Bull. Environm. Contam. Toxicol. 29, 68–75 (1982)

Uptake and Toxicity of Toxaphene to Cell Cultures Derived from Goldfish (*Carassius auratus*)

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Until recently toxaphene had been the most widely used organochlorine insecticide in the United States (CASIDA et al. 1974). Numerous studies have documented the poisoning of fish by relatively low environmental concentrations of toxaphene (WORKMAN & NEUHOLD 1963, HEMPHILL 1954, LAWRENCE 1950, DOUDOROFF et al. 1953, HOOPER & GRZENDA 1957). This phenomenon is based both on the biological stability of toxaphene (WEBER & ROSENBERG 1980, EPA TOXAPHENE STATUS REPORT 1971) and the ability of fish to substantially concentrate the insecticide from the surrounding water. Goldfish, for example, have been shown to attain a tissue concentration of toxaphene of 200-fold over that of the environment (HUNT & KEITH 1960). In comparative tests of the relative toxicity of insecticides to representative members of common fish families, goldfish as a species were consistently the least susceptible (MACEK & McALLISTER 1970).

As a preliminary investigation of a general study, the uptake and toxicity of toxaphene to cells derived from goldfish were examined.

MATERIALS AND METHODS

Toxaphene: stock solutions of toxaphene (obtained from Hercules Corp., Delaware) were prepared in 100% ethanol at 0.2g/mL and serially diluted with medium to final concentrations of 200, 100, 50, 45, 40, 35, 30, 25, 20, 15, 10, and 1 parts per million (ppm). These dilutions resulted in a maximum of 0.1% ethanol (in the 200ppm-treated cultures, with further dilutions thereafter); incorporation of 0.1% or less ethanol into culture medium had no effect as measured by the parameters utilized in this study.

Medium: culture medium consisted of Medium 199 supplemented with 10% fetal bovine serum and 100 units of penicillin and streptomycin per mL and 0.25mcg of Fungizone per 100 mL (Gibco).

Cells: Car cells (ATCC CCL-71), originally derived from goldfish fin, were obtained from the American Type Culture Collection. Cells used in these studies were between the 40th and 60th passage from repository stocks. Cells were incubated at 25°C.

Acute toxicity studies: cells were planted in 24-well trays (Linbro) at a density of 9×10^4/well. Twenty-four hr later, medium was decanted and cultures received medium containing toxaphene at the concentrations listed above, with 4 replicate cultures per concentration. At 24hr intervals for 10 days following addition of toxaphene, cultures were harvested and examined for cytopatho-

0007-4861/82/0029-0068 $01.60

genic effect (CPE), number of cells per culture, total protein per culture, and pH of culture medium. CPE of all 4 replicate cultures was judged by cytoplasmic granulation, rounding of cells, and detachment from the culture well, and scored according to the following scheme:

+/-	less than 10% of cells exhibiting granulation
1+	mild overall granulation, 10-25% of cells rounding, less than 10% detachment
2+	50-75% accumulation of granules (numerous granules crowding cytoplasm), 25-50% rounding, 10-25% detachment
3+	90-100% accumulation of granules, 50-75% rounding, 25-50% detachment
4+	greater than 75% detachment, remainder of cells rounding.

Medium was aspirated from replicate cultures, pooled, and the average pH determined. Two cultures were trypsinized and the number of cells counted in duplicate for each. Total protein per culture was determined by duplicate readings of the two remaining cultures according to the method of OYAMA & EAGLE (1956). All above tests were repeated on three separate occasions.

Determination of TD50: the dosage of toxaphene which resulted in a 50% inhibition of treated cultures versus controls (TD50) was determined by dividing the mean of 10 treated cultures by the mean of 16 controls for both numbers of cells and total protein per culture, and the average CPE scored for 10 cultures. Cultures were assayed at 48hr post addition of toxaphene at concentrations from 1-50ppm as described above.

Uptake of toxaphene by CAR cells: cells were planted in 25cm^2 flasks (Corning) at a density equivalent (7x) to that used in the acute toxicity test. To approximate the toxaphene concentration used in the 10ppm acute toxicity test, a total of 70ppm was introduced into the flasks at time 0 in 5mL medium without serum. Cultures were examined for CPE at 0, 2, 16, 24, 36, 48, and 60hrs post addition of toxaphene and harvested at these times for assay of toxaphene as follows: medium was decanted, monolayers rinsed with 5mL Hank's Balanced Salt Solution (HBSS), with medium and rinse then combined as a single sample; cells were removed with 1 mL trypsin and aspirated, and the flask was rinsed with 9mL HBSS, with cells and rinse combined; the flask was retained for assay. The above three samples (defined as medium, cells, and flask, respectively) were assayed for toxaphene by adding 1mL hexane, agitating vigorously, decanting the hexane phase and performing thin-layer chromatography according to the method of WEBER & ROSENBERG (1980). As medium containing serum resulted in a sudsy emulsion upon shaking after hexane introduction, with no extractable hexane phase, medium without serum was employed. In a separate experiment, medium containing serum was utilized; extraction of the cell and flask samples revealed toxaphene chromatographs identical to experiments without serum, and cultures exhibited identical CPE to those containg serum. In these experiments CPE was scored simply as the percentage of cells detached from the vessel: 1+, 25% or less; 2+, 25-50%; 3+, 50-75%; 4+, 75% or more detached.

Intracellular localization of toxaphene: this was performed by a modification of the method of MURAKAMI & FUKAMI (1979). 24hr-old cultures in 75cm^2 flasks (Corning) received 10mL of medium containing 10ppm toxaphene per mL. 48hr post addition of toxaphene, medium was decanted and cultures rinsed with 10mL HBSS. Cells were fractionated into acid-soluble, alcohol-soluble, and acid- and alcohol-insoluble fractions. The HBSS and cell fractions were assayed for the presence of toxaphene by thin-layer chromatography. Cultures showed no CPE at the time of harvest.

Chronic toxicity test: cells were planted in 150cm^2 flasks (Corning) at a density of 2x10^6/flask. 24hr later, medium was replaced with 20mL of medium containing 0, 5, 10, or 25ppm toxaphene per mL in duplicate. At 10 day intervals for a maximum of 100 days thereafter, cultures were subcultured, with the above treatment repeated. At each subculturing, cells from each concentration were planted in 24-well trays and exposed to 0, 10, 15, 25, 35 or 50ppm toxaphene in a manner identical to the acute toxicity test (above), with 4 replicate cultures examined at each acute concentration from each chronic population. In experiments involving increase of chronic dosage, surviving cells of the acute toxicity test were repassaged in 24-well trays at their respective acute concentrations.

RESULTS AND DISCUSSION

A representative dose-response curve of CAR cells to toxaphene, as measured by numbers of cells per culture, is presented in Fig. 1. In the absence of toxaphene, cell numbers showed slightly less than a doubling within 2 days and essentially leveled off by 7 days. Presence of 1ppm gave results virtually equal to control cultures. On day 2, 10ppm, 15ppm, and 20ppm were essentially equal to control levels; by day 7, 10ppm showed less inhibition than 15 and 20ppm, which were equally inhibited. Cultures treated with 25ppm showed limited initial growth followed by a decrease. Cultures treated with 30 and 35ppm did not increase in numbers, with 35ppm-treated cultures exhibiting a net loss of cells by day 10. There was a shrp demarcation between cultures treated with 35ppm as compared to 40ppm. 40ppm-treated cultures exhibited a decrease in cells by day 2, with continued decrease until termination of the experiment. Concentrations above 40ppm resulted in more abrupt decreases in cell numbers.

The results obtained for total protein per culture (Fig. 2) demonstrated essentially identical relationships as did those obtained for numbers of cells.

Examination of cultures for toxaphene-induced CPE gave results consistent with the above two assays (Table 1). Cultures treated with 1ppm were indistinguishable from controls. 10ppm induced minor but detectable effects. Cultures treated with 15 to 35ppm showed progressively increasing CPE, but with some cells apparently unaffected. 40ppm and above resulted in destruction of the cultures by day 7 or sooner.

A determination of the TD50 by the above three methods using a larger sample size is presented in Fig. 3. Based on all three methods, the TD50 at 48hrs of toxaphene to CAR cells lies between 30 and 35ppm. Several reports have documented LD100, LD50 and

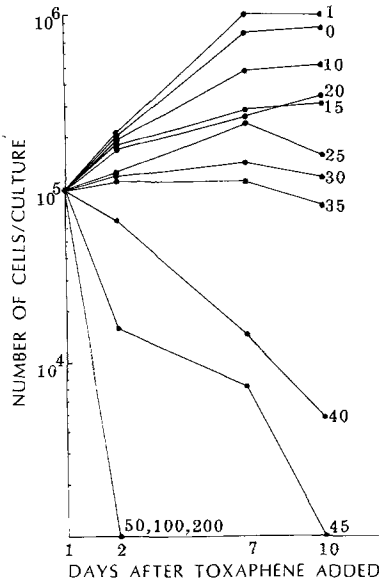

Fig. 1

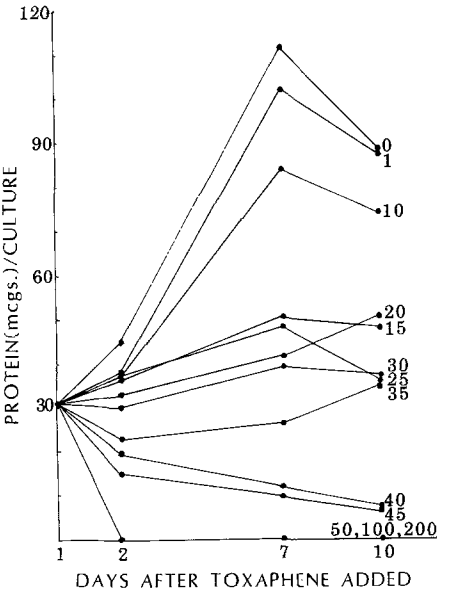

Fig. 2

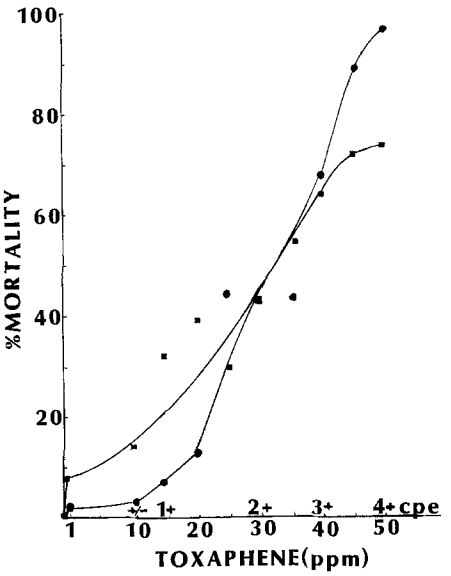

Fig. 3

Fig.1: Dose-response of CAR cells to toxaphene measured by cell numbers. Curves are labelled with ppm toxaphene.

Fig.2: Dose-response of CAR cells to toxaphene measured by total protein.

Fig.3: Percent mortality of CAR cells exposed to varying amounts of toxaphene. ●,cell numbers; ■,total protein. CPE annotated along base.

TABLE 1: Dose-resonse of CAR Cultures to Toxaphene as Measured by CPE

Toxaphene (ppm)	Cytopathogenic Effect		
	Day 2	Day 7	Day 10
0	-	-	-
1	-	-	-
10	+/-	+/-	+/-
15	1+	1+	1+
20	1+	1+	1+
25	1+	2+	2+
30	2+	2+	2+
35	2+	2+	3+
40	3+	4+	4+
45	3+	4+	4+
50	3+	4+	4+
100	4+	4+	4+
200	4+	4+	4+

maximum tolerated dosages of toxaphene to intact goldfish (MACEK & McALLISTER 1970, WORKMAN & NEUHOLD 1963, HEMPHILL 1954, HOOPER & GRZENDA 1957, MEHRLE & MAYER 1975a,b). In these in vivo studies, the LD50 was at a level less than 1ppm, with the exception of MACEK & McALLISTER (1970), who determined a 96hr LD50 of 7ppm. Under the conditions used in the present study, CAR cells are clearly less sensitive to toxaphene than is the intact organism from which they were derived.

Table 2 presents pH values of medium from cultures treated with toxaphene. pH of medium from control cultures and cultures exposed to 1ppm remains virtually unchanged. A progressive decrease in final pH was observed from 10ppm to 35ppm. Further increase in toxaphene resulted in progressive loss of this effect and eventual elevation of pH beyond control levels. Medium containing 0 or 30ppm toxaphene was deposited into wells without cells and assayed; pH values obtained were virtaully identical, and both were higher than values for control cultures. Thus presence of the compound alone is not responsible for the differential pH values. Correlation of the hyperbolic nature of the pH levels with numbers of cells per culture suggests an interaction of cells with insecticide, possibly metabolic, with 30-35ppm as the optimal concentration for this interaction. These are the concentrations which are essentailly static for culture growth (Fig.1,2; Table 1). Organochloride insecticides have been shown to alter steroid metabolim and to induce increased ascorbic acid production in vivo (CONNEY et al. 1967). Biochemical examinations of the mechanism(s) responsible for the alteration of pH will be conducted.

The uptake of toxaphene by CAR cells is shown in Fig. 4. Virtually all of the toxaphene introduced into the medium at time 0 had localized in the cells within 2hrs and remained there until 60hrs post addition. At 60hrs, lysis of approximately 50% of the culture ensued; toxaphene was approximately equally distributed throughout the culture system (medium, cells and flask) at this point. Focal areas cleared of cells were observed upon lysis, and toxaphene released from lysed cells probably adsorbed to the now-exposed plas-

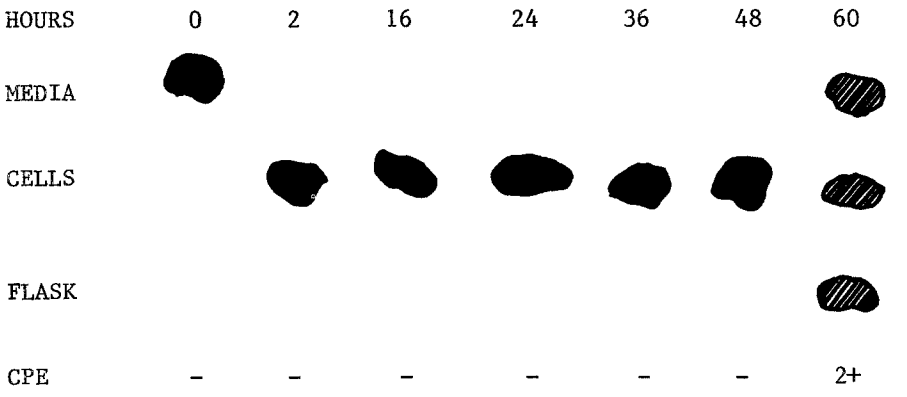

FIG. 4: Cryptogram of Localization of Toxaphene in CAR Cultures as Shown by TLC

HBSS PERCHLORIC ACID ETHANOL FORMIC ACID

FIG. 5: Cryptogram of Intracellular Localization of Toxaphene as Shown by TLC

TABLE 2: pH of Media of CAR Cultures Exposed to Toxaphene

Toxaphene (ppm)	pH Day 2	Day 7	Day 10	difference from control on Day 10
C	7.00	7.02	7.05	-----
1	7.09	7.03	7.07	+0.02
10	7.05	6.69	6.56	-0.49
15	6.43	6.40	6.35	-0.71
20	6.52	6.39	6.35	-0.71
25	6.75	6.37	6.36	-0.70
30	6.73	6.29	6.31	-0.69
35	6.62	6.28	6.32	-0.74
40	6.89	6.61	6.54	-0.51
45	6.87	6.53	6.77	-0.28
50	7.11	7.00	7.06	+0.01
100	7.28	7.50	7.55	+0.50
200	7.47	7.55	7.55	+0.50
medium(w/o cells)	7.41	7.23	7.25	+0.20
medium w 30ppm (w/o cells)	7.31	7.29	7.30	+0.25

tic surface. The solubility of toxaphene in water is approximately 0.4ppm (COHEN et al. 1960). It is not expected that the solubility would be drastically altered in our culture medium. Solutions of 10ppm and above resulted in increasingly turbid suspensions in which material would settle out over time. However, mild agitation easily suspended this material, and the amount of toxaphene extractable from medium in the absence of cells remained constant over at least 60hrs at 25°C.

To test whether toxaphene was simply non-specifically adsorbing to the surface of the cells, subcellular fractionation of the cells was performed at 48hrs post addition of toxaphene. Fig. 5 shows exclusive localization of toxaphene in the alcohol-soluble fraction. It is entirely possible, however, that toxaphene first non-specifically adsorbed to the cell surface and was subsequently internalized.

Stock cultures were chronically exposed to 0, 5, 10, 25ppm with subculturing and retreatment with respective levels of toxaphene at 10 day intervals. Cultures chronically treated with 25ppm were lost on the 2nd passage; growth had ceased and progressive detachment was observed. Cultures chronically treated with 10ppm were lost in similar fashion on the 3rd passage. Cultures chronically treated with 5ppm exhibited mild granulation but growth was comparable to controls. Acute toxicity tests of cells chronically exposed to 5ppm showed these cells to have increased susceptibility to toxaphene versus controls (Table 3).

TABLE 3: Representative Acute Toxicity Test of Cells Chronically Exposed to 5ppm Toxaphene

Chronic Exposure	Day	Toxaphene (ppm)					
		0	10	15	25	35	50
5ppm-treated	2	+/-	1+	1+	2+	3+	4+
	7	+/-	1+	2+	3+	4+	4+
	10	-	1+	2+	3+	4+	4+
0ppm (control)	2	-	+/-	+/-	1+	2+	3+
	7	-	+/-	1+	2+	3+	4+
	10	-	+/-	1+	2+	3+	4+

Cells were chronically exposed to 5ppm for 100 days with no further detectable changes. All attempts to increase chronic dosage resulted in destruction of the cultures. Based on the localization of toxaphene within cells (above), accumulation and (at least temporary) intracellular retention of sequential dosages could account for the increased sensitivity observed with chronically treated cells.

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Accepted May 12, 1982