of either the cows or the hens due either to the high percentage of alfalfa in the feed or the Kerb residues.

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Metabolism and Photoalteration of 2-*sec*-Butyl-4,6-dinitrophenol (DNBP Herbicide) and Its Isopropyl Carbonate Derivative (Dinobuton Acaricide)

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Metabolism of 2-*sec*-butyl-4,6-dinitrophenyl isopropyl carbonate (dinobuton) in mice and rats involves rapid hydrolysis to form 2-*sec*-butyl-4,6-dinitrophenol (DNBP) which, in turn, undergoes oxidation of either of the two methyl groups on the *sec*-butyl side chain, conjugation of the phenolic products, formation of many uncharacterized metabolites, and, in rats only, reduction of either of the two nitro groups and acetylation of the metabolically formed *p*-amino group. The most active esterases for dinobuton hydrolysis are present in liver and blood, but those in blood possibly are most important in the release of the actual toxicant, DNBP, and in determining the relative toxicity of dinobuton to various mammalian species. Carbaryl protects the rat from poisoning by dinobuton, probably by inhibiting its hydrolysis to DNBP. Microsomal enzymes of rat liver and housefly abdomens hydrolyze dinobuton and reduce the *o*-nitro group of DNBP. Dinobuton and DNBP are not highly systemic in bean plants, but they are metabolized in or photodecomposed on plants to yield many products by hydrolysis, reduction, and other types of reactions.

Dinobuton (2-*sec*-butyl-4,6-dinitrophenyl isopropyl carbonate) is a promising acaricide and fungicide chemical, especially for the control of mites resistant to organophosphorus compounds (Martin, 1968; Pianka, 1966; Pianka and Smith, 1965). 2-*sec*-Butyl-4,6-dinitrophenol (DNBP), the phenol moiety of dinobuton, is widely used as a nonselective herbicide chemical (Martin, 1968). Dinobuton is much less toxic to mammals than DNBP; the acute oral LD_{50} values (mg/kg) are 265–460 and 40, respectively, for rats, and 2100–3500 and 65, respectively, for mice (Pianka and Smith, 1965). The toxic action of dinobuton probably results from uncoupling of oxidative phosphorylation, after being hydrolyzed to DNBP (Ilivicky and Casida, 1969; Pianka, 1966); so, the rates and pathways of metabolism probably contribute to the selective toxicity of dinobuton.

An understanding of the metabolism and photoalteration of dinobuton and DNBP is of importance in determining safe and effective conditions for their use. Alkyl dinitrophenols are known to be metabolized in mammals and locusts by several pathways, including reduction of one or both of the aromatic nitro groups, acetylation of the metabolically formed amino groups, oxidation of a methyl group in the alkyl side chain, and conjugation of the phenolic hydroxyl or other metabolically formed reactive groups (Ernst and Bär, 1964; Guerbet and Mayer, 1932; Henneberg, 1964; Kikal and Smith, 1959; Parker, 1952; Smith *et al.*, 1953; Truhaut and de Lavaur, 1967; Yasuda, 1957). Photodecomposition of dinobuton possibly involves ester hydrolysis, nitro reduction, alkyl side chain oxidation, and hydroxyalkyl dehydration (Matsuo and Casida, 1970).

This paper deals with the fate of dinobuton and DNBP in certain mammals, insects, and plants, as well as with their photoalteration chemistry.

MATERIALS

Chemicals. Pure dinobuton (colorless prisms, mp 52–53°, from hexane) was isolated from the technical material (about 98% purity) (Union Carbide Chemical Co., South Charleston, W. Va.) by chromatography on a Florisil column, eluting with benzene, and recrystallization. Pure DNBP (yellow leaflets, mp 45–46°, from hexane; 60% yield) was obtained by hydrolysis of dinobuton in methanolic ammonium hydroxide, followed by chromatography on Florisil, using benzene as the elutant (to remove small amounts of 4-*sec*-butyl-2,6-dinitrophenol which remain on the column), and recrystallization (Bandal, 1971).

Dinobuton-carbonyl-^{14}C (0.8 mCi/mmol) and dinobuton-ring-^{14}C (uniform ring label, 3.5 mCi/mmol), both having greater than 99% radiochemical purities, were provided by Union Carbide Chemical Co. DNBP-^{14}C (0.59 mCi/mmol, >99% radiochemical purity) was prepared by hydrolysis of dinobuton-ring-^{14}C, followed by chromatography and recrystallization as described above.

Eight unlabeled derivatives of DNBP were provided by Union Carbide Chemical Co. as follows: 2-*sec*-butyl-4-amino-6-nitrophenol hydrochloride (4-NH_2-NBP); 2-*sec*-

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butyl-4-nitro-6-aminophenol hydrochloride (6-NH_2-NBP); 2-*sec*-butyl-4-acetamido-6-nitrophenol; 2-*sec*-butyl-4-nitro-6-acetamidophenol; 3-(2-hydroxy-3,5-dinitrophenyl)butyric acid (DNBP-3-COOH); 2-(2-β-butenyl)-4,6-dinitrophenol; 2-(hydroxy-*sec*-butyl)-4,6-dinitrophenol.

2-(2-Hydroxy-3,5-dinitrophenyl)butyric acid [DNBP-2-COOH; appropriate infrared (ir) spectrum] was prepared from phenol *via* the following intermediates: 2-phenoxybutyric acid [mp 70–71°, from hexane; appropriate ir, nuclear magnetic resonance (nmr), and mass spectra (ms)] from phenol and 2-bromobutyric acid; photochemical rearrangement to 2-(2-hydroxyphenyl)butyric acid (appropriate ir and nmr), using the procedure of Kelly and Pinhey (1964) and isolating the product as the methyl ester by reaction with diazomethane and preparative thin-layer chromatography (tlc); 2-(2-hydroxy-3,5-dinitrophenyl)butyric acid methyl ester (appropriate ir spectrum) by nitration in fuming nitric acid and glacial acetic acid, and preparative tlc isolation; hydrolysis in methanolic potassium hydroxide. For characterization, the final product was methylated with diazomethane to produce 2-(2-methoxy-3,5-dinitrophenyl)butyric acid methyl ester, which was identified by ir, nmr, and ms (Bandal, 1971).

Biochemicals. The following enzymes and cofactors were used: reduced nicotinamide-adenine dinucleotide (NADH) and its phosphate derivative (NADPH) (Calbiochem, San Diego, Calif.); β-glucuronidase (bacterial, Type I), β-glucosidase (almond emulsin), aryl sulfatase (Type III), and glusulase (100,000 units of β-glucuronidase and 50,000 units of sulfatase per milliliter) (Endo Laboratories, Inc., Garden City, N. Y.).

METHODS

Effect of Carbaryl on the Acute Oral Toxicity of Dinobuton and DNBP to Rats. The symptoms of poisoning and mortality were observed following oral administration by stomach tube of dinobuton, DNBP, carbaryl, and varying doses of carbaryl–dinobuton and carbaryl–DNBP mixtures to male albino rats (200 g, Sprague–Dawley strain). The anticholinesterase (anti-ChE) symptoms resulting from carbaryl poisoning and the uncoupler symptoms from dinobuton or DNBP poisoning were those described by Carpenter *et al.* (1961) and Ilivicky and Casida (1969), respectively.

Thin-Layer Chromatography. Silica gel F_{254} precoated chromatoplates (20 × 20 cm) (Merck AG, Darmstadt, Germany) were used at 0.25- and 0.5-mm gel thickness for analysis and at 2.0-mm gel thickness for preliminary cleanup and resolution of metabolites. The solvent systems used and their alphabetical designations are as follows: A, benzene (saturated with formic acid)–ether mixture (10:3); B, chloroform–methanol mixture (9:1); C, ether–hexane mixture (2:1); D, benzene (saturated with formic acid)–ether mixture (5:1); E, *n*-butanol–acetic acid–water mixture (3:1:1); F, benzene; and G, acetonitrile. To avoid streaking, the organic extracts were applied as bands (12 × 2 mm) at the origin of the tlc plate. On two-dimensional development, the components are resolved as bands after the first development and these bands usually become well-defined circular areas during the second development. Owing to the unstable nature of certain metabolites, all chromatoplates were developed in the dark.

The unlabeled compounds were detected by spraying the chromatoplates with 1 *N* of aqueous sodium hydroxide, producing yellow spots with dinitrophenols, dinitrophenyl esters, and 2-*sec*-butyl-4-nitro-6-acetamidophenol, and orange-red spots with 2-*sec*-butyl-4-acetamido-6-nitrophenol, 4-NH_2-NBP, and 6-NH_2-NBP. Cochromatography of a labeled metabolite or photoproduct, detected by radioautography, with the authentic unlabeled compound in at least four different solvent systems was considered to constitute tentative identification of the metabolite or photoproduct. R_f values for the authentic compounds in the various tlc solvent systems are given by Bandal (1971). Quantitative data were obtained after scraping the radioactive gel regions, detected by radioautography, into scintillation vials for direct counting.

Esterase Activity Studies. The esterase activity of whole heparinized blood of six mammalian species and of tissue homogenates and homogenate fractions (Hogeboom, 1955) prepared from male albino mice (Swiss–Webster strain) was determined by monitoring the liberation of $^{14}CO_2$ from dinobuton–carbonyl-^{14}C. The tissue homogenates (10%, w/v) and homogenate fractions (reconstituted to 10% fresh tissue weight equivalent) were prepared in phosphate buffer (0.1 *M*, pH 6.5). Three assay procedures were used, each involving the addition of dinobuton–carbonyl-^{14}C (0.006 μmol in 5–10 μl of ethanol) to the esterase source in phosphate buffer (1.0 ml, equilibrated at 37°), and each giving essentially the same results. In the first method, the assay chamber consisted of two liquid scintillation vials joined together (one above the other, with a sleeve made from two screw caps, each with a large hole drilled in the top) that were cemented to each other in an air-tight back-to-back arrangement. The lower vial contained the incubation mixture and the upper inverted vial contained glass wool impregnated with 0.2 ml of 20% aqueous potassium hydroxide. Dinobuton–carbonyl-^{14}C was added to the esterase preparation in the lower vial; the upper vial (complete with impregnated glass wool) was quickly attached to the lower one by the gas-tight sleeve and, following incubation, the upper vial was removed and the potassium carbonate-^{14}C contained in it was determined by the addition of 15 ml of 0.55% (w/v) 2,5-diphenyloxazole in toluene–2-methoxyethanol mixture (2:1) and direct liquid scintillation counting. In the other two methods, the dinobuton–carbonyl-^{14}C remaining unhydrolyzed in the incubation mixture was determined by liquid scintillation counting of a 0.2-ml aliquot or, usually, by extraction directly in the scintillation vial with 15 ml of 0.55% (w/v) 2,5-diphenyloxazole in toluene. The extraction involved vigorous shaking for 1 min, followed by centrifugation to completely separate the toluene layer containing the radioactive dinobuton from the aqueous phase. With appropriate settings of the lower and upper levels of the energy spectrum to minimize background chemiluminescence, the dinobuton–carbonyl-^{14}C was determined without interference from constituents in the aqueous phase. Certain potential inhibitors and cofactors were tested for their effect on the system, using a 1-min preincubation period with the enzyme before the substrate was added.

Metabolism in Microsomal Systems. Microsomes prepared from rat liver (400-mg equivalent of fresh liver weight) and housefly abdomens (ten abdomen equivalents from adult females, *Musca domestica*, R-Baygon strain, Shrivastava *et al.*, 1969) according to Oonnithan and Casida (1968) and Tsukamoto and Casida (1967), respectively, were incubated with NADPH (0 or 2 μmol), bovine serum albumin (1.5%, w/v, in the case of houseflies only), and substrate (dinobuton–ring-^{14}C, dinobuton–carbonyl-^{14}C, or DNBP-^{14}C, 0.07 to 0.30 μmol, added in 5 μl of ethanol as the last reaction component) in phosphate buffer (0.1 *M*, pH 7.4, 2.0 ml). Following incubation for 90 min in a shaking incubator at 37° in the case of rat microsomes and at 30° for housefly microsomes, the metabolites were recovered by extraction with ether (5 ml ×

3), before and after the aqueous phase was acidified to pH 1 with hydrochloric acid. In each case, the combined ether extracts were evaporated under nitrogen and analyzed by two-dimensional tlc using solvent systems C and D. In one study, the metabolites were reacted with diazomethane in ether to observe shifts in R_f values, if any, for individual metabolites. In a separate set of experiments, the microsome–substrate–cofactor mixture was incubated in a Warburg flask containing an atmosphere of nitrogen, for 90 min at 30 or 37°, and the metabolites were analyzed as above.

Tissue Levels of Dinobuton and Metabolites Following Oral Administration of Dinobuton-^{14}C to Mice. Dinobuton–ring-^{14}C or carbonyl-^{14}C was given orally to male mice (20 g) at 1500 mg/kg, using ethanol for administration. Severe symptoms of poisoning appeared 2–3 hr after the treatment. The radiocarbon content of heparinized whole blood, taken by heart puncture, and of various tissues, after they were washed with distilled water, was determined by combustion and liquid scintillation counting according to Krishna and Casida (1966). The blood was also extracted with anhydrous ether (3 × 2.5 vol), and the ether-soluble labeled products were cochromatographed on tlc with unlabeled dinobuton and DNBP, using solvent systems A and C.

Metabolism in Living Mammals. Male albino mice (20 g) and male albino rats (180 g) were treated orally by stomach tube with 8–10 μmol/kg of dinobuton–ring-^{14}C, dinobuton–carbonyl-^{14}C, or DNBP-^{14}C administered in dimethyl sulfoxide or ethanol. The treated animals were held in individual metabolism chambers for 72 hr while receiving food and water *ad libitum*, and the radiocarbon content of the expired $^{14}CO_2$, urine, feces, and certain tissues was determined according to Krishna and Casida (1966). The container for urine collection was always maintained at 5°.

The organosoluble components of the urine samples were analyzed by the same procedure used for the microsomal metabolites (above). The aqueous fraction of the extracted urine was partially evaporated under nitrogen to remove residual ether, chromatographed on a column of Bio-Rad SM-2 beads (Bio-Rad Lab., Richmond, Calif.; 2.5 g of beads/ml of urine; Kamienski and Casida, 1970), first washing with water and then, second, eluting greater than 95% of the radiocarbon with methanol, and resolving the products in the methanol eluate by preparative tlc using solvent system E. The radioactive gel regions from chromatography of the methanol eluate fraction and the material remaining at the origin on tlc of the organosoluble fraction were individually scraped and extracted with methanol or an ether–methanol mixture (1:1), and selected extracts were used for enzymatic hydrolysis with β-glucuronidase (3 mg in 2.0 ml of 0.1 *M* sodium acetate, pH 4.5, incubated for 12 hr at 38°) and, subsequently, with aryl sulfatase (4 mg in 2.0 ml of 0.1 *M* sodium acetate, pH 5, incubated for 20 hr at 37°). After the incubation period, each reaction mixture was extracted with anhydrous ether (5 ml × 3), the radiocarbon content of the ether and aqueous fractions was determined, and the components of the ether fraction were analyzed by tlc using solvent systems C and D.

The feces samples (1–10 g) were dried over phosphorus pentoxide at 30° under reduced pressure, extracted with methylene chloride (2 × 3 vol), and the combined methylene chloride extracts were analyzed by tlc as described above. The extracted feces, after evaporation of the residual methylene chloride under nitrogen, were homogenized in water (5 vol) and centrifuged to obtain the water-soluble and water-insoluble products. The aqueous fraction was acidified to pH 1 with concentrated hydrochloric acid, passed through a Bio-Bead column, and the methanol eluate (>90% radiocarbon recovery) was analyzed by preparative tlc in the same manner described for the urine samples. The water-extracted residue was hydrolyzed in sealed ampoules with base or acid (sodium hydroxide or hydrochloric acid, 6 *N*, 90°, 12 hr in each case) and the hydrolyzates were extracted with ether to determine acid- and base-labile metabolites.

In a separate set of experiments, male albino rats (250 g) were treated orally with large, single, or multiple doses of dinobuton–ring-^{14}C in ethanol in an attempt to obtain large amounts of the individual metabolites for chemical identification. One group of animals received orally administered dinobuton at 250 mg/kg at 12-hr intervals. Another group, receiving single oral doses of 0.25–400 mg/kg of dinobuton, was placed in individual metabolism chambers, observed occasionally for toxicity symptoms, and the urine and feces which were collected for each successive 24-hr interval over a total period of 72 hr were radioassayed.

Metabolism in Growing Bean Plants. Radiolabeled dinobuton (40 μg in 30 μl of ethanol) or DNBP-^{14}C (10 μg in 30 μl of ethanol) was injected into the stem of Contender variety bean plants (*Phaseolus vulgaris*) as described by Abdel-Wahab *et al.* (1966). The treated plants were held in a greenhouse under a "normal" May sunlight cycle until they were sacrificed for analysis. Dinobuton-^{14}C injected at up to 400 μg per plant occasionally produces some localized tissue necrosis in the region of the injection site, whereas 10-μg injections of DNBP-^{14}C usually produce necrosis of this type; however, the plants survived these treatments and appeared to grow normally during a 7-day period.

The radiocarbon distribution in bean plants 7 days after treatment with dinobuton–ring-^{14}C or DNBP-^{14}C was determined by radioautography, using a piece of household Saran Wrap placed between the plant and the X-ray film to prevent darkening of the film by the plant tissue itself. Also, the total radiocarbon content was separately determined by combustion for the root, stem, and leaf regions 7 days after injection with each labeled compound.

For metabolite analysis the treated plants were cut into small pieces and extracted by homogenization, first in acetone (30 ml) and second in chloroform (30 ml × 2), obtaining a soluble fraction and an organo-insoluble residue. The organo-insoluble residue was dried and its radiocarbon content was determined by combustion and radioactivity counting. The soluble fraction was separated, by the addition of water and mixing, into an aqueous fraction and an organosoluble (acetone–chloroform) fraction, and their radiocarbon contents were determined. The organosoluble fraction was subjected to the clean-up procedure of Abdel-Wahab *et al.* (1966) except that, in preparing the charcoal–Florisil column, the alternate layers of charcoal and Florisil were doubled in number and halved in volume. The eluate, containing more than 95% of the radiocarbon, was analyzed on tlc chromatoplates of 2.0-mm gel thickness by developing first, in two dimensions, with solvent systems C and D and then, after breaking off the portion of the chromatoplate containing the materials at the origin, with solvent system E to resolve the materials at the origin.

In the case of bean plants, 7 days after receiving stem injections of dinobuton–ring-^{14}C, the organo-insoluble residue was homogenized in distilled water (5 vol) and the homogenate was centrifuged to obtain water-insoluble and water-soluble fractions. The water-soluble fraction was freeze dried, and the residue, dissolved in 0.1 *M* citrate–0.2 *M* phosphate buffer, was subjected to hydrolysis with β-glucosidase

Table I. Effect of Carbaryl on Acute Oral Toxicity of Dinobuton and DNBP to Rats

Dose of compounds administered, mg/kg			Mortality, %	Symptoms	
Dinobuton	DNBP	Carbaryl		Type	Severity[a]
0	0	75 or 100	0	Anti-ChE	++
0	0	250	0	Anti-ChE	+++
250	0	50	0	Anti-ChE	+
250	0	100 or 250	0	Anti-ChE	+++
250	0	0	100	Uncoupler	+++
0	100	0 or 50	100	Uncoupler	+++

[a] Most severe, +++.

Table II. Activity of Homogenates of Various Mouse Tissues and of Mammalian Blood in Hydrolyzing Dinobuton–Carbonyl-^{14}C

Tissue, fraction, or species	Esterase activity[a]
Mouse tissues	
Brain homogenate	3
Kidney homogenate	67
Liver homogenate	1670
Microsomal fraction	1430
Mitochondrial fraction	250
Nuclear fraction	250
Soluble fraction	833
Small intestine homogenate	333
Spleen homogenate	59
Blood	
Guinea pig	400
Hamster	5
Human	42
Mouse	182
Rabbit	59
Rat	833

[a] Reciprocal of the amount of tissue fresh weight, g, required to effect 50% hydrolysis (10 min, 37°); highest value represents greatest activity.

(6 mg in 8.0 ml of buffer, pH 4.4, incubated for 20 hr at 35°) and, subsequently, with glusulase (0.5 ml in 10 ml of buffer, pH 4.5, incubated for 24 hr at 38°). The products of β-glucosidase and glusulase cleavage, recovered by extraction with ether after each incubation period before and after acidification to pH 1 with hydrochloric acid, were analyzed by tlc. The radiocarbon remaining in the aqueous phase was regarded as the unextractable water-soluble fraction.

In order to study the degradation of topically applied dinobuton–ring-^{14}C or carbonyl-^{14}C, the primary leaves of bean plants were treated on the dorsal surface with uniform application of the compound (10 μg/cm^2) and the plants were held in a greenhouse for the desired length of time (Abdel-Wahab *et al.*, 1966). Each treated leaf was cut off at the petiole, washed with anhydrous ether (5 ml × 2) in a beaker containing anhydrous sodium sulfate (3 g), and homogenized in acetone. The insoluble residue was filtered off, dried, and combusted, and the combustion products were radioassayed to determine the unextractable radioactivity. The combined ether washes were analyzed by two-dimensional tlc, using solvent systems C and D. The acetone homogenates, which had intense green color, were chromatographed as bands (55 × 2 mm) using solvent systems C and D on separate 0.5-mm tlc plates. To examine the extent of translocation of the applied radiocarbon from the treated leaf to other parts of the plant, the opposite untreated primary leaf was cut at the petiole for total radiocarbon assay.

Persistence of Dinobuton-^{14}C Deposits on Bean Leaves and Glass Surfaces. Deposits of dinobuton–ring-^{14}C and carbonyl-^{14}C (10 μg/cm^2) on the dorsal surfaces of the primary leaves of bean plants and on glass cover slips were exposed to direct sunlight for appropriate time intervals. For analysis, each treated leaf was cut off at the petiole and washed with ether (10 ml × 2), and the combined ether extracts were assayed for radiocarbon content. The residual radioactivity on the cover slips was determined by placing them directly into scintillation vials for radioanalysis.

Sensitized Photoalteration of Dinobuton on Bean Leaves. The primary leaves of bean plants were treated first with dinobuton–ring-^{14}C or carbonyl-^{14}C (2.5–3 μg/cm^2), then with xanthen-9-one or rotenone (level twice that of the dinobuton), and, following exposure to direct sunlight, each treated leaf was cut off at the petiole, washed with anhydrous ether, and analyzed by tlc as described above. The ether-washed leaf and the opposite untreated leaf were, in each case, subjected to further analyses, as described above, in order to evaluate the penetration and translocation of dinobuton-^{14}C in the presence of photosensitizers.

RESULTS

Antagonistic Effect of Carbaryl on the Acute Oral Toxicity of Dinobuton to Rats. An orally administered dose of carbaryl, at a level that produces signs of acute anti-ChE poisoning, protects rats from intoxication by dinobuton but not DNBP (Table I). This antagonistic effect probably results from the inhibition by carbaryl of dinobuton-hydrolyzing esterases because (1) blood taken from rats 1–2 hr after oral administration of carbaryl at 75 mg/kg is about 50% inhibited in its dinobuton-hydrolyzing activity (Bandal, 1971) and (2) the rate of evolution of $^{14}CO_2$ from orally administered dinobuton–carbonyl-^{14}C is markedly reduced in carbaryl-treated rats compared with untreated rats. Thus, dinobuton is probably activated in living rats by esteratic cleavage of the carbonate group. Dinobuton does not undergo significant spontaneous hydrolysis when held at low concentrations in aqueous media of pH 5–8 at 37° for 1 hr (Bandal, 1971) so, any hydrolysis occurring in a biological material is likely to be the result of esterase action.

Distribution and Properties of Esterases Hydrolyzing Dinobuton. Of the mouse tissues assayed, liver is the most active in hydrolyzing dinobuton and its activity resides largely in the microsomal and soluble fractions (Table II). Brain is the least active tissue. The small intestine homogenate is quite active (Table II); however, the contents of the digestive tract are not active and their pH value is below that where dinobuton undergoes appreciable spontaneous hydrolysis (Bandal, 1971). The blood esterases hydrolyzing dinobuton are most active from rat and least from hamster blood (Table II).

The dinobuton-hydrolyzing activity of liver microsomes is unaltered by NADH and NADPH at 1.4×10^{-3} *M*, but it is inhibited more than 50% by coumaphoxon and *m*-isopropylphenyl methylcarbamate at 2.2×10^{-5} *M*. At 2×10^{-5} *M*, eserine has no inhibitory effect and dichlorvos almost completely inhibits dinobuton hydrolysis by either mouse liver homogenate or microsomal fraction (Bandal, 1971). Thus, the dinobuton-hydrolyzing esterases are sensitive to inhibition by some organophosphates and methylcarbamates, but are not very sensitive to inhibition by eserine.

Levels of Dinobuton and Metabolites in Tissues of Orally-Treated Mice and Rats. Following oral administration of dinobuton–ring-^{14}C to mice at 1500 mg/kg, the peak radio-

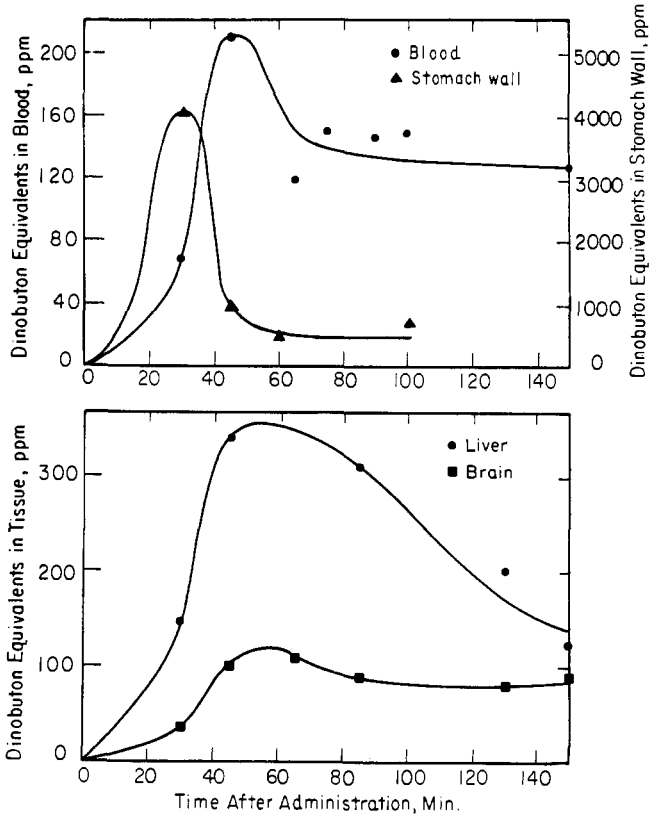

Figure 1. Levels of dinobuton and its labeled metabolites in tissues of mice following oral administration of dinobuton-ring-^{14}C at 1500 mg/kg

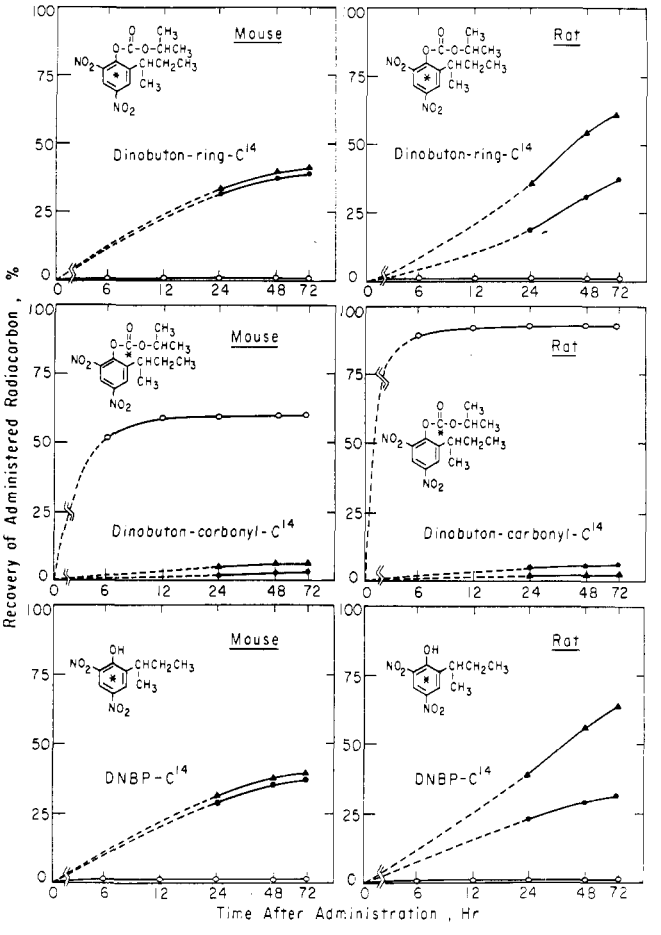

Figure 2. Excretion of labeled products by mice and rats following oral administration of dinobuton-ring-^{14}C, dinobuton-carbonyl-^{14}C, and DNBP-^{14}C at 8–10 μmol/kg. ▲, urine; ●, feces; ○, $^{14}CO_2$. The dotted line for radiocarbon excreted in the urine and feces during the first 24 hr and as $^{14}CO_2$ during the first 6 hr indicates that the exact elimination rate is not known, the first samples being taken at 24 hr for the urine and feces, and at 6 hr for $^{14}CO_2$

carbon content appears first in the stomach wall and then, at about the same time, in blood, liver, and brain, the peak level of dinobuton equivalents being quite different for each tissue (Figure 1). To a greater extent than the other tissues, the blood and particularly the brain appear to retain the dinobuton-derived metabolites at levels above 55% of the respective maximum level attained. The organosoluble radiocarbon in the blood at 120 min after treatment with dinobuton-ring-^{14}C, at a dosage producing severe poisoning symptoms at this time, is mostly DNBP (93%), while the nature of the remainder which does not move from the tlc origin is not known. Also dinobuton-carbonyl-^{14}C does not yield detectable dinobuton or radiolabeled metabolites in the brain, kidney, liver, or spleen of poisoned animals at the time of severe symptoms. In confirmation, colorimetric analysis of blood by the method of Bough *et al.* (1965) shows no difference between free and total DNBP content (Bandal, 1971).

Rats treated orally with dinobuton-ring-^{14}C at 8 μmol/kg contain 0.03, 0.05, 0.19, and 0.09%, respectively, of the radiocarbon in the brain, heart, kidney, and liver after 72 hr.

Metabolites Formed in Living Mammals. Dinobuton-carbonyl-^{14}C is quickly hydrolyzed in orally treated mice and rats yielding, within 24 hr, approximately 60 and 92%, respectively, of the radiocarbon as $^{14}CO_2$; the rat hydrolyzes dinobuton more completely (Figure 2) and much more rapidly than the mouse. The level of urinary radiocarbon with either mice or rats is similar for dinobuton-ring-^{14}C and DNBP-^{14}C, and is much higher than that for dinobuton-carbonyl-^{14}C. The radiocarbon from dinobuton-ring-^{14}C is almost completely excreted within 72 hr in the urine and feces by the rat, but excretion by the mouse is lower (74%).

The urine from rats and mice receiving dinobuton-ring-^{14}C contains a large number of labeled products resolvable by tlc. Preliminary studies indicated little or no difference in the distribution and nature of the products in the 0–24, 24–48, and 48–72-hr urine samples; so, only the 0–24 hr urine sample was analyzed in more detail. The organosoluble fraction of rat urine contains at least 13 components, five of which are tentatively identified by cochromatography (Figure 3A). Two of the metabolites (DNBP-3-COOH and DNBP-2-COOH) are not resolved in solvent systems C and D (Figure 3A) or in solvent systems A, B, E, and F; however, they are separated in solvent system G (R_f values of 0.50 and 0.90, respectively). The remaining aqueous fraction, after cleanup, yields at least five radioactive bands (in solvent system E); each of these bands represents a mixture of two or more conjugates [probably glycosides, based on the extent of cleavage and products released on β-glucuronidase treatment (Bandal, 1971)]. The unknown aglycones 1 through 4 are obtained from these five bands after cleavage with β-glucuronidase; aglycones 1 and 2 possibly are metabolites D and F, based on their tlc characteristics. The uncleaved portion from each of these bands, together with that from the unknown I (origin of chromatogram, Figure 3A), is designated as the unknown complex (Table III); thus, β-glucuronidase fails to cleave a large proportion of the metabolites in the aqueous fraction. These metabolites are also not extensively cleaved by aryl sulfatase, with less than 5% of the radiocarbon being ether-extractable after the enzyme treatment.

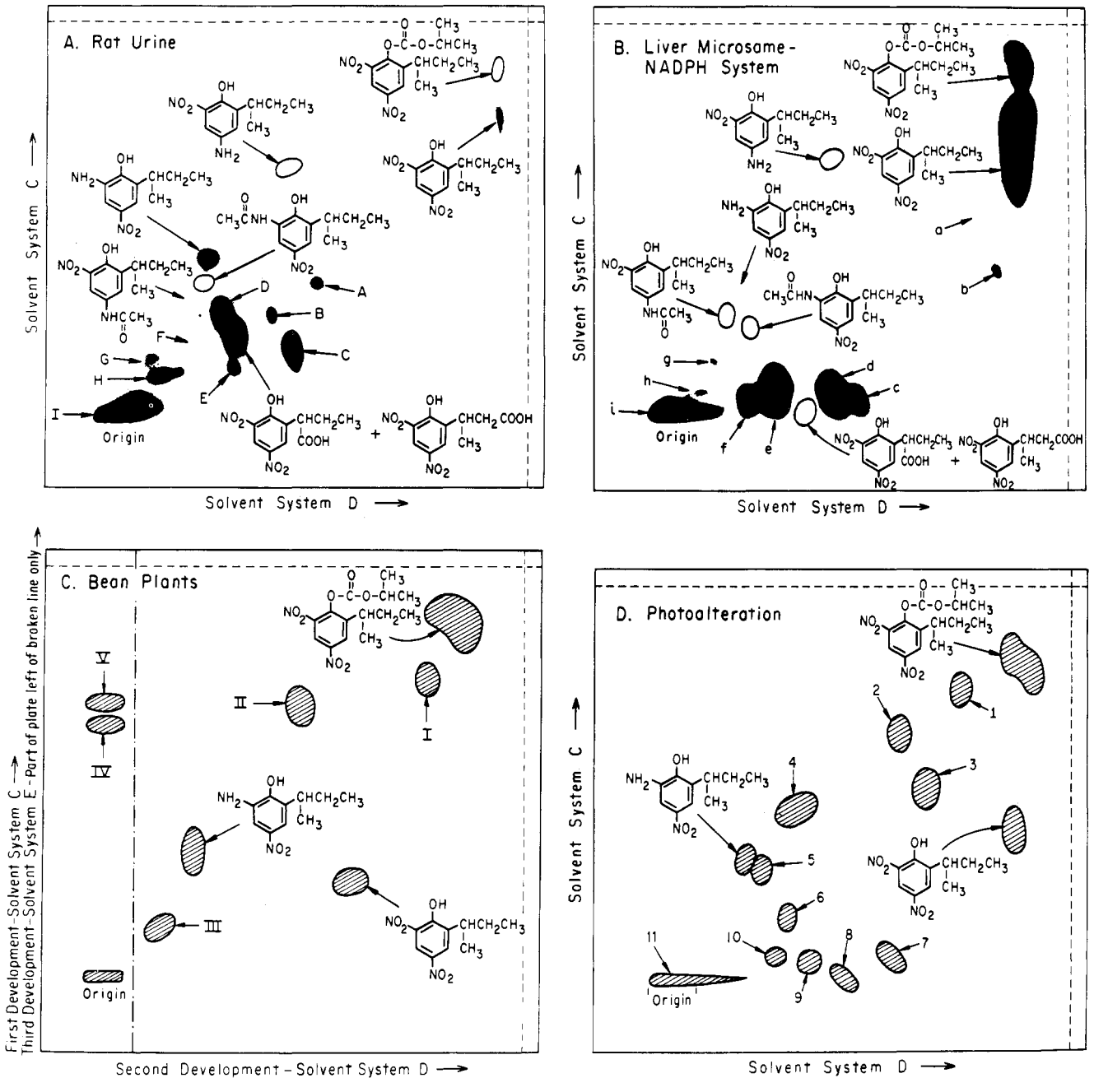

Figure 3. Tlc chomatographic positions for metabolites and photoalteration products of dinobuton–ring-^{14}C and the structures for compounds cochromatographing with individual components. The positions for metabolites and photoalteration products are shown in dark or cross-hatched areas; those for known compunds not cochromatographing with metabolites are shown as open circles in diagrams A and B. A, organosoluble metabolites from rat urine; B, organosoluble metabolites from rat liver microsome–NADPH system; C, organosoluble metabolites from bean plants 7 days after stem injection; D, organosoluble photoalteration products from rotenone-treated bean leaves. Photographs of actual radioautograms are shown in diagrams A and B and illustrative representations of the chromatograms are shown in diagrams C and D. In diagram C, the portion of the chromatoplate to the left of the broken line (–·–·–), containing metabolites remaining at the origin in solvent systems C and D, is that which is broken off and redeveloped in solvent system E

The products detected by tlc on urinalysis of mice and rats receiving DNBP-^{14}C are similar to or are the same as the majority of those obtained from dinobuton–ring-^{14}C administration (Bandal, 1971). In similar studies with dinobuton–carbonyl-^{14}C, the urine contains at least four labeled components, each having a R_f value below 0.25 in solvent systems C and D; two of these components chromatograph in the manner expected for metabolites G and H, indicating that these metabolites retain the ester linkage. A portion of the metabolite content of region I (Figure 3A) also arises from structural modification(s) other than ester hydrolysis because more than 50% of the ether-soluble radiocarbon remains at the origin on tlc in solvent systems C and D.

Urinalysis establishes that dinobuton metabolism in living mammals involves hydrolysis and subsequent modifications of DNBP, including conjugation. The metabolites formed by oxidation, reduction, and acetylation reactions are detected in relatively minor quantities (Table III). Unchanged dinobuton appears in mouse but not in rat urine, and the following five metabolites appear only in rat urine: 6-NH_2-NBP; 2-*sec*-butyl-4-acetamido-6-nitrophenol; and unknown metabolites A, E, and G.

Table III. Products in Urine of Mice and Rats 24 Hr after Oral Administration of Dinobuton-ring-^{14}C

Product designation[a]	Amount of urinary radiocarbon recovered, %, in indicated species: Mouse	Rat
Dinobuton	0.8	0.0
DNBP, free	1.1	1.2
DNBP, conj[b]	...[c]	0.7
6-NH_2-NBP, free	0.0	1.7
6-NH_2-NBP, conj[b]	...[c]	1.7
2-*sec*-Butyl-4-acetamido-6-nitrophenol, free	0.0	0.4
DNBP-3-COOH, free	2.7	5.5
DNBP-3-COOH, conj[b]	...[c]	1.7
DNBP-2-COOH, free	0.8	1.6
Unknown A, free	0.0	0.6
Unknown B, free	0.8	0.6
Unknown C, free	6.0	5.3
Unknown D, free	8.8	3.8
Unknown E, free	0.0	0.8
Unknown F, free	1.1	0.5
Unknown G, free	0.0	0.4
Unknown H, free	1.6	1.0
Unknown 1, conj[b,d]	...[c]	2.4
Unknown 2, conj[b,d]	...[c]	2.0
Unknown 3, conj[b,d]	...[c]	2.4
Unknown 4, conj[b,d]	...[c]	11.2
Unknown complex	69.6[e]	52.4[e]
Total	93.3	97.9

[a] The tlc positions of the organosoluble products are given in Figure 3A. [b] The "conjugated metabolites" are obtained and cleaved to their corresponding aglycones by: (1) reextraction of the products remaining at the origin (designated as I, Figure 3A) followed by β-glucuronidase hydrolysis; and (2) cleanup of the ether-extracted urine on a Bio-Bead column, preparative tlc of the methanol eluate in solvent system E, and subsequent β-glucuronidase hydrolysis of individual radioactive gel regions. [c] β-Glucuronidase hydrolysis was not done with the mouse urine; therefore, the unknown complex includes the radiocarbon in area I (Figure 3A), which is 33.9%, and in the ether-extracted urine (35.7%). [d] R_f values for the unknown aglycones 1, 2, 3, and 4 are: 0.20, 0.09, 0.64, and 0.00 in solvent system C, and 0.33, 0.25, 0.65, and 0.00 in solvent system D, respectively. [e] Represents at least five methanol-soluble products uncleaved by β-glucuronidase hydrolysis; R_f values on preparative tlc in solvent system E are: 0.96, 0.85, 0.68, 0.60, and 0.49.

Rat feces collected 24 hr after dinobuton-ring-^{14}C administration contains 10–12% of its radiocarbon in a form extractable with methylene chloride; this consists of about one part of DNBP (identified by tlc cochromatography) to 19 parts of material remaining at the origin in tlc solvent systems C and D. The labeled products in the methylene chloride-extracted feces are partially recovered on additional solvent extractions, first with water (35% of the feces radiocarbon) and then by acid or base hydrolysis and extraction with ether (25% of the feces radiocarbon). The water extract contains two major radioactive components (bands in solvent system E, R_f values of 0.77 and 0.85) which are not identified. Attempts to resolve the components released on acid or base hydrolysis were unsuccessful by tlc.

Increased dosages of dinobuton were administered orally to rats in order to obtain, if possible, milligram quantities of urinary and fecal metabolites for spectral examination. The rats did not survive two doses of 250 mg/kg administered 13 hr apart, and a single oral dose of 400 mg/kg resulted in the death within 72 hr of two of the three rats treated. With single oral doses, the rats void a greater percentage of the administered radiocarbon in the feces as the dose increases and the percentage of radiocarbon excreted in the urine decreases (Figure 4). It appears that there is a maximum rate of urinary elimination of products derived from orally administered dinobuton, the excess amount being eliminated *via* the feces; with doses of 50 mg/kg or more, the maximum appears to be approximately 26 mg equivalent of dinobuton/kg in a period of 72 hr (Figure 4).

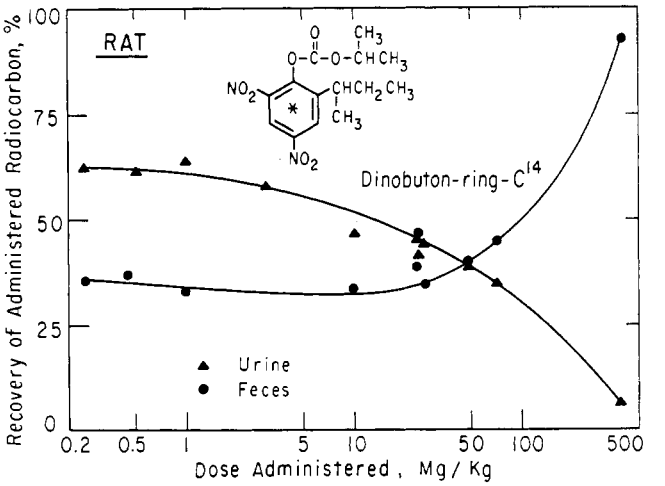

Figure 4. Effect of dosage level of orally administered dinobuton-ring-^{14}C on 72-hr excretion of radiocarbon in the urine and feces of rats

Table IV. Metabolites of Dinobuton-ring-^{14}C and DNBP-^{14}C Formed on Incubation with Microsomes from Rat Liver and Housefly Abdomens Fortified with NADPH

Product or fraction designation	Amount of initial radiocarbon recovered, %, with microsomes from indicated animal: Dinobuton-ring-^{14}C, Rat	Fly	DNBP-^{14}C, Rat	Fly
Organosoluble products[a]				
Dinobuton	3.1	0.5	...	...
DNBP	60.7	83.8	81.4	84.8
6-NH_2-NBP	0.1	0.5	0.0	0.2
Unknowns a, b, c, f, and g[b,c]	1.0	1.1	0.4	0.2
Unknown d	5.4	0.2	0.5	0.0
Unknown e	8.6	2.9	1.0	0.3
Unknown h	0.2	0.0	0.8	0.1
Unknown i[c]	5.2	1.6	6.9	0.9
Unknown j[c,d]	0.0	0.9	0.0	0.0
Water-soluble fraction	12.0	8.3[c]	8.4	6.3
Total	96.3	99.8	99.4	92.8

[a] The tlc positions are given in Figure 3B. [b] The amount of each individual product in no case exceeds 0.5% of the initial radiocarbon. [c] Unknowns g and j and major portions of unknown i and the water-soluble metabolites from the fly system retain the carbonate ester grouping based on comparable studies with dinobuton-ring-^{14}C and carbonyl-^{14}C. All the other unknowns are not detected with dinobuton-carbonyl-^{14}C, so they are phenols. [d] Unknown j forms only with fly microsomes; it gives R_f values of 0.18 and 0.60 in solvent system C and D, respectively.

Metabolites Formed by Microsome–NADPH Systems of Rat Liver and Housefly Abdomens. The metabolic reactions of dinobuton and DNBP carried out by rat liver and housefly microsomes are dependent on fortification with NADPH, with the exception of the hydrolysis of dinobuton. In the absence of NADPH, DNBP and dinobuton are metabolized to an average extent of only 0.7 and 0.5% (excluding DNBP formation), respectively. A portion of the DNBP detected on incubation of dinobuton-ring-^{14}C with the housefly abdomen system may result from the esteratic activity of the albumin in the reaction mixtures. The microsome–NADPH systems form the same organosoluble metabolites from either dinobuton or DNBP (Table IV), with the exception of unknowns g and j (which appear to be esters) and of unknown i (apparently a mixture of phenols and esters) (Figure 3B). Apparently, only the *o*-nitro group is reduced to give 6-NH_2-

Table V. Fractionation of Radiolabeled Products from Growing Bean Plants Injected with Dinobuton–ring-^{14}C, Dinobuton–carbonyl-^{14}C, or DNBP-^{14}C into the Stem

Compound	Time after injection, days	Amount of injected radiocarbon recovered, %, in indicated fraction: Organo-soluble	Aqueous	Organo-insoluble	Total
Dinobuton–ring-^{14}C	0	89.7	0.0	2.1	91.8
	1	42.8	2.7	18.8	64.3
	7	16.5	7.5	29.4	53.4
Dinobuton–carbonyl-^{14}C	0	70.5	0.0	2.5	73.0
	1	42.1	0.6	2.8	45.5
	7	20.8	0.9	15.6	37.3
DNBP-^{14}C	0	68.4	1.0	3.7	73.1
	1	0.7	17.5	26.6	44.8
	7	1.9	26.6	20.6	49.1

Table VI. Products Recovered from Growing Bean Plants Injected with Dinobuton–ring-^{14}C into the Stem

Product or fraction designation	Amount of injected radiocarbon recovered, %, at indicated days after injection: 0	1	7
Tlc resolved products[a]			
Dinobuton	89.7	41.9	15.9
DNBP, free	0.0	0.3	0.1
DNBP, conj	...	...	0.6
4-NH_2-NBP, conj	...	...	0.4
6-NH_2-NBP, free	0.0	0.0	0.1
6-NH_2-NBP, conj	...	...	0.6
Unknowns I, II, III ,IV, and V, free	0.0	0.6	0.4
Unknown VI, conj	...	...	0.1
Unknown VII, conj	...	...	0.4
Unknown VIII, conj	...	...	5.3
Tlc unresolved fraction[b]	2.1	21.5	29.5
Total	91.8	64.3	53.4

[a] The tlc positions of the organosoluble products are given in Figure 3C. For products designated as conjugates, the aglycones were obtained by treating the water extract of the organo-insoluble fraction with β-glucosidase and glusulase. The R_f values for the unknown aglycones VI, VII, and VIII are 0.78, 0.11, and 0.00, respectively, in solvent system A; it is not known whether these aglycones retain the carbonate ester grouping. Hydrolytic studies with enzymes were not done except on 7-day samples. [b] Represents the radiocarbon in uncleaved water soluble and insoluble metabolites.

Table VII. Products Recovered from Bean Leaves Treated Topically with Dinobuton–ring-^{14}C in the Presence and Absence of Rotenone and Exposed to Light

Product or fraction designation	Amount of applied radiocarbon recovered, %, with indicated exposure conditions[a]: Rotenone absent; exposure, 168 hr	Rotenone present; exposure, 1 hr
Surface residue products[b]		
Dinobuton	40.3	51.4
DNBP	0.0	2.3
6-NH_2-NBP	0.0	0.7
Ester unknown 1–6[c]	1.3	4.9
Phenol unknowns 7–10[d]	0.3	2.4
Ester and phenol mixture–unknown 11	2.6	19.5
Penetrated residue products[b]		
Dinobuton	1.2	2.4
Ester unknown 12[e]	0.7	1.0
Insoluble portion	3.8	3.3
Total	50.2	87.9

[a] When rotenone was added as photosensitizer, the plants were exposed to direct sunlight; those not treated with rotenone were kept in a greenhouse. [b] The surface residue was recovered by washing with ether and the penetrated residue was recovered by subsequent homogenization in acetone. The tlc positions for products in the surface residue are given in Figure 3D. [c] The amount of individual products varies from 0.0 to 0.6% without and 0.5 to 1.3% with rotenone; each product is also detected in a comparable experiment with dinobuton–carbonyl-^{14}C. [d] The amount of individual products varies from 0.0 to 0.3% without and 0.2 to 1.2% with rotenone; not any of these products are detected in a comparable experiment with dinobuton–carbonyl-^{14}C. [e] Remains at the origin in solvent systems C and D.

NBP; the 4-NH_2 derivative, if produced, apparently is unstable. With a nitrogen atmosphere, the reductive metabolic attack is enhanced considerably, producing 10.3 and 3.5% of 6-NH_2-NBP with rat liver and housefly abdomen microsomes, respectively. The unknowns d and e partially react with diazomethane.

Metabolites in Bean Plants after Stem Injection. Dinobuton-^{14}C and DNBP-^{14}C are not highly systemic in plants, with only a small fraction (<1.2%) of the injected radioactivity translocating to the leaves and not any to the root region within a period of 7 days. The translocated radiocarbon, when present, is uniformly distributed throughout the primary and trifoliate leaves. Metabolism of these materials, which must occur largely in the stem under the experimental conditions, results in rapid loss of radiocarbon from the organosoluble fraction, and an increase of radiocarbon in the aqueous and organo-insoluble fractions (Table V).

In the case of dinobuton-^{14}C, the labeled material recovered in the organosoluble fraction at 0 days is almost entirely dinobuton-^{14}C (based on tlc analysis) but, at 1 and 7 days after injection, it consists of at least eight tlc-resolvable areas (Figure 3C), even though the unchanged parent compound still predominates. Less than 1% of the dinobuton is hydrolyzed to give persisting DNBP, even after 7 days (Table VI). Metabolites I, II, III, and IV apparently retain the ester linkage, while metabolite V apparently is a product of ester hydrolysis. It appears that a large proportion of the metabolites in the organosoluble fraction arise from nonhydrolytic reactions. (Due to the extremely low radiocarbon content in each metabolite area, work was not done to reveal their chemical identity.)

The metabolites of dinobuton in the aqueous and organo-insoluble fractions are mostly products from ester hydrolysis (Table V). From the studies with DNBP-^{14}C, it is reasonable to expect that the *sec*-butyl and/or *o*-nitro groups are modified and that conjugates subsequently form which remain in the aqueous fraction or are tightly bound to the plant tissues. The radioactive components in the aqueous fraction (Table V) remain unidentified.

The knowledge about the possible formation of glycosides is based only on studies with the 7-day samples of the organo-insoluble fraction (Table V) from plants treated with dinobuton–ring-^{14}C. Homogenization of the organo-insoluble residue (from acetone–chloroform extraction) in water releases 60% of its radiocarbon into water-soluble material. Treatment with β-glucosidase cleaves approximately 30% of the water-soluble metabolites, and tlc analysis (solvent system A) of the hydrolytic products reveals the presence of four compounds; three of them are tentatively identified, by cochromatography, as DNBP, 4-NH_2-NBP, and 6-NH_2-NBP (Table VI). Glusulase incubation of the water-soluble fraction following β-glucosidase incubation gives further cleavage of conjugates and approximately 15% of the water-soluble radiocarbon becomes ether extractable. Tlc and cochromatography of the combined ether extracts reveal the presence, as aglycones, of DNBP, 4-NH_2-NBP, 6-NH_2-NBP, and at least three other unknown products (Table VI). Thus, some of the water-soluble metabolites are cleaved by β-glucosidase and glusulase to ether-extractable compounds which

have phenolic and/or amino moieties consistent with the requirement for glycoside formation.

The organo-insoluble residue from bean plants receiving dinobuton–carbonyl-^{14}C contains a high level of labeled compounds even after 7 days (Table V). Although this material was not subjected to enzymatic cleavage studies, it seems likely that it consists of a variety of materials incorporating the $^{14}CO_2$ released on hydrolysis because it is known (Kuhr and Casida, 1967) that 57% of the radiocarbon injected as sodium carbonate-^{14}C appears in the insoluble fraction after 6 days. Alternatively, it is possible that nonhydrolytic modification of dinobuton and subsequent conjugation results in metabolites that are bound firmly to plant constituents.

Preliminary experiments indicate that the mere storing of the treated plants in a freezer prior to analysis results in extensive decomposition of dinobuton. Bean plants that are frozen immediately (0 days) after treatment with dinobuton–ring-^{14}C and stored at freezing temperature for approximately 1 month show the presence of 6–7 organosoluble products, 35% of the administered radiocarbon being in the organo-insoluble residue; there is a significant loss of the injected radioactivity (about 27%), possibly as a result of volatilization. Accordingly, the results reported are based on plants that were not stored but were analyzed immediately after completion of each phase of the studies.

Products in Dinobuton-Treated Bean Leaves. The radiocarbon of topically applied dinobuton–ring-^{14}C is not translocated to the opposite untreated leaf, but a portion penetrates the treated leaf and is metabolized, appearing in the acetone homogenate and insoluble residue (Table VII). The products in the surface ether wash are regarded as mainly due to photodecomposition (Table VII) and are discussed below. The acetone homogenate of plants grown for 7 days in the greenhouse after treatment with dinobuton–ring-^{14}C contains unchanged dinobuton as the major component, but 37% of the radiocarbon consists of polar products that remain at the origin on tlc in solvent systems C and D. A comparison of the results of studies with dinobuton–carbonyl-^{14}C and ring-^{14}C reveals that a considerable portion of these polar products retains the ester grouping. The amount of these polar materials is significantly higher in the presence of a photosensitizer, especially rotenone, and they seem to form more rapidly; so, the presence of the photosensitizer possibly changes the balance of products available for metabolism.

The acetone homogenate of bean leaves, analyzed 4 hr following topical treatment with dinobuton–ring-^{14}C in the presence of xanthen-9-one, contains additional compound(s) not evident in the surface ether wash; this material chromatographs on tlc as expected for one of the isomeric 2-(hydroxy-*sec*-butyl)- and 2-(2-β-butenyl)-4,6-dinitrophenols, but the radioactivity level is not sufficient for cochromatography. It is not known if this product originates from metabolism or sensitized photoalteration (Bandal, 1971).

Persistence of Dinobuton Deposits on Bean Leaves and Glass Surfaces. The half-life of the dinobuton–carbonyl-^{14}C derived products is approximately 16 hr on glass surfaces and 60–75 hr on bean leaves; the longer persistence on leaves indicates that there is some penetration, decreased volatility, and/or less ester hydrolysis. The loss of dinobuton–ring-^{14}C derived products from bean leaves and glass surfaces proceeds at a considerably slower rate than observed with the carbonyl-labeled material, and the difference is greater on glass than leaf surfaces; thus, hydrolysis is a significant factor in the loss of dinobuton, and the photo-induced hydrolysis of dinobuton probably proceeds faster on glass surfaces than on bean leaves.

Photoalteration Products on Dinobuton-Treated Bean Leaves. The ether-soluble surface residue from plants held in a greenhouse for 1 day after application of dinobuton consists almost entirely of unchanged dinobuton but there are trace levels of DNBP (0.2%) and at least eight other radioactive products (Bandal, 1971). Seven days after the treatment, dinobuton remains as the major radioactive component, DNBP is not detected, and other products appear in increased amounts (Table VII). Thus, under greenhouse conditions, dinobuton does not undergo extensive photoalteration and any DNBP that forms is photodecomposed to other product(s).

Two photosensitizers were tested as a possible means to obtain larger amounts of photoalteration products for investigation of their nature. Rotenone and xanthen-9-one increase the rate at which the ether wash constituents are lost. This takes place to such an extent that the loss of ether-soluble surface residue of dinobuton on bean leaves is as great in 4 hr in direct sunlight, in the presence of the photosensitizers, as it is in 7 days, under greenhouse conditions, in the absence of such sensitizers (Bandal, 1971). Xanthen-9-one is effective and rotenone is highly effective in accelerating the rate of dinobuton photoalteration (Bandal, 1971). Both photosensitizers result in the formation of 10–13 products present at levels greater than 0.2% (Figure 3D). There are differences in the products detected with xanthen-9-one and rotenone; 6-NH_2-NBP and unknown products 2 and 9 are detected only with rotenone sensitization; polar unknown 11 is in much greater amount with rotenone; ester cleavage is more prominent with rotenone, yielding increased amounts of DNBP and an accelerated loss of labeled products from dinobuton–carbonyl-^{14}C; xanthen-9-one is the more effective sensitizer in accelerating the loss of labeled products from dinobuton–ring-^{14}C (Figure 3D; Table VII; Bandal, 1971).

On the basis of comparative studies with dinobuton–ring-^{14}C and carbonyl-^{14}C, it appears that unknown products 7–10 are phenols, unknowns 1–6 are esters, and the products remaining at the origin (unknown 11, Table VII) are mixtures of esters and phenols (Table VII). The tlc position of product 8 (see Figure 3D) is similar to that of authentic unlabeled DNBP-3-COOH; however, direct comparison of the R_f values may be misleading because of interference from plant extractives. Photoproducts 3 and 4 possibly are DNBP esters with modifications in the isopropyl group, such as at the methyl substituents, because, on hydrolysis with 10% aqueous sodium hydroxide, they yield $^{14}CO_2$ (the label is lost from the carbonyl-^{14}C preparation) and DNBP (tlc cochromatography of the products from dinobuton–ring-^{14}C).

DISCUSSION

Figure 5 gives the tentative metabolic and photoalteration pathways for dinobuton-^{14}C and DNBP-^{14}C in various organisms and on bean leaves, respectively.

Dinobuton metabolism in mice and rats involves, for the major portion of the dose, initial hydrolysis of the carbonate group liberating carbon dioxide, followed by oxidation of either methyl group in the *sec*-butyl moiety, conjugation of phenolic metabolites and, in rats only, reduction of the *o*- and *p*-nitro groups and acetylation of the metabolically formed *p*-amino group. The data found in the present study confirm and extend the results of an earlier investigation on metabolism of DNBP and certain of its esters in rats (Ernst and Bär, 1964). They are also consistent with findings on the

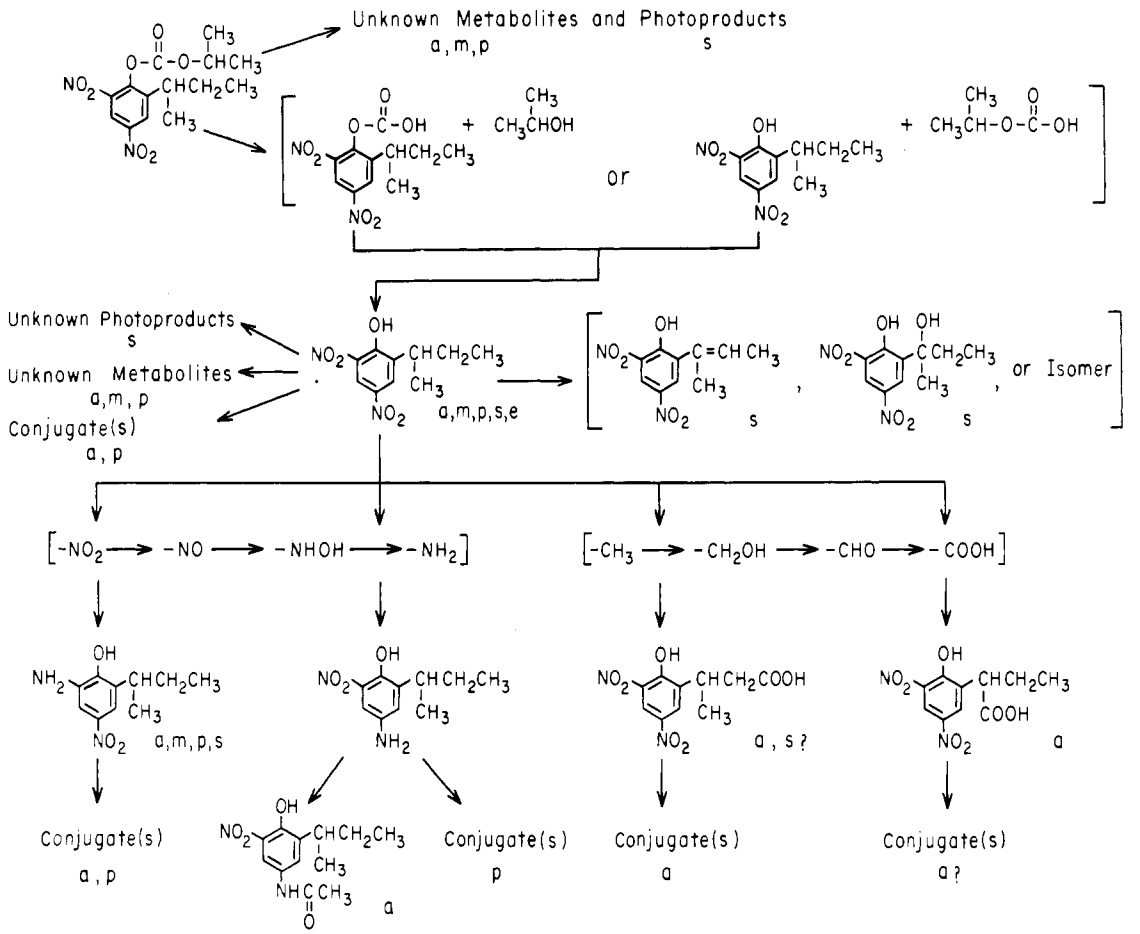

Figure 5. Tentative pathways for dinobuton metabolism in mice, rats, bean plants, and enzymatic systems, and for dinobuton photoalteration on exposure to sunlight. a, rat or mouse *in vivo;* m, microsome–NADPH; p, bean plant; s, sunlight; e, esterase

metabolism of other dinitrophenols in insects and mammals (Guerbet and Mayer, 1932; Henneberg, 1964; Kikal and Smith, 1959; Parker, 1952; Smith *et al.*, 1953; Truhaut and de Lavaur, 1967; Yasuda, 1957).

In the case of insects, the metabolic change to DNBP is an activation process but the formation of the oxidized and reduced derivatives of DNBP apparently represents detoxification reactions; the metabolites other than DNBP have, in general, lower toxicity than dinobuton to houseflies and milkweed bugs (Bandal, 1971).

A previous study established that DNBP and not dinobuton is the actual uncoupler of oxidative phosphorylation and that uncoupling in brain mitochondria is probably most significant in initiating symptoms of acute poisoning (Ilivicky and Casida, 1969). The present study shows that there is a very low dinobuton-hydrolyzing activity in the brain tissue and that the radiocarbon recovered from the brain of mice treated with dinobuton–ring-^{14}C is approximately one-third that recovered from the liver at the time of severe symptoms or death. *In vitro* and *in vivo* studies indicate that dinobuton is probably not extensively hydrolyzed in the gut before absorption, that hydrolysis occurs, in part, on passage through the digestive tract wall and is almost complete on entering the blood stream or shortly thereafter, and that little or no dinobuton remains to be hydrolyzed by the time it reaches the liver. The persistence of DNBP in the blood may result from formation of a DNBP–albumin complex, on analogy with the study in rats by Henneberg (1964), and the persistence in the brain may be due to slow metabolism and very slow removal from brain tissue. These findings indicate that hydrolysis of dinobuton in the blood or other tissues and subsequent transport of DNBP to the brain is probably involved in its toxic action to mammals.

The relative toxicity of various 2-alkyl-4,6-dinitrophenyl esters to different mammalian species appears to depend, in part, on the activity of their esterases for liberating the actual uncoupling agents. The rate of dinobuton cleavage by blood esterases from various species differs greatly and probably contributes to the selective toxicity of dinobuton. Thus, rats are about ten times more sensitive than mice to acute poisoning by orally administered dinobuton (Bandal, 1971; Gaines, 1969; Kenaga and Allison, 1969; Martin, 1968; Pianka and Smith, 1965) and the hydrolysis of dinobuton to DNBP occurs more rapidly in rats than in mice and with rat blood than with mouse blood. The selective toxicity of other esters of DNBP and of related compounds (Bough *et al.*, 1965; Gaines, 1969; Kenaga and Allison, 1969; Martin, 1968) also possibly results from their relative ease of *in vivo* hydrolysis. Carbaryl protects rats from acute poisoning by dinobuton but not by DNBP, probably because carbaryl inhibits the esterase(s) responsible for the hydrolysis of dinobuton to DNBP. Carbaryl also causes inactivation of dinobuton in some other species (Martin, 1968).

Liver microsomes are highly active in dinobuton cleavage but their activity is not dependent upon NADH or NADPH, indicating that oxidative processes are not involved. The cleavage involves esterases other than cholinesterases because some organophosphates and methylcarbamates, but not eserine, are inhibitory under *in vitro* conditions. It is not known whether the initial hydrolysis occurs at the isopropyl

or phenyl ester group of the carbonate moiety, or both, but the intermediate carbonate cleavage product probably would be unstable, in either case. Dinobuton metabolism by housefly abdomen microsomes is similar to that in rat liver microsomes, both involving mainly ester hydrolysis. Significant oxidation of the methyl groups in the *sec*-butyl moiety occurs in intact mammals, but the terminal oxidation products are not recovered in *in vitro* assays, probably due to incomplete *in vitro* oxidation of the *sec*-butyl side chain. The reduction of the *o*-nitro group takes place noticeably in air but occurs more rapidly under anaerobic conditions.

Dinobuton and DNBP are not highly systemic in bean plants and their metabolic pathways are generally the same in plants as in mammals, with the exceptions that the side chain oxidation and acetylation of the metabolically formed *p*-amino group, which occur in rats, possibly do not take place in bean plants. The hydrolytic pathway appears to be relatively slower, or of less importance, in plants than in mammals. The water-soluble metabolites in plants are formed, in part, by ester hydrolysis, reduction of the nitro groups, and subsequent conjugation possibly to form *O*-glycosides and/or *N*-glycosides. Relatively small amounts of DNBP and 6-NH_2-NBP occur as unconjugated aglycones in the organo-soluble extracts of plants, whereas 4-NH_2-NBP does not occur as an unconjugated metabolite. Any 4-NH_2-NBP formed as a plant metabolite appears to be reactive, rapidly conjugating or decomposing in the plant or in the extraction process. It appears that the rate-limiting reactions in plant metabolism of dinobuton possibly are the hydrolysis and reduction steps rather than the formation of conjugates. It is possible that the aglycones identified, especially DNBP, are conjugated not only with a monosaccharide moiety but also with a disaccharide or trisaccharide moiety; however, the identity of the sugar components of the dinobuton metabolite glycosides is not known.

Dinobuton residues persist for only a relatively short time on treated areas and, therefore, very little of such residues on topically treated bean leaves penetrates the leaves and comes into contact with the plant metabolic systems. When they do occur, the penetrated residues consist of unchanged dinobuton and unknown polar metabolites. The photodecomposition reactions occurring in the presence or absence of photosensitizers on the surface of bean leaves involve ester hydrolysis, reduction of the *o*-nitro group, oxidation of the *sec*-butyl side chain, and possibly attack on the isopropyl carbonate moiety without hydrolysis. The sensitized photoalteration of dinobuton primarily involves polymerization or other reactions yielding significant amounts of polar products. In the presence of xanthen-9-one, photoalteration possibly also involves modification of the *sec*-butyl side chain to yield small quantities of the isomeric 2-(hydroxy-*sec*-butyl)- and 2-(2-β-butenyl)-4,6-dinitrophenols.

The carbonate group confers selective toxicity to dinobuton because this group is cleaved to liberate the actual toxicant at varying rates in different organisms. Once liberated, DNBP is metabolically unstable. Many unstable intermediates are generated in the course of metabolism and photoalteration of dinobuton and DNBP, forming terminal residues that have not been characterized.

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