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2 February 1980; revised 5 May 1980

Metabolism of Propachlor by the Germfree Rat

Abstract. *Three major metabolites of propachlor were isolated from the excreta of germfree rats given ^{14}C-labeled propachlor orally. In contrast, 11 urinary metabolites, six of which were 2-methylsulfonylacetanilides not present in excreta of germfree rats, were isolated from control rats given ^{14}C-labeled propachlor orally. Enterohepatic circulation and microbial metabolism in the intestine were necessary for production of the methylsulfonyl-containing and other metabolites of propachlor in the conventional rat.*

The metabolism in vivo of several xenobiotics to methylthio-, methylsulfoxyl-, and methylsulfonyl-containing metabolites has been reported (*1*), and metabolic pathways for their production have been proposed. DeBaun *et al.* (*2*) described one pathway in which methionine supplied the methylthio group in the formation of methylmercapto-2-acetylaminofluorenes. Sumino and Mio (*3*) proposed a similar pathway in which methionine reacted with an arene oxide to form methylsulfide-containing metabolites from 2,5,2′,5′-tetrachlorobiphenyl. Colucci and Buyske (*4*) and Tateishi *et al.* (*1*) showed that rat liver contained an enzyme system, cysteine conjugate β-lyase, that produced thiols from the cysteine conjugates of certain xenobiotics. Tateishi *et al.* showed that the corresponding methylthiols were formed when liver microsomes and *S*-adenosylmethionine were added to their β-lyase system.

Propachlor (**1**, Fig. 1) is also metabolized to methylsulfonyl-containing metabolites by the rat (*5*). Studies on the metabolism of this herbicide in rats have indicated that an intestinal *C-S* lyase participated in the formation of the methylsulfonyl-containing metabolites that were excreted in the urine (Table 1).

In control rats, propachlor was metabolized to 11 urinary metabolites and to fecal residues that could not be solubilized by common solvents or by treatment with dilute acid or base (*5*). The intractable characteristics of these fecal residues indicated that the metabolites were covalently bound. The major urinary metabolite was the mercapturate; six of the metabolites (23 percent of the dose) were 2-methylsulfonylacetanilides. In a separate study, the sulfur from the cysteine and glutathione conjugates of propachlor was shown to be a precursor to the methylsulfonyl sulfur (*6*).

Rats with cannulated bile ducts secreted 67 percent of oral doses of propachlor in the bile as the glutathione conjugate (**2**), the cysteine conjugate (**3**), the mercapturate (**4**), and the mercapturate sulfoxide (**5**); no other ^{14}C-labeled metabolites were detected in the bile (*7*). The discrepancy between the quantities of ^{14}C secreted in the bile of cannulated rats and that excreted in the feces by the conventional rats indicated that the biliary metabolites must have been reabsorbed from the intestine and therefore that enterohepatic circulation of the ^{14}C from the precursors of the mercapturate (*8*) or from mercapturate (or both) participated in the metabolism of propachlor in rats. The identities of the biliary metabolites showed not only that the reabsorbed ^{14}C came from the mercapturate and its precursors, but also that these conjugates were available as substrates for the intestinal flora. Therefore, the metabolism of propachlor was studied in germfree rats and the results were compared with those obtained from control rats to determine what effect the absence of the intestinal flora would have on the metabolic fate of this compound (Table 1).

The germfree rats (*9*) given [^{14}C]-propachlor orally excreted 98.6 percent of the dose in the urine and the feces within 48 hours. Three metabolites were isolated from the excreta, and the fecal radioactive metabolites were water-soluble. The major metabolite was the mercapturate (**4**, Fig. 1), which accounted for 66.8 percent of the dose. The cysteine conjugate (**3**, Fig. 1) was present only in the feces and was the major fecal metabolite. The other metabolite (15.1 percent of the dose) was assigned structure **5** (Fig. 1) from mass spectral data (*10*). This mercapturate sulfoxide is not limited to germfree rats because it has also been isolated from rat bile and from the excreta of chickens given propachlor orally (*7*). The metabolism of propachlor in germfree rats is summarized in Fig. 2.

All of the propachlor was metabolized by the mercapturic acid pathway in the germfree rat. No methylsulfonyl-containing metabolites were formed, and none of the other metabolic transformations of the propachlor molecule found in the control rat had occurred. These transformations included aromatic and aliphatic hydroxylations, *N*-dealkylation, amide hydrolysis, and glucuronide formation. We concluded that enterohepatic circulation and metabolism of the

Table 1. Comparison of the excretion of single oral doses of [^{14}C]propachlor by control rats, control rats with fistulated bile ducts, and germfree rats.

Metabolite	Recovery of ^{14}C (percent of dose)				
	Control		Bile-fistulated	Germfree	
	Urine	Feces	Bile	Urine	Feces
Glutathione conjugate (**2**)*			37		
Cysteine conjugate (**3**)			13		19
Mercapturate (**4**)	17		12	63.1	3.7
Mercapturate sulfoxide (**5**)			4	5.7	9.4
Nonextractable residues		19			
Other metabolites	51†				
Total	68	19	66	68.8	32.1

*Metabolite designations are those used in the figures. †At least ten; see (*5*). Blank spaces indicate that none of the labeled metabolite was detectable.

0036-8075/80/1024-0433$00.50/0

mercapturate or its precursors by the intestinal flora were necessary for the production of the methylsulfonyl-containing metabolites and the other metabolites of propachlor that were excreted by the control rat. From studies with the germfree rat, we propose the pathway outlined in Fig. 3 to describe the metabolic fate of propachlor in control rats.

The tissue in which propachlor enters the mercapturic acid pathway has not been determined. The liver is an obvious site for the glutathione conjugation, but the intestinal mucosa cannot be disregarded because everted sacs of rat small intestine convert propachlor to the cysteine conjugate in vitro (*11*), and preparations of rat gastrointestinal mucosa have been shown to contain a glutathione-*S*-transferase system capable of using xenobiotics as substrates (*12*).

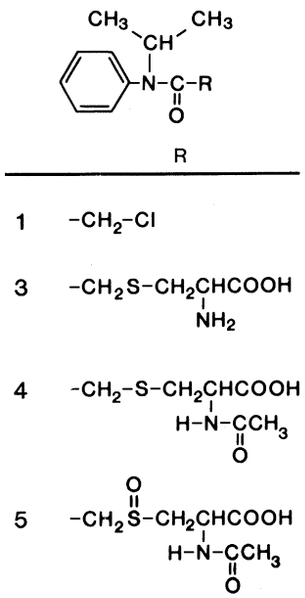

Fig. 1. Structures of propachlor (**1**), the cysteine conjugate (**3**), the mercapturate (**4**), and the mercapturate sulfoxide (**5**).

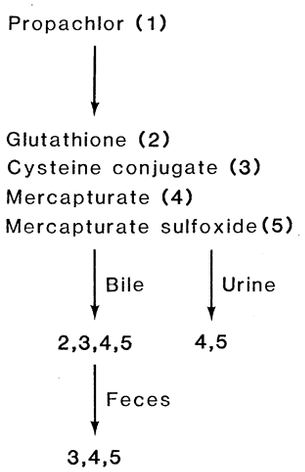

Fig. 2. Summary of the metabolism of propachlor in germfree rats.

In the first cycle of the enterohepatic circulation, the mercapturate and its precursors are secreted in the bile and become substrates for the intestinal microflora. The product of the microbial metabolism (possibly 2-thiolo-*N*-isopropylacetanilide) then proceeds by two pathways, one part reacting to form the metabolite (possibly 2-methylthioisopropylacetanilide) that is reabsorbed into the blood, and the other part producing the nonextractable residues that are excreted with the feces.

The binding of the radioactive carbon from propachlor in the feces was attributed to microbial metabolism because, in the absence of the intestinal microflora, only water-soluble metabolites from the mercapturic acid pathway were present. If the biliary secretion of propachlor metabolites in the germfree rat is qualitatively similar to that in the control rat, the presence of the three water-soluble fecal metabolites indicates that a system is present in the germfree intestine that is capable of releasing the glutamyl and glycyl residues from the glutathione conjugate of propachlor, the glutathione conjugate being the major biliary metabolite. Also, if the first-pass metabolism in germfree and control rats is quantitatively similar (70 percent of the dose being secreted in the bile), there must also be reabsorption of the mercapturate and its precursor from the gut of the germfree rat, because only 30 percent of the dose was excreted in the feces.

In the second cycle of the enterohepatic circulation, the reabsorbed microbial metabolites are further metabolized to form, in part, the two glucuronides (**6** and **7**) shown in Fig. 3. These glucuronides were secreted in the bile (31 percent of the dose) when the cysteine conjugate was given orally to control rats with cannulated bile ducts (*7*). These biliary glucuronides subsequently initiate the third cycle in the enterohepatic circulation of propachlor metabolites after the release of the aglycones by microbial glucuronidase systems. In the simulated third cycle, 65 percent of an oral dose of the aromatic glucuronide (**6**, Fig. 3) was secreted in the bile, again as glucuronide **6** (*7*).

This study of the metabolism of propachlor in germfree rats shows that the presence of the intestinal flora in the control rat greatly complicates the metabolism of this herbicide. The flora, in effect, creates from the products of the mercapturic acid pathway new nonpolar compounds that are reabsorbed into the blood. These new compounds must again be converted to polar compounds so they can be excreted. The subsequent metabolism to form these excretable compounds requires at least two more cycles in the enterohepatic circulation system and metabolism of all parts of the original propachlor molecule.

Our results show that the concept of xenobiotic detoxication by conjugation with glutathione must be modified to include the microbial metabolism of those mercapturates and mercapturate precursors secreted in the bile. This consideration is especially important for carcinogenic xenobiotics, because the metabolic pathway we described leads to the production, in the colon, of absorbable metabolites that must again be detoxified. These metabolites represent new

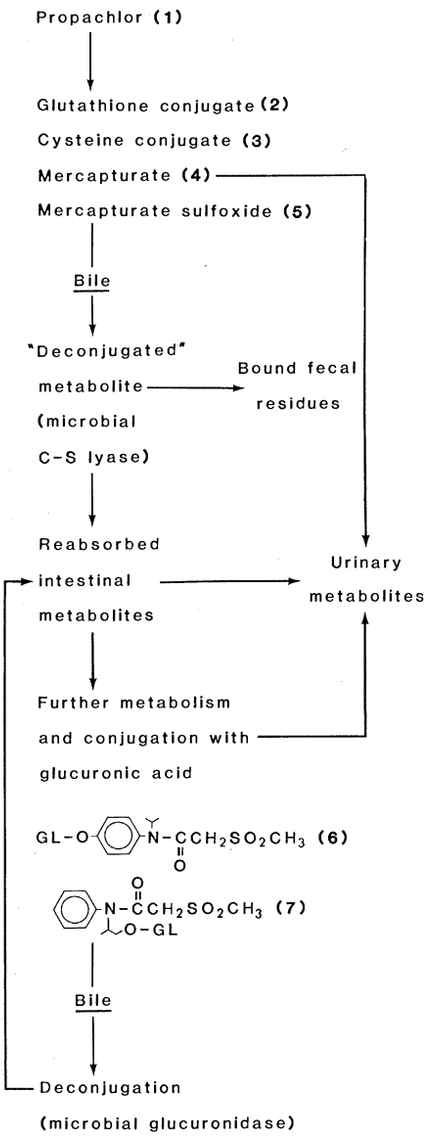

Fig. 3. A diagrammatic representation of the metabolism of propachlor in control rats. Metabolites **6** and **7** are glucuronides.

xenobiotics which, if they are carcinogenic, could act directly in the colon or, after reabsorption, in other tissues. The significance of the microbial production of carcinogens in the colon has been discussed (*13*).

The presence of this pathway for the metabolism of mercapturate-forming xenobiotics shows that a complete study of the metabolic fate of a compound should include a determination of the biliary secretion of its metabolites and study of its metabolism in germfree animals. The study in the germfree animal would define the role of the flora in the metabolism of the compound and would provide the ultimate model for determination of the effect that antibiotic feeding or therapy could have on the metabolic fate of xenobiotics. These studies could be of practical importance where antibiotics are added to animal feed and to patients on antibiotic therapy. Rats treated with antibiotics show measurable germfree characteristics for prolonged periods after antibiotic administration (*14*).

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1 February 1980; revised 4 June 1980

Acetylcholine Synthesis by Displaced Amacrine Cells

Abstract. *The ganglion cell layer of the rabbit retina contains neurons that synthesize acetylcholine. To identify these neurons, the ganglion cells were labeled by retrograde transport of a fluorescent dye, and the acetylcholine-synthesizing cells of the same retinas were labeled by exposing the tissue to tritiated choline. Autoradiographs inspected by fluorescence microscopy showed that tritiated acetylcholine and the dye accumulated in different cells. Optic nerves of other animals were sectioned, causing degeneration of many neurons of the ganglion cell layer. This loss affected neither the retina's overall rate of acetylcholine synthesis nor the number of acetylcholine-containing cells in the ganglion cell layer. The acetylcholine-synthesizing neurons thus appear to be displaced amacrine cells.*

Acetylcholine is almost certainly a neurotransmitter used in mammalian retinas. The enzymes of its metabolism are present in substantial amounts; it is synthesized by the tissue and released in response to photic stimulation; and, when it is applied to the retina, many ganglion cells are excited. The retinal pathways in which acetylcholine is involved are selected ones. Ganglion cells of some functional classes are stimulated by acetylcholine and depressed by cholinergic antagonists, while ganglion cells with other functional characteristics are unaffected (*1*). The narrow role of acetylcholine in the retina's physiology is matched anatomically by its restriction to a small subset of retinal neurons. These cells are sparsely scattered along both margins of the inner plexiform layer, and they appear to have processes that are confined to two thin planes in the neuropil of that layer (*2*, *3*).

The acetylcholine-synthesizing cells of the inner nuclear layer have the soma size and position of amacrine cells. In the rabbit they make up about 5 percent of the cells that line the inner margin of the layer. The acetylcholine-synthesizing cells of the ganglion cell layer are approximately equal in absolute number to those of the inner nuclear layer, but in the ganglion cell layer they make up more than 20 percent of the total cell population. These neurons must be either ganglion cells or displaced amacrines—neurons that cannot be securely distinguished by ordinary histological methods (*4*).

The acetylcholine-synthesizing neurons of the ganglion cell layer, because they are so numerous, would at first appear to be ganglion cells. On the other hand, there are indications that the number of amacrine cells located in the ganglion cell layer may be larger than was once thought. We carried out two experiments designed to resolve the ambiguity. (i) We labeled the ganglion cells by retrograde transport of a marker dye and examined the localization of acetylcholine in the same retinas by autoradiography. (ii) We sectioned the optic nerve and, after ganglion cell degeneration, examined the retina's acetylcholine synthesis and its cellular localization.

In order to identify the ganglion cells, we needed a marker that is retrogradely transported and is compatible with the dry process used for the localization of acetylcholine. This precluded the use of horseradish peroxidase because the histochemical reaction used to visualize the enzyme would extract acetylcholine from the tissue. Instead, we used a mixture of fluorescent compounds introduced for pathway tracing by Kuypers *et al*. (*5*). These dyes retain fluorescence in the presence of the chemicals used in our autoradiographic method. In addition, they accumulate in the nucleus of the cell and provide a focal region that is easily visible in sections of 2 to 4 μm.

The dorsal thalamus of New Zealand rabbits was exposed by removal, through suction, of overlying brain structures. A slit was made across the total width of the lateral geniculate body, extending slightly ventral and medial to it. The cut thus severed optic tract fibers destined for tectal regions as well as many fibers in the geniculate. A piece of Gelfoam that had been soaked in a solution of 1.3 percent 4,6-diamidino-2-phenylindole (Serva), 1.3 percent primuline (Lachat), and 3 percent lysolecithin (Sigma) (*6*) was inserted into the slit. After 4 days the contralateral retina was removed from the eye and incubated in vitro (*7*). After a 10-minute preliminary incubation in control medium, the retina was transferred to medium containing 0.3 μM [*methyl*-^{3}H]choline (84 Ci/mmole; New England Nuclear), incubated for 15 minutes, and then rinsed for 10 minutes in control medium. High-voltage electrophoresis (*8*) of acid extracts from dye-labeled retinas, homogenized after such incubations, demonstrated that they contain approximately 9.5×10^{-14} mole of [^{3}H]acetylcholine per milligram (wet weight). After incubation, the retina was divided in half. As a check of the number of ganglion

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