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Teratogenesis of Polychlorocycloalkane Insecticides in Chicken Embryos Resulting from Their Interactions at the Convulsant Recognition Sites of the GABA (Pro)Receptor Complex

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There is an increasing concern for the effects of toxicants expressed during embryonic development. This paper considers the teratogenicity of polychlorocycloalkane insecticides. Polychlorocycloalkanes present a special toxicological problem due to their high environmental persistence and ability to accumulate in lipid tissues. They are neurotoxicants which act at the convulsant recognition site(s) of the GABA receptor-chloride ionophore (Lawrence and Casida, 1984) and antagonize GABA-mediated inhibitory neurotransmission. Since the convulsant [picrotoxinin or t-butylbicyclophosphorothionate (TBPS)] recognition sites develop early in embryogenesis with pharmacological properties similar to those in adult brains (Seifert 1988), the embryo is a vulnerable target for convulsive GABA antagonists, polychlorocycloalkanes included.

This study uses the chicken embryo as a model organism. It focuses on malformations related to interactions of polychlorocycloalkane insecticides with the convulsant recognition sites in the brain. Information provided by this study is important for investigations on the teratogenicity of insecticidal GABA antagonists in mammals.

MATERIALS AND METHODS

Polychlorocycloalkanes were provided by Dr. M. Sherman, Dept. Entomol. of Univ. Hawaii. Their identity and purity were examined with corresponding standards obtained from the EPA; endosulfan contained mostly β-isomer, chlordane 26% of α- and γ-isomers, other preparations more than 90% of an active component. [^{3}H]TBOB ([^{3}H]t-butylbicycloortho - benzoate) was obtained from Amersham Corp., IL., USA. Picrotoxinin and other chemicals were purchased from Sigma, MO., USA.

Test compound(s) in methoxytriglycol (triethylene glycol dimethyl ether) (20 µl) was injected into the yolk of fertile chicken eggs (Gallus domesticus, White leghorns, obtained from a local hatchery) (Seifert and Casida 1981) and the eggs were incubated at 37.5°C and 70-80% relative humidity. In a typical experiment, a dozen eggs per dose were injected on day 4-6 of incubation (the time optimal for producing the most severe embryonic deformities) and the embryos examined at days 16-18 for gross malformations. Control eggs were injected with carrier solvent; 20 µl of methoxy - triglycol did not produce any malformations. The candidate alleviating agents were administered together with the teratogen in methoxytriglycol solution.

Methoxytriglycol solution (20 µl) of varying picrotoxinin or endrin concentrations was administered intraperitoneally into 5-day old chicks. Chicks were observed for toxic effects for 24 hrs.

Embryonic brain membranes were prepared from whole brains (cerebra and cerebella) by sequential centrifugation (1,000 and 10,000xg) of a brain homogenate in EDTA, then dialyzed against water. 5 nM [^{3}H]TBOB was incubated with brain membranes for 30 min at 37°C. The binding of the radioligand to membranes was determined by the fast filtration method. Nonspecific binding was defined with picrotoxinin (32 µM) (Seifert 1988).

In vitro inhibitory potency of test compounds was determined in membranes prepared from brains of 16-day old embryos. Test compound (2 µl of dimethylsulfoxide solution with final concentration 10^{-7} M) was added to the reaction mixture containing only radioligand just before addition of brain membranes. The reaction mixture was incubated for 30 min at 37°C and the binding of the radioligand to membranes determined by the fast filtration method.

The effects of in ovo treatment of embryos with polychlorocycloalkanes and picrotoxinin on [^{3}H]TBOB binding to brain membranes were examined in preparations obtained from 16-day old embryos. The eggs were injected with test compound(s) on day 6 of incubation, brain membranes prepared from 16-day old embryos and the binding of [^{3}H]TBOB determined as described earlier.

Table 1. Malformations produced by polychlorocycloalkane insecticides, picrotoxinin and 3-mercaptopropionic acid

Compound (number of embryos)	Defects (% incidence)[a] major (≥ 5%)	minor (< 5%)
Endrin (151)	leg deformations (100) (micromelia, arthrogryposis)	edemas
	cervicocerebral edema (100)	crossed beak
	coelosoma (55)	encephalocele
	hemorrhages (30)	microsomia
	exophthalmos (5)	perocormus
		perodactylia
		wry neck
Other PC[b] (20 per compound)	leg deformations (40-90)	crossed beak
	coelosoma (19)	edemas
	exophthalmos (8)	encephalocele
	wry neck (7)	perocormus
picrotoxinin (83)	leg deformations (100)	edemas
	cervicocerebral edema (70)	hemorrhages
	coelosoma (18)	microsomia
	wry neck (7)	perocormus
3-mercapto-propionic acid (36)	cervicocerebral edema (60)	exophthalmos
		hemorrhages
		microsomia

The test compound (3 mg/egg except for 3-mercaptopropionic acid - 1.0 mg/egg) was injected into fertile chicken eggs on day 6 of incubation. The embryos were removed from the eggs on day 17 for examination of malformations.

[a] incidence for the control was ~ 1%; [b] aldrin, chlordane, dieldrin, endosulfan , γ-HCH, heptachlor

RESULTS AND DISCUSSION

Polychlorocycloalkane insecticides (3 mg/egg) cause leg deformations (arthrogryposis and micromelia), cervicocerebral edema, coelosoma, hemorrhages (primarily of the skull but also appearing over all the body) and exophthalmos in chicken embryos (Table 1). Endrin is a distinctly potent teratogen producing observable defects at 0.1 mg dose per egg. Deformations occuring with minor incidence are listed in Table 1. Embryo size (crown to rump), the length of legs and wings as measured on days 16-18, are reduced by endrin (3 mg/egg) to 92±4, 87±8 and 85±12% of control values, respectively. Other parameters, e.g. embryo, brain and liver weights are unaffected by endrin.

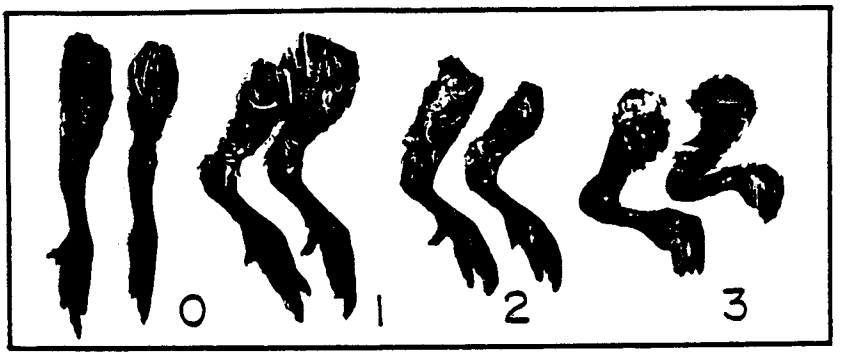

Figure 1. Leg deformations induced by endrin. Endrin was administered into fertile chicken eggs on day 5 of incubation. The embryos were removed from eggs on day 17 for evaluation. Based on severity of defects, leg deformations were scored from 1 (least severe) to 9 (most severe). 0-control, 1-2 points (0.5 mg endrin), 2-5 points (1.0 mg endrin), 3-8 points (3 mg endrin).

Leg defects and cervicocerebral edema were used in a semiquantitative evaluation of malformations. A scale from 1 (least severe) to 9 points (most severe symptoms) was used for evaluation of leg deformations while 1 to 2 (mild and severe) for cervicocerebral edema in embryos treated with 0.1-3 mg of endrin per egg (Figs. 1 and 2). The value of the scoring system is illustrated in its application for study of the timing of endrin administration (Fig. 3), or in a search for protective agents (Table 2).

The timing of endrin administration shows two important features (Fig. 3). First, the period for inducing significant embryonic defects is relatively large, from day 5 up to day 10 (the intensity of malformations produced at day 10 is about 70% of the maximal value at day 5). Second, there is a delay in development of malformations, i.e. seven days after endrin injection on day 5. Increased calcium metabolism (e.g., due to secretion of parathormone from day 10, or absorption of calcium from the shell from day 12) and leg flexing (Freeman and Vince 1974) are two events which may be associated with defects caused by endrin. Negligible changes are observed in embryos treated on day 13 of incubation.

The toxicity of endrin is low in chicken embryos during the preimplantation and organogenesis period (12% mortality at 3 mg of endrin/egg). Apparently, the GABA-dependent system which is also responsive to the benzodiazepine, diazepam, and to picrotoxinin (Wee and Zimmerman 1983) is not crucial for vital functions of the embryo at this stage.

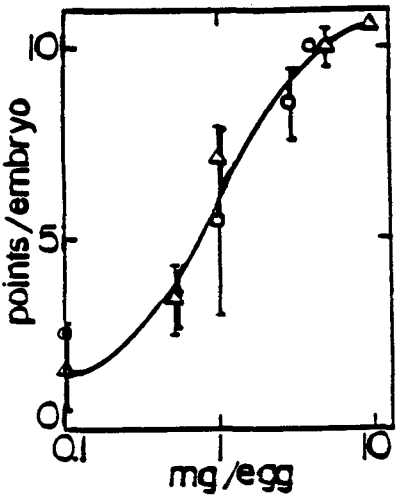

Figure 2. Effects of varying endrin and picrotoxinin concentrations on severity of malformations produced in chicken embryos. Endrin (o) or picrotoxinin (Δ) (0.1-10.0 mg/egg) was administered into fertile chicken eggs on day 5 of incubation. The embryos were removed from the eggs on day 17 for evaluation of leg deformations and cervicocerebral edema. A group of 12 embryos was evaluated for each dose with points averaged per embryo. The final score is the arithmetic average ± S.D. of five sets of independent experiments.

Table 2. Effects of various drugs for alleviation of endrin-induced malformations in chicken embryos

Compounds (mg/egg)	Malformations compound(endrin only) [points/embryo[a]]
Endrin(1)+riboflavin(6)	4.6(5.0)
Endrin(1)+nicotinamide(1)	4.6(4.9)
Endrin(3)+GABA(1)	9.2(9.2)
Endrin(3)+diazepam(2.6)	7.2±4.1(9.6±1.6)[b]
Endrin(3)+phenobarbital(3)	0.5±0.6(8.4±2.5)[c]
Endrin(3)+phenobarbital(1)	5.2±2.8(8.4±2.5)[c]
Endrin(3)+phenobarbital(0.5)	7.1±3.0(8.4±2.5)
Endrin(3)+phenobarbital(0.1)	6.3±2.9(8.4±2.5)

[a]See Fig. 1; values are arithmetic average±S.D. (shown for diazepam and phenobarbital) of evaluation of 9-12 embryos; [b]elimination of the most severe defects; [c]values are significantly different ($p < 0.02$)

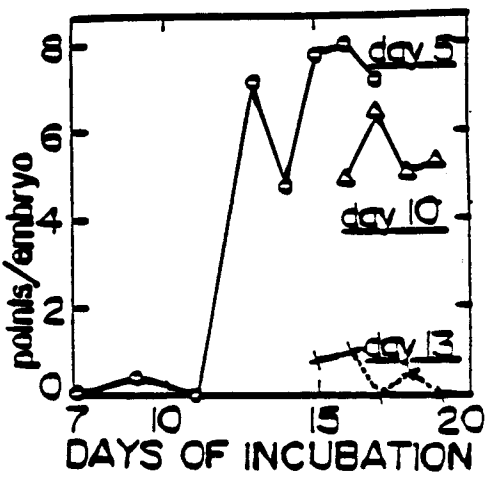

Figure 3. Effects of timing of endrin administration on severity of malformations produced in chicken embryos. Endrin (3 mg/egg) was administered into fertile chicken eggs on day 5, 10 or 13, respectively. Severity of leg deformities and cervicocerebral edema in chicken embryos were evaluated by using the scoring system (see Fig. 1 and 2) on indicated days of incubation.

Toxicity increases during late stages of embryonic development (to 50% mortality) and after hatching (LD_{50} 0.7-0.9 mg of endrin/kg), possibly in response to the onset of functional synaptic GABA-mediated neurotransmission. In 5-day old chicks endrin induces symptoms typical for convulsive GABA antagonists.

Three pieces of evidence link the malformations produced by endrin to its interactions with the embryonic GABA (pro)receptor complex. First, the model GABA antagonist picrotoxinin which acts at the convulsant recognition site (Ticku 1986) causes similar defects in chicken embryos with severity comparable to endrin (Fig. 2, Table 1). Bicyclophosphorous esters, other potent convulsive GABA antagonists, also cause leg abnormalities in chicken embryos (Casida et al. 1976). 3-Mercaptopropionic acid which inhibits GABA biosynthesis induces formation of cerebrocervical edema in chicken embryos. This suggests that the toxic effects of endrin may include more mechanisms than a direct interaction at the convulsant recognition sites, *e.g.* limited availability of GABA. Further evidence for involvement of the GABA receptor complex in the teratogenic action of endrin is provided by alleviation of endrin-induced

Table 3. Teratogenicity and acute toxicity of polychlorocycloalkane insecticides and picrotoxinin, and their effects on [^{3}H]TBOB binding to embryonic brain membranes

Compound (mg/egg)	Teratogenicity [points/embryo]	Acute toxicity [LD_{50}, mg/kg]	[^{3}H]TBOB [% of control] Bind.	Inhib.
Potent teratogens				
Endrin(3)	9.3±0.9	0.3-3.0	31±13[a]	88
Endrin(1)	5.5±2.5		59[b]	-
Endrin(0.5)	3.4±0.9		83	-
Picrotoxinin	8.5±0.5	3-8	106	11
Poor teratogens				
aldrin(3)	1.1	38-60	98	10
chlordane(3)	2.5	500-1100	-	12
dieldrin(3)	2.0±0.5	47	98	36
endosulfan(3)	2.2±1.4	76-240	110	0
γ-HCH(3)	1.5±0.7	40	-	16
heptachlor(3)	1.9±0.5	100-162	92±19	38[c]
Inactive as teratogens				
α-HCH(2.2)	0.4	200	-	11
β-HCH(3)	0.3	200	-	10

Teratogenicity - compounds were administered on day 6 of incubation and embryos removed on day 17 for evaluation-values are arithmetic averages±S.D. of two sets of independent experiments (each set 12 embryos) except for endrin and picrotoxinin (3-6 sets)

Acute toxicity - LD_{50}'s for endrin and picrotoxinin were determined in chicks (5-day old)-other values were taken from Lawrence and Casida (1984)

[^{3}H]TBOB binding - brain membranes (day 16) were prepared from embryos treated with a test compound on day 6

[^{3}H]TBOB inhibition - 10^{-7} M compound

[a] 5.1±0.8 μg endrin and 76±26 ng 12-ketoendrin/brain, yielding 70 nM endrin in membrane preparations

[b] 1.1±0.8 μg endrin/brain; [c] heptachlor-epoxide

malformations by phenobarbital and diazepam (Table 2). Both drugs are used for treatment of acute poisoning by convulsive GABA antagonists, e.g. chlorinated cyclodienes, with a mechanism involving interactions at the GABA receptor complex (Haefely and Polc 1986). The ineffectiveness of GABA and aminooxyacetic acid, an inhibitor of GABA degradation, was probably due to poor distribution and stability of GABA in the egg and embryo while use of aminooxyacetic acid was limited by its embryotoxicity. Neither of the two cofactors essential for the embryonic development, riboflavin and nicotinamide, ameliorated the defects caused by endrin. Thus, the mechanisms by which riboflavin alleviates deformed legs in chicks (Scott et al. 1976)

or the defects alleviated by nicotinamide (e.g., micromelia due to diminished NAD levels-Seifert and Casida 1981) do not appear relevant. Third, polychlorocycloalkane and picrotoxinin teratogenesis correlates with their acute toxicity in adult animals, i.e. endrin and picrotoxinin are much more toxic than the other compounds and distinctly potent as teratogens (Table 3). There is essentially the same discrepancy between teratogenic potency of endrin and picrotoxinin and *in vitro* affinity of the target recognition sites for the toxicants as in the case of their acute toxicity. While endrin and picrotoxinin are equally teratogenic in chicken embryos, the affinity for endrin binding in embryonic brain membranes is more than thirty times higher than that for picrotoxinin (Seifert 1988).

Binding of [^{3}H]TBOB or [^{35}S]TBOB to embryonic brain membranes during the critical period for development of malformations is very low (Seifert 1988). Therefore attempts have been made to correlate teratogenicity with binding of the radioligand to brain membranes prepared from *in ovo*-treated 16-17 day old embryos. Except for endrin, polychlorocycloalkane insecticides and picrotoxonin do not alter the binding capacity of brain membranes (Table 3). In embryos treated with varying amounts of endrin, [^{3}H]TBOB binding was reduced in a manner dependent on the dose of endrin administered into the eggs. However, GLC analysis revealed residual endrin and its metabolite 12-ketoendrin in brains and brain membrane preparations (Table 3) indicating that the residual endrin is the cause of the diminished radioligand binding rather than alteration of the convulsant recognition sites. Accordingly, *in ovo* treatment with phenobarbital at doses which alleviated the endrin-induced malformations did not alter the reduced [^{3}H]TBOB binding caused by residual endrin. Endrin is also the most potent *in vitro* inhibitor of [^{3}H]TBOB binding to embryonic brain membranes, followed by heptachlor epoxide and dieldrin (Table 3).

The results obtained with chicken embryos provide basic information for extending this research to mammals due to the similarity in pharmacological properties of convulsant recognition sites within animal species (Cole et al. 1984). The role of the GABA (pro)receptor complex in embryonic development prior to establishment of the neural synaptic systems deserves special attention in continuing research. A variety of insecticides which act at the GABA receptor complex will be investigated in further efforts to understand the mechanisms of their toxic action and assessment of their toxicity for humans.

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