REPRINT OF PAPER

PRESENTED

AT THE

MEETING OF

THE NORTHEASTERN WEED CONTROL CONFERENCE

VOLUME 15

[redacted]

JANUARY 4,5,6, 1961

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THE METABOLISM OF CERTAIN HERBICIDES BY PLANTS--A FACTOR IN THEIR BIOLOGICAL ACTIVITY[1,2,4]

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Since ancient times, man has sought chemicals to abate disease, the disease of his livestock and to control pests that exact their toll of his food crops. The rise of exact scientific studies in chemistry and biology permitted man to turn his attention to the effect of these chemicals on the organism exposed to them. It was early noted in animal experiments that the animals were able to tolerate a prolonged exposure to low concentrations of many of the organic chemicals tested. In attempting to discover the reason for this tolerance, it was found that the animals were able to detoxify or metabolize these drugs (4). The metabolism was found to take many forms in animals ranging all the way from the formation of a simple conjugate to a complete metabolism of the administered drug. It was found that the compound administered might be conjugated to simple amino acids, sugars or proteins, it may be coupled with sulfur-containing amino acids to form the mercapturic acids, the compound might be hydroxylated, via oxidation or the compound might be oxidized completely to carbon dioxide and water (4). Very often a combination of these detoxification mechanisms were found. Subsequently, it was shown that microorganisms, insects and other lower animals possess the same ability to metabolize organic substances. It is now known that microorganisms particularly have a wide range of abilities to deal with organic materials introduced into their environment.

Although it may have been surmised that plants possess the same ability to metabolize drugs, it remained for the workers at Boyce Thompson Institute (15,16, 17) to demonstrate this phenomena. These workers studying the effects of various fumigants on plants discovered that ethylene chlorohydrin was rapidly converted to a glycoside. Subsequently, other compounds were shown to be metabolized by plants, such as the dinitrophenols, where it was demonstrated that plants possess the ability to reduce one of the nitro groups of the dinitrophenol.

The introduction of growth-regulating compounds for weed control and for horticultural purposes greatly stimulated interest in the metabolism of chemicals by plants. The theory has been advanced that the mode of action of certain of these compounds may depend upon the metabolic conversion to an active form (14, 18). That such may be the case in certain instances was demonstrated by the workers at Boyce Thompson Institute who showed in 1947 that the omega phenoxy alkyl carboxylic acids could be converted by beta oxidation to 2,4-D (21). There remained for Wain (22) and his colleagues (2) in England, however, to make practical application of this discovery.

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1. Based in part on manuscripts submitted to Weeds.

2. Supported in part by a grant from Geigy Agricultural Chemicals, New York.

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4. Published as Oregon Agricultural Experiment Station Miscellaneous Paper #101.

The introduction of synthetic organic compounds such as 2,4-D for practical application in agriculture led to a renewed interest in determining the mode of action of the natural occurring plant hormones. A good deal of attention was focused on indole-3-acetic acid (IAA) resulting in the discovery of its destruction in the plant by the indoleacetic acid oxidase (2). It was demonstrated that the IAA was destroyed by oxidation which fact was thought to be related to the manner in which the chemical induced certain plant responses. It was felt that since the indoleacetic acid oxidase was a riboflavin-containing material, that this system would be light sensitive and hence on unilateral illumination of a plant stem, a greater amount of the indoleacetic acid would be destroyed on the lighted side. With destruction of the IAA, a loss of stimulation of cellular activity would be suffered with greater growth occurring on the dark side causing a bending of the stem. In addition to undergoing oxidation, it was found that IAA may also be conjugated to amino acids and proteins of the plant. Andreae (1) has been able to isolate free indoleacetyl aspartic acid from plants exposed to IAA.

It was only natural that considerable attention should be devoted to the mode of action of 2,4-D. In the course of such studies, it was found that this compound underwent modification after absorption by the plant. This modification could clearly be demonstrated to be the result of the action of various metabolic activities by the plant. For example, Holley and his co-workers (11,12) found a new derivative of 2,4-D following administration to a plant which he indicates is a hydroxylated form of the parent compound. Additional metabolism of 2,4-D by the plant was demonstrated by Jaworski (13) in finding that 2,4-D is conjugated to peptides. These conjugates were found to be biologically inactive when isolated and then applied to a plant. More complete breakdown of 2,4-D by the plant's metabolism is illustrated in the findings of Weintraub, et al. (24,25) and Butts and Fang (3,5) that $C^{14}O_2$ was evolved from a plant treated with C^{14} labeled 2,4-D. The rate of evolution of $C^{14}O_2$ varied between the carboxyl and methylene labeled 2,4-D with the greatest evolution occurring in the carboxyl labeled material. While the exact mechanism of the oxidation is not clearly understood, it may be a riboflavin requiring enzyme is involved as has been shown in the case of microorganisms (2). In this instance, metabolism results in cleavage of the ether bond between the ring and side chain giving rise to the corresponding phenol.

Subsequently, it was found that other herbicides are metabolized by plants. For example, monuron had been demonstrated to conjugate in the plant (7). The more recently discovered triazines have been demonstrated by Roth (18) to undergo metabolism in the plant. The U.S.D.A. workers have shown also that 2,2-dichloropropionic acid may enter the plant metabolic system, competing with β alanine (20).

This laboratory has been interested for a number of years in the metabolism of herbicides by plants. This interest stems in part from studies on the mode of action of these compounds and in part from the relationship of this important phenomena to chemical residue problems. Clearly if the compound is being destroyed by the plant, the amount of chemical remaining to serve as a residue is markedly lessened. It has been found that the plant may utilize several metabolic pathways for detoxifying these chemicals. The experimental results will be presented according to the type of metabolism suffered by the compound.

1. Conjugation.

As pointed out earlier, Jaworski (13) working at Oregon State College, discovered that 2,4-D was conjugated to a peptide by plants. Several different

conjugates were found and the amount of the 2,4-D being conjugated into one or more of these materials varied with the species of plants. The more tolerant grass plants produced so called "unknown 3" conjugate in substantial quantity (3).

Another compound found to undergo conjugation was monuron. Like 2,4-D, this material was conjugated to a peptide or a low molecular weight protein. In this instance the C^{14} labeled material was applied to the plant and at varying intervals subsequent to this the plant harvested, sectioned and the radioactivity extracted. This radioactivity was then fractionated by paper chromatography and an attempt made to determine the nature of the radioactive spot on paper (7). Table 1 shows the changes in the concentration of various radioactive components of the plant extract.

Table 1*

Distribution of Major Radioactive Compounds in 80% Ethyl Alcohol Extract of Bean Leaves

(After treatment with 50 γ of carbonyl-C^{14}-labeled CMU. Plants harvested after varying intervals.)

Harvested, Days after treatment	R_f 0.62-0.66, CMU Complex, %	R_f 0.84-0.87, Free CMU, %
1 hour	0	100
1	5	93
2	11	87
4	13	85
8	19	80
12	19	81

2. Hydrolysis.

The hydrolytic enzymes of plants are fully capable of hydrolytic cleavage of bonds in herbicides. Hagen et. al. (10) early demonstrated the ability of plant lipase to hydrolyze the ester of 2,4-D. Recently very good evidence has been brought forth to show that the various esters of 2,4-D are rapidly hydrolyzed in the plant and that the active compound is, therefore, 2,4-D acid. It would be expected similarly, that other ester-type compounds such as the carbamates might probably undergo hydrolysis in the plant. Such is the case with EPTC which we have recently been able to show does undergo hydrolysis according to the following reaction:

$$(C_3H_7)_2\,N - \overset{\overset{O}{\|}}{C} - S\,C_2H_5 + H_2O \xrightarrow{\text{Enzymes}} (C_3H_7)_2\,NH + C_2H_5\,SH + CO_2$$

*From Fang, Freed, et. al., J. Ag. Food Chem., 3, 400, 1955.

The components of the foregoing reaction may further be metabolized as is shown by the distribution of S^{35} of S^{35} EPTC, Fang and Theisen (6).

3. Oxidation.

Of particular interest is the ability of plants to metabolize herbicides via oxidation. By this means the compound may be radically altered or completely destroyed by the plants metabolism. In view of the complexity of the plants enzyme system it is not surprising to find that they possess the ability to oxidize herbicides nor is it startling to find that the carbon dioxide arising from this oxidation is further incorporated into plant constituents.

One of the first compounds coming under investigation was endothal. This compound is an effective pre-emergence herbicide for the chenopod crops such as sugar beets and spinach. Their tolerance for this compound is well known and it was thought possible that these plants might possess the ability to metabolize the material giving rise at least in part to the observed tolerance. Accordingly experiments were undertaken using C^{14}- labeled endothal to determine precisely the nature of this relationship.

Beets were seeded in soil and a pre-emergence application of radioendothal made to the soil. The plants after emergence were harvested at intervals and the amount of radioactivity in the plant determined. It was discovered that an appreciable amount of radioactivity could be detected in the plant.

It was not known from the measurement of total radioactivity in the plant whether or not this existed in the form of the parent radioendothal or whether it represented products arising from metabolic attack by either the plant or microorganisms of the soils. Accordingly attempts were made to fractionate the radioactivity by solvent extraction. This was accomplished by taking dried plant tissue and solvent extracting the material, first with ether and then with alcohol. It was demonstrated that the nonpolar solvent, diethyl ether was incapable of extracting endothal whereas the alcohol was an efficient solvent for this purpose. The following table shows a distribution of radioactivity in the different fractions.

Table 2

Crop	% of Total Radioactivity in Ether Extract	Ethanol Extract	Tissue Residue
Beets	6.8	33.5	59.7
Spinach	8.9	29.7	61.4

Table 3
Ion Exchange Chromatography of Alcohol Extracts of Beets and Spinach

	Beets #1 CPM/0.5 ml	Beets #2 CPM/0.5 ml	Spinach CPM/0.5 ml	Radioendothal CPM/0.5 ml
Before column	13.2	13.0	15.6	53.5
After column	14.5	11.6	14.1	0.9

10.

It will be noted that while an appreciable amount of radioactivity exists in fractions, other than that which could be endothal, the significant amount of C^{14} is found in the alcohol extract. In order to learn more about the nature of the compounds containing C^{14} in the alcohol extract, it was decided to attempt to fractionate the radioactivity of this extract using ion exchange columns. The anion exchange columns such as Dowex-1 were found to adsorb endothal quantitatively. Upon passing the radioactive extract through such ion exchange columns, it was found that a considerable quantity of C^{14} passed through the column indicating that it was no longer in the form of endothal. This is shown in Table 3. Chemical tests on the effluent of these columns indicated that the radioactivity was in the form of carbohydrates.

Undoubtedly a considerable amount of the radioendothal was decomposed by soil microorganisms. However, it is felt that the plant itself is exposed to some radioendothal which was probably metabolized by the plant and the radioactive carbon arising from this metabolism incorporated into plant constituents. Of particular importance to us at this juncture is the fact that this study provided us with effective techniques for studies on other compounds.

A compound of rather recent introduction that is showing considerable promise as pre-emergence herbicide is amiben (2,5-dichloro-3-aminobenzoic acid). This material is particularly effective for weed control in soybeans as well as other crops. The stability of the compound suggests that as a matter of necessity the plant must be exposed to the chemical after germination and emergence from the soil. An attempt was made to determine whether or not amiben was oxidized by soybeans. This was accomplished by exposing a soybean plant to a solution of carboxyl C^{14}-labeled amiben in a closed system that permitted the trapping of the CO_2 evolved in sodium hydroxide. This system consists essentially of a train through which air is passed. The train having a wash bottle of sodium hydroxide to remove the carbon dioxide of the air and this CO_2-free air is then passed through the chamber containing the plant with its roots immersed in the nutrient solution containing the labeled chemical. The air is then swept from the chamber to another wash bottle containing sodium hydroxide which traps the CO_2 evolved in the plants respiration. The carbonate produced by the plant and trapped in the sodium hydroxide is then precipitated with barium chloride and plated and counted.

In performing this experiment with carboxyl C^{14}-labeled amiben and soybeans, it was found that a measurable amount of $C^{14}O_2$ was produced. In attempts to localize or to determine which portion of the plant was responsible for production of this radioactive CO_2, the plant was separated into tops and roots and given exposure. By this means it was clearly demonstrated that the oxidation occurred in the roots of the plant.

Another compound of considerable interest is amitrole. This material has proved to be highly effective for the control of many weeds and while active on a wide spectrum of plants, does show a few instances of interesting selectivity between plants. One such case of this selectivity is the surprising tolerance of certain varieties of oats to low levels of application of amitrole. In contrast the other small grains are decidedly sensitive. In the course of studying the amount of amitrole in the different small grain plants, it occurred to us to determine the difference in oxidative pattern between two of the small

rains. We early found that the oat plant possessed a marked ability to evolve $^{14}O_2$ following exposure to carbon 14 labeled amitrole. Figure 1 presents a clear demonstration of the difference in the abilities of the two small grains, oats and barley, to metabolize this compound. The oat plant clearly is able to continue metabolism of the compound whereas the other small grain showing an initial ability to metabolize it rapidly loses this ability perhaps largely through inhibition.

The compound 4(2,4-dichlorophenoxy) butyric acid (2,4-DB) is of particular interest from the standpoint of metabolism. The Boyce Thompson workers had shown that only the phenoxy alkyl acids containing an even number of carbon atom chains group gave rise to an active compound probably through Knoops beta oxidation. Wain and his co-workers (2,22) made brilliant application of this theory and were able to show by means of paper chromatography that 2,4-D did arise in plants exposed to 2,4-DB. The various intermediates that arise through the beta oxidation of phenoxybutyric acids have been isolated from cultures of microorganisms exposed to this chemical (23). However, as yet no one has isolated a system of enzymes from the plant and shown that they are capable of oxidizing this material.

In the course of studies with this compound, it became of interest to us to attempt to prepare a system of enzymes that could be tested for their ability to oxidize 2,4-DB. Accordingly, a number of attempts were made to isolate some such system. It was discovered that an acetone powder preparation from Laxton Progress peas could be used as a source of soluble enzymes that appeared to possess the ability to oxidize this chemical. It was reasoned that since TPN was reduced in the course of fat oxidation, that it should be possible to couple this reduction to a dye and thus enable us to follow the course of the reaction. The tetrazolium compounds are particularly good to couple to such a system since they accept electrons and hydrogen from DPN and TPN, and in so doing form colored compounds. Upon testing this theory, it was found to work admirably.

Utilizing the enzyme system described above with suitable cofactors, it was possible to demonstrate that it used 2,4-DB as a substrate. In fact 2,4-DB proved to be a more satisfactory substrate than did butyrate. The following table presents these findings.

Table 4

The Oxidation of Substrates by Enzyme Preparation from

Chemical	Concentration/Tube	Sample/Std.
2,4-DB	10 γ	1.34
2,4-DB	10 γ	1.66
Butyrate	10 γ	1.10
2,4-D	10 γ	0.45

When the enzyme system was incubated with radioactive 2,4-DB a number of radioactive compounds were produced. This was demonstrated by means of paper chromatography where a time course study demonstrated that the R_f value of the bulk of the radioactivity was changing. In addition, there was a

tendency for the radioactivity to become smeared out over the paper indicating the development of a number of radioactive compounds.

The triazine herbicides have been of particular interest in the study of metabolism of herbicides by plants. These recently developed materials (9) have shown some amazing differences in selectivity between plants. The selectivity of simazine and atrazine for corn despite their broad activity against other grasses is especially striking. The tolerance of the corn plant for simazine and atrazine can be accounted for on one of three basis: (a) the compound is not taken up by the corn plant, (b) the enzyme systems of the plant are unaffected by the presence of the chemical, or (c) the plant is able to metabolize the compound to innocuous products. Shortly after the selectivity of simazine and atrazine toward corn was demonstrated, we undertook to study this phenomena using C^{14}-labeled compounds.

The very early experiments clearly indicated, by the presence of C^{14} in the sample, that the plant was capable of taking up readily detectable amounts of simazine and atrazine. Of course it would be argued that the C^{14}-labeled material absorbed by the plant was not simazine or atrazine but something to which they had been converted by soil microorganisms. However, chromatography of soil extracts demonstrated the presence of large quantities of the parent triazine herbicide. Clearly then, at least one of the C^{14}-containing compounds taken up by the corn was the parent triazine.

The question then arose as to whether the C^{14} found in the plant represented the parent herbicide or whether this herbicide had been altered by the plants metabolism. Since it was known that simazine and atrazine could be quantitatively extracted from plant tissue with chloroform samples of the plant tissue were taken at time intervals and exhaustively extracted with chloroform. The following table presents the results of this study.

Table 5
The Percentage of Total C^{14} in Treated Corn which was Chloroform Extractable

	Simazin		Atrazine	
Rate lb/A	Days following treatment	% C^{14} chloroform extractable	Days following treatment	% C^{14} chloroform extractable
2	49	41.0	21	83
8		42.4		89
2	91	33.8	56	75.4
8		39.8		71.2
2	119	18.1	113	72.3
8		28.7		54.9
2	119, ears	53.8	113, ears	48.8
8		39.4		59.6

The fact that there was a change in the percentage of the total radioactivity in the plant extractable with chloroform gave evidence that these compounds were being altered by the plant's metabolism. Clearly it would not be unreasonable to

assume that the C^{14} extracted by chloroform would not all necessarily be in the form of the parent triazine. In this instance, the concentration of chemicals in the chloroform is very low so that even compounds normally considered insoluble in this solvent could appear at these low concentrations. An attempt was made, therefore, to further fractionate this radioactivity to determine whether or not a C^{14} in the chloroform represented the parent compound or metabolic products of the parent compound. It was found that the nitrogen of the alkylamino substituent of simazine and atrazine was sufficiently basic so that these compounds could be chromatographed on a cation exchange resin from a chloroform solution. Thus, it was possible to further fractionate the radioactivity by adsorption on Dowex-50. When this was done, it was clearly demonstrated that the C^{14} in the chloroform extract was not all in the form of parent triazine. This is illustrated in the next table.

Table 6

Adsorption of C^{14} of Atrazine or Chloroform Extracts of Plants by Dowex-50 Column.

Solution	Atrazine rate/A	Days following treatment	cpm/0.5 ml Before	cpm/0.5 ml After	% Retained
Atrazine	---	---	374	3.3	99.1
Plant extract	2	119	221.7	6.3	72.2
Plant extract	8	119	207.7	28.1	86.5
Extract of ear	8	119	61.8	32.0	48.2

If the triazine is being degraded by the plant's metabolic activities, the question arises as to how far this degradation is carried. If the degradation is complete, then it would be expected that for radioactivity carbon dioxide should be evolved from the plant. In order to test whether or not this was the case, the closed chamber metabolism experiment as described previously was attempted. In this case, corn plants were germinated in sand and then taken as the test plant. Table 7 clearly demonstrates that the plant is capable of complete degradation of simazine and atrazine.

Table 7

Metabolism of C^{14} Simazin and Atrazine by Corn Plants as Measured by the Release of $C^{14}O_2$

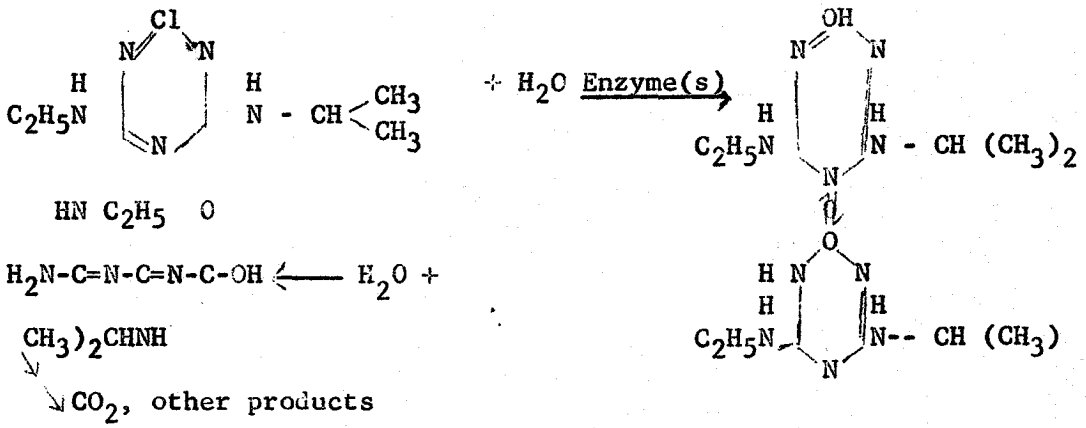
Experiment	Duration hours	µg Triazine metabolized	µg Triazine remaining in plant	% Triazine metabolized of that taken up
Simazine 1	50	.48		
2	48	.19	2.0	8.7
3 (in dark)	68	.91	.53	63.2
Atrazine 1	54	.67	3.05	18.0
2	72	.30	1.49	16.8
3 (in dark)	70	.28	.73	27.7

Since the corn plant is clearly capable of complete oxidation of the triazines as shown above, it now becomes of interest to determine by what mechanism this is accomplished. This was investigated using expressed sap of corn plants to ascertain whether or not the reaction was the result of metabolic activities.

Roth (19) had speculated as a result of his studies on this metabolism that certain polyphenol substances which he found in the sap were the agents responsible for this metabolism. Gysin (8) had suggested from chemical considerations that the first product of metabolism would most likely be the hydroxy triazine. To investigate this possibility, the expressed sap of corn buffered to an appropriate pH was used to incubate with the solution of atrazine. One sample of the juice was boiled to destroy any enzymes that might be present; the other was used fresh. After an appropriate incubation period, the water phase was extracted with chloroform and the chloroform extract then chromatographed on paper. With the fresh corn sap two radioactive peaks were found, one of which was atrazine, the other which we were able to show was hydroxy atrazine by comparing the R_f of a synthetic sample of this produce to that found in the incubated mixture. With the boiled sap, however, only one peak was found and that corresponded to the original atrazine.

From these studies it was possible to demonstrate that the first product of the metabolic attack on the triazine herbicides was the hydroxy compound. It then became of interest to attempt to follow out this process in order to learn more about subsequent products of metabolism of the hydroxy compound. We speculated that the next step in the breakdown involved the oxidation of the hydroxy compound to a keto form resulting in a structure which would readily undergo hydrolytic cleavage of the ring. Careful study of the infrared adsorption spectra of the hydroxy compound clearly revealed the presence of a carbonyl stretching group at 1680 cm^{-1}. If the hydroxy compound existed only in the OH form, this particular band would not be expected. However, if rearrangement or oxidation occurred on this particular compound, then the carbonyl group would appear producing a structure susceptible to attack by hydrolysis.

It now appears possible to write some of the chemical reactions and structures of the compounds up through breakage of the ring. This is seen in the following reaction scheme.



It will be noted that the structure resulting from the hydrolysis of the ring would be highly unstable and susceptible to further attack giving release to radioactive carbon dioxide and affording products that would be further incorporated into the plant by enzymatic action.

In concluding the presentation of information on metabolism of triazines, it may be of interest to note that considerable variation in the ability of plants to metabolize these compounds has been found. It appears that the certain susceptible plants are much less able to metabolize the triazine than some of those that are resistant. In perennial plants tolerant to these compounds, one does

not find an abundant production of $C^{14}O_2$ but rather an incorporation of the radiocarbon into plant constituents. This was brought home forcibly in the study of the metabolism of the triazine herbicides by trees and shrubby plants. Appreciable quantities of radiocarbon would be found in the leaves but upon extraction with the use of solvents to fractionate the radioactivity, the C^{14} was found to be widely distributed in many fractions.

Similarly differences in the ability of a given plant species to metabolize different triazines was noted. A major difference in rates of $C^{14}O_2$ production by corn when exposed to the different chlorine containing alkylamino triazines has been found.

Summary and Conclusions

Scattered published reports clearly indicate that plants like animals and microorganisms are fully capable of metabolic attack on organic chemicals to which they are exposed in their environment. Again, parallel with animals or microorganisms, the plant may carry out this metabolic attack by one of several means. The metabolism may take the form of a conjugation of the externally applied chemical with proteins or carbohydrates or with simpler compounds such as amino acids and the sulfur-containing amino acids. Similarly the compounds may undergo hydrolysis, hydroxylation and other chemical modifications. Of particular interest in this paper has been the matter of the plant's ability to carry out oxidation of externally applied materials. It has been shown by the experimental work reported that plants possess the ability to oxidize such chemicals as amitrole and amiben, 2,4-DB and the triazine herbicides. The evidence for this oxidation rests on the evolution of the radioactive carbon dioxide and the appearance of the C^{14} in normal plant constituents.

Metabolism of herbicides may be shown to be related to the mode of action of the drug. This comes about in the first instance where an innocuous compound is converted by the plant's metabolism to a compound of high biological potency, thus killing the plant or modifying its growth. In the second instance, the metabolism helps to reduce the toxicity of the compound and may serve in part at least as a basis of the tolerance of the plant toward the chemical. Complete oxidation of the compound to innocuous products is of importance at the practical level in that this serves to reduce the residue of that compound. The initial steps in the sequence of reactions leading to complete oxidation of the triazine herbicides has been partially worked out. The reactions have been shown to be dependent upon enzymes leading to organically unstable molecules.

This preliminary report attempts to summarize some of the information on metabolism of herbicides. It is hoped that this modest effort will serve as a stimulus to additional work in this field and the elucidation of complete mechanisms by which many of the herbicides are metabolized by plants.

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Figure 1. Production of $C^{14}O_2$ from C^{14} Amitrol by Oats and Barley