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Variability of Lindane Toxicity in *Tetrahymena pyriformis* with Special Reference to Liposomal Lindane and the Surfactant Tween 80®

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Bacteria and algae are commonly used in toxicity testing of chemical pollutants. Protozoa, on the other hand, have received less attention in such studies despite the fact that they constitute an important group in freshwater ecosystems. Tetrahymena pyriformis is widely distributed in nature being normally found in unpolluted lakes, ponds and slow moving streams (Carter and Cameron 1973). Furthermore, the physiology and biochemistry of Tetrahymena have been extensively studied. The present paper describes the effects of lindane on the growth of T. pyriformis (Ciliata) and the modifying effects of liposomes and Tween 80 on the toxicity of lindane.

MATERIALS AND METHODS

The gamma isomer of 1,2,3,4,5,6-Hexachlorocyclohexane (γ-HCH = lindane,analytical grade) was obtained from E. Merck, Darmstad, FDR, and Tween 80 (technical grade) from Koch-Light Laboratories Ltd., Colnbrooks Bucks, England).

Stock cultures of Tetrahymena pyriformis strain GL were maintained axenically at 20°C in 2% proteose peptone (Difco), 0.1% yeast extract (Difco), plus 0.018 mM ferrous ammonium sulphate. Streptomycin sulfate and penicillin G (250 μg/ml each) were added to the autoclaved medium immediately prior to use. The pH was approximately 7.0. A volume of medium containing cells grown exponentially in a continuous culture was inoculated so as to yield an initial density of approximately 10,000 cells per ml, unless otherwise stated. Culture growth was carried out at 20 1°C in 10 ml of medium in glass petri dishes placed on a rotary shaker generating approximately 60 cycles per min. Duplicate cultures were used in all experiments.

The growth rates of the cultures were monitored by counting the number of cells in a 1 ml sample, diluted 1:25 with a balanced electrolyte solution, with a Coulter Counter, Model ZB Industrial, fitted with a 140 μm orifice tube (Coulter

Electronics Inc., Dunstable, Bads, U.K.). Five counts per sample were calculated. To express the effects of the test substances on the growth of Tetrahymena, the 24 h median lethal concentration, LC50, was employed.

Corn oil liposomes were used in the present study. The test chemicals were first dissolved in the oil and appropriate volumes of these solutions and/or appropriate volumes of toxicant-free oil were added to the medium to yield the desired final concentration of toxicant per ml medium. Liposomes were prepared as follows: Oil plus 0.25% Tween 80 was added to the medium, were sonicated for 4 min in a 30 ml centrifuge tube using a 19 mm titanian probe of a Soniprep (MSE) 200 W sonicator. The control media were also sonicated for 4 min. All liposomes contained Tween 80 except when stated otherwise. The size of the liposomes were examined using both the Coulter Counter and a microscope.

Lindane medium without any organic solvents was saturated with lindane. This was centrifuged and the supernatant was filtered using a 1 µm millipore filter. Pure medium was added to the saturated medium to yield the following: undiluted, 50, 10 and 5%. These concentrations were equivalent to 8.5, 2.1, 0.85 and 0.44 mg lindane/l, respectively, as measured by gas chromatography. T. pyriformis was exposed to these concentrations of lindane in order to test for its effect on growth rate.

In order to test whether or not the growth inhibition due to lindane exposure was reversible, T. pyriformis was exposed to the following concentrations of lindane-saturated medium for 24 h: 100%, 25%, 10%, 5% and 0%. The cells were then washed twice in toxicant-free medium, the densities were adjusted to 31,000-37,000 cells per ml and grown in toxicant-free medium for 53 h.

The effect of liposomal lindane on the growth of T. pyriformis was studied. Organisms were cultured in medium containing lindane in liposomes (1% corn oil in medium) at final concentrations equivalent to 2 and 20 mg/l lindane. Control cultures consisted of toxicant-free and liposome medium. Since liposomes contain both oil and Tween 80, T. pyriformis was grown for 96 h under the following conditions in order to test for any effects associated with these substances. The four treatment groups consisted of medium control, medium + 0.25% Tween 80, medium + 1% corn oil, and medium + 0.25% Tween 80 + 1% corn oil. All media were sonicated as described earlier. Tween 80 (0.25%) was added to medium containing 0, 0.5, 1.0, 2.5 and 10.0 mg/l lindane in order to test for the influence of this surfactant on lindane toxicity in T. pyriformis during a 46 h period. Controls consisted of the same concentrations of lindane without Tween 80.

RESULTS AND DISCUSSION

The solubility of lindane in water is approximately 10 mg/l at 20°C (Ulmann 1972). In the present study it was found that the solubility of lindane in 2% proteose-peptone medium averaged 8.2 ± 0.5 mg/l (n=8). Exposure of T. pyriformis to lindane in saturated medium, approximately 8.5 mg/l, was cytotoxic to most of the organisms. Microscopic examination revealed many dead, but intact cells, which were also counted in the Coulter Counter, thus giving an over-estimation of population density. No appreciable cytotoxic effects were observed at the other concentrations. When the cultures were allowed to grow for an additional 4 to 5 days, all treatments, except saturated lindane, reached cell densities similar to the control population. The effects of lindane on the morphology and growth of unicellular organisms including T. pyriformis were studied by Jeanne-Levain (1974). Exposure to 10 mg lindane/l in 0.2% acetone caused complete inhibition of cell division and 5 mg/l led to a reduced growth rate compared to control cultures. These results are in close agreement with the present findings.

The concentration at which population growth is inhibited by 50% after 24 h exposure was calculated to be 1.9 mg/l. This value is the mean for 4 experiments, with a range of 1.8 - 2.0 mg/l. The toxicity of lindane has previosly been tested on other species of Protozoa. Jeanne-Levain (1974) found that the flagellate, Euglena gracilis Z, was quite resistant to lindane since they tolerated concentrations up to 60 mg/l. The sensitivity of Colpidium campylum, (Ciliata) to lindane was similar to that of Tetrahymena (Dive et al. 1980). The effects of lindane have also been studied on unicellular algae. The growth of Dunaliella bioculata was partially inhibited at 5 mg/l, and completely inhibited at 10 mg/l. Thus, the sensitivity was similar to T. pyriformis. The most sensitive species to lindane was Amphidinium carteri, a dinoflagellate, which revealed complete inhibition of growth in 2 mg/l (Jeanne-Levain 1974) and 0.3 mg/l prevented cell multiplication (Jeanne 1979).

It appears that a typical response of Tetrahymena to the presence of toxic concentrations of xenobiotics is to become progressively more spherical. Inhibition of growth was only observed in the presence of lindane. When the organisms were washed twice, transferred to toxicant-free medium at initial densities between 31,000 and 37,000 cells per ml, all treatments had similar densities after 53 h. The organisms previously cultured in lindane-saturated medium showed only a slight lag phase but the final densities were within the same range as the other treatments. Jeanne-Levain (1974) made similar observations when T. pyriformis were transferred from medium containing lindane to toxicant-free medium. He also reported that even the inhibitory effects associated with 3 d exposure to 10 mg/l lindane were reversible when the organisms were transferred to fresh medium.

Table 1. The effects of lindane on the growth of Tetrahymena pyriformis.

Lindane treatment (% saturated medium)	Exposure Period (h)		
	0	24	77
		cells/ml	
100% (8.5 mg/l)	10,600	36,680[1]	33,500[1]
25% (2.1 mg/l)	"	137,630	239,150
10% (0.85 mg/l)	"	269,920	564,350
5% (0.44 mg/l)	"	301,720	670,400
0%	"	312,000	735,400

[1] Cell densities include dead cells.

The mechanisms by which Tetrahymena adapt to the inhibitory effects on growth rate which was observed in the present study appears to be typical for protozoans in general. For example, Silberstein and Hooper (1974) found that the effects of 2,4,5-trichlorophenoxyacetic acid on exponentially growing cells could be summarized as follows: The population growth is slowed (I) and then stopped (II). This was followed by recovery phases in which, after an initial burst of rapid division (III), the growth rate returned to that of the uninhibited cells (IV). Phase IV lasted until the culture reached the stationary phase. Similar responses were observed when T. pyriformis was exposed to cycloheximide (Frankel, 1970), lead acetate (Nilsson, 1978), or when Euglena viridis, a photosynthetic flagellate was exposed to various pesticides (Poorman, 1973).

Exposure of T. pyriformis to toxic compounds, such as phenol, have triggered a general mucocyst discharge and caused a rounding up of the cells (Schultz and Dumont, 1977). These appear to be nonspecific protective mechanisms. The mucous discharge could serve to protect the cell surface as well as to bind toxic substances.

Lindane dissolved in liposomes in concentrations ranging from 2 to 20 mg/l medium, caused no detrimental effects on the growth of T. pyriformis after 1, 2 or 4 d exposure (data not shown). However, the growth rates of the cultures exposed to lindane in liposomes were identical with liposome control cultures (Table 2).

In a study having a similar design, Onaga and Baillie (1980) found that the 4 h LC50 for liposomal chloramphenicol (CAP) was 1.5 mg/l compared to 600 mg/l for CAP dissolved in the medium. However, the water solubility of lindane and CAP is an important factor to be considered since it predicts the ability of a

dissolved substance to move from ingested liposomes into the surrounding food vacuoles. In attempting to understand why liposomal lindane failed to cause toxic effects on Tetrahymena, a comparison of Onaga and Baillies' (1980) with the present results may help to clarify some of the important factors which should be considered. Dissolved lindane is much more toxic to exponentially growing Tetrahymena than CAP. The water solubility of CAP is approximately 250 times greater than for lindane. Thus, if both substances are dissolved in water at equal concentrations, then much more lindane will enter the fat phase (=Tetrahymena) than CAP. On the other hand, with equal concentrations in a fat phase, surrounded by water, CAP will be distributed in the water phase much more so than lindane. Therefore, the amount of CAP entering the food vacuole from ingested liposomes, and thus becoming biologically available, will be much greater than for lindane. It is presumed that very little of the lindane in the liposomes becomes biologically available where it could exert toxic effects intracellularly.

Not only did lindane-liposomes fail to exert toxic effects, but stimulated the cells to grow faster and reach higher, final densities than medium controls or controls containing 0.25% Tween 80. After 2 d the densities of T. pyriformis grown in the presence of liposomes, either with or without lindane, were approximately 1.3 x 10^6/ml compared to 807,000 for the controls (Table 2). These findings resemble unpublished results which were obtained when HCB or DDT liposomes were tested, since ingestion of liposomes containing these organochlorines failed to exert toxic effects, and the final densities of Tetrahymena cultured in the presence of liposomes were greater than for controls.

Since the liposome controls in the experiments also contained 0.25% Tween 80, two additional control cultures were included in order to assess the significance of these variables. It was found that the growth rate and/or final densities in medium containing oil only was = oil + Tween 80, and > Tween 80 > control medium after 1, 2 and 4 d. Microscopic examination of the cultures revealed that even after 4 d, the liposomes without Tween 80 were still in suspension although there was a greater tendency for these liposomes to fuse together to form small oil droplets.

Tween 80 is a synthetic fatty acid-containing surfactant. It has been previously shown that the rate of cell division in T. pyriformis is more rapid during the early phases of growth in the presence of Tween 80 than in its absence (Dewey and Kidder, 1978). They felt that the presence of Tween 80 during the rapid phase of growth provided the cells with an additional lipid source at a time in the cell cycle when the stored triglycerides are quickly metabolized. The growth rate in the presence of Tween 80 in the present study (Table 2) confirms the anabolic effect of this surfactant.

Table 2. The effects of adding 0.25% Tween 80 and/or 1% corn oil liposomes to proteose-peptone medium on the growth rate and density of Tetrahymena pyriformis.

Treatment	Time (h)		
	24	48	96
	cells/ml x 10^3		
Control	307	807	619
Control + Tween 80	296	895	783
Control + oil	459	1,481	1,349
Control + oil + Tween 80	411	1,369	1,336

The increased growth rate associated with the presence of liposomes may be an effect of the added nutritive value of corn oil and/or Tween 80. However, the liposomes are in themselves physical, ingestible particles which may stimulate the formation of food vacuoles thereby increasing the uptake of dissolved nutrients from the medium, as was observed by Rasmussen (1973). Further evidence which suggests that stimulation of growth by liposomes is related to physical stimulation of internalized particles and not due to the addition of potentially digestible fatty acids, was observed in an earlier unpublished study (DDT) which used mineral oil liposomes. These liposomes, which are non-digestible, stimulated growth rate during the first 48 h compared to control cultures of T. pyriformis. At present, no explanations can be given as to why the final densities of Tetrahymena are higher in proteose-peptone medium containing liposomes than in control medium. However, it is not because liposomes represent an additional nutritive source since mineral oil liposomes (unpublished data), and indigestible polystyrene beads and/or ferric hydroxide particles (Rasmussen 1973) were all associated with higher, final population densities compared to controls.

The toxicity of lindane was reduced by the addition of 0.25% Tween 80 to medium (Figure 1). After 24 h almost all of the organisms exposed to approximatley 10 mg lindane/l were dead. In comparison, Tetrahymena exposed to the same concentration of lindane but in the presence of Tween 80 remained viable, and after 24 h the density was equivalent to 37% of the control cultures containing Tween 80. The 24 h LC50 for lindane in the culture containing Tween 80 was calculated to be 8.2 mg/l compared to 2.0 mg/l for the lindane control medium. It can also be seen in Figure 1 that T. pyriformis cultured in medium containing 2.5 mg/l lindane plus Tween 80 grew equally well as the control cultures. In an earlier study, Epstein and Niskanen (1967) reported that the addition of Tween 60 to the medium reduced the toxicity of Benzo(a)pyrene (BP) in both T. pyriformis and isolated rat mitochondria. This was associated with a

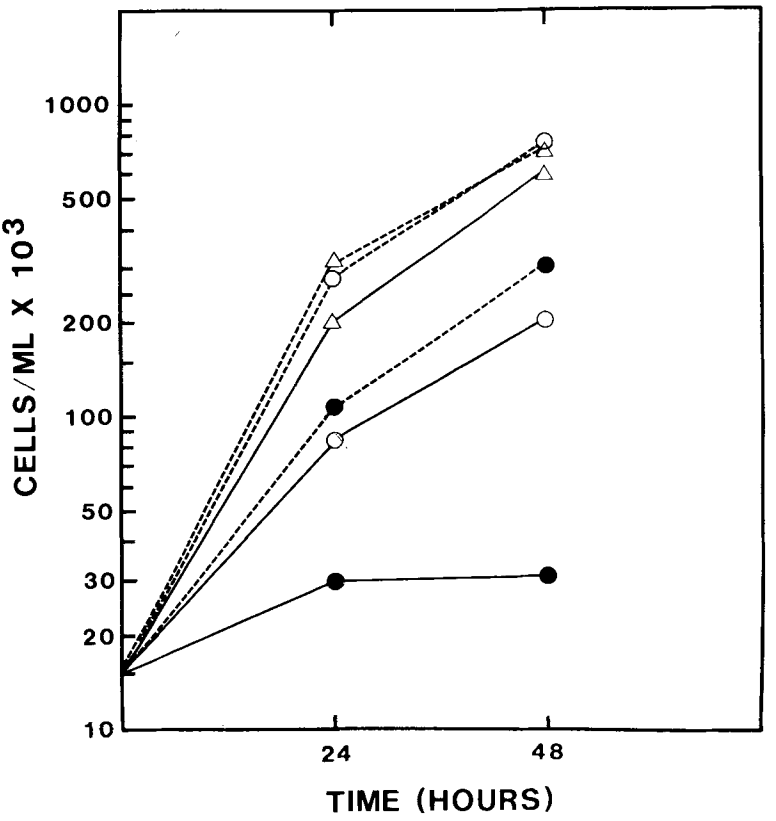

Figure 1. The effects of Tween 80 on the toxicity of lindane. (●=10 mg/l lindane,○=2.5 mg/l lindane, △=control medium, ——— medium without Tween 80, ------ medium with Tween 80).

decreased photodynamic response. They also reported that other non-ionic surfactants (Tween®) markedly protected another ciliate, Paramecium caudatum against the phototoxicity of BP. They concluded that the protection afforded by Tween 60 was the elution of BP from both T. pyriformis and isolated liver mitochondria.

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REFERENCES

Carter JW, Cameron IL (1973) Toxicity bioassay of heavy metals in water using Tetrahymena pyriformis. Water Research 7: 951-961

Dewey VC, Kidder GW (1978) Influence of sphingolipid bases, fatty acids and Tween 80 on the folate requirement of Tetrahymena pyriformis. J Protozool 25: 270-273

Dive D, Leclerc H, Persoone G (1980) Pesticide toxicity on the ciliat protozoan Colpidium campylum: possible consequences of the effect c pesticides in the aquatic environment. Ecotoxicol Environ Safety 4: 129-133

Epstein SS, Niskanen EE (1967) Effects of Tween 60 on benzo(a)pyrene uptake by Tetrahymena pyriformis and by isolated rat liver mitochondria. Experimental Cell Res 46: 211-234

Frankel, J (1970) An analysis of the recovery of Tetrahymena from the effects of cycloheximide. J Cellular Physiol 76: 55-64

Jeanne N (1979) Effets du lindane sur la division, le cycle cellulair et les biosynthèses de deux algues unicellulaires. Can J Bot 57: 1464-1472

Jeanne-Levain N (1974) Etude des effets du lindane sur la croissance et le développement de quelques organismes unicellulaires. Bull Soc zool de France 99: 105-109

Nilsson JR (1978) Retention of lead within the digestive vacuole in Tetrahymena. Protoplasma 95: 163-173

Onaga IC Baillie AJ (1980) The toxicity of liposomal chloramphenicol for Tetrahymena pyriformis. Int J Pharm 7: 89-98

Poorman AE (1973) Effects of pesticides on Euglena gracillis. I Growt studies. Bull Environm Contam & Toxicol 10: 25-28

Rasmussen L (1973) On the role of food vacuole formation in the uptak of dissolved nutrients by Tetrahymena. Exptl Cell Res 82: 192-196

Schultz TW Dumont JN (1977) Cytotoxicity of synthetic fuel products on Tetrahymena pyriformis. I. Phenol. J Protozool 24: 164-172

Silberstein GB Hooper AB (1974) The effect of the herbicide 2,4,5 Trichlorophenoxy acetic acid (245T) on the growth and metabolism of Tetrahymena pyriformis. J Cell Physiol 85: 331-338

Ulmann E (1972) Lindane: A monograph of an insecticide. Schillinger, Freiberg im Breisgau

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