Effects of Organochlorine Insecticides on Bacterial Growth, Respiration and Viability

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SUMMARY

The growth of a range of Gram-positive bacteria was inhibited by organochlorine insecticides while that of Gram-negative organisms was unaffected. Growing cultures of *Bacillus subtilis* (ATCC 9372) treated with 20 p.p.m. technical chlordane ceased to grow and showed a decline in viable count and respiration rate, both being eliminated after about 3 h. A delayed release of incorporated L-[U-¹⁴C]leucine and L-malate dehydrogenase occurred concomitant with a fall of *E*₅₈₀. It is suggested that these phenomena are a result of disruption of membrane-associated metabolism, including electron transport and cell wall biosynthesis, which leads to cell lysis. No effect on these parameters occurred with growing cultures of *Escherichia coli* (ATCC 8739). The chlordane sensitivity of succinate oxidation by sphaeroplasts of *E. coli* indicates that the intact cell wall prevents penetration of pesticide to sensitive sites within the walls of Gram-negative bacteria.

INTRODUCTION

The long-term persistence of organochlorine insecticides (Alexander, 1968; Kearney, Plimmer & Helling, 1969) is an indication of their resistance to degradation by soil micro-organisms. Limited slow modifications of cyclodiene insecticides in the biosphere have been reported (Korte, Ludwig & Vogel, 1962; Lichtenstein, Schulz & Cowley, 1963; Korte, 1967; Matsumura & Boush, 1967, 1968; Tu, Miles & Harris, 1968; MacRae, Raghu & Bautista, 1969).

Because of their extended persistence, slow rate of decomposition and the fact that the bulk of any insecticide application almost invariably reaches the soil, it is desirable that actual or potential effects upon the soil microflora be investigated. Reported results of experiments, in which growth and metabolic activity of soil bacteria *in situ* has been investigated following organochlorine insecticide application, range from descriptions of increased activity (Fletcher & Bollen, 1954; Sinha, 1961) or of a total lack of effect (Eno & Everett, 1958; Martin, Harding, Cannell & Anderson, 1959; Shaw & Robinson, 1960) to inhibition of growth and metabolic activity (Bollen, Morrison & Crowell, 1954*a, b*; Sinha, 1961; MacRae, Raghu & Castro, 1967; Pramer & Bartha, 1968).

More consistent results have been obtained with pure cultures of micro-organisms growing on synthetic media. Gray (1954) found that, of fifty species of heterotrophic bacteria incubated on nutrient agar, growth of 37 was inhibited by γ -hexachlorocyclohexane at 250 p.p.m. Gray & Rogers (1955) observed the growth only of Gram-negative organisms when mixed cultures from soil samples were incubated on nutrient agar with γ -hexachloro-

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cyclohexane as a surface film (50 $\mu\text{g./cm.}^2$). Duda (1958) found that chlordane and hexachlorocyclohexane incorporated into nutrient agar at 500 and 1000 p.p.m. inhibited the growth of *Bacillus mycoides*, *Bacillus subtilis*, *Sarcina lutea* and *Streptomyces griseus* while the growth of *Escherichia coli* and other Gram-negative organisms was unaffected. Collins & Langlois (1968) demonstrated that addition of 50 p.p.m. heptachlor to liquid culture media inhibited the growth of *Staphylococcus aureus* while growth of *Pseudomonas fluorescens* was unaffected. Lyr (1969) has reported the inhibition of growth of *B. subtilis* by 30 p.p.m. δ -hexachlorocyclohexane.

We now extend these observations, demonstrate some of the phenomena associated with toxicity towards Gram-positive bacteria and indicate the characteristic which confers immunity upon Gram-negative organisms. A preliminary report of some of this data has already appeared (Trudgill & Widdus, 1970).

METHODS

Organisms. The sources of all the bacteria used are shown in Table 1. Stock cultures were maintained on nutrient agar slopes, with the exception of *Streptococcus faecalis* for which the nutrient agar was fortified with 3 % (w/v) D-glucose.

Growth media. Organisms were grown on nutrient agar solid media or in liquid culture on nutrient broth (Oxoid Ltd, London) when typically 100 ml. of medium in a 250 ml. Erlenmeyer was shaken at 30° and 200 rev./min. on a New Brunswick gyrotary shaker (New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.). For growth of *Streptococcus faecalis* D-glucose, 3 % (w/v), was incorporated into the medium.

Table 1. *Sources of the bacteria*

Gram-positive bacteria	
<i>Bacillus cereus</i>	Laboratory strain isolated from milk
<i>B. megaterium</i>	NCTC 9848
<i>B. subtilis</i>	ATCC 9372
<i>Corynebacterium</i> sp. TI	Kay, Conrad & Gunsalus (1962)
<i>Microbacterium flavum</i>	NCIB 8707
<i>Micrococcus lysodeikticus</i>	NCIB 9278
<i>Nocardia</i> sp. A	Laboratory strain isolated from soil
<i>Nocardia</i> sp. B	Laboratory strain isolated from soil
<i>Sarcina lutea</i>	NCIB 8553
<i>Staphylococcus albus</i>	Laboratory strain
<i>Staphylococcus aureus</i>	Laboratory strain
<i>Streptococcus faecalis</i>	NCIB 8661
<i>Streptomyces antibioticus</i>	NCIB 8504
Gram-variable bacteria	
<i>Arthrobacter simplex</i>	NCIB 8929
<i>Pseudomonas iodinum</i>	NCIB 8179 Wilkinson (1968)
Gram-negative bacteria	
<i>Achromobacter butyri</i>	NCIB 9404
<i>Achromobacter</i> sp. PC 4	(Dagley, Chapman & Libson, 1963)
<i>Escherichia coli</i>	ATCC 8739
<i>Klebsiella aerogenes</i>	Laboratory strain
<i>Pseudomonas acidovorans</i>	ATCC 17454
<i>P. aureofaciens</i>	NCIB 9030
<i>P. dehalogens</i>	NCIB 9061
<i>P. fluorescens</i>	(Trudgill & Widdus, 1966)
<i>P. fluorescens</i>	NCIB 9392
<i>P. multivorans</i>	NCIB 9092
<i>P. putida</i>	(Trudgill & Widdus, 1966)

Measurement of bacterial growth. Bacterial growth was monitored at 580 nm. with an Hitachi Perkin-Elmer model 139 spectrophotometer (Hitachi Ltd, Tokyo). Where necessary dilutions of culture samples were assayed so that the measured extinction did not exceed 0.7.

Viable cell counts. Viable cells in growing and experimental cultures were estimated, as colony-forming units, by appropriate serial dilution followed by sample plating on nutrient agar.

Measurement of respiration. Oxygen uptake by bacterial suspensions or sphaeroplasts was determined using a Clark-type oxygen electrode in an agitated and thermostatically controlled reaction vessel (Oxygen Monitor, model 53; Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, U.S.A.).

Preparation of sphaeroplasts. Sphaeroplasts of *Escherichia coli* in which cell wall removal was not complete (Martin, 1963; Salton, 1964) were prepared by the method of Lederberg (1956) in which benzyl penicillin, 100 units/ml., was added to growing cultures in the presence of sucrose. Sphaeroplasts were collected by centrifuging at 9000 *g* and 4° for 1 h. and resuspended in 30 mM tris-HCl buffer, pH 8.0, containing 0.5 M-sucrose and 5 mM-MgCl₂.

Organochlorine insecticides. Technical grade chlordane (1,2,4,5,6,7,8,8-octachloro-3,4,4,7,7-*a*-tetrahydro-4,7-methanoindane), technical grade endrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4,4,5,6,7,8,8-*a*-octahydro-*endo*-1,4-*endo*,5,8-dimethanonaphthalene), γ -hexachlorocyclohexane (1,2,3,4,5,6-hexachlorocyclohexane) and telodrin (1,3,4,5,6,7,8,8-*a*-octachloro-1,3,3,4,4,7,7-*a*-hexahydro-4,7-methanoisobenzofuran) were donated by Lankro Chemical Co., Eccles, Manchester. Technical grade chlordane, analytical grade α -chlordane, analytical grade γ -chlordane, technical grade bandane (4,5,6,7,8,8-*a*-hexachloro-3,4,4,7,7-*a*-tetrahydro-4,7-methanoindane) and technical grade heptachlor (1,4,5,6,7,8,8-*a*-heptachloro-3,4,4,7,7-*a*-tetrahydro-4,7-methanoindane) were donated by Velsicol Chemical Corp., Chicago, U.S.A. Technical grade aldrin (1,2,3,4,10,10-hexachloro-1,4,4,4,5,8,8-*a*-hexahydro-*endo*-1,4-*exo*-5,8-dimethanonaphthalene), technical grade dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4,4,5,6,7,8,8-*a*-octahydro-*endo*-1,4-*exo*-5,8-dimethanonaphthalene) and technical grade thiodan (6,7,8,9,10,10-*a*-hexachloro-1,5,5,6,9,9-*a*-hexahydro-6,9-methano-2-4,3-benzodioxathiopen-3-oxide) were donated by Shell Research Ltd, Sittingbourne, Kent. DDT[2,2-bis(*p*-chlorophenyl)-1,1,1-trichloro-ethane] was obtained from Kodak Ltd, London.

Gas-liquid chromatography of chlordane. Technical chlordane was analysed with a Perkin-Elmer F. 11 Gas Chromatograph (Perkin-Elmer Ltd, Beaconsfield, Buckinghamshire) using a stainless steel column (1.5 m. long and 2 mm. internal diameter) packed with 5 % S.E. 30 on Chromosorb W, 60 to 80 mesh, acid washed and treated with dimethyldichlorosilane. Argon was employed as carrier gas and the detector was of the hydrogen flame ionization type.

Addition of organochlorine insecticides to aqueous media. Because of the near complete insolubility of these compounds in aqueous solution our methods of administration aimed at producing finely divided suspensions, without resorting to the use of emulsifying or carrier agents which are frequently used commercially. Typically, additions to experimental systems were made in acetone solution. Final pesticide concentrations of 2 to 1000 p.p.m. could be achieved without allowing the acetone concentration to exceed 0.5 % (v/v). Applications to solid media were made by allowing an appropriate volume of an acetone or light petroleum (b.p. 40 to 60°) solution of the pesticide to evaporate on the surface of the medium leaving behind a thin film of the compound.

Radioactive materials and their assay. L-[U-¹⁴C]leucine was obtained from the Radiochemical Centre, Amersham, Buckinghamshire. Incorporation of radioactive leucine into bacterial cultures was estimated by aseptically withdrawing samples from the culture,

followed by collection of the cells on Oxoid membrane filters (mean pore diameter 0.5μ), thorough washing of the filters with deionized water, transfer to planchets, drying and counting on a Nuclear Chicago model 8705 Automatic Gas-Flow Counter.

Enzyme assays. L-Malate:NAD oxidoreductase (ED 1.1.1.37) (L-malate dehydrogenase) activity of *Bacillus subtilis* was assayed by observing the decrease in E_{340} subsequent to the addition of oxaloacetate ($2 \mu\text{moles}$) to a 1 cm. light path cuvette which contained, in 3 ml., the test extract, phosphate buffer (pH 7.1), and $0.5 \mu\text{mole}$ of NADH.

RESULTS

Sensitivity of plate-cultured bacteria to organochlorine insecticides. Technical grade insecticides were applied to nutrient agar plates as surface films (0.5 mg./cm.^2). Plates were inoculated from stock agar slopes and growth assessed visually after 24 and 48 h. (Table 2). Differences in susceptibility to inhibition of growth can be correlated with the Gram-stain reaction. Gram-negative organisms are, almost without exception, resistant while

Table 2. *The effects of organochlorine insecticides on bacterial growth*

Micro-organism	Insecticide									
	Bandane	Chlordane (V)	Chlordane (L)	Heptachlor	Dieldrin	Aldrin	Endrin	Telodrin	γ -HCH	DDT
Gram-positive										
<i>Bacillus megaterium</i>	—	—	—	—	—	—	—	—	—	—
<i>B. subtilis</i>	—	—	—	—	—	—	—	—	+	+
<i>Streptomyces antibioticus</i>	—	—	—	—	—	—	—	—	—	+
<i>Nocardia</i> sp. B	—	—	—	—	—	—	—	—	(+)	+
<i>Corynebacterium</i> sp. T 1	—	—	—	—	—	—	—	+	+	+
<i>B. cereus</i>	—	—	—	—	—	—	—	(+)	+	+
<i>Nocardia</i> sp. A	—	—	—	—	—	—	+	+	+	+
<i>Microbacterium flavum</i>	—	—	—	—	—	—	+	+	+	+
<i>Micrococcus lysodeikticus</i>	—	—	—	—	—	—	+	+	+	+
<i>Staphylococcus albus</i>	—	—	—	—	—	—	+	+	+	+
<i>Staphylococcus aureus</i>	—	—	—	—	—	+	+	+	+	+
<i>Sarcina lutea</i>	—	—	(+)	+	+	+	+	+	+	+
<i>Streptococcus faecalis</i>	—	—	—	—	—	—	—	—	—	—
Gram-variable										
<i>Arthrobacter simplex</i>	—	—	—	—	—	+	+	+	+	+
<i>Pseudomonas iodinum</i>	—	—	—	—	—	—	—	—	—	—
Gram-negative										
<i>Achromobacter butyri</i>	+	+	+	+	+	+	+	+	+	+
<i>Achromobacter</i> sp. PC 4	+	+	+	+	+	+	+	+	+	+
<i>Escherichia coli</i>	+	+	+	+	+	+	+	+	+	+
<i>Klebsiella aerogenes</i>	+	+	+	+	+	+	+	+	+	+
<i>P. acidivorans</i>	+	+	+	+	+	+	+	+	+	+
<i>P. aureofaciens</i>	+	+	+	+	+	+	(+)	+	+	+
<i>P. dehalogens</i>	+	+	+	+	+	+	+	+	+	+
<i>P. fluorescens</i>	+	+	+	+	+	+	+	+	+	+
<i>P. fluorescens</i>	+	+	+	+	+	+	+	+	+	+
<i>P. multivorans</i>	+	+	+	+	+	+	+	+	+	+
<i>P. putida</i>	+	+	+	+	+	(+)	+	+	+	+

+, Growth not inhibited; (+), growth slightly inhibited; —, growth severely or completely inhibited.

Fig. 1. The effect of technical chlordane (20 p.p.m. final concentration), administered in acetone solution, upon the growth (E_{580}), respiration rate and incorporation of radioactive leucine by *Bacillus subtilis* growing in nutrient broth. Additions of acetone to control flasks (C) and chlordane in acetone to the test flasks (T) are indicated by the arrows. For details of the techniques employed in measuring radioactive leucine incorporation and respiration see the Methods section.

Table 3. The effects of various concentrations of technical chlordane on bacterial growth

Micro-organism	Chlordane (mg./cm. ²)			
	1.0	0.1	0.01	Vapour*
Gram-positive				
<i>Bacillus megaterium</i>	—	—	—	—
<i>B. subtilis</i>	—	—	—	—
<i>Corynebacterium</i> sp. T 1	—	—	—	—
<i>B. cereus</i>	—	—	(+)	—
<i>Nocardia</i> sp. B	—	—	(+)	—
<i>Staphylococcus aureus</i>	—	—	—	—
<i>Sarcina lutea</i>	—	—	(+)	—
<i>Streptococcus faecalis</i>	—	—	(+)	—
Gram-negative				
<i>Achromobacter butyri</i>	+	+	+	+
<i>Escherichia coli</i>	+	+	+	+
<i>Klebsiella aerogenes</i>	+	+	+	+
<i>Pseudomonas acidovorans</i>	+	+	+	+
<i>P. fluorescens</i>	+	+	+	+
<i>P. multivorans</i>	+	+	+	+
<i>P. putida</i>	+	+	+	+

+, Growth not inhibited; (+), growth slightly inhibited; —, growth severely or completely inhibited.

* A drop of chlordane was placed in the lid of each inverted Petri dish.

Gram-positive organisms show a variation of susceptibility. *Bacillus* species are the most sensitive, judged by the range of insecticides which inhibit their growth, while staphylococci and sarcinas are much less so. The two Gram-variable organisms studied probably occupy an intermediate position. All the cyclodiene insecticides are of similar potency with the exception of endrin and telodrin. γ -Hexachlorocyclohexane and DDT inhibited growth of only a few of the Gram-positive organisms under investigation.

Fig. 2. The effect of technical chlordane (20 p.p.m. final concentration), administered in acetone solution, on a growing culture of *Bacillus subtilis* showing the delayed release of malate dehydrogenase from the cells, which is associated with the fall in E_{580} . For details of the malate dehydrogenase assay see the Methods section.

Sensitivity of plate-cultured bacteria to a range of technical chlordane concentrations. The effects of technical chlordane further emphasizes the relative sensitivity of Gram-positive organisms (Table 3). Growth of all Gram-positive bacteria tested was inhibited at concentrations 100-fold less than those shown to have no detectable effect upon Gram-negative organisms.

For a more detailed investigation of these phenomena *Bacillus subtilis* (ATCC 9372) and *Escherichia coli* (ATCC 8739) were selected as representative susceptible and resistant strains while technical chlordane was chosen as the test compound because, being a syrup, it is relatively easily dispersed in aqueous media.

Effects of technical chlordane on the growth and respiration of Bacillus subtilis. The growth of *B. subtilis* on nutrient broth containing L-[U- 14 C]leucine in shake culture was completely inhibited by chlordane at 20 p.p.m. Respiration virtually ceased 3 h. after administration of

Fig. 3. The effects of various levels of technical chlordane on the growth (E_{580}) and respiration of *Bacillus subtilis* in nutrient broth culture. Additions of acetone to a control culture, Fig. 3(a); 20 p.p.m. chlordane, Fig. 3(b); 10 p.p.m. chlordane, Fig. 3(c); and 5 p.p.m. chlordane, Fig. 3(d) in acetone were made at times indicated by the arrows.

Fig. 4. Gas chromatographic analysis of technical grade chlordane (10 μ g.) was performed on a column of silicone gum rubber (S.E.-30) supported on Chromosorb W (60 to 80 mesh) at a temperature of 200° with other conditions as described in the Methods section. Peaks, identified by comparison with authentic pure compounds or from appropriate literature references (see text) are: A, heptachlor; B, bandane; C, γ -chlordane; D, α -chlordane; E, nonachlor (1, 2, 3, 4, 5, 6, 7, 8, 8-nonachloro-3a, 4, 7, 7a-tetrahydro-4, 7-methanoindane).

chlordane (Fig. 1) and the rapid fall in E_{580} coupled with the release of [^{14}C]-labelled material from the bacteria suggest that marked changes in cell permeability or even cell lysis may be occurring. The leakage of L-malate dehydrogenase from the cells which commences, following a time lag of $1\frac{1}{2}$ h., after administration of chlordane and is associated with the rapid fall in E_{580} , supports this view (Fig. 2). No viable bacteria remained 3 h. after addition of 20 p.p.m. chlordane.

Fig. 5. Effect of pure isomers of chlordane upon the growth (E_{580}) of *Bacillus subtilis* in nutrient broth. Additions, indicated by the arrows, were made to parallel cultures (●) as follows: (○), acetone, as a control; (□), α -chlordane; (■), γ -chlordane. The final concentration of both chlordane isomers was 20 p.p.m.

While essentially similar results were obtained with 100 p.p.m. chlordane, 10 and 5 p.p.m. had progressively decreasing effects upon growth and respiration (Fig. 3).

When exponential phase broth-grown bacteria were harvested, thoroughly washed and resuspended in phosphate buffer, pH 7.1, in the absence of a nitrogen source but with 22 mM-glucose present to suppress sporulation, decrease in E_{580} (initial value 1.0) and release of L-malate dehydrogenase did not occur on addition of 20 p.p.m. technical chlordane. Respiration was progressively inhibited, 50 % inhibition being achieved 2 h. after administration of chlordane.

Technical chlordane is a complex mixture of closely related compounds (Fig. 4) a number of which have been identified in the sample used either by reference to retention times of analytical samples or to gas chromatography data obtained by other workers (Bevenue & Yeo, 1969). Though it is appreciated that technical chlordane is the material used for pest

control purposes it seemed desirable to see whether or not the pure α and γ isomers are themselves actively antibacterial. A very similar pattern of growth inhibition is obtained when the pure isomers are added to growing cultures of *Bacillus subtilis* (Fig. 5). Therefore the cyclodiene itself is an effective antibacterial compound and the phenomena observed when the technical material is used are not due to some chemically unrelated impurity present.

Fig. 6. Effect of technical chlordane upon the incorporation of radioactive leucine by *Escherichia coli* growing aerobically in nutrient broth. Additions, indicated by the arrows, were made to parallel cultures as follows: Δ , acetone as a control; $\blacktriangle$, technical chlordane in acetone, final concentration 1000 p.p.m.

Table 4. *The effect of technical chlordane on succinate oxidation by sphaeroplasts of Escherichia coli*

Oxygen monitor vessels contained, in 3 ml., 0.03 M-tris buffer (pH 8.0) with 0.5 M-sucrose and 5 mM-MgCl₂, benzyl penicillin sphaeroplasts obtained from 10 mg. dry wt of *E. coli* and 17 mM-disodium succinate. Reactions were carried out at 30° with omissions or additions as indicated.

	Oxygen con- sumed (nmoles/min.)	Inhibition (%)
Succinate omitted	14.6	—
Complete system	58.5	—
Acetone added (15 μ l)	50.5	12
*Chlordane added (20 p.p.m.)	20.4	74.5
Chlordane added (100 p.p.m.)	20.5	74.0

* Additions of chlordane were made in 15 μ l. of acetone.

Such an observation does not, of course, alter the likelihood that the bandane, heptachlor and other components of technical chlordane have antibacterial activity.

Effects of technical chlordane on the growth and respiration of Escherichia coli. Neither the growth of *E. coli* (ATCC 8739) in nutrient broth nor incorporation of L-[U-¹⁴C]leucine by growing cultures is inhibited by technical chlordane at concentrations up to 1000 p.p.m. (Fig. 6), thus confirming the resistance of Gram-negative organisms. Respiration of whole organisms was not inhibited by chlordane concentrations up to and including 660 p.p.m.

Benzyl penicillin-induced sphaeroplasts of *Escherichia coli* (Lederberg, 1956) display a chlordane sensitive succinate respiration (Table 4) though whole organisms suspended in the same sucrose-buffer system are still completely resistant, implying that the complex wall of this organism acts as an effective barrier to chlordane penetration.

DISCUSSION

The inhibition of growth of Gram-positive bacteria by organochlorine insecticides and the lack of effect upon Gram-negative organisms (Tables 1 and 2) is a general extension of the observations of Gray & Rogers (1955), Duda (1958) and Lyr (1969).

Within the cyclodiene group the syrupy preparations, technical chlordane and technical bandane, show a wider range of toxicity than, for example, the slightly more polar and crystalline telodrin (Table 2). Whether this is significant or simply a reflexion of the greater ease with which the syrups form a continuous film on an agar surface is not clear. Toxicity of the lowest concentration used on agar plates (0.01 mg./cm.²) is difficult to relate to concentrations in liquid medium used in later experiments, but it does clearly demonstrate the variation in response of Gram-positive bacteria, *Sarcina lutea* and *Streptotoccus faecalis* being significantly less sensitive to technical chlordane than *Bacillus subtilis* (Table 3).

Addition of technical chlordane to a growing culture of *Bacillus subtilis* causes several changes the most rapid of which is a marked decrease in respiration rate, probably a result of attraction of the hydrophobic pesticide molecules to the lipid core of the lipoprotein membrane with a consequent disruption of its electron transport ability. This view is reinforced by the lack of effect of technical chlordane upon the activity of soluble cytoplasmic enzymes such as L-malate dehydrogenase. Less rapid, but equally dramatic loss of incorporated [¹⁴C]leucine and L-malate dehydrogenase from the bacteria (Fig. 2) occurs together with a fall in E_{580} and loss of viability. Since such observations all indicate eventual cell lysis following inhibition of respiration a plausible explanation of this is worthy of consideration.

If we take the rapid crippling of respiration as an indication of impairment of membrane function then it is likely that a number of essential membrane-dependent activities are also disrupted. This may include such activities as protein synthesis and, interestingly, cell wall biosynthesis since Anderson, Matsushashi, Haskin & Strominger (1965) have suggested that murein precursors formed within the cell are attached to lipid moieties in the membrane for translocation to the exterior. Such a chlordane-induced disruption of cell wall biosynthesis may trigger cell lysis, a view reinforced by the observation that treatment of resting *Bacillus subtilis* suspensions (in 22 mM-glucose to suppress sporulation) with chlordane at 20 p.p.m. fails to promote a decrease in E_{580} or leakage of L-malate dehydrogenase.

Koch (1969*a, b*) has reported inhibition of both the Na⁺/K⁺ dependent transport ATPase and the Mg²⁺ dependent oxidative phosphorylation ATPase of rat brain homogenates by aldrin, chlordane, dieldrin, γ -hexachlorocyclohexane and DDT. Koch, Cutkamp & Do (1969) and Patil, Matsumura & Bratkowski (1969) have reported similar observations with insect tissues. Since bacterial ATPase enzymes are known to be associated with the bacterial

cell membrane it is possible that cyclodiene insecticides may interfere with the functioning of these enzymes interrupting transport and oxidative phosphorylation and disturbing permeability barriers. Since *Streptococcus faecalis* (NCIB 8661) possesses no membrane-bound cytochrome electron transport chain and is a lactate producing fermenter with substrate-level phosphorylation, electron transport cannot be a target site in this organism. Perhaps this is reflected in its decreased sensitivity to technical chlordane, relative to *Bacillus subtilis* (Table 3), the pesticide still being lethal because of the disruption of the other membrane associated phenomena that have been discussed.

The demonstration that Gram-positive bacteria obtained from culture collections and grown under laboratory conditions are sensitive to organochlorine pesticides at concentrations roughly equivalent to those found in the soil (Stringer & Pickard, 1963) is not of itself proof that agricultural application of these compounds will result in a selection of resistant Gram-negative organisms. However, as an indication that this may indeed occur it has been shown by Kokke (1970) that the proportion of DDT-resistant bacteria is markedly elevated in DDT-containing environments. Cyclodiene insecticide-resistant Gram-positive strains do exist as illustrated by the reported contamination of aldrin samples by *Bacillus cereus* and *Bacillus subtilis* (Fletcher & Bollen, 1954).

To test the theory that the cell walls of resistant Gram-positive organisms act as a barrier to organochlorine insecticides it is not strictly necessary to know first the primary site, if any, of action of the compound. Any activity internal to the wall and sensitive to the pesticide can serve as an indicator. Succinoxidase of *Escherichia coli* (ATCC 8739) is just such an indicator (Trudgill & Widdus, 1970) and is known to be membrane bound (Gel'man, Lukoyanova & Ostrovski, 1967). Since modification of cell wall permeability of *E. coli* by penicillin renders succinoxidase sensitive to technical chlordane (Table 4) a cell wall dependent resistance of Gram-negative organisms to cyclodiene pesticides is postulated. Other known instances where the cell walls of Gram-negative organisms prevent access of antibacterial compounds occur with hexachlorophene (Joswick, 1961), tetrachlorosalicylanilide (Hamilton, 1968) and anacardic acids (Gellerman, Walsh, Werner & Schlenk, 1969). While it can be appreciated that the more complex cell wall of a Gram-negative organism may be able to act as a barrier to cyclodiene penetration it is also, and perhaps paradoxically, interesting that in general Gram-negative cell walls contain more lipid than those of Gram-positive organisms and so might be expected to attract these very hydrophobic compounds. Indeed, such a suggestion is supported by observations of Chacko & Lockwood (1967) who demonstrated that *Agrobacterium tumefaciens* was much more effective at accumulating dieldrin and DDT than was *Bacillus subtilis*.

Factors which confer resistance to other antibacterial compounds upon some organisms, such as the development of detoxifying enzymes or absence of a specific target site are probably not relevant in the case of cyclodiene insecticides. In the first place the rapid death of Gram-positive bacteria, as judged by the decline in respiration as well as viability, makes it unlikely that a resistant Gram-negative organism could produce a detoxifying enzyme rapidly enough to ward off the effects of technical chlordane. A view reinforced by the known very slow metabolic conversion of the compound by bacteria and the failure of chlordane to inhibit growth of *Escherichia coli* even at concentrations as high as 1000 p.p.m. Though the possibility of a specific target site being absent from Gram-negative bacteria and thus rendering them immune has not been categorically excluded it is unlikely in view of the relatively high concentrations of chlordane needed to inhibit Gram-positive bacterial growth, about 1.5×10^7 molecules/organism added to the growth medium. Though we have not yet established what fraction of the added chlordane is taken up by the cells a useful comparison

is provided by the nonspecific physicochemically membrane-active antibacterial compounds such as monochlorophenoxy-salicylanilide, 7.2×10^5 molecules of which have to be taken up by each cell of *Staphylococcus aureus* to achieve bacteriostasis (Hamilton, 1968). By contrast antibacterial compounds for which specific target sites have been demonstrated exert their effects at uptake levels several orders of magnitude lower than that observed with monochlorophenoxy-salicylanilide. The inference we draw from this is that chlordane may exert a relatively non-specific but lethal 'swamping effect' which deforms the structural integrity of the cell and would, but for the barrier action of the cell wall, have the same effects upon *E. coli* as are observed with *Bacillus subtilis*; the respiratory sensitivity of *E. coli* sphaeroplasts being an indication of this.

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