Studies with Dairy Cows and Laying Hens Fed Alfalfa Containing Field-Aged Residues due to 3,5-Dichloro-*N*-(1,1-dimethyl-2-propynyl)benzamide

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Groups of dairy cows and laying hens were fed specially prepared diets containing alfalfa with field-aged residues due to the herbicide 3,5-dichloro-*N*-(1,1-dimethyl-2-propynyl)benzamide. Residues greater than 0.01 ppm were found only in milk from cows fed 7.5 ppm of herbicide residues and in eggs from chickens fed 1.8 ppm of herbicide residues. No evidence for a continual accumulation of residues was observed in either eggs or milk. Detectable residues were found only in cow liver and kidney, and hen liver, kidney, gizzard, and fat.

Kerb (Rohm & Haas, formerly RH-315) [3,5-dichloro-*N*-(1,1-dimethyl-2-propynyl)benzamide] is a selective herbicide used to control grasses in alfalfa (Viste *et al.*, 1970). Since alfalfa hay may be present in farm animal rations, studies were conducted to determine the residue levels in milk, eggs, and selected tissues of dairy cows and laying hens which may result from repeated ingestion of feed containing fixed concentrations of residues due to Kerb. Studies using ^{14}C-labeled Kerb with rats and a cow have been conducted by Wargo *et al.* (1972).

EXPERIMENTAL SECTION

Materials. To produce a substrate with relatively high concentrations of field-aged residues due to Kerb, alfalfa (Vernal variety) was sprayed with the herbicide at a rate of 3 lb/acre, 54 days prior to cutting. The nature of residues on alfalfa following a similar application of ^{14}C-labeled Kerb has been described (Yih and Swithenbank, 1971). Normalized residue values resulting from the use of Kerb on alfalfa have been reported (Adler *et al.*, 1972).

The alfalfa was cut, field-dried, baled, and stored in a barn pending completion of necessary preliminaries prior to the actual feeding studies. The storage time was 108 days. A commercial mill was used to grind and mix the dried alfalfa hay. Subsamples of the hay were blended with ground control hay to produce lots of different residue content, as shown in Table I.

Subsamples of the control and residue-containing alfalfa hay lots were further formulated and pelletized to produce a caged-layer ration containing *ca.* 20% alfalfa by weight.

Four groups of 15 mature white Leghorn hens *ca.* 1-year-old were used in one experiment. Four groups of three cows each (two Holsteins, one Guernsey) were used in the other experiment. The cows used produced at least 20 lb of milk daily.

Methods. The laying hens were individually housed in metal cages for the duration of the experiment. Water and grit were available *ad libitum*. Each bird received 0.25 lb of feed daily. During the first 2 weeks of the experiment, only control feed was used. After a 2-week acclimation period, three of the groups were fed pelletized rations containing Kerb residues, while the fourth group was continued on control feed.

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After 3 weeks on test feed, three hens from each group were sacrificed. After 5 and 7 weeks of feeding, six hens per group were sacrificed.

The cows were housed in a standard milking stanchion barn, and were maintained there for the duration of the experiment. An empty stanchion was kept between cows in order to eliminate the possibility of one animal eating another's food. The daily supply of food consisted of 25 lb of milled alfalfa hay and 8 lb of grain concentrate. Half of the hay and half of the grain was provided at each of two daily feedings. Water was available *ad libitum* from automatic drinking cups. For 1 week the hay fed to each group was free of residue. Following this period, the hay used for three of the groups contained aged residues due to Kerb. After 2 weeks of residue feeding, one cow from each of two of the treated groups and the control group was sacrificed. This was repeated after 3 and 4 weeks of feeding. The remaining three cows (in the low test feed group) were maintained on their diet for 3 weeks. At this point they were fed another (higher) residue containing feed. One cow from this group was sacrificed after 2, 3, and 4 weeks of feeding on the supplementary diet.

Collection of Samples. Eggs from the hens were collected daily and suitably identified with the number of the hen and date of collection. At sacrifice, samples of liver, kidney, skin, fat, gizzard, heart, breast muscle, thigh muscle, and a long bone were removed from each hen. During one 24-hr period in the third week of residue feeding, all hen excreta were collected separately from each test group.

Milk from each cow was collected daily. Subsamples of the combined morning and evening milking were taken for analysis. At sacrifice, samples of liver, kidneys, muscle (three sites), and fat (three sites) were removed from the cows. During one 24-hr period at the conclusion of the second week of residue feeding, urine and feces from each cow were collected separately.

Analysis of Samples. All analyses were based on the conversion of Kerb and/or metabolites to methyl 3,5-dichlorobenzoate (MDCB) which was detected and quantitated by electron capture gas chromatography using a ^{63}Ni electron capture detector. A detailed description of the method (sensitive to 0.01 ppm of Kerb) has been reported (Adler *et al.*, 1972).

The following modification of the method was used to handle egg samples. Each egg was cracked into a Waring blender jar containing two drops of Dow Antifoam Emulsion Y. The mixture was blended at high speed for 30 sec and poured into a 2-oz glass vial for storage. Samples were kept

Table I. Kerb Residues in Substrates Used for Feeding Studies

Feed group	Residue content (ppm calculated as Kerb): Milled alfalfa hay	Cow diets[a]	Chicken feed
Control	NDR[b]	NDR	NDR
Low	0.88	0.7[c]	0.22
Intermediate	2.34	1.8	0.51
Supplementary	4.58	3.5[c]	...
High[d]	9.92	7.5	1.82

[a] Assuming a daily ration of 25 lb of milled alfalfa hay and 8 lb of grain concentrate. [b] NDR = no detectable residue. [c] After receiving the low (0.7 ppm) test feed for 3 weeks, these cows were administered a supplementary (3.5 ppm) test feed for 2, 3, and 4 weeks. [d] Not diluted with control hay. The other lots were prepared by mixing approximately one part of this lot with 9, 3, and 1 part of control hay, respectively.

Table II. Recovery of Kerb Fortifications

Sample type	No. of fortifications	Fortification range, ppm Kerb	Average recovery, % ± σ
Eggs	35	0.01–0.1	68.8 ± 6.6
Milk	28	0.005–0.04	76.0 ± 7.3
Tissues[a]	56	0.01–1.1	70.6 ± 9.1
Fat[b]	17	0.02–0.26	59.0 ± 8.6
Cow feces	4	0.1–1.2	74.3 ± 8.7
Cow urine	7	0.4–9.0	72.0 ± 13.2

[a] This includes liver, kidney, muscle, etc., from cows and hens. [b] From cows and hens.

Table III. Residue Levels in Laying Hen Tissues

Test group	Duration of feeding, weeks	Average residues (ppm calcd as Kerb): Kidney	Liver	Gizzard	Fat
Low	3	0.02	NDR[a]	NDR	NDR
	5	NDR	NDR	NDR	NDR
	7	NDR	NDR	NDR	NDR
Intermediate	3	0.02	0.01	NDR	0.01
	5	0.04	NDR	NDR	NDR
	7	0.02	0.01	0.01	0.01
High	3	0.03	0.03	0.02	0.02
	5	0.02	0.02	NDR	NDR
	7	0.09	0.04	0.02	0.03

[a] No detectable residue.

refrigerated until analyzed. Ten grams of the homogenate was used for analysis.

Control samples of eggs, milk, excreta, and tissues were fortified with known quantities of Kerb and then taken through the analytical procedure. Recovery data are listed in Table II. Results of analyses of alfalfa, eggs, milk, tissues, and excreta have been corrected for recovery.

RESULTS AND DISCUSSION

The lots of milled alfalfa hay which were used for the cows and for preparing the pelleted poultry rations were analyzed for Kerb residues. Table I displays the results of the analyses.

Analysis of 94 eggs from each of the control, low, and intermediate test groups in the laying hen study failed to detect residues at a sensitivity of 0.01 ppm of Kerb. Eggs from the high test group did contain detectable residues which reached a plateau between 0.01 and 0.02 ppm after 1 week of the test period. The plateau remained stable for the duration of the feeding. No obvious differences were found in residue levels in eggs from the various hens in the group.

Several eggs from the high test group were separated into yolk and egg white. Residue analysis of the whites (at a sensitivity of *ca.* 0.002 ppm, due to larger sample size) detected between 0.003 and 0.008 ppm.

Analysis of *ca.* 36 milk samples from each of the low, intermediate, and supplementary feed groups did not find residues exceeding the method sensitivity of 0.01 ppm. The milk samples analyzed were roughly equally distributed among the three cows in each group. The high group milk displayed residues in about 70% of the samples analyzed, but never exceeded 0.02 ppm. The plateau was reached after 3 days of feeding and remained stable throughout the study. No obvious differences were observed in the residue levels in milk from cows of different breeds.

Table III details the residues found in the various laying hen tissues. Light meat, dark meat, skin, heart, and bone samples were free of residues. For the hen tissue analyses the results in general are reported for six hens. These six hens included two from each of three sacrifice dates. All livers and kidneys from the low test group hens were analyzed. All livers and kidneys from hens fed residues for 7 weeks in the remaining two test groups were analyzed.

Table IV details the residues found in the cow livers and kidneys. No detectable residues were found in any samples of muscle or fat from the cows.

A material balance was calculated from the known feed intake and the residue levels found in the excreta of the cows and hens of each group. Table V indicates that over 60% of the material fed daily was found in the excreta taken once during each experiment. The natural fluctuation in daily excretion and the practical difficulties in sample collection may be reasons for the only moderately successful material balance.

No effects were observed in general health or productivity

Table IV. Residue Levels in Cow Tissues

Test group	Duration of feeding, weeks	Residue found (ppm calcd as Kerb)[a]: Liver	Kidney
Intermediate	2	0.02	NDR[b]
	3[c]	0.03	NDR
	4	0.05	0.02
Supplementary	2	0.06	NDR
	3[c]	0.08	0.04
	4	0.06	0.03
High	2	0.12	0.04
	3[c]	0.16	0.04
	4	0.17	0.03

[a] No detectable residues at a sensitivity of 0.01 ppm were found in any samples of muscle or fat. [b] No detectable residue. [c] These were Guernsey cows. All others were Holstein.

Table V. Material Balance

Test group	Percent of residue fed eliminated in: Hen excreta	Cow urine	Cow feces	Total cow excreta
Control	...	...	...	...
Low	61.3	52.3	20.3	72.6
Intermediate	66.7	57.2	15.8	73.0
High	59.8	35.3	17.4	52.7

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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