition of the suspending medium did not affect pesticide uptake. Furthermore, the amount of DDT taken up from each medium did not vary as the DDT concentrations were increased from 1 ng/ml to 5 ng/ml.

Johnson and Kennedy (1973) apparently were oblivious to the research of Sodergren (1968). Sodergren conducted studies with DDT and came to a number of conclusions about the behavior of the compound in the presence of a *Chlorella* sp.: (i) 0.6 ng/ml was the maximum concentration of DDT that could be used in experiments where the separation of DDT from cells in aqueous suspension by centrifugation or filtration was necessary, since precipitation could result at higher concentrations, (ii) 1.2 mg cells could accumulate 0.38 µg DDT from aqueous surroundings, and the rate of uptake was identical to the rate of diffusion of DDT in water and (iii) DDT uptake is most probably a form of absorption and not adsorption, as evidenced by the irreversible nature of the concentrating effect.

Vance and Drummond (1969) performed uptake experiments by growing four algal species in the presence of 1 µg DDT/ml. The algae and DDT were separated by filtration. The results of these experiments suggested that maximal uptake of DDT occurred after 30 min of exposure

and that DDT was concentrated in large but undetermined quantities in the algae. Cell weights or numbers were not given, thus precluding any quantitative estimate of uptake. Similarly, Kiel and Priester (1969) found that the diatom *Cylindrotheca closterium* concentrated the pesticide 265 times above the ambient concentration when exposed to 0.1 ng DDT/ml. Reportedly, the cells were separated from the suspended DDT by filtration.

Lower concentrations of DDT (0.274 to 0.362 µg per unknown amount of H_2O) were used by Cox (1970a) to illustrate that marine algae will accumulate 16 to 54 per cent of the initial DDT levels from seawater. Filtration was employed to remove residual DDT from the environment of the cells. Using similar techniques, Gregory *et al.* (1969) found that *Anacystis nidulans* (a blue-green alga), *Scenedesmus obliquus* (a green alga), *Euglena gracilis* and two *Paramecium* spp. contained from 99 to 953 times the amount of DDT to which they were exposed (1.0 µg/ml); again, filtration reportedly separated the DDT and cells.

An entirely different method was employed by Kokke (1970a,b) to determine relative amounts of DDT concentrated by microorganisms. Cells isolated from various microbial environments were grown on agar plates impregnated with 0.8 µg ^{14}C-DDT/ml. After incubation, autoradiography was performed in the cold over the surface of the plates. Colonies with heavy concentrations of

DDT showed as dark spots on the photographic film. Fewer than 1 per cent of the colonies arising from a tap water sample accumulated the DDT, but DDT was concentrated by 2 to 7, 60 to 70, or 80 to 90 per cent of the colonies from, respectively, polluted surface water, garden soil or nursery soil heavily enriched with organic matter. Kokke also established a direct correlation between the ability of the bacteria to concentrate pesticide and the ability to grow on agar containing DDT. Other effects were also observed: (i) the percentage of organisms able to accumulate DDT increased when other pesticides were sprayed on the soil prior to isolation, (ii) microorganisms that were sensitive to 1 µg DDT/ml of peptone agar could be induced to grow on the agar by sequential transfer from other plates containing 0.6 µg and 0.8 µg DDT/ml. From these data Kokke suggested that bacteria in nature may have adapted to pesticide-contaminated environments, as the amount of DDT available to microorganisms in water is less than that available to bacteria in garden or nursery soil.

Apparently, Cox (1970a) performed the only *in situ* assessment of long term accumulation of DDT by marine phytoplankton. Levels of DDT or DDT metabolites found within the marine algae increased from about 0.25 ppm (1955) to about 0.55 ppm (1970). Controls indicated that the increase was not an apparent one due only to advancements in analytical technology.

The concentration of DDT at various trophic levels and in various nonmicrobial elements of food chains has been extensively investigated *in situ*. All reported uptake values for bacteria and for most other microorganisms are from *in vitro* experiments which only represent the potential of the cells to concentrate DDT. The few *in situ* determinations relating to DDT uptake by microorganisms are listed in Table 2 along with DDT residues in other organisms. The data in Table 2, representative of several publications (Woodwell, 1967; Cade, 1971; Goodman, 1974; Reichel *et al.*, 1969; Cox, 1970b; Woodwell *et al.*, 1971; Burnett, 1971; Deichmann, 1972), are presented to relate uptake by microorganisms to uptake by other organisms, and to illustrate the biological magnification of the compound as it moves through various trophic levels.

Though the data from the above papers vary in magnitude, it is evident that microorganisms in general will accumulate and concentrate large amounts of DDT from aqueous or soil environments. Apart from the study by French and Hoopingarner, little was known of the precise interactions of microorganisms and DDT; is DDT absorbed primarily on the surface of the cell or do large quantities of the insecticide actually enter the cell? Pfister (1972) states:

Table 2. DDT concentrations in biological systems from European and North American regions (data are representative of several reports)

		Σ DDT (ppm)[a]
Woodwell (1967)	water	0.00001-0.00005
Goodman (1974)	rain over United Kingdom	0.0001
Woodwell (1971)	biota of swamps and marshes	0.001
Goodman (1974)	untreated soils, remote areas of U.K.	0.001
Woodwell (1971)	biota of lakes and streams	0.01
Woodwell (1967)	plankton (mostly zooplankton)	0.04
Woodwell (1971)	open ocean algae	0.1
Goodman (1974)	fat of antarctic seals and penguins	0.1
Woodwell (1971)	ocean protozoa, coelenterates, annelids, nematodes, mollusks, echinoderms (avg.)	0.1
Goodman (1974)	human milk	0.1
Cox (1970)	marine algae (coastal)	0.55
Goodman (1974)	lake trout fry (high embryonic mortality)	1.0
Woodwell (1971)	continental shelf algae	1.0
Woodwell (1967)	flounder	1.28
Woodwell (1967)	estuary bottom samples	1.28
Woodwell (1967)	shore birds (gulls, terns, herons, cormorants, mergansers)	1.48-1.75

Table 2 (continued)

		Σ DDT (ppm)[a]
Burnett (1971)	*Emerita analoga*--sand crab (California coast) --avg.	3.62
Deichmann (1972)	"normal" human cadaver body fat	9.7
Deichmann (1972)	human cadaver body fat; cause of death was atherosclerosis, leukemia, carcinoma, or hypertension	17.3-24.8 (avg. 21.1)
Reichel (1969)	pesticide poisoned bald eagle (Florida)	
	carcass	33.5
	liver	85.0
	brain	31.6
Cade (1971)	peregrine eggs	167.0-899.0
Goodman (1974)	herring gull with impaired reproduction	1000.0

[a]Σ DDT = DDT + DDD + DDE. Data based from samples taken in late 1960s and early 1970s. PPM = µg/ml or µg/gm.

> Whether this [DDT] storage ability is actually inside the cell or merely the entrapment of halogenated compound in polymer matrices on the external cell surface remains to be seen. In either case, [the] extensive surface area that the microbial system presents for adsorption places them [sic] in an important position with respect to a larger ecology.

Effects of DDT or DDT Metabolites on Various Physiological Processes of Microflora

Nitrogen Transformations

One microgram DDT/ml is sufficient to inhibit nitrification in *Nitrobacter agilis* (Garretson and San Clemente, 1968). However, nitrification of ammonium sulfate was not affected in soil treated with DDT (Bollen *et al.*, 1954; Martin *et al.*, 1959) at concentrations that approximate those found in soil immediately after application for agricultural purposes (20 lbs/acre--Martin *et al.*, 1959). Eno and Everett (1958) demonstrated that DDT initially will increase nitrification (12.5 ng DDT/g soil) in soil planted with legumes, but after sixteen months nitrification was decreased. Bollen (1961) reported a decrease in clover root nodulation when DDT was applied to soil at 10 lbs/acre. Jones (1956) reported that DDT had few or no effects on ammonifying organisms in culture unless very high concentrations of DDT were used (0.1%, the approximate equivalent of 1 ton/acre). Wilson and Choudhri (1946) reported that up to 0.5 per cent DDT had no effect on ammonification in soil, and had no effect

on the growth of leguminous bacteria in pure culture. In the tradition of true sages, Wilson and Choudhri stated in 1946 that more investigation involving DDT "seemed useless."

In blatant disregard of this advice, Jones (1952) found that nitrifiers in soil were affected only at a DDT concentration of 0.1% (1000 ppm) as determined by plate counts, whereas 1.0% DDT slightly stimulated growth of nitrogen-fixing bacteria. Similarly, Bartha *et al.* (1967) found no effect of 150 ppm DDT on nitrate production in soil but 1500 ppm DDT increased nitrate production somewhat. DDT did not inhibit nitrogen fixation or growth of *Azotobacter vinelandii*, even at 50 times the DDT concentration normally applied to agricultural areas (Mackenzie and MacRae, 1972).

DDD at 10 µg/ml interferes with growth and, thus, with nitrite oxidation in pure cultures of *N. agilis* (Winely and San Clemente, 1970), whereas nitrite consumption was only halved in cells suspended in non-nutritive medium containing 125 µg DDT/ml. These authors suggested that growth studies may be a better indicator of some pesticide effects. Nitrite oxidation studies show only differences in energy assimilation, whereas growth studies allow elucidation of biosynthetic reaction inhibition.

Photosynthesis

The effects of DDT on nitrogen transformations may be sketchy, contradictory and inconclusive, but little doubt exists that DDT dramatically reduces photosynthesis in marine algae. DDT concentrations as low as 5 ng/ml reduced carbon assimilation in pure algal cultures and, significantly, produced similar results in a mixed phytoplankton culture taken directly from coastal regions (Wurster, 1968; Ware and Roan, 1970). In another study Stadnyk *et al.* (1971) found that 0.1 µg DDT/ml culture medium decreased the photosynthetic rate and the biomass of a freshwater alga. Studies on inhibitory actions of DDT in microbial photosynthesis apparently are not available. However, Rogers *et al.* (1971) indicate two sites of inhibition of electron transport in barley chloroplasts: one before photosystem II and the other in the electron transport chain linking the two photosystems. Stadnyk *et al.* (1971) did suggest that inhibition of photosynthesis may be secondary to inhibition of growth or acceleration of death in the alga studied. Indeed, the most extensively investigated effect of DDT on microorganisms has been that of possible growth inhibition.

Growth Effects

Analysis of data from growth experiments is difficult due to the nature of the experiments. As the biomass in

culture containing DDT increases, the ratio of pesticide to biomass naturally changes, thus eliminating the possibility of obtaining dose-response data. Ordinarily, one would expect that the effective concentration of a compound in solution would not exceed the solubility limit and the precipitated or excess compound merely would replenish the solute as it was removed from solution. If this were the case, the volume of the culture medium would affect the time in which the individual DDT molecules could be solubilized and could reach the microbial cell. Adsorption of DDT to surfaces presents yet another problem to be dealt with, and this may be the key contributor to the many discrepancies between *in situ* experiments conducted in soil or gut and *in vitro* determinations involving only glassware. Lastly, the solubility of DDT in aqueous environments varies with the composition and the pH of the suspending medium, the carrier solvent used to introduce the DDT to the medium, and the surface area and chemistry of particles to which DDT may be absorbed (Shin *et al.*, 1970). With all these factors considered, it is of little wonder that the experimental results of three decades are so varied, and in some cases appear to be so contradictory and inconclusive. However, analysis of all research reports reveals several most definite, albeit only qualitative, trends.

Bollen *et al.* (1964) detected no increase in CO_2 evolution or glucose decomposition in soil treated with

an unspecified amount of DDT. Another field study (Martin *et al.*, 1959) indicated that DDT had no effect on the decomposition of organic matter as measured by CO_2 evolution, nor on the relative numbers or kinds of microflora present, as measured by plate counts. Technical grade DDT was used in this work and was applied to the soil at a rate of 20 lbs/acre/6 inch soil surface, a concentration that would approximate maximal normal agricultural use. Eno and Everett (1958) suggested that 12.5 to 100 ppm DDT did not appreciably affect numbers of fungi in soil as determined by plate counts, but CO_2 evolution was increased. Varshney and Guar (1972) reported that many species of fungi were inhibited by 1 µg DDD/g soil, but noted that the addition of manure protected the fungi against adverse effects.

Jones (1956) found that concentrations of DDT sufficient to inhibit nitrogen transformations would increase microbial numbers in soil. Dalton *et al.* (1971) found that growth rates of aquatic hyphomycetes were enhanced when the culture media contained DDT above concentrations of 2 µg/ml. The authors suggested that the cause of these increases may have been that DDT was metabolized, was acting as a cofactor in metabolic processes, or had altered the permeability of the cells. They further implied that DDT may be beneficial ecologically because decomposition of organic matter may be stimulated, leading

to a slowing of the eutrophication process. Wurster (1968) indicated that 5 ng DDT/ml would affect growth of photosynthetic organisms which in turn would increase the eutrophication rate.

Ko and Lockwood (1968a) found that both DDT and DDD were highly toxic to soil microorganisms in cultures containing from 0.1 to 10 µg pesticide/ml, but DDD appeared to affect a greater number of species. However, neither DDT nor DDD at similar concentrations seemed to have any effect on microorganisms in soil. Ko and Lockwood suggested that these pesticides could have ecological implications in areas of high microbial activity such as the gut or the rhizosphere. However, Braunberg and Beck (1968) found no differences in flora of the alimentary canals of rats maintained on a constant diet of normal food plus "100 ppm" DDT.

The growth of *Pseudomonas fluorescens* or *Staphylococcus aureus* was inhibited at 50 µg DDT/ml trypticase soy broth, but inhibition was detected only as the stationary growth phase was attained; no inhibition of similar organisms in DDT treated skim milk was detected (Collins and Langlois, 1968; Langlois and Collins, 1970).

Menzel *et al.* (1970) found that DDT would inhibit cell division in two of the four species of marine algae tested. One hundred nanograms DDT/ml culture were added each day to the algae in an attempt to maintain some degree

of dose-response relationship. The authors concluded that DDT may "...exert a dramatic influence on the succession and dominance of individual forms."

Several other researchers used very high DDT concentrations to screen the growth effects of this compound on bacteria. Although growth inhibition at high DDT levels would be somewhat academic, a lack of inhibition would be significant. In the most extensive of these experiments, Trudgill *et al.* (1971) found that DDT would not inhibit growth of any organism tested (except *Bacillus megaterium*) on agar containing a surface film of DDT of unspecified concentration but probably 0.5 mg/cm^2. Dougherty *et al.* (1971) found that 0.01 M DDT was the minimum inhibitory concentration for *B. thuringiensis* spores on agar.

Mode of Action of DDT or Its Metabolites on Biological Systems

Winely and San Clemente (1970) found that low DDD concentrations interfere with nitrite oxidation in *N. agilis* not from direct inhibition of nitrite oxidation but rather through growth inhibition.

Apart from the above study little or no work has been performed to elucidate the precise biochemical effects of DDT or DDT analogs on prokaryotic cells. Janicki and Kinter (1971) report that 1 to 50 µg DDT/ml will partially inhibit the Na^+, K^+, Mg^{2+}-ATPase in homogenates of gills

or intestinal mucosae of flounder. This enzyme is believed to be instrumental in sodium transport across membranes, and its inhibition would affect the osmotic differential required in marine fish. Janicki and Kinter believe that this may explain the sensitivity of many fish to low environmental concentrations of DDT, but they add that pesticide concentrations in *in vitro* studies are essentially meaningless unless the uptake patterns of the lipid-rich membrane homogenates are known.

Matsumura and Patil (1969) also observed DDT-induced inhibition of a Na^+, K^+, Mg^{2+}-ATPase but emphasized that the enzyme may not be involved in a sodium-potassium pump because DDT action on a neuron is quick (e.g., Hilton *et al.*, 1971) and inhibition of this enzyme likely would produce a gradual effect on the neuron. Hence, the authors suggested that the ATPase was a secondary target of DDT.

Hilton *et al.* (1973) reported that the lethal action of DDT in arthropods probably results from an overexcitation of neurons caused by an extended increase in sodium conductance. Using a lecithin bilayer (Tinsley *et al.*, 1971, had previously reported a strong association between DDT and lecithin *in vitro*) with valinomycin added to induce K^+ conductivity across the layer, the authors found K^+ conductance was decreased by 10^{-6} M DDT, was decreased less with 10^{-6} M DDE, and showed an uncertain

response with DDD. However, the effects did not correlate with those observed in arthropod neurons. The authors speculated that DDT may increase the fluidity of the artificial membrane.

Microbial Interactions with 2,4,5-T

The herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) has been used extensively throughout the world to control broadleaf woody vegetation. Unlike most other herbicides, 2,4,5-T is somewhat recalcitrant under natural conditions. The compound may remain stable from three weeks to 270 days, depending on the location and type of microbial environment (DeRose and Newman, 1948; Walker and Newman, 1957; Loos, 1969a). Technical grade and even more highly purified preparations of 2,4,5-T may contain varying amounts of chlorodibenzodioxin and chlorodibenzofuran isomers (Woolson *et al.*, 1972). These substances have been implicated strongly as the cause of various diseases in man and as teratogenic agents in animals. One dioxin isomer, 2,3,6,7-tetrachlorodibenzodioxin,[1] is one of the most toxic compounds known (Kimbrough, 1972). Nearly all scientific investigations of 2,4,5-T preparations have emphasized the effects of the contaminants and relatively little has been focused on the

[1]The designation 2,3,7,8-tetrachlorodibenzodioxin also is used to describe an identical compound.

herbicide proper as compared to other previously used pesticides. Moreover, the literature pertaining to 2,4,5-T studies on microorganisms is essentially restricted to studies on metabolism or cometabolism of the compound (e.g., Horvath, 1970, 1972; Loos, 1969, 1969b).

Several authors (e.g., Okey and Bogan, 1965) have suggested that the molecular structure of 2,4,5-T is responsible primarily for the recalcitrance of 2,4,5-T. However, Horvath (1971) found that a *Brevibacterium* sp. will degrade the molecule *in vitro*, by cometabolism, to 3,5-dichlorocatechol, an intermediate in the metabolism of 2,4-D, a 2,4,5-T analog (e.g., Bollag *et al.*, 1968; Tiedje *et al.*, 1969).

The effects of 2,4,5-T on microbial processes such as photosynthesis, nitrogen transformations, and other specific biochemical properties evidently are unknown. Dougherty *et al.* (1971) reported that the minimum inhibitory concentration for *B. thuringiensis* is 0.01 M (2.56 mg/ml). Loos (1969b) studied soil that originally deactivated 2,4,5-T and found that inhibition of biological activity arose when the perfusing solution contained more than 200 ppm herbicide. Bell (1960) reported increased respiratory activity of an *Achromobacter* sp. after exposure to 2,4,5-T, which was interpreted to indicate substantial oxidation of the compound. Sometime later, Horvath (1971) claimed that he was the first to detect oxidation of 2,4,5-T.

In an earlier study, Bell (1957) found that 2,4,5-T inhibited an unidentified soil bacterium growing in liquid medium containing 3 mM 2,4,5-T. At lower concentrations, the bacterium showed an increased endogenous respiratory pattern.

There is no evidence to indicate that 2,4,5-T is magnified within food chains (e.g., Leng, 1972; Southwick, 1974) and there is evidence suggesting that some ruminants will not retain ingested 2,4,5-T within body tissues for more than one week (Leng, 1972). No research has been reported on the uptake of 2,4,5-T by microorganisms, nor on the distribution of 2,4,5-T within prokaryotic or eukaryotic cells.

Woolson (1972) has developed reliable and relatively simple methods to extract contaminants from 2,4,5-T preparations. This represents a critical advance in the techniques needed to evaluate independently the biological effects of 2,4,5-T, dioxin, or other contaminants.

Summary

From the many studies on higher organisms and from the work of Kokke, it is evident that microflora can concentrate DDT to an indeterminate but many-fold amount over environmental concentrations. The results of studies that attempted to elucidate uptake of the water-insoluble compound by filtering, centrifuging, or directly extracting DDT from pesticide-bacteria mixtures should be

interpreted carefully; the precise quantity of DDT a bacterium is able to accumulate is entirely unknown. The intracellular disposition of DDT similarly is unknown, either in bacteria or in higher cells.

The potential of DDT to be metabolized or cometabolized by microorganisms has been determined to some extent, but the artificial conditions (e.g., cell-free extracts) under which many experiments were conducted leads only to speculation about the mechanisms of degradation under natural conditions. Most researchers who studied DDT degradation by whole cells reported detecting only DDE or DDD as end products. However, a few workers observed rather extensive DDT degradation in cultures that approached *in situ* conditions, but reports of photoconversion cast considerable doubt about the ecological importance of microbially-mediated DDT degradation in the biosphere.

DDT severely affects photosynthesis in algae but results from studies of nitrogen transformations are so contradictory as to preclude even an intelligent guess on the effect of DDT in nitrogen cycles. Similarly, growth effects on microorganisms are as varied in *in vitro* studies as they are in soil studies. Kokke's work illustrates that microbial response to DDT may vary and can be altered; other workers have shown that the organic matter content of soil may at least temporarily affect the response of soil organisms to DDT. Studies

involving dose-response effects of DDT on the growth of microorganisms essentially are meaningless unless the precise DDT content of the target cell is known; availability of DDT to the cell, affected by solubility of DDT and adsorption to nonliving particles, most probably will affect the uptake characteristics of the cell which in turn will affect the response of the microbial community.

In no paper reviewed here was there an attempt to integrate these factors, either experimentally or intellectually, in determining the gross interactions of cells and DDT. It is evident that the results of DDT interactions with microflora, reported over the last decade, fit only into the broadest of patterns. The diversified research techniques, good or bad, combine with the uncertainties of DDT solubility, with the wide range of organisms and with a wide range of microbial environments to give widely scattered or conflicting results.

The herbicide is not considered to be a recalcitrant compound in the context of many other organochlorine pesticides such as DDT. Possibly because of its relatively short half-life or due to research emphasis placed on contaminating molecules, 2,4,5-T has been largely neglected by microbiologists or cytologists interested in subcellular distribution or effects. Considerable

evidence indicates that 2,4,5-T does not become concentrated or magnified within food chains but as yet no author has reported uptake characteristics of this compound by any of the microflora.

A series of experiments performed under constant conditions with one organism would provide some insight on biologically mediated pesticide transformations, be they physical or chemical, and on pesticide mediated biological effects. First, however, techniques must be developed to remove suspended DDT from mixtures of the pesticide and cells, and to evaluate 2,4,5-T uptake as well as retention of 2,4,5-T by cells. These experiments and technique development have been done, some have been published, and all are reported in this dissertation.

This dissertation is organized into two major sections which include (i) a critical literature analysis of all phases of DDT or 2,4,5-T interaction with microflora, and (ii) experimental results. The latter incorporates a published paper, a manuscript prepared for publication, and other sections containing pertinent experimental data.

REFERENCES

REFERENCES

Ahmed, M., and D. D. Focht. 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol. 19:47-52.

Alexander, M. 1965. Biodegradation: Problems of molecular recalcitrance and microbial fallibility. Adv. Appl. Microbiol. 7:35-80.

Anderson, J. P. E., and E. P. Lichtenstein. 1971. Effect of nutritional factors on DDT-degradation by *Mucor alternans*. Can. J. Microbiol. 17:1291-1298.

Barker, P. S., and F. D. Morrison. 1964. Breakdown of DDT to DDD in mouse tissue. Can. J. Zool. 42:324-325.

Barker, P. S., F. O. Morrison, and R. S. Whitaker. 1965. Conversion of DDT to DDD by *Proteus vulgaris*, a bacterium isolated from the intestinal flora of a mouse. Nature 205:621-622.

Bartha, R., R. P. Lanzilotta, and D. Pramer. 1967. Stability and effects of some pesticides in soil. Appl. Microbiol. 15:67-75.

Bell, G. R. 1957. Some morphological and biochemical characteristics of a soil bacterium which decomposes 2,4-dichlorophenoxyacetic acid. Can. J. Microbiol. 3:821-840.

Bell, G. R. 1960. Studies on a soil *Achromobacter* which degrades 2,4-dichlorophenoxyacetic acid. Can. J. Microbiol. 6:325-337.

Bitman, J., H. C. Cecil, and G. F. Frier. 1971. Non-conversion of o,p'-DDT to p,p'-DDT in rats, sheep, chickens, and quail. Science 174:64-66.

Bollag, J. M., G. C. Briggs, J. E. Dawson, and M. Alexander. 1968. 2,4-D metabolism. Enzymatic degradation of chlorocatechols. J. Agr. Food Chem. 16:829-833.

Bollen, W. B. 1961. Interactions between pesticides and soil microorganisms. Annu. Rev. Microbiol. 15: 69-92.

Bollen, W. B., H. E. Morrison, and H. H. Crowell. 1954. Effect of field and laboratory treatments with BHC and DDT on nitrogen transformations and soil respiration. J. Econ. Entomol. 47:307-312.

Bowman, M. C., F. Acree, and M. K. Corbett. 1960. Solubility of carbon-14 DDT in water. J. Agr. Food Chem. 8:406-408.

Braunberg, R. C., and V. Beck. 1968. Interaction of DDT and the gastrointestinal microflora of the rat. J. Agr. Food Chem. 16:451-453.

Burge, W. D. 1971. Anaerobic decomposition of DDT in soil. Acceleration by volatile components in alfalfa. J. Agr. Food Chem. 19:375-378.

Burnett, R. 1971. DDT residues: Distribution of concentrations in *Emerita analoga* (Stimpson) along coastal California. Science 174:606-608.

Cade, T. J., J. L. Lincer, C. M. White, D. C. Roseneau, and L. G. Schwartz. 1971. DDE residues and eggshell changes in Alaska falcons and hawks. Science 172:955-957.

Castro, C. E. 1954. The rapid oxidations of iron (II) porphyrins by alkyl halides. A possible mode of intoxication of organisms by alkyl halides. J. Am. Chem. Soc. 86:2310-2311.

Chacko, C. I., and J. L. Lockwood. 1967. Accumulation of DDT and dieldrin by microorganisms. Can. J. Microbiol. 13:1123-1126.

Chacko, C. I., J. L. Lockwood, and M. Zabig. 1966. Chlorinated hydrocarbon pesticides: Degradation by microbes. Science 154:893-894.

Collins, J. A., and B. E. Langlois. 1968. Effect of DDT, dieldrin, and heptachlor on the growth of selected bacteria. Appl. Microbiol. 16:799-802.

Cope, O. B. 1966. Contamination of the freshwater ecosystem by pesticides. J. Appl. Ecol. 3(Suppl): 33-36.

Cox, J. L. 1970a. Low ambient level uptake of ^{14}C-DDT by three species of marine phytoplankton. Bull. Environ. Contam. Toxicol. 5:218-221.

Cox, J. L. 1970b. DDT residues in marine phytoplankton: Increase from 1955 to 1969. Science 170:71-72.

Dalton, S. A., M. H. Jodkinson, and K. A. Smith. 1970. Interactions between DDT and river fungi. I. Effects of p,p'-DDT on the growth of aquatic hyphomycetes. Appl. Microbiol. 20:662-666.

Deichmann, W. B. 1972. The debate on DDT. Arch. Toxikol. 29:1-27.

deKoning, H. W., and D. C. Mortimer. 1971. DDT uptake and growth of *Euglena gracilis*. Bull. Environ. Contam. Toxicol. 6:244-248.

DeRose, H. R., and A. S. Newman. 1948. The comparison of the persistence of certain plant growth regulators when applied to soil. Proc. Soil Sci. Soc. Am. 12:222-226.

Dinamarca, M. L., L. Levinbook, and E. Valdes. 1971. DDT-dehydrochlorinase. II. Subunits, sulfhydryl groups, and chemical composition. Arch. Biochem. Biophys. 147:374-383.

Dougherty, E. M., C. F. Reichelderfer, and R. M. Faust. 1971. Sensitivity of *Bacillus thuringiensis* var. *thuringiensis* to various insecticides and herbicides. J. Invert. Pathol. 17:292-293.

Ecobichon, D. J., and P. W. Saschenbrecker. 1967. Dechlorination of DDT in frozen blood. Science 156:663-665.

Eno, C. F., and P. H. Everett. 1958. Effects of soil applications of 10 chlorinated hydrocarbon insecticides on soil microorganisms and the growth of stringless black valentine beans. Proc. Soil Sci. Soc. Am. 22:235-238.

Focht, D. D. 1972. Microbial degradation of DDT metabolites to carbon dioxide, water and chloride. Bull. Environ. Contam. Toxicol. 7:52-55.

Focht, D. D., and M. Alexander. 1970. DDT metabolism and analogs: Ring fission by *Hydrogenomonas*. Science 170:91-92.

French, A. L., and R. A. Hoopingarner. 1970. Dechlorination of DDT by membranes isolated from *Escherichia coli*. J. Econ. Entomol. 63:756-759.

Galston, A. W. 1972. Science and social responsibility, a case history. *In* The social responsibility of scientists. Ann. N. Y. Acad. Sci. 196:223-235.

Garretson, A. L., and C. L. San Clemente. 1968. Inhibition of nitrifying chemolithotrophic bacteria by several insecticides. J. Econ. Entomol. 61:285-288.

Goodman, G. T. 1974. How do chemical substances affect the environment? Proc. Roy. Soc. London, Ser. B, 185:127-148.

Gregory, W. W., J. K. Reed, and L. E. Priester. 1969. Accumulation of parathion and DDT by some algae and protozoa. J. Protozool. 16:69-73.

Guenzi, W. D., and W. E. Beard. 1967. Anaerobic biodegradation of DDT to DDD in soil. Science 156: 1116-1117.

Harrison, H. L., O. L. Loucks, J. W. Mitchell, D. F. Parkhurst, C. R. Tracy, D. G. Watts, and V. J. Yannacone, Jr. 1970. Systems studies of DDT transport. Science 170:503-508.

Hilton, B. D., T. A. Bratkowski, M. Yamada, T. Narahashi, and R. D. O'Brien. 1973. The effect of DDT analogs upon potassium conductance in synthetic membranes. Pesticide Biochem. Physiol. 3:14-19.

Hilton, B. D., and R. D. O'Brien. 1970. Antagonism by DDT of the effects of valinomycin on a synthetic membrane. Science 168:841-842.

Horvath, R. S. 1972. Microbial co-metabolism and the degradation of organic compounds in nature. Bacteriol. Rev. 36:146-155.

Horvath, R. S. 1971. Microbial cometabolism of 2,4,5-trichlorophenoxyacetic acid. Bull. Environ. Contam. Toxicol. 6:537-541.

Janicki, R. H., and W. B. Kinter. 1971. DDT inhibits Na^+, K^+, Mg^{2+}-ATPase in the intestinal mucosae and gills of marine teleosts. Nature New Biol. 233:148-149.

Johnson, B. T., R. N. Goodman, and H. S. Goldberg. 1967. Conversion of DDT to DDD by pathogenic and saprophytic bacteria associated with plants. Science 157:560-561.

Johnson, B. T., and J. O. Kennedy. 1973. Biomagnification of p,p'-DDT and methoxychlor by bacteria. Appl. Microbiol. 26:66-71.

Jones, L. W. 1952. Stability of DDT and its effect on microbial activities of soil. Soil Sci. 73:237-241.

Jones, B. R., and J. B. Moyle. 1963. Population of plankton animals and residual chlorinated hydrocarbons in soils of six Minnesota ponds treated for control of mosquito larvae. Trans. Am. Fish Soc. 92:121-124.

Kaiser, K. L. E., and P. T. S. Wong. 1974. Bacterial degradation of polychlorinated biphenyls. I. Identification of some metabolic products from *Arochlor* 1242. Bull. Environ. Contam. Toxicol. 11:291-296.

Kallman, B. J., and A. K. Andrews. 1963. Reductive dechlorination of DDT to DDD by yeast. Science 141:1050-1051.

Kapoor, I. P., R. L. Metcalf, R. L. Nystrom, and G. K. Sangha. 1970. Comparative metabolism of methoxychlor, methiochlor and DDT in mouse, insects and a model ecosystem. J. Agr. Food Chem. 18:1145-1152.

Keil, J. E., and L. E. Priester. 1969. DDT uptake and metabolism by a marine diatom. Bull. Environ. Contam. Toxicol. 4:169-173.

Kimbrough, R. D. 1972. Toxicity of chlorinated hydrocarbons and related compounds. A review including chlorinated dibenzodioxins and chlorinated dibenzofurans. Arch. Pathol. 94:125-131.

Ko, W. H., and J. L. Lockwood. 1968a. Conversion of DDT to DDD in soil and the effect of the compounds on soil microorganisms. Can. J. Microbiol. 14:1069-1073.

Ko, W. H., and J. L. Lockwood. 1968b. Accumulation and concentration of chlorinated hydrocarbon pesticides by microorganisms in soil. Can. J. Microbiol. 14: 1075-1078.

Kokke, R. 1970a. DDT: Its action and degradation in bacterial populations. Nature 226:977-978.

Kokke, R. 1970b. Pesticide and herbicide interaction with microbial ecosystems. Antonie van Leeuwenhoek 36:580-581.

Langlois, B. E. 1967. Reductive dechlorination of DDT by *Escherichia coli*. J. Dairy Sci. 50:1168-1170.

Langlois, B. E., and J. A. Collins. 1970. Growth of selected bacteria in liquid media containing DDT, dieldrin, and heptachlor. J. Dairy Sci. 53:1666-1670.

Langlois, B. E., J. A. Collins, and K. G. Sides. 1970. Some factors affecting degradation of organochlorine pesticides by bacteria. J. Dairy Sci. 53:1671-1675.

Ledford, R. A., and J. H. Chen. 1969. Degradation of DDT and DDE by cheese microorganisms. J. Food Sci. 34:386-388.

Lemire, R., and V. Fredette. 1961. Notes Préliminaires Action due DDT sur les bactéries. Rev. Can. Biol. 20:833-836.

Leng, M. L. 1972. Residues in milk and meat and safety to livestock from the use of phenoxy herbicides in pastures and rangeland. Down to Earth 28:12-20.

Lichtenstein, E. P., and K. R. Schulz. 1970. Volatilization of insecticides from various substrates. J. Agr. Food Chem. 18:814-818.

Loos, M. A. 1969a. Phenoxyalkanoic acids, p. 1-49. *In* P. C. Kearney and D. D. Kaufman (ed.), *Degradation of Herbicides*. Marcel Dekker, Inc., New York.

Loos, M. A. 1969b. Degradation of phenoxyalkanoic acid herbicides by soil microorganisms. J. Suid-Afrikaanse Chem. Inst. 22:571-578.

Mackenzie, K. A., and I. C. MacRae. 1972. Tolerance of the nitrogen-fixing system of *Azotobacter vinelandii* for four commonly used pesticides. Antonie van Leeuwenhoek 38:529-535.

Malone, T. C. 1970. *In vitro* conversion of DDT to DDD by the intestinal microflora of the northern anchovy, *Engraulis nordax*. Nature 227:848-849.

Matsumura, F., and K. C. Patil. 1969. Adenosine triphosphatase sensitive to DDT in synapses of rat brain. Science 166:121-122.

Maugh, T. H. 1973. DDT: An unrecognized source of polychlorinated biphenyls. Science 180:578-579.

Mendel, J. L., and M. S. Walton. 1966. Conversion of p,p'-DDT to p,p'-DDD by intestinal flora of the rat. Science 151:1527-1528.

Menzel, D. W., J. Anderson, and A. Randtke. 1970. Marine phytoplankton vary in their response to chlorinated hydrocarbons. Science 167:1724-1725.

Metcalf, R. L. 1973. A century of DDT. J. Agr. Food Chem. 21:511-519.

Miskus, R. P., D. P. Blair, and J. E. Casida. 1965. Conversion of DDT to DDD by bovine rumen fluid, lake water, and reduced porphyrins. J. Agr. Food Chem. 13:481-483.

Miyazaki, S., and A. J. Thorsteinson. 1972. Metabolism of DDT by fresh water diatoms. Bull. Environ. Contam. Toxicol. 8:81-83.

Martin, J. P., R. B. Harding, G. H. Cannell, and L. D. Anderson. 1959. Influence of five annual field applications of organic insecticides on soil biological and physical properties. Soil Sci. 87:334-338.

Okey, R. W., and R. H. Bogan. 1965. Apparent involvement of electronic mechanisms in limiting the microbial metabolism of pesticides. J. Water Pollut. Contr. Fed. 37:692-712.

Palmer, K. A., S. Green, and M. S. Legator. 1972. Cytogenic effects of DDT and derivatives of DDT in a cultured mammalian cell line. Toxicol. Appl. Pharmacol. 22:355-364.

Patil, K. C., F. Matsumura, and G. M. Boush. 1970. Degradation of endrin, aldrin, and DDT by soil microorganisms. Appl. Microbiol. 19:879-881.

Peterson, J. E., and W. H. Robison. 1964. Metabolic products of p,p'-DDT in the rat. Toxicol. Appl. Pharmacol. 6:321-326.

Pfaender, F. K., and M. Alexander. 1973. Effects of nutrient additives on the apparent cometabolism of DDT. J. Agr. Food Chem. 21:397-399.

Pfaender, F. K., and M. Alexander. 1972. Extensive microbial degradation of DDT *in vitro* and DDT metabolism by natural communities. J. Agr. Food Chem. 20:842-846.

Pfister, R. M. 1972. Interactions of halogenated pesticides and microorganisms: A review. CRC Crit. Rev. in Microbiol. 2:1-33.

Plimmer, J. R., V. I. Klingebiel, and B. E. Hummer. 1970. Photo-oxidation of DDT and DDE. Science 167:67-69.

Plimmer, J. R., P. C. Kearney, and D. W. von Endt. 1968. Mechanism of conversion of DDT to DDD by *Aerobacter aerogenes*. J. Agr. Food Chem. 16:594-597.

Reichel, W. L., T. G. Lamont, E. Crozartie, and L. N. Locke. 1969. Residues in two bald eagles suspected of pesticide poisoning. Bull. Environ. Contam. Toxicol. 4:24-30.

Reynolds, L. M. 1969. Polychlorbiphenyls (PCBs) and their interference with pesticide residue analysis. Bull. Environ. Contam. Toxicol. 4:128-143.

Risebrough, R. W., D. B. Peakall, S. G. German, M. N. Kirven, and P. Reiche. 1968. Polychlorinated biphenyls in the global system. Nature 220:1098-1102.

Robinson, J., A. Richardson, A. N. Crabtree, J. C. Coulson, and G. R. Patts. 1967. Organochlorine residues in marine organisms. Nature 214:1307-1311.

Rogers, L. J., W. J. Owen, and M. E. Delaney. 1971. Sites of inhibition of photosynthetic electron transport by 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane (DDT). 2nd International Congress on Photosynthesis, pp. 689-699.

Schwartz, H. G. 1967. Microbial degradation of pesticides in aqueous solutions. J. Water Poll. Contr. Fed. 39:1701-1707.

Shin, Y., J. J. Chodan, and A. R. Wolcott. 1970. Adsorption of DDT by soils, soil fractions, and biological materials. J. Agr. Food Chem. 18:1129-1133.

Singh, J., and M. Malaiyandi. 1969. Dechlorination of p,p'-DDT in aqueous media. Bull. Environ. Contam. Toxicol. 6:337-342.

Sink, J. D., H. Varela-Alvarez, and C. Hess. 1972. Metabolism of ^{14}C-DDT by ovine rumen fluid *in vitro*. J. Agr. Food Chem. 20:7-9.

Sodergren, A. 1968. Uptake and accumulation of C^{14}-DDT by *Chlorella* sp. (Chlorophyceae). Oikos 19:126-138.

Southwick, L. 1974. 2,4,5-T chronology, an update (1972-1974). Down to Earth 29:21-24.

Spencer, W. F., and M. M. Cliath. 1972. Volatility of DDT and related compounds. J. Agr. Food Chem. 20:645-649.

Stadnyk, L., R. S. Campbell, and B. T. Johnson. 1971. Pesticide effect on growth and ^{14}C assimilation in a freshwater alga. Bull. Environ. Contam. Toxicol. 6:1-8.

Stenersen, J. H. V. 1965. DDT metabolism in resistant and susceptible stable flies and in bacteria. Nature 207:660-661.

Su, G. C. G., and M. Zabig. 1972. Photochemistry of bioactive compounds. Photolysis of arlymidine derivatives in water. J. Agr. Food Chem. 20: 320-323.

Tiedje, J. M., J. M. Duxbury, M. Alexander, and J. E. Dawson. 1969. 2,4,-D metabolism: Pathway of degradation of chlorocatechols by *Arthrobacter* sp. J. Agr. Food Chem. 17:1021-1026.

Tinsley, I. J., R. Haque, and D. Schmedding. 1971. Binding of DDT to lecithin. Science 174:145-147.

Trudgill, P. W., R. Widdus, and J. S. Rees. 1971. Effects of organochlorine insecticides on bacterial growth, respiration and viability. J. Gen. Microbiol. 69:1-13.

Vance, B. D., and W. Drummond. 1969. Biological concentration of pesticides by algae. J. Amer. Water Works Assoc. 61:360-362.

Varshney, T. N., and A. C. Gaur. 1972. Effect of DDT and Sevin on soil fungi. Acta Microbiol. Acad. Sci. Hung. 19:97-102.

Walker, R. L., and A. S. Newman. 1957. Microbial decomposition of 2,4-dichlorophenoxyacetic acid. Appl. Microbiol. 3:201-205.

Ware, G. W., and C. C. Roan. 1970. Interaction of pesticides with aquatic microorganisms and plankton. Residue Rev. 33:15-45.

Wasserman, M., D. Wasserman, E. Kedar, M. Djavaherian, S. Cucos, and S. Ventura. 1973. Effect of organochlorine compounds on serum proteins. Bull. Environ. Contam. Toxicol. 10:42-50.

Wedemeyer, G. 1966. Dechlorination of DDT by *Aerobacter aerogenes*. Science 152:647.

Wedemeyer, G. 1967. Dechlorination of 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane by *Aerobacter aerogenes*. I. Metabolic products. Appl. Microbiol. 15:569-574.

Wilson, J. K., and R. S. Choudhri. 1946. Effects of DDT on certain microbial processes in the soil. J. Econ. Entomol. 39:537-538.

Winely, C. L., and C. L. San Clemente. 1970. Effects of pesticides on nitrate oxidation by *Nitrobacter agilis*. Appl. Microbiol. 19:214-219.

Woodwell, G. M., C. F. Wurster, and P. A. Isaacson. 1967. DDT residues in an east coast estuary: A case of biological concentration of a persistent insecticide. Science 156:821-824.

Woodwell, G. M., P. P. Craig, and H. A. Johnson. 1971. DDT in the biosphere: Where does it go? Science 174:1101-1107.

Woolson, E. A., R. F. Thomas, and P. D. J. Ensor. 1972. Survey of polychloro-p-dioxin content in selected pesticides. J. Agr. Food Chem. 20:351-354.

Wurster, C. F. 1968. DDT reduces photosynthesis by marine phytoplankton. Science 159:1474-1475.

EXPERIMENTAL RESULTS

SECTION 1. THE SEPARATION OF DDT FROM CELLS IN SUSPENSION AND THE CHANGES IN SOLUBILITY OF DDT ON EXPOSURE TO CELLS

Introduction

The various techniques previously reported to ascertain uptake of insoluble pesticide by cells are inadequate for quantitative determinations. Such techniques include (i) autoradiography of microbial colonies grown on medium containing DDT, (ii) direct extraction with hexane of a suspension of DDT and cells, (iii) centrifugation of the suspension prior to extraction, (iv) filtering the suspension prior to extraction; (v) washing mycelia with water prior to extraction; and (vi) a combination of (iii), (iv), and (v). The researchers who performed these techniques apparently did not check the validity or reliability of results stemming from their use. Moreover, the aqueous solubility of the pesticide has been reported to be 5 or less ng/ml. Interactions of DDT with other biological materials in effect may change the solubility of the compound, which in turn may alter the efficiency of the technique used to separate DDT and cells. The possible changes in the solubility of

the DDT after exposure to cells could have a profound effect on the fundamental interactions of biological systems with the pesticide. In addition, all suspended and soluble DDT must be removed from the suspended cells before any accurate estimates of uptake, by intact cells or subcellular fractions, can be determined.

After initial trials it became apparent that both differential centrifugation and filtration of pesticide-cell mixtures were entirely unsuitable procedures for achieving good separation. Microscopic examination of the suspensions suggested that many DDT particles were as large as, or larger than, the bacterial cells in the immediate environment. Moreover, the density of DDT is about 1.5, whereas the density of the bacterial cell is about 1.1. Thus a program was launched to develop sucrose density gradient techniques to achieve this separation. By use of this procedure one could see, even without chemical analysis, the degree of gross separation of DDT and cells, a factor which was highly desirable in expediting the screening of initial parameters involved in the separation.

Materials and Methods

Most methods used here are outlined in Article 1. The procedures used to remove supernatant DDT from cells are illustrated in Figure 2. When extra detail or emphasis is appropriate, the methods are reiterated here.

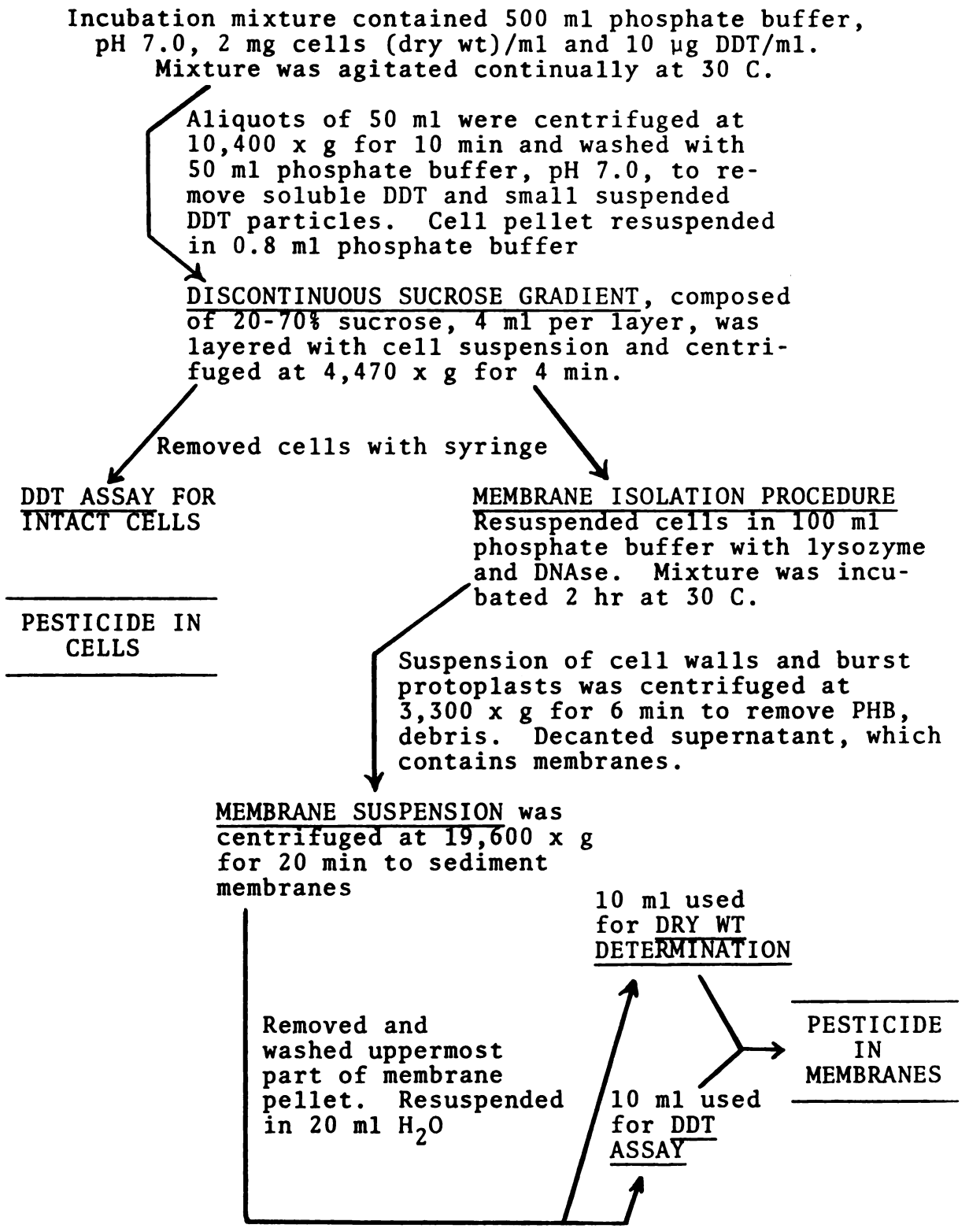

Figure 2. Procedure developed to prepare intact cells or membranes for DDT assay.

Cellulose nitrate centrifuge tubes were used to hold all gradients because they are very transparent and are not susceptible to the actions of organic solvents used to extract DDT that may have been adsorbed to the walls of the tube. Gradients were prepared immediately before use because the 70% sucrose solution in the bottom layer tended to precipitate if stored more than about three hours. Many trials consisting of different centrifuge speeds or various cell preparation procedures were needed before optimal separation of the DDT and cells was obtained. In all sucrose phases of the gradient, these were followed by extraction and and analysis over 24 h. After long exposure of cells to DDT these analyses tested for efficiency of separation and also elucidated the changing physical state of the DDT. In addition, prior to sucrose gradient centrifugation, the amounts of DDT in cell washes and in incubation mixture supernatants were determined to evaluate total recovery of the pesticide.

Results

The optimum relative centrifugal force and time were found to be about 4 min at a force that would just exceed the vibration point of the centrifuge (this is stated in Article 1 to be 4,470 x g, but slightly smaller forces were also satisfactory). The cells formed a wide band in the 40% sucrose layer, whereas particulate DDT

sedimented into the lowermost (70%) sucrose layer. The cells did not reach an equilibrium point in the 40% sucrose; centrifugation for longer periods eventually would drive the cells into the 70% sucrose. Total recovery of pesticide, expressed as percent DDT plus DDD extracted divided by the amount of DDT introduced to the incubation medium, was always in excess of 88%. Thus, little pesticide was lost through vaporization. In addition, no evidence was found of any degradation product other than DDD. Occasionally small but irreproducible extraneous peaks appeared during gas chromatographic analysis.

Most of the pesticide in the upper five layers (e plus f in Figure 3) was associated with the intact cells (f) in the 40% sucrose layer. Successive washing of cells with phosphate buffer prior to gradient centrifugation reduced somewhat the amount of DDT found apart from the cells within the gradient. Since the gradient itself apparently duplicated this washing effect, preliminary washes were eliminated from the procedure.

Good separation of cells and DDT was achieved over the entire 24 h test period. Surprisingly, much of the DDT became more soluble in the aqueous medium as the exposure time increased (Figure 3). There was an initial increase in cell associated DDT (up to 1.5 h) followed by a marked decrease of pesticide in the gradient with a

Figure 3. Distribution of DDT in aqueous media, phosphate buffer washes and sucrose density gradients over a 24 h exposure period.

Closed circles denote total measured recovery of pesticide from 500 µg in each sample. Pesticide unaccounted for is indicated by a. Centrifugation removed increasingly large amounts of free DDT (▽) and washing the cells continued the trend (■). The pesticide in the supernatants (b) and cell washes (c) was discarded. Most DDT sedimented to the 70% sucrose of the gradient (d) and remaining pesticide (□) was free in the other sucrose layers (e) or was bound to the cells (△) in the 40% sucrose layer (f). Data are composite averages of many determinations.

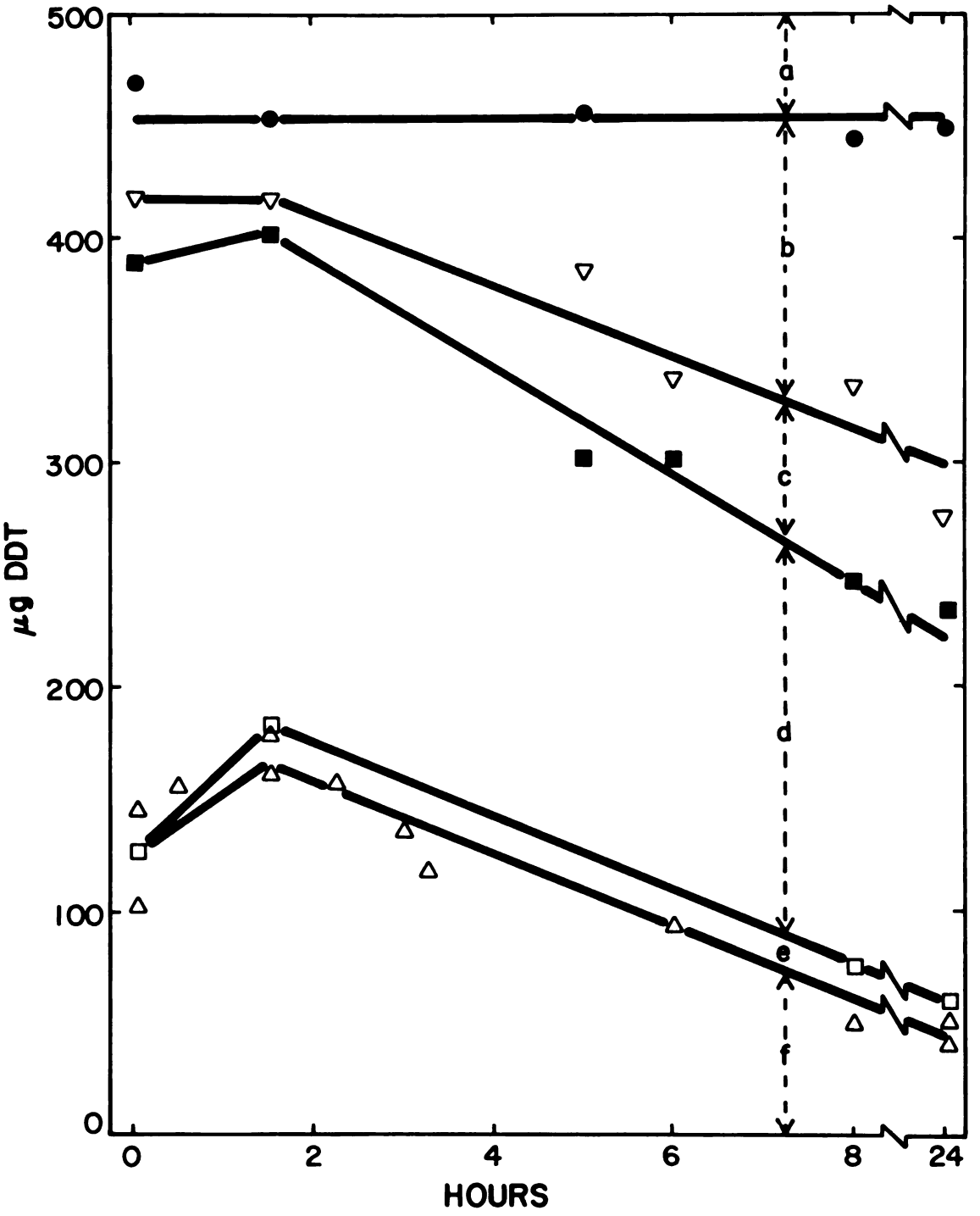

Figure 3

concomitant increase in unsedimentible pesticide in the supernatant of the incubation mixture. The quantity of pesticide in the cell washes also increased over 24 h. To confirm this apparent increase in pesticide solubility, supernatant fluids of the incubation mixture were subjected to ultracentrifugation which removed 26 to 50% of the DDT. Nevertheless, extended exposure caused more DDT to remain in solution even at high centrifuge speeds (Table 3).

Crystalline material, presumably pesticide, was observed microscopically in the supernatants of the incubation mixture, as well as in the buffer used to wash the cells. Whereas the DDT in the 70% sucrose layer of the gradient was primarily macroscopic, microscopic particles, some of which were smaller than *B. megaterium* cells, were also included within this layer. Thus, the gradient was effective in separating relatively small as well as large particles from the cells.

Discussion

It is clear that the centrifugation of a suspension containing DDT plus cells does allow suspended free DDT to enter the cell pellet. However, the described sucrose gradient technique effectively and efficiently removes the particulate DDT associated with the cell pellet after initial centrifugation and washes. Thus, the technique seemed appropriate for use in preparing cells for precise

Table 3. Changes in DDT sedimentation characteristics during exposure to cells

	Time (h)				
	0	1.5	5	8	24
μg Σ DDT[a] in cell supernatant after centrifugation at 10,400 x g for 10 min	48	30	78	107	172
μg Σ DDT sedimented from supernatant by ultracentrifugation (100,000-280,000 x g; 1 h)	22	15	28	34	44
% Σ DDT sedimented by ultracentrifugation	48	50	36	34	26

[a] Σ DDT = DDT + DDD.

measurements of pesticide uptake by intact cells and, indirectly, subcellular parts.

Development and testing of the gradient technique unexpectedly revealed that the apparent solubility of the pesticide changed markedly upon continued exposure to the bacterial cells. Consequently there exists no single value to express DDT solubility during the course of the experiments to be described below.

The *B. megaterium* strain used here forms particularly large cells which tend to form chains containing two to four bacteria. Therefore, the functional units of this organism are considerably larger than many other bacteria. As a sucrose gradient separates particles on the basis of size as well as density, it would appear that efficiency of separation of a somewhat smaller bacterium from DDT particles would be even greater; a smaller bacterium most probably would not move as fast through the gradient. Other microbial species or other insoluble particles were not tested but, in light of its practicability, the method seems entirely suitable for other systems.

SECTION 2 (ARTICLE 1)

LOCATION AND CONSEQUENCES OF 1,1,1-TRICHLORO-2,2-BIS(p-CHLOROPHENYL)ETHANE UPTAKE BY *BACILLUS MEGATERIUM*

By

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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. Winely 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) Bactopeptone (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 10^7 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 10^7 bacteria. Bacterial growth was determined visually.

About 3×10^8 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 were 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 monitored 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 Gas 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 20% (wt/wt) sucrose solutions, were employed to separate cells from sedimentible DDT. Cells, suspended at 2.0 mg of cell dry weight/ml in 50 ml of buffer containing 10 μg of DDT/ml, were centrifuged (10,400 × *g*, 10 min), washed with 100 ml of buffer, and resedimented. This procedure eliminated virtually all acetone and nonsedimentible pesticide. The pellet was thoroughly suspended in 0.8 ± 0.1 ml of buffer and layered on the gradient. Buffer was added to bring the total volume of the layer to 1.0 ml. The gradient was then centrifuged (4,470 × *g*, 4 min) in a swinging-bucket rotor (Sorvall, HS-4), which was allowed to coast to a stop. The cells 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 acetone 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 spectrophotometer, model DB-G, was used for these determinations. Observations of $KMnO_4$-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 Mallinckrodt 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-

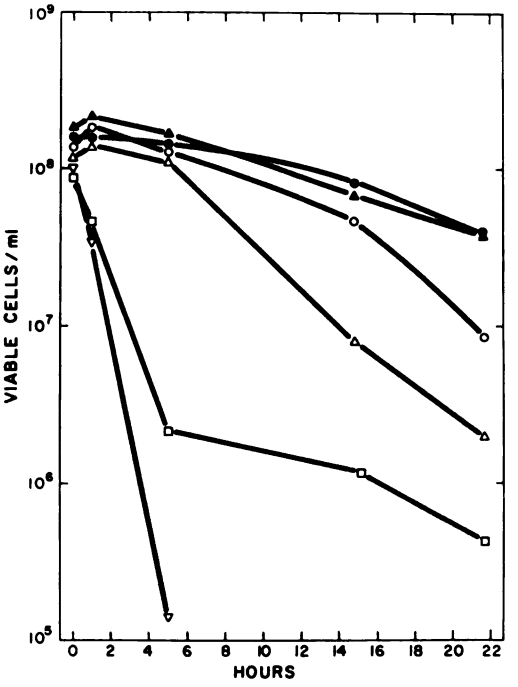

FIG. 1. *Effect of DDT on the viability of B. megaterium. Cells (2.0 mg of dry weight/ml) were agitated in buffer containing 1 μg of DDT/ml (○), 10 μg of DDT/ml (△), 50 μg of DDT/ml (□), 100 μg of DDT/ml (▽), acetone alone (▲), or no addition (●). Values are representative of six experiments.*

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 discounted 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*[a]

Exposure time (h)	Q_{O_2} (μliters of O_2 per hr per mg of cell dry wt)											
	Endogenous						Exogenous					
	Control	Acetone	DDT (μg/ml)				Control	Acetone	DDT (μg/ml)			
			1	10	50	100			1	10	50	100
0	38	35	38	39		36	110	118	105	107		105
4	15	17	15	14	18	16	117	112	110	112	100	
8	11	11	12	9		12	90	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	55

[a] 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.

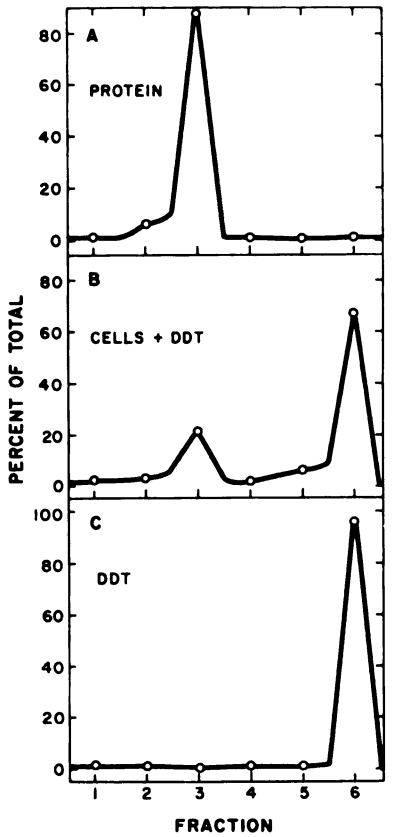

FIG. 2. *Relative amounts of DDT and protein found in each of six fractions of the sucrose gradients. Percentages are based on the total pesticide recovered, which varied from 89 to 96% of the DDT originally added. Figures 2A and 2C show the distribution of protein or DDT, respectively, when cells only or DDT only were subjected to gradient centrifugation. Figure 2B illustrates the typical DDT distribution obtained from preparations containing cells treated for 4 h with DDT.*

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

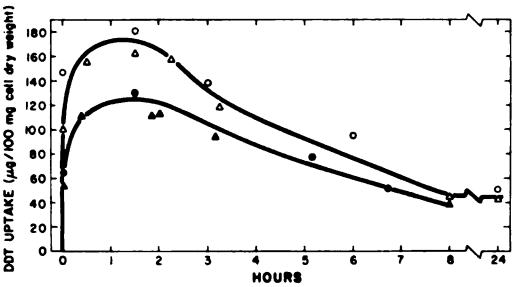

FIG. 3. *Uptake of DDT by resting cells of B. megaterium (open symbols) or by membranes isolated from similar cells (closed symbols) after exposure to 10 μg of DDT/ml. Amounts of 2 mg dry weight of cells/ml of buffer (containing 10 μg of DDT/ml) were exposed for 24 hr. The circles and triangles denote two separate experiments for each curve.*

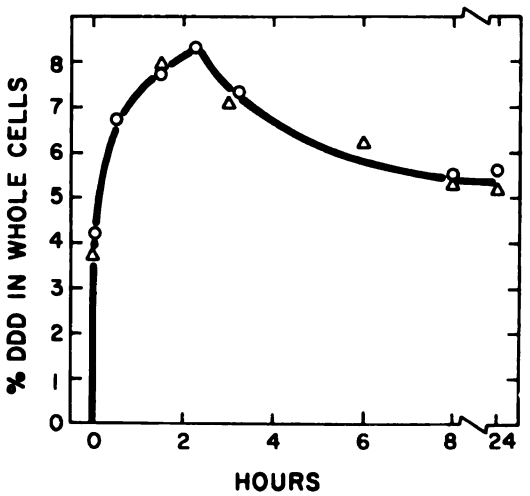

FIG. 4. *DDD content of the total pesticide recovered from whole cells of B. megaterium. Different symbols denote different experiments.*

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/cm^2, 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

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 repartitioned 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 suprising 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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LITERATURE CITED

1. Alexander, M. 1961. Introduction to soil microbiology. John Wiley & Sons, New York.
2. Chacko, C. I., and J. L. Lockwood. 1967. Accumulation of DDT and dieldrin by microorganisms. Can. J. Microbiol. **13**:1123-1126.
3. Dalton, S. A., M. Hodkinson, and K. A. Smith. 1970. Interactions between DDT and river fungi. I. Effects of *p,p'*-DDT on the growth of aquatic hyphomycetes. Appl. Microbiol. **20**:662-666.
4. de Koning, H. W., and D. C. Martinez. 1971. DDT uptake and growth of *Euglena gracilis*. Bull. Environ. Contam. Toxicol. **6**:244-248.
5. Gerhardt, B., and H. Beevers. 1968. Influence of sucrose on protein determination by the Lowry procedure. Anal. Biochem. **24**:337-338.
6. Harrison, H. L., O. L. Loucks, J. W. Mitchell, D. F. Parkhurst, C. R. Tracy, D. G. Watts, and V. J. Yannacone, Jr. 1970. Systems studies of DDT transport. Science **170**:503-508.
7. Janicki, R. H., and W. B. Kinter. 1971. DDT inhibits Na^+,K^+,Mg^{2+}-ATPase in the intestinal mucosae and gills of marine teleosts. Nature N. Biol. **233**:148-149.
8. Ko, W. H., and J. L. Lockwood. 1968. Accumulation and concentration of chlorinated hydrocarbon pesticides by microorganisms in soil. Can. J. Microbiol. **14**:1075-1078.
9. Ko, W. H., and J. L. Lockwood. 1968. Conversion of DDT to DDD in soil and the effects of these compounds on soil microorganisms. Can. J. Microbiol. **14**:1069-1073.
10. Kondo, T., and M. Tomizawa. 1969. Hemolytic actions of short-chain alkylamine hydrochlorides. J. Pharm. Sci. **58**:255-256.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
12. Salton, M. R. J. 1967. Structure and function of bacterial cell membranes. Annu. Rev. Microbiol. **21**:417-422.
13. Salton, M. R. J., and M. D. Schmitt. 1967. Effects of diphenylamine on carotenoids and menaquinones in bacterial membranes. Biochim. Biophys. Acta **135**:196-207.
14. Shin, Y. O., J. J. Chodan, and A. R. Wolcott. 1970. Adsorption of DDT by soils, soil fractions, and biological materials. J. Agr. Food Chem. **18**:1129-1133.
15. Storck, R., and J. T. Wachsman. 1957. Enzyme localization in *Bacillus megaterium*. J. Bacteriol. **73**:784-790.
16. Trudgill, P. W., R. Widdus, and J. S. Rees. 1971. Effects of organochlorine insecticides on bacterial growth, respiration and viability. J. Gen. Microbiol. **69**:1-13.
17. Umbreit, W. W., R. J. Burris, and J. F. Stauffer. 1964. Manometric techniques, 4th ed. Burgess Publishing Co., Minneapolis.
18. Winely, C. L., and C. L. San Clemente. 1970. Effects of pesticides on nitrite oxidation by *Nitrobacter agilis*. Appl. Microbiol. **19**:214-219.
19. Wodzinski, R. S., and D. Bertolini. 1972. Physical state in which naphthalene and bibenzyl are utilized by bacteria. Appl. Microbiol. **23**:1077-1081.
20. Wodzinski, R. S., and M. J. Johnson. 1968. Yields of bacterial cells from hydracarbons. Appl. Microbiol. **16**:1886-1891.
21. Woodwell, G. M., P. P. Craig, and H. A. Johnson. 1971. DDT in the biosphere: where does it go? Science **174**:1101-1107.
22. Yudkin, M. D. 1966. Isolation and analysis of the protoplast membrane of *Bacillus megaterium*. Biochem. J. **98**:923-928.

SECTION 3 (ARTICLE 2)

DISTRIBUTION AND EFFECTS OF 2,4,5-T IN CELLS OF *BACILLUS MEGATERIUM*

By

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Distribution and Effects of 2,4,5-T in Cells of *Bacillus megaterium*[1]

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ABSTRACT

Cell death in a resting population of *Bacillus megaterium* was accelerated by ambient concentrations of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) equal to or greater than 10 µg/ml, or 5 µg/mg of cells (dry weight), but only after prolonged exposure. Conversely, populations of growing cells were not markedly influenced even at 100 µg/ml. Effects on cell respiration were not manifest until the ambient concentration reached 1000 µg of 2,4,5-T/ml, or 500 µg/mg. Cells of *B. megaterium* did, however, accumulate 2,4,5-T passively to a level approximately 2-fold above the ambient concentration. Most of the accumulated compound was easily washed from the cells but, of the firmly bound herbicide, about 0.5 µg/mg of cells (dry weight), nearly 60% by weight, was localized in the protoplast membrane. The foregoing results, obtained with a purified preparation of 2,4,5-T, were also elicited by 2,4,5-T analytical standards. The extracted contaminants did not produce the results alone, nor did they influence the results when present in combination with 2,4,5-T.

(INTRODUCTION)

The herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) has been used extensively to control broadleaf woody vegetation throughout the world. Some 2,4,5-T preparations contain various amounts of chlorodibenzo-p-dioxins (CDDs) and chlorodibenzofurans, principally the extremely toxic 2,3,7,8-tetrachlorodibenzo-p-dioxin that has been an inherent contaminant in the manufacturing process (Kimbrough, 1972). The occupational and environmental hazards of 2,4,5-T preparations of various degrees of purity have been investigated (see Lee and Falk, 1973). But, apart from degradation, the interactions of 2,4,5-T with bacterial cells are largely unknown (loos, 1969).

Dougherty *et al.* (1971) reported that 2,4,5-T inhibits growth of *Bacillus thuringiensis*. The minimum inhibitory concentration depended on the solvent system used to deliver the agent but was not less than 0.01 M (2.55 mg/ml). By means of a soil perfusion system, Loos (1969b) attempted to select for microflora that would decompose 2,4,5-T. A perfusing solution containing 200 µg 2,4,5-T per ml caused the soil to lose biological activity.

Although 2,4,5-T has been reported to persist in the environment for up to 270 days (Loos, 1969), it is nevertheless subject to slow biodegradation. The pathway may be similar to that by which the much less recalcitrant

2,4,-dichlorophenoxyacetic acid is degraded (Horvath, 1971, 1972).

The subcellular distribution of 2,4,5-T apparently has not been examined. Since the molecule contains both hydrophilic and hydrophobic regions, it is amphipathic and might well lodge in membranes. Such accumulations could be important in the physiology of cells and might influence the way in which the herbicide moves through the biosphere.

In this paper, we show that binding of 2,4,5-T to cells of *B. megaterium* is easily reversible. However, small quantities of 2,4,5-T are firmly sequestered by cell membranes.

MATERIALS AND METHODS

Organism and growth conditions. Cells of *B. megaterium*, strain KM, were grown and harvested as described previously (Hicks and Corner, 1973).

Stock 2,4,5-T preparation and extraction of contaminants. A preparation of 2,4,5-T (K and K Laboratories, Plainview, N.Y.) was cleaned by recrystallization three times from benzene, washing with n-hexane in a separatory funnel, and employing the methods of Woolson *et al.* (1972) to remove possible CDD contaminants. The resulting solution was dried under nitrogen to provide a pesticide-free

extract of contaminants for determination of interference or synergistic effects in the reported experiments.

Stock solutions of 2,4,5-T (1 mg/ml) were made by dissolving K_2HPO_4 (6.80 g) and NaOH (0.02 g) in about 900 ml of distilled water to which solution 2,4,5-T (1 g) was added. Following agitation for approximately 3 h, or until all the 2,4,5-T was dissolved, KH_2PO_4 (8.71 g) and distilled water to one liter were added to yield 1 g herbicide per liter of 0.1 M potassium phosphate buffer, pH 7.0. For long term storage, the stock solution was filtered and sterilized by repetitive steaming. This process did not affect the herbicide as determined by comparison with an identical but unheated solution.

Growth experiments. The medium used in the growth experiments consisted of 20 g Oxoid peptone (Flow Labs, Rockville, Md.), 900 ml distilled H_2O and 100 ml potassium phosphate buffer. Sterilized buffer alone or buffer containing herbicide was added to the previously autoclaved peptone solution. Final concentrations of herbicide in the media were 1, 10, 50 or 100 µg per ml. Media were inoculated with about 10^8 cells taken from the late exponential phase of growth. The cultures were incubated at 30 C with aeration by means of shaking. Growth of bacteria in media containing herbicide was monitored both by following changes in optical density at 700 nm and by microscopic observation over a 30 h period.

Survival measurements. Cells (2 mg dry weight/ml) were suspended in buffer containing 1, 10, 50 or 100 µg of 2,4,5-T per ml. Flasks containing these mixtures were agitated at 30 C for periods up to 24 h. Periodically, measurements of colony-forming units were made by appropriately diluting culture samples in 1.2% (weight/vol) peptone (Difco) and spreading the diluted samples on 3 Trypticase soy agar (BBL) plates.

Respiratory measurements. Respiration of cells was measured using conventional Warburg respirometry (Umbreit *et al.*, 1964). Cells were exposed to 10, 50, 100, 500 or 1000 µg of 2,4,5-T per ml of cell suspension which contained 2 mg of cells (dry weight)/ml in phosphate buffer.

Analysis of 2,4,5-T retention by intact cells. Intact cells were placed in a 2-liter flask containing 500 ml of buffer (pH 7.0) and 10 µg of 2,4,5-T per ml. The final biomass concentration in the flask was 2 mg of cells (dry weight) per ml. Hence the effective concentration of the herbicide was 5 µg per mg of cells. At selected intervals, 50 ml samples of cell suspension were withdrawn from the flask, sedimented in the cold (10,400 x g; 10 min) and washed once by centrifugation with approximately 100 ml of buffer and twice with 100 ml of distilled H_2O. The 2,4,5-T was then extracted from the cells in

preparation for analysis by gas chromatography. A control flask without added herbicide was treated identically to check for substances that might have interfered with the herbicide assay.

Analysis of 2,4,5-T retention by membranes. Cells were treated as described above, but membranes were isolated from cells exposed to 2,4,5-T prior to extraction of the herbicide. Cells were washed twice with buffer (10,400 x g; 10 min) and resuspended in buffer. Lysozyme (mucopeptide-N-acetylmuramylhydrolase; EC 3.2.1.17) was added at 0.5 μg per mg of cells (dry weight) and the mixture was allowed to incubate for 2 h at 30 C. Five milligrams of deoxyribonucleate oligonucleotidohydrolase (EC 3.1.4.5) were then added to reduce the viscosity of the mixture. The preparation was centrifuged once at low speed (3,300 x g; 6 min) to remove debris and most poly-β-hydroxybutyrate granules. The membranes were sedimented (19,000 x g; 30 min) and washed once with buffer and twice with distilled H_2O; one-half was assayed for herbicide content and the other half was used for a dry weight determination. The latter was necessary because membrane yield tended to decrease in older cells.

Determination of absorptive capacity of the cells to 2,4,5-T. The basic methods of Scherrer and Gerhardt (1971), the so-called space or thick suspension technique,

were followed except that equilibration periods ranged from 0.5 to 10 h. In addition, the amounts of pesticide in the cell pellets as well as those in the supernatant solutions were determined. Known wet weights of previously washed cells were therefore exposed to 10 µg of 2,4,5-T per ml (initial concentration) and, at selected intervals, the cells were sedimented by centrifugation. The pesticide contents of both the cell pellets and the supernatants were determined by gas chromatography. Little or no herbicide absorbed to the walls of the centrifuge tube and temperature-induced precipitation did not occur.

Calculation of amounts of 2,4,5-T taken up by cells. The interstitial space (the aqueous volume between the packed *B. megaterium* cells in the pellet) is 21.6% of the pellet volume (Scherrer and Gerhardt, 1971). The void volume within the cell pellet, which includes the space within the peptidoglycan cell wall and the interstitial space, is between 43.4% (pellet space infiltrated by stachyose) and 50.5% (pellet space infiltrated by sucrose); neither sucrose nor stachyose penetrate the protoplast membrane in significant levels (Corner and Marquis, 1969). These data are in general agreement with those of Marquis (1968), who reported that about 36% of the total cell volume of a nonplasmolyzed cell consisted of "sucrose space" of the cell wall. The sucrose space

calculated from the data of Scherrer and Gerhardt (1971), is 28.9%. The 36% wall space value and the 21.6% interstitial space value were used in our calculations in order to minimize the possibility of overestimating the magnitude of 2,4,5-T absorption. The ambient pesticide concentration in the supernatant after equilibration was considered to approximate the concentration of pesticide in the void volume. This quantity was subtracted from the total pesticide found in the entire cell pellet; the remaining pesticide in the pellet was associated with the membrane, cytoplasm or cell wall materials, exclusive of peptidoglycan void space.

Extraction and analysis of 2,4,5-T. Samples containing herbicide were suspended in 10 ml of distilled H_2O, acidified to pH 2.0 with 2 N HCl and extracted three times with several volumes of diethyl ether in a separatory funnel. Samples containing cells or membranes frequently formed emulsions that would not break spontaneously. When this occurred, about 1 gm of anhydrous Na_2SO_4 (previously washed with diethyl ether) was added to the emulsion and the mixture was allowed to stand for 24 h. This procedure resulted in good separation of the ether and H_2O phases. The spent Na_2SO_4 was then extracted three times with diethyl ether and all ethereal extracts were pooled and appropriately reduced in volume by evaporation at room temperature under N_2. The Na_2SO_4

did not affect the extraction efficiency or introduce measurable contaminants.

The herbicide extracted from each sample was esterified by dropwise addition of diethyl ether solution of diazomethane. Diazomethane was freshly prepared from Diazold (Aldrich Chemical Co., Inc., Milwaukee, Wisc.) by distilling an ethereal Diazold solution in the presence of 95% ethanol and KOH. Analytical standards of 2,4,5-T and standards made from our cleaned preparation were esterified as above. Actual quantitative analysis of the 2,4,5-T ester was made by use of a Beckman GC-4 gas chromatograph equipped with an electron capture detector and a column containing 10% DC 200 on Gas Chrom Q, 60/80 mesh. Helium was the carrier gas and the column temperature was 180 C.

RESULTS

Purity of herbicide and effects of contaminants. The cleaned herbicide preparation produced no extraneous peaks when analyzed by electron capture gas chromatography. However, several relatively small unidentified peaks were detected in the analysis for CDD contaminants in the uncleaned preparation. The contaminants extracted from the uncleaned preparation did not affect the growth rate, the survival of resting cells, cell respiration or cause fluctuations in uptake values when used alone or in

combination with the cleaned preparation. Furthermore, when respiration or survival of resting cells was determined, 2,4,5-T analytical standards of high but unknown purity yielded results identical to those produced by the cleaned preparation. No evidence of 2,4,5-T degradation or cometabolism was found during the course of the reported experiments. Therefore, effects reported here are attributable solely to 2,4,5-T rather than to degradation products or contaminants.

Action of 2,4,5-T on resting cells. Exposure of cells, aerated in phosphate buffer, to various concentrations of 2,4,5-T revealed that levels as low as 10 µg/ml (5 µg/mg of cells, dry weight) did accelerate death in the population (Figure 1). However, the acceleration was only slight at 10 µg/ml but became greater when the 2,4,5-T concentration was raised to 50 or 100 µg/ml. Moreover, the major increases in death rate occurred only after about 24 h of exposure.

Action of 2,4,5-T on growing cells. Although exposure to 10 µg/mg was at least marginally lethal to resting cells and higher concentrations did accelerate death, growing cells were not affected by exposure to concentrations less than 100 µg/ml (Figure 2). Even at this particular concentration, the inhibition was expressed as a slight increase in the length of the lag

Figure 1. Survival of cells of *B. megaterium* (2 mg of cells, dry weight, per ml) in buffer containing 10 µg/ml (□), 50 µg/ml (Δ), 100 µg/ml (●) or no addition (0). Cells were incubated at 30 C with shaking. Data averaged from 5 experiments.

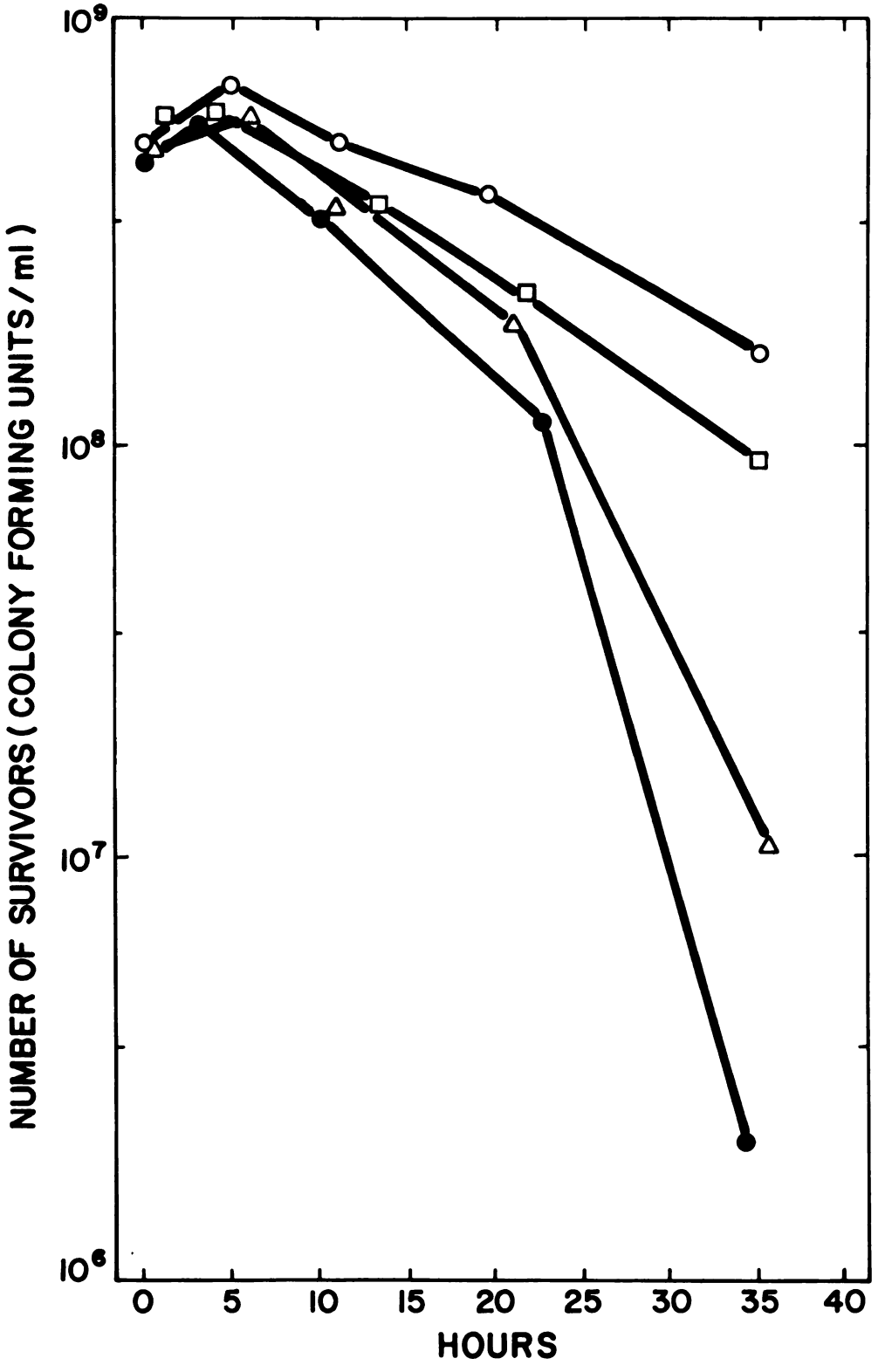
10⁹
10⁸
10⁷
10⁶
NUMBER OF SURVIVORS (COLONY FORMING UNITS / ml)
0
5
10
15
20
25
30
35
40
HOURS

Figure 1

Figure 2. Effects of 2,4,5-T on cells of *B. megaterium* grown in buffered peptone. Optical densities at 700 nm were determined during the growth cycles. The upper curve represents cell growth at 2,4,5-T concentrations of 1 µg/ml (O), 10 µg/ml (□), 50 µg/ml (Δ) and no herbicide (●); the lower curve illustrates cell growth at 100 µg 2,4,5-T/ml (▲). Data averaged from 6 experiments.

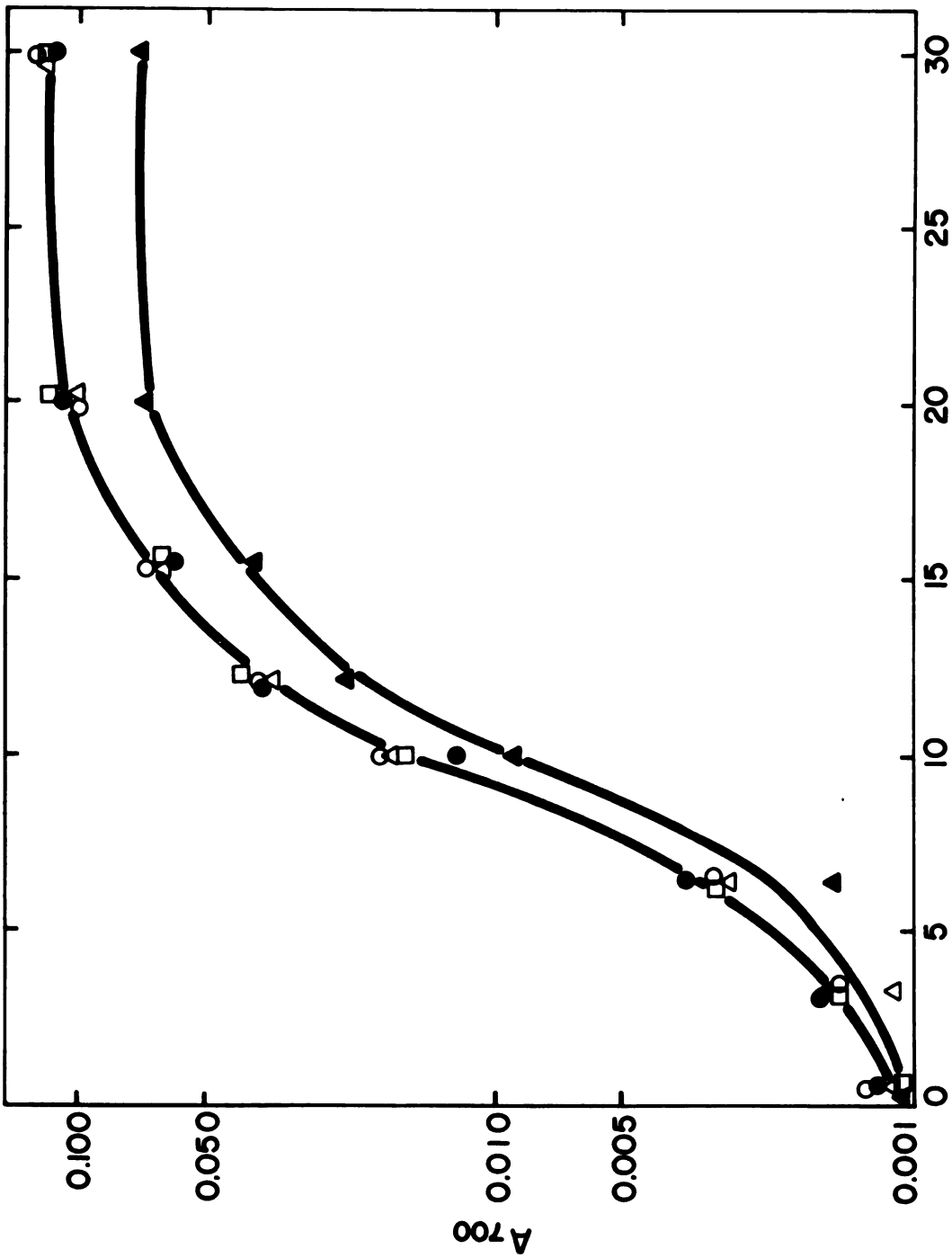

Figure 2

period and a reduced total cell yield. The exponential growth rate was not markedly affected by the presence of 100 µg of 2,4,5-T/ml.

Effect of 2,4,5-T on cell respiration. The rates of endogenous or exogenous (glucose) metabolism were not significantly different among cells exposed to up to 500 µg 2,4,5-T/ml (250 µg/mg of cells, dry weight) and unexposed cells (Figure 3). The rate of endogenous metabolism was markedly reduced in cells exposed to 1000 µg/ml and the rate at which glucose was oxidized was somewhat less than that exhibited by unexposed cells.

Uptake characteristics. The 2,4,5-T found in the cell pellet under static conditions, exclusive of the interstitial space and the peptidoglycan, was about 2-fold higher than the theoretical ambient concentration that would have existed in cells freely permeable to the herbicide (Table 1). However, after washing, equivalent cells retained only about 0.49 µg of the herbicide (Table 2) so that much of the bound compound was easily removed from the cells. Although this herbicide retention was well below the theoretical ambient concentration of about 1 µg/100 mg cells (dry weight), the amount retained by membranes, about 0.3 µg, was greater than the theoretical ambient level of 0.065 µg/mg of membranes (dry weight). The amount of herbicide bound to membranes of exposed

Figure 3. Oxygen uptake at 30 C by cells of *B. megaterium* (2 mg of cells, dry weight, per ml), exposed to 10 µg 2,4,5-T/ml (Δ), 50 µg/ml (□), 100 µg/ml (●), 500 µg/ml (▽), 1000 µg/ml (■) or no addition (0). Arrow indicates addition of glucose.

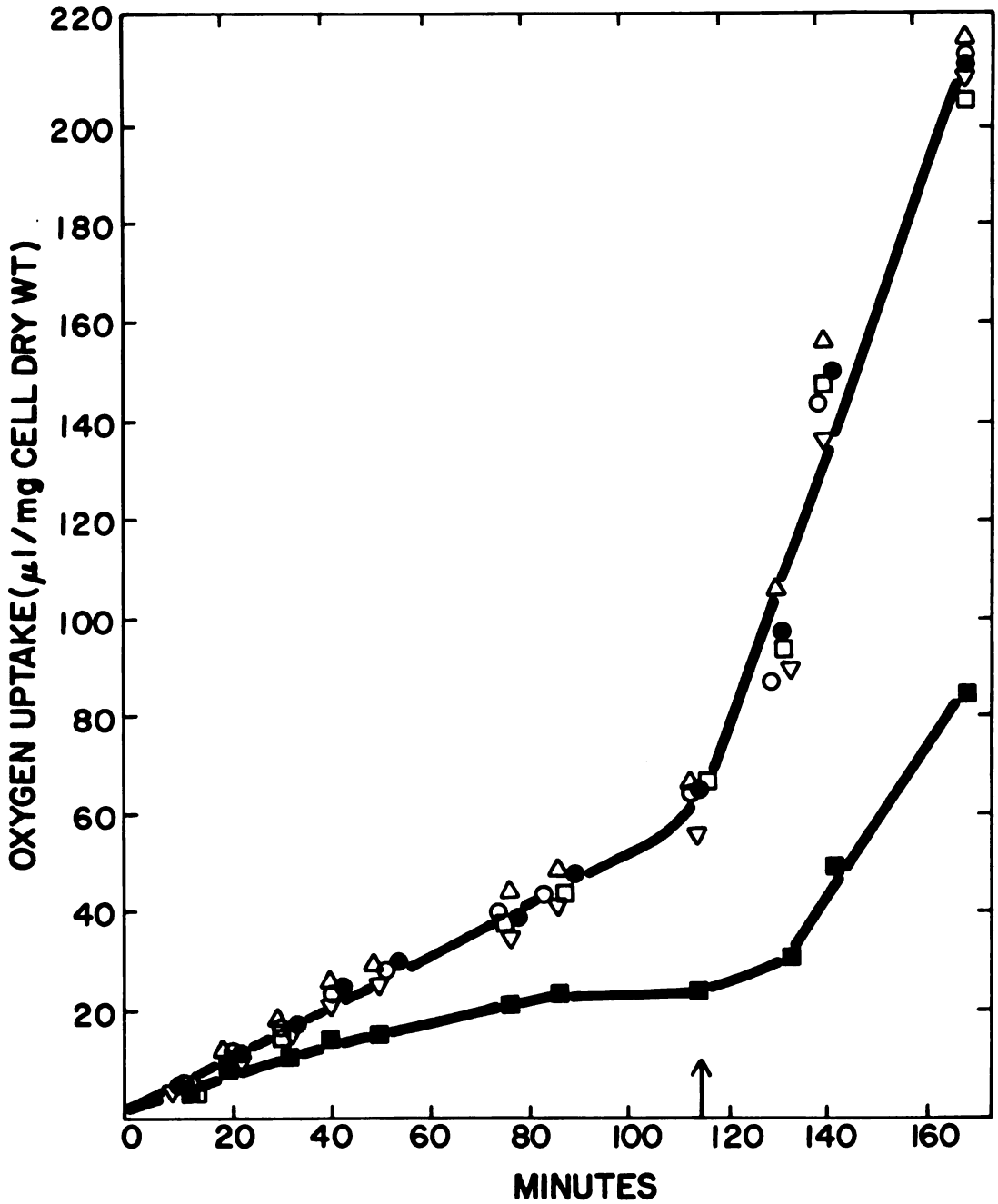
OXYGEN UPTAKE(μl/mg CELL DRY WT)
220
200
180
160
140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
160
MINUTES

Figure 3

Table 1. Uptake of 2,4,5-T by cells of *Bacillus megaterium*

Equilibration time (b)	Predicted[a] value (µg)	Experimental[b] value (µg)	Fold increase
0.5	2.1	4.3	2.0
1	2.7	5.2	1.9
3	2.2	4.6	2.1
10	2.2	5.1	2.3

[a]Theoretical ambient concentration of 2,4,5-T in 500 mg (wet weight) cells.

[b]Uptake as determined by the thick suspension technique (Scherrer). Data averaged from four experiments.

Table 2. Retention of 2,4,5-T by 100 mg of cells (dry weight) of *Bacillus megaterium* or by membranes

Exposure period (h)	Residual 2,4,5-T (µg)	
	In cells[a]	In membranes[b]
0-3	0.49	0.30
4-9	0.48	0.29
10-13	-	0.34
23-24	0.49	0.29

[a]Residual herbicide in 100 mg cells (dry weight) after exposure to 10 µg 2,4,5-T/ml (5 µg/mg cells). Data averaged from three experiments.

[b]Residual herbicide in membranes isolated from 100 mg exposed cells. Membranes were considered to comprise 6.5% of cell dry weight. Data averaged from four experiments.

cells represented about 60% of the total sequestered pesticide.

DISCUSSION

The experimental conditions used here were not designed to simulate conditions in the natural environments of organisms in soil or water but rather to permit elucidation of the potential responses of a model bacterium to 2,4,5-T. Moreover, the pesticide concentrations were not typical of 2,4,5-T concentrations normally found in contaminated soils, waters or sediments.

The methods used to remove possible contaminants from the crude 2,4,5-T preparation were adequate to minimize possible influence of the contaminants on the experimental results. However, the reported extreme toxicity of some CDD isomers (Kimbrough, 1972) necessitates exhaustive analytical and toxicological studies on each individual 2,4,5-T preparation before making judgments about absolute purity or toxicity to higher organisms.

Low concentrations of 2,4,5-T accelerated the death of resting cells, but similar concentrations did not kill cells in growing cultures wherein the biomass was increasing. In accord with observations on the lethal effects of other organochlorine biocides such as DDT (Hicks and Corner, 1973) and hexachlorophene (Silvernale *et al.*, 1971), this finding supports the contention that the detrimental effects of a pesticide depend largely on the

ratio of pesticide to biomass rather than on the ambient concentrations in the immediate microbial environment (Wurster, 1968).

Ko and Lockwood (1968) and Chacko and Lockwood (1968), among others, have demonstrated that water-insoluble compounds such as DDT and dieldrin may become highly concentrated within the soil microflora many-fold above ambient concentrations. Previously Hicks and Corner (1973) reported that DDT is taken up by *B. megaterium*, with most of the accumulated DDT sequestered in the limiting membranes of the cells. Similarly, hexachlorophene was found to be concentrated in membranes of *B. megaterium* (Silvernale *et al.*, 1971). Thus, the uptake of 2,4,5-T by membranes is not a singular phenomenon. However, the quantities taken up were much smaller compared to the amounts of DDT or hexachlorophene. The residual content of the cells may have gone unnoticed or thought insignificant had not herbicide assays been performed on membranes isolated from exposed whole cells. The trace amounts of 2,4,5-T in the intact cells suggest that the herbicide does not partition into biological systems to the extent necessary for biological magnification within food chains. Indeed, to our knowledge there have been no reports of 2,4,5-T accumulation in higher predators.

In addition to the actual sequestering of 2,4,5-T by *B. megaterium* membranes, a more subtle interaction may

have occurred. The 2,4,5-T molecule possesses hydrophobic (the substituted benzene ring) and hydrophilic (the carboxyl group) regions. Thus, it may act analogously to detergents and selectively associate with those parts of the cell that are composed of adjacent hydrophobic and hydrophilic areas. This interaction may partially account for the relatively large quantities of herbicide that become reversibly associated with the cells under static conditions. Since the active concentrating mechanisms of a mesophile operate slightly, if at all, at the low temperature (0C) at which uptake was studied, the association is probably entirely passive. Moreover, this organism has not undergone the selective pressure needed to develop transport mechanisms specific for this man-made compound, though the 2,4,5-T molecule could conceivably act as an analog of a biomolecule of similar structure, such as phenylalanine or tyrosine.

However, much of the herbicide may not, in fact, have penetrated the cell membranes, as membranes are relatively impermeable to dissociated acids (Bell *et al.*, 1959). Consequently, the difference between the residual herbicide in the whole cells and residual herbicide in membranes isolated from exposed intact cells may represent 2,4,5-T bound to the cell wall. The affinity of 2,4,5-T for cells of *B. megaterium*, at least, may be primarily restricted to membranes and cell walls.

Lockwood and his colleagues (1968a,b) have suggested that the concentration of certain chlorinated pesticides in biological material may facilitate the persistence of these compounds in soil. The degree of 2,4,5-T accumulation exhibited by the cells studied here reduces the likelihood of this mechanism as a major contributor to 2,4,5-T persistence. Nevertheless, that amount of 2,4,5-T sequestered in the cells would be less available to degradative organisms.

ACKNOWLEDGEMENTS

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LITERATURE CITED

1. Bell, T. A., J. L. Etchells, and A. F. Borg. 1959. Influence of sorbic acid on the growth of certain species of bacteria, yeasts, and filamentous fungi. J. Bacteriol. 77:573-580.

2. Chacko, C. I., and J. L. Lockwood. 1967. Accumulation of DDT and dieldrin by microorganisms. Can. J. Microbiol. 13:1123-1126.

3. Corner, T. R., and R. E. Marquis. 1969. Why do bacterial protoplasts burst in hypotonic solutions? Biochim. Biophys. Acta 183:544-558.

4. Dougherty, E. M., C. F. Reichelderfer, and R. M. Faust. 1971. Sensitivity of *Bacillus thuringiensis* var. *thuringiensis* to various insecticides and herbicides. J. Invert. Pathol. 17:292-293.

5. Hicks, G. F., and T. R. Corner. 1973. Location and consequences of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane uptake by *Bacillus megaterium*. Appl. Microbiol. 25:381-387.

6. Horvath, R. S. 1971. Microbial co-metabolism of 2,4,5-trichlorophenoxyacetic acid. Bull. Environ. Contam. Toxicol. 5:537-541.

7. Horvath, R. S. 1972. Microbial co-metabolism and the degradation of organic compounds in nature. Bacteriol. Rev. 36:146-155.

8. Kimbrough, R. D. 1972. Toxicity of chlorinated hydrocarbons and related compounds. Arch. Pathol. 94:125-131.

9. Ko, W. H., and J. L. Lockwood. 1968. Accumulation and concentration of chlorinated hydrocarbon pesticides by microorganisms in soil. Can. J. Microbiol. 14:1075-1078.

10. Lee, D. H., and H. L. Falk (ed.). 1973. *Environmental Health Perspectives*, Issue No. 5. U.S. Department of Health, Education, and Welfare.

11. Loos, M. A. 1969. Degradation of phenoxyalkanoic acid herbicides by soil microorganisms. J. Suid-Afrikaanse Chem. Inst. 22:S71-S78.

12. Loos, M. A. 1969. Phenoxyalkanoic acids, p. 1-49. *In* P. C. Kearney and D. D. Kaufman (ed.), *Degradation of Herbicides*. Marcel Dekker, Inc., New York.

13. Marquis, R. E. 1968. Salt-induced contraction of bacterial cell walls. J. Bacteriol. 19:775-781.

14. Scherrer, R., and P. Gerhardt. 1971. Molecular sieving by the *Bacillus megaterium* cell wall and protoplast. J. Bacteriol. 107:718-735.

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

16. Umbreit, W. W., R. J. Burris, and J. F. Stauffer. 1964. *Manometric Techniques*, 4th ed. Burgess Publishing Co., Minneapolis.

17. Woolson, E. A., R. F. Thomas, and P. D. J. Ensor. 1972. Survey of polychlorodibenzo-p-dioxin content in selected pesticides. J. Agr. Food Chem. 20:351-354.

18. Wurster, C. F. 1968. DDT reduces photosynthesis by marine phytoplankton. Science 159:1474-1475.

19. Yudkin, M. D. 1966. Isolation and analysis of the protoplast membrane of *Bacillus megaterium*. Biochem. J. 98:923-928.

SECTION 4. MEMBRANE MODIFICATION INDUCED BY DDT

Introduction

DDT and 2,4,5-T accumulate in the limiting membranes of *Bacillus megaterium* (Hicks and Corner, 1973; Hicks and Corner, manuscript in preparation). Therefore, the pesticide effects on cells may be exerted at the level of the membrane. Others have suggested that DDT may exert toxic effects on organisms by inhibiting processes associated with membranes (Hilton and O'Brien, 1970; Janicki and Kinter, 1971; Matsumura and Patil, 1969; Rogers *et al.*, 1971; Tinsley *et al.*, 1971).

Both DDT and 2,4,5-T inhibit cell division in *B. megaterium* at concentrations of 5 µg pesticide/mg of cells (dry weight), but no well defined biochemical or physiological event could be implicated in the gradual killing effect of these compounds, even at concentrations as high as 50 µg pesticide/mg of cells (dry weight) (Hicks and Corner, 1973; Hicks and Corner, manuscript). In addition, membranes isolated from cells exposed to DDT had atypical macroscopic appearances, which suggested that

the membranes were chemically modified (Hicks and Corner, 1973).

The suggestion that DDT affects the gross chemical composition of membranes is confirmed by the results reported below. However, membranes from cells exposed to 2,4,5-T were identical to control membranes.

Materials and Methods

Membrane Isolation

Membranes were isolated from the cells of *B. megaterium* by the methods of Hicks and Corner (1973). Preparations of membranes were obtained direclty from cells taken from (i) the late exponential phase of growth (control) and from the following mixtures after 1.5 h exposure in phosphate buffer, pH 7.0; (ii) buffer alone; (iii) buffer amended initially with 0.1% (vol/vol) acetone; (iv) buffer containing 10 µg 2,4,5-T/ml; and (v) buffer containing 0.1% (vol/vol) acetone and 10 µg DDT/ml. A sixth preparation was made from cells subjected to a sucrose density gradient procedure (Hicks and Corner, 1973) after exposure in buffer containing 0.1% acetone. After isolation, the various membrane preparations were frozen at about -35 C and then lyophilized. Yields varied from 40 to 110 mg membrane material.

Lipid Extraction

Dried membrane materials (5, 10 or 20 mg) were extracted with about 10 ml chloroform:methanol (2:1) in the cold with agitation under a partial vacuum for periods that ranged from 8 h to 40 h. The membrane residue was then washed by filtration and the chloroform:methanol extracts were dried and weighed. As a reference for insoluble material that may have adhered to or passed through the filter, the following alternate method was used. Insoluble material in the chloroform:methanol solvent was allowed to settle to the bottom of the extraction vessel and the supernatant was removed with a pipette. The insoluble residue was washed several times with chloroform:methanol and all supernatants were pooled.

Fatty Acid Analysis of Lipid Extracts

The DDT incorporated in the membranes severely interfered with resolution of conventional thin layer chromatographic phospholipid assays, whereas DDT did not markedly distort the flame ionization characterization of component fatty acids. Hence, fatty acids were used as indicators of phospholipid and thus approximated relative amounts of gross lipid in the membranes. One-half milliliter of 15% BF_3 (vol/vol) in methanol (Supelco or Applied Sciences) was added to the dried chloroform:methanol residue and boiled for about 3 min. The esterified fatty acids in the boiled mixture were partitioned into 30 ml

petroleum ether in a separatory funnel containing the petroleum ether and 20 ml H_2O. The aqueous bottom layer was discarded and the ether was evaporated to dryness. Subsequently the residue was dissolved in petroleum ether and analyzed by flame ionization gas chromatography. Fatty acid standards were obtained from Applied Sciences.

Protein Determinations

Protein was measured using the procedure of Lowry *et al.* (1951) with bovine serum albumin as a standard. Protein determinations were performed on the intact membranes and on the membrane residues left after lipid extraction. This latter procedure was necessary to equate the effects that DDT or 2,4,5-T may have had on the protein determinations of the intact membranes. The remaining protein fractions and lipid extracts were assayed for DDT by use of procedures previously described (Hicks and Corner, 1973). This served to confirm the DDT content of the membranes and to determine how much DDT, if any, would be available to interfere with the Lowry procedure.

Poly-β-hydroxybutyric Acid (PHB) Analysis

A routine PHB analysis was performed on all membranes. The methods of Law and Slepecky (1961) were used in addition to the PHB extraction technique of Bishop *et al.* (1967). Subjective microscopic measurements of the final

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membrane preparations were also used to estimate PHB content.

Results

Of the six membrane preparations studied, only membranes prepared from cells exposed to DDT showed any marked difference in gross composition. The DDT in the membranes or in the exposure medium did not measurably affect the protein content (Table 1). The fatty acid content of the chloroform:methanol extracts of the exposed membranes was markedly lower than that of all other samples (Table 2). Hence, the DDT but not 2,4,5-T replaced or eluted some lipid material from the membranes.

DDT interfered with conventional thin layer chromatographic analysis of phosopholipids in both the membrane preparation and similar test preparations with an equivalent amount of DDT added. However, DDT at concentrations found in the membranes had no detectable effect on gas chromatographic resolution of fatty acids. Hence, the fatty acid content of these membranes was used as an indicator of relative lipid content exclusive of PHB. No qualitative differences among the fatty acids were evident in the membrane preparations.

The Lowry procedure performed on intact membranes produced erratic and irreproducible results when DDT was present; similar results were observed when DDT was added to bovine serum albumin. Chloroform:methanol

Table 1. Summary of gross membrane composition

Environment of cells (membrane source)	Protein % of total[a]	% CCL_4/MeOH extractable material			Total %
		% lipid[b]			
		PHB	Other	% pesticide	
Buffer only	71 (66-74)	1±1	20 (16-23)	0	92
Acetone in buffer	68 (64-72)	1±1	21 (18-24)	0	90
DDT, acetone in buffer	72 (65-74)	1±1	15 (13-18)	2±0	88
2,4,5-T	71 (67-74)	1±1	22 (18-27)	0	93
Buffer, acetone; sucrose gradient	68 (64-72)	1±1	19 (16-23)	0	88
Control	70 (65-75)	1±1	21 (18-25)	0	92

[a]Determined by the methods of Lowry *et al.* (1951); range of values given in parentheses.

[b]Total lipid determined by weight of chloroform:methanol extracts.

Table 2. Relative percent fatty acid and pesticide in chloroform:methanol extracts of purified membranes

Membranes isolated from cells exposed to	Fatty acid %[a]	Pesticide %
Buffer only	91	0
Acetone in buffer	91	0
DDT, acetone in buffer	77	9
2,4,5-T	105	0
Sucrose gradient	95	0
Control	100	0

[a]Control = 100%.

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extraction removed the DDT and 2,4,5-T from the membranes which were then measured for total protein content.

Discussion

Direct protein determination with the Lowry method, and membrane phospholipid analysis with thin layer chromatography, were clearly unsuitable for studies with membranes isolated from DDT-exposed cells. The alternate methods devised to determine protein and lipid content of membranes were illustrative and adequate for relative comparison of membrane chemical composition. These methods may not, however, precisely measure absolute quantities of any one component.

DDT enters the membrane slowly, attaining a maximal concentration of about 2% after 1.5 h exposure (Hicks and Corner, 1973). Of the six membrane preparations only the one containing DDT appeared to have a somewhat different texture and color in the hydrated state. The apparent solubility of DDT, as determined by sedimentation characteristics, increased with increased time of exposure to cells. Thus, presumptive evidence suggested that DDT became associated with extracellular biological material. Since DDT in its crystalline form is soluble in solvents with hydrophobic character, membrane lipid or other hydrophobic cell components could well play a role in changing the physical state of DDT.

This is the first report of DDT-induced physical modification of a cell. Though 10 µg DDT/ml is considerably higher than environmental concentrations, it is clear from these results that DDT does have the physical potential to disrupt the cell membrane without the outward display of dramatic effects such as immediate cell lysis or instantaneous death of cells.

Literature Cited

Bishop, D. G., L. Rutenberg, and B. Samuelson. 1967. The chemical composition of the cytoplasmic membrane of *Bacillus subtilis*. Euro. J. Biochem. 2:448-453.

Hicks, G. F., and T. R. Corner. 1973. Location and consequences of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane uptake by *Bacillus megaterium*. Appl. Microbiol. 25:381-387.

Hilton, H. D., and R. D. O'Brien. 1970. Antagonism by DDT of the effects of valinomycin on a synthetic membrane. Science 168:841-842.

Janicki, R. H., and W. B. Kinter. 1971. DDT inhibits Na^+, K^+, Mg^{2+}-ATPase in the intestinal mucosae and gills of marine teleosts. Nature New Biol. 233:148-149.

Law, J. H., and R. A. Slepecky. 1961. Assay of poly-β-hydroxybutyric acid. J. Bacteriol. 82:33-36.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Matsumura, F., and K. C. Patil. 1969. Adenosine triphosphatase sensitive to DDT in synapses of rat brain. Science 166:121-122.

Tinsley, I. J., R. Haque, and D. Schmedding. 1971. Binding of DDT to lecithin. Science 174:145-147.

Yudkin, M. D. 1966. Isolation and analysis of the protoplast membrane of *Bacillus megaterium*. Biochem. J. 98:923-928.

DISCUSSION

The trend expressed in the scientific literature was to characterize DDT degradation and movement hypothetically in accord with fundamental experimental observations: some DDT from the insoluble pesticide pool in the biosphere slowly partitions into microorganisms, further magnifies with predation and, after much cycling, enters anaerobic environments where a mixed microbial community could presumably degrade the compound.

The present results, in combination with past observations, allow refinement of the above hypothesis. Many microorganisms, including *B. megaterium*, are not eliminated from a viable population by low environmental DDT concentrations. This would enable DDT to become sequestered, delaying degradation and providing direct entrance through predation to other lower organisms. However, long exposure to the insecticide could result in high or threshold concentrations within autonomous cells and the DDT could elute in a more soluble form, thus increasing the mobility of the compound in aqueous environments. This provides a hypothetical mechanism whereby DDT or DDD could partition more extensively from

sediments and associated microbial populations into ambient waters, thereby reducing the quantities accessible for anaerobic biodegradation while concurrently increasing concentrations in surface waters where UV-mediated photodecomposition can occur. Obviously, a thorough study to resolve the nature of DDT degradation *in situ* is critical to ascertain the validity of this hypothesis.

The precedents and accuracy of the DDT analytical procedures led to the finding that the membrane of *B. megaterium* is the primary site of very low but detectable 2,4,5-T accumulation. However, the water soluble herbicide is not eluted in the fashion of DDT even as viability reduction occurs. Although this physical entrapment may contribute somewhat to the recalcitrance of the compound in nature, analogously to DDT, it is evident from these and previous studies that 2,4,5-T will not partition into biological systems over the observed trace amounts.

The development of gradient centrifugation techniques and the application of the space technique to the study of pesticide interactions with microflora represent an advance in analysis of pesticide residues at the microbial or subcellular level. These procedures should aid investigations of pesticide uptake *in situ* and resolve some questions about the movement and fate of recalcitrant compounds in the biosphere. Similarly, chemical modification of membranes apparently is the first

evidence presented to date about the precise physical effects that DDT may have at the subcellular level. Applicability of this observation specifically or generally to the mode of action of DDT on target organisms remains to be seen.