BIOLOGIJA. 2006. Nr. 3. P. 25–29

Effects of the herbicide pendimethalin on mitochondrial functions

A. Četkauskaitė,

A. Zimkus*,

J. Borovik

Department of Biochemistry and Biophysics, Faculty of Natural Sciences, Vilnius University, M. K. Čiurlionio 21, LT-03100 Vilnius, Lithuania

Two main functions of rat liver mitochondria namely the respiration rate and generation of membrane potential, were analysed simultaneously to evaluate the toxicity of the dinitroaniline herbicide pendimethalin. The chromatography grade pendimethalin enhanced the mitochondrial respiration in a concentration-dependent manner and markedly decreased the membrane potential starting from 8.2×10^{-5} M up to 5.47×10^{-4} M (23–154 mg/ml). A higher toxicity of technical grade pendimethalin (Stomp – a mixture of pure ingredient and adjuvant(s)) was observed at lower concentrations of the pure ingredient (pendimethalin) than those of the single chromatography grade chemical. These data indicate that: 1) pure and technical grade pendimethalin preparations act as uncouplers of oxidative phosphorylation in mitochondria (enhance respiration and diminish membrane potential), 2) these effects are weaker but comparable (of the same concentration order) to those of 2,4-dinitrophenol, and 3) technical grade pendimethalin is more toxic to oxidative phosphorylation in mitochondria than pure chemical pendimethalin (active ingredient) itself. These data clearly disclaim the statement presented in the manual "Recognition and Management of Pesticides Poisonings" (US EPA 1999) that pendimethalin does not act as an uncoupler of oxidative phosphorylation.

Key words: mitochondria, respiration, membrane potential, herbicide pendimethalin, Stomp, uncoupling of oxidative phosphorylation

INTRODUCTION

Pendimethalin, CAS No. 40487-42-1, chemical name (IUPAC) N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidene, is used as a selective herbicide in both preemergence (before the sprouting of weed seeds) and early postemergence (within 7 days) to control most of annual grasses and certain broadleaf weeds. It is recommended to protect field corn, potatoes, rice, cotton, soybeans, tobacco, peanuts, sunflower and a variety of vegetables [1, 2]. Products of technically different compositions and products containing pendimethalin (Stomp 400SC, Pendimethalin 400SC, Ruck, Terapur, Prowl) are produced by different companies [2–4]. The main manufacturer, American Cyanamid Company, produces technical herbicide Stomp containing 33% of the active ingredient pendimethalin. Other products of technical pendimethalin can contain up to 42.3% and even 92.6% (Stomp of BASF Co. and Technical Pendimethalin of DOW Agrosciences LLS) [2, 4] of the active substance. Stomp has been confirmed in Lithuania as suitable for field applications [3]. The most important physico-chemical properties of pendimethalin are: low water solubility (up to 0.3 ppm at 20 ^{0}C) and high hydrophobicity (log Po/w = 5.18 – experimental at pH 7); both minimize its leaching from soil to groundwater [5, 6]. The high adsorption coefficient (5.000) favours relative accumulation of pendimethalin in sediments [1]. Its toxicity and relatively poor or better magnification even in individual food webs during a long degradation half-life in water bodies is influenced by the accumulation and metabolic rate ratio [7, 8]. More than 1080 entries on pendimethalin are presented in the TOXNET [6] and other databases [1, 9, 10]. These data show a relatively low acute oral toxicity for rats (LD_{50} > 5.000 mg/kg), mice (LD_{50} = 3.189 mg/kg) and mallard ducks (LD_{50} = 1.421 mg/kg). Critical effects include short-term toxicity for the liver and thyroid of rats [6, 8]. Acute (eco)toxicity data show pendimethalin to be toxic at lower concentrations than those of its solubility in water: 1) highly acute, 96 h toxicity to fish (LC_{50} = 0.138 mg/l, *Oncorhynchus mykiss*), 2) crustaceans (LC_{50} = 0.28 mg/l, *Daphnia magna*), and 3) algae (EC_{50} 0.006 mg/l, *Selenastrum capricornutum*). Chronic (30 days) toxicity to sediment-dwelling organisms (*Chironomus riparius*) was found at a concentration of >0.138 mg/l (higher than NOEC) [1, 8]. Direct overspray of a water body with a usual application rate of

* Corresponding author. E-mail: Aurelijus.Zimkus@gf.vu.lt

pendimethalin (2.4 kg/ha) can result in the concentrations severely toxic to algae, crustaceans, fish at a depth of 0.15 m (up to 1.6 mg/l) [11]. Because pendimethalin is a hydrophobic substance whose bioaccumulation depends on the metabolism and excretion rates [1, 6] and has a long degradation half-life in soil (30–150 days), it is topical to analyse its mode of action in cell biomembranes and the organelles of terrestrial biota, including mammals. Despite quite a rapid excretion of the compound from the rat body (up to 90% within 24 h), about 70% of this hydrophobic compound goes through the enterohepatic pathway [1, 6]. The universal model system such as eukaryotic liver mitochondria could reveal the energetic mode of action during an acute poisoning of the metabolising liver tissue. The aim of the present study was to evaluate the toxicity of the dinitroaniline herbicide phendimethalin to the oxidative phosphorylation function of rat liver mitochondria by comparing the strength of its action with the dinitrophenol structure uncoupler and with the technical grade herbicide (Stomp) preparation.

MATERIALS AND METHODS

Reagents. The chromatography grade pendimethalin (99.9% purity) and its technical formulation Stomp (33% of purity) were the products of American Cyanamid Company (kindly donated by Dr. O. Šakalienė, Lithuanian Institute of Agriculture, Vokė Branch). Other reagents were: ethanol (redistilled), NaCl, KCl, $MgCl_2$, KH_2PO_4, Na_2HPO_4, Tris-HCl, saccharose from ROTH (Germany), $CuSO_4 \times 5H_2O$, $NaKC_4H_4O_6 \times 4H_2O$, KJ and NaOH from Reachim (Russia), EGTA and ADP, bovine serum albumin, BSA from Serva (USA), 2,4-dinitrophenol, tetraphenyl-phosphonium bromide from Aldrich.

Preparation of mitochondria. Wistar rats weighing 275–300 g were sacrificed according to the rules defined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (License No. 0006 of State Veterinary Service for working with laboratory animals). Liver mitochondria were isolated using standard differential centrifugation procedures [12]. The liver was quickly removed and placed into ice-cold isotonic (0.9%) KCl solution. The tissue was cut into small pieces and homogenized in an ice-cold Teflon pestle-glass Potter–Elvehjem homogeniser with a medium containing 10 mM Tris-HCl, 250 mM sucrose, 3 mM EGTA, and 4 mg/ml bovine serum albumin (BSA), pH 7.4 (at 2 ˚C). The homogenate was centrifuged at 750× *g* for 5 min, and the supernatant was centrifuged at 7000× *g* for 10 min. at 0 ˚C. The pellet of the first low-speed centrifugation step was resuspended in A medium and recentrifuged to recover the mitochondria retained in the low-speed pellet. The final wash of the mitochondrial pellet was done in a buffer containing 250 mM sucrose, 5 mM Tris-HCl, pH 7.3 (at 2 ˚C). The mitochondrial pellet was resuspended in the same medium, the protein was adjusted to 50 mg/ml, and the preparation was stored on ice until use. Protein concentration was determined by the biuret method, using 50 µl of mitochondria suspension dissolved in 5% sodium deoxycholate [13].

The respiration and membrane potential ($\Delta\Psi$) of mitochondria were measured at 30˚C in a closed, stirred and thermostated 1.0 ml glass vessel fitted with both a Clark oxygen electrode (Rank and Brothers, Cambridge, UK) and a tetraphenylphosphonium (TPP^+)-selective electrode [14]. The incubation medium contained 20 mM Tris·HCl, pH 7.2, 5 mM KH_2PO_4, 110 mM KCl, 1 mM $MgCl_2$. TPP^+ was added up to 300 nM for membrane potential measurements. Incubations were carried out in the presence of the following substrates and inhibitor: 3.8 mM mM succinate (plus 1 mM rotenone), 2 mM ADP. Mitochondria were added in 50 µl volume (final protein concentration: (–1.3–1.5 mg/ml). 2,4-DNP (10^{-1}–10^{-3} M) and pendimethalin 10 mg/ml stock solutions diluted with solvents were added 10 µl volume. The mitochondria membrane potential was calculated according to [16]. The means presented in Tables were calculated from three independent measurements.

RESULTS AND DISCUSSION

Methods used for the evaluation of oxidative phosphorylation in mitochondria and the value of combined measurements. Several toxic effects of different chemicals in eukaryotic mitochondria were reported in the literature in 1991–1998. The effects of 22 pesticides were monitored by separate measurements of mitochondrial respiration [16]. The membrane potential was analysed to evaluate the effects of DDT, 2,4-D, dinoseb, paraquat, cationic detergents [17–20]. In this work, we present a simultaneous analysis of the inhibitory effects of certain chemicals on the respiration and membrane potential of rat liver mitochondria. This enables to collect more information on the membrane state in general and on the process kinetics with such threshold concentrations, particularly when one of the functions is decaying and the other still survives.

Toxicity of herbicide active ingredient pure pendimethalin, technical herbicide Stomp and 2,4-dinitrophenol to rat liver mitochondria. Data of control samples indicated that the initial $\Delta\Psi$ value generated by an intact respiring mitochondria in Energy State 4 (or 2) (respiration in the presence of substrate succinate and the absence of ATP synthesis), was constantly decreased by the addition of 2 mM ADP (Energy State 3). This usual effect indicated the switching of the mitochondrial ADP phosphorylation to ATP (Table 1). The increasing concentrations of pure pendimethalin, its technical grade preparation herbicide Stomp and 2,4-dinitrophenol (2,4-DNP) caused inhibition of the initial membrane potential ($\Delta\Psi$) of intact respiring mitochondria. The inhibitory action of these three substances was not limited to a decrease of the initial $\Delta\Psi$ value in State 4 (2).

The changes were observed in the ΔΨ response to ADP, i. e. the decrease in ΔΨ, caused by ADP was also diminished. This effect was developing with increasing the herbicide and 2,4-DNP concentrations up to the critical moment when the addition of ADP caused a total ΔΨ fall at certain concentration of the herbicide or 2,4-DNP. In general, this indicated that ADP addition in the presence of these substances lead to an additional exhaustion of ΔΨ, which the organelles were unable to restore. Data of simultaneous measurements of oxygen consumption (Table 2) showed that the respiration rate of rat liver mitochondria in State 4 increased with increasing the concentrations of pendimethalin, Stomp and 2,4-DNP. In agreement with the early classification of the mode of action of chemical substances in biomembranes [21], this indicates that all these substances act at least as uncouplers of oxidative phosphorylation. Respiration inhibition in State 3 (the presence of ADP and succinate) indicated that these three chemicals could also be inhibitors of the electron transporting chain. Despite the understanding of the classical uncoupler action as of a substance that enhances respiration but decreases and finally stops ATP synthesis, experimental facts show different mixed modes of action of classical uncouplers (substituted phenols, hydrazone derivatives, nitrosalicylanilides), and the chemicals affecting the oxidative phosphorylation are subjected to a few classification schemes of their modes of action [21, 22]. There is a consensus that the inhibition of microtubles, or tubulin formalion, like the inhibition of protein and nucleic acid synthesis, is a general toxic action of pendimethalin in plants [23]. Contradictory characteristics of pendimethalin mode of action in eukaryotic mitochondria may be found in literature. Pendimethalin, like other dinitroaminobenzenes (butralin, oryzalin), is characterised as having some known or suspected adverse effects such as not uncoupling oxidative phosphorylation or generate methemoglobin [10]. In contrast to these statements, our data revealed that: 1) preparations of pure and technical grade pendimethalin act as uncouplers of oxidative phosphorylation in mitochondria (enhance the respiration and diminish membrane potential), and 2) these effects are weaker but comparable (of the same concentration order) to that of 2,4-dinitrophenol. Yamano and Morita showed that among the 22 pesticides analysed by them, pendimethalin uncoupled State 4 (2) respiration in rat liver mitochondria at least by 20% and depleted the non-protein sulfhydryl content in hepatocytes by 20% at a minimum concentration of 1 mM [16]. Our data indicated that the concentrations of pendimethalin as low as 0.08 mM enhanced over 40% the respiration of mitochondria, but not affected the ΔΨ value.

Table 1. **Comparison of inhibition of mitochondrial membrane potential by pure pendimethalin, technical herbicide Stomp and uncoupler 2,4-dinitrophenol**

Exp. No.	Concentration of pendimethalin (A and B) and of 2,4-DNP (C), M or ppm (mg/l)	**Energy State 4 (or 2)** Initial ΔΨ (mV) (± SD)*	**Energy State 3** Decrease of ΔΨ, caused by ADP (%), (± SD)
	A. Effects of pure ingredient pendimethalin		
1.	0	200 ± 7	100 ± 5.1
2.	0.82×10^{-4} M (23 ppm)	199 ± 7	98 ± 6.0
3.	3.28×10^{-4} M (92.3 ppm)	197 ± 8	89.1 ± 6.3
4.	4.65×10^{-4} M (131 ppm)	150 ± 6	– ***
5.	5.47×10^{-4} M (154 ppm)	145 ± 7	– ***
	B. Effects of total technical Stomp (S), (ppm) concentrations and real concentrations (M, ppm) of pure pendimethalin in technical herbicide Stomp**		
1.	**0**	200 ± 6	100 ± 7.4
2.	**(77 ppm S)****; 0.91×10^{-4} M (25.4 ppm)	186 ± 6	83.5 ± 7.4
3.	**(95.6 ppm S)****; 1.16×10^{-4} M (31.6 ppm)	165 ± 8	56.4 ± 6.8
4.	**(115 ppm S)****; 1.41×10^{-4} M (38 ppm)	145 ± 9	30.8 ± 8.3
	C. Effects of uncoupler 2,4-dinitrophenol		
1.	0	204 ± 7	100 ± 5.5
2.	1.0×10^{-5} M	186 ± 6	98.7 ± 5.7
3.	3.8×10^{-5} M	150 ± 8	66.2 ± 6.3
4.	3.8×10^{-4} M	106 ± 9	14.9 ± 8.7

* – mean values of three repetitions are given with standard deviations;

** – concentrations written in bold are of technical herbicide Stomp; other concentrations of lower values (in M, ppm) are recalculated to pure, active ingredient, making up 330 g/kg of technical preparation of herbicide;

*** – irreversible decrease of ΔΨ

Table 2. **Comparison of stimulation of mitochondrial respiration by pure pendimethalin, technical herbicide Stomp and uncoupler 2,4-dinitrophenol**

Exp. No.	Concentration of pendimethalin (A and B) and of 2,4-DNP (C), M	Respiration rate in Energy State 4 (%), (± SD)*	Respiration rate in Energy State 3 (%), (± SD)
	A. Pure ingredient pendimethalin		
1	0	100 ± 7.9	100 ± 8.1
2	0.82×10^{-4} M (23 ppm)	141.7 ± 6.7	113.4 ± 5.6
3	3.28×10^{-4} M (92.3 ppm)	166.7 ± 7.0	83.6 ± 6.8
4	4.65×10^{-4} M (131 ppm)	200 ± 10.3	74.6 ± 5.6
5	5.47×10^{-4} M (154 ppm)	241.7 ± 11.5	35.8 ± 4.0
	B. Effects of total technical Stomp (S), (ppm) concentrations and real concentrations (M, ppm) of pure pendimethalin in technical herbicide Stomp**		
1	**0**	100 ± 6.0	100 ± 7.3
2	**(77 ppm S)****; 0.91×10^{-4} M (25.4 ppm)	141.7 ± 5.4	80.9 ± 6.7
3	**(95.6 ppm S)****; 1.16×10^{-4} M (31.6 ppm)	190.0 ± 8.2	57.4 ± 5.1
4	**(115 ppm S)****; 1.41×10^{-4} M (38 ppm)	241.7 ± 8.1	33.8 ± 3.0
	C. Uncoupler 2,4-dinitrophenol		
1	0	100 ± 7.3	100 ± 6.7
2	1.0×10^{-5} M	400 ± 12.5	85.7 ± 5.9
3	3.8×10^{-5} M	375 ± 13.2	64.3 ± 4.5
4	3.8×10^{-4} M	338 ± 13.4	37.5 ± 3.8

*, ** – the same as in Table 1.

Comparison of active ingredient mode of action with that of technical grade herbicide. The data presented in Tables 1 and 2 show that both pendimethalin preparations had a similar effect on respiration: they enhanced respiration in State 4 (or 2) by more than 40% at relatively low concentrations (0.82×10^{-4} M of pure pendimethalin and 0.91×10^{-4} M of pendimethalin ingredient in mixture of the technical herbicide Stomp). However, a concentration of pure pendimethalin up to 3.9 times higher was needed (5.47×10^{-4} M) as compared to the active ingredient (1.41×10^{-4} M) in the technical mixture with adjuvants in order to achieve the same respiration rate (241.7%) in State 4 (2). The question arises which chemical substances could cause a higher toxicity of the technical herbicide preparation. The characteristic examples of adjuvants used for the dissolution of hydrophobic chemicals in an aqueous environment are: surfactant-type technical mixtures which contain, for example, 4.48 to 8% of oleic acid, a mixture of alkylaryl polyethoxylene glycols + free fatty acids + isopropanol, alkyl phenols used (up to 90%) as non-ionic spreaders, activators, with insecticides, herbicides, acaricides [2, 4]. It was shown earlier by our group that oleic acid enhanced the acute toxic effects of chlorinated phenylurea herbicides (monuron, diuron) to *Vibrio fischeri* bioluminescence [24]. Thus, these data show that the technical grade pendimethalin is more toxic to oxidative phosphorylation in mitochondria than pure chemical pendimethalin (active ingredient) itself. This means that at equal active chemical concentrations non-target species will be affected much more by the technical herbicide Stomp than by chemically pure pendimethalin.

CONCLUSIONS

1. Pure and technical grade pendimethalin preparations act as uncouplers of oxidative phosphorylation in mitochondria: they enhance the respiration and diminish the membrane potential.

2. These concentrations and effects are weaker but comparable (of the same order) to that of 2,4-dinitrophenol.

3. Technical grade pendimethalin is more toxic to oxidative phosphorylation in mitochondria than the pure chemical pendimethalin (active ingredient) itself.

Received 7 March 2006
Accepted 23 June 2006

References

1. EXTOXNET (Extention Toxicology Network of Cornell, Michigan State, Oregon State and California at Davies Universities, USA).1996. Pesticide Information Profile (PIP). Pendimethalin. http://pmep.cce.cornell.edu/profiles/extoxnet/metiram-propoxur/pendimethalin-ext.html, and http://extoxnet.orst.edu/pips/pendimet.htm. Accessed February 21, 2006.
2. Sine C. (ed.). Farm Chemicals Handbook' 92. Pesticides Dictionary. Part C. Meister Publ. Comp. 1997.

3. State Plant Protection Service of the Republic of Lithuania: http:/www.vaat/lt. Accessed February 14, 2006.
4. US EPA OPP. Pesticide Ingredient Database of the Office of Pesticides Programs. http://www.cdpr.ca.gov/cgi-bin/epa/mkrep3.pl
5. htttp://esc.syrres.com. Accessed February 14, 2006, or chemIDplus, TOXNET database (see 7).
6. TOXNET 2003. http://toxnet.nlm.nih.gov/cgi-bin/sis/ search. Accessed February 14, 2006.
7. Zulalian J. J. Agricult Food Chem 1990; 38(8): 1743–54.
8. European Commission. Health and Consumer Protection Directorate General. Directorate E – Food Safety: plant health, animal health and welfare. Review Report for the active substance pendimethalin 7477/VI/98-final. 13 January 2003. europa.eu.int/comm/food/plant/protection/ evaluation/existactive/list1-35_en.pdf. Accessed February 21, 2006.
9. US EPA, IRIS. United States Environmental Protection Agency. Integrated Risk Information System 2004. Pendimethalin. http://www.epa.gov/iris/subst/0292.htm, Accessed February 21, 2006.
10. Reigart R, Roberts J (Eds.). Chapter 13. Other Herbicides. In: Recognition and Management of Pesticide Poisonings. Fifth edition. US EPA Office of Pesticides Programs. Washington DC 1999 118–24. http://npic.orst.edu/RMPP or http://ww.epa.gov/pesticides/safety/healthcare/handbook/handbook.pdf
11. CICAD OAS. The Toxicology of Selected Substances Used in the Production and Refining of Cocaine and Heroin: A Tier-Two Assessment. Washington DC, USA, CICAD, Organization of American States, Report Number OAS/CICAD 2005-02.
12. Mildaziene V, Baniene R, Marcinkeviciute A, Nauciene Z, Kalvenas A, Zimkus A. Mol Cell Biochem 1997; 174: 67–70.
13. Gornal AG, Bardavill GJ, David MM. J Biol Chem 1949; 177: 751–66.
14. Zimkus A, Chaustova L. Chemija 2004; 15(2): 27–30.
15. Daugelavicius R, Bamford JK, Bamford DH. J Bacteriol 1997; 179(16): 5203–10.
16. Yamano T, Morita S. Arch Environ Contam Toxicol 1995; 28: 1–7.
17. Moreno AJM, Madeira VMC. Biochim Biophys Acta 1991; 1060: 166–74.
18. Palmeira CM, Moreno AJ, Madeira VMC. Biochim Biophys Acta 1995; 1229: 187–92.
19. Bragadin M, Dell'Antone P. Arch Environ Contam Toxicol 1996; 30: 280–4.
20. Da Silva EM, Soares AM, Moreno AJ. Chemosphere 1998; 36(10): 2375–90.
21. Ogata M, Mori T, Izushi F, Etoh K, Sakai R, Meguro T, Inoue B. Physiol Chem Physics Medicinal NMR 1983; 15(3): 229–32.
22. Rottenberg H, Hashimoto K. Biochemistry 1986; 25: 1747–55.
23. Fedtke K. Biochemistry and Physiology of Herbicides Action. Moscow, Agropromizdat (Agro-Industry Publishers), 1985 (in Russian).
24. Četkauskaitė A, Bražėnaitė J. Biologija 2004; 4: 37–40.

A. Četkauskaitė, A. Zimkus, J. Borovik

HERBICIDO PENDIMETALINO BEI JO TECHNINIO PREPARATO „STOMP“ POVEIKIS ŽIURKĖS KEPENŲ MITOCHONDRIJŲ FUNKCIJOMS

S a n t r a u k a

Dvi pagrindinės žiurkės kepenų mitochondrijų funkcijos – kvėpavimo greitis ir membranos *potencialo* dydis – vienu metu analizuotos vertinant dinitroanilinų grupės herbicido – pendimetalino – toksiškumą. Chromatografinio švarumo pendimetalinas didino mitochondrijų kvėpavimą priklausomai nuo koncentracijos ir gerokai mažino membranos potencialą nuo $8{,}2\times10^{-5}$ M iki $5{,}47\times10^{-4}$ M (23–154 mg/ml). Didesni techninio švarumo pendimetalino (t. y. „Stomp“ – grynos veikliosios medžiagos mišinio su adjuvantais(-u)) toksiniai efektai buvo pastebėti žemesnėse grynosios veikliosios medžiagos (pendimetalino) koncentracijose palyginus su tomis pačiomis vieno chromatografinio švarumo cheminio junginio. Šie duomenys rodo, kad: 1) grynasis (chromatografinis) ir techninio švarumo pendimetalinas veikia kaip oksidacinio fosforilinimo skyrikliai mitochondrijose, t. y. didina kvėpavimą ir slopina membranos potencialą; 2) šios koncentracijos ir efektai yra mažesni, bet palyginami (tos pačios eilės) su 2,4-dinitrofenolio koncentracijomis ir efektais ir 3) techninio švarumo pendimetalinas yra toksiškesnis oksidaciniam fosforilinimui mitochondrijose už chromatografiškai gryną pendimetaliną (aktyvusis ingredientas). Šie duomenys aiškiai paneigia tvirtinimą, pateiktą JAV EPA 1999 žinyne „Recognition and Management of Pesticides Poisonings“, kad pendimetalinas neveikia kaip oksidacinio fosforilinimo skyriklis.