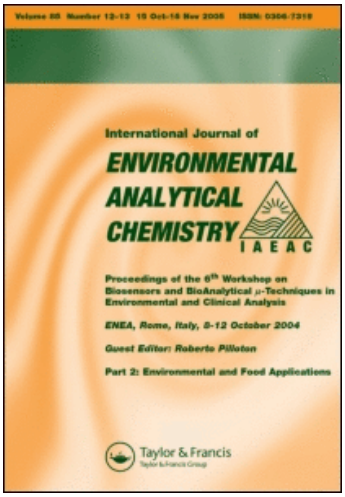

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In Vitro Biological Systems as Models to Evaluate the Toxicity of Pesticides

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IN VITRO BIOLOGICAL SYSTEMS AS MODELS TO EVALUATE THE TOXICITY OF PESTICIDES

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Recently the use of *in vitro* systems has gained acceptance for toxicological research, since they offer several advantages. We have used primary and immortalized cell cultures to evaluate the effect of the fungicide Methyl 1-(butylcarbamoyl)benzimidazole-2-carbamate (Benomyl), the insecticide 0-2-diethylamino-6-methylpyrimidine-4-yl—0,0-dimethyl phosphorothioate (Pirimiphos-methyl), the herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA). Benomyl resulted responsible for microtubular disorganization in time-dose dependent manner, and for glutathione depletion. Pirimiphos-methyl, alone or combined with Benomyl, had no effect on microtubule organization, but reinforced glutathione depletion. MCPA exerted its primary effect on mitochondria, increasing the mitochondrial membrane potential and inhibiting the ATP-synthesizing ATPase. Flow cytometric analysis suggests that MCPA affects C3H cell cycle and induces the formation of type II foci in standard transformation assay. The results presented demonstrate that cultured cells represent a rapid, controlled and useful method to test pesticides both individually and in combination.

KEY WORDS: Primary rat hepatocytes, immortalized cell line, Benomyl, Pirimiphos-methyl, MCPA, cytological effects.

INTRODUCTION

Various approaches and methodologies are used in toxicological research to identify hazards and to verify the processes which influence the expression of responses of living organisms to pesticides[1–3]. The response of an organism to a given exposure of a chemical results from a diffuse array of interdependent processes at the molecular, cellular and organism levels. Thus toxicokinetics and toxicodynamic interactions underscore the most obvious advantages of whole animal testing. Exploring possible alternative methodology, *in vitro* toxicity testing has gained acceptance during the last ten years[4]. This term includes a battery of living systems such as bacteria, cultured cells, fertilized chicken eggs, frog embryos, that can be used for the evaluation of toxicity of chemicals in view of their previsional effect on human health.

This paper is dedicated to Roberto Marchetti, Professor of Ecology, who is no more with us.

Frog embryo and cultured cells are the *in vitro* systems used in our laboratories. Frog embryos can be obtained by *in vitro* fertilization to have a large number of individuals at synchronized developmental stages[5], are easy to be used under standard conditions[6], allow the evaluation of metabolic activation, express cytochrome P-450[7]. The procedure to standardize this model is in progress in U.S.A.[8] and in some European laboratories[5], who are compiling data base, so that better predictions can be drawn from interlaboratory results[9].

Primary cell cultures as well as immortalized cell lines represent suitable models for toxicological research[4,10,11] Experience over the last years with isolated mammalian hepatocytes has demonstrated that such *in vitro* systems provide insight into the mechanism of action of chemical toxins[12]. Cultured animal cells present some obvious disadvantages, since they are devoid of structural organization, have lost their histotipic architecture and often the associated biochemical properties, do not achieve a steady state unless special conditions are employed. Nevertheless the advantages of this *in vitro* model are significative as they can be propagated and divided into identical replicates, can be characterized and preserved by freezing, can be grown in selective media, can be separated genotypically to have cell strain with considerable uniformity, can be treated with measured and controlled dose of chemicals. They are used for studies of screening toxicity of compounds, and viability, respiration, membrane integrity are analyzed. Primary cultured cells are used to study the effects of specific toxins and specialized cell functions such as enzymatic activities, membrane receptors and proteins involved in the processes of compound bioconversion.

Two international projects have been activated during the last ten years to validate these systems, the FRAME (Fund for Replacement of Animal in Medical Experiments) which is studying the correlation of LD_{50} with *in vitro* test and the MEIC (Multicenter Evaluation of *in vitro* Cytotoxicity, Scandinavian Society Cytotoxicology), which utilizes a mathematic model to correlate the *in vitro* data with those of acute toxicity in man. Even *in vitro* experimental conditions are very different from those produced in animals, today the validity of *in vitro* test is accepted for preliminary investigations such as screening of genotoxic or cytotoxic potential of compounds.

The severity of the damage caused by pesticides depends on their bioavailability, metabolism and tissue turn-over rate. At high concentration they cause general cytotoxicity and cell death. At low and intermediate concentrations they may impair specific functions. Identification of cellular target may yield valuable information for prediction of toxicity at low or intermediate doses, for selected appropriate biomarkers and for understanding their action mechanism. Pesticides can affect plasma membrane structure and function, DNA synthesis, the level of peroxisomal enzymes and biochemical indices. Recently significative results have been obtained in testing the herbicides: 2,4,5-T[13], 2,4-D[14], chlorosulfuron, glyphosate[15] and the insecticides: diazinon[16], deltametrin, DDT, lindane[17], malathion[18] on immortalized cell lines and primary cultures.

We present the results obtained on primary cultured rat hepatocytes and on the immortalized cell line C3H testing the effect of the fungicide Benomyl alone and combined with the organophosphorous insecticide Pyrimiphos-methyl and of the clorophenoxy herbicide MCPA.

The results obtained stress the validity of *in vitro* systems for understanding the mechanism of action of pesticides on living organisms, moreover suggest that the knowledge of the compound metabolites allows the identification of the affected cell functions.

EXPERIMENTAL

Chemicals

Methyl-1-(butylcarbamoyl)benzimidazol-2-yl carbamate (Benomyl) and 0-2-diethylamino-6-methylpyrimidin-4-yl—0,0-dimethyl phosphorothioate (Pirimiphos-methyl) were obtained from ICI Soplant S.p.A. and their purities were 98 and 92.8%. 4-chloro-2-methylphenoxyacetic acid (technical MCPA) was purchased from Aldrich-Chemical Company, U.S.A. Benzo[a]pyrene (B[a]P), used as positive control and diluted in acetone and ribonuclease were purchased from Sigma. All other chemicals were of analytical quality.

Cell lines and culture conditions

Rat hepatocytes were obtained by the modified method of collagenase perfusion[19]. The cell suspension obtained after perfusion was filtered through cotton gauze and washed twice with culture medium by sedimentation.

C3H10T1/2 (clone 8) cells (C3H)[20], a mouse embryo immortalized cell line highly sensitive to postconfluence inhibition of cell division has been used. C3H treated with chemical carcinogens develops foci of transformed cells overlying the normal cell monolayer. C3H express a subfamily of cytochrome P-450[21]. Cells were grown at 37°C in basal minimal Eagle's medium supplemented with 25 μg/ml gentamicin, 10 mM Hepes, 2 mM glutamine and 10% heat inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2 in air.

C3H and hepatocyte viability was tested by trypan-blue (0.4%) exclusion after treatment with the compounds.

Benomyl (B), Pirimiphos-methyl (P) and MCPA addition

Hepatocytes were treated, 20–24 hr after plating, with the single molecules and with a mixture (ratio 6:1) of B and P (25 μgB/ml + 4.1 μgP/ml or 50 μgB/ml + 8.3 μgP/ml); exposure times were 15 min., 30 min. and 2 hr. The single compounds were used at the same doses and times as the mixture.

Hepatocytes were treated 1, 2, 5, 10 mM MCPA for 10 and 20 min. C3H were treated 24 hr after seeding with 2.5 mM MCPA for 7, 15 and 25 hr.

Fluorescence assay

0.5 ml of the hepatocyte suspension were loaded with 2 μM Rhodamine-123 (Rho-123) and after 10 min. 2 μM propidium iodide (PI) were added to evaluate Mitochondrial Membrane Potential (MMP) and viability with a flow cytometric analysis. Cells were then incubated with increasing concentrations (1, 2, 5 and 10 mM) of MCPA and 2,500 cells were measured in a FACScan flow cytometer (Becton-Dickinson Immunocytometry System, San Jose, Ca, U.S.A.) with a focused argon laser. Moreover

cells were loaded with 2 μM Rho-123 and MMP was measured. 150 μM digitonin was used to permeabilize the mitochondrial membrane; 250 μM carbonyl cyanide p-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler, was used as positive control.

Microtubule cytoskeleton was analyzed with the indirect immunofluorescence technique[22].

Glutathione assay

Cells, plated on collagen-coated dish at a density of 100,000 cells/cm^2 were treated and processed for glutathione (GSH) assay according to the method of Hissin and Hilf[23] with minor modifications. The samples obtained were washed and immediately stored at –80°C. The frozen sample was sonicated, proteins precipitated with a solution containing 25% H_2PO_3 and this mixture centrifuged (100,000 g, 30 min. at 4°C). The pellet was stored at –20°C for protein determination[24] and GSH was measured in the supernatant using o-phthalaldehyde as a fluorescent reagent. GSH was expressed as the amount of GSH/mg proteins.

Flow cytometric analysis

C3H were seeded at a concentration of 40,000 cells/dish, after exposure to MCPA. Treated and control cells were trypsinized, centrifuged and resuspended in 200 μl PBS, then counted with a hemocytometer. Samples were harvested by centrifugation (200 g, 5 min.), washed twice in 3 ml PBS and resuspended in 2 ml cold PBS. To prevent cell clumping during fixation 6 ml ethanol were vortexed during the drop addition of cell suspension (final ethanol concentration was 95%). Cells were stored at 4°C. Ethanol fixed samples were washed by centrifugation (200 g, 7 min.) and resuspended in 2 ml PBS containing 1 mg/ml ribonuclease A for 30 min. at 37°C.

Denatured samples were washed by centrifugation (200 g, 7 min.) and incubated for 30 min. at 25°C with fluorescein isothiocyanate (FITC) staining solution to a final concentration of 0.1 μg/ml FITC in PBS. Cells were centrifuged (200 g, 7 min.), washed once in 3 ml PBS, centrifuged (200 g, 7 min.), and resuspended in a solution containing 50 μg/ml PI. The cell suspensions were kept on ice for approximately 20 min. and analyzed with the flow cytometer (FACScan, Becton-Dickinson, San Jose, Ca). Cells from each sample were tested for relative DNA and protein contents by red fluorescence of PI and green fluorescence of FITC.

Standard transformation assay

The procedure reported[25] with minor modifications was followed. 700 cells were plated in 60 mm dish, 5 or 10 dishes per group to obtain 40% of plating efficiency. 24 hr after seeding MCPA was added to the medium. At 6 weeks, dishes for the transformation assay and the controls were washed in PBS, fixed in methanol, stained with Giemsa and scored for transformed foci.

RESULTS AND DISCUSSION

Effects of Benomyl and Pirimiphos-methyl on isolated hepatocytes

Benzimidazole derivatives have been introduced as wide spectrum systemic fungicides. In mammals these compounds have low acute toxicity, but various toxic effects have been reported after repeated administration[26,27]. Benomyl acts as an antimitotic agent in fungi, slime molds, higher plants and mammalian cells[28]. We have demonstrated that it interferes with cytoskeletal assembly[29] and one of us has recently emphasized the role of GSH in the maintenance of intact microtubule in isolated hepatocytes for secretion of low density lipoprotein[30]. Benomyl is used in Italian agriculture and it is found in food-stuffs, thus the mechanism of action of this compound for its implication in human health needs further investigation.

Cell viability after treatment with both the mixture and Benomyl alone was decreased by 20% after 2 hr with the highest dose, while viability was not affected by Pirimiphos-methyl (Figure 1). Cells recovered in control media presented the same viability as controls (Figure 2). Tubulin cytoskeleton was disorganized after treatment of Benomyl alone and of the mixture at the highest dose within 2 hr, the effect was time-dose dependent (Table 1 a). Pirimiphos-methyl alone had no effect. When cells treated with 25 μg/ml Benomyl were left to recover in control media, the microtubule assembly was restored (Table 1 b). GSH was depleted in time and dose dependent manner by Benomyl, it was affected by 8.3 μg/ml of Pirimiphos-methyl. 4.1 μg/ml of the compound mixed with the lower dose of Benomyl potentiated GSH depletion (Figure 3). The level of total GSH descended on the cytotoxic threshold 3% of the control value after 30 min treatment with the mixture. GSH, which accounts for 90% of the non-protein SH groups, resulted to be oxidized by the drug. The identification of a reactive carbamoylating agent S-(n-Butyilcarbamoyl)glutathione as a metabolite of Benomyl[31] (Figure 4) furnishes a possible explanation of the mechanism of action of this compound on the cells analysed.

Effects of MCPA on isolated hepatocytes

MCPA belongs to the phenoxyalkanoic acids, which are considered to be potential contaminants of water where they have the highest solubility. They are present in soil mainly in the dissociated form. These herbicides have been found in groundwater in many countries[32]. MCPA does not produce acute toxicity to the aquatic organisms[33]. When administered to groups of rats at doses 80 ppm in the diet for 24 months it result to be toxic to the target organs: kidney, liver and red cells[34]. Recently[35] it has been reported that there are no evidences implicating chlorophenoxy herbicides as human carcinogens, while cytotoxic effects such as peroxisome proliferation and oxidative stress are described[36]. We have analysed the effect of MCPA on primary cultured hepatocytes to evaluate the modification of intracellular parameters. As it has been shown[37] ester hydrolysis was the initial step of MCPA metabolism and the resulting free phenoxyacetic acid was hydroxilated and conjugated with mono- and di-carbohydrates (Figure 5), we have addressed our experiments to the evaluation of mitochondrial membrane potential (MMP). Viability of cells treated with 2.5 mM was maintained within 24 hr. MMP values were increased in a time-dose dependent way (Figure 6). The effect produced by

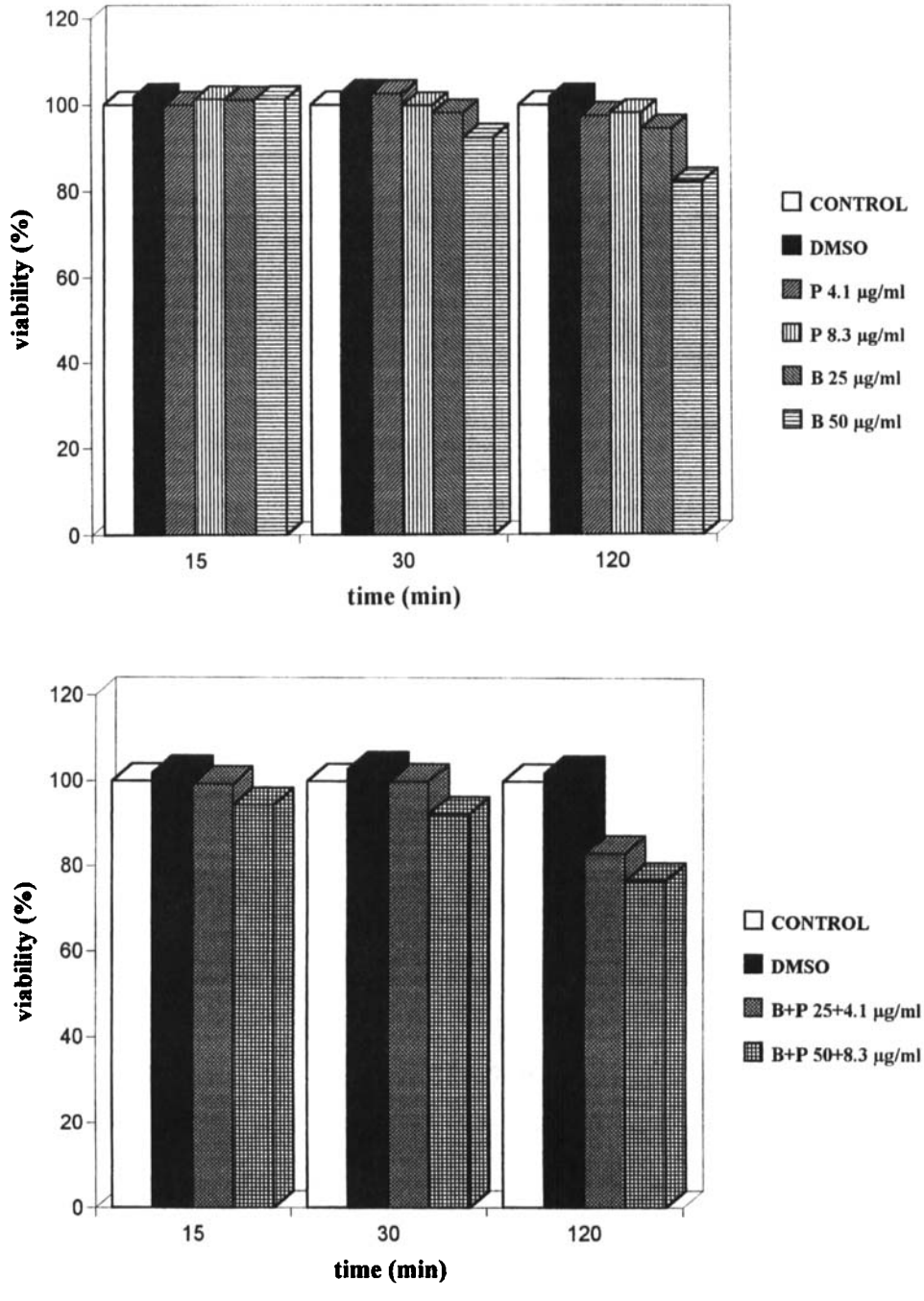


Figure 1 Cell viability in control and treated rat hepatocytes determined by trypan-blue exclusion. B = Benomyl; P = Pirimiphos-methyl.

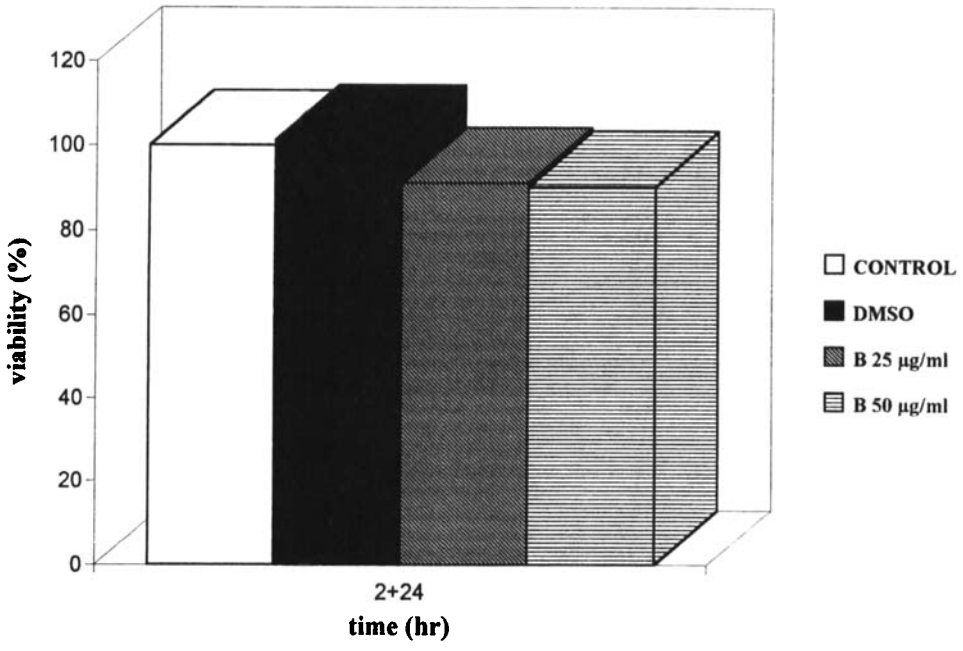


Figure 2 Cell viability in control and treated rat hepatocytes recovered in culture medium for 24 hr treatment. B = Benomyl.

Table 1 Microtubule disorganization in rat hepatocytes treated with Benomyl alone and in mixture (a) and recovered for 24 hr in culture medium (b). Data are expressed as% of affected cells ± SD.

(a) *Time (min)*	*B 25 µg/ml*	*B 50 µg/ml*	*B + P 25 µg/ml + 4.1 µg/ml*	*B + P 50 µg/ml + 8.3 µg/ml*
15	–	20.1 ± 1.3	–	20.5 ± 0.38
30	29.9 ± 0.61	59.7 ± 0.52	30.1 ± 0.22	50.5 ± 0.39
120	90.2 ± 1.4	99.8 ± 0.49	88.4 ± 0.69	99.9 ± 0.17
(b) *Time (hr)*	*B 25 µg/ml*	*B 50 µg/ml*		
2 + 24	100 ± 0	96.1 ± 0.26		

10 mM MCPA on MMP (Figure 7 a) was compared to the one produced by oligomycin, which elevates the MMP (Figure 7 b). CCCP and digitonin, that completely abolish the fluorescence signal (Figure 7 c) were used to demonstrate that the fluorescence increase was due to the increase of MMP. From these results it emerges that MCPA exerts its primary effect on mitochondrial membrane potential, probably as a consequence of the metabolic pathway of this compound (Figure 5).

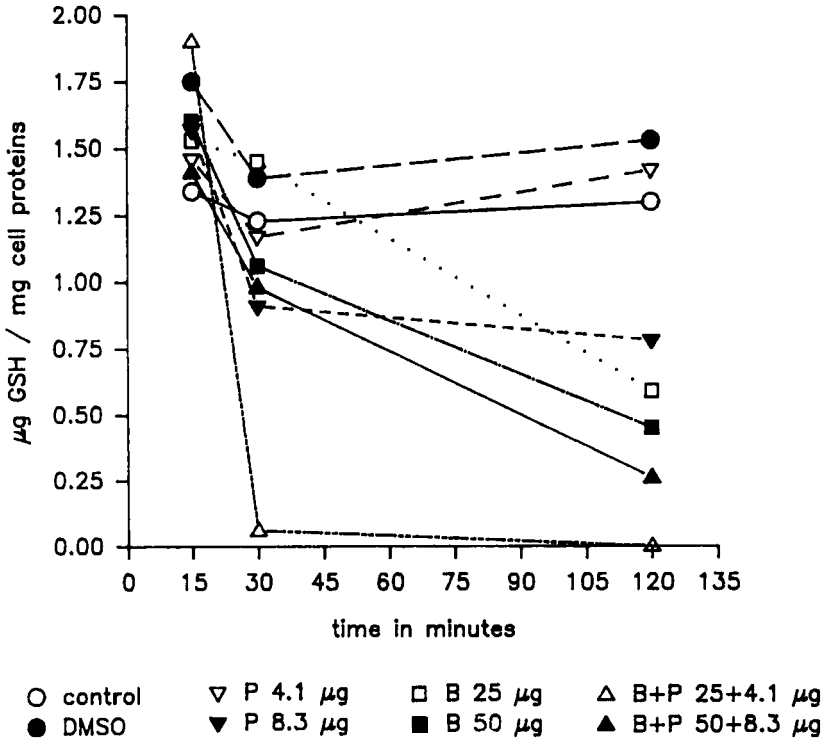


Figure 3 Time course of intracellular glutathione depletion in rat hepatocytes treated for 15, 30 and 120 min. B = Benomyl; P = Pirimiphos-methyl.

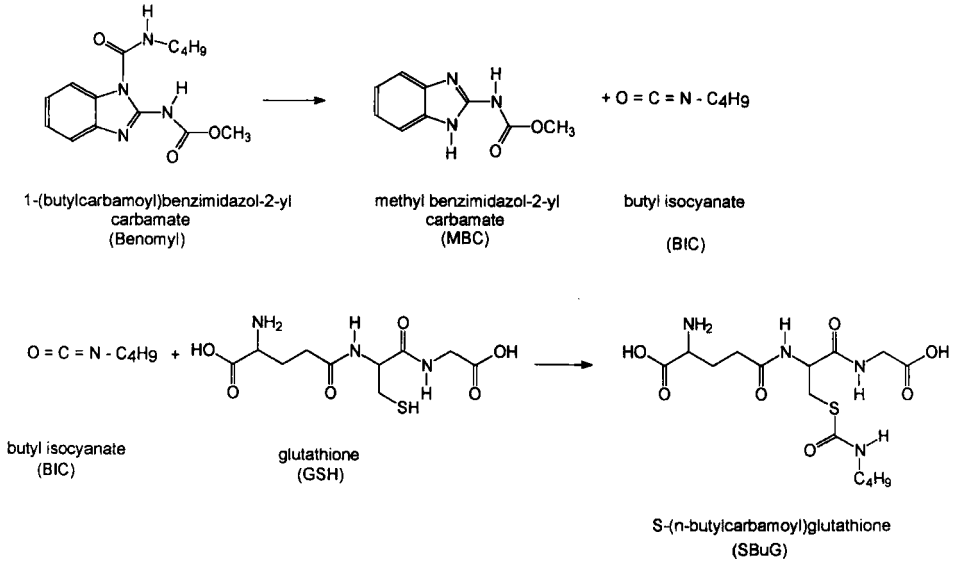
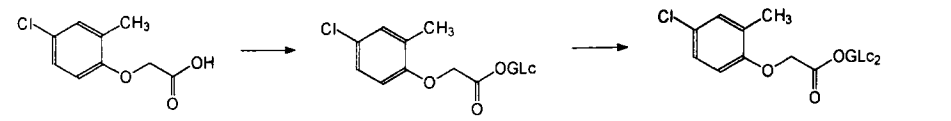


Figure 4 Chemical structure of 1-[butylcarbamoyl]benzimidazol-2-yl carbamate (Benomyl) and cell metabolic reactions.



4-chloro-2-methylphenoxyacetic acid (MCPA) | 4-chloro-2-methylphenoxyacetic acid-Glc (MCPA-Glc) | 4-chloro-2-methylphenoxyacetic acid-Glc2 (MCPA-Glc2)

Figure 5 Chemical structure of 4-chloro-2-methylphenoxyacetic acid (MCPA) and cell metabolic pathway. Glc = glucose.

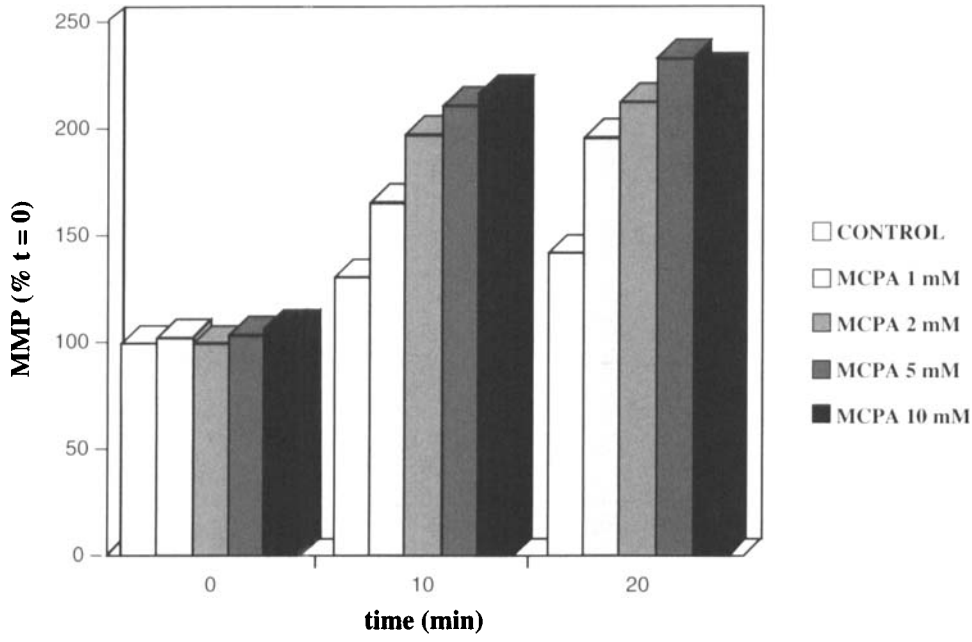


Figure 6 Mitochondrial membrane potential (MMP) of hepatocytes treated with increasing concentrations of MCPA. MMP is expressed as average Rhodamine-123 fluorescence corresponding to the average MMP in living cell (propidium iodide negative).

Effects of MCPA on C3H immortalized cell line

C3H cell line was used to test MCPA on cell proliferation and on cell transformation. The flow cytometric technique[38,39] allows to analyse DNA and protein contents during cell cycle so to score the most sensitive phase of the cycle to the compound. Viability of cells was maintained within the 25 hr with 2.5 mM MCPA (Figure 8). The analysis of cell proliferation evidenced that cell number in control and treated experiments was unchanged at 7 hr, while the number of treated cells at 15 hr and 25 hr was significantly decreased respect to the control (Figure 9). The protein histograms showed that they were progressively incremented at 7 hr and at 25 hr (Figure 10, lane 1), while cell number was reduced. DNA histograms showed a reduced number of cell in S-phase

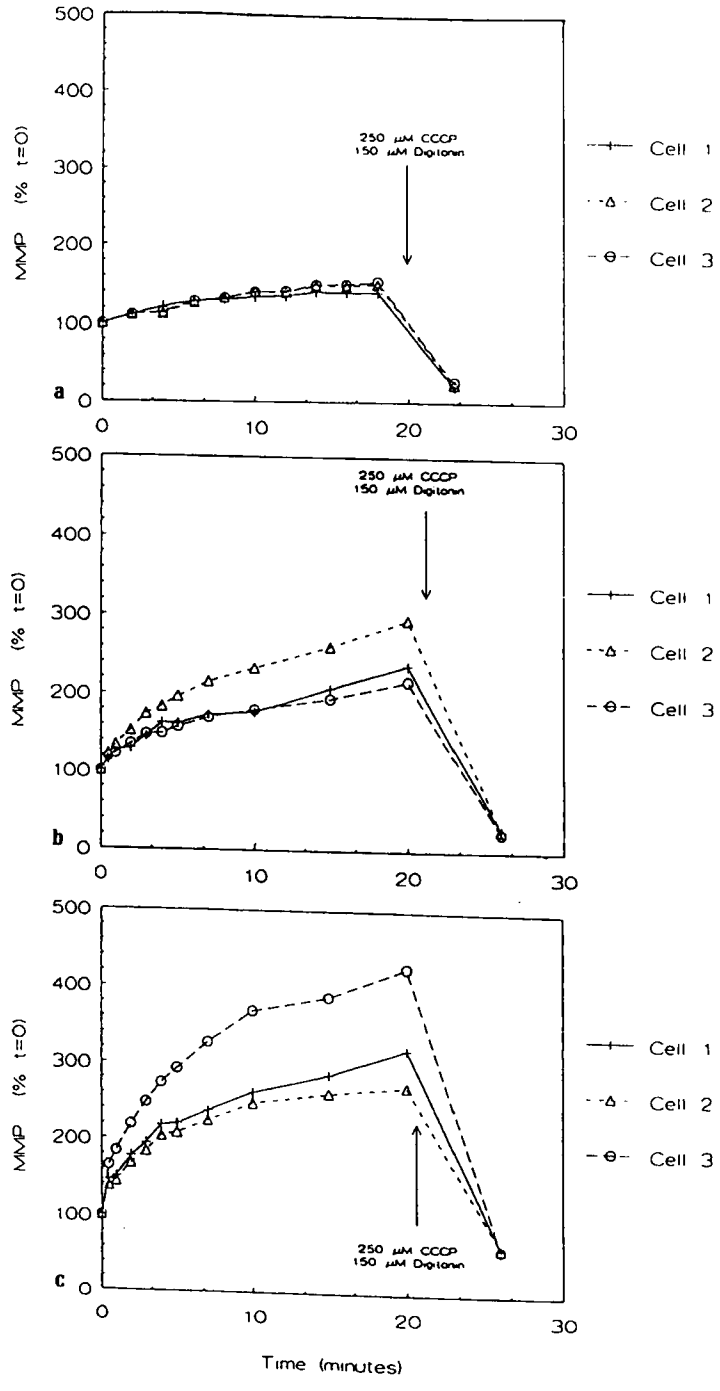


Figure 7 Mitochondrial membrane potential (MMP) in hepatocytes treated with 10 mM MCPA measured by the video-intensified fluorescence microscopy (VIFM). Fluorescence intensities, measured in single living cells, are expressed in graph-form: (a) control cells, (b) hepatocytes treated with 10 µM oligomycin and (c) hepatocytes treated with 10 mM MCPA.

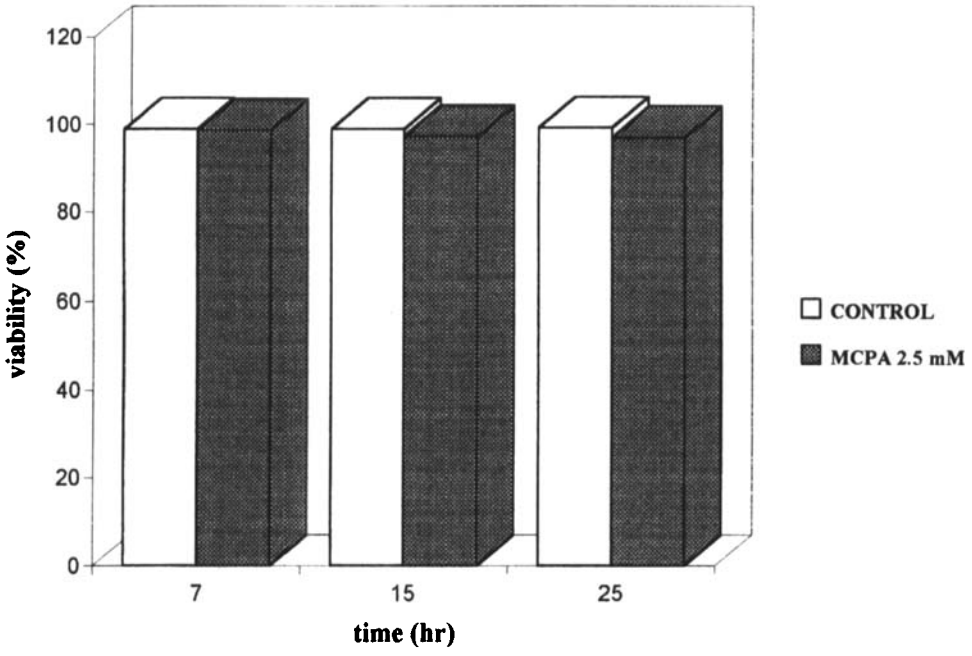


Figure 8 Cell viability in control and treated C3H10T1/2CL8 determined by trypan-blue exclusion.

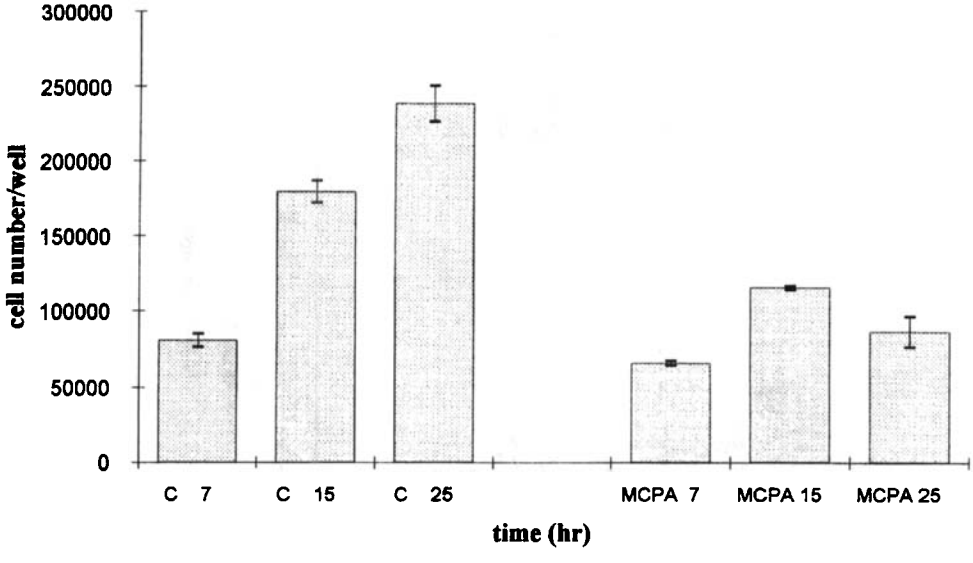


Figure 9 Cell proliferation in control and 2.5 mM MCPA treated C3H10T1/2CL8. Average and standard deviation of 3 experiments are indicated.

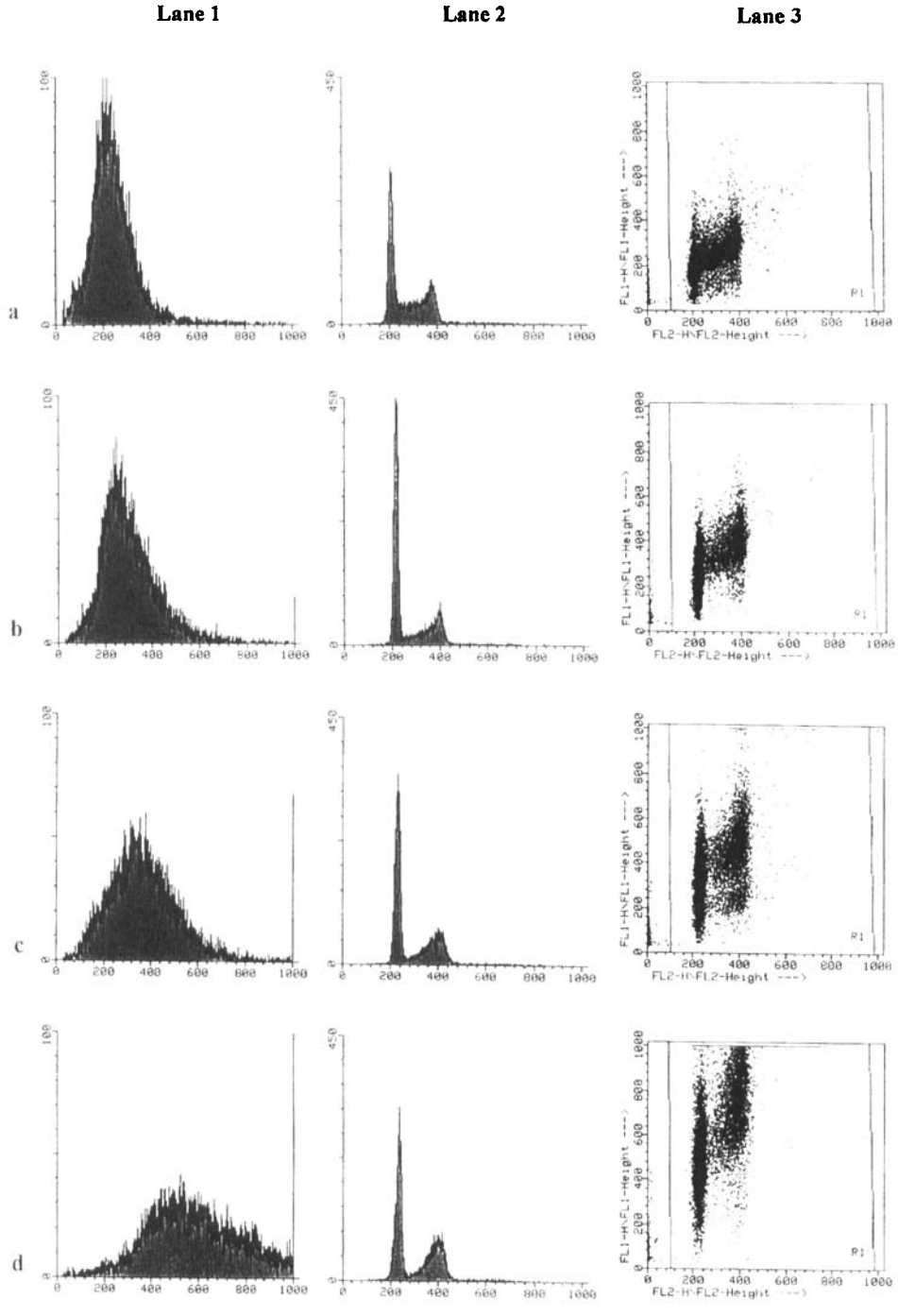


Figure 10 Distribution of total protein content (Lane1) and DNA content (Lane 2) in control (a) and C3H10T1/2CL8 treated with 2.5 mM MCPA after 7 hr (b), 15 hr (c) and 25 hr (d). Lane 3: two parameter DNA-protein profiles. Lane 1 abscissa: protein content; ordinate: cell frequency. Lane 2 abscissa: DNA content; ordinate: cell frequency. Lane 3 abscissa DNA content; ordinate: protein content.

Table 2 Effect of acetone, MCPA and B[a]P on survival (expressed as % vs control) and transformation (expressed as n° of foci/n° of dishes scored) in C3H10T1/2CL8.

Chemical	*Concentration*	*% Survival*	*Transformation* *Total n° of foci/n° of dishes scored* *Type II*	*Type III*
Control		100	0/10	0/10
acetone (control)	0.55	97.3	0/5	0/5
MCPA	2.5 mM	82	2/9	0/9
B[a]P	1 μg/ml	75	6/5	1/5

(Figure 10, lane 2). These preliminary results demonstrate that MCPA affects cell proliferation and cell cycle, suggesting that the cell cycle analysis is a valid and innovative method to be used for testing the cellular mechanism of action of pesticides. Moreover immortalized cell lines are suitable models to test compounds suspected to be oncogenic. Induction of malignant transformation in response to several carcinogenic hydrocarbons has been proved[40,41]. The presence of foci, which may be formed in petri dish containing treated cells after 6 weeks and retained to be an index of carcinogenicity, was identified in MCPA treated cells (Table 2) suggesting that this compound needs further investigation.

CONCLUSIONS

The results obtained with cultured cells show that a general evidence for a cytological toxic effect of pesticides is furnished by the analysis of cytoskeletal organization. Specific biochemical parameters allow the identification of their mechanism of action. Benomyl resulted to be a potent glutathione depleting agent, and its metabolic pathway indicates a carbamoylated derivative of GSH. The organophosphorous insecticide Pirimiphos-methyl, which in the Italian agricultural practice is used mixed with Benomyl, is ineffective on the cell parameters tested, while it potentiates the GSH depleting effect when mixed with Benomyl. This result points the attention to the chemical and biological interactions which may occur when pesticides mixture are used with different consequence for final cell toxicity. Flow cytometric analysis demonstrated that MCPA exerts its primary effect on mitochondrial membrane potential and flow cytometric studies showed that MCPA has a growth modulating effect on cell cycle. The presence of transformation foci in immortalized cells suggests that this compound needs further investigations to clarify its effect on human health.

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