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EFFECTS OF DDT AND POLYCHLORINATED BIPHENYLS ON CELLULAR METABOLISM AND ULTRASTRUCTURE OF CRITHIDIA VASCICULATA, A FLAGELLATED PROTOZOAN

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Research was conducted according to the principles enunciated in the
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20. ABSTRACT (continued)

transient inhibition and then stimulation after 6 and 24 hours, respectively. After 6 hours exposure to DDT or PCB, inhibition of both uptake and incorporation of thymidine and uridine, but not of L-leucine, was observed. By 24 hours of PCB exposure, uptake and incorporation of these three radioprecursors was two to three times greater than control. After 24 hours DDT exposure, only thymidine uptake and incorporation exhibited such an increase. Ultrastructural changes induced by these compounds included cellular and mitochondrial swelling, disruption of the mitochondrial genophore, and failure of the mitochondrion to replicate in synchrony with cell division. It is concluded that the initial DDT or PCB inhibition of nucleic acid biosynthesis and subsequent loss of cell regulatory capacity causes the decrease in cell population growth. An inexpensive and rapid screening method for toxic chemical compounds used by military organizations is of great importance. To meet these requirements, such a method was developed using the protozoan Crithidia fasciculata.

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PREFACE

We are grateful to Dr. F. E. Guthrie, North Carolina State University, for the p,p'-DDT and to W. B. Papageorge of Monsanto Industrial Chemical Company for the Aroclors 1221 and 1254. The technical assistance of W. G. Ewald is gratefully acknowledged. Our appreciation is expressed to W. J. Flor for his critical review of this paper. This work was supported in part by USPHS Grant ES-00083 to North Carolina State University.

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INTRODUCTION

The mechanisms of toxicity of p,p'-DDT and polychlorinated biphenyls (PCBs) are poorly understood although these compounds have been shown to affect directly or indirectly several subcellular systems. In vitro inhibition of oxidative phosphorylation by DDT has been reported in vertebrates, and invertebrates,^{7, 8, 23, 26} except in cockroach subcellular preparations where no significant effects were noted.²⁹ PCBs have also been shown to inhibit in vitro oxidative phosphorylation of beef heart mitochondria.²² Such relatively large doses of DDT were required to inhibit glycolytic enzymes that it was concluded to be an indirect effect.¹ However, at sublethal doses of DDT, gluconeogenic enzymes in rat liver are stimulated.¹⁰ These compounds have also been shown to inhibit protein and nucleic acid biosynthesis in cultured mammalian cells. DDT and dieldrin inhibit or stimulate protein or nucleic acid synthesis in both cellular and subcellular preparations of HeLa cells, depending on the dose.^{3, 4} However, in another study 125 ppm DDT inhibited DNA, RNA and protein synthesis in HeLa cells, while 125 ppm NaCl stimulated DNA synthesis.¹⁵ Ehrlich ascites carcinoma cells responded to 35 ppm DDT exposure by alteration of purine synthesis, and DNA, RNA and protein synthesis inhibition.³⁰

DDT has been shown to alter the axonal action potential of nerve cells by impairing cation (K^+ , Na^+) transport.^{9, 20} This change in membrane permeability has been related to DDT-induced charge transfer complexes.^{18, 19} One effect of this membrane interaction has been indicated by the recent evidence that both DDT and PCBs inhibit in vitro both Mg^{++} and Na^+ , K^+ -dependent adenosine triphosphatases (ATPases).^{5, 13, 17, 32} This mechanism has not been completely defined, but the evidence indicates that DDT interacts with membrane lipoprotein¹⁹ resulting in ATPase inhibition and alteration of membrane permeability. EPR-spin labeling experiments²⁵ support the concept of membrane perturbations resulting in loss of membrane fluidity. It has been suggested that this phenomenon could also disrupt the transport of essential nutrients in cells by steric rearrangement of membrane structure.²⁷ This hypothesis could

account for many of the effects observed in vivo and in vitro as described above. We have previously shown that DDT or PCB depresses cell population growth of Crithidia fasciculata,⁶ thus making it a suitable model cell for studying the biochemical mechanism of DDT and PCB toxicity.

It was the objective of these investigations to determine further the feasibility of using C. fasciculata as a model cell system for in vitro xenobiotic toxicity studies. The present studies of the effects of p,p'-DDT or PCB on energy metabolism, protein and nucleic acid biosynthesis and fine structure by a time course analysis during exponential growth were undertaken to elucidate the mechanism(s) of this inhibition.

MATERIALS AND METHODS

The culture of C. fasciculata, p,p'-DDT or PCB (Aroclor 1254) exposure, collection, cell density and protein determinations were carried out as described previously.⁶

Radioisotope measurement. Under aseptic conditions measurement of [¹⁴C] carbon dioxide evolution from 0.1 μ Ci [¹⁴C] D-glucose (1 μ Ci/ml, 240 mCi/mole, Schwartz-Mann, Orangeburg, New York) per 0.5 ml reaction medium (cells in Krebs-Ringers phosphate with glucose (KRP/G) as described previously⁶) was carried out by trapping CO₂ on Hyamine saturated paper in a sealed Erlenmeyer flask (Kontes Glass Company, Vineland, New Jersey) during a 60-min incubation at 27°C in a shaking water bath. Uptake and incorporation of 0.5 μ Ci [³H] L-leucine (15 Ci/mole), [³H] thymidine (6 Ci/mole) or [³H] uridine (29 Ci/mole) (Schwartz-Mann, Orangeburg, New York) per 0.5 ml reaction medium were measured individually after 30 min incubation at 27°C in a shaking water bath. Reactions were stopped by placing vessels on ice for uptake measurement or by adding an equal volume of 10 percent trichloroacetic acid (TCA) for incorporation measurements. Incorporation of radioprecursors is defined here to include only that radioactivity in the acid insoluble macromolecular fraction. Uptake is defined to include this fraction plus the radioactivity in

the acid soluble precursor pool. Uptake and incorporation are determined by sampling incubate or acid precipitate and collection on membrane filters (0.45 μm , Millipore Corporation, Bedford, Massachusetts) followed by rigorous washing with KRP/G (including unlabeled precursor in question at physiological levels) or 5 percent TCA, respectively. Radioactivity of paper or dried filters was determined in a liquid scintillation spectrometer (Packard Tri-Carb, Model 314EX, Palo Alto, California or Mark-II, Nuclear-Chicago, Chicago, Illinois) in a scintillation fluor consisting of 6 g 2,5-diphenyloxazole (PPO) and 75 mg 1,4-bis[2-(4-methyl-5-phenyloxazole)] benzene (POPOP) per liter of toluene (Amersham/Searle, Arlington Heights, Illinois).

Electron microscopy. Cells were suspension-fixed in 3 percent glutaraldehyde (in 0.1 M cacodylate, pH 7.4, with 7.5 percent sucrose) and postfixed in 2 percent osmium tetroxide (in 0.1 M cacodylate) at 4°C for 2 hours each. Cells were dehydrated and embedded in Epon 812¹⁶ (Ladd Research Industries, Burlington, Vermont). Thin sections were obtained with a diamond knife (Dupont, Wilmington, Delaware) on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall Corporation, Norwalk, Connecticut) and stained with uranyl acetate³¹ and lead citrate.²⁴ Specimens were examined on a Siemens Elmiskop I at an accelerating voltage of 80 kV.

Statistical analysis. Analysis of variance was used to determine statistical significance. Mean values were tested for significant differences at the 95 percent confidence level of Duncan's new multiple range test.²⁸

RESULTS

Energy metabolism. As an index of energy metabolism, [¹⁴C] carbon dioxide evolution from [U¹⁴C] D-glucose was measured. There were no significant dose-response effects after 12 hours DDT exposure. Exposure to 250 ppm DDT for 3, 6 and 12 hours also showed no significant differences (Table 1). However, evolution of CO₂ after 6 hours PCB exposure at all doses examined

was significantly ($p \leq 0.05$) decreased and was then significantly ($p \leq 0.05$) elevated above controls at all doses after 24 hours exposure (Figure 1).

Table 1. Effect of p,p'-DDT on [^{14}C] Carbon Dioxide Evolution from [^{14}C] D-Glucose by *Crithidia fasciculata*. I. Dose-Response After 12 Hours Postinoculation Exposure. II. Time Course Analysis After Exposure to 250 ppm. Mean Percent Control \pm SE of Three Replications.

	Dose (ppm)			
	0.35	3.5	35	355
I.	98 \pm 9	126 \pm 13	99 \pm 21	122 \pm 13
	Time (hours postinoculation)			
	0	3	6	12
II.	86 \pm 6	114 \pm 10	113 \pm 12	96 \pm 6

Nucleic acid and protein metabolism. Incorporation values of the radioprecursors, [^3H] thymidine, [^3H] uridine and [^3H] L-leucine, were used as indices of the effects of DDT or PCB on nucleic acid and protein metabolism. Uptake values are defined here as the transported radioprecursors in both the acid soluble and insoluble fractions of washed cells, whereas incorporation values refer to the label in the insoluble fraction only (as described in Materials and Methods).

There was a significant ($p \leq 0.05$) PCB and DDT dose-related inhibition of both uptake and incorporation of [^3H] thymidine after 6 hours exposure, except at the lowest DDT dose (0.35 ppm). Only after 12 hours exposure to the lowest PCB level (0.5 ppm) did the inhibition of incorporation persist. At all other

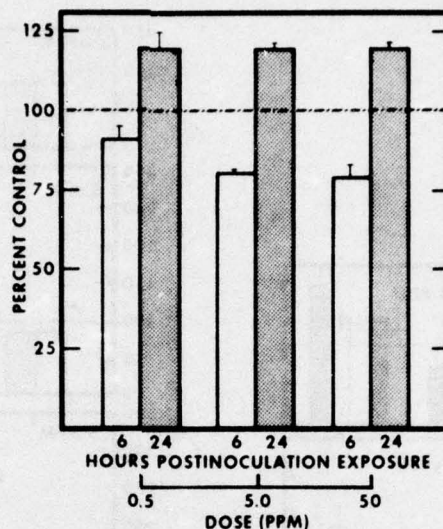


Figure 1. The effect of Aroclor 1254 on [^{14}C] carbon dioxide evolution from U^{14}C D-glucose in *Crithidia fasciculata* after 6 and 24 hours of exposure. Mean percent control \pm SE of three replications.

concentrations and later times, uptake and incorporation increased significantly ($p \leq 0.05$), relative to controls (Figures 2 and 3).

DDT significantly ($p \leq 0.05$) inhibited [^3H] uridine uptake and incorporation at all doses, except the lowest (0.35 ppm) after 6 hours exposure (Figure 4). Although [^3H] uridine uptake recovered and increased significantly ($p \leq 0.05$) above controls at both 12 and 24 hours, incorporation was still inhibited at 12 hours, but returned to slightly above control level by 24 hours. In contrast, only the greatest level of PCB (50 ppm) tested exhibited significant inhibition of [^3H] uridine uptake and incorporation after 6 hours exposure (Figure 5). After 12 hours PCB exposure, [^3H] uridine uptake and incorporation were within control levels at all doses. After 24 hours PCB exposure, for all except the lowest dose (0.5 ppm), [^3H] uridine uptake and incorporation increased two to three times above the control levels.

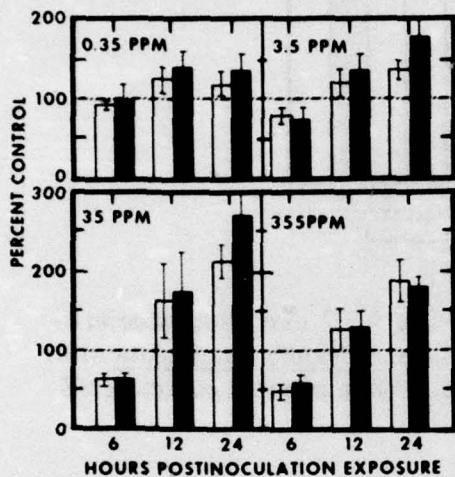


Figure 2. The effect of p,p'-DDT on the uptake and incorporation of [^3H] thymidine into *Crithidia fasciculata*. Mean percent control \pm SE of three replications. □ = uptake; ■ = incorporation.

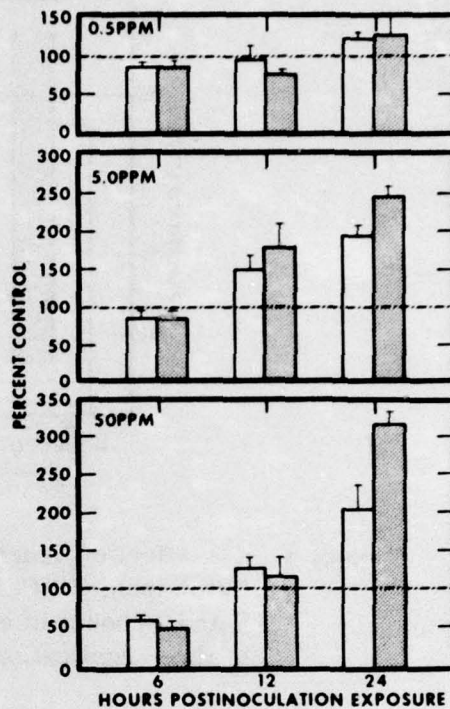


Figure 3. The effect of Aroclor 1254 on the uptake and incorporation of [^3H] thymidine into *Crithidia fasciculata*. Mean percent control \pm SE of three replications. □ = uptake; ▨ = incorporation.

Uptake of [^3H] L-leucine was increased (not significantly $p > 0.05$) at 12 and 24 hours exposure to all levels of DDT tested, except after 24 hours exposure to the greatest dose (355 ppm; Figure 6). Incorporation of [^3H] L-leucine after DDT exposure remained at control levels at all doses and times tested, except for inhibition after 6 and 24 hours exposure at the greatest dose tested (355 ppm). PCB exhibited no inhibitory effects on [^3H] L-leucine uptake or incorporation at any dose level (Figure 7). Both uptake and incorporation were increased after 6 hours exposure to the lowest PCB level (0.5 ppm), only incorporation was significantly increased at all dose levels after 12 hours exposure, and higher PCB

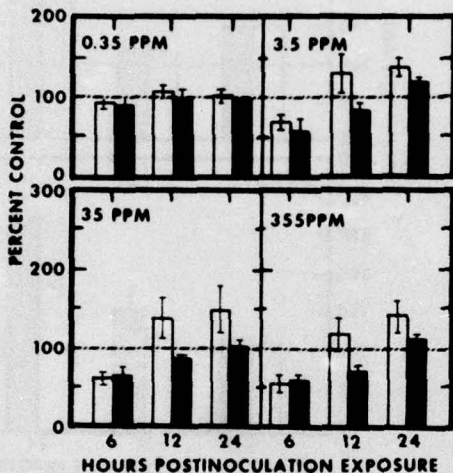


Figure 4. The effect of p,p'-DDT on the uptake and incorporation of [^3H] uridine into *Crithidia fasciculata*. Mean percent control \pm SE of three replications. \square = uptake; \blacksquare = incorporation

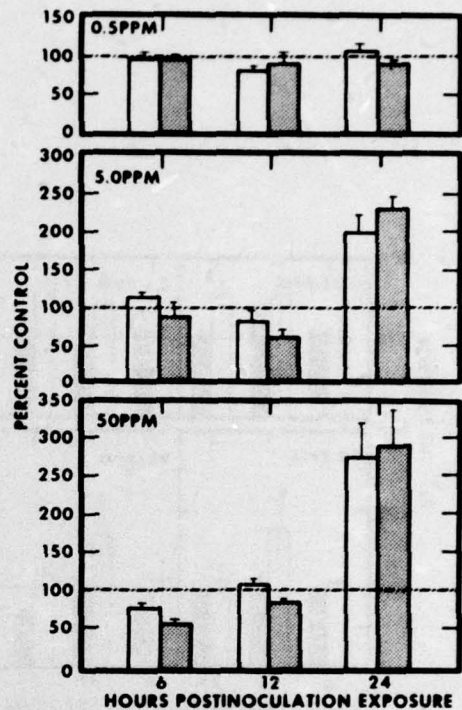


Figure 5. The effect of Aroclor 1254 on the uptake and incorporation of [^3H] uridine into *Crithidia fasciculata*. Mean percent control \pm SE of three replications. \square = uptake; \blacksquare = incorporation.

levels (5.0 and 50 ppm) elicited a twofold to threefold increase of both uptake and incorporation after 24 hours.

Ultrastructure. The fine structure of *C. fasciculata* is distinctly altered after 6 to 12 hours exposure to DDT or PCB. In Figures 8-10, representative micrographs illustrate consistent and characteristic morphological changes to moderate doses of DDT or PCB (35 and 50 ppm, respectively). Control cells grown in the presence of 0.1 percent (v/v) DMSO are characterized by perinuclear swelling and occasional (<10 percent, relative to DMSO-free controls) concentric membrane arrays (CA) in the cytoplasm. The kinetoplast (a specialized

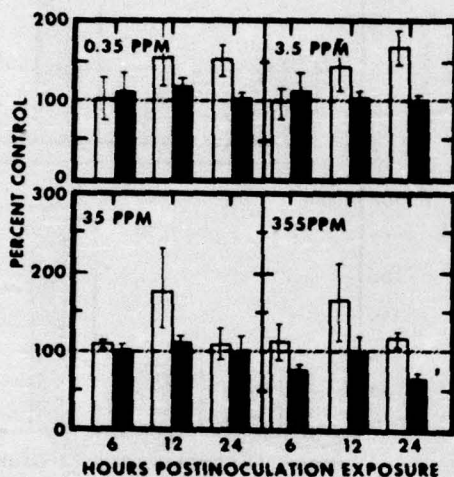


Figure 6. The effect of p,p'-DDT on the uptake and incorporation of [^3H] L-leucine into *Crithidia fasciculata*. Mean percent control \pm SE of three replications. \square = uptake; \blacksquare = incorporation.

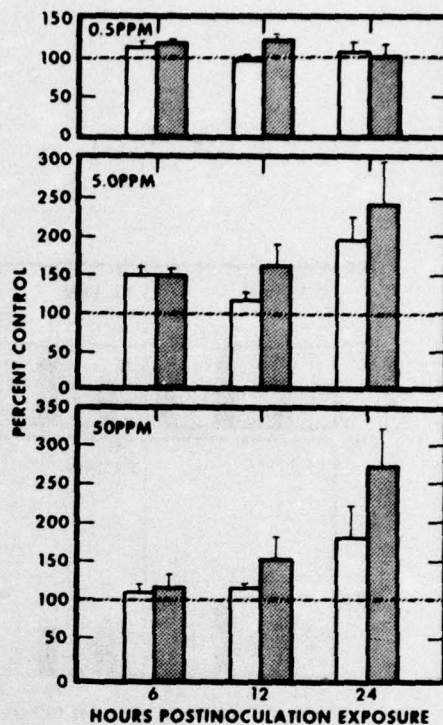


Figure 7. The effect of Aroclor 1254 on the uptake and incorporation of [^3H] L-leucine into *Crithidia fasciculata*. Mean percent control \pm SE of three replications. \square = uptake; \blacksquare = incorporation.

portion of the mitochondrion containing genetic material)¹⁴ and associated mitochondrial elements were not altered by this low concentration of DMSO. Elongation of the kinetoplast and genophore (the electron-dense core containing the mDNA) in synchrony with flagellar division appeared normal (Figure 8A, B).

Exposure to DDT (35 ppm) for 6 to 12 hours caused several morphological changes (Figure 9A, B). The genophore of the kinetoplast was disorganized after 6 hours exposure (100 percent of all cells where the kinetoplast was visible in the thin section). Consistently, this disorganization was associated with cells where flagellar division was completed before elongation and division of the kinetoplast. Evacuated areas (E, areas of electron lucency that are not

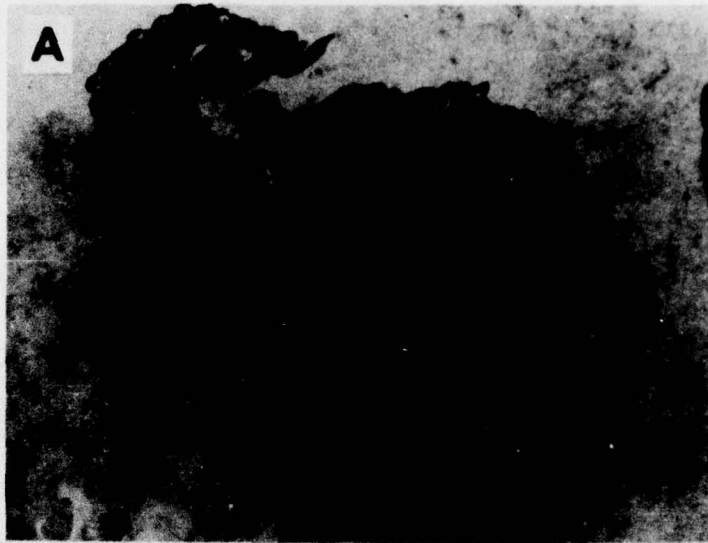


Figure 8. The ultrastructure of *Crithidia fasciculata* grown in DMSO. A. Control (0.1 percent v/v DMSO) after 6 hours exponential growth. Cross section through the midregion of the cell illustrating occasional concentric membrane array. X 16,000. B. Control (0.1 percent v/v DMSO) after 12 hours exponential growth. Tangential section through the anterior region illustrating normal appearing kinetoplast and flagella. Perinuclear swelling is also seen in control cultures. X 44,400. N, nucleus; M, mitochondrion; F, flagella; FP, flagellar pocket; CA, concentric membrane array; K, kinetoplast.



Figure 9. The effect of p,p'-DDT on the ultrastructure of *Crithidia fasciculata*. A. DDT (35 ppm) after 6 hours exposure during exponential growth. A longitudinal section through the anterior region of the cell showing a disrupted kinetoplast and an evacuated area due to intracellular swelling. X 44,400. B. DDT (35 ppm) after 12 hours exposure during exponential growth. A similar section to A illustrating a more pronounced disruption and failure of the kinetoplast to elongate in synchrony with the flagella. X 44,400. N, nucleus; K, kinetoplast; F, flagella; E, evacuated area.

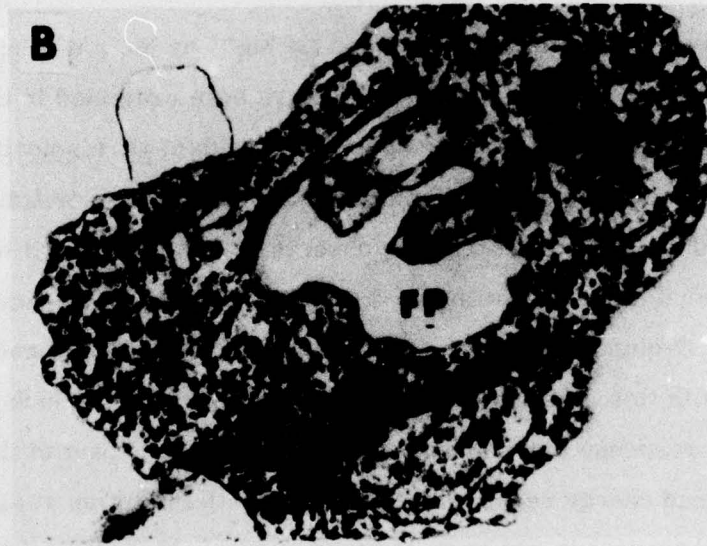
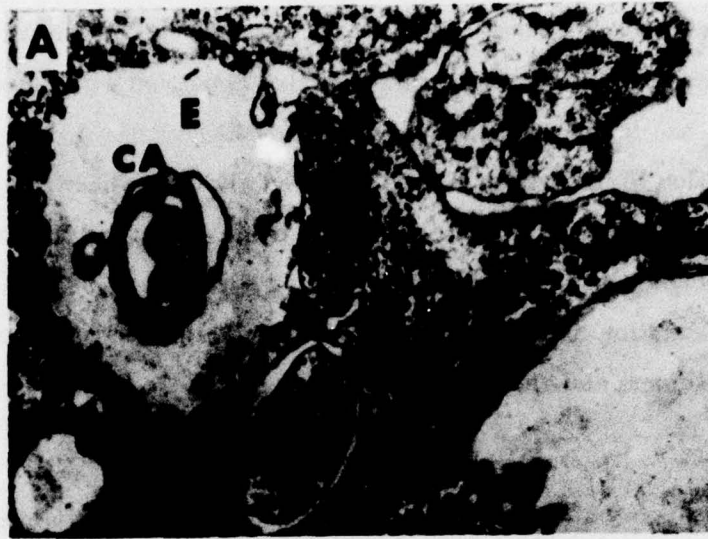


Figure 10. The effect of Aroclor 1254 on the ultrastructure of *Crithidia fasciculata*. A. PCB (50 ppm) after 6 hours exposure during exponential growth. A cross section illustrating characteristic concentric membrane arrays in an evacuated area. X 26,600. B. PCB (50 ppm) after 12 hours exposure during exponential growth. A tangential section through the anterior region of the cell showing deterioration and failure of the kinetoplast to elongate in synchrony with the flagella prior to cell division. A characteristic swelling of the mitochondrion is also seen in this section. X 28,000. M, mitochondrion; K, kinetoplast; F, flagella; FP, flagellar pocket; E, evacuated area; CA, concentric membrane array.

membrane bound) were frequently seen (56 percent) in cells at all doses and times examined after DDT exposure. CA also appeared with greater frequency (22 percent) than in controls exposed only to DMSO.

In addition to finding these DDT-induced alterations, PCB-induced morphological changes were characterized also by an increased incidence (49 percent) of mitochondrial swelling after 12 hours exposure to 50 ppm (Figure 10A, B). No condensation or contraction of the inner mitochondrial membrane was seen at these doses and exposure times.

DISCUSSION

Cellular metabolism. Previous reports have indicated several possible sites of direct or indirect DDT and/or PCB induced subcellular lesions. These possible sites are (1) energy metabolism,^{1, 7, 8, 10, 22, 23, 26, 29} (2) protein and nucleic acid synthesis,^{3, 4, 15, 30} and (3) Mg⁺⁺ or Na⁺, K⁺-dependent ATPases.^{5, 13, 17, 32} The first two sites have been examined in this study in order to explain inhibition of cell population growth of *C. fasciculata*.⁶

Energy metabolism. No significant effects with dose or time (up to 12 hours) of DDT on CO₂ evolution were observed. However, PCB inhibited CO₂ evolution after 6 hours and stimulated it after 24 hours. The evolution of ¹⁴CO₂ from [U¹⁴C] D-glucose involves a number of pathways and the contribution of each pathway in this organism is not known. Therefore, the lack of a marked dose-related response could mean: (1) inhibition of only part of the total system, or (2) a reduced energy requirement due to growth inhibition at another lesion site. The subsequent increase at 24 hours suggests that alteration of cellular energy requirements may be an explanation. Since the DDT effects at 24 hours were not determined, it is not known if the energy requirement was altered at this time. Also, it is important to note that the technique we used measured the activity from intact cells and not from subcellular mitochondrial preparations, which show DDT and PCB inhibitory effects on energy metabolism^{1, 7, 8, 22, 23, 26}

but do not include possible transport and extramitochondrial effects of these agents on energy metabolism.

Nucleic acid and protein metabolism. Previous studies^{3, 4, 15, 30} have not attempted to distinguish between uptake and incorporation of radioprecursors of nucleic acids and of protein as indices of inhibition at the membrane level and of macromolecular synthesis mechanisms, respectively. At least two sites for protein synthesis and three sites for nucleic acids must be considered in lesion induction: (1) uptake, possible inhibition of ATPases which could result in the upset of ionic balance and transport processes, and alteration of carrier molecules for different protein or nucleic acid substrates; (2) polymerases, inhibition or interruption of replication, transcriptional and/or translational processes in protein and nucleic acid synthesis; and (3) kinases, inhibition of nucleotide formation for synthesis of nucleic acids.

The method utilized in this study provides an initial attempt to distinguish between effects on uptake or incorporation mechanisms. Because of the size fluctuation of the soluble precursor (cytoplasmic) pool in response to synthesis rates and due to the inherent difficulty in pulse label experiments of this type, definitive explanations are not possible from these data. However, some meaningful observations can be made.

DDT and dieldrin exposure for 24 hours reportedly inhibited L-leucine, uridine, and thymidine incorporation at 5-10 ppm, but stimulated uridine incorporation at 50 ppm in HeLa cells.³ Other studies with DDT or dieldrin doses in this range with HeLa cells¹⁵ or Ehrlich ascites carcinoma cells³⁰ indicate precursor incorporation is still inhibited after 48-hour exposures. Due to the longer generation times of these mammalian cell lines, the change from inhibition to stimulation may not occur until stationary growth phase is approached. It is not possible to state that the C. fasciculata response differs from cultured mammalian cells because a time course analysis over the population growth cycle, to our knowledge, has not been described in the literature for mammalian cells.

The inhibition after 6 hours of DDT and/or PCB exposure of CO₂ evolution, uptake and/or incorporation of thymidine and uridine correlates strongly with the time of the greatest concentration of DDT within the cellular compartment.⁶ If it is assumed that PCB partitioning kinetics are similar to those of DDT, the metabolic effects observed may change as the redistribution of DDT or PCB occurs in the cell population with time. After this transient inhibition, these cells apparently lose the ability to regulate uptake and/or incorporation of nucleic acid or protein precursors. If the lesion(s) causing these phenomena was repaired or reduced by cell redistribution of DDT or PCB due to cell division, uptake and incorporation values would be expected to return to control levels, and not to increase two to three times in magnitude, as observed. Although these results could be influenced by excess substrates and cell crowding, these experiments were designed to minimize such collateral effects.

Recently Roubal²⁵ has described EPR-spin labeling experiments that indicate plasma membrane perturbations caused by DDT and PCB exposure. These data indicate that DDT and PCB intercalate in the membrane, resulting in membrane rigidity. Disruption of membrane geometry could explain the multiple effects exhibited during DDT and PCB exposure, if transport mechanisms were altered and resulted in a pyramidal chain reaction of alterations in cellular functions.

Ultrastructure. The characteristic fine structure changes after DDT or PCB exposure include the disorganization of the kinetoplast, mitochondrial and cellular swelling, and formation of concentric membrane arrays (CA). Acriflavin, an inhibitor of DNA synthesis, induces similar effects in the kinetoplast as observed in this investigation.^{2,14} CA have been reported in DDT and PCB treated rat hepatocytes^{11, 12, 21} and appear to be a consistent cellular response to chemical injury. Cellular swelling also suggests hyperosmotic effects due to the loss of cell regulatory capacity at the membrane level. Failure of the gonophore and kinetoplast to elongate in synchrony with the flagella in preparation for cell division also suggests possible inhibition of mitochondrial DNA synthesis.

Inhibition of mitochondrial DNA synthesis, whether by effect on precursor transport or directly on the biosynthesis mechanisms, would inhibit cell population growth.

In summary, we have shown that the cell population growth inhibition of C. fasciculata by DDT or PCB is initially due to inhibition of uptake and/or incorporation of thymidine and uridine. This transient inhibition is followed by a loss of cell regulatory capacity and continued cell population growth inhibition. Further investigation into the specific nature of the lesion(s) induced by DDT or PCB in C. fasciculata will be required. DDT or PCB effects on transport kinetics of nutrients, effects on cyclic AMP and Mg^{++} and Na^+ , K^+ -dependent ATPases should all be examined. The loss of cell regulatory control as demonstrated in this study has important consequences for homeostatic mechanisms of organisms adjusting to rapidly changing environments.

The sensitivity of C. fasciculata to low levels of DDT or PCB, the magnitude of the biological response, and facility of culture make it an appropriate cell model for screening toxic substances and for studying the mechanisms of xenobiotic effects on eukaryotic cells.

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