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# Metabolites of the Prototype Insecticide (2*E*,4*E*)-*N*-Isobutyl-6-phenylhexa-2,4-dienamide. 2. Formation in Mouse and Rat Liver Microsomal Systems, Rat Hepatocytes, and Houseflies

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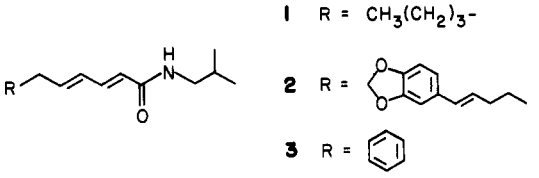
The metabolism of (2*E*,4*E*)-*N*-isobutyl-6-phenylhexa-2,4-dienamide is examined as a prototype of the natural and synthetic isobutylamide insecticides. Nine metabolites from mouse and rat liver microsomal systems, rat hepatocytes, and/or houseflies are identified by HPLC, GC, and GC–MS comparisons with synthetic standards. The parent isobutylamide yields the corresponding unsubstituted amide on *N*-methylene hydroxylation in the microsomal oxygenase system. Both of these amides are readily hydrolyzed by rat but not mouse amidases. The unsubstituted amide in mouse microsomes appears to undergo sequential enzymatic oxidation and hydrolysis to the corresponding carboxylic acid; the presumed hydroxamic acid intermediate is not detected. Additional metabolites are the β-hydroxyisobutyl, 6-hydroxy, 6-keto, and *p*-hydroxy derivatives of the parent isobutylamide and the 6-hydroxy derivatives of the *N*-(β-hydroxyisobutyl) compound and of the unsubstituted amide and carboxylic acid. Hepatocytes conjugate some of these metabolites. The persistence and toxicity of this prototype insecticide are limited by oxidative metabolism at multiple sites in the isobutyl and benzyl moieties.

More than 20 insecticidal isobutylamides [*N*-(2-methylpropyl)amides] are identified from plants of the families Piperaceae, Compositae, and Rutaceae (Jacobson, 1971; Elliott, 1985; Su, 1985; Elliott et al., 1987). They include the *N*-isobutyl-2,4-dienamides pellitorine (**1**) and pipercide (**2**) (one of the isobutylamide constituents of dietary black pepper) (Crombie, 1952; Su and Horvat, 1981; Miyakado et al, 1983; Crombie and Denman, 1984).

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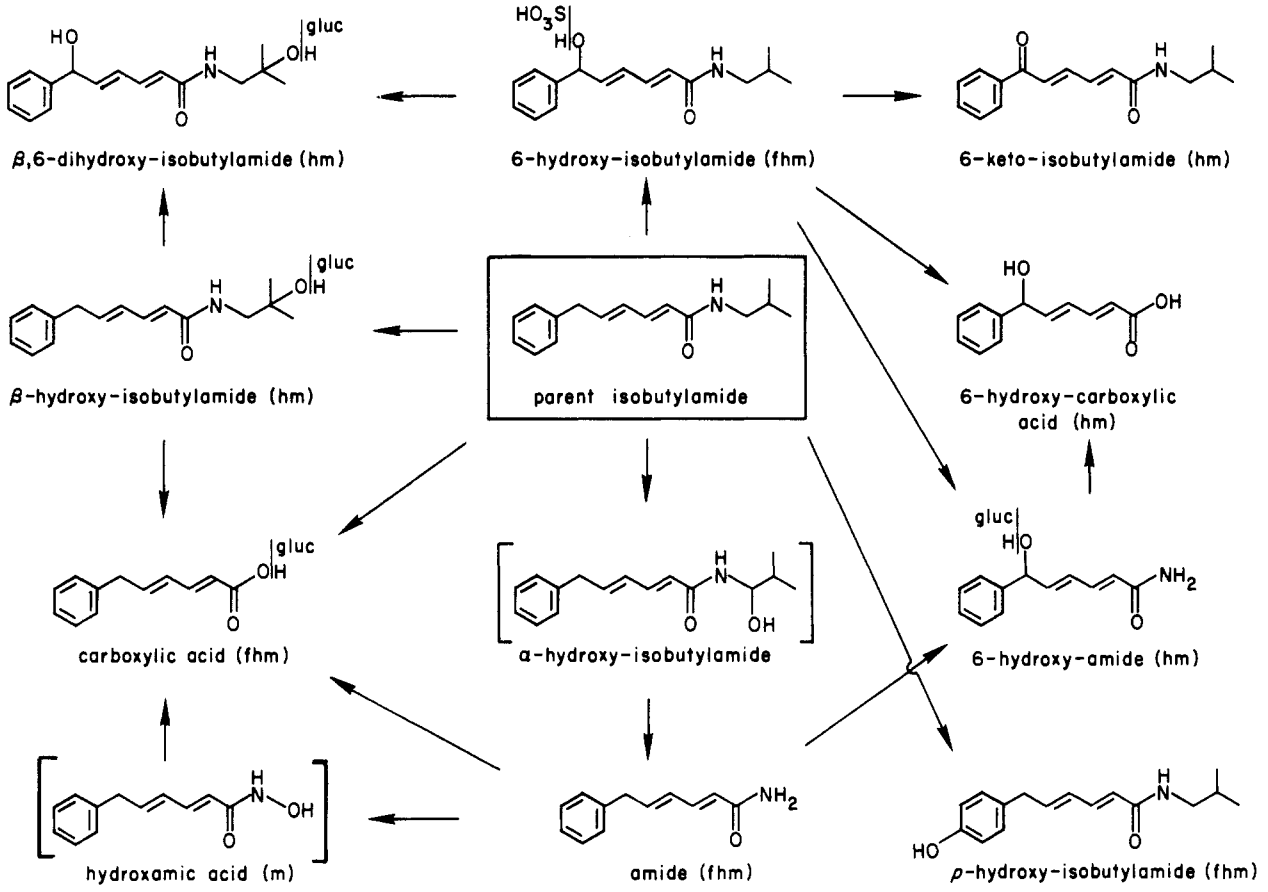


**Figure 1.** Metabolic pathways of (2*E*,4*E*)-*N*-isobutyl-6-phenylhexa-2,4-dienamide, indicating the trivial names used for various compounds and the metabolites detected in mouse and rat liver microsome (m) systems, rat hepatocytes (h), and houseflies (f). Conjugates are designated as gluc for β-glucuronides and $SO_3H$ for a sulfate. The glucuronide of the β,6-dihydroxy isobutylamide is arbitrarily shown at the β-position.

These botanicals have served as prototypes for the synthesis of candidate insecticides leading to (2*E*,4*E*)-*N*-isobutyl-6-phenylhexa-2,4-dienamide (**3**) (Elliott, 1985; Elliott et al., 1986, 1987). Compound **3**, due to its relatively simple structure and substantial potency to houseflies and mustard beetles, can be considered as a lead or prototype chemical in the optimization of lipophilic amides for potential practical use as insecticides (Elliott et al., 1987).

Knowledge of molecular sites sensitive to metabolism may suggest structural modifications for enhanced potency and stability. This proved to be the case following the first definitive studies on metabolism of the methylcarbamate (Dorough et al., 1963) and pyrethroid (Yamamoto and Casida, 1966) insecticides.

This report considers the first investigation on metabolism of an insecticidal *N*-isobutyl-2,4-dienamide. Prototype insecticide **3** (referred to in this paper as the parent isobutylamide) was selected for study for three reasons: It is the simplest compound of this type with substantial insecticidal activity. The ultraviolet (UV) absorption characteristics of **3** [$\lambda_{max}$ (methanol) 258 (30000) nm] and its potential metabolites facilitate their analysis by high-performance liquid chromatography (HPLC). Advances in synthesis make it feasible to prepare authentic standards of candidate metabolites (Horsham et al., 1988). The metabolic systems utilized are mouse and rat liver microsomal enzymes, rat hepatocytes, and houseflies in vivo, often using metabolic inhibitors to differentiate oxygenase and amidase pathways.

## MATERIALS AND METHODS

**Chemicals.** The parent isobutylamide and compounds synthesized as candidate metabolites are referred to by the trivial names given in Figure 1. Their synthesis and chromatographic and spectroscopic characteristics are reported by Horsham et al. (1989). Phenylsaligenin cyclic phosphonate (PSCP), a potent amidase inhibitor, was provided by C. J. Palmer of this laboratory. Piperonyl butoxide (PB), a microsomal mixed-function oxygenase (MFO) inhibitor, was from Fluka Chemical Corp. (Ronkonkoma, NY). Desferal mesylate was a gift from CIBA-Geigy Corp. (Suffern, NY).

**Analyses.** Several analytical methods were used for comparison of the authentic standards (Horsham et al., 1989) with the metabolites.

Metabolites were separated by HPLC and tentatively identified by cochromatography with authentic standards using an Altex Ultrasphere-Si column (4.6 mm × 25 cm; Altex Scientific Inc., Berkeley, CA). Two Beckman Model 112 solvent delivery modules and a Beckman Model 421 controller were used. The gradient employed was 38% methanol (HPLC grade) in water (HPLC grade) to 100% methanol over a period of 50 min followed by 100% methanol for an additional 30 min with a constant flow rate of 0.8 mL/min at 25 °C (Horsham et al., 1989). Metabolites were quantitated by comparison of their peak heights at 258 nm with those of synthetic standards on the Hitachi Model 100-40 spectrophotometer. Polar metabolites were also examined with a solvent system consisting of 0.01% (w/v) desferal mesylate in 55% methanol buffered to pH 3.5 with 0.01 M potassium phosphate (Corbett and Chipko, 1979) giving retention times ($R_t$) of 14.0, 17.5, and 62.0 min for the 6-hydroxy carboxylic acid, hydroxamic acid, and carboxylic acid, respectively.

Gas chromatography (GC) utilized a Hewlett-Packard 5840A gas chromatograph equipped with a fused silica capillary column (SE 54, 0.25-μm film thickness, 0.25 mm × 15 m; Supelco Inc., Bellefonte, PA) and a flame ionization detector (FID) and was temperature-programmed as follows: 90 °C for 3 min, 25 °C/min to 165 °C, 165 °C for 1 min, and 3 °C/min to 240 °C. Helium was used as the carrier gas at 40 cm/s (Horsham et al., 1989). Metabolites were identified by comparison of retention times and

coinjection with synthetic standards or their methyl esters or ethers (prepared by treatment with diazomethane).

GC–mass spectrometry (GC–MS) involved the Hewlett-Packard 5985B GC–MS system with a fused silica capillary column (SPB5, 0.25-μm film thickness, 0.32 mm × 15 m; Supelco) with a sequential temperature program of 90 °C for 3 min, 25 °C/min to 160 °C, 160 °C for 1 min, and 2 °C/min to 240 °C and helium as the carrier gas (30 cm/s) for separation. Chemical ionization (CI) using methane (0.9 Torr) as the reagent gas resulted in intense $[M + 1]^+$ and $[M + 29]^+$ signals; hydroxylated metabolites revealed additional $[M - 17]^+$ fragments. CI was preferred over electron-impact ionization (70 eV) because it resulted in better sensitivity and selectivity. The metabolites and standards or their methylated derivatives were detected in the selected ion monitoring mode. $R_t$ and MS characteristics are reported by Horsham et al. (1989).

**Microsomal Metabolism.** Liver microsomes from male albino Swiss–Webster mice and male albino rats (Simonsen Laboratories, Gilroy, CA) were prepared by centrifugation of a 20% (w/v) liver homogenate in phosphate buffer (0.1 M, pH 7.4) at 15000*g* for 15 min, recovery of the supernatant fraction, and centrifugation at 105000*g* for 1 h. The microsomal pellet was washed once with 0.25 M sucrose, suspended in this medium at a concentration of 10 mg of protein/mL (Bradford, 1976), and stored frozen at −80 °C.

Incubation mixtures consisted of the microsomal preparation (15 mg of protein in 1.5 mL of 0.25 M sucrose) and NADPH (18 μmol) plus 8.5 mL of buffer (115 mM potassium chloride and 50 mM Tris at pH 7.4) in a 50-mL Erlenmeyer flask. The parent isobutylamide (10 μmol) or synthetic candidate metabolite (3 μmol) was added last in dimethylformamide (25 μL). These incubations were sometimes varied by deleting the MFO cofactor (NADPH) or adding the MFO inhibitor (PB) (15 μmol) or the amidase inhibitor (PSCP) (0.1 μmol) in dimethylformamide (25 μL). After incubation for 60 min at 37 °C, the reaction mixtures were extracted with dichloromethane (20 mL × 2), adjusted to pH 3.0 with 0.1 M hydrochloric acid, and extracted again with dichloromethane (20 mL). The organic extracts were pooled, dried (magnesium sulfate), concentrated on a rotary evaporator, transferred to a small vial, and evaporated to dryness under a gentle stream of nitrogen at 25 °C. The residue was then dissolved in dichloromethane (750 μL), and aliquots (100 μL) were analyzed by HPLC. This procedure provides 100 ± 5% recovery of the parent isobutylamide from the mouse microsomes, alone, with extraction prior to incubation. Recovery of metabolites was not improved by an additional extraction with dichloromethane (20 mL).

Two other procedures were also used to analyze aliquots (100 μL) of the microsomal incubate extracts as above. Following a preliminary cleanup in which the parent isobutylamide was removed by TLC on silica gel with ethyl acetate ($R_f$ 0.60), the products of lower $R_f$ were analyzed by GC–FID and GC–MS after methylation with diazomethane in diethyl ether for 20 min at 25 °C. Possible epoxide metabolites were evaluated by TLC (silica gel, ethyl acetate) and detection with 4-(*p*-nitrobenzyl)pyridine (Hammock et al., 1974).

A modified procedure was used to test for the presence of the hydroxamic acid, as a metabolite of the amide, in incubations with mouse microsomes, NADPH, and PSCP. The incubation system was selected to favor the detection of the hydroxamic acid, i.e., 2 mL of buffer as above containing 2 mg of protein, 0 or 2 μmol of NADPH, 0.004 μmol of PSCP, and 0.2 μmol of amide added in 20 μL of ethanol. As a control, the amide was replaced with 0.02 μmol of hydroxamic acid. After incubation for 20 min at 37 °C, 0.2 μmol of the parent isobutylamide in 20 μL of ethanol was added as an internal standard and the reaction mixtures were saturated with sodium chloride and extracted with ethyl acetate (2 mL × 2). The organic extracts were dried (magnesium sulfate) prior to analysis by GC–FID for quantitation. The extract was then further concentrated under nitrogen to 0.1 mL and treated with diazomethane in 0.3 mL of ether at −15 °C for 12 h. Further concentration to 25 μL preceded GC–MS analysis for the amide, the methyl ester of the carboxylic acid, and the dimethyl derivative of the hydroxamic acid.

**Hepatocyte Metabolism.** Hepatocytes were isolated from rats as above by collagenase–hyaluronidase digestion of liver slices (Fry, 1981). The viable hepatocytes were recovered by centrifugation at 60*g* for 1.5 min in pH 7.4 Hank's balanced salt solution (Gibco Laboratories, Grand Island, NY) containing 35% Percol (Sigma Chemical Co., St. Louis, MO). Cell viability was greater than 95% by trypan blue dye exclusion. Hepatocytes were suspended at $1 \times 10^6$ cells/mL in Leibovitz's L-15 media (Gibco) fortified with 5% v/v fetal bovine serum (Sigma), 10% v/v tryptose phosphate broth (Gibco), and 20 mM glutamine.

Cell suspensions (10 mL) were incubated for 1 h at 37 °C following the addition of 1.23 μmol of parent isobutylamide in 20 μL of dimethylformamide. Controls were treated identically except that the incubation medium contained no hepatocytes. The incubates were extracted with dichloromethane and analyzed by HPLC as previously described for the microsomal reactions. In an additional experiment, isobutylamide-containing and control hepatocyte preparations were analyzed for conjugates after removal of the nonconjugated products by extraction with dichloromethane as above, but without acidification, and rotary evaporation to remove the residual dichloromethane. These extracted cell suspensions were acidified to pH 5 by adding 0.2 M acetate buffer and then treated with either 8 units of sulfatase (Type V, Sigma) and 20 mg of D-saccharic acid 1,4-lactone (to inhibit any β-glucuronidase present) or 5000 units of Glucurase (β-glucuronidase) (Sigma). After incubation for 8 h at 37 °C, they were extracted and analyzed for metabolites as previously described, comparing incubations with hepatocytes and with incubation medium only.

**Housefly Metabolism.** Three hundred adult houseflies (*Musca domestica* L., SCR strain) were individually treated with the parent isobutylamide (7 μg) in acetone (0.5 μL) applied topically to the ventrum of the abdomen to give a dose of 350 μg/g. After 4 h at 25 °C, each group of flies was rinsed with acetone (40 mL) to remove the unabsorbed compound. Metabolites were then recovered by homogenizing the "rinsed flies" in acetone (2 × 30 mL) followed by ethyl acetate (30 mL), and the combined organic extracts were dried (magnesium sulfate) and concentrated by rotary evaporation and then under a stream of nitrogen at 25 °C. Control flies were heated to 90 °C for 5 min to stop their metabolic activity (Casida et al., 1968) prior to application of the isobutylamide. In both cases an aliquot equivalent to the extract from 60 flies was subjected to HPLC analysis.

**Toxicity to Houseflies and Mice.** The parent isobutylamide was applied topically to the ventrum of the abdomen of adult female houseflies as above. Similar tests were made with houseflies pretreated topically with PB or PSCP at 250 μg/g 1 h before administering the isobutylamide. $LD_{50}$ values were determined after 24 h at 14, 23, or 27 °C.

Male Swiss–Webster mice (18–22 g) were treated intraperitoneally (ip) with the parent isobutylamide in methoxytriglycol with mortality determinations after 24 h. Similar tests were made with mice pretreated 1 h with PB or PSCP administered ip at 150 and 50 mg/kg, respectively.

**Mutagenicity Assays.** Ames assays were performed on the synthetic hydroxamic acid using *Salmonella typhimurium* strain TA 100 both with and without S-9 activation (Arochlor-1254-induced microsomes) (Maron and Ames, 1983). These assays were conducted by the Microbial Mutagenesis Program at SRI International (Menlo Park, CA).

## RESULTS

**Microsomal Metabolism of the Parent Isobutylamide (Table I; Figure 2).** Rat liver microsomes are more effective than mouse liver microsomes in hydrolyzing the parent isobutylamide to the carboxylic acid without forming other metabolites. PSCP inhibits formation of the acid by 50% in rat microsomes. Nine metabolites are identified when mouse or rat liver microsomes are fortified with NADPH, the major ones being the amide, the carboxylic acid, the β-hydroxy isobutylamide, the 6-hydroxy isobutylamide, and the 6-hydroxy carboxylic acid. Minor metabolites are the *p*-hydroxy, 6-keto, and β,6-dihydroxy isobutylamides and the 6-hydroxy amide. These metabolites are identical with the corresponding synthetic

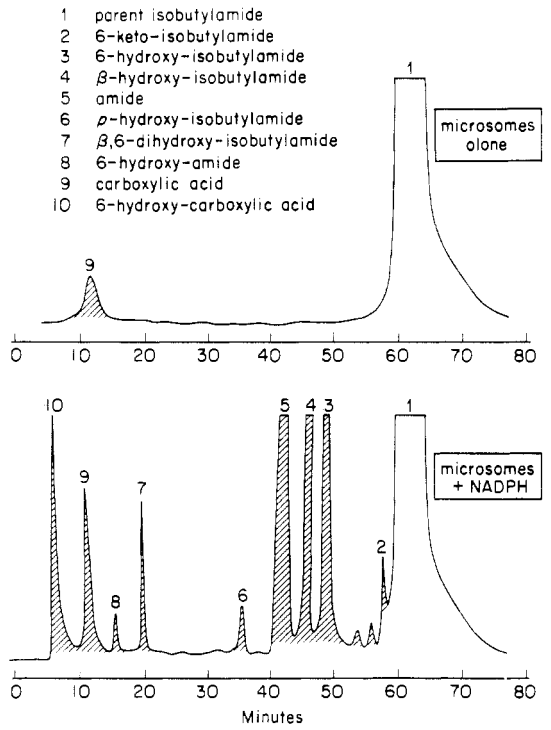


**Figure 2.** HPLC pattern of mouse liver microsomal metabolites of (2*E*,4*E*)-*N*-isobutyl-6-phenylhexa-2,4-dienamide. Metabolites are designated as darkened peaks.

**Table I. Metabolism of (2*E*,4*E*)-*N*-Isobutyl-6-phenylhexa-2,4-dienamide (Parent Isobutylamide) by Mouse and Rat Liver Microsomes Alone and Fortified with NADPH**

| metabolite | mouse microsomes,[a] nmol | | rat microsomes,[a] nmol | |
|---|---|---|---|---|
| | alone | +NADPH | alone | +NADPH |
| carboxylic acid | 2 | 84 | 48 | 68 |
| amide | <0.5 | 136 | <0.5 | 276 |
| β-hydroxy isobutylamide | <0.5 | 44 | <0.5 | 84 |
| *p*-hydroxy isobutylamide | <0.5 | 1.4 | <0.5 | 3 |
| 6-hydroxy isobutylamide | <0.5 | 46 | <0.5 | 30 |
| 6-keto isobutylamide | <0.5 | 3 | <0.5 | 15 |
| 6-hydroxy carboxylic acid | <0.5 | 40 | <0.5 | 32 |
| 6-hydroxy amide | <0.5 | 28 | <0.5 | 10 |
| β,6-dihydroxy isobutylamide | <0.5 | 14 | <0.5 | 14 |

[a] Almost all of the substrate (10 μmol) not accounted for as the tabulated metabolites is recovered as the parent isobutylamide. Mean of two experiments differing by ~10%.

standards with respect to HPLC in one or two different solvent systems. GC–FID and GC–MS (CI) confirm the presence of the first six of the tabulated metabolites in incubation mixtures fortified with NADPH. The carboxylic acid and *p*-hydroxy isobutylamide in these cases were examined as their methyl ester and ether, respectively. There is no evidence for any epoxide metabolites based on the detection procedure with 4-(*p*-nitrobenzyl)-pyridine after TLC.

Quantitation of the metabolites in the mouse and rat liver microsome–NADPH systems establishes that the carboxylic acid and the amide account for about 70% of the metabolism. Benzylic hydroxylation to form the 6-hydroxy compounds appears to be more important in the mouse than in the rat microsome–NADPH systems. Fortification with NADPH increases the yield of the carboxylic acid 1.4-fold with rat microsome and 42-fold with mouse microsomes. Aromatic hydroxylation is the least efficient pathway for both species.

**Table II. Metabolism of (2*E*,4*E*)-6-Phenylhexa-2,4-dienamide to the Corresponding Carboxylic Acid by Mouse and Rat Liver Microsomes Alone and Fortified with NADPH**

| metabolic system | nmol carboxylic acid[a] | |
|---|---|---|
| | mouse microsomes | rat microsomes |
| alone | 4 | 172 |
| NADPH | 172 | 172 |
| NADPH + PB | <0.5 | 116 |
| NADPH + PSCP | <0.5 | <0.5 |

[a] Almost all of the substrate (3 μmol) not accounted for as the carboxylic acid is recovered as the unmetabolized amide. Mean of two experiments differing by ~10%.

PB strongly inhibits formation of all metabolites in the mouse microsome–NADPH system and of all metabolites except a small amount of carboxylic acid in the rat microsome–NADPH system. Thus, with PB the metabolite composition approximates that formed in these systems without NADPH fortification. Addition of PSCP to the microsome–NADPH system significantly decreases the amount of carboxylic acid formed without greatly changing the yield of other metabolites.

**Microsomal Metabolism of the Amide and the β-Hydroxy and 6-Hydroxy Isobutylamides.** Mouse microsomes are much less efficient than rat microsomes in hydrolyzing the amide to the carboxylic acid, but they are equally effective in forming the carboxylic acid when fortified with NADPH (Table II). Carboxylic acid formation is strongly inhibited in the mouse microsome–NADPH system by either PB or PSCP and in the rat microsome system (with or without added NADPH) by PSCP but not by PB. Additional metabolites in the mouse microsome–NADPH system are the 6-hydroxy carboxylic acid and 6-hydroxy amide.

Incubation of the 6-hydroxy isobutylamide with mouse microsomes and NADPH gives the 6-keto isobutylamide, 6-hydroxy carboxylic acid, 6-hydroxy amide, and β,6-dihydroxy isobutylamide.

The β-hydroxy isobutylamide as a substrate is metabolized to the β,6-dihydroxy isobutylamide, carboxylic acid, and 6-hydroxy carboxylic acid in decreasing amounts.

**Hydroxamic Acid as a Possible Intermediate in Metabolism of the Amide.** The requirement for both oxygenase and amidase activities in mouse microsomes to convert the isobutylamide and the unsubstituted amide to the carboxylic acid (Tables I and II) suggests the possibility of a hydroxamic acid intermediate. The hydroxamic acid should be present in largest amount from the unsubstituted amide substrate in the mouse microsomal oxygenase system containing PSCP as an amidase inhibitor to minimize its subsequent hydrolysis to the carboxylic acid. This metabolite mixture was examined by HPLC with the dichloromethane extract of the acidified incubation mixture and by GC–MS with the ethyl acetate extract of the metabolites following methylation with diazomethane. The standard HPLC system used for other metabolites gave very low sensitivity and a broad early peak with the standard hydroxamic acid [perhaps due to its strong chelating properties; see Corbett and Chipko (1979)]. Another HPLC system recommended for hydroxamic acids (Corbett and Chipko, 1979) was not appropriate in the present case since the standard hydroxamic acid coeluted with interfering microsomal extractives. On the other hand, the hydroxamic acid is readily analyzed by GC–MS (CI) as its major dimethyl derivative ($R_t$ 11.3 min; Horsham et al., 1989). This method has sufficient sensitivity to detect the hydroxamic acid if it is present at a level of 3% relative to that of the added unsubstituted

**Table III. Metabolism of (2E,4E)-N-Isobutyl-6-phenylhexa-2,4-dienamide (Parent Isobutylamide) by Rat Hepatocytes**

| metabolite | incubn medium,[a] nmol | hepatocytes,[a] nmol | |
|---|---|---|---|
| | | conj[b] | conj[b] |
| carboxylic acid | <1 | 43 | 5 |
| amide | <1 | 101 | <1 |
| β-hydroxy isobutylamide | <1 | 27 | 9 |
| p-hydroxy isobutylamide | <1 | 15 | <1 |
| 6-hydroxy isobutylamide | <1 | 162 | <1 |
| 6-keto isobutylamide | <1 | 8 | <1 |
| 6-hydroxy carboxylic acid | <1 | 17 | <1 |
| 6-hydroxy amide | <1 | 17 | <1 |
| β,6-dihydroxy isobutylamide | <1 | 1 | 4 |

[a] Almost all of the substrate (1.23 μmol) not accounted for as the tabulated metabolites is recovered as the parent isobutylamide. Mean of two experiments differing by ~15%. [b] Liberated on incubation with β-glucuronidase but not with sulfatase, except for the 6-hydroxy isobutylamide with 7-nmol cleavage by β-glucuronidase and 2 nmol by sulfatase.

**Table IV. Metabolism of (2E,4E)-N-Isobutyl-6-phenylhexa-2,4-dienamide (Parent Isobutylamide) in Houseflies.**

| metabolite | heated control,[a] nmol | normal,[a] nmol |
|---|---|---|
| carboxylic acid | 43 | 117 |
| amide | <2 | 37 |
| 6-hydroxy isobutylamide | <2 | 8 |
| p-hydroxy isobutylamide | <2 | 15 |

[a] Almost all of the parent isobutylamide applied (8.6 μmol) appears unmetabolized in the surface wash (unabsorbed) and the extract of the "rinsed flies". The tabulated metabolites appear only in the latter extract. Mean of two experiments differing by <5%.

amide. Under conditions in which 17% of the unsubstituted amide is metabolized, the hydroxamic acid was not detected.

The synthetic hydroxamic acid is hydrolyzed to the carboxylic acid by rat microsomes but not by rat microsomes pretreated with PSCP.

**Rat Hepatocyte Metabolism of the Parent Isobutylamide (Table III).** Rat hepatocytes give the same four major metabolites and five minor metabolites discussed earlier for rat microsomes with NADPH. The carboxylic acid, β-hydroxy isobutylamide, and β,6-dihydroxy isobutylamide appear to be conjugated as glucuronides and the β-hydroxy isobutylamide also as a sulfate based on their cleavage reactions with β-glucuronidase and sulfatase, respectively.

**Housefly Metabolism of the Parent Isobutylamide.** The major housefly metabolites are identified by HPLC cochromatography as the carboxylic acid and amide. Small amounts of the p-hydroxy and 6-hydroxy isobutylamides are also detected (Table IV).

**Toxicity of the Parent Isobutylamide.** The toxicity of the parent isobutylamide to houseflies at 23 °C is not synergized by PSCP but is synergized 21-fold by PB, i.e., $LD_{50}$ values of 17 μg/g alone and with PSCP and 0.8 μg/g with PB. Increasing the temperature from 14 to 27 °C results in about a 2-fold decrease in housefly toxicity of the parent isobutylamide with PB whereas in the absence of PB this temperature change has no effect on the $LD_{50}$. Thus, in contrast to pyrethroid insecticides, the parent isobutylamide does not have a negative temperature coefficient.

The IP $LD_{50}$ of the parent isobutylamide in mice is 3.2 mg/kg with 2-fold synergism by PB and 1.5 by PSCP.

**Mutagenicity of the Hydroxamic Acid.** The hydroxamic acid at 5–1000 μg/plate is not mutagenic to *S. typhimurium* strain TA 100 either with or without S-9 activation. Toxicity is evident at 500 and 1000 μg of hydroxamic acid/plate both by itself and with S-9.

## DISCUSSION

The parent isobutylamide is metabolized in microsomal systems, hepatocytes, and houseflies by both hydrolytic and oxidative mechanisms (Figure 1). The hydrolytic pathway is clearly evident with rat but not mouse liver microsomes cleaving the parent isobutylamide to the carboxylic acid by the action of an amidase(s). Several types of evidence establish the importance of oxidative pathways in microsomal metabolism: Metabolite formation in mouse and rat microsomes is dependent on or greatly enhanced by fortification with NADPH. Several mono- and dihydroxylated metabolites are formed. PB almost completely inhibits the NADPH-dependent metabolism. The β-hydroxy isobutylamide and 6-hydroxy isobutylamide are each further metabolized to the 6-hydroxy carboxylic acid and β,6-dihydroxy isobutylamide. Additional metabolites are the carboxylic acid from the β-hydroxy isobutylamide and the 6-keto isobutylamide and 6-hydroxy amide from the 6-hydroxy isobutylamide. Several of the metabolites with hydroxy substituents undergo conjugation in hepatocytes to form glucuronides and a sulfate.

The isobutyl group is very sensitive to oxidative metabolism at the α- and β-positions. Oxidative conversion of the parent isobutylamide to the amide undoubtedly involves α-hydroxylation to yield the unstable α-hydroxy compound, which spontaneously decomposes to the amide and presumably isobutyraldehyde. Thus, the amide is the major metabolite in the mouse microsomal–oxygenase system, and its formation is dependent on NADPH and is inhibited by PB. The β-hydroxy compound is an additional major metabolite, and its formation is also dependent on cytochrome P-450.

Isobutyl hydroxylation may also be important in plants since N-(β-hydroxyisobutyl)-2,4,8,10-dodecatetraenamide is naturally occurring in fruits of *Zanthoxylum piperitum* (Aihara, 1951). It is also possible that plants hydroxylate the α-position of the isobutyl moiety, thereby yielding the primary amide, but the authors are not aware of any amide coexisting with the corresponding isobutylamide in plants (Greger, 1984).

The carboxylic acid is formed from the parent isobutylamide directly by amidase hydrolysis or indirectly by biooxidation and then hydrolysis, with the latter pathway primarily involving the amide as an intermediate. Carboxylic acid formation is almost completely via the oxidative pathway in mouse microsomes and by both hydrolysis and oxidation in the rat microsome–NADPH system. As with the isobutylamide, the unsubstituted amide is more extensively hydrolyzed by rat than mouse microsomes; this portion of the metabolism is sensitive to inhibition by PSCP but is insensitive to NADPH and PB. In mouse microsomes both the MFOs and amidases are critical to acid formation from the unsubstituted amide since the cleavage requires NADPH and is inhibited by PB and PSCP. These observations are rationalized as the oxidative conversion of the unsubstituted amide to the hydroxamic acid, which in turn undergoes amidase-catalyzed hydrolysis. However, if the hydroxamic acid does exist as a metabolite, such existence appears to be transient, since it could not be detected by a method (based on GC–MS) shown to be sensitive to low levels. Although some N-hydroxy compounds are mutagens in the Ames assay (Rinkus and Legator, 1979), this is not the case for the hydroxamic acid examined here. The synthetic hy-

droxamic acid undergoes PSCP-sensitive and therefore amidase-catalyzed hydrolysis.

Liver hepatocytes yield all of the metabolites detected in the microsome–NADPH system, supporting the relevance of the enzyme studies. The hepatocytes also yield conjugates tentatively identified as the glucuronides of several metabolites with hydroxy and carboxy substituents and, in small amount, the sulfate of the β-hydroxy isobutylamide.

Housefly metabolism of the parent isobutylamide is probably similar to the pathways described above for liver microsomes and hepatocytes. The principal products are the amide and the carboxylic acid whereas some 6-hydroxy and *p*-hydroxy isobutylamides are also detected. Each of the metabolite standards is of lower toxicity than the parent isobutylamide to mice and to PB-pretreated houseflies (Horsham et al., 1989). It therefore appears that the parent isobutylamide is very sensitive to detoxification by oxidation at both the benzylic methylene and isobutyl substituents.

This is the first study on metabolism of an *N*-isobutyl-2,4-dienamide. Two related *N*-isobutyl-2-enamides, with additional unsaturation at other positions, undergo epoxide formation and hydrolysis and allylic hydroxylation in a microsomal oxygenase system and in houseflies, but metabolic attack on the *N*-isobutyl-2-enamide moiety is not observed (Brealey, 1988).

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