Bull. Environ. Contam. Toxicol. (1992) 49:520–526

Environmental Contamination and Toxicology

Esterase Activities during Chick Embryonic Development and Its Relationship with the Metabolism of 2,4-Dichlorophenoxyacetic Acid Butyl Ester

Stella Castro de Cantarini,[1] Ricardo Duffard,[2] and Ana Evangelista de Duffard[2]

[1]Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Enlaces de rutas 8 y 36 Km 601, (5800) Río Cuarto, Córdoba, Argentina and [2]Laboratorio de Toxicología Experimental, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 570, (2000) Rosario, Santa Fe, Argentina

2,4-Dichlorophenoxyacetic acid (2,4-D) is an organic chemical developed in the mid-1940's as a herbicide for the control of broad leaf weeds. This compound was formulated for use as butyl ester which is more volatile than the parent acid and therefore easier to apply in aerial spraying (Sanders 1981). Taking into account the importance of knowing the fate in the environment and the toxicological effects of herbicides on living organisms, Duffard et al. (1982) demonstrated that external application of 2,4-D butyl ester on fertile hen eggs on day 0 of incubation resulted in a 50% of mortality. Chicks hatched from these treated eggs exhibited postural and motor disfunctions and other alterations as hypomyelination and changes in the nervous system lipid composition (Mori et al. 1985, 1986). The same embryotoxic effects of 2,4-D butyl ester were found when the herbicide was applied on day 5 and 10 of incubation, but the 15 day group improved the hatching percentage (Duffard et al. 1987a).

Several studies have also been conducted on the metabolic fate of 2,4-D rather than applied butyl ester. Hydrolysis of the esters with formation of the free 2,4-D acid is the major metabolic pathway. Ester hydrolysis can be carried out by the actions of the many esterase enzymes found in the liver, blood and other organs of different animal species (Matsumura 1975). Erne (1966b) found that upon oral administration of 2,4-D butyl ester to rats, pigs and calves, this compound was hydrolyzed in the body to form 2,4-D and excreted by via the urine. The same results were demonstrated by subcutaneous administration of 2,4-D butyl ester to rats (Schulze et al. 1985). However, we did not find 2,4-D as metabolite of 2,4-D butyl ester; only the ester was detectable in all analyzed organs and tissues from one-day-old chicks hatched from treated eggs (Duffard et al. 1987b) as well as during chick embryonic development (Castro de Cantarini et al. 1989). This fact could be due to metabolism of 2,4-D butyl ester, and specially to change of esterase activities. The present study was designed to investigate the esterase activities during chick embryonic development and its relationship with the metabolism of 2,4-D butyl ester.

Send reprint requests to Duffard A.M. at the above address.

MATERIALS AND METHODS

Commercial formulation of 2,4-Dichlorophenoxyacetic acid butyl ester (31.6% w/v) was supplied by Atanor, Argentina and its purity was checked by gas liquid chromatography (GLC). (^{14}C)2,4-dichlorophenoxyacetic acid (specific activity 56 mCi/mmol) was purchased from Sigma (MO, USA) and used to prepare (^{14}C)2,4-D butyl ester for esterification with n-butyl alcohol (Horner et al. 1974). The efficiency of esterification was approximately 92%. All the other chemicals were purchased either from Merck Química Argentina or from Sigma, MO, USA.

Fertile hen eggs of an Arbor Acres strain were obtained from a commercial hatchery. They were treated externally in narrow bands, excluding the air compartment, with an ether solution of commercial 2,4-D butyl ester (3.1 mg/egg) before starting incubation, as previously described (Duffard et al. 1981). Control eggs were treated with ether. After drying, the eggs were placed in an incubator maintained at 38 °C and 60% relative humidity. They were rotated twice a day.

Twenty-four hours after hatching, chicks were decapited and livers removed and immediately homogenized in 0.1 M phosphate buffer pH 7.4 at 0-4 °C. The homogenates were then centrifuged for 10 min at 6000 g in a refrigerated centrifuge. The resulting supernants were diluted with 10 volumes of 0.1 M phosphate buffer pH 7.4 and used as enzyme source.

The metabolism of (^{14}C)2,4-D butyl ester was measured using the following medium: 0.1 M phosphate buffer pH 7.4; 1 mM 2,4-D butyl ester; 0.025 µCi (^{14}C)2,4-D butyl ester and approximatly 10 µg of protein content in a final volume of 0.3 ml. The reaction was started at the addition of enzyme preparation. After 30 min of incubation at 30 °C, the assay was stopped by adding of trichloroacetic acid (TCA) to a final volume of 10%. Control incubations without supernant livers were made to discount autohydrolysis of 2,4-D butyl ester. The metabolites were separated and detected by thin layer chromatography (TLC) using 20 x 20 cm silica gel G plates and ethylacetate: hexane: formic acid (20:80:0.3, v/v/v) as solvent system (Erne 1966a). Radioactive spots were visualized with ultraviolet radiation at 254 nm and identified by direct comparison authentic nonradioactive compounds (2,4-D butyl ester, 2,4-D). Metabolites were then quantified by scraping off the spots from the TLC plates, and counting them by scintillation spectrophotometry using Triton X-100 : Toluene (1:3), 0.02% 1,4-bis (5-phenyl-2-oxazyl) benzene (POPOP), 0.4% 2,5-diphenyl-oxazole (PPO) as scintillation medium, in a LS-100 Beckman Scintillation Counter. The external standard method for quenching correction was used.

Esterase activity was determined by Gomori's method with some modifications using α-naphthylacetate (α-NA) and Fast Violet B, during chick embryonic development and in different organs and tissues of one-day-old chicks. The samples were homogenized in

0.1 M phosphate buffer pH 7.4 and then centrifuged for 10 min at 6000 g. Dilutions of these supernants were prepared as required, and immediately used as enzyme source. The substrate solutions were prepared by diluting a stock solution of α-NA in acetone (0.03) in 0.1 M phosphate buffer pH 7.4. Enzymatic activity was measured by the change in absorbance at 500 nm and 30 °C of a reaction mixture with the following composition: 0.5 mM substrate-solution; 0.3 ml bidistilled water; 0.2 mg/ml Fast Violet B and 0.1 ml a suitable dilution of the enzyme in a final volume of 3 ml. Enzyme units (U) were arbitrarily defined as the amount of enzyme which produced 0.05 absorbance change at 500 nm min^{-1}.

All protein determinations were carried out by the Bradford's method using bovine serum albumine as standard.

Statistical analyses were performed using the Student's t-test (Bancroft 1960). Levels of significance are shown on tables.

RESULTS AND DISCUSSION

Owing to the fact that the *in vitro* system has the advantage that the conditions can be manipulated to maximize formation of metabolites and considering also that the liver is the organ with the largest metabolic capacity, the table 1 shows that 2,4-D butyl ester underwent hydrolysis to form 2,4-D by the action of esterase enzymes found in the one-day-old chick livers hatched from control and treated eggs. After separation of 2,4-D and 2,4-D butyl ester by TLC, with the following values of R_f 2,4-D (R_f=0.40), 2,4-D butyl ester (R_f=0.65) the radioactivity levels of these compounds did not show significant differences between chick livers hatched from control and 2,4-D butyl ester treated eggs. Therefore, approximately 70% of the radioactivity level was 2,4-D.

Table 1. *In vitro* metabolism of ^{14}C-2,4-D butyl ester by chick liver

	% Radioactivity recovered[a] as	
Treatment	2,4-D	2,4-D butyl ester
Control	71.33 ± 0.88	17.67 ± 0.89
2,4-D butyl ester	73.33 ± 2.40	20.67 ± 4.10

a: After separation by TLC.
Results are mean ± S.E.M. of 3 determinations.
For details see "Materials and Methods".

Considering that there was a discrepancy with our previous *in vivo* studies, where 2,4-D was none detectable in one-day-old chicks hatched from 2,4-D butyl ester treated eggs, we decided

to determine the esterase activities during embryonic development. The most striking finding was the inhibition of esterase activities of yolk from 2,4-D butyl ester treated eggs (Table 2). In fact, we found that the esterase activities of yolk eggs were diminished to values of 53% of control (on 1st incubation day) and 36% of control (on 3rd incubation day). Bearing in mind the kinetics of the penetration of 2,4-D butyl ester into egg (Castro de Cantarini et al. 1989), the level of 2,4-D butyl ester detected in yolk treated eggs was 1.55 ± 0.16 µg/g.

Table 2. Esterase activities of yolk eggs on 1st and 3rd incubation days.

Incubation days	Specific Activity (U/mg protein) Control	2,4-D butyl ester
1	3.70 ± 0.78	1.74 ± 0.37 [a]
3	1.05 ± 0.05	0.67 ± 0.15 [b]

Results are mean ± S.E.M. of 3 determinations.
[a] $p < 0.05$; [b] $p < 0.025$
Note: 2,4-D butyl ester was applied to fertile hen eggs before starting incubation.

Interestingly, when the specific enzyme activities were analyzed in tissues from 15-day-old chick embryos, we discovered that the esterase activities showed a diminution of 38% in liver and 15% in brain with respect to the controls. In addition, there were statistically differences in the weight of the tissues (Table 3), so the total enzyme activities were also diminished about 27% in liver and 18% in brain.

Table 3. Wet weight and Esterase activities in tissues from 15-day-old chick embryos.

Tissues		Wet Weight (g)	Specific Activity (U/mg protein)
Liver	Control	0.16 ± 0.01 [b]	110.46 ± 13.61 [a]
	2,4-D butyl ester	0.12 ± 0.008	68.04 ± 7.06
Brain	Control	0.30 ± 0.02 [c]	8.37 ± 0.36 [b]
	2,4-D butyl ester	0.16 ± 0.03	7.13 ± 0.53

Results are mean ± S.E.M. of 3 determinations.
[a] $p < 0.05$, [b] $p < 0.025$, [c] $p < 0.005$.

However, esterase activity determinations indicated that there were no statistically significant differences between tissues from chicks hatched from control and treated eggs. The largest enzyme activities were found in liver followed by kidney, brain and serum (Table 4). Taking into account that there were no differences in the weight of the different tissues (Duffard et al. 1987b, Mori et al. 1985), the total enzyme activity remained unchanged in the treated groups.

Table 4. Esterase activities in tissues from one-day-old chicks hatched from 2,4-D butyl ester treated eggs.

Tissues	Specific Activity (U/mg protein) Control	2,4-D butyl ester
Liver	207.27 ± 45.63	200.81 ± 35.39
Kidney	38.35 ± 6.20	28.50 ± 4.84
Brain	17.73 ± 1.66	16.10 ± 1.56
Serum	4.52 ± 0.57	3.36 ± 0.62

Results are mean ± S.E.M. of 3-4 determinations.

The present results, specifically the *in vitro* assay, imply that esterase enzymes found in chick liver are capable of hydrolyzing the 2,4-D butyl ester to 2,4-D. Moreover, the esterase activity is normal at the hatching time whereas it is diminished during embryonic development. In consequence of the normal esterase activity at the hatching time, we could not discard the possibility that some quantity of 2,4-D butyl ester was hydrolysed to 2,4-D without being detected in one-day-old chicks (Duffard et al. 1987b). This could be due to the fact that the newly produced 2,4-D was immediately bound to tissue macromolecules (Evangelista et al. unpublished results; Kelley and Vessey 1987) or it was rapidly metabolized being a transient metabolite. So, 2,4-D was nonextractable from the macromolecules by the extraction method used in the residue studies.

On the other hand, the phenoxyherbicides appear to have some effects on hepatic drug metabolism enzymes of chick embryo which can not be easily interpreted (Kelly and Vessey 1987; Santagostino et al. 1991). The latter also described vacuolization and biliary stasis of the chick hepatocyte by MCPA, therefore they suggest the inhibitory effect on microsomal enzymes result from the detergent effect of hepatic accumulation on bile acids, how Schaffner et al. (1971) informed. In our case, two possibilities to explain the toxic effects of the 2,4-D butyl ester on the chick embryonic development are feasible,

a) a direct action and b) an indirect, as it was suggested by Duffard et al. (1990) in muscles of chicks hatched from 2,4-D butyl ester treated eggs, where free fatty acids accumulation would play an important role in the 2,4-D butyl ester toxic mechanism, *per se* or by producing small lipid droplets and vacuolization. Furthermore, Argüello et al. (1990) demonstrated one Ca^{2+} homeostasis alterations suggesting that the primary effect could be an alteration of Ca^{2+} permeability.

Acknowledgments. This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Consejo de Investigaciones de la Provincia de Córdoba (CONICOR).

REFERENCES

Argüello JM, Evangelista de Duffard AM, Duffard RO (1990) Ca^{2+} homeostasis alterations induced by 2,4-dichlorophenoxyacetic butyl ester and 2,4-dichlorophenoxyacetic acid on avian skeletal muscle. Biochem Pharmacol 40:2441-2448

Bancroft H (1960) Introducción a la Bioestadística. EUDEBA, Buenos Aires, p 205-211

Bradford MM (1976) A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Castro de Cantarini SM, Evangelista de Duffard AM, Duffard RO (1989) Penetration studies and residue determinations of 2,4-dichlorophenoxyacetic acid butyl ester in fertile hen eggs and chicks hatched from treated eggs. Drug Chem Toxicol 12(2):137-146

Duffard RO, Traini L, Evangelista de Duffard AM (1981) Embryonic and teratogenic effects of phenoxyherbicides. Acta Physiol Latinoam 31:39-42

Duffard RO, Mori de Moro GB, Evangelista de Duffard AM (1982) Hatching and lipid composition of chicks brain from eggs treated with 2,4-dichlorophenoxyacetic butyl ester. Toxicology 24:305-311

Duffard RO, Mori de Moro GB, Evangelista de Duffard AM (1987a) Vulnerability of myelin development of the chick to the herbicide 2,4-Dichlorophenoxyacetic butyl ester. Neurochem. Research 12:1077-1080

Duffard RO, Fabra de Peretti AI, Castro de Cantarini SM, Mori de Moro GB, Arguello JM, Evangelista de Duffard AM (1987b) Nucleic acid content and residue determinations in tissues of chicks born from 2,4-Dichlorophenoxyacetic butyl ester treated eggs. Drug Chem Toxicol 10:339-355

Duffard RO, Argüello JM, Evangelista de Duffard AM (1990) Biochemical alterations in skeletal muscle induced by 2,4-dichlorophenoxyacetic butyl ester during chick embryonic development. Biochem Pharmacol 40:2433-2440

Erne K (1966a) Determination of phenoxyacetic herbicide residues in biological materials. Acta Vet Scand 7:77-86

Erne K (1966b) Studies on the animal metabolism of phenoxyacetic

herbicides. Acta Vet Scand 7:264-271
Evangelista de Duffard AM, Fabra de Peretti AI, Castro de Cantarini SM, Duffard RO (1991) Effects of 2,4-dichlorophenoxyacetic acid butyl ester on chick liver.(unpublished results)
Gomori G (1953) Human esterases. J Lab Clin Med 42:445-453
Horner J, Que Hee S, Sutherland R (1974) Esterification of (2,4-Dichlorophenoxy) acetic acid - A quantitative comparison of esterification techniques. Anal Chem 46:110-112.
Kelley M, Vessey D (1987) The effects of pretreatment with 2,3,7,8,-tetrachlororodibenzo-p-dioxin on the hepatic metabolism of 2,4,5,-trichlorophenoxyacetate (2,4,5-T) and 2,4-dichlorophenoxyacetate (2,4-D). Toxicol. and Applied Pharmacol 91:295-298
Matsumura F (1975) Toxicology of insecticides. Plenum Publishing Corp. New York, p 171
Mori de Moro GB, Duffard RO, Evangelista de Duffard AM (1985) Chick brain hypomyelination produced by 2,4-dichlorophenoxyacetic butyl ester . Neurotoxicology 6:133-138
Mori de Moro GB, Duffard RO, Evangelista de Duffard AM (1986) Changes in fatty acid composition of myelin lipids after 2,4-dichlorophenoxyacetic butyl ester. Neurotoxicology 7:173-180
Sanders HJ (1981) Herbicides: a special report. Chemical and Engineering News 59:20-35
Santagostino A, Leone MP, Maci R, Casale A, Marabini L (1991) Effects of phenoxyacetic acid herbicides on chicken embryo liver drug metabolizing enzymes. Pharmacol. and Toxicol. 68:110-114
Schaffner F, Hutterer F, Scharnbeck H, Sarkozi L, Bacchin PG, Denk H, Popper H (1971) Mechanism of cholestasis. 4. Structural and biochemical changes in the liver and serum in rats after bile aduct ligation. Gastroenterology 60:888-897
Schulze GE, Blake JW, Dougherty JA (1985) The metabolic fate of 2,4-dichlorophenoxyacetic acid n-butyl ester in the wistar rat. Arch Toxicol 57:231-236

Received September 9, 1991; accepted April 10, 1992.