Enhancement of Lysosomal Enzymes by the Pyrethroids,
Fenvalerate and \textit{trans}-Cypermethrin

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We looked for a direct correlation between the suggested effect of fenvalerate and \textit{trans}-cypermethrin as tumor promoters, and the documented elevations in the activities of lysosomal enzymes in various types of tumors. Results of this study demonstrate that the pyrethroids, fenvalerate and \textit{trans}-cypermethrin, significantly enhanced the activities of the hepatic lysosomal enzymes, arylsulfatase A (ASA), arylsulfatase B (ASB), \(\beta\)-glucuronidase and cathepsin D in live mice. These increased activities correlate directly with fenvalerate or \textit{trans}-cypermethrin treatment, in a significantly dose-dependent manner. Hepatic ASA, ASB and \(\beta\)-glucuronidase from untreated mice gave significantly elevated relative activity values when preincubated with both pyrethroids as compared to those of controls. Moreover, heat stability study showed that the specific activities of these enzymes from mice that were treated with fenvalerate or \textit{trans}-cypermethrin correlated well with the specific activities of their corresponding controls from vehicle-treated animals. These findings may show the possible tumor promotion effect of the pyrethroids, fenvalerate and \textit{trans}-cypermethrin.

\textbf{Key words} — lysosomal; arylsulfatase; \(\beta\)-glucuronidase; cathepsin D; pyrethroids

\textbf{Introduction}

Lysosomes encapsulate a group of hydrolyzing enzymes that may be classified, according to substrates, into: (a) polysaccharide-hydrolases, such as arylsulfatas (EC 3.1.6.1) and \(\beta\)-glucuronidase (EC 3.2.1.31), (b) protein-hydrolases, such as cathepsins, (c) nucleic acid-hydrolases, (d) lipid-hydrolases and (e) phosphatases. Lysosomal arylsulfatas are differentiated into A and B forms. Both arylsulfatase A and B were reported to be glycoproteins with high mannose-type sugar chains. Arylsulfatase A hydrolyzes the sulfate esters of cerebroside 3-sulfate and ascorbic acid 2-sulfate, while arylsulfatase B catalyzes the hydrolysis of \(N\)-acetylgalactosamine 4-sulfate. On the other hand \(\beta\)-glucuronidase catalyzes the hydrolytic cleavage of the glucuronide bond at the non-reducing termini of glycosaminoglycans. Human \(\beta\)-glucuronidase has several mannose-type oligosaccharide chains. Cathepsin D (EC 3.4.23.5) is one of the major
proteolytic enzymes of animal tissues and is known as an aspartic acid protease.⁹

Elevated activities of arylsulfatase A and B were demonstrated in human malignant neoplasms such as gastrointestinal,¹⁰ bladder,¹¹ lung¹² carcinomas and in peripheral leukocytes from patients with chronic myelogenous leukemia,¹³ as well as in the urine of bladder tumor patients,¹⁴ myeloid leukemia¹⁵,¹⁶ and acute nonlymphocytic leukemia.¹⁷

Pyrethroids are very potent neurotoxicant insecticides that have achieved widespread agricultural and varied environmental health applications. They are synthetic derivatives of natural pyrethrins.¹⁸,¹⁹ Pyrethroids toxicity appears to be due to sodium channels modification that causes a disturbance in the action potential generation in neural tissues.²⁰ Although some studies failed to describe pyrethroids as genotoxic or carcinogenic potential substances, others reported contradictory findings, testing fenvalerate²¹ and deltamethrin.²² Moreover, it was demonstrated that fenvalerate²³,²⁴ as well as cypermethrin²⁴ is a potential tumor promoter.

Little information is available about the effects of fenvalerate and cypermethrin on lysosomal enzymes, although it has been stated that cypermethrin has a short-term pharmacological effect on β-glucuronidase.²⁵

In an attempt to reveal the possible tumor promotion effect of pyrethroids, we demonstrate the effect of fenvalerate and cypermethrin on the activity of the lysosomal enzymes, arylsulfatase A and B, β-glucuronidase and cathepsin D. In the present study, we have tried to directly correlate the suggested effect of fenvalerate²³,²⁴ and cypermethrin²⁴ as tumor promoters, with the documented increased activities of lysosomal enzymes such as arylsulfatase A and B in different carcinomas.¹⁰–¹⁷

**Materials and Methods**

**Chemicals and Reagents** — Technical-grade fenvalerate (purity 96.6%), from Sumitomo Chemical Co., Ltd. was kindly provided by Dr. Ashraf Moustafa (Department of Applied Medical Chemistry, Medical Research Institute, Alexandria University, Egypt). Technical grade trans-cypermethrin (purity 96.6%) was purchased from Ciba-Geigy Co. p-nitrochatechol sulfate, p-nitrophenyl β-D-glucuronide and bovine hemoglobin were from Sigma. DEAE-cellulose powder (DE 52) was from Whatman. Other reagents used were of analytical grade.

**Animals and in Vivo Treatments** — Female Swiss albino mice, weighing 20—30 g, were obtained from the animal house of the Medical Research Institute (Alexandria, Egypt). Mice were housed 4/cage (60×30×20 cm) and had free access to food and water. They were kept under conventional conditions of temperature, humidity and 12 h photoperiod. Pyrethroids were prepared in different doses for the treatment groups in a corn oil base (six mice were used per group for each dose). Animals were challenged by gavage with pyrethroids in 200 µl of corn oil. Each group was treated once a d for two d and mice were then sacrificed 24 h after the second dose was given. Control groups received vehicle only. The used doses of fenvalerate were 15,
20, 25 and 30 μg/g body wt, while those of trans-cypermethrin were 6, 12 and 18 μg/g body wt. The highest used dose of either pyrethroids was less than 15% of its LD₅₀ value.

**Preparation of Enzymes** — Enzymes were prepared from the liver of mice, individually, as described by Gasa et al. Mice were sacrificed and the liver was removed immediately and homogenized, using a Brinkmann homogenizer, in 10 volumes of ice-cold buffer, containing 0.01% Triton X-100 and 0.25 M sucrose in 10 mM Tris-HCl buffer, pH 7.4. The homogenate was then centrifuged at 500 × g for 10 min. The resulant supernatant was then centrifuged at 20000 × g for 20 min. All the preparatory steps were done at 4°C. The supernatant obtained was used as an enzyme preparation. Arylsulfatase A and B were separated on DEAE-cellulose column.¹³

**In Vitro Treatment of Enzymes** — Enzyme preparations from untreated mice were incubated with different concentrations of either fenvalerate (0—1.5 μg/ml) or trans-cypermethrin (0—1.42 μg/ml), at 37°C for 5 min before being assayed for enzymatic activities as described below. Pyrethroids were dissolved in acetone which was added, in the same amount, to control preparations. The final concentration of acetone in the assay medium was 2.5% (v/v).

**Assay of Arylsulfatase A and B Activities** — The colorimetric method of Baum et al. was used to assay the activity of arylsulfatase A and B separately, using p-nitrocatechol sulfate as a substrate. For the assay of arylsulfatase A activity, the incubation mixture contained 10 mM p-nitrocatechol sulfate and 0.5 mM sodium pyrophosphate in 0.5 M sodium acetate buffer, pH 5.0. The incubation mixture for the assay of arylsulfatase B activity contained 50 mM p-nitrocatechol sulfate and 0.1 mM barium acetate in 0.5 M sodium acetate buffer, pH 6.0. Amounts of the liberated p-nitrocatechol were measured at 515 nm. One unit of enzyme activity refers to 1 nmol p-nitrocatechol produced per h at 37°C.

**Assay of β-Glucuronidase Activity** — The enzyme activity was assayed using 1 mM p-nitrophenyl, β-D-glucuronide, as a substrate, in 0.1 M sodium acetate buffer, pH 4.0. Amounts of the liberated p-nitrophenol were measured at 420 nm. One unit of β-glucuronidase activity represents the activity that liberates 1 μmol of p-nitrophenol per min at 37°C.²⁷

**Assay of Cathepsin D Activity** — In the assay of cathepsin D activity, 1% (w/v) bovine hemoglobin was used as a substrate in 0.5 M formate buffer, pH 3.3. The unreacted protein was precipitated by trichloroacetic acid.²⁸ One activity unit of the enzyme is defined as the supernatant extinction at 280 nm (corrected for the blank) giving a net extinction value of 1.0 under the conditions of assay in 20 min at 37°C.

**Heat Stability of Enzymes** — Enzyme preparations from mice treated in vivo with 30 μg/g fenvalerate or 18 μg/g trans-cypermethrin, as described under Animals and in Vivo Treatments, and those from vehicle-treated control subjects were heated at various temperatures (25, 37, 50, 60 and 70°C) for 10 min and enzymatic activities were assayed as described above. Temperatures were measured by a thermocouple connected to a digital monitor (Model Bat-12, Bailey Instruments, Saddlebrook, NJ).
**Protein Measurement** — Protein concentrations were determined according to the method of Lowry et al. using bovine serum albumin as standard.  

**Statistical Analysis** — Significant differences among comparable parameters were estimated using ANOVA with a post-test of Bonferroni.

### Results

**In Vivo Effect of Fenvalerate on the Activities of Lysosomal Enzymes**

Activities of the examined lysosomal enzymes from mice treated with fenvalerate increased significantly in a dose dependent manner, compared to those of controls from vehicle-treated animals ($p < 0.05$). As shown in Table 1, arylsulfotase A and B, $\beta$-glucuronidase and cathepsin D from mice treated with fenvalerate (30 $\mu$g/g body wt), displayed relative activity values of 350, 220, 278 and 191%, respectively. $\beta$-Glucuronidase showed a relative activity of 650% (6.5 fold increase) at a dose of 25 $\mu$g/g.

**In Vivo effect of trans-Cypermethrin on the Activities of Lysosomal Enzymes**

All tested lysosomal enzymes from the trans-cypermethrin-treated mice recorded significantly increased activities compared to those from vehicle-treated controls ($p < 0.05$). The increased activities paralleled the increased doses of the test compound. Table 1 shows that arylsulfotase A and B, $\beta$-glucuronidase and cathepsin D from mice treated with trans-cypermethrin (18 $\mu$g/g body wt) displayed relative activity values of

<table>
<thead>
<tr>
<th>Fen (\mu g/g body wt)</th>
<th>Specific Activity (unit/mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>Arylsulfotase A</td>
</tr>
<tr>
<td>0</td>
<td>12.2±1.3</td>
</tr>
<tr>
<td>15</td>
<td>17.0±0.3</td>
</tr>
<tr>
<td>20</td>
<td>29.3±2.8</td>
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<tr>
<td>25</td>
<td>32.6±1.0</td>
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<tr>
<td>30</td>
<td>42.8±6.7</td>
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<tr>
<td>6</td>
<td>31.4±0.2</td>
</tr>
<tr>
<td>12</td>
<td>32.7±1.2</td>
</tr>
<tr>
<td>18</td>
<td>44.7±1.1</td>
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Each group of mice was treated once a d for two d with the indicated dose of fenvalerate (Fen) or trans-cypermethrin (Cyper) in 200$\mu$l of corn oil base by gavage. Mice were sacrificed 24 h after the second dose was given. Control groups received vehicle only. The highest dose of fenvalerate used was less than 15% of its LD$_{50}$ value. Enzymes were prepared from the liver of each animal and assayed separately for their specific activities as described in Methods. Data are expressed as mean±S.D., n=6. *a* ) Significantly different from control value ($p<0.001$).
366, 204, 443 and 302%, respectively.

**In Vitro Effect of Fenvalerate on the Activities of Lysosomal Enzymes**

Fig. 1A demonstrates the significant increases in the relative activities of all tested lysosomal enzymes, except cathepsin D, that were incubated with fenvalerate, as compared to those of control enzymes ($p < 0.05$). The significantly elevated relative activities paralleled the increases in the fenvalerate concentrations used for the *in vitro* enzymatic treatments. Arylsulfatase A and B and $\beta$-glucuronidase gave relative activity values of $144 \pm 6.4$, $125 \pm 5.0$ and $117 \pm 3.5\%$, respectively, when incubated with $0.25 \, \mu g/ml$ fenvalerate (lowest concentration used), and gave relative activity values of $334 \pm 5.0$, $231 \pm 1.7$ and $245 \pm 4.5\%$, respectively, when incubated with $1.50 \, \mu g/ml$ fenvalerate (highest concentration used).

**In Vitro Effect of trans-Cypermethrin on the Activities of Lysosomal Enzyme**

The relative activities of all examined lysosomal enzymes except cathepsin D that were incubated with *trans*-cypermethrin displayed significant elevations compared with their corresponding values of controls ($p < 0.05$). As seen in Fig. 1B, arylsulfatase A and B and $\beta$-glucuronidase that were treated with $0.47 \, \mu g/ml$ *trans*-cypermethrin, the lowest effective concentration used with all tested enzymes, gave the relative activity values of $142 \pm 3$, $103 \pm 2$ and $125 \pm 1\%$, respectively, while those that were incubated with $1.42 \, \mu g/ml$, the highest concentration used of *trans*-cypermethrin, gave the relative activity values of $177 \pm 3.5$, $112 \pm 0.6$ and $139 \pm 4.6\%$, respectively. Cathepsin D activity

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**Fig. 1A. In Vitro Effect of Fenvalerate on the Activities of the Lysosomal Enzymes:**

Arylsulfatase A (ASA), Arylsulfatase B (ASB) and $\beta$-Glucuronidase ($\beta$-Glu)

Enzyme preparations from livers of untreated mice were incubated with different concentrations of fenvalerate at $37^\circ C$ for 5 min before being assayed for enzymatic activity. Data are presented as % relative enzymatic activity (ratio of the specific activity of the pyrethroid treated enzyme to that of the control vehicle treated enzyme). Each value is the mean±S.D. of 6 preparations. *denotes significant difference from control at $p < 0.05$.

**Fig. 1B. In Vitro Effect of trans-Cypermethrin on the Activities of the Lysosomal Enzymes:**

Arylsulfatase A (ASA), Arylsulfatase B (ASB) and $\beta$-Glucuronidase ($\beta$-Glu)

Enzyme preparations from livers of untreated mice were incubated with different concentrations of *trans*-cypermethrin at $37^\circ C$ for 5 min before being assayed for enzymatic activity. Data are presented as % relative enzymatic activity (as explained in the legend of Fig. 1 A). Each value is the mean±S.D. of 6 preparations. *denotes significant difference from control at $p < 0.05$. 

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could not be determined in vitro due to the interference of the UV absorption of the pyrethroids with the enzyme product assay at 280 nm.

**Heat Stability Studies on Lysosomal Enzymes from Fenvalerate or trans-Cypermethrin in Treated Mice**

All the tested enzymes in preparations from mice treated with either fenvalerate, trans-cypermethrin or vehicle and subjected to a temperature range of 25–70°C, displayed the same pattern of variation in specific activities with different temperatures, i.e., were equally heat sensitive. As shown in Fig. 2 (I, II, III and IV), the changes in specific enzymatic activities of arylsulfatase A and B, β-glucuronidase and cathepsin D, from mice that were treated with either fenvalerate (30 μg/g body wt) or trans-cypermethrin (18 μg/g body wt), correlate well with those of their corresponding control enzymes from the vehicle-treated subjects. The correlation coefficient of each peak

![Fig. 2. Effect of Heat on the Activities of the in Vivo Pyrethroid Treated Lysosomal Enzymes.](image)

Enzymes were prepared from mice that were treated in vivo with fenvalerate (30 μg/g) or trans-cypermethrin (18 μg/g) or vehicle (control subjects). Preparations from pyrethroid-treated and control mice were heated at various temperatures (25, 37, 50, 60 and 70°C for 10 min and enzymatic activities were determined. Means±S.D. (n=6) are given. The statistically calculated correlation matrix for variables is unity. Cont., control; Fen., fenvalerate; Cyper., trans-cypermethrin.
with its control was equal to 1.

Discussion

Chemicals that possess the ability to enhance altered hepatic foci and inhibit intercellular communication are likely to be classified as tumor promoters. Based on these suggested criteria, pyrethroids were evaluated as tumor promoters. Hemming et al. reported that all the pyrethroids they tested can act as tumor promoters. They found that fenvalerate, cypermethrin and flucyathrin enhanced the development of N-nitrosodiethylamine-initiated γ-glutamyltranspeptidase (GGT)-positive foci in liver of live rats and also inhibited gap junctional intercellular communication. We previously reported that fenvalerate and cypermethrin enhanced the activity of GGT in mouse kidney. Moreover, Flodstrom et al. and Warngard and Flodstrom showed that fenvalerate and flucyathrin inhibited intercellular communication between V 79 cells, while cypermethrin was inactive.

Induction of 7-pentoxyresorufin O-dealkylation has been suggested as a qualitative marker for tumor promotion. However, fenvalerate and cypermethrin induced 7-pentoxyresorufin O-dealkylation to only a minor extent as compared to dichlorodi-phenyltrichloroethane (DDT).

Although some pyrethroids were considered as tumor promoters, several studies failed to evaluate their possible carcinogenic effect. Moreover, pyrethroids like fenvalerate and cypermethrin were found to be non-genotoxic. Taken together, the results of other studies evaluating pyrethroids for their genotoxicity and for their tumor promotion potential were contradictory and inconsistent. Accordingly, we have been prompted to shed more light on the possible tumor promotion potential of pyrethroids. In the present study, we tried to find a direct correlation between the suggested effect of fenvalerate and cypermethrin as tumor promoters and the documented elevation in the activities of some lysosomal enzymes like arylsulfatase A and B in different carcinomas. Our presented data indicate that the observed significant increases in activities of the lysosomal enzymes, arylsulfatase A and B, β-glucuronidase and cathepsin D, correlate directly with the treatment of the pyrethroids, fenvalerate and cypermethrin, in a dose dependent manner (Table 1). These findings will likely support findings of other studies, in which it was demonstrated that fenvalerate and cypermethrin can act as tumor promoters.

In order to show whether this increased enzymatic activity in vivo is a result of conformational change of the area of the enzyme active site, we performed a heat stability study as most enzymes lose some of their activities when they are heated. Heating easily disrupts the folded structure of an enzyme by increasing the vibrational and rotational motions of atoms. The tested lysosomal enzymes followed the same pattern of activities with changing temperature after and before treatment with pyreth-
roids. If there were conformational changes due to the pyrethroid treatment, the pattern of activities with changing temperature would be different. Thus, from the results of Fig. 2 we can conclude that the observed increased activities may not be a result of changes in the enzyme protein.

To determine whether there is a direct interaction between pyrethroids and the tested lysosomal enzyme, the assay was done in vitro on pyrethroid-treated and vehicle-treated enzymes, as well as in vivo. The activities of lysosomal enzymes were significantly enhanced in both studies (Table 1 and Fig. 1). Accordingly, we suggest that there may be a direct interaction between pyrethroids and the tested enzymes. Furthermore, the observed elevated activities of the tested enzymes may be due to molecular modifications. In some tumors, arylsulfatases A and B were known to be modified by sialylation and phosphorylation\textsuperscript{39–42} and β-glucuronidase was also known to be phosphorylated on both carbohydrate and protein moieties.\textsuperscript{43,44} Therefore, one can suggest the possibility that these types of modification occur through in vivo enhancement of the tested lysosomal enzymes by pyrethroids. However, the study of the effect of pyrethroids on sialylation and/or phosphorylation itself is currently underway to clarify this.

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References

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