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Perspective

Aldrin epoxidation and dihydroisodrin hydroxylation as probes of *in vivo* and *in vitro* oxidative metabolic capability of some caterpillars

Robert I Krieger*

Personal Chemical Exposure Program, 10 Chapman Hall, Department of Entomology, Environmental Toxicology Graduate Program, University of California, Riverside, CA 9252, USA

Abstract: Comparative biochemical studies are productive means to study factors that limit both beneficial and harmful effects of chemicals. Reactions such as aldrin epoxidation and dihydroisodrin hydroxylation are valuable assays of oxidative metabolism in scientific studies of chemical biology in insects, subhuman primates and other living things. The tissue distribution of activity in caterpillars may have functional significance. Localization of relatively high concentrations of these cytochrome P450 monooxygenases in gut tissue of lepidoptera may represent an important means to minimize absorption of lipophilic foreign chemicals in food. Some polychlorocycloalkanes permit *in vivo* and *in vitro* studies owing to their stability, acceptable toxicity and relatively simple pattern of metabolism. *In vivo* studies to assess the significance of *in vitro* findings are feasible with substrates such as aldrin, dihydroisodrin (DHI) and oxidative methylenedioxyphenyl inhibitors such as piperonyl butoxide (PBO) or carbon monoxide. Biphasic dose-dependent decreased and increased DHI-OH formation resulted from PBO pretreatment by gut, fat body, head and Malpighian tubule homogenates of cutworms and gut and fat body (the only tissues tested) of cabbage looper *Trichplusia ni* (Hübner) and black cutworm *Agrotis ipsilon* (Hüfnagel). The biphasic *in vivo* responses of caterpillars to PBO are a reminder of the complexity of biochemical and physiological responses of organisms coexposed to chemicals that are classified, often glibly, as *toxic substances* and *metabolic inhibitors and inducers*. Knowledge of dose and time relationships demands very careful evaluation in living things in the environment.

Keywords: aldrin; dihydroisodrin; piperonyl butoxide; cytochrome P450; epoxidation; xenobiotic metabolism; Lepidoptera

1 INTRODUCTION

The synthesis of chlorinated cyclodienes, the discovery of their toxicities, cyclodiene insecticide use in treatment and control of pests, persistence of residues in air, water, soil and biota, including humans, and the politics of pollution all continue to make the polychlorocycloalkanes rich resources for those who study pesticide science and toxicology.[1] The present objective is to show how some of Gerry Brooks' sterling contributions to pesticide science have enabled some of the research with which the author is familiar – there are, of course, many other studies and advances that are citable.

The environmental stability of insecticidal cyclodienes and pest management issues stemming from insecticide resistance and later insecticide synergism were part of the research of Gerry Brooks and colleagues at the Agricultural Research Council, Pest Infestation Laboratory, Slough, United Kingdom. There, Ray[2] and Lewis[3] unequivocally demonstrated the cytochrome P450 dependence of aldrin epoxidation in houseflies. The microsomal localization of an NADPH-oxygen dependent aldrin epoxidase was reported by Ray.[2] Carbon monoxide inhibition was shown, implicating cytochrome P450. Lewis took advantage of houseflies' tolerance of carbon monoxide atmospheres in a classic series of *in vivo* experiments.[3] He showed that dieldrin formation was inhibited following topical application of aldrin, elegantly demonstrating the cytochrome P450 dependence of aldrin epoxidation. Wilkinson continued his earlier research on methylenedioxyphenyl synergism of insecticides at Slough and used aldrin at Cornell University as an important monooxygenase substrate. A series of

* Correspondence to: Robert I Krieger, Personal Chemical Exposure Program, 10 Chapman Hall, Department of Entomology, Environmental Toxicology Graduate Program, University of California, Riverside, CA 92521, USA
E-mail: bob.krieger@ucr.edu
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studies at Cornell that investigated the properties of microsomal monoxygenases in several insect species were ultimately published.[4,5]

Brooks periodically visited Wilkinson's Cornell laboratory as an extension of their earlier collaboration and friendship. In 1968, aldrin epoxidation and *p*-chloro-*N*-methylaniline demethylation were being used as cytochrome P450 dependent substrates in studies of the oxidative metabolic capability of the southern armyworm *Spodoptera eridania* (Stoll) (formerly *Prodenia eridania*). At that time, all attempts to measure hydroxylation using aniline as a substrate had been unsuccessful. Earlier, Brooks and Harrison[6] reported that sesoxane, a methylenediphenyl insecticide synergist, inhibited housefly dihydroisodrin hydroxylation both *in vitro* and *in vivo*. Brooks suggested that dihydroisodrin (DHI), which lacked the olefinic double bond, would be monohydroxylated in the 6-(7-)position. The reduced substrate was prepared, and the monohydroxylated product (DHI-OH) was isolated and characterized.[7]

2 SUBSEQUENT WORK

The simple metabolism of aldrin and DHI, the stability of the reaction products and the relative ease of analysis of dieldrin and DHI-OH by electron capture gas chromatography make these cyclodiene derivatives useful research tools. Three additional aspects related to these early studies will be overviewed in this section.

2.1 Selection and optimization of sources of monooxygenase activity

Xenobiotic disposition processes, including oxidative metabolism, can profoundly limit the biological activity of chemicals in living things. Comparative biological studies are productive means to study factors that limit both beneficial and harmful effects of chemicals. Reactions such as aldrin epoxidation and DHI hydroxylation (Fig. 1) are valuable tools in scientific studies of chemical biology. Availability of purified and stable starting materials and products, simple metabolism, specificity and sensitivity of analysis and responsiveness of the cytochrome P450 monooxygenases to known inducers and inhibitors add value to polychlorocycloalkanes as these tools in research.

Since simple tissue (or 'crude') homogenates and microsomal preparations are suitable enzyme sources that require minimal amounts of tissue, relatively small numbers of organisms at particular life stages are required. This has been especially useful in comparative studies with insects. Insecticide biochemistry and toxicology research progress, coupled with progress in general biochemistry and pharmacology, has rapidly advanced understanding of disposition processes during the past 50 years as investigators utilized similar methods and techniques.

The early studies at Cornell with caterpillars had a corollary with respect to the use of small tissue samples and aldrin and DHI as substrates.[4,7] Studies in subhuman primates at the University of California, Davis, took advantage of both the small tissue requirement and cyclodienes as monooxygenase substrates.[8,9] Rhesus monkeys, *Macaca mulatto* (Zimm.), were given measured dosages of ethanol, phenobarbital and DDT during 6 years of studies of the metabolic capability of liver. Under ketamine anesthesia, monkey liver needle biopsies (yielding about 10 mg tissue) served as enzyme source for

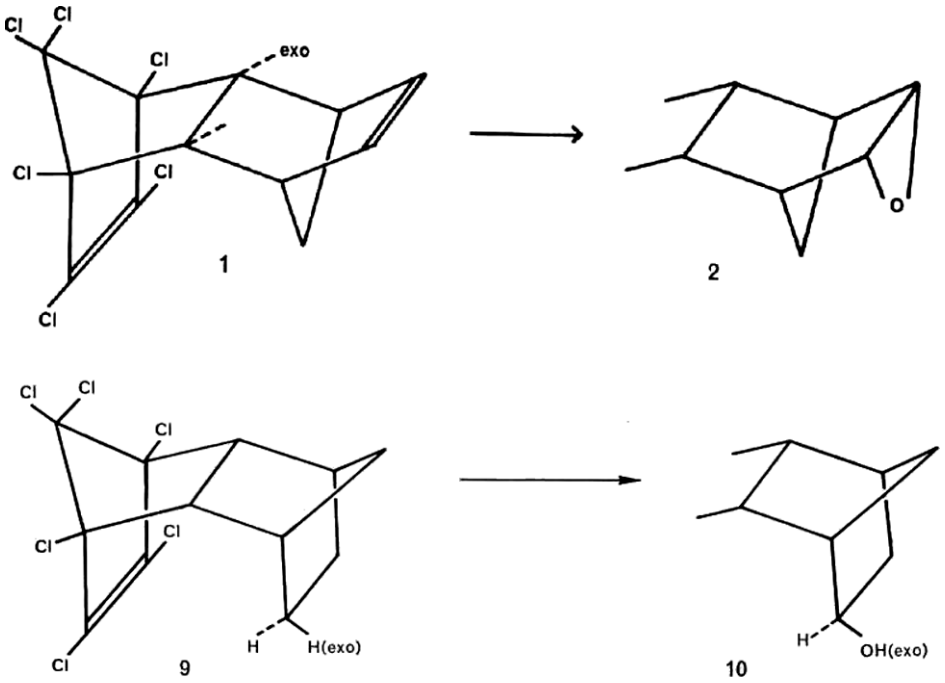

Figure 1. Aldrin (**1**) epoxidation and dihydroisodrin (**9**) hydroxylation reactions catalyzed by monooxygenases can be used to estimate the oxidative metabolic capability of insects and many other living things.

aldrin epoxidation, dihydroisodrin hydroxylation and *p*-chloro-*N*-methylaniline demethylation assessments of P450-catalyzed oxidations. During this extended period of study, which involved multiple drug and DDT treatments, the animals served as their own controls. This preserved an invaluable human model for metabolic and toxicology research.

2.2 Distribution of activity may have functional significance in caterpillars

Localization of relatively high concentrations of cytochrome P450 monooxygenases in gut tissue may represent an important means to minimize absorption of lipophilic foreign chemicals in food.

In general, in lepidopterous larvae, including the well-studied southern armyworm, cytochrome P450 monooxygenase activities are most concentrated in gut tissues.[4,7] There are numerous known examples of lepidopterous larvae with the highest P450 monooxygenase activities in their gut tissues.[10,11] More recent studies concerning the tissue distribution of aldrin epoxidation activity and cytochrome P450 in cotton bollworms *Helicoverpa armigera* (Hübner)[12] also indicate that midgut preparations are better enzyme sources than fat body, another useful enzyme source in some lepidoptera.[10,13,14] Gut and fat body are active enzyme sources in dogwood sawfly *Macremphytus varianus* (Norton) (Hymenoptera)[15] and in aquatic caddis fly larvae (Tricoptera).[16] A similar distribution has also been reported in *Pergagrapta polita* (Leach) sawfly feeding on two *Eucalyptus* sp.[17]

Whether the pattern of distribution in these insect larvae represents preparative biochemical convenience or whether high specific activity in gut tissue is truly of functional significance should not be assumed. Available evidence indicates that, in caterpillars, the gut probably has a first-pass role in protecting caterpillars from accumulation of lipophilic chemicals in ingesta. More convincing data concerning the *in vivo* functions of monooxygenase systems in the disposition of endogenous chemicals, including secondary plant substances in their native states, should be sought. This type of research may take advantage of the physical and biological properties of some stable polychlorocycloalkane biomarkers in future research.

On the basis of the concentration of aldrin epoxidation activity in the midgut of lepidopterous larvae, Krieger *et al*.[11] suggested that the gut monooxygenase systems might be a determinant of the oxidative metabolic capability of lepidoptera. Data in Table 1 have been augmented by additional data collected at UC Davis by Thongsinthusak using DHI.[18]

Caterpillars with a more limited host plant range were found to have lower oxidase activity in gut tissue homogenates than larvae that were known to feed upon more plant families. Later research demonstrated enzyme induction as an additional feature of potential importance in plant herbivory.[13]

Multiple forms of cytochrome P450 are known in insects. Their roles in insecticide resistance, tolerance of secondary plant substances and metabolism of xenobiotics have been demonstrated or hypothesized. This subject is too vast to be further addressed here.

2.3 *In vivo* studies to assess the significance of *in vitro* findings are feasible with substrates such as aldrin, dihydroisodrin (DHI) and oxidative metabolic inhibitors such as piperonyl butoxide (PBO)

PBO is the best-known methylenedioxyphenyl insecticide synergist. In addition to its importance as an investigative tool in toxicity testing and biochemical studies of P450-dependent processes, PBO is of increasing economic importance as a pyrethrin and pyrethroid insecticide synergist. It is coadministered with pyrethrins and pyrethroid insecticides, where PBO to insecticide mole ratios vary, for example, from 2.5 (Permanone® mosquito adulticide) to 13.5 (0.33% synergized pyrethrins for head lice) respectively. Table 2 lists the mole ratios of several commercial synergized pesticides as well as some levels

Table 1. Gut aldrin epoxidase or dihydroisodrin hydroxylase activity of caterpillars: a possible role in defense against secondary plant chemicals

	Number of host plant families		
Caterpillar family (species represented)	Monophagous 1	Oligophagous 2–10	Polyphagous 11 or more
Saturniidae (6)	0	4	2
Lasiocampidae (2)	1	0	1
Geometridae (1)	0	1	0
Sphingidae (1)	1	0	0
Notodontidae (1)	1	0	0
Lymantridae (1)	0	0	1
Noctuidae (27)	3	18	6
Arctiidae (4)	0	2	2
Nymphalidae (1)	1	0	0
Danaidae (1)	1	0	0
Pyraldiae (1)	1	0	0
n	9	25	12
Mean oxidase pmol mg^{-1} protein min^{-1} ($N = 2$ or more per family)	19.9	86.4	294.4

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Table 2. Piperonyl butoxide occurrence in selected products

Source	Occurrence	Piperonyl butoxide (%)	Pyrethroid	Approximate mole ratio	Response
Drione	Structural pest control	10	1% pyrethrins	11	Synergistic
Scourge	Mosquito adulticide	54	18% resmethrin	3	Synergistic
Permanone® 31–66	Mosquito adulticide	66	31%	2	Synergistic
Pyrenone 5–25	Mosquito adulticide	2.5	0.5% pyrethrins	6	Synergistic
RID® maximum strength	Head lice medication	4	0.33% pyrethrins	13	Synergistic
Thongsinthusak and Krieger[18]	Experimental *in vivo* and *in vitro* black cutworm and cabbage looper	–	–	0.01	Inhibition and induction

of synergists used in experimental studies and others detected in environmental sampling. The high ratios of synergist to active ingredient found in commercial products do not occur by happenstance. They are required to obtain 'synergism' – increasing the toxicity of the insecticide with which it is coadministered under normal conditions of use.

In sharp contrast, PBO was a much more potent inducer than inhibitor of DHI hydroxylation in comparative studies with two species of Lepidoptera. The following summary of a series of experimental studies reveals an additional biological activity of PBO that may be of importance in long-term, low-level PBO exposures that may occur in the environment.

The responses to PBO of black cutworm *Agrotis ipsilon* (Hüfnagel) and cabbage loopers *Trichoplusia ni* (Hübner) were time and dose dependent and biphasic with respect to enzyme inhibition and induction.[14,18] *In vitro*, the PBO I_{50} of DHI hydroxylation (DHI-OH) by gut homogenate was 6.4×10^{-5} M. Caterpillars of each species were treated topically with three dosages of PBO ranging from 0.2 to 2.7 $\mu g\,g^{-1}$. After 0, 2, or 3 h, 5 h, 15 h and 25 or 30 h, tissue homogenates of gut, fat body, Malpighian tubules and head capsules were prepared. Transient, modest inhibition was time and dose dependent in both species at the first time interval. Following PBO, about 50% inhibition of *in vitro* DHI-OH was measured in tissue homogenates. The inhibition may have resulted from residual PBO in the homogenates.

Much more dramatic, dose-dependent increases in DHI-OH formation resulted from PBO pretreatment by gut, fat body, head and Malpighian tubule homogenates of cutworms and gut and fat body (the only tissues tested) of cabbage loopers. The greatest relative increases in activity were measured in gut (92%) and fat body (147%) homogenates of cutworms. The greatest induction was observed *in vitro* in loopers, in which specific activity was 1600% greater in gut homogenates of PBO-treated loopers than in acetone-treated controls. These responses of lepidopterous larvae were also assessed *in vivo* in concurrent studies using DHI and PBO.

The *in vivo* fate of topically applied, fed and intracoelomically injected DHI was assessed in control and PBO-pretreated larvae. The extent of DHI-OH formation was greater in cutworms both *in vivo* and *in vitro* at dosages (14–1400 μg DHI g^{-1}) that produced no adverse effects. Intracoelomic injection yielded the greatest extent of metabolism in cutworms [49%, the lowest dosage with virtually quantitative recovery (DHI + DHI-OH)]. At 140 $\mu g\,g^{-1}$, feeding, topical application and injection yielded respectively 9.4, 15.1 and 55.9 nmol DHI-OH. Three hours after PBO (2.7 $\mu g\,g^{-1}$) injection, DHI metabolism was reduced 26%, but after 15 h DHI-OH was 25% greater than untreated controls ($n = 4$; $P < 0.005$). This biphasic inhibition and stimulation of DHI metabolism was much less than the response seen with tissue homogenate-catalyzed hydroxylation, but PBO induction was unexpected. PBO induction and inhibition have been studied in mammals,[19] but possible biphasic responses have been virtually ignored in insects.[18,20] Lower levels of aldrin epoxidation were not induced by PBO diets (0.05% w/w) in fall armyworm *Spodoptera frugiperda* (Smith JE) larvae.[20] Further study of time–PBO concentration relationships will be needed to determine responses of insects to coexposures to PBO and other environmental chemicals.

In these experiments, the mole ratio of PBO to DHI was only 0.01, the reciprocal of some synergist to insecticide ratios of commercial mixtures (Table 2). This dosage of PBO, which biphasically altered both *in vivo* and *in vitro* DHI metabolism, did not alter the acute toxicity of either carbaryl or carbofuran to the caterpillars. The oxidative metabolic capability of larvae measured using DHI paralleled the *in vitro* observations. The results require further investigation of the biology of monooxygenase activity in insects exposed to low doses of PBO and other chemicals under environmental conditions.

3 THE FUTURE

These scientific studies in which the cyclodienes have been extensively used provide strong evidence of the dynamic oxidative metabolic capability of some Lepidoptera and living things in general. Ever-expanding lists of substrates transformed by oxidations catalyzed by isoforms of Omura and Sato's cytochrome P450[21] now number in the hundreds.[22] Monooxygenases are of ubiquitous distribution among living things.[22] When drugs, pesticides and other

chemicals of economic importance are considered, the role of the enzyme systems is readily made apparent. Clear evidence of their evolutionary importance and function in the disposition of endogenous chemicals is much less clear with issues such as synergism and resistance attributable to changes in metabolic capability. Substrates such as aldrin and DHI can be used *in vivo* and *in vitro* by experimental biologists who seek better understanding of important homeostatic mechanisms that include chemical disposition processes.

The biphasic *in vivo* responses of caterpillars to PBO are a reminder of the complexity of biochemical and physiological responses of organisms coexposed to chemicals that are classified, often glibly, as *toxic substances* and *metabolic inhibitors and inducers*. Knowledge of dose and time relationships demands very careful evaluation in living things in the environment. Currently there is great concern over environmental exposures of aquatic organisms to insecticide synergists and pyrethroid insecticides. Piperonyl butoxide is currently only being considered 'for its ability to inhibit the mixed-function oxidase enzymes, blocking natural detoxification pathways'.[23] That viewpoint is understandable but may be irrelevant to what occurs when low synergist to substrate ratios are encountered by organisms in sediments, based upon findings with PBO and DHI.[18] In fact, PBO at environmental levels might enhance the breakdown of chemicals such as pyrethroids.

Environmental toxicologists and other investigators must be encouraged to adopt a more Koch-like rigor.[24] Speculation about health, which lacks an ample scientific foundation, may launch media, players-of-the-internet and susceptible parts of the regulatory community on hazard-based missions linked to the occurrence of detectable (or even measurable) chemicals in air, water or sediment. The link to causation, before launching the scientific and regulatory communities' societal responses, based upon environmental monitoring alone, is frequently driven by incomplete data (when much more complete data are possible). The fabled Chicken Little concern about a falling sky is easily recognized in many contemporary responses to long lists of uncertainties based upon hazard identification studies and preliminary range finding. The practice results in a hoary reality created by media and other mongering opportunists. And in short order, advocates and campaigners (including grant writers of many scientific persuasions) pressure funding agencies and decision-makers for regulatory action. It remains paradoxical that the benefits that are sought can never be demonstrated, as they represent dosages that produce (or are *associated* or *linked with*) 'no observed adverse effects!'. What more could be asked?

This contribution closes with an important but less well-known reminder from Professor Ernest Hodgson,[25] taken from the author's hard-bound copy of *Enzymatic Oxidations of Toxicants*, the proceedings of a 1967 meeting at North Carolina State University. In opening the meeting, he called to mind that 'After the organism has been reduced to its constituent molecules, it is necessary to put it back together again . . .'. The cyclodienes are tools of continuing value and proven probes in scientific studies to understand the disposition of chemicals in living things. Gerry Brooks, his many colleagues and coworkers and numerous other investigators have used and continue to use cyclodienes or polychlorocycloalkanes in scientific research in comparative toxicology. As always, our imaginations, energies and means are the only limitations of the future.

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