Degradation of 4-(2,4-Dichlorophenoxy)-butyric Acid [4-(2,4-DB)] in Plants

D. L. LINSCOTT
Crops Research Division,
Agricultural Research Service,
U. S. Department of Agriculture,
Cornell University, Ithaca, N. Y.

Studies on beta oxidation of fatty acids and research leading to the development of 4-(2,4-DB) as a herbicide are reviewed. In greenhouse studies, 85 to 93% of C^{14}-labeled herbicide applied to legumes disappeared within 30 days after treatment. Simulated rainfall accelerated herbicide removal. Herbicide applied to legumes was not found in regrowth after a clipping. Fermentation of legumes for 45 days in small tubes resulted in substantial decomposition (67%) of 4-(2,4-DB).

THE USE of chemicals to eliminate undesirable vegetation will and must increase if long-term, world requirements for low-cost, high-quality food and fiber are to be fulfilled. The need for determining the quantities of herbicides appearing in food products and for understanding the processes of herbicide degradation is evident. First, scientists must determine that residues of chemicals and degradation products that appear in the food and water are either safe or of a sufficiently low level to ensure consumer safety. On the other hand, the public should not lose the tremendous saving in cost of food and clothing by the imposition of unwarranted or unrealistic chemical tolerance levels. Second, producers of the world's food products should be provided with safe, reliable, and low-cost methods of controlling plant pests. Finally, herbicide metabolism is of academic interest. Fundamental studies often lead to major advances in technology. Indeed, the basic research of Wain and Wightman (*19–21*) in the early 1950's led to the development of 4-(2,4-dichlorophenoxy)butyric acid [4-(2,4-DB)] and 4-(2 methyl, 4-chlorophenoxy)butyric acid [4-(MCPB)] as herbicides for the selective control of dicotyledons in legume crops. This paper will discuss previous investigations involving 4-(2,4-DB) and beta oxidation and will summarize some of the work of the author's laboratory.

Wain and Wightman (*21*), while studying growth-regulating properties of chlorophenoxy alkyl carboxylic acids, observed that 4-(2,4,5-trichlorophenoxy)-butyric acid [4-(2,4,5-TB)] was active in the wheat coleoptile straight growth test but was inactive in a split pea test. Previously, they had confirmed earlier investigations of Grace (*9*) and Synerholm and Zimmerman (*18*) by establishing that a beta-oxidative system was operative on the aliphatic moiety of aromatic alkane carboxylic acids. The Wye college group realized that if certain plants, in contrast with others, had the ability to oxidize the aliphatic side chain of chlorinated phenoxy butyric acids to an active form, a new principle of selective weed control would be possible. Further experimentation in the laboratory and greenhouse proved that plants differed in their susceptibility to chlorinated phenoxy butyric acids. Legumes as a class were highly resistant to these compounds.

Several investigations proved the practicality and potential usefulness of 4-(2,4-DB) and 4-(MCPB) in selective weed control in such legumes as alfalfa (*Medicago sativa* L.), birdsfoot trefoil (*Lotus corniculatus* L.), clovers (*Trifolium sp.*), and field peas (*Pisum sativum* L.) (*1*, *3–5*, *15*, *20*). Directed spray applications have been used successfully to control weeds on soybeans (*Glycine max* L.) (*17*). At present, 4-(2,4-DB) is approved for use on seedling or established legumes with the restriction that the forage cannot be fed to livestock within 30 days after herbicide application. The compound is also approved for weed control in peas and soybeans. Of course, 4-(2,4-DB) does not solve all the problems of weed control in legumes as many troublesome dicotyledons such as yellow rocket (*Barbarea vulgaris* R. Br.), bedstraw (*Galium mollugo* L.), and almost all monocots are relatively insensitive to this compound. However, one can hardly question that a great advance has been made by the introduction of 4-(2,4-DB).

Review of Beta Oxidation

Early work of Knoop (*12*) established the principle of beta oxidation of phenyl alkane carboxylic acids in animals. Since Knoop's original studies, considerable effort has been spent in developing the mechanism of beta oxidation, an example of which is the research of Lynen (*13*). Most of the studies of beta oxidation have been conducted with animal tissue. However, work of Stumpf (*16*) and others with peanut mitochondria indicates that the process is essentially the same in plants. Therefore, the author has postulated that beta oxidation of 4-(2,4-DB) in plants may be similar to beta oxidation mechanisms proposed by Lynen (*13*) and others. Assume that R (Figure 1) represents a 2,4-chlorinated phenoxy moiety with a butyric side chain. Coenzyme A in the presence of adenosine triphosphate (ATP) reacts with 4-(2,4-DB) to form dichlorophenoxy butyryl thio Co-A (Figure 1). With flavin adenine dinucleotide (FAD) as electron acceptor, and the reaction catalyzed by an acyl Co-A dehydrogenase, two hydrogens are lost and the formation of dichlorophenoxy crotonyl Co-A results. This compound undergoes hydrolysis to form dichlorophenoxy beta hydroxy butyryl Co-A. Dichlorophenoxy vinyl acetyl Co-A may be formed by the dehydration of the beta hydroxy butyryl derivative. The next hydrogen loss involving dichlorophenoxy butyryl Co-A is dependent on diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) and is catalyzed by a beta hydroxy acyl Co-A dehydrogenase. The resulting compound is dichlorophenoxy beta keto butyryl Co-A. Cleavage occurs at the beta carbon, resulting in acetyl Co-A and, in this case, 2,4-dichlorophenoxy acetic acid (2,4-D). Therefore, if chlorinated phenoxy butyric acids are considered, a plant may have a tolerance potential to the compound if it lacks the capacity to carry out a beta

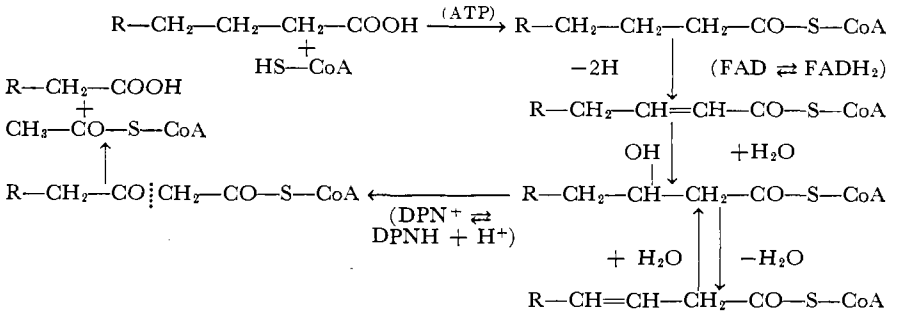

Figure 1. Beta oxidation of chlorinated butyryl phenoxy carboxylic acids

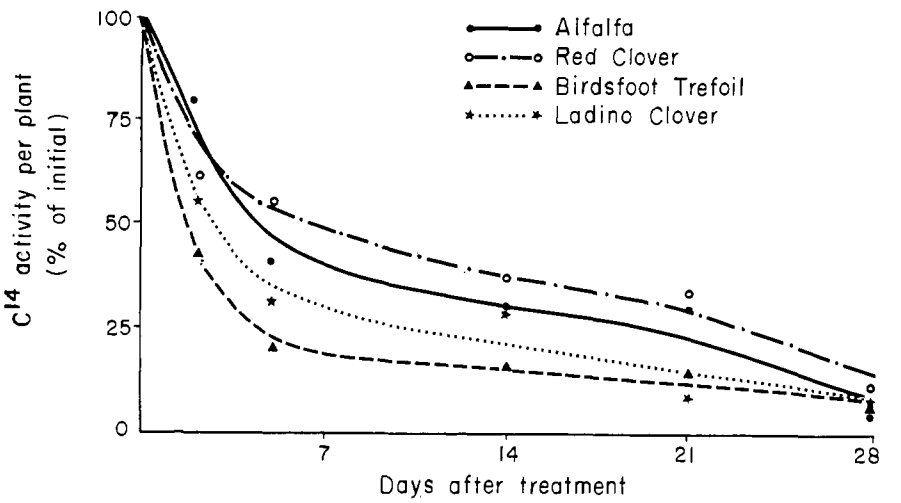

Figure 2. Disappearance of 4-(2,4-DB)-C^{14} from legumes

oxidation reaction, the reaction proceeds too slowly, or the plant rapidly detoxifies the herbicide. Plants having capabilities of beta oxidizing 4-(2,4-DB) to active 2,4-D may have a susceptibility potential.

Most research available indicates that beta oxidation is the primary mechanism for conversion of 4-(2,4-DB) to the toxic 2,4-D form (*4*, *5*, *22*). Up to this time, the most direct evidence of beta oxidation has been shown by Webley *et al.* (*22*), who found beta hydroxy acids formed after exposure of chlorophenoxy butyric acids to *Nocardia* sp. Kief (*11*) used a triphenyl tetrazolium chloride as an electron acceptor in 4-(2,4-DB) oxidation studies. Acetone powder preparations of pea seeds were used as the enzyme source. Good evidence of beta oxidation was found in that 4-(2,4-DB) and possible beta oxidation intermediates, such as dichlorophenoxy crotonic, beta hydroxy butyric, and beta keto butyric acids, served as substrates for dye reduction. The herbicide 4-(2,4-DB) is a selective herbicide for weed control in field peas. Peas are quite susceptible to 2,4-D. Therefore, Kief's findings appear to be inconsistent. However, the oxidation rate may have been accelerated or an enzymatic block allowing accumulation of 2,4-D may have occurred in vitro. It is difficult on occasion to relate in vitro and in vivo research.

Recent research by MacRae *et al.* (*14*) indicates cleavage of 4-(2,4-DB) at the ether linkage by *Flavobacterium* sp. and the direct formation of dichlorophenols. Dichlorophenols are inactive as plant growth regulators. The possibility of omega oxidation as a detoxification mechanism in plants therefore cannot be overlooked and may be an explanation for the ineffectiveness of 4-(2,4-DB) in killing certain dicotyledons.

Methods and Materials

Nine milligrams (SA 2.96 μc. per mg.) of 4-(2,4-DB)-C^{14} labeled in the carboxyl position was dissolved in 6 ml. of the following solvent: 3.0 ml. of acetone, 2.7 ml. of H_2O, 0.3 ml. of 1% isoöctyl phenyl polyethoxy ethanol (Triton X-100).

Ten microliters of the solution containing labeled 4-(2,4-DB) was applied to the second and third trifoliate leaves of alfalfa (*Medicago sativa* L.) variety Narragansett, birdsfoot trefoil (*Lotus corniculatus* L.) variety Viking, and to a single trifoliate leaf of red clover (*Trifolium pratense* L.) variety Dollard and ladino clover (*Trifolium repens* L.) variety Pilgrim. Just prior (within 5 minutes) to applying tagged 4-(2,4-DB), the legume plants were sprayed with 1 pound per acre of the commercial dimethyl amine salt of 4-(2,4-DB) to make the quantity of herbicide per plant equivalent to that commonly received in field applications. A continuous belt spray table was used. The legumes were about 6 inches tall at time of treatment and were growing rapidly. Treated plants were harvested 0, 2, 5, 14, 21, and 28 days after treatment, frozen with dry ice, and kept in the freezer until analyzed.

In a second study, alfalfa, birdsfoot trefoil, red clover, and ladino clover were treated only with labeled 4-(2,4-DB) as above. Seven days after treatment half of the plants were clipped 1½ inches above the soil line. Eighteen days after treatment, all pots were harvested and the plant material was quick-frozen. Plants were analyzed for C^{14} as above. The study was repeated with alfalfa and birdsfoot trefoil only.

Alfalfa and birdsfoot trefoil were treated as in the second study and subjected to heavy surface sprinklings of water (simulating rainfall) 1 and 2 days after treatment. Plants were harvested 0, 7, 14, 21, and 28 days after treatment, and before and after the simulated rainfall.

For analysis, plants were separated into components, immersed in isopropanol (1:5 w./v. plant material–alcohol) and blended for 10 minutes at the ambient temperature in a Lourdes blender. The blend was filtered under vacuum through Whatman No. 1 paper and washed several times with isopropanol. The resulting solution was made to volume with isopropanol. Aliquots were placed in stainless steel planchets, evaporated to dryness under heat lamps, and counted for C^{14} with a thin window gas flow Geiger detector. Controls or initials in all cases were determined from plants harvested immediately after herbicide application. Gross accumulation of C^{14} was determined by autoradiography using methods of Crafts (*2*).

Alfalfa and birdsfoot trefoil plants were chopped to ¼-inch pieces and mixed with carboxyl-labeled 4-(2,4-DB)-C^{14}. The mixture was tamped into small glass vials (30 grams of forage) and sealed. Initial herbicide concentration in the forage was 15 p.p.m. in two trials. Specific activity of the labeled herbicide was 2.96 μc. per mg. In a third trial initial herbicide concentration in the forage was adjusted to 76 p.p.m. Specific activity of the labeled herbicide in this case was 50.6 μc. per mg. Control forages containing herbicide were placed in a freezer immediately after packing in vials. Others were allowed to ferment for 45 days. Plant materials were blended for 10 minutes with a small Waring Blendor in 60 ml. of isopropanol

(1:1 w./v. forage/alcohol). The blend was filtered and analyzed for C^{14} as above.

All plants and plant materials in this study were grown in open-sided, plastic-roofed greenhouses during June and July of 1961 and 1962. All studies were replicated four times per entry.

Results and Discussion

Disappearance of 4-(2,4-DB) from Legumes. After 28 days, extractable compounds containing C^{14} varied from 7 to 15 % of the quantity initially applied to legumes (Figure 2). The difference in C^{14} concentration among species at this time was insignificant. Disappearance of the herbicide was more rapid initially in birdsfoot trefoil than in the other species. Degradation proceeded most slowly in red clover. Rapid degradation immediately after treatment is indicated by the relative steepness of the degradation curves.

Most of the activity was associated with treated leaves. Lesser quantities appeared in newly developing leaves, stems, and roots (Table I). The 4-(2,4-DB) was not translocated readily in these legume plants. Still, a limited quantity of the herbicide (C^{14}) was extracted from roots after treatment. Although extractable C^{14} diminished with time, autoradiographic distribution patterns changed very little. No gross changes in C^{14} deposition (gross autoradiograms) occurred during the entire experimental period. Therefore, considerable quantities of the carboxyl carbon were fixed or immobilized. Several mechanisms, including fixation of intact herbicide molecules or of fragments resulting from alpha or beta oxidation could have been responsible.

The author was interested in whether the herbicide could be detected in regrowth after cutting (Table II). In all treatments, negligible amounts of herbicides (C^{14}) appeared in the regrowth. The observation agrees with work of Freed (*7*). The practical significance of this study is obvious. As only limited amounts of herbicide (C^{14}) have translocated into roots and even less retranslocated into regrowth, removal of forage receiving 4-(2,4-DB) should negate a residue problem. The economic feasibility of this procedure is another matter, however.

The degradation or disappearance curve of 4-(2,4-DB) may be different under field conditions. Indeed, heavy applications of water (simulating 1-inch rainfall) 1 and 2 days after 4-(2,4-DB) treatment resulted in removal of 80 to 85% of the herbicide (Figure 3). It is significant that degradation proceeded slowly after the heavy watering. Twenty-eight days after treatment, the plants, whether in a greenhouse or in the open

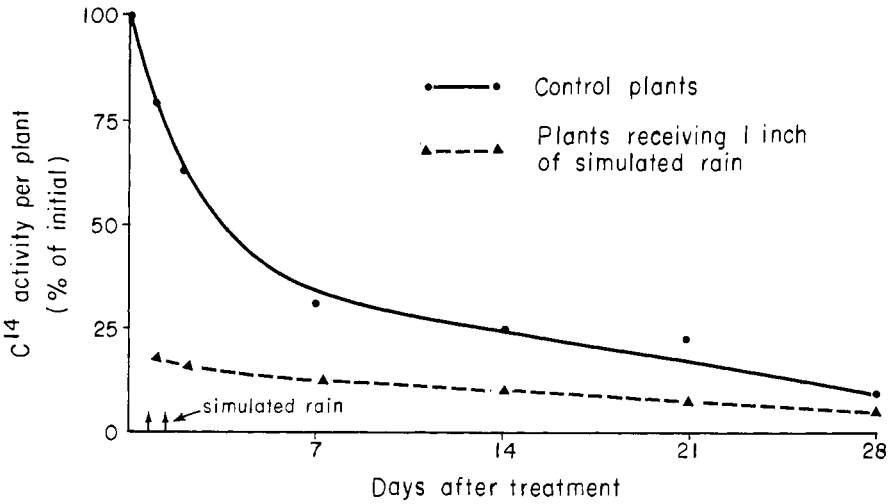

Figure 3. Effect of surface watering on disappearance of 4-(2,4-DB)-C^{14} from alfalfa and birdsfoot trefoil

Table I. Distribution of Extractable C^{14} in Treated Leaves, Leaves Plus Stems, and Roots of Alfalfa and Birdsfoot Trefoil after Treatment with 4-(2,4-DB)-C^{14}

Forage	Counts per Minute per Indicated Period (Days) after Treatment				
	0	2	5	14	28
Alfalfa					
Treated leaves	15,500	10,100	4200	4575	>725
Leaves plus stems	0	734	1550	200	
Roots	0	1,900	600	125	100
Birdsfoot trefoil					
Treated leaves	15,500	4,850	2433	2725	>1225
Leaves plus stems	0	200	666	425	
Roots	0	1,575	167	25	0

Table II. Disappearance of 4-(2,4-DB)-C^{14} from Four Legumes As Affected by Time and Clipping Treatment

Forage	Days after Treatment	% C^{14} Remaining	
		Trial I	Trial II
Control	0	100	100
Plants not clipped			
Alfalfa	7	89	79
Birdsfoot trefoil	7	94	50
Red clover	7	98	
Ladino clover	7	95	
Alfalfa	18	8	15
Birdsfoot trefoil	18	13	12
Red clover	18	16	
Ladino clover	18	16	
Regrowth following clipping			
Alfalfa	7[a]	<1	<1
Birdsfoot trefoil	7	<1	<1
Red clover	7	<1	
Ladino clover	7	<1	

[a] Tops were removed 7 days after herbicide application. The entire plant was harvested 12 days later.

exposed to additional rainfall and other weathering effects, still contained approximately 5% of the initial C^{14}. Other investigators have observed similar effects of rainfall in decreasing herbicide residues in plants (*8*, *10*). There remains for speculation the question of whether the removal effected by rainfall is a simple physical process caused by dissolving and washing away of surface herbicide or whether a more complex mechanism is also involved. For example, high humidity or light rain could result in an increased movement of

herbicide from leaf surfaces to sites of metabolism. Further, possible inhibitors of chemical reaction on and in leaves could be leached away resulting in accelerated herbicide breakdown.

Regarding field applications, 4-(2,4-DB) applied to legumes during the seedling stage probably will be dissipated or diluted to insignificant amounts by the first cutting. Fertig (*6*) found that samples of legumes taken 65 days after treatment for weed control in the seedling stage had insignificant residual herbicide. Research by Gutenmann and Lisk (*10*) indicated that 4-(2,4-DB) applied to an established stand of birdsfoot trefoil/timothy (*Phleum pratense* L.) disappeared after 48 days of generally dry weather. They detected 2,4-D in the forage as a metabolite but did not speculate as to which species was involved in the breakdown. There remains some question as to whether 4-(2,4-DB) was oxidized to 2,4-D under the influence of plant enzymes or was assimilated as 2,4-D through the roots after microbial breakdown in soil.

Disappearance of 4-(2,4-DB) from Silage. What can be done about reducing the chemical residues which may be present in forage after herbicide treatment? The fermentation involved in ensiling forage materials may be a partial answer for some herbicides. In each of three trials, 4-(2,4-DB) was partially decomposed after ensiling for 45 days (Table III). Decomposition varied considerably among trials and among forages. However, up to 55% of the herbicide applied to birdsfoot trefoil was degraded in one trial and 67% in an alfalfa trial. The organisms and mechanisms involved in degradation of 4-(2,4-DB) in silage have not been determined at this time. Whether ensiling will degrade 4-(2,4-DB) under field and farm conditions must be determined by further investigation.

Research Needs. Relatively few reports have been published on metabolism and degradation of 4-(2,4-DB). In general, pathways of herbicide degradation in plants and identification of degradation products have not been elucidated and should receive more attention. Much of the information now available is based on bioassay, paper chromatography, and radioisotope detection. All of these techniques are valuable but all have limitations. Probably the most serious hindrance to research has been the lack of suitable analytic methods for herbicides and metabolites. Recent developments in gas chromatography and other analytic methods will stimulate research with 4-(2,4-DB) and other chlorinated phenoxy-alkyl carboxylic acids. The key to real progress in future research is the development of sensitive analytic methods specific for a given herbicide in a given plant. Methods must be of a type which allows rapid, yet accurate, analyses.

Table III. Degradation of 4-(2,4-DB)-C^{14} on Legume Forage When Fermented as Silage

	Trial I		Trial II		Trial III	
Forage	P.P.M.	% Degraded	P.P.M.	% Degraded	P.P.M.	% Degraded
Alfalfa						
Control silage	15.0		15.0		76.0	
Silage (fermented 45 days)	13.1	13	9.5	37	25.1	67
Birdsfoot trefoil						
Control silage	15.0		15.0			
Silage (fermented 45 days)	7.7	49	6.8	55		

Literature Cited

(1) Carpenter, K., Soundy, M., *Proc. Brit. Weed Control Conf.* **1,** 327 (1954).
(2) Crafts, A. S., *Hilgardia* **26,** No. 6, 335 (1956).
(3) Fawcett, C. H., Ingraham, J. M. A., Wain, R. L., *Proc. Roy. Soc. London, Ser. B* **142,** 60 (1954).
(4) Fawcett, C. H., Pascal, R. M., Pybus, M. B., Taylor, H. F., Wain, R. L., Wightman, F., *Ibid.*, **150,** 95 (1959).
(5) Fawcett, C. H., Taylor, H. F., Wain, R. L., Wightman, F., *Ibid.*, **148,** 543 (1958).
(6) Fertig, S. N., Cornell University, Ithaca, N. Y., private communication, 1963.
(7) Freed, V. H., Oregon State University, Corvallis, Ore., private communication, 1962.
(8) Glastonbury, H. A., Stevenson, M. D., Ball, R. W. E., *Weeds* **7,** 362 (1959).
(9) Grace, N. H., *Can. J. Res.* (C) **17,** 247 (1939).
(10) Gutenmann, W. H., Lisk, D. J., J. Agr. Food Chem. **11,** 277 (1963).
(11) Kief, M. M., M.S. thesis, Oregon State University, Corvallis, Ore. 1961.
(12) Knoop, F., *Beitr. Chem. Physiol. Path.* **6,** 150 (1904).
(13) Lynen, F., *Ann. Rev. Biochem.* **24,** 653 (1955).
(14) MacRae, I. C., Alexander, M., Rovira, A. D., *J. Gen. Microbiol.* **32,** 69 (1963).
(15) Shaw, W. C., Gentner, W. A., *Weeds* **71,** 75 (1957).
(16) Stumpf, P. K., *Ann. Rev. Plant Physiol.* **10,** 197 (1959).
(17) Sylvester, E. P., *Res. Rept. N. Central Weed Control Conf.* 72 (1962).
(18) Synerholm, M. E., Zimmerman, P. W., *Contrib. Boyce Thompson Inst.* **14,** 369 (1947).
(19) Wain, R. L., *Ann. Appl. Biol.* **42,** 151 (1955).
(20) Wain, R. L., *Proc. Brit. Weed Control Conf.* **1,** 311 (1954).
(21) Wain, R. L., Wightman, F., *Proc. Roy. Soc. London, Ser. B* **142,** 525 (1954).
(22) Webley, O. M., Duff, R. B., Farmer, V. C., *Nature* **179,** 1130 (1957).

Received for review September 3, 1963. Accepted December 10, 1963. Division of Agricultural and Food Chemistry, 144th Meeting, ACS, Los Angeles, Calif., April 1963. Cooperative investigations of the Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture and Cornell University, Ithaca, N. Y.