The Effects of Technical Chlordane on Growth and Energy Metabolism of *Streptococcus* *faecalis* and *Mycobacterium phlei*: a Comparison with *Bacillus subtilis*

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SUMMARY

The growth of *Mycobacterium phlei* and *Bacillus subtilis* either on plate culture or in liquid medium is completely inhibited by technical chlordane at identical concentrations. NADH oxidation by membrane fragments and oxidative phosphorylation by crude extracts of *M. phlei* are only partially inhibited by incubation with chlordane at concentrations equal to or greater than the minimum levels required to completely inhibit growth.

Streptococcus faecalis is relatively insensitive to technical chlordane, bacteriostasis only being achieved at chlordane concentrations some five- to tenfold greater than those required with *Bacillus subtilis* and *Mycobacterium phlei*. The energy metabolism of *S. faecalis* is relatively simple since it contains neither a cytochrome-mediated electron transport chain nor a functional TCA cycle. Fermentation of D-glucose by soluble protein extracts, Na-K-ATPase and cation permeability of the plasma membrane are not sensitive to chlordane at concentrations that stop growth in liquid culture. Inhibition of fermentation by growing cultures, upon addition of chlordane, may be a reflection of the inhibition of biosynthetic reactions that recycle ATP.

INTRODUCTION

Organochlorine insecticides are inhibitory to the growth of Gram-positive bacteria (Gray & Rogers, 1955; Duda, 1958; Lyr, 1969; Trudgill, Widdus & Rees, 1971). A range of sensitivity has been observed, *Bacillus subtilis* being prevented from growth by lesser chlordane concentrations than are required to produce similar results with *Sarcina lutea* and *Streptococcus faecalis*. Effects observed with chlordane treated *B. subtilis* include cessation of growth, inhibition and eventual cessation of respiration, loss of viability coupled with a delayed release of polymeric molecules from within the bacteria (Trudgill *et al.* 1971). Inhibition of whole-cell respiration is paralleled by inhibition of electron-transport capability of subcellular membrane fragments. Disruption of this and other membrane and mesosome associated phenomena has been postulated as being the primary cause of cessation of growth and loss of viability (Widdus, Trudgill & Maliszewski, 1971).

In the present work extracts of *Mycobacterium phlei* have been used to assess the possible importance of oxidative phosphorylation as a chlordane-sensitive metabolic activity. An attempt has also been made to assess the importance of the TCA cycle and electron transport as chlordane sensitive target sites by reference to *Streptococcus faecalis*. Though *S. faecalis*

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contains the respiratory components necessary for oxidative phosphorylation in the flavin region (Baum & Dolin, 1965) it is devoid of cytochromes (Dolin, 1955) and is only capable of limited oxidative phosphorylation associated with NADH dehydrogenase (Gallin & VanDemark, 1964; Faust & VanDemark, 1970) when grown under aerobic conditions or provided with a suitable electron acceptor during anaerobic growth.

METHODS

Organisms. *Streptococcus faecalis*, strain 10 C 1 (NCIB8661) and *Mycobacterium phlei* (ATCC354) were obtained from the respective culture collections. Stock cultures of *M. phlei* were maintained on nutrient agar. For *S. faecalis* the agar was fortified with 3% (w/v) D-glucose and 5% (w/v) K_2HPO_4 . Cultures were incubated under static conditions at 37° or, when fully aerobic conditions were required, in a New Brunswick Gyrotary shaker (New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.) at 200 rev./min. and 37°. Growth in fermenter vessels was rendered anaerobic when required by passage of oxygen-free N_2 through the medium.

Mycobacterium phlei was grown on nutrient agar or in liquid medium (45° and 150 rev./min.) which contained (g./l.): proteose peptone, 13; sodium fumarate, 5; Na_2HPO_4 , 1; KH_2PO_4 , 0.5; $MgSO_4 \cdot 7 H_2O$, 0.3; $FeSO_4 \cdot 7 H_2O$, 0.02.

Harvesting and disruption of organisms. Bacteria were harvested and disrupted by the methods described by Widdus *et al.* (1971).

Measurement of bacterial growth. Growth of *Streptococcus faecalis* in liquid culture was followed as previously described (Trudgill *et al.* 1971).

Addition of technical chlordane to aqueous growth media. Additions of chlordane to both liquid culture media and agar plates were made as described previously by Trudgill *et al.* (1971).

Measurement of acid production by growing cultures of Streptococcus faecalis. Acid production was measured either by following the decrease in pH subsequent to the addition of a 10% (v/v) inoculum to 750 ml. of medium in a 1 l. fermenter pot on a Type 1 fermenter (L. H. Engineering, Stoke Poges, Buckinghamshire) or by addition of a 10% (v/v) inoculum to 150 ml. medium maintained at pH 7.5 by use of an Autotitration Controller (Pye Unicam Ltd, Cambridge). In the latter case consumption of 1.5 N-KOH was monitored manually at suitable time intervals. Growth media were maintained at 37°.

Preparation of crude extracts. Broken bacterial suspensions were centrifuged at 0° and 20,000g for 10 min. The supernatant crude extract was subjected to further fractionation where required.

Preparation of high-speed supernatant fluid and membrane fraction. The supernatant fluid from the 20,000g centrifugation was centrifuged at 125,000g for 1 h. The high-speed supernatant fluid was decanted and the pellet, washed by resuspension in a suitable buffer followed by further centrifugation at 125,000g, was designated the crude membrane fraction.

Protein estimations. The protein content of extracts was determined by the modified biuret procedure of Gornall, Bardawill & David (1949).

Measurement of acid production by Streptococcus faecalis extracts. The fermentation of D-glucose by extracts of *S. faecalis* was monitored by the controlled addition of 1 mM-NaOH to an unbuffered reaction system containing the necessary additional reaction components. The pH was maintained at 7.5 by the Autotitration Controller and alkali consumption monitored manually at suitable time intervals.

NADH oxidase activities. NADH oxidases and peroxidases of high-speed supernatant

fluid and membrane fractions of *Streptococcus faecalis* were assayed by following the fall in E_{340} when 0.5 μ mole of NADH was added to a suitable amount of protein contained in a final vol. of 3 ml. 0.01 M-tris-HCl buffer, pH 8.0. Cytochrome chain mediated NADH oxidation by crude extracts or membrane fractions from *Mycobacterium phlei* was measured by following the stimulation of O_2 consumption that occurred when 2 μ mole NADH were added to a 3 ml. reaction volume that contained 1 to 5 mg. of protein and appropriate buffer. Determinations were carried out in a conventional Warburg apparatus or with a Clark-type oxygen electrode in an agitated and thermostatically controlled vessel (Oxygen Monitor, model 53; Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio, U.S.A.).

Measurement of oxidative phosphorylation. Oxygen consumption by extracts of *Mycobacterium phlei* was measured as described and residual inorganic phosphate estimated by the method of Fiske & SubbaRow (1925) subsequent to the denaturation of protein by addition of 10% (w/v) trichloroacetic acid and its removal by centrifugation.

Adenosine triphosphatase (ATPase) activity. Na-Ka-ATPase of *Streptococcus faecalis* was assayed according to the method of Harold, Baarda, Baron & Abrams (1969).

RESULTS

Mycobacterium phlei

Effects of chlordane on growth of Mycobacterium phlei. The growth of *M. phlei* on nutrient agar plates was inhibited by technical chlordane added as a surface film and displayed the same sensitivity as *Bacillus subtilis* (Table 1). Because of the granular growth characteristics of *M. phlei* in liquid culture it was not possible effectively to monitor growth by following turbidity at 580 m μ . Accordingly the effects of chlordane on the growth of *M.*

Table 1. *The sensitivity of Mycobacterium phlei growing on agar plates to technical chlordane: a comparison with Bacillus subtilis, Streptococcus faecalis and Sarcina lutea*

Micro-organism	Chlordane (mg./cm. ²)			
	1.0	0.1	0.01	Vapour*
<i>M. phlei</i>	—	—	—	—
<i>B. subtilis</i> †	—	—	—	—
<i>S. faecalis</i> †	—	—	+	—
<i>S. lutea</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.

† Data from Trudgill *et al.* (1971).

Table 2. *Inhibition of growth of Mycobacterium phlei in liquid culture by technical chlordane*

Erlenmeyer flasks containing 50 ml. medium were inoculated with *M. phlei* followed by additions as indicated. After 72 h. growth at 45° on a gyrotary shaker, cultures were harvested on tared 5 μ pore size membrane filters and dried to constant weight.

Addition	Bacteria dry wt (mg.)
Acetone (control)	40.7
Chlordane (5 p.p.m.)	46.6
Chlordane (10 p.p.m.)	32.3
Chlordane (20 p.p.m.)	2.6
Chlordane (100 p.p.m.)	0.6

phlei were assayed by harvesting cells from experimental and control culture flasks on pre-dried and weighed membrane filters. The dry weights of bacteria obtained (Table 2) confirm the growth sensitivity of *M. phlei* to technical chlordane as being of the same order as that of *Bacillus subtilis*. It was therefore considered that *M. phlei* was an ideal organism in which to test the effects of chlordane on oxidative phosphorylation since sub-cellular systems of *M. phlei* capable of catalysing effective oxidative phosphorylation with high P/O ratios have been well described by Brodie & Gray (1956). The NADH oxidase activity of crude membrane preparations is chlordane sensitive as shown in Table 3. The inhibition obtained was

Table 3. *Effects of technical chlordane on NADH oxidase activity of crude membrane preparations from Mycobacterium phlei*

NADH oxidase activity was measured as described in the Methods. The oxygen monitor vessel contained 2 μ mole NADH, 2 mg. crude membrane protein and 280 μ mole tris-HCl buffer pH 7.4. Reactions were followed at 37°.

Addition	NADH oxidase* activity (μ mole O ₂ /mg. protein/h.)	Inhibition (%)
None	2.4	—
Acetone	1.75	0
Chlordane (20 p.p.m.)	1.14	35
Chlordane (100 p.p.m.)	0.74	58

* Assay systems were incubated with acetone or chlordane for 1 h. at 37° prior to addition of NADH.

Table 4. *Effect of technical chlordane on oxidative phosphorylation by crude extract of Mycobacterium phlei*

Respiration was measured with the oxygen monitor as described in the Methods section. Reaction systems contained the following: KH₂PO₄, 5 μ mole; MgCl₂, 10 μ mole; D-mannose, 20 μ mole; ADP, 5 μ mole; KF, 20 μ mole; hexokinase, 2.8 units and 10 to 15 mg. crude extract protein. Volumes were made to 3 ml. with tris/HCl buffer pH 7.4 and reactions started by addition of 2 μ mole NADH.

Reaction system	Addition	O ₂ consumed (μ atom)*	P _i esterified (μ mole)	P/O ratio
(a) NADH absent	.	0.117	0.00	0
Complete	.	0.863	1.62	1.87
Complete	10 ⁻³ M-dinitrophenol†	0.805	0.00	0
(b) Complete	Acetone	1.00	1.05	1.05
Complete	Chlordane (20 p.p.m.)	1.08	0.75	0.70
Complete	Chlordane (100 p.p.m.)	1.06	0.57	0.54

* Reactions were stopped after consumption of the amounts of O₂ indicated. Dinitrophenol caused no significant change in the rate of O₂ consumption. Addition of chlordane at 20 and 100 p.p.m. caused 22 and 45 % inhibition of the rate of O₂ uptake, respectively, in comparison with the acetone control. (a) and (b) were conducted with different bacterial extracts.

in general less than that observed with membrane preparations from *B. subtilis* (Trudgill *et al.* 1971) and showed some variation with the age of the membrane preparation. Oxidative phosphorylation by crude extract with NADH as electron donor yielded P/O ratios in the range of 0.8 to 1.8 and was completely inhibited by 10⁻³ M-dinitrophenol. Incubation of crude extract with chlordane at 20 and 100 p.p.m. resulted in significant inhibition of oxidative phosphorylation (Table 4), though the data show that under the experimental conditions employed, electron transport by the cytochrome chain is more chlordane sensitive than the phosphorylation step so that oxidative phosphorylation cannot be regarded as a particularly sensitive target site.

Streptococcus faecalis

Sensitivity of growth and fermentation by Streptococcus faecalis to technical chlordane. The growth of *S. faecalis* on nutrient agar plates fortified with D-glucose has been shown to be sensitive to the presence of technical chlordane though the organism is not so sensitive as other Gram-positive bacteria, for example *Bacillus subtilis* (ATCC9372) (Trudgill *et al.* 1971). This lower sensitivity of *S. faecalis* was demonstrated during growth in liquid culture (Fig. 1), 200 p.p.m. chlordane being required to cause a near complete inhibition of cell growth ($+ \Delta E_{580}$) while complete inhibition of growth of *B. subtilis* cultures at a similar cell density was achieved at 20 p.p.m. chlordane. The loss of viability and lysis observed with *B. subtilis* (Trudgill *et al.* 1971) were not evident when *S. faecalis* was treated with 200 p.p.m. chlordane. Concomitant with the near cessation of growth of *S. faecalis* which was usually delayed until some 30 min. after the addition of chlordane, a marked inhibition of D-glucose fermentation was suggested by the abrupt inflexion in the pH curve.

Fig. 1

Fig. 2

Fig. 1. Effect of technical chlordane on growth of *Streptococcus faecalis* and medium pH profile under anaerobic conditions. Δ , $\blacktriangle$, E_{580} ; $\circ$, $\bullet$, pH of growth medium. Chlordane (200 p.p.m.) was added to test experiment ($\blacktriangle$, $\bullet$) and acetone to the control (Δ , $\circ$) at the time indicated by the arrow.

Fig. 2. The effect of technical chlordane on glycolysis by high-speed supernatant fluid from *Streptococcus faecalis*. Reaction mixtures on the autotitration controller in deionized water, final vol. 10 ml., pH 7.5 contained: ADP, 40 μ mole; KH_2PO_4 , 40 μ mole; NAD, 5 μ mole; ATP, 5 μ mole; 68 mg. protein. Reaction was started by the addition of glucose (20 μ mole) and acid production followed as described in Methods. High-speed supernatant fluid was incubated for 1 h. with acetone as control ($\circ$) and chlordane at 200 p.p.m. ($\bullet$).

The decreased fermentation rate that occurred with growing cultures was not reproduced with a cell-free glycolytic system maintained at pH 7.5. When acid production from D-glucose was monitored in the presence of the necessary cofactors, incubation of crude

extract with 200 p.p.m. technical chlordane for 1 h. did not inhibit glycolysis (Fig. 2). This suggested that inhibition of glycolysis observed with growing cultures was not due to any direct effect of the chlordane upon the glycolytic enzymes but rather that it might be due to inhibition of associated or coupled phenomena in whole cell metabolism. Such phenomena include biosynthetic reactions that utilize ATP and the cation transporting ATPase present in the plasma membrane (Harold *et al.* 1969). It was also possible that such effects might be compounded by modification of membrane permeability and uncoupling of cation transport from membrane ATPase so that the intracellular pH was not maintained more alkaline than

Fig. 3. Effect of technical chlordane on growth and acid production by *Streptococcus faecalis* maintained at constant pH. Acid production by *S. faecalis* growing at 37° was followed by monitoring addition of 1.5 N-KOH required to maintain the culture at pH 7.5 as described in Methods. Δ , $\blacktriangle$, E_{580} ; $\circ$, $\bullet$, addition of alkali. Chlordane (200 p.p.m.) was added to the test culture ($\blacktriangle$, $\bullet$) and acetone to the control (Δ , $\circ$) at the time indicated by the arrow.

that of the growth medium by energy-dependent proton excretion (Harold, Pavlasova & Baarda, 1970). Under the experimental conditions (Fig. 1) a decrease of intracellular pH value to that of the growth medium at the time of chlordane addition would itself contribute to the inhibition of glycolysis. Since a similar pattern of inhibition of glycolysis was observed when chlordane at 200 p.p.m. was administered to a growing culture maintained at pH 7.5 on the pH stat (Fig. 3) lack of control of intracellular pH as a result of changes in membrane permeability was not a significant factor in the inhibition of glycolysis.

Effect of chlordane on Na-K-ATPase of Streptococcus faecalis. The membrane-bound ATPase of *S. faecalis* is involved in the energy dependent transport of cations across the plasma membrane (Abrams, 1960; Zarlengo & Schultz, 1966; Harold *et al.* 1969). Since this enzyme hydrolyses ATP generated at substrate level during glycolysis, inhibition of ATPase may interrupt glycolysis by restricting the recycling of ATP as was observed with *N,N*-

dicyclohexylcarbodiimide (Harold *et al.* 1970). Incubation of crude membrane preparations from *S. faecalis* with 200 p.p.m. technical chlordane for 1 h. did not inhibit Na-K-ATPase, suggesting that the observed inhibition of glycolysis was not due to interference with this ATP recycling reaction.

Effect of chlordane on NADH oxidizing enzymes of Streptococcus faecalis. The mixture of NADH oxidases and peroxidases responsible for the respiration of intact *S. faecalis*, devoid of haeme pigments, under aerobic conditions have been variously reported as being coupled to oxidative phosphorylation (Gallin & VanDemark, 1964) and not involved in generation of ATP (Bryn-Jones & Whittenbury, 1969). In view of the possibility that these enzymes may be of importance in the energy metabolism of aerobically grown *S. faecalis* the effect of technical chlordane was investigated as described in the Methods section. Incubation of subcellular fractions for 1 h. with 200 p.p.m. chlordane inhibited NADH oxidation by a somewhat variable amount which ranged from 6 to 29 % from preparation to preparation. Membrane bound activity was not significantly more sensitive than the soluble enzymes.

DISCUSSION

Attempts to elucidate the nature of the cyclodiene insecticide sensitivity of *Bacillus subtilis* (ATCC9372) that is responsible for inhibition of growth and respiration and for loss of viability have been only partially successful (Trudgill *et al.* 1971; Widdus *et al.* 1971). Inhibition of bacterial respiration is paralleled by inhibition of NADH oxidase of isolated membrane fragments, indicating that, in common with other biochemical systems studied (Morrison & Brown, 1954; Colvin & Phillips, 1968; Wineley & San Clemente, 1970), organized lipid-containing membranous structures are targets that attract hydrophobic cyclodiene insecticides. However, because cessation of bacterial growth precedes the complete inhibition of respiration it may be argued that the observed effects upon respiration are secondary phenomena (Widdus *et al.* 1971). The possibility also exists that the phosphorylation steps coupled to cytochrome mediated electron transport may be chlordane sensitive. To test this suggestion crude extract preparations from *Mycobacterium phlei* (ATCC 354) that are capable of effective oxidative phosphorylation (Brodie & Gray, 1956) were used. Though growing cultures of *M. phlei* exhibited the same sensitivity to technical chlordane as *B. subtilis* (Tables 1, 2) oxidative phosphorylation by *in vitro* systems is only partially inhibited and displays a sensitivity no greater than that shown by NADH oxidase of uncoupled membrane fragments (Tables 3, 4). In *M. phlei*, and perhaps *B. subtilis*, neither electron transport nor coupled oxidative phosphorylation would appear to be a critically disrupted primary site of chlordane action.

By comparison with *Bacillus subtilis* the relative insensitivity of *Streptococcus faecalis* could be attributed to a lack of cytochromes and TCA cycle in the latter organism. Such an argument is reinforced by the studies of Nelson & Williams (1971) who showed that aerobically grown *Saccharomyces cerevisiae* displayed a time-dependent inhibition of respiration in the presence of chlordane. Only when the organism was grown aerobically on non-fermentable substrates, with an obligatory requirement for oxidative phosphorylation, did chlordane inhibit growth. The view that electron transport, oxidative phosphorylation and the TCA cycle are primary targets of cyclodiene action is however probably an oversimplification of the situation with respect to bacteria and does not explain the variation in sensitivity exhibited by organisms within the same genus and even different strains of the same species (Fletcher & Bollen, 1954; Trudgill *et al.* 1971).

Sarcina lutea, which has a cytochrome complement very similar to that of *Bacillus subtilis*

(Gel'man, Lukyanova & Ostrovskii, 1967), was the least sensitive of the Gram-positive organism tested (Trudgill *et al.* 1971). Clearly other factors such as differences in cell wall structure and composition (the factor responsible for resistance of Gram-negative bacteria) and, in this case perhaps the association of carotenoids with the plasma membrane (Mathews & Siström, 1959), might play a role in conferring a degree of resistance to the effects of technical chlordane.

Technical chlordane is bacteriostatic to *Streptococcus faecalis* but only at relatively high concentrations (Fig. 1). The effect of chlordane is not due to inhibition of the membrane-bound ATPase with a resulting disruption of energy-dependent ion transport across the plasma membrane nor does it appear to involve any aspect of the energy metabolism of glucose-grown bacteria (Fig. 2).

The inhibition of glycolysis by whole bacteria that accompanies virtual cessation of growth (Fig. 3) may reflect the inhibition of energy-dependent biosynthetic processes with a consequent decrease in the rate of turnover of ATP imposing a limitation on glycolysis. Our failure to show inhibition of glycolysis in high-speed supernatant fluids is compatible with the relative insensitivity of soluble enzymes as reported for *Bacillus subtilis* (Widdus *et al.* 1971).

As previously suggested (Widdus *et al.* 1971) and not contradicted by the data presented here, the cyclodiene insecticides probably exert their effects by deformation of membrane integrity the primary results of which are a cessation of membrane associated aspects of cell division and biosynthesis while effects upon energy metabolism may appear as closely associated secondary phenomena.

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