Nature of Toxic Metabolites Formed in Mammals, Insects, and Plants from 3-(Dimethoxyphosphinyloxy)-*N,N*-dimethyl-*cis*-crotonamide and Its *N*-Methyl Analog

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Bidrin, 3-(dimethoxyphosphinyloxy)-*N,N*-dimethyl-*cis*-crotonamide, is metabolized to yield trace amounts of 3-(dimethoxyphosphinyloxy)-*N*-methyl-*N*-hydroxymethyl-*cis*-crotonamide and larger amounts of 3-(dimethoxyphosphinyloxy)-*N*-methyl-*cis*-crotonamide (SD 9129). SD 9129 is further metabolized to yield 3-(dimethoxyphosphinyloxy)-*N*-hydroxymethyl-*cis*-crotonamide and 3-(dimethoxyphosphinyloxy)-*cis*-crotonamide. The toxicity to both insects and mammals increased upon successive *N*-demethylation. Balance studies on the fate of the P^{32} and C^{14} from Bidrin-P^{32}, Bidrin-*N*-methyl-C^{14}, SD 9129-P^{32}, and SD 9129-*N*-methyl-C^{14} are considered. Studies on milk residues, urinalysis, and metabolism in houseflies (*Musca domestica* L.) and bean plants are reported. An unusual pattern of synergism of the toxicity of the Bidrin metabolites in houseflies by sesamex [2-(2-ethoxyethoxy)ethyl-3,4-(methylenedioxy)phenyl acetal of acetaldehyde] was noted.

Two vinyl phosphates which have recently been found very effective as agricultural insecticides are 3-(dimethoxyphosphinyloxy) - *N,N* - dimethyl - *cis* - crotonamide (Bidrin, Shell Chemical Co. trade-mark) and 3-(dimethoxyphosphinyloxy) - *N* - methyl-*cis*-crotonamide (SD 9129, code designation, Shell Development Co.). Related vinyl phosphates already in use are mevinphos, where the active ingredient is 3 - (dimethoxyphosphinyloxy)methyl *cis*-crotonate, and phosphamidon, which consists of the mixed isomers of 3-(dimethoxyphosphinyloxy) - *N,N* - diethyl-2-chlorocrotonamide. Studies on the metabolism of mevinphos have established that hydrolysis can occur at the vinyl phosphate, P—*O*-methyl, or carbomethoxy group; these hydrolysis products are further degraded; and only the unhydrolyzed ester is highly toxic (*9, 10, 25*). Degradation of phosphamidon in plants involves the formation of the more toxic 3-(dimethoxyphosphinyloxy)-*N* - ethyl - 2 - chlorocrotonamide (des-*N*-ethyl phosphamidon), which must be considered when evaluating residues of this pesticide. Other degradation products identified include *N,N*-diethyl-2-chloroacetoacetamide and *N*-ethyl-2-chloroacetoacetamide. The unsubstituted amide of phosphamidon [3-(dimethoxyphosphinyloxy) - 2 - chlorocrotonamide] and the des-*O*-methyl derivative [3-(hydroxymethoxyphosphinyloxy) - *N,N* - diethyl - 2-chlorocrotonamide] were not detected in plants (*3, 16*).

Studies with *O,O*-dimethyl 2,2-dichlorovinyl phosphate or dichlorvos have established as metabolites in mammals the following compounds which lack the toxicity of the original molecule: inorganic phosphate, monomethyl phosphate, dimethyl phosphate, *O*-methyl 2,2-dichlorovinyl phosphate, and dichloroethanol conjugates (*11, 15*).

The metabolism of Bidrin and SD 9129 was investigated in mammals, plants, and insects in relation to the mode of action and residue problems that might be anticipated. Only the *cis*-crotonamide isomers were considered, as these isomers are those of the greatest biological activity, and only small amounts, if any, of the *trans*-isomers are present in Bidrin or SD 9129. The nature of the phosphorus-containing hydrolysis products is not considered here, since a study on their nature following Bidrin administration to rats, cotton plants, and insects has been reported (*5*). The present investigation deals primarily with the fate of the dimethylamide or methylamide group on that portion of the administered dose that has not been hydrolyzed at any given time after administration. It appeared possible that *N*-demethylation might occur and that intermediates in the reactions leading to successive *N*-demethylation would be toxic (*5, 18*).

Experimental

Materials. The following chemicals were provided by R. R. Whetstone, Shell Development Co., Modesto, Calif.: Bidrin, which was further purified by column chromatography; SD 9129, m.p. 49° C.; the unsubstituted amide analog of Bidrin and SD 9129, or 3 - (dimethoxyphosphinyloxy) - *cis*-crotonamide, m.p. 101° C.; Bidrin acid, or 3 - (dimethoxyphosphinyloxy) - *cis*-crotonic acid, prepared by hydrolysis of the *cis*-crotonate isomer of mevinphos according to the method of Spencer *et al.* (*26*); des-*O*-methyl Bidrin, or 3-(hydroxymethoxyphosphinyloxy) - *N,N* - dimethyl - *cis* - crotonamide, sodium salt, m.p. 141° C., prepared by reaction of *cis*-Bidrin with sodium iodide according to Spencer *et al.* (*26*); *N,N*-dimethylacetoacetamide, b.p. 83–85° C./0.2 mm.; *N*-methylacetoacetamide, m.p. 44° C.; 3 - hydroxy - *N,N* - dimethylbutyramide, b.p. 88–89° C./0.75 mm.; and 3-hydroxy-*N*-methylbutyramide, b.p. 110–11° C./0.1 mm.

Radiolabeled Bidrin and SD 9129 samples were also provided by the Shell Development Co. at the following specific activities: Bidrin-P^{32} at 2.6, 2.8, 3.0, and 4.6 mc. per mmole; Bidrin-*N*-methyl-C^{14} at 1.0 mc. per mmole; Bidrin-*O*-methyl-C^{14} at 0.6 mc. per mmole; SD 9129-P^{32} at 2.1 mc. per mmole; SD 9129-*N*-methyl-C^{14} at 2.0 mc. per mmole; and SD 9129-*O*-methyl-C^{14} at 0.2 mc. per mmole. All radiolabeled compounds were purified on either silica gel or Celite partitioning columns. Only the *cis*-crotonamide isomers of the labeled materials were used after ascertaining their composition and purity by infrared spectra and cochromatography with authentic nonlabeled materials.

Attempted Synthesis of *N*-Hydroxymethyl Analogs of Bidrin and SD 9129. SD 9129 (3.2 mmoles), formaldehyde (25.1 mmoles in 2.0 ml. of water), and enough sodium bicarbonate to raise the pH to 7.0 were held 20 hours at 45° to 50° C., and the reaction mixture was then extracted with chloroform. The chloroform was evaporated and the residue chromatographed on the Celite column as described later.

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Two products in addition to the starting material were eluted, one before the position of Bidrin and the second in the position indicated as unknown A (Figure 2). The two products other than SD 9129 yielded formaldehyde as detected with a sulfuric acid solution of chromotropic acid (*8*) and inorganic phosphate following digestion with perchloric acid according to the method of Allen (*2*). The formaldehyde-yielding functional groups were present in organophosphorus compounds with both new products, since the phosphate- and formaldehyde-yielding materials cochromatographed in both cases. The material from the first peak contained no bands associated with hydroxyl functions in its infrared spectrum, while the material chromatographing in the unknown A region yielded the spectrum indicated in Figure 1. The yield of unknown A was 20%. This material is proposed to be 3-(dimethoxyphosphinyloxy)-*N*-methyl-*N*-hydroxymethyl-*cis*-crotonamide, on the basis of the synthetic route, infrared spectrum, chromatographic characteristics, and degradation to yield formaldehyde and phosphate from the same compound. An NMR spectrum was also consistent with the proposed structure.

The unsubstituted amide analog of Bidrin (1.34 mmoles), formaldehyde (25.1 mmoles in 2.0 ml. of water), and enough sodium bicarbonate to raise the pH to 7.0 were held 20 hours at 28° C., and the reaction mixture was then extracted with chloroform. The extract was purified in a manner similar to that for unknown A to yield the unreacted unsubstituted amide and only one additional product, which chromatographed in the position indicated for unknown B in Figure 2. This product, as with unknown A, contained groups which yielded both phosphate and formaldehyde upon degradation. The yield of unknown B was 84%. This material is proposed to be 3-(dimethoxyphosphinyloxy) - *N* - hydroxymethyl-*cis*-crotonamide based on the same criteria applied to unknown A.

These routes of synthesis were suggested in part by Whetstone (*30*). The infrared and NMR spectra were prepared and interpreted at the Shell Development Co., Modesto, Calif., by G. E. Pollard. Based on rechromatography of the samples, the mono-*N*-hydroxymethylamide appeared to be stable up to 2 weeks at 4° C., but the *N*-methyl-*N*-hydroxymethylamide was almost entirely decomposed after 4 weeks to yield SD 9129. The sample of *N*-methyl - *N* - hydroxymethylamide had partially decomposed to SD 9129 during shipment prior to preparation of the infrared spectrum of Figure 1. In view of the known ability of hydroxymethyl compounds to decompose with loss of formaldehyde (*29*), this is not surprising.

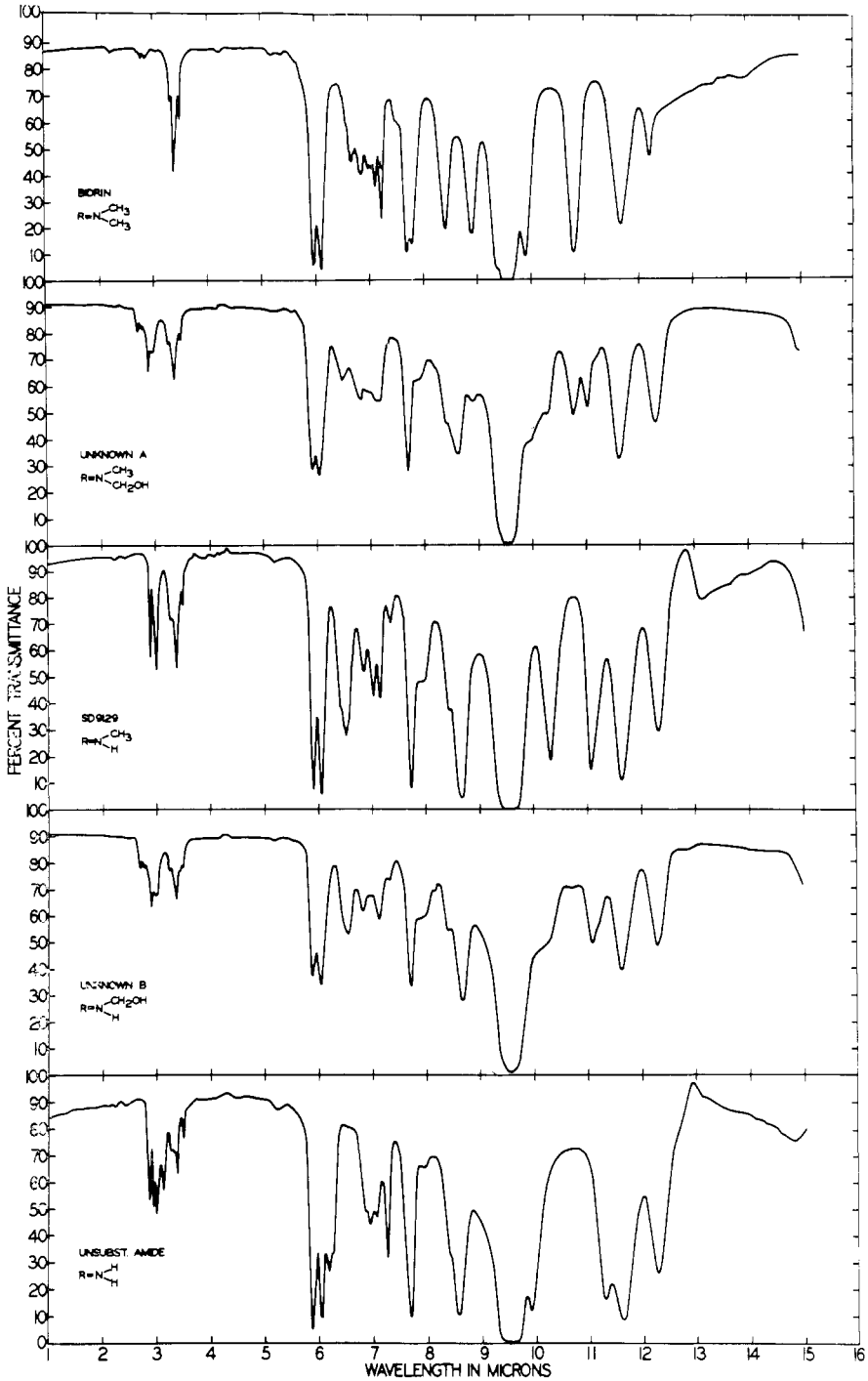

Figure 1. Infrared spectra on *cis*-$(CH_3O)_2P(O)OC(CH_3)$=$CHC(O)R$ compounds

Spectra determined from 10% solutions in methylene chloride using Beckman Model IR4 infrared spectrophotometer. Compounds were of known structure except for unknowns A and B, synthetic materials of the proposed structures. Unknown A contained appraximately 50% SD 9129; unknown B contained little, if any, of the unsubstituted amide

Partitioning Characteristics of Bidrin and Potential Metabolites. Partition coefficients for the phosphorus-containing compounds between chloroform and water were determined by either total phosphate or radioactivity analysis. Other partition coefficients were estimated on the basis of the weight of materials recovered upon extraction with chloroform. The following partition coefficients (chloroform/water) at 25° C. were obtained: Bidrin, 25; SD 9129, 2.5; the *N*-methyl-*N*-hydroxymethylamide analog of Bidrin, 0.97; the unsubstituted amide, 0.40; the *N*-hydroxymethylamide analog of SD 9129, 0.12; *N*,*N*-dimethylacetoacetamide, 1.15; *N*-methylacetoacetamide, 0.17; 3-hydroxy-*N*,*N*-dimethylbutyramide, 0.80; and 3-hydroxy-*N*-methylbutyramide, 0.017.

Chloroform was used to extract metabolites from aqueous solution for analysis of residues in plants and urine as described later. Three extractions

with chloroform volumes equal to the aqueous volume were used. Recoveries anticipated from this extraction procedure if the partitioning were unaffected by the biological material would be 99.99% for Bidrin, 97.7% for SD 9129, 86.9% for the *N*-methyl-*N*-hydroxymethylamide analog of Bidrin, 63.6% for the unsubstituted amide, 28.8% for the *N*-hydroxymethylamide analog of SD 9129, 89.9% for *N,N*-dimethylacetoacetamide, 37.5% for *N*-methylacetoacetamide, 82.8% for 3-hydroxy-*N,N*-dimethylbutyramide, and 4.9% for 3-hydroxy-*N*-methylbutyramide.

Neither Bidrin nor SD 9129 was extracted to any extent into *n*-hexane from water. These partitioning characteristics suggested the use of a partitioning column and mixtures of chloroform and hexane for resolving these potential Bidrin metabolites.

Phosphorus-containing hydrolysis products of the Bidrin analogs would be expected to remain in neutral aqueous solution on extraction with chloroform. Based on such a partitioning procedure and total phosphorus analyses, the following half-life values were obtained for the organophosphates at 0.1% in 0.037*N* sodium hydroxide at 28° C.: 64 minutes for Bidrin, 42 for SD 9129, and 35 for the unsubstituted amide.

Chromatography and Detection of Metabolites. Celite or silica gel was used in 2- × 30-cm. columns for separation of metabolites or 3.5- × 30-cm. columns for larger scale purifications. For the standard 2- × 30-cm. columns, water was added to silicic acid (Mallinckrodt chromatographic grade, 100-mesh) in a ratio of 1 to 2 (v.:w.) and mixed until the silica gel was homogeneous. For the other type of column, 25 ml. of water were added to 40 grams of Celite (Johns Manville's Hyflo Super-Cel) and mixed until the damp powder was homogeneous. The prepared stationary phase was then slurried in *n*-hexane and used in the packing of the columns. Silica gel was used in the early stages of this study, but in changing to different lots of silicic acid variable decomposition of metabolites and poorer resolution resulted. In most of the studies reported and for all quantitative data tabulated, Celite columns were utilized. Compounds were eluted from both silica gel and Celite columns with hexane, followed by hexane-chloroform mixtures, then chloroform, and finally a wash of methanol to clear the columns of any material that might have remained after elution with the hexane and chloroform mixtures. Resolution on the Celite columns is indicated in Figure 2. The ratio of eluting solvents was slightly different for the silica gel columns, although the basic relationships and order of compound elution remained the same. Twenty-milliliter fractions were collected at a flow rate of approximately 2 ml. per minute.

In some cases P^{32}- and *N*-methyl-C^{14}–labeled compounds were mixed for treatment of the organisms. The energy

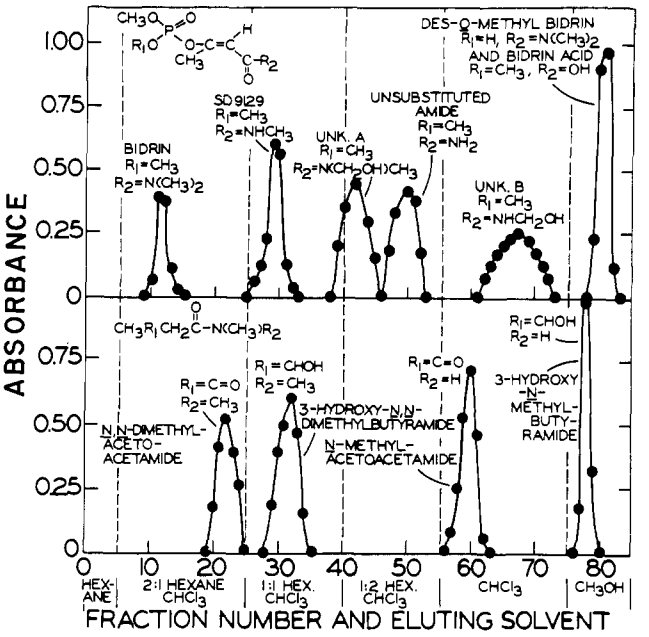

Figure 2. Chromatographic characteristics of some potential Bidrin metabolites on a Celite column based on partitioning between water and hexane-chloroform mixtures

Mixture of 50 mg. of each phosphorus-containing compound was chromatographed and 0.2 ml. from each 20-ml. fraction was analyzed for total phosphorus content (*2*). 50 mg. of each non-phosphorus-containing compound were similarly chromatographed and 1.0 ml. from each 20-ml. fraction was analyzed by a colorimetric procedure based on the 2,4-dinitrophenylhydrazones of the acetoacetamides, while the elution positions of the 3-hydroxybutyramides were determined by weights on the residues following evaporation of the solvents. All compounds were of known structure and high purity except unknowns A and B, which were synthetic materials

difference in the beta particles of P^{32} and C^{14} made it possible to assay with a liquid scintillation spectrometer each of these isotopes separately with little interference from the other in a mixed label sample. In this manner metabolism of both the phosphate and the *N*-methylamide portions of the molecule could be investigated simultaneously under identical biological conditions. Known nonradioactive compounds suspected of being metabolites were added to columns along with radiolabeled samples from treated plants or animals to aid in establishing the identity of the radiolabeled compounds with known materials eluting from the columns. Colorimetric procedures were used for total phosphate (*2*), formaldehyde (*8*), and 2,4 - dinitrophenylhydrazones derived from the known compounds.

For determination of the *N,N*-dimethylacetoacetamide and *N*-methylacetoacetamide which might result from hydrolysis of Bidrin and SD 9129, respectively, a rapid qualitative test was used, followed by quantitative analysis on the appropriate regions of the column eluates. To locate the regions where these two acetoacetamides were eluted, a 0.5-ml. aliquot of each chloroform or hexane-chloroform fraction was transferred to a small test tube and 2 drops of a 1.0% solution of ferric chloride in chloroform were added. Upon the addition of 1 drop of pyridine, a purple color was developed if either of the acetoacetamides was present (*24*). The acetoacetamides were then quantitatively determined as colored derivatives formed by degradation of the 2,4-dinitrophenylhydrazones (*15*). The 2,4-dinitrophenylhydrazones were prepared by adding 1.0 ml. of a 0.1% solution of 2,4-dinitrophenylhydrazine in 2*N* hydrochloric acid to the residue from evaporation of 1.0-ml. aliquots from column fractions, followed by heating at 90° C. for 30 minutes. The addition of 7.0 ml. of 0.75*N* sodium hydroxide formed a reddish-purple material which tended to precipitate. An equal volume of 2-methoxyethanol dissolved the precipitate. The absorbance of the resulting solution was read at 443 mμ.

The chromatographic positions of the reduction products of the two acetoacetamides, 3-hydroxy-*N,N*-dimethylbutyramide and 3-hydroxy-*N*-methylbutyramide, were determined with ceric ammonium nitrate reagent which was changed from yellow to deep red in their presence (*24*), or by evaporation of the fractions and determination of weights and infrared spectra of the residues.

Formation of Radiolabeled Derivatives from Degradation Products of Metabolites. To determine the nature of the amide portion of certain C^{14}-metabolites of Bidrin and SD 9129, the compounds under investigation were subjected to hydrolysis, derivative formation, and reversed isotope dilution procedures. Metabolites of Bidrin-*N*-methyl-C^{14} were used for determination of radio-labeled methylamine and form-

aldehyde, or dimethylamine; metabolites of SD 9129-*N*-methyl-C^{14}, for determination of radio-labeled formaldehyde or methylamine. Following chromatography, the solvent was evaporated from a known quantity of resolved metabolite contained in a round-bottomed flask. For the determination of labeled methylamine or dimethylamine, 10 ml. of 2*N* sodium hydroxide were added to the flask, which was then fitted with a water-cooled distillation condenser leading to a cooled receiving tube. The sodium hydroxide solution of the metabolite was heated until nearly all of the water containing hydrolyzed C^{14} compounds had distilled. To the distillate were added 20 μl. of a 40% aqueous solution of methylamine, followed by 42 μl. of phenyl isothiocyanate. This mixture was vigorously shaken. Methyl phenylthiourea crystallized (m.p. 113° C., 70% yield) from solution when held 18 hours at 4° C. The C^{14} content of a portion of the crystals was determined, and the remaining thiourea was subjected to thin layer chromatography on silica gel plates (Silica Gel G, Brinkmann Instruments, Inc., Great Neck, N. Y.) using a 2 to 1 ether-hexane mixture. Methyl phenylthiourea, R_f 0.5, and dimethyl phenyl thiourea, R_f 0.2 in a separate determination, were detected by spraying with 1.0% aqueous ferric chloride followed by 1.0% aqueous potassium ferricyanide to yield blue spots (*12*). The coincidence of the radioactive material with methyl phenylthiourea was determined by radioautography.

A procedure was developed for determination of labeled formaldehyde, methylamine, and dimethylamine recovered on acid hydrolysis of a single sample of *N*-methyl-C^{14}-metabolite. For C^{14}-formaldehyde determination the initial procedure was the same as above, except that 2.0% hydrochloric acid was used instead of 2*N* sodium hydroxide. To the distillate were added 5 μl. of a nonradioactive 37% aqueous solution of formaldehyde and 25 ml. of a hot solution of 0.25% aqueous 4-hydroxycoumarin, and the mixture was refluxed for 1 hour. The white crystals of dicoumarol (m.p. 285° C., yield 68%) were removed by filtration of the hot solution (*22*), and the radioactivity was determined. Unlabeled methylamine and dimethylamine (0.10 mmole each) were then added to the filtrate from the dicoumarol preparation, and the solution was evaporated to about 1 ml. with a stream of air and an infrared lamp. A sufficient quantity of 10% sodium hydroxide just to neutralize the solution was added, followed by 0.2 mmole of 3-nitrophthalic anhydride. The mixture was warmed to effect solution and then evaporated to dryness with a stream of air. The residue was heated for 30 minutes at 145° C. in order to convert the *N*-methylphthalamic acid to *N*-methylphthalimide (*1*). After cooling, 3 ml. of a saturated solution of sodium bicarbonate were added to dissolve the *N,N*-dimethylphthalamic acid and to decompose and dissolve any unreacted 3-nitrophthalic acid or anhydride. The phthalimide (60% yield) was filtered off, dried, and counted. The phthalamic acid was recovered (25% yield) from the bicarbonate solution by acidification, repeated extraction with 5-ml. portions of ethyl ether, and crystallization. The radioactivity was then determined. Quench correction for scintillation counting of the nitrophthalamic acid and the nitrophthalimide was 30% with a 6-mg. sample.

Classical procedures were used to prepare the semicarbazones and 2,4-dinitrophenylhydrazones of *N,N*-dimethyl- and *N*-methylacetoacetamides and the phenylurethanes of 3-hydroxy-*N,N*-dimethyl- and 3-hydroxy-*N*-methylbutyramides (*24*). Severe quenching in scintillation counting resulted with the 2,4-dinitrophenylhydrazones, but not with the semicarbazones or phenylurethanes.

Melting points and elemental analyses of the new derivatives are:

N-methyl-3-nitrophthalimide, m.p. 113° C.; calcd. for $C_9H_6N_2O_4$, C 52.43, H 2.93, N 13.59; found, C 52.57, H 3.07, N 13.74; *N,N*-dimethyl-3-nitrophthalamic acid, m.p. 186° C.; calcd. for $C_{10}H_{10}N_2O_5$, C 50.42, H 4.23, N 11.76; found, C 47.11, H 4.81, N 12.95; the semicarbazone of *N*-methylacetoacetamide, m.p. 163° C.; calcd. for $C_6H_{12}N_2O_4$, C 41.85, H 7.02, N 32.54; found C 41.89, H 6.91, N 32.31; the semicarbazone of *N,N*-dimethylacetoacetamide, m.p. 189° C.; calcd. for $C_7H_{14}N_4O_2$, C 45.15, H 7.58, N 30.09; found, C 41.89, H 5.04, N 30.18; the phenylurethane of 3-hydroxy-*N*-methylbutyramide, m.p. 89° C.; calcd. for $C_{12}H_{16}N_2O_3$, C 61.00, H 6.83, N 11.86; found, C 61.24, H 7.03, N 11.67; and the phenylurethane of 3-hydroxy-*N,N*-dimethylbutyramide, m.p. 111° C.; calcd. for $C_{13}H_{18}N_2O_3$, C 62.30, H 7.25, N 11.19; found, C 62.44, H 7.39, N 11.33.

Treatment of Plants and Animals. Garden snap bean seedlings (*Phaseolus vulgaris* L.) of the Contender variety in the trifoliate stage (average weight, 4.6 grams) were treated with 200 μg. of Bidrin-P^{32} or SD 9129-P^{32} by injection into the stems. Bidrin-P^{32} was introduced in 50 μl. of aqueous solution through glass capillary tubes (*12*). A more rapid and quantitative method of injection (suggested by R. J. Kuhr) was used with SD 9129-P^{32}. A small hole was made in the base of the stem of each plant, after which 20 μl. of the insecticide aqueous solution were injected directly into the stem about 2 inches above the initial hole, using a Hamilton 50-μl. microsyringe.

A 65.5-kg. Saanen goat was catheterized and treated orally with Bidrin-P^{32} at the rate of 1.0 mg. per kg. and, 4 days later, at 2.0 mg. per kg. Another goat, weighing 57.0 kg., was treated with a mixture of SD 9129-P^{32} and *N*-methyl-C^{14} and, 3 weeks later, with a mixture of Bidrin-P^{32} and *N*-methyl-C^{14} at 1.0 mg. per kg. The labeled compound was distributed equally among four gelatin capsules containing crushed corn to absorb the small amount of chloroform involved in the transfer. A balling gun was used in administering the capsules. Neither goat showed any symptoms of toxicity after the doses of organophosphate. Milk, urine, and feces were collected for analysis at frequent intervals up to 72 hours after treatment. The level of radioactivity secreted into the milk or excreted was at an insignificant level following the first treatment with labeled compound before the second dose was administered.

Rats, mice, rabbits, and a dog were also treated at 1 mg. per kg. with aqueous solutions of labeled compounds, primarily for urinalysis. White rats of both sexes (140 to 160 grams) from Rolfsmeyer Farms, Madison, Wis., were treated by stomach tube and held in metabolism cages to allow for separate collection of urine and feces. In one experiment two rats treated with Bidrin-*N*-methyl-C^{14} were held in metabolism cages designed for total collection of expired $C^{14}O_2$ in a 2 to 1 mixture of 2-methoxyethanol–ethanolamine. This mixture was counted directly in a scintillation counter (*17*). Female white mice (25 grams, Rolfsmeyer Farms) were treated intraperitoneally, held in metabolism cages, and handled in a manner identical with that for the rats. Two male rabbits (3.2 and 2.8 kg.) were treated intraperitoneally, and the total urine was collected for 48 hours. A catheterized female dog (18 kg., studied in cooperation with R. C. Herrin, University of Wisconsin Medical School) was treated orally by stomach tube and the total urine collected for 48 hours.

Adult American cockroaches [*Periplaneta americana* (L.)] were treated at 20 μg. of Bidrin-P^{32} per cockroach by injection through the ventral abdominal wall. The cockroaches showed severe poisoning symptoms but were still alive after 4 hours, at which time they were killed and the whole animals extracted. Adult female houseflies (*Musca domestica* L., C.S.M.A. 1948 strain, 3 days after emergence) were treated on the tip of the abdomen with 1.0 μl. of acetone containing Bidrin- or SD 9129-*O*-methyl-C^{14} per fly to yield 1.0 mg. of toxicant per kg. of flies. A portion of the flies were treated on the thorax with 10 μg. of sesamex [2-(2-ethoxyethoxy)-ethyl-3,4-(methylenedioxy)phenyl acetal of acetaldehyde] per fly in 1.0 μl. of acetone immediately before application of the labeled toxicant. The flies were extracted 4 hours after treatment. No symptoms of poisoning were evident at 4 hours with Bidrin or SD 9129 in the absence of sesamex, but 25% mortality occurred with Bidrin and 10% with SD 9129 in the presence of sesamex.

Extraction and Analysis Procedures. Plants were extracted by a procedure designed to differentiate the nonhydrolyzed products from the labeled phosphoric or crotonic acid metabolites. The whole plant was macerated with 50 ml. of acetone in either a glass homogenizer or a Lourdes Multi-Mix. If the glass homogenizer was used, anhydrous sodium sulfate equal to one half the weight of the plant was added; otherwise, the plant was macerated with

acetone alone. The aqueous-acetone solution was separated from the plant residue by filtering through a Büchner funnel. If sodium sulfate had been added, the plant residue was washed with 10 ml. of water, and the radioactivity of both the water and the residue was counted. The aqueous-acetone solution was evaporated to a small volume with the aid of a gentle stream of air and an infrared lamp, after which the remaining aqueous solution was made up to 4.0 ml. with water. This water was extracted with an equal volume of hexane, which removed most of the plant pigments but none of the radioactivity. Extraction of the water with an equal volume of chloroform was then used to ascertain the percentage of the administered compound remaining in the unhydrolyzed form. The data were corrected for the known partitioning characteristics of the unhydrolyzed compounds analyzed. Extraction of samples to which a known quantity of labeled Bidrin-P^{32} was added immediately before extraction yielded 85% recovery of the Bidrin. The recovery from extraction immediately after injection of Bidrin-P^{32} or SD 9129-P^{32} was considered to be 100% for preparation of residue dissipation curves. In some cases, the chloroform extract was chromatographed to determine the identity of labeled compounds that might be present and in these studies the aqueous metabolite solution was extracted three times with equal volumes of chloroform for more complete recovery.

The goat milk was extracted according to a described procedure within 1 hour after milking (*28*). One hundred fifty milliliters of acetonitrile warmed to 40° C. were added to 100 ml. of milk, except with the earliest samples, where only between 50 and 100 ml. of milk were available for analysis. The acetonitrile addition resulted in protein precipitation to yield a "solids fraction" which was then washed with 150 ml. of chloroform. Combining all solvents and mixing gave two layers, a chloroform and acetonitrile layer and a second phase consisting of the water from the milk. Radioactivity in the solids (100-mg. aliquot) and aqueous (0.2-ml. aliquot) fractions was determined by liquid scintillation counting. The chloroform-acetonitrile, or "organo-extractable fraction," was counted, the solvent stripped, and the residue chromatographed to determine the residues of Bidrin or SD 9129 and their metabolites.

Urine samples were extracted three times with an equal volume of chloroform, using centrifugation to separate the phases. The proportion of P^{32} in the two fractions of the first extraction was used to calculate the percentage of hydrolyzed products in the urine of Bidrin-treated animals, while the sum of the P^{32} in the three chloroform extracts was compared to that in the water for SD 9129-treated animals. The combined chloroform from the three extractions was evaporated onto a small amount of Celite and placed directly on columns for chromatography.

The treated cockroaches were macerated with sodium sulfate and chloroform in a Lourdes Multi-Mix, then filtered through a Büchner funnel; the chloroform was evaporated onto Celite for chromatography. The residue containing sodium sulfate was washed with water to recover the hydrolysis products for counting. Prior to maceration the houseflies were subjected to a preliminary surface wash with acetone to remove labeled compounds which had not penetrated the cuticle. The radioactive materials which penetrated were extracted for determination of hydrolysis products or for chromatography in the same manner as with the cockroaches.

Other Methods. Anticholinesterase assays with fly head homogenates and human plasma were made as previously described (*12*). Acetylcholine was used as the substrate for fly head homogenates, and butyrylcholine was used for plasma. Toxicity of the compounds to 25-gram female white mice was evaluated 24 hours after intraperitoneal injection in aqueous solutions. LD_{50} values for Bidrin analogs with adult female houseflies in the presence and absence of sesamex were determined under the same conditions of topical application used in the metabolism studies with flies. Mortality observations were made at 24 hours. No mortality resulted from sesamex alone, even at twice the level used.

The Packard Tricarb Model 314EX liquid scintillation spectrometer was used for most of the radioactivity measurements. The scintillation mixture consisted of 0.55% 2,5-diphenyloxazole in a 2 to 1 toluene–2-methoxyethanol mixture. Corrections for quenching were made when appropriate, except with the solids fraction from goat's milk. Studies with P^{32}-labeled compounds involving partitioning between chloroform and water for determining hydrolysis rates in plants and direct counting for determining the per cent excretion of metabolites in feces of rats and goats involved counting with the Nuclear-Chicago No. D-47 gas flow counter using a Mylar window.

Results

Chemical Nature of Bidrin-P^{32} and SD 9129-P^{32} Metabolites. Bidrin-P^{32} yielded from three to six peaks attributable to Bidrin or its metabolic products based on chromatography on Celite columns of organic solvent extracts of urine from various animals, milk from goats, whole insects, and bean plants. In these same extracts from animals that had been treated with a mixture of Bidrin-P^{32} and *N*-methyl-C^{14}, one P^{32}-containing peak lacked C^{14}, two very minor peaks contained C^{14} but no P^{32}, and the other five peaks contained both P^{32} and C^{14}. When similar extracts from SD 9129-P^{32}–treated animals were chromatographed, only three to four peaks were evident. When the mixture of SD 9129-P^{32} and *N*-methyl-C^{14} was administered, one P^{32}-containing peak lacked C^{14}, and the other two peaks contained both P^{32} and C^{14}. The chromatographic positions for the peaks from SD 9129-P^{32} were identical to those of the last metabolites eluted with Bidrin-P^{32}. Figure 2 illustrates the elution positions for some known compounds, along with two synthetic materials for which structures are proposed. All compounds can be satisfactorily resolved, except SD 9129 from 3-hydroxy-*N*,*N*-dimethylbutyramide and the mixture of materials finally eluted with methanol. The presence of 3-hydroxy-*N*,*N*-dimethylbutyramide in the SD 9129 peak would be evidenced in mixed label studies by an increase in the C^{14} content relative to the P^{32} content as compared with the ratio of the labels administered.

Bidrin, SD 9129, and the unsubstituted amide were identified, when present, by cochromatography of the radioactive unknowns with the nonlabeled known compounds. Bidrin and SD 9129 from plants treated with Bidrin were also identified by examination of infrared spectra of the fractions isolated by column chromatography. The Bidrin peak was present only when Bidrin was administered, while the SD 9129 peak and the unsubstituted amide peak appeared with either Bidrin or SD 9129 treatment. When P^{32}- and *N*-methyl-C^{14}-Bidrin were simultaneously administered, the Bidrin recovered from chromatography contained the same C^{14}:P^{32} ratio as the administered compound, the SD 9129 peak contained one half that ratio, and the amide peak lacked any C^{14}. When P^{32}- and *N*-methyl-C^{14}-SD 9129 were simultaneously administered, the SD 9129 peak from chromatography contained the same isotope ratio administered, but the amide peak lacked any C^{14}. Unknown A, which appeared only following Bidrin administration, contained the same isotope ratio as the Bidrin when the mixed label was used. Acid degradation of unknown A yielded 1 mole of formaldehyde-C^{14} per mole of metabolite as determined by formation of dicoumarol-C^{14}. The amine liberated on hydrolysis of the metabolite was monomethyl-C^{14}-amine, not dimethyl-C^{14}-amine, based on formation of the *N*-methyl-C^{14}-3-nitrophthalimide. Furthermore, alkaline hydrolysis and reaction with phenyl isothiocyanate yielded methyl-C^{14}-phenylthiourea but no dimethyl-C^{14}-phenylthiourea. Unknown A cochromatographed with the *N*-methyl-*N*-hydroxymethylamide analog of Bidrin formed by reaction of SD 9129 with formaldehyde. Unknown A is therefore proposed to be 3-(dimethoxyphosphinyloxy) - *N* - methyl - *N* - hydroxymethyl-*cis*-crotonamide. Unknown B was formed from both Bidrin and SD 9129 and contained one half the C^{14}:P^{32} ratio administered when derived from

Table I. Metabolites of Bidrin-P^{32} and SD 9129-P^{32} in Urine Following Treatment of Mammals at 1.0 Mg. per Kg.

(All results expressed as per cent of administered P^{32})

Treatment and Hours	No. of Animals	Total Excreted	Hydrolysis Products[a]	cis-$(CH_3O)_2P(O)OC(CH_3)$=$CHC(O)R$[b]					CH_3OH Fraction[c]
				$N(CH_3)_2$	$N(CH_2OH)CH_3$	$NHCH_3$	$NHCH_2OH$	NH_2	
Rats, Bidrin (Oral by Stomach Tube)									
♂ 0–6	6	51.4	37.9	3.0	1.3	7.9	0.68	0.00	0.67
♀ 0–6	5	44.8	33.8	3.0	0.40	5.4	0.23	0.00	2.0
♂ 6–12	6	12.5	10.8	0.39	0.25	0.84	0.07	0.00	0.12
♀ 6–12	5	11.0	10.3	0.31	0.08	0.16	0.01	0.00	0.15
♂ & ♀ 12–24	11	0.40	0.38	0.003	0.002	0.01	0.001	0.00	0.003
♂ & ♀ 24–48	11	2.1	2.05	0.02	0.0006	0.03	0.00	0.00	0.003
♂ 0–48	2	70.1	60.5	2.8	2.8	3.0	0.35	0.00	0.68
♀ 0–48	2	64.5	53.1	3.2	0.08	5.3	0.09	0.00	2.7
Rats, SD 9129 (Oral by Stomach Tube)									
♂ 0–6	6	53.6	34.5	...	...	11.6	1.8	0.24	5.5
♀ 0–6	6	57.8	34.6	...	...	17.3	1.6	0.06	4.2
♂ 6–12	6	9.0	7.5	...	...	0.81	0.07	0.03	0.6
♀ 6–12	6	13.4	11.1	...	...	1.3	0.17	0.03	0.8
♂ & ♀ 12–24	12	5.1	4.4	...	...	0.47	0.07	0.00	0.2
♂ & ♀ 24–48	12	3.0	2.5	...	...	0.37	0.04	0.00	0.1
♂ 0–48	2	71.3	52.2	...	...	13.0	1.6	0.20	4.3
♀ 0–48	2	62.8	48.2	...	...	12.1	0.64	0.27	1.6
Mice, Female, Bidrin (Intraperitoneal)									
0–48	19	71.7	57.0	1.2	0.43	9.9	1.0	0.00	2.2
Rabbits, Male, Bidrin (Intraperitoneal)									
0–48	2	72.1	67.7	1.0	0.18	2.8	0.04	0.04	0.36
Dogs, Female, Bidrin (Oral by Stomach Tube)									
0–48	2	97.6	92.2	0.76	0.28	3.3	0.31	0.00	0.77
Goat, Female, Bidrin (Oral by Capsule)[d]									
0–1	1	0.05	0.023	0.0006	0.00	0.014	0.00	0.003	0.01
1–2	1	10.6	7.8	0.10	0.00	1.5	0.16	0.53	0.48

[a] Hydrolysis products considered to be P^{32} compounds remaining in water on extraction with an equal volume of chloroform in Bidrin studies, or three extractions with equal volumes of chloroform in SD 9129 studies.

[b] Bidrin is the $—N(CH_3)_2$ analog and SD 9129, the $—NHCH_3$ analog. Percentages based on column chromatography of chloroform extracts as indicated in [a]. Results corrected for incomplete extractions of certain metabolites based on their partitioning characteristics as indicated in experimental section.

[c] Methanol fraction eluted from columns contained a mixture of unidentified materials.

[d] Urine from Bidrin-treated goat collected at intervals greater than those indicated contained all P^{32} compounds as hydrolysis products. All urine samples from SD 9129–treated goat contained no chloroform-extractable P^{32} compounds.

Bidrin and the same C^{14}:P^{32} ratio administered when derived from SD 9129. Acid hydrolysis yielded 1 mole of formaldehyde-C^{14} per mole of compound, but no labeled amine. Unknown B cochromatographed with the *N*-hydroxymethylamide analog of SD 9129 formed by reaction of the unsubstituted amide with formaldehyde. Unknown B is therefore proposed to be 3-(dimethoxyphosphinyloxy) - *N* - hydroxymethyl-*cis*-crotonamide. The final peak eluting with methanol, present in extracts of organisms treated with either Bidrin or SD 9129, yielded variable C^{14}:P^{32} ratios, depending on the biological system. It therefore was not a homogeneous fraction. Known compounds eluting in this position are Bidrin acid, des-*O*-methyl Bidrin, and 3-hydroxy - *N* - methylbutyramide. The methanol column wash may contain some of these compounds, and perhaps others of a hydrolytic nature, but no attempt was made to characterize these materials further.

Chemical Nature of Bidrin- and SD 9129-*N*-methyl-C^{14} Metabolites. Hydrolysis of Bidrin-*N*-methyl-C^{14} should yield *N*,*N*-dimethyl-C^{14}-acetoacetamide, and of SD 9129-*N*-methyl-C^{14} should yield *N*-methyl-C^{14}-acetoacetamide. In vivo reduction might subsequently form 3 - hydroxy - *N*,*N* - dimethyl - C^{14} - butyramide and 3-hydroxy-*N*-methyl-C^{14}-butyramide. The chromatographic resolution was adequate to investigate the potential presence of the two acetoacetamides as components separated from phosphorus-containing materials, but was less satisfactory for the 3-hydroxybutyramides since the *N*,*N*-dimethyl analog eluted in the approximate position of SD 9129 and the *N*-methyl analog eluted in the methanol fraction (Figure 2). Studies with mixtures of labeled materials were particularly useful with 3-hydroxy-*N*,*N*-dimethylbutyramide since a higher than anticipated C^{14}:P^{32} ratio for this peak indicated the presence of the butyramide as a contaminant. The methanol fraction might consist of a mixture of phosphorus- and non-phosphorus-containing metabolites. The heterogeneity of metabolities in the methanol fraction was confirmed with mixed label studies where the C^{14}:P^{32} ratio recovered varied from that administered as follows: almost the same for urine from SD 9129–treated rats; 1.5 times for urine from Bidrin-treated rats; 0.4 times for urine from a Bidrin-treated goat; 0.5 times for urine from Bidrin-treated mice; and 1.8 to 16.3 times for milk from a goat treated with Bidrin or SD 9129, as discussed later.

In early samples of urine from rats treated with Bidrin-*N*-methyl-C^{14}, evidence was obtained of the presence of *N* - methyl - C^{14} - acetoacetamide and 3 - hydroxy - *N*,*N* - dimethyl - C^{14}-butyramide. There was no indication of *N*,*N* - dimethyl - C^{14} - acetoacetamide in urine of Bidrin-treated rats, nor of *N*-methyl-C^{14}-acetoacetamide in urine of SD 9129–treated rats. None of these materials appeared in other urine samples or in the milk samples following either Bidrin- or SD 9129-*N*-methyl-C^{14} administration. In the 0- to 8-hour sample of urine from male rats treated with Bidrin-*N*-methyl-C^{14}, a shoulder on the last-eluting portion of the SD 9129 peak was suspected of being 3-hydroxy-*N*,*N*-dimethyl-C^{14}-butyramide. This labeled compound cochromatographed

Table II. Bidrin, SD 9129, and Organoextractable Metabolites in Milk of a Goat Following Oral Administration of Bidrin-P^{32} or SD 9129-P^{32} in Capsules at 1.0 Mg. per Kg.

Hr. after Treatment	Parts per Million Based on Column Chromatography[a]						Total P.P.M. Organo-extractable[b]
	$N(CH_3)_2$[a]	$N(CH_2OH)CH_3$	$NHCH_3$[a]	$NHCH_2OH$	NH_2	CH_3OH froc.	
			Bidrin Administration				
1	0.0208	<0.0001	0.0328	0.0038	<0.0001	0.0030	0.095
2	0.0184	<0.0001	0.0760	0.0026	0.0011	0.0252	0.234
4	0.0053	<0.0001	0.0376	0.0113	0.0028	0.0296	0.165
6	0.0069	<0.0001	0.0203	0.0146	0.0013	0.0084	0.098
8	0.0004	<0.0001	0.0010	0.0001	<0.0001	0.0005	0.064
12	0.0011	<0.0001[c]	0.0043	0.0029	<0.0001	0.0018	0.055
16	0.0004	<0.0001	0.0015	0.0009	<0.0001	0.0008	0.011
20	0.0003	<0.0001	0.0008	0.0003	<0.0001	0.0004	0.025
24	0.0003	<0.0001	0.0006	<0.0001[c]	<0.0001	0.0013	0.021
			SD 9129 Administration				
1	...	...	0.0451	0.0001	<0.0001[c]	0.0058	0.067
2	...	...	0.0320	0.0086	0.0007	0.0056	0.061
4	...	...	0.0335	0.0086	0.0015	0.0103	0.074
6	...	...	0.0233	0.0105	0.0013	0.0109	0 057
8	...	...	0.0116	0.0099	0.0007	0.0029	0.042
12	...	...	0.0073	0.0044	0.0006	0.0018	0.025
16	...	...	0.0058	0.0020	0.0001	0.0040	0.012
20	...	...	0.0116	0.0009	0.0003	0.0147	0.022
24	...	...	0.0014	0.0007	<0.0001	0.0005	0.007

[a] Designations refer to *cis*-$(CH_3O)_2P(O)OC(CH_3)=CHC(O)R$ where R = $N(CH_3)_2$ for Bidrin and $NHCH_3$ for SD 9129. Fortification of milk with known P^{32} compounds followed by described extraction and chromatography procedures yielded 80.7% recovery for Bidrin and 94.3% recovery for SD 9129. Recovery analyses not made on other materials. All p.p.m. values based on fresh weight of whole milk.

[b] A variable proportion of the total organoextractable-P^{32} was recovered following column chromatography. Difference between total p.p.m. of organoextractable P^{32} and sum of fractions recovered from column probably resulted in part from unknown metabolites not eluted from column.

[c] Metabolites present at less than 0.0001 p.p.m. are divided into those in which trace amounts were evident as designated by [c] and those where no evidence of their presence was obtained and no letter is used.

with known 3-hydroxy-*N*,*N*-dimethylbutyramide and formed a labeled phenylurethane from the mixed unlabeled known and labeled unknown compounds. The amount of 3-hydroxy-*N*,*N* - dimethyl - C^{14} - butyramide was 5 to 10% that of the SD 9129 in this urine sample. In this same urine sample, a peak containing C^{14} but no P^{32} appeared in the position associated with *N*-methylacetoacetamide and contained 18% of the C^{14}-content of the Bidrin-C^{14} peak. This labeled metabolite peak was recovered and found to cochromatograph with known *N*-methylacetoacetamide and to form a labeled semicarbazone of the expected specific activity.

Excretion of Metabolites from Bidrin- and SD 9129-Treated Mammals. RATS. Sixty-three to 71% of the P^{32} from an oral dose of Bidrin-P^{32} or SD 9129-P^{32} to male or female rats was excreted in the urine within 48 hours. No marked sex or compound difference in total excretion was apparent and the majority of this radioactivity appeared in the urine within the first 6 hours (Table I). An additional 6% appeared in the feces within 48 hours following Bidrin-P^{32} treatment and 5% with SD 9129-P^{32}. Residual P^{32} in the tissues 48 hours after Bidrin-P^{32} treatment did not vary with sex. The total Bidrin-P^{32} equivalents based on whole tissue counting without fractionation as averaged from 11 animals were as follows (p.p.m.): blood, 0.049; bone, hind femur, 0.287; brain, 0.038; fat, omental, 0.000; heart, 0.045; liver, 0.620; kidney, 0.340; and muscle, 0.010. Female rats yielded 14% more of the administered C^{14} from Bidrin- or SD 9129-*N*-methyl-C^{14} in the urine than with P^{32} compounds, while the increased excretion of C^{14} with males was only 3 to 5% compared with P^{32}, and again did not vary with compound. An additional 12% was recovered as $C^{14}O_2$ within 48 hours after treatment of male rats with Bidrin-*N*-methyl-C^{14}.

With both compounds and both labels, the majority of the radioactivity in the urine was not extractable into chloroform, and that portion which was extracted was present largely as compounds containing both phosphorus and at least one *N*-methyl carbon based on the mixed isotope studies. The major chloroform-extractable product in the urine following treatment with either Bidrin or SD 9129 was SD 9129 (Table I). It accounted for about 5% of the administered Bidrin dose and about 12% of the administered SD 9129. Significant amounts of the *N*-hydroxymethylamide analog of SD 9129 appeared following treatment with either compound, while the *N* - methyl - *N* - hydroxymethylamide compound was present only following Bidrin treatment, and the unsubstituted amide, only following SD 9129 treatment.

GOATS. Goats treated orally with Bidrin or SD 9129 also excreted in the urine a high proportion of the administered P^{32}- or *N*-methyl-C^{14} labels. The percentages of the administered radioactivity appearing in the urine were, with one goat: for Bidrin, 27 P^{32} and 22 C^{14} at 4 hours, 56 P^{32} and 41 C^{14} at 8 hours, 78 P^{32} and 60 C^{14} at 16 hours, and 90 P^{32} and 84 C^{14} at 72 hours; for SD 9129, 12 P^{32} and 14 C^{14} at 4 hours, 42 P^{32} and 45 C^{14} at 8 hours, 58 P^{32} and 65 C^{14} at 16 hours, and 67 P^{32} and 90 C^{14} at 72 hours. Another goat treated with Bidrin-P^{32} alone yielded the following percentages of the administered radioactivity in the urine, the first value being that for a 1 mg. per kg. dose, and the second, that for a 2 mg. per kg. dose: 21 and 4 at 4 hours, 55 and 45 at 8 hours, 72 and 65 at 16 hours, and 78 and 82 at 72 hours. Feces elimination accounted for 0.77% of the P^{32} from the administered 1 mg. per kg. dose and 1.31% of the 2 mg. per kg. dose. Extraction of the urine collected at frequent intervals after SD 9129-P^{32} and *N*-methyl-C^{14} administration yielded no radioactivity in the chloroform, even with three extractions, indicating that less than 0.01% of the administered dose was excreted in the urine as SD 9129, its *N*-hydroxymethylamide analog, or the unsubstituted amide. The P^{32}-compounds in the urine following Bidrin-P^{32} administration were extractable into chloroform only to the extent of 52% for the 0- to 1-hour sample, 23% for the 1- to 2-hour sample, less than 5% for the

2- to 4-hour sample, and even less thereafter when most of the total P^{32} was excreted. Trace amounts of Bidrin, SD 9129, the *N*-hydroxymethylamide analog of SD 9129, and the unsubstituted amide were present in the urine. Their combined amounts accounted for less than 2.6% of the administered dose (Table I).

OTHER ANIMALS. Urinalysis results for dogs, mice, and rabbits, compared with those for rats and goats (Table I), indicated that species variation exists in the rate at which a Bidrin-P^{32} dose is metabolized and the extent of excretion of the metabolites in the urine. In mice, Bidrin-P^{32} yielded 71.7% of the label in the urine, while Bidrin-*N*-methyl-C^{14} gave only 60% from the same animals.

Residues in Milk Following Treatment of Goats with Bidrin and SD 9129. Treatment of goats with 1.0 mg. per kg. of P^{32}- and C^{14}-labeled Bidrin or SD 9129 resulted in the rapid appearance of labeled compounds in the milk with Bidrin, but a much slower secretion into the milk with SD 9129 (Figure 3). The percentage of the administered radiolabel appearing in the milk within 72 hours was 0.72 for Bidrin-P^{32} and 1.4 for SD 9129-P^{32}, while the *N*-methyl-C^{14}-label gave 3.2 for Bidrin and 2.9 for SD 9129. Both compounds and both labels yielded the majority of the radioactivity (the difference between the "whole milk" and the "organoextractable" in Figure 3) in the solids and aqueous fractions after extraction with chloroform and acetonitrile.

No method was found for separating the metabolites from these fractions for further investigation. A larger proportion of the P^{32} appeared in the solids than in the aqueous fraction with SD 9129 compared with Bidrin, although most of the radioactivity in the milk with both compounds was in the solids fraction after 48 hours. Only within the first 24 hours after treatment did the organoextractable fraction account for more than 0.01 p.p.m. of P^{32} equivalents or 0.1 p.p.m. of C^{14} equivalents of the administered compound in the milk, and this fraction reached a peak level of 0.234 p.p.m. with Bidrin-P^{32} at 2 hours, 0.470 p.p.m. with Bidrin-*N*-methyl-C^{14} at 2 hours, 0.074 p.p.m. with SD 9129-P^{32} at 4 hours, and 0.208 p.p.m. with SD 9129-*N*-methyl-C^{14} at 6 hours. More C^{14} than P^{32} compared to the ratio administered appeared in the organoextractable fraction with both compounds, but especially with Bidrin. These additional C^{14} compounds were eluted with hexane alone coincident with the milligram amounts of milk lipids where no P^{32} compounds were present, and with methanol, where both C^{14} and P^{32} compounds were eluted. No C^{14}- or P^{32}- labeled materials, other than those previously described, were eluted with hexane-chloroform mixtures or chloroform alone. This additional C^{14} fraction which contained no P^{32} was recovered along with the lipids from goat milk following either Bidrin or SD 9129 mixed label treatments, but was not present in any other case examined. The early C^{14}-"lipid" component reached levels of 0.003, 0.012, 0.021, 0.024, and 0.041 Bidrin-C^{14} p.p.m. equivalent at 1, 4, 8, 16, and 24 hours, respectively, and 0.008, 0.006, 0.013, 0.007, and 0.004 p.p.m. SD 9129-C^{14} equivalent at 1, 4, 8, 16, and 24 hours, respectively. This C^{14} fraction may be the result of incorporation of the released formaldehyde into the one-carbon pool (*19*).

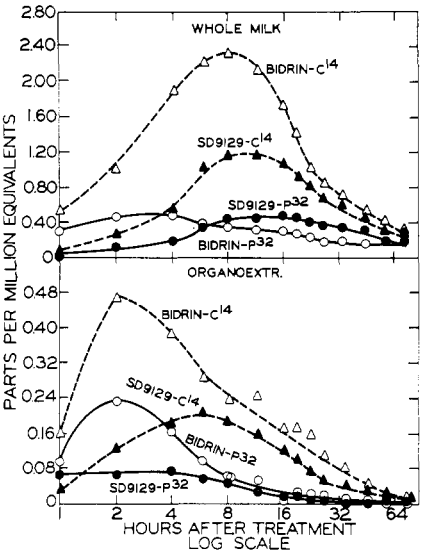

Figure 3. Bidrin-P^{32} and -C^{14} and SD 9129-P^{32} and -C^{14} in whole goat milk and organoextractable fraction following oral administration in capsules of 1.0 mg. per kg.

Addition of acetonitrile to whole milk precipitated solids fraction. Extraction of supernatant with chloroform yielded organoextractable and aqueous fractions. P.p.m. equivalents in whole milk were generally greater than summation of fractions. Losses resulted from errors in detecting total radioactivity in the solids fraction. Results from chromatography of organoextractable fraction indicated in Table II

In the methanol fraction from columns, the C^{14}:P^{32} ratio was 1.8 times that administered during the first 6 hours and 8.6 times thereafter with Bidrin, but was 3.5 times during the first 6 hours and 16.3 times thereafter with SD 9129. The parts per million values for the C^{14} in this methanol fraction were 0.0054, 0.0533, 0.0043, 0.0069, and 0.0112 Bidrin-C^{14} equivalent at 1, 4, 8, 16, and 24 hours, respectively, and 0.0203, 0.0361, 0.0473, 0.0652, and 0.0082 SD 9129-C^{14} equivalent at 1, 4, 8, 16, and 24 hours, respectively. No *N*,*N* - dimethylacetoacetamide, *N*-methylacetoacetamide, or 3-hydroxy-*N*,*N*-dimethylbutyramide was present in the milk, and the nature of the C^{14} materials in the methanol fractions from columns remains unknown. The major organoextractable P^{32} material in the milk was SD 9129 following both Bidrin and SD 9129 treatment (Table II). Less than 0.0001 p.p.m. of the *N*-methyl-*N*-hydroxymethylamide compound appeared in the milk with Bidrin treatment, and less than 0.003 p.p.m. of the unsubstituted amide following treatment with either Bidrin or SD 9129, assuming that the recovery of these materials with the extraction procedure used was satisfactory. The level of the hydroxymethylamide analog of SD 9129 was always lower than that of SD 9129. These P^{32} results for Bidrin were confirmed as to proportional milk levels and compounds present with another goat treated with a 1.0 mg. per kg. dose followed later by a 2.0 mg. per kg. oral dose (*18*).

Metabolism of Bidrin and SD 9129 by Insects. Houseflies and American cockroaches rapidly metabolized Bidrin and SD 9129 to form the same intermediate *N*-hydroxymethyl and *N*-demethylated derivatives detected in mammalian urine and milk (Table III). Sesamex inhibited the penetration of the organophosphate through the cuticle of houseflies since the percentage of the applied dose that penetrated was 94.9 for Bidrin, 78.7 for Bidrin plus sesamex, 87.8 for SD 9129, and 76.0 for SD 9129 plus sesamex. This penetration difference with sesamex may have been due in part to the more toxic nature of the organophosphate-synergist combination resulting in poisoning symptoms not evident with the organophosphate alone. The synergist did not greatly affect the percentage of the compounds hydrolyzed at 4 hours nor the residual level of SD 9129, but greatly increased the percentage of the Bidrin dose that persisted. Sesamex had a marked inhibitory effect on the formation of the metabolites associated with the oxidative demethylation of the *N*,*N*-dimethylamide or *N*-methylamide group. Comparatively large amounts of the *N*-hydroxymethylamide compounds were detected in flies treated with Bidrin or SD 9129 alone, but they were absent from the extracts of flies treated with the toxicant plus synergist, and only minute amounts of SD 9129 were detected in flies treated with Bidrin plus sesamex.

Metabolism of Bidrin and SD 9129 by Bean Plants. Bidrin-P^{32} and SD 9129-P^{32} injected into the stems of bean plants were rapidly translocated into the foliage and persisted for several weeks under greenhouse conditions. Residue dissipation curves indicating similar over-all rates with foliage only or whole plant analysis were obtained following Bidrin or SD 9129 injection. Half-life values were 9 days for Bidrin and 14 days for SD 9129, although in comparing the results it must be appreciated that the compounds were investigated at dif-

Table III. Metabolites of Bidrin and SD 9129 in *Musca domestica* and *Periplaneta americana* 4 Hours after Treatment

Compound and Insect	% of Injected or Penetrated Radioactivity						
	Hydrolysis Products[a]	cis-$(CH_3O)_2P(O)OC(CH_3)=CHC(O)R$[b]					CH_3OH Fraction[b]
		$N(CH_3)_2$	$N(CH_2OH)CH_3$	$NHCH_3$	$NHCH_2OH$	NH_2	
Bidrin, *M. domestica*[c]							
No sesamex	90.7	5.3	0.3	2.3	0.9	0.1	0.4
10 μg. sesamex per fly	85.3	14.1	<0.1	0.4	<0.1	<0.1	0.2
SD 9129, *M. domestica*[c]							
No sesamex	81.2	...	...	13.4	3.8	0.2	1.4
10 μg. sesamex per fly	86.2	...	...	12.5	<0.1	<0.1	1.3
Bidrin, *P. americana*[d]	78.6	14.3	<0.1	4.0	0.2	<0.1	2.8

[a] Radioactive material not extracting and chromatographing in manner anticipated for neutral phosphate esters.
[b] Designations refer to labeled compounds recovered by macerating whole insects with sodium sulfate and chloroform and subjecting chloroform extract to chromatography on Celite column. R = $N(CH_3)_2$ for Bidrin and $NHCH_3$ for SD 9129. Methanol fraction eluted from columns contained a mixture of unidentified materials.
[c] Topical treatment with Bidrin- or SD 9129-*O*-methyl-C^{14} at 1.0 mg. per kg. with and without simultaneous treatment of flies with 10 μg. sesamex per fly. Prior to maceration whole flies rinsed with acetone, so only labeled compounds which had penetrated into insect are considered.
[d] Adult American cockroaches injected into abdomen with an aqueous solution of Bidrin-P^{32} to yield dose of 20 mg. per kg.

ferent times of the year (Figure 4). The nature of the hydrolysis products and their rate of incorporation into the plant constituents may have varied between the two compounds, since Bidrin treatment yielded both Bidrin and hydrolysis products in the aqueous-acetone extract, while SD 9129 treatment yielded SD 9129 but little or no hydrolysis products in the aqueous-acetone extract. The radioactivity incorporated into the solids fraction of the plant was also much greater with SD 9129 than with Bidrin (22% for Bidrin *vs.* 39% for SD 9129 at 12 days). Administration of Bidrin yielded some SD 9129, trace amounts of the *N*-methyl-*N*-hydroxymethylamide analog, but no mono-*N*-hydroxymethylamide analog or unsubstituted amide. The SD 9129 was present at 10 to 14% of the Bidrin level at 8, 10, and 12 days following Bidrin treatment.

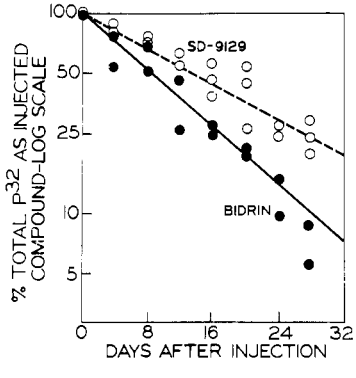

Figure 4. Persistence of Bidrin-P^{32} and SD 9129-P^{32} in bean seedlings following injection into stem to yield 43.5-p.p.m. initial dose based on weight of whole plant

Studies conducted under greenhouse conditions on Bidrin-P^{32} in October 1962 and on SD 9129-P^{32} in April 1964. Chromatography of chloroform extracts yielded less than 0.14% of unknown B and the unsubstituted amide following injection of SD 9129, and up to 14% of SD 9129 following injection with Bidrin. Four replicates of plants used for both treatments

A group of 105 bean plants treated with a total of 311 mg. of Bidrin yielded 2.2 mg. of SD 9129 and 15.7 mg. of Bidrin from the leaves after 8 days following chromatography, with considerable loss of compounds in order to achieve purity. The metabolite recovered was identical in infrared spectrum with known SD 9129. Following SD 9129 administration, trace amounts of the mono-*N*-hydroxymethylamide compound and the unsubstituted amide (0.14 and 0.10% of the SD 9129, respectively) were present at 8 days, but only SD 9129 was evident after 20 and 32 days (less than 0.05% *N*-hydroxymethylamide or unsubstituted amide).

Biological Activity of Bidrin and SD 9129 Metabolites. Bidrin, SD 9129, the unsubstituted amide, and the proposed *N*-hydroxymethylamide analogs of Bidrin and SD 9129 were assayed for toxicity to mice and houseflies and for anticholinesterase activity in vitro with fly head and human plasma cholinesterase (Table IV). Houseflies were used both with and without sesamex (10 μg. per fly) as a synergist. Bidrin was synergized about 38 times (LD_{50} without synergist/LD_{50} with synergist) by sesamex, SD 9129, eight times, and the unsubstituted amide was essentially unaffected in its toxicity by sesamex. The high degree of synergism of Bidrin by sesamex has been noted (*27*). The *N*-hydroxymethylamide compounds were intermediate in their toxicity to houseflies, and were also strongly synergized as with the *N*-methylamide compounds. Bidrin, SD 9129, and the unsubstituted amide were progressively more toxic with successive *N*-demethylation to both mice and houseflies. With sesamex all the compounds were of similar toxicity to houseflies, which resulted in a very favorable increase in the selective toxicity (milligram per kilogram LD_{50} for mice/LD_{50} for flies) upon demethylation. The *N*-hydroxymethylamide compounds were less toxic to both mice and houseflies than the corresponding amides formed on loss of the hydroxymethyl groups. All of the compounds were more potent inhibitors of fly head cholinesterase than of human plasma cholinesterase, and the potency as inhibitors for both enzymes decreased somewhat on *N*-demethylation.

Table IV. Biological Activity of Bidrin Analogs

cis-$(CH_3O)_2P(O)OC(CH_3)=CHC(O)R$	LD_{50}, Mg./Kg.			pI_{50}[b]	
	Mouse, ♀, i.-p.	Housefly, ♀, Topical[a]			
		−Sesamex	+Sesamex	Fly ChE	Plasma ChE
R = $N(CH_3)_2$ [Bidrin]	14	38	1.0	7.2	6.8
R = $N(CH_2OH)CH_3$[c]	18	14	1.2	7.0	6.5
R = $NHCH_3$ [SD 9129]	8	6.4	0.8	6.8	6.5
R = $NHCH_2OH$[c]	12	30	3.4	6.9	5.9
R = NH_2	3	1.0	0.9	6.5	5.6

[a] Fly LD_{50} values for organophosphates determined with and without simultaneous treatment with 10 μg. sesamex per fly. Slopes for log dose–probit mortality lines were similar for all compounds with and without synergist.
[b] Negative logarithm of molar organophosphate concentration for 50% inhibition with a preincubation of fly head homogenate with organophosphate for 20 minutes at 28° C. and 20 minutes at 37.5° C. prior to addition of acetylcholine for residual cholinesterase activity assay. For assay of human plasma cholinesterase, similar conditions were used, except that butyrylcholine was used as substrate and 0.05 ml. whole plasma was used per flask. Concentration of organophosphate indicated is that in final incubation mixture.
[c] Impure reaction products as discussed in experimental section.

Discussion

Four organophosphate metabolites from Bidrin and two from SD 9129 were recovered from organic solvent extracts of biological materials. The time sequence for appearance of these metabolites suggests the following scheme of successive *N*-methyl oxidation and *N*-dehydroxymethylation reactions for the R group where the substrate is *cis*-$(CH_3O)_2P(O)OC(CH_3)=CHC(O)R$:

$$\underset{\text{Bidrin}}{-N(CH_3)_2} \rightarrow \underset{\substack{N\text{-Methyl-}N\text{-hydroxy-}\\ \text{methylamide of Bidrin}}}{-N(CH_2OH)CH_3} \rightarrow \underset{\text{SD 9129}}{-NHCH_3} \rightarrow \underset{\substack{N\text{-Hydroxymethyl-}\\ \text{amide of SD 9129}}}{-NHCH_2OH} \rightarrow \underset{\substack{\text{Unsub-}\\ \text{stituted}\\ \text{amide}}}{-NH_2}$$

This pathway occurs, at least in part, in all of the organisms considered. It has also been reported in hen eggs injected with Bidrin, where all products except the *N*-methyl-*N*-hydroxymethylamide of Bidrin were detected (*21*) and the *N*-hydroxymethylamide of SD 9129 and the unsubstituted amide were the most persistent metabolites (*20*). The metabolites in this sequence which appeared in the greatest amount depended on the experimental organism and the administered compound—for example, the largest percentage of SD 9129 appearing in the urine from a Bidrin-treated animal was with mice, while the largest percentage of the unsubstituted amide excreted was in goat urine; the largest amount of the *N*-methyl-*N*-hydroxymethylamide was found in early urine samples from rats treated with Bidrin, while the *N*-hydroxymethylamide was found in largest amounts in the urine from rats treated with SD 9129. However, the stability of the *N*-hydroxymethylamide compounds during sample preparation and analysis is not known. A portion of the apparent SD 9129 residues may have resulted from degradation of the *N*-methyl-*N*-hydroxymethylamide of Bidrin during work-up, and in a similar manner the observed levels of the *N*-hydroxymethylamide of SD 9129 and the unsubstituted amide may have been influenced by degradation of the *N*-hydroxymethylamide. Losses might also have occurred from hydrolysis or reaction of the hydroxymethyl group with another chemical as well as from *N*-dehydroxymethylation.

The organophosphate metabolites of Bidrin and SD 9129 recovered on extraction with organic solvents represented only a small portion of the total dose administered. No attempt was made to characterize other metabolites formed, except for the *N*-substituted acetoacetamides and 3-hydroxy-*N*-substituted butyramides. The problems involved in attempting to provide a total accounting for metabolites can be appreciated by considering the potential fate of the tagged positions of the molecules administered. Studies with Bidrin-P^{32} would yield five neutral phosphate esters, including the administered compound as indicated above. Each of these might be susceptible to enzymatic cleavage of one or both P-*O*-methyl groups to yield an additional 10 metabolites. Other P^{32}-containing metabolites might be: H_3PO_4, $CH_3OP(O)(OH)_2$, $(CH_3O)_2P(O)OH$, $(CH_3O)_2P(O)OC(CH_3)=CHC(O)OH$, $(HO)(CH_3O)P(O)OC(CH_3)=CHC(O)OH$, and $(HO)_2P(O)OC(CH_3)=CHC(O)OH$.

Each of these potential products is based on analogy with reported studies on the metabolism of other vinyl phosphates (*3*, *6*, *9–11*, *15*, *25*).

It is likely that relatively few of these metabolites would actually be formed since the rate of hydrolysis at either the amide, vinyl phosphate, or P-*O*-methyl group would probably predominate for any particular intermediate. SD 9129 would potentially yield 14 P^{32}-labeled metabolites rather than the 20 listed for Bidrin. The incorporation of orthophosphate^{32} into the metabolic pool would also result in a variety of other labeled materials. The situation is even more complex with the *N*-methyl-C^{14}–labeled materials, where 11 of the Bidrin metabolites and five of the SD 9129 metabolites would contain this label in addition to the P^{32} label. Non-phosphorus-containing metabolites might include *N*,*N*-dimethylacetoacetamide, *N*-methylacetoacetamide, 3-hydroxy-*N*,*N*-dimethylbutyramide, 3-hydroxy-*N*-methylbutyramide, various conjugates of these hydroxybutyramides, and various labeled compounds derived from the substituted amine fragments released on hydrolysis and of the formaldehyde released during *N*-dehydroxymethylation. Many of these fragments would also appear with SD 9129-*N*-methyl-C^{14}. Using only paper chromatography for identification, it has been reported that Bidrin-P^{32} yields H_3PO_4, $CH_3OP(O)(OH)_2$, $(CH_3O)_2P(O)OH$, $(HO)(CH_3O)P(O)OC(CH_3)=CHC(O)N(CH_3)_2$, $(CH_3O)_2P(O)OC(CH_3)=CHC(O)OH$, $(HO)(CH_3O)P(O)OC(CH_3)=CHC(O)OH$, $(CH_3O)_2P(O)OC(CH_3)=CHC(O)NHCH_3$, $(CH_3O)_2P(O)OC(CH_3)=CHC(O)N(CH_2OH)CH_3$, and an unidentified material with some toxic properties, with certain insects, cotton plants, or in the urine of treated rats (*5*). The present study indicates that trace amounts of *N*-methylacetoacetamide and 3-hydroxy-*N*,*N*-dimethylbutyramide appear in rat urine after Bidrin administration. Of the compounds listed as possible metabolites, only those indicated in the depicted metabolic scheme would be expected to have appreciable anticholinesterase activity, and each might contribute to the poisoning of animals by Bidrin and SD 9129. The rates of the reactions leading to *N*-demethylation relative to those involving hydrolysis are felt to be critical in the biological activity and the potential hazard from persisting residues of Bidrin and possibly also of SD 9129.

Bidrin-P^{32} metabolites excreted in urine and eliminated in feces accounted for 70 to 76% of the administered dose with rats, and 78, 82, and 90% with goats. Excretion in the urine alone accounted for 98% of the Bidrin-P^{32} dose with dogs, 72% with mice, and 72% with rabbits. Comparable figures with SD 9129 as per cent excretion of the administered P^{32}-dose were 76 for rats in urine and feces and 67 in the urine of a goat. Tissues of Bidrin-P^{32}–treated rats, including bone, contained considerable residual P^{32} 48 hours after treatment, when little further P^{32} was being excreted, indicating considerable incorporation of hydrolysis and phosphorylation products into certain tissues, and partially accounting for the incomplete excretion noted. These observations are similar to a variety of studies that have been made with other organophosphates labeled with P^{32} following administration to mammals. The percentage of the administered *N*-methyl-C^{14} dose recovered in urine was 74 to 78 for Bidrin-treated rats, 84 for a Bidrin-treated goat, 60 for Bidrin-treated mice, 75 to 85 for SD 9129-treated rats, and 90 for an SD 9129-treated goat. An additional 12% of the *N*-methyl-C^{14} administered as Bidrin appeared as $C^{14}O_2$ with rats. Although tissues were not analyzed for C^{14} residues, it is known that methylamine-C^{14} yields relatively high levels of persistent radioactivity in several rat tissues (*7*).

Bidrin and SD 9129 are water-soluble organophosphates that might be expected to partition poorly from aqueous media into fatty materials. Bidrin-P^{32} did not appear to persist in the fat of treated rats. Lacking storage depots, the administered compound or its water-soluble metabolites were excreted relatively rapidly. The in vivo stability of Bidrin and SD 9129 appears to be greater than that of other vinyl phosphates which have been studied, and therefore a portion of the dose is excreted in the urine or secreted into milk as unhydrolyzed metabolites. A similar situation in relation to excretion of some unhydrolyzed organophosphate in the urine has also been noted with octamethyl pyrophosphoramide and tetramethylphosphorodiamidic fluoride (*4*).

The toxicity to houseflies of Bidrin and all of its neutral organophosphate metabolites except the unsubstituted amide analog was greatly increased by sesamex. Sun and Johnson (*27*) sur-

mised that this effect might result from blocking the biological oxidations attacking the *N*-methylamide grouping. The failure of sesamex to synergize the unsubstituted amide further supports this relationship, as did the studies on metabolism of labeled Bidrin and SD 9129 in flies. Sesamex increased the toxicity of all the metabolites to about the same level as for the unsubstituted amide, but inhibited the mechanisms for *N*-demethylation to form the amide. It is not clear from the available data whether the same metabolite served as the effective fly toxicant with each compound in both the presence and absence of sesamex, but it appears likely that *N*-demethylation in flies is not a necessary preliminary step in the poisoning process.

Plant metabolism of Bidrin and SD 9129 was much slower than that in mammals, as with all other organophosphates that have been investigated. The pathway appears to be at least in part similar to that in mammals, in that Bidrin yields SD 9129, and SD 9129 yields trace amounts of the *N*-hydroxymethylamide of SD 9129 and the unsubstituted amide. Hydrolysis products from SD 9129 appeared to be more readily incorporated than those from Bidrin into the solids fraction from plants, indicating that the primary hydrolysis sites may differ for Bidrin and SD 9129 in plants. The hydrolysis rate of SD 9129 in beans appeared to be slower than that of Bidrin, but both were more persistent than other vinyl phosphates that have been studied under somewhat comparable conditions (*6*).

Bidrin and SD 9129 may be converted in low amounts to compounds of comparable or increased toxicity relative to the insecticide as applied. SD 9129 formed metabolically from Bidrin may be present in greater amounts than Bidrin itself a few hours after administration to mammals, as evidenced from urine and milk analyses. Tissue residues of Bidrin and SD 9129 are possibly dissipated within 24 hours in rats and goats since none of the unhydrolyzed metabolites appeared in milk or urine after that time. The anticholinesterase procedure seems most appropriate of the available methods for residue analysis but careful attention must be given to the choice of a cholinesterase source sensitive to the series of potentially toxic metabolites that may be formed.

N-Hydroxymethyl derivatives have been postulated as intermediates in a variety of *N*-demethylation reactions. These intermediates appear to be more stable in the case of amides than with amines, since evidence for their existence as compounds of sufficient stability for isolation has been obtained only with *N,N* - dimethylphosphoramides, *N*-methylcarbamates, and *N,N*-dimethylcarbamates (*12–14*). Only in the case of the *o*-isopropoxyphenyl *N*-hydroxymethylcarbamate formed enzymatically from the *N*-methylcarbamate analog has the material been isolated for comparison of infrared spectra with material from an attempted synthesis (*12*). An unidentified metabolite of dimethoate, *O,O*-dimethyl-*S*-(*N*-methylcarbamoylmethyl) phosphorodithioate, might be formed by oxidation of the *N*-methyl group (*23*). The present study constitutes the only case known to the authors where the full series of intermediates in the total demethylation of an *N,N*-dimethylamide or an *N*-methylamide grouping have been demonstrated and their properties described.

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