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EFFECTS OF BUTACHLOR, PARAQUAT, SATURN AND AZODRIN ON THE KILLING AND BIOCHEMISTRY OF CELL LINES FROM TILAPIA, LOACH AND TOP MINNOW

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H. C. Wang and F. H. Yew (1987) Effects of butachlor, paraquat, saturn and azodrin on the killing and biochemistry of cell lines from tilapia, loach and top minnow. *Bull. Inst. Zool., Academia Sinica* **26**(1): 95-106. Butachlor, Paraquat, Saturn and Azodrin are widely used in agriculture for weeds and pests control. The toxicities of these drugs were assessed by established cell lines from warm water fish tilapia, loach and top-minnow. The sensitivities of the cell lines towards the drugs varied extensively; from null effects of Azodrin to extreme sensitivity of Butachlor on topmimmow cells. The differential responses of the cell lines towards the drugs revealed the high tolerant level of tilapia cells, which might support the finding that population of the species increased even in polluted environment. To use cell lines for toxicity test is a quicker and more sensitive method. It also provides information on biochemical actions of the drugs at cellular levels.

Herbicides and insecticides as potential mutagens and carcinogens have alerted the general public. Workers in factories and farms in contact with the drugs were reported to have higher rates of sister chromatid exchange and more incidences of neoplastic growth (Yoder *et al.*, 1973; Axelson and Sundell, 1974). Experiments with live animals have been conducted with fish in either LC50 measurement and embryo developement (Wang, 1984). The results were definitive and sensitive but the experiments were time comsuming and difficult to keep the conditions constant. Toxicity test using cultured mammalian cells has been applied in assessing mutagenic and carcinogenic potential of a great number of chemicals (Ahmed *et al.*, 1977). Short term screening tests for carcinogens using perment cell lines has also been reported (Bridges, 1976). Recently, three cell lines from organs of warm water fish, loach fin (Chen, 1983), tilapia kidney (Chen *et al.*, 1983) and top-minnow (Wang, 1985), have been developed in this institute. We have chosen these cell lines for drug experiments because the existing animal test showed considerable difference in response. In this study we tried to assess the biochemical changes exerted on the cells by the drugs and their potential hazard towards human beings.

MATERIALS AND METHODS

Cell lines

Tilapia kidney cell line (TK) was developed from the hybrid of *Sarotherdon mossambicus* and *Sarotherodon niloticus* (Chen *et al.*, 1983). Loach fin cell line (LF) originated

from *Misgunus anguillicandatus* (Chen, 1983). Top minnow cell line (TM) was developed from tissue homogenate of *Gambusia patruelis* (Wang, 1985). Three cell lines were all cultured in Lebovitz medium 15 (Flow Laboratory, USA), supplemented with 10% fetal calf serum (Flow Laboratory, USA) together with antibiotics (50 I. U. penicillin and 50 μg/ml streptomycin). The optimum temperature for the growth was 31°C. Cells were subcultured within two or three days; TK and LF were divided in 1:4 and TM in 1:2.

Chemicals

Azodrin alias Monocroptophos, or Nuvacron (dimethylophate of 3-hydroxy-*N*-methyl-cis-crotonamide, 77% purity) was produced by Shen Hong Co., Taiwan. Butachlor alias Machat (2-chloro-2, 6-dimethyl-*N*-(butoxymethyl)-acetanilide, 90% purity) were purchased from Monsanto Co., USA. Saturn alias Benthiocarb (*S*-(4-chlorobenzyl) *N*, *N*-dimethylthiocarbamate, 94.57%) was obtained from Kumiai Co., Japan. Paraquat alias Cramoxoe (1, 1′-dimethyl-4, 4′-bipyridylium dichloride) was recrystallized from a 24% product from Chia Tai Co., Taiwan. All four chemicals were prepared as 50 mM solution, (Azodrin and Paraquat in water, Butachlor and Saturn in dimethylsulfoxide) swinnexed and stored at 4°C. All other chemicals were from Sigma.

Toxicity test

The toxicity test was performed according to Fernandez-Pol *et al.* (1982). The cells in monolayer were washed with phosphate buffer saline (PBS) followed by 0.1% trypsin in PBS. The cells came off the flask on second trysin treatment, and were diluted immediately with medium to appropriate cell concentrations. The cells were again seeded in 25 cm^2 culture flask, incubated for 24 hours to let them attach firmly to the flask. Drugs were added into the flasks at various concentrations. For Butachlor and Saturn DMSO not over 0.4% v/v were added to the control group. For the following three days after treatment the flasks were shaken gently to detach dead cells, and the attached cells were washed with PBS, trysinized and cell numbers were counted with a haemocytometer. The cells collected were over 98% living according to trypan blue test.

Determination of DNA, RNA and protein synthesis

To monitor the number of living cells ^{14}C-thymidine (0.02 μCi/ml, 50 mCi/m mole; ICN Chemical and Radioisotope, USA) were added to the culture flask and incubated for 48 hours. The cells were then trysinized and reseeded into a 24-well culture plate in fresh media for 12 hours. The media in each well was replaced with various concentrations of drugs in culture medium and treated for one hour. The cells were washed twice with PBS and resupplied with fresh medium. After 0, 0.5, 1, 1.5, 2 and 3 hours ^{3}H-thymidine (10 μCi/ml; 70 Ci/m mole; ICN, USA) was added to pulse label the DNA for 10 min and were washed again with cold PBS. The cells were then lysed with 0.5 ml of alkaline solution (0.3 M NaOH, 0.5 M NaCl, 5% sucrose) for 5 min. A drop of 50% trichloroacetic acid was added to each well to precipitate the DNA. The samples were left in the cold overnight and the precipitates were filtered with glass fibre filter (Whatman GF/C, 2.4 cm). The filters were dried and immersed in the scintillation fluid (4 g PPO, 0.1 g POPOP in 1 litre toluene) and the radioactivities were counted in a LKB 1217 Rackbeta scintillation counter. The ratio of ^{3}H and ^{14}C counts of the control was taken as 100% rate of DNA synthesis.

The rate of RNA synthesis was measured according to a similar protocol as that of DNA synthesis. A pulse label of ^{3}H-uridine (1 μCi/ml, 39 Ci/m mole, ICN, USA) was given to the cells at various times after drug treatment. Prior to the pulse label the cells were given 10 mM hydroxyurea to block the reduction of ribonucleotides into deoxyribonucleotides. Actinomycin D was used as

positive control for the inhibition of RNA synthesis.

The rate of protein synthesis was measured at various intervals after drug treatment similar to that of DNA synthesis. Radioactive ^{3}H-leucine (2 μCi/ml. 120 Ci/m mole) was used. Cycloheximide treatment of cells was taken as positive control of inhibition.

RESULTS

The effect of drugs on cell survival

LF cells (Fig. 1): The cells did not respond to Azodrin (50 μM to 800 μM) after three days exposure (Fig. 1a). Butachlor at 25 μM killed all the cells in one day, at 2.5 μM reduced the surviving population to 30% (Fig. 1b). The toxicity of paraquat was less; the minimal concentration to kill 90% of the cells in two days was 50 μM, however, lower concentrations would kill all the cells in three days (Fig. 1c). Saturn had moderate effect on the survival of LF cells; at 800 μM for three days would reduce the population to 28% (Fig. 1d).

TK cells (Fig. 2): Azodrin from 50 to 800 μM depressed the cell growth slightly on the second day, but recovered on the third day (Fig. 2a). Butachlor at 100 μM completely

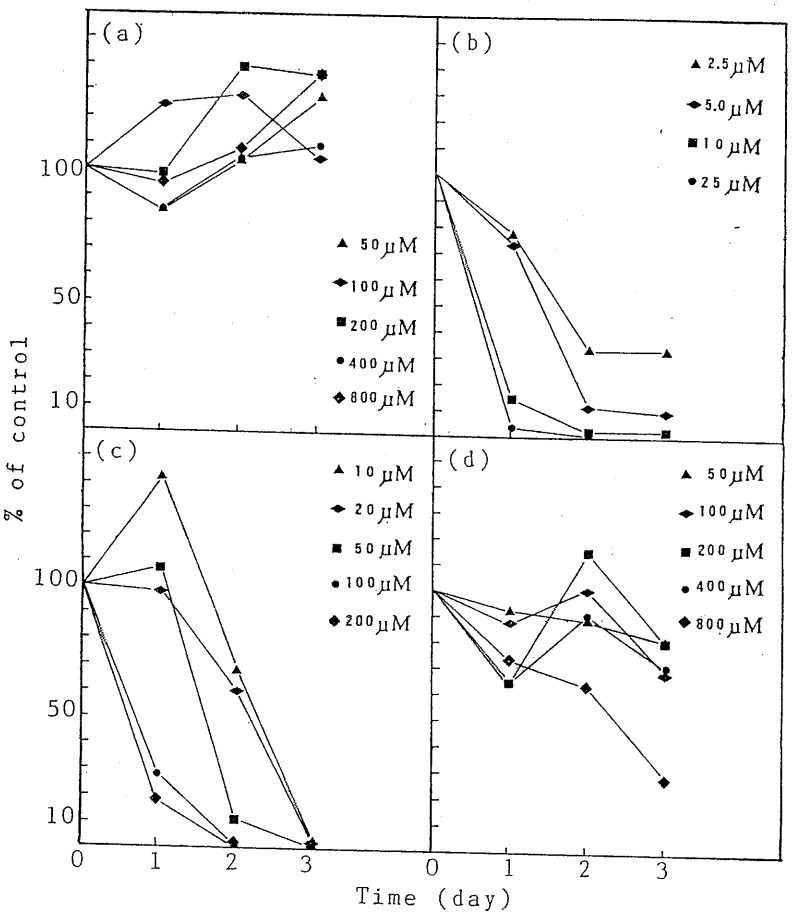

Fig. 1. Dose dependence of growth inhibition in LF cells treated with (a) Azodrin (b) Butachlor (c) Paraquat (d) Saturn. Cells were plated about 2×10^{5} cells/flask; 24 hr later pesticides at the indicated concentrations were added. The control cultures were treated identically but without the drug. Cell counts were determined by haemocytometer at the indicated times. Each data point is the mean of two determinations.

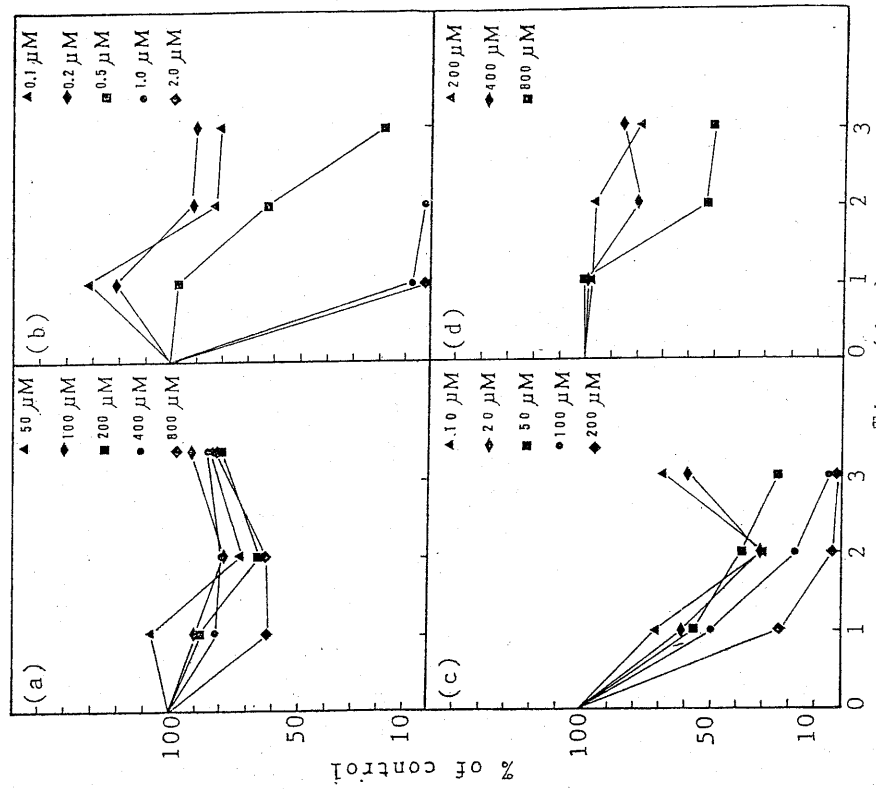

Fig. 2. Dose dependence of growth inhibition in TK cells treated with (a) Azodrin (b) Butachlor (c) Paraquat (d) Saturn. Cells were plated about 2×10^5 cells/flask; 24 hr later pesticides at the indicated concentrations were added. The control cultures were treated identically but without the drug. Cell counts were determined by haemocytometer at the indicated times. Each data point is the mean of two determinations.

Fig. 3. Dose dependence of growth inhibition in TM cells treated with (a) Azodrin (b) Butachlor (c) Paraquat (d) Saturn. Cells were plated about 2×10^5 cells/flask; 24 hr later pesticides at the indicated concentrations were added. The control cultures were treated identically but without the drug. Cell counts were determined by haemocytometer at the indicated times. Each data point is the mean of two determinations.

killed the cells in one day. Yet TK cells seemed to be able to overcome the toxicity at lower concentration of 25 μM; the growth was reduced to 40% after one day but resumed after three days (Fig. 2b). Paraquat from 50 to 800 μM killed the cells in a dose responsive way (Fig. 2c), whereas Saturn in the same concentration range produced a shouldered survival curve at 50 and 100 μM (Fig. 2d), suggesting that the cells had certain level of resistance to the toxicity.

TM cells (Fig. 3): The cells were extremely sensitive to Butachlor (Fig. 3b); at 1.0 μM the cells were completely killed in one day, below 0.5 μM the cells showed capacity to resist the toxicity. The effect of paraquat on TM cells was similar to that on TK cells (Fig. 3c), except at concentrations below 20 μM the cells showed some recovery in growth. Saturn affected the cell growth moderately at high concentration of 400 μM (Fig. 3d) while Azodrin remained ineffective (Fig. 3a).

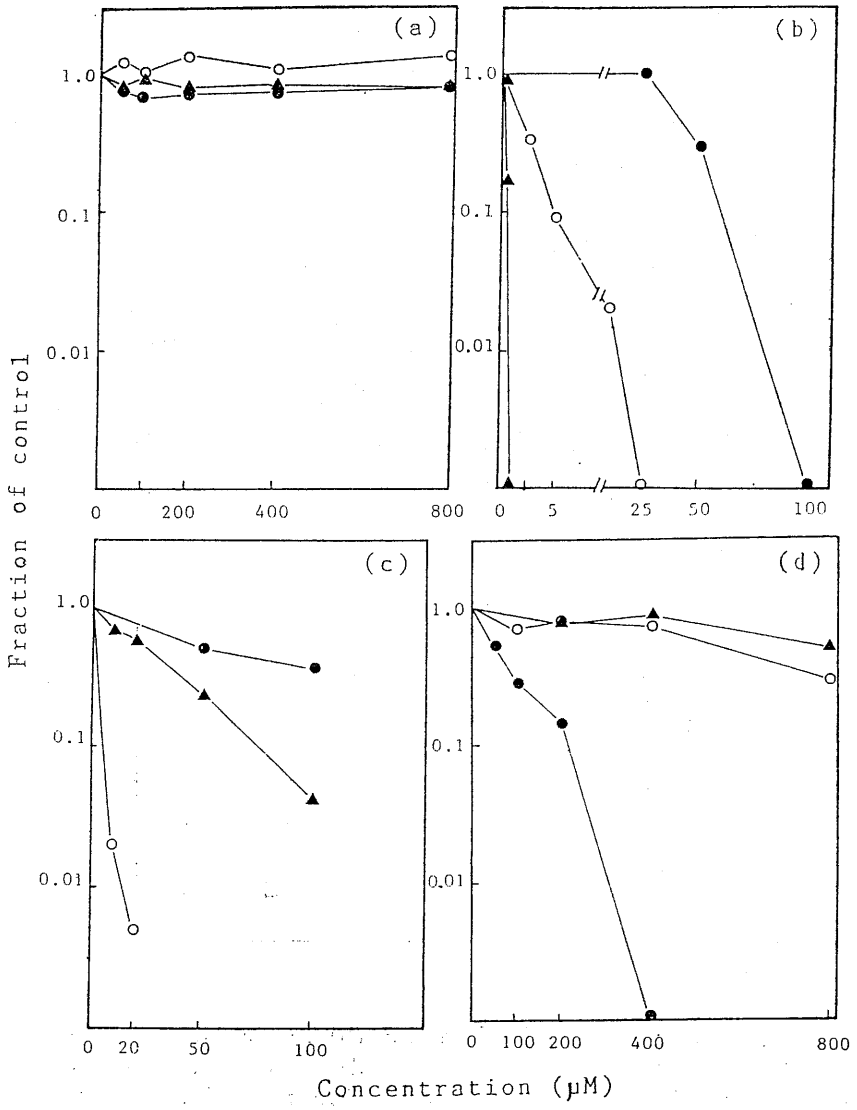

Fig. 4. Cytotoxicities of (a) Azodrin (b) Butachlor (c) Paraquat (d) Saturn to LF (○), TK (●) and TM (▲) cells after three days treament. The experimental conditions are described in the legend to Fig. 1. Cell number of control flask was taken as 1.

To compare the sensitivities of different cell lines against the drugs administered we found that the variability was extensive. To obtain the differential sensitivities of cell lines against the drugs, we plotted the survival population percentage after three-day drug treatment. In Fig. 4a we might conclude that Azodrin had no effect on any of the cell lines. Fig. 4b shows the extremities of the toxicity of Butachlor on cells from different origins. The resistance of TK cells up to 50 μM and the complete extermination of TM cells at 1 μM suggest that the metabolism of Butachlor in the two cell lines must be very different. The toxicities of Paraquat towards TK, LF and TM were in decreasing order (Fig. 4c); again TK cells were most resistant. Saturn only showed significant

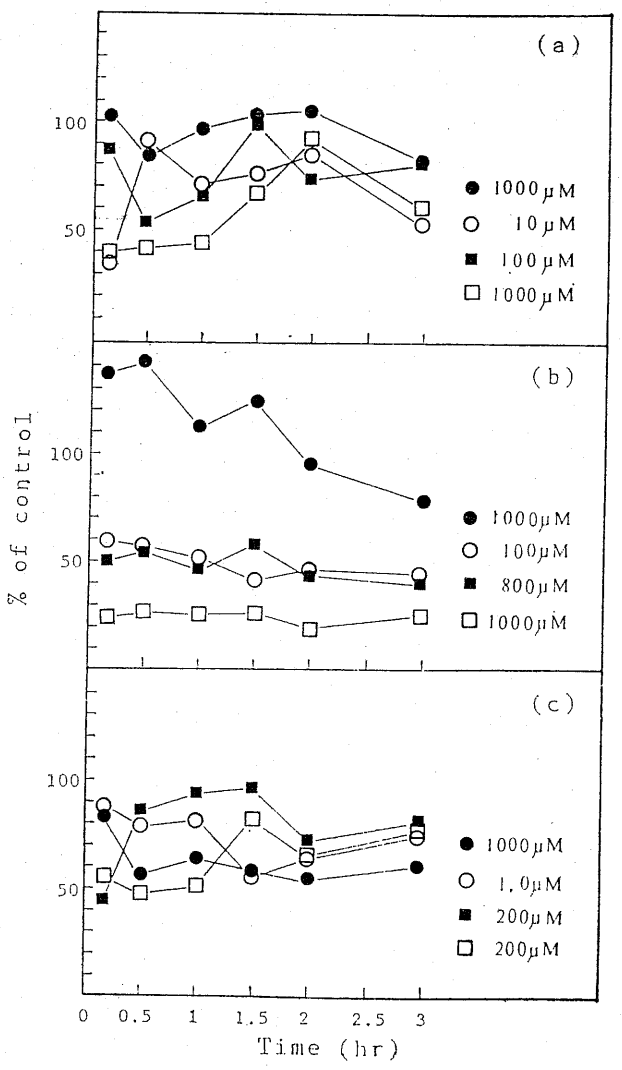

Fig. 5. The rate of DNA synthesis of (a) LF (b) TK (c) TM cells after Azodrin (●) Butachlor (○) Paraquat (■) and Saturn (□) treatment. Cells were generally labeled with ^{14}C-thymidine, then replated into 24-well plate in subconfluence for 12 hr without ^{14}C. Pesticides at the indicated concentrations were treated 1 hr, then 10 min pulse labeled with ^{3}H-thymidine in complete media at 0, 0.5, 1, 1.5, 2 and 3 hr after treatment. The ratio of ^{3}H/^{14}C of control cell were taken as 100%.

toxicity towards TK cells (Fig. 4d), whereas TM and LF cells were only affected at high concentrations of 800 μM.

Rates of DNA synthesis

Drugs which affect DNA replication are potential mutagens, and cells failed to respond to clastogenic challenges by reducing the rate of DNA synthesis may be defective in protective mechanism (Painter and Young, 1980). The effects of the drugs on each cell lines are described as follows:

LF cells (Fig. 5a): Azodrin suppressed DNA synthesis to 70% after 30 min treatment but recovered within an hour. Butachlor had strong initial effects by reducing the rates to 40% but resumed the control rate in 30 min. Paraquat and Saturn both had initial effects then gradually recovered but not to the control level.

TK cells (Fig. 5b): Azodrin when given to the cells seemed to boost the rate of DNA synthesis by 30%; then came down to about 20% below the untreated cells. Butachlor at 100 μM had a persistance effect in reducing the rate below 50% of the control value. The effect of Paraquat was also of persistant suppression. Saturn showed strong suppressive effect at 1000 μM by decreasing the rate to 20%.

TM cells (Fig. 5c): Azodrin at 1000 μM had slight effects, and Butachlor affected the cells in a similar level. Paraquat had little effect on the cells right after treatment, whereas Saturn treated cells recovered their rate of DNA synthesis gradually after the initial depression.

RNA synthesis

The response of the cell lines on the inhibition of RNA synthesis was first tested by treating cells with actinomycin D, 10 μg/ml.

TABLE 1
Inhibition of RNA synthesis after pasticides treatment[a]

Pesticide	Concen-tration (μM)	% of control		
		LF	TK	TM
Azodrin	100	88.8	97.3	66.7
	500	89.9	107.8	69.0
	1000	94.9	100.5	51.2
Butachlor	0.1	ND	ND	61.9
	0.5	ND	ND	52.4
	2.5	98.2	ND	b
	5.0	136.5	ND	ND
	10.0	96.9	ND	ND
	100.0	ND	93.3	ND
	400.0	ND	55.5	ND
Paraquat	10	99.1	ND	ND
	50	94.4	ND	71.0
	100	87.2	88.8	76.2
	200	ND	ND	84.1
	400	ND	98.1	ND
	800	ND	67.2	ND
Saturn	50	ND	ND	104.3
	100	114.4	105.1	76.1
	500	76.6	48.4	b
	1000	83.6	43.4	ND

a. The data based upon 0.5 hr after pesticides treatment.
b. Cell survival was too low to determine.
ND. Not determined.

The inhibition was 50% throughout the three hour incubation period (data not shown). The results of the drug treatments are summarized in Table 1. Among the cell lines TK seemed to be the least affected. Although Saturn made a suppression about 50% at 500 μM and more, the concentration was so high that other side effects were probably involved. TM cells were most affected especially by Butachlor.

Protein synthesis

The inhibition of protein synthesis was first tested with cycloheximide on all cell lines. The levels were reduced to 40% of control as saturation (data not shown). The responses of cell lines towards individual drug treatment were described as follows:

Azodrin (Fig. 6a): Protein synthesis was not affected in all cells.

Butachlor (Fig. 6b): LF cells was most affected, the level of protein synthesis was down to 20% at 100 μM. For TK and TM cells the inhibition was about 30%.

Paraquat (Fig. 6c): LF cells were not affected whereas TK and TM cells were slightly depressed by 10 and 20% respectively.

Saturn (Fig. 6d): LF cells responded most strongly, the inhibition was about 70%. TM cells and TK cells less sensitive; for

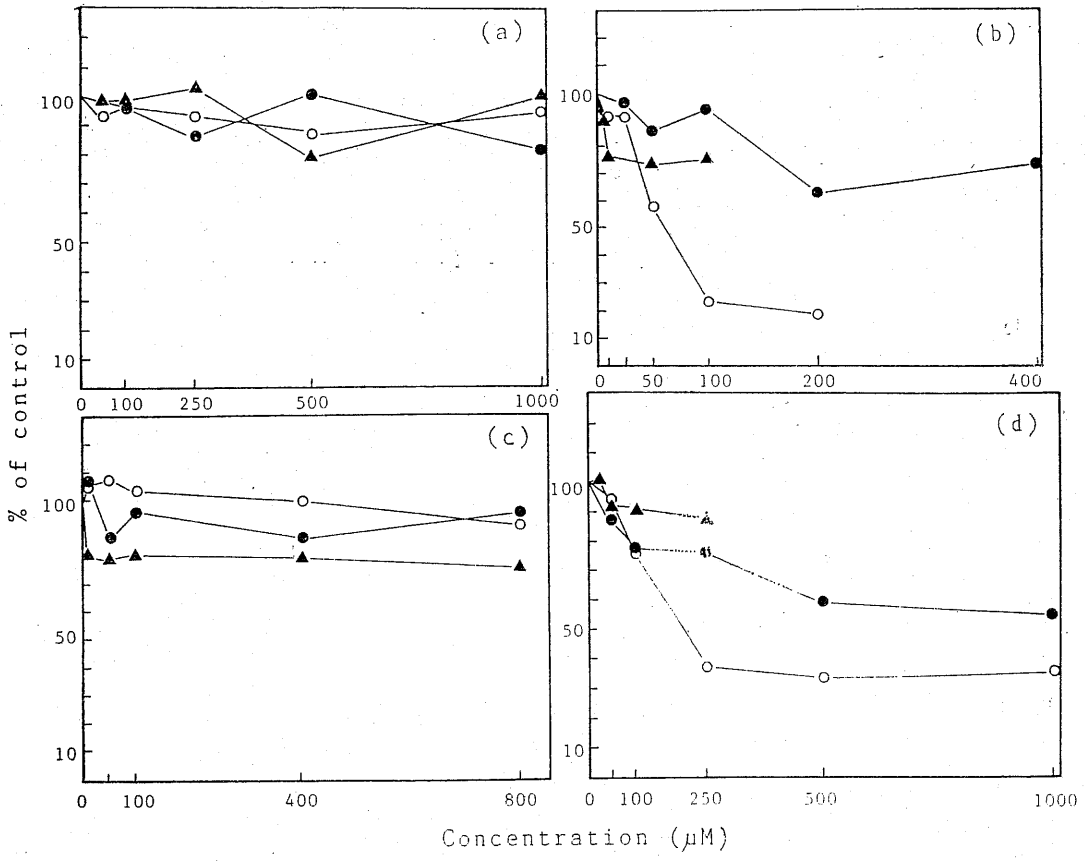

Fig. 6. The rate of protein synthesis after (a) Azodrin (b) Butachlor (c) Paraquat and (d) Saturn treatment. Cells were generally labeled with ^{14}C-thymidine, then replated with 24-well plate in subconfluence for 12 hr without ratioactive label. Various concentrations of pesticides were treated 1 hr and pulse labeled with ^{3}H-leucine during the last 30 min. The ratio of ^{3}H/^{14}C of control were taken as 100%. Each point was average of 4 experiments. LF (○) TK (●) TM (▲).

250 μM the depressions were 15 and 25% respectively.

DISCUSSION

Toxicity test using established cell lines have been reviewed by Bridges (1976), recently it has been adopted for the analysis of pollutant-induced damages in cultured mammalian cells (Packham *et al.*, 1982). Because the propagation of cell lines can be carefully controlled, information such as chromsome aberration, sister chromatid exchange and phenotype transformation which cannot be drawn from live animals were readily obtainable, especially when the damages are sublethal and the effects chronical. Though results obtained from cultured cells does not always correspond to those from animal test, yet they are often complementary. The widely used Ames test for screenig carcinogens (Ames *et al.*, 1973) is an example.

Pollution of river and coast has serious consequenses on fish population. The biologic effects with emphasis on neoplasia of aquatic animals were extensively reviewed (Kraybill, 1977). Surveys of fish population revealed that the sensitivities of fish towards pollutants were so varied that the sizes of each individual group changed as the quality of the water deteriorated.

In this study we reported the tests of four most widely used agricultural chemicals on pest and weed control on three different fish cell lines developed in this institute. The test not only provide information on the toxicities of the chemicals but also revealed certain physiological responses of the cells which may help to probe into the basic defensive mechanism of cells against environmental challenges.

Cell survival on drug treatments

Among the four drugs tested Azodrin was least toxic; the cell growth was not inhibited up to 800 μM drug concentration. Saturn was also non toxic to TM and LF cell up to 400 μM, only at 800 μM the growth was slightly depressed; yet for TK cells there was a continuous suppression of growth, at 400 μM the cells were completely killed. The toxicities of Butachlor and Paraquat towards three cell lines were apparent but differentiated; among them TM was most sensitive, at 1 μM no surviving cells were found; LF cell showed a near linear response against the drug concentration, the shoulderless curve suggests that the cells did not have a capacity of tolerance against the drug toxicity; TK cells was the least sensitive one among the three, a broad shoulder at 25 μM revealed that the cells could resist the toxicity of Butachlor better than other cells. Paraquat also showed differential toxicities towards three different cell lines, but the effects were not as extreme as that of Butachlor; however, the order of sensitivity was reversed for TM and LF.

Rate of DNA synthesis

When cells were challenged with DNA damaging agent such as radiation and chemicals, the rate of DNA synthesis declined immediately reflecting that initiation of new replicons for DNA synthesis had been turned off (Cleaver *et al.*, 1983; Rudé and Friedberg, 1977). The declination level was proportional to the dose administered (Edenberg, 1976). Usually the rate of DNA synthesis would recover to normal level within 24 hours if the challenge was sublethal (Swenson and Setlow 1966; Cleaver, 1965). Painter (1977) further demonstrated that although the suppression of DNA synthesis was a common effect of cells suffered form challenge, nevertheless after removal of the agents DNA synthesis rate would remained suppressed if the agents were DNA damaging such as mutagens and carcinogens otherwise the rate would recover immediately.

In this work we chose the doses of four drugs when administered to the cells would maintain over 90% survival. The results are summarized in Table 2. The degrees of inhibition were defined as: a. negative, the

TABLE 2
Inhibition of DNA, RNA and protein synthesis after pesticides treatment

Pesticide	LF			TK			TM		
	DNA	RNA	Prot.	DNA	RNA	Prot.	DNA	RNA	Prot.
Azodrin	+[a]	−	−	−	−	−	++	++	−
Butachlor	+++	−	+++	++[b]	++	+	++	++	+
Paraquat	++	−	−	++[b]	+	−	++	+	+
Saturn	++	+	+++	+++[b]	++	++	++	+	+

a. −, negative; +, weak inhibition (80-60% of control rate); ++, moderate inhibition (60-40% of control rate); +++, strong inhibition (40-10% of control rate).
b. rate of DNA synthesis did not recover within 3 hr.

rate was within the control limit; b. weak inhibition, 80-60% of control rate; c. moderate inhibition, 60-40% of control rate; and d. strong inhibition, 40-10% of the control rate. Most of the drugs did not maintain the inhibitory effect after they were washed off, yet for those rates which did not return to control level within three hours after the removal of the drugs were specially indicated. All four drugs suppressed DNA synthesis on three cells lines but to various extent. The most potent ones were Butachlor and Saturn. TM and LF cells recovered from the suppression immediately after the removal the drugs, but TK failed to do so with Butachlor, Paraquat and Saturn.

Rate of RNA and protein synthesis

The measurements help to probe whether drugs affect the processes of gene expression (Wang and Rao, 1984; Johnston and Singer, 1978). The cells were first tested with actinomycin D and cycloheximide to ascertain that fish cell lines responded to the inhibitory drugs to the same extent as mammalian cells (Stryer, 1981; Kornberg, 1980; Reich *et al.*, 1961). We also used hydroxyurea to inhibit ribonucleotide reductase in order to reduce the flow of ^{3}H-uridine into DNA (Sjoeberg, 1977). The results showed that the fish cells used in this study responded normally. The effects of the drugs on RNA synthesis showed differential sensitivities towards three cell lines; whether it is because the DNA template activity was affected (Nierlich, 1978) or the associated protein factors were altered remains to be investigated. The only known mechanism of the inhibitory effect on protein synthesis was on Butachlor reported by Hayasaka and Wakimoyi (1981). Saturn was known to suppress the plant growth which was associated with reduced protein synthesis (Ichizen, 1981). Our results also demonstrated the inhibitory effects of Butachlor and Saturn especially on LF cells.

The chemistry of drug effects on cellular level is complex. Here we only tried to find how three different fish cell lines responded to these potential environmental hazardous chemicals. The survival data corresponded well those findings in wild animal populations; in polluted streams loach population diminished in an astounding rate while tilapia population stayed unchanged (Chen, unpublished data). It is therefore possible to evaluate other potential environmental pollutants by cell culture test.

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三種魚細胞對四種農藥敏感度之研究

王 錫 杰　游 復 熙

近年來環境污染日趨嚴重，爲測量河川污染程度，常選用魚類的存活率作爲指標，本實驗以三種魚類細胞株對四種農藥的毒性進行測試，三種魚細胞爲大肚魚 (top-minnow)、吳郭魚腎 (tilapia kidney) 和泥鰍鰭 (loach fin)，四種農藥是巴拉刈 (Paraquat)，丁基拉草 (Butachlor)，亞速靈 (Azodrin) 和掃坍 (Saturn)，都是最常見的魚類和最常用的農藥，我們發現農藥中除亞速靈外，其它三種都有毒性，而魚細胞對農藥的敏感性差異也很大，其中以吳郭魚的抗毒性最高，與實際污染水域中吳郭魚族羣不減反增的實例相吻合，同時細胞中高分子合成也受到農藥處理的影響。