Use of Isolated Leaf Cells of *Abutilon theophrasti* to Localize the Action of Two Aminotriazinone Herbicidal Derivatives

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The effects of the aminotriazinone herbicides metribuzin [4-amino-6-*tert*-butyl-3-(methylthio)-1,2,4,-triazin-5(4H)one] and metamitron [4-amino-3-methyl-6-phenyl-1,2,4-triazin-5(4H)-one] on the metabolism of enzymatically isolated leaf cells of *Abutilon theophrasti* Medic. were examined. Photosynthesis, protein, ribonucleic acid (RNA), and lipid synthesis were assayed by the incorporation of radioactive substrates such as $NaH^{14}CO_3$, [^{14}C]leucine, [^{14}C]uracil, and [^{14}C]acetate, respectively, into the isolated cells. Photosynthesis was the most sensitive process inhibited by both herbicides, but metribuzin was a more potent inhibitor than metamitron. Protein synthesis was not affected by any herbicide. RNA synthesis was inhibited significantly by both herbicides and may be involved in their ultimate herbicidal action. Lipid synthesis was inhibited significantly only by the high concentrations of metribuzin, and it was not affected by any concentration of metamitron at any incubation time. Inhibition of lipid synthesis may be involved in the action of metribuzin as a herbicide.

Introduction

Almost two decades ago, Dornow *et al.* [1] were the first to report on the synthesis of a new class of heterocyclic compounds, the 4-amino-1,2,4-triazin-5-ones. Following this discovery, numerous studies on the structure-activity relationships of substituted aminotriazinones have demonstrated the herbicidal potential of these compounds [2–6]. In particular, two aminotriazinone derivatives known by the common names metribuzin and metamitron have attracted considerable interest and are used commercially as herbicides. Metribuzin [4-amino-6-*tert*-butyl-3-(methylthio)-1,2,4-triazin-5(4H)-one] is a useful preemergence herbicide for controlling some of the troublesome broadleaved weeds of soybeans [*Glycine max* (L.) Merr.] [7]. Metamitron [4-amino-3-methyl-6-phenyl-1,2,4-triazin-5(4H)-one] is used primarily in Europe as a selective herbicide for controlling many broadleaved and some grass weeds of sugar beets (*Beta vulgaris* L.) following preemergence or postemergence applications [8]. Rapid metabolism of metribuzin by soybeans and of metamitron by sugar beets, leading to the formation of metabolites with reduced phytotoxicity, has been documented in numerous studies as the basis for the observed selectivity of these two herbicides [9–14].

Aminotriazinones are known to be strong photosynthetic inhibitors. Studies with isolated chloroplasts have demonstrated that both metribuzin and metamitron inhibit photosynthesis by blocking the flow of electrons at the reducing side of photosystem II [3, 6, 12, 13]. However, apart from their effect on photosynthesis, the effects of metribuzin and metamitron on nucleic acid synthesis as well as on other physiological processes of plants, such as protein and lipid synthesis, have not been examined and need to be evaluated for their contribution to the ultimate herbicidal action of these compounds.

The purpose of the present study was to determine the primary metabolic site of metribuzin and metamitron by examining the effects of these herbicides on photosynthesis and RNA, protein, and lipid synthesis of enzymatically isolated leaf cells of *Abutilon theophrasti* under various time-course and concentration condition.

Materials and Methods

Plant growth conditions

Seeds of *Abutilon theophrasti* collected from a field located in Blacksburg, Virginia (USA) were planted in greenhouse soil mixture, one seed/473 ml plastic

Abbreviations: MES, [2-(N-morpholino)-ethanesulfonic acid]; HEPES, [N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid].

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cup. The soil mixture was a 2 : 2 : 1 mixture of potting medium (Weblite Corporation, Blue Ridge, Virginia), vermiculite and sphagnum peat moss containing a controlled-release fertilizer (14-14-14) and limestone. The emerged seedlings were grown at 28 klux in a growth chamber with a 16-h photoperiod and 30 C day/20 C night temperature for 3 weeks. Then, the photoperiod of the chamber was changed to 6 hours. A short-day treatment has been reported to be important for high photosynthesis rates of isolated leaf cells of cotton [15] and of soybean [16], since it reduces the amount of starch in chloroplasts. After one week of short-day treatment, mature leaves of *Abutilon theophrasti* were used for enzymatic isolation of mesophyll cells. On the day of cell isolation, mature leaves were detached from the plant after they had been illuminated for at least one hour.

Cell isolation

All procedures were carried out at room temperature. The detached leaves were rinsed with distilled water, blotted, deveined and cut into 1 mm × 1 cm strips with a razor blade. Two or three grams of tissue were then infiltrated under vacuum with 30 ml of infiltration medium until the tissue was fully infiltrated. The infiltration medium for all preparations contained 20 mM MES, pH 5.8, 2% macerase (Calbiochem-Behring Corp., La Jolla, California) and 0.3% potassium dextran sulfate (Calbiochem-Behring Corp., La Jolla, California). After vacuum infiltration, the leaf tissue was filtered through a 242 μm nylon net, the filtrate was discarded and the leaf tissue was transferred to a beaker with 30 ml of a maceration medium containing 20 mM MES, pH 5.8, 2% macerase, 0.3% potassium dextran sulfate and 0.6 M sorbitol. The tissue was stirred slowly on a magnetic stirrer for 10 min. The suspension was again filtered through the nylon net and the filtrate discarded. The leaf tissue remaining was transferred to 30 ml of fresh maceration medium and stirred for a period of 50 to 60 min. The cells released during this period were filtered again through the nylon net and then washed three times with 10 ml aliquots of a wash medium by centrifugation at 80 × *g* for 3 min. The wash medium contained 0.5 M sorbitol, 5 mM KNO_3, 2 mM $Mg(NO_3)_2$, 1 mM $CaCl_2$ and it was buffered with 50 mM HEPES, pH 7.8 for photosynthesis or with 50 mM MES, pH 5.8 for protein, RNA and lipid synthesis assays. After each washing, the supernatant solution was removed by suction and finally the cells were made up to volume with incubation medium. The incubation medium was identical to the wash medium. For the chlorophyll determination, 1 ml of the cell suspension was added to 4 ml of 80% acetone and mixed thoroughly. The supernatant fluid was then assayed spectrophotometrically for its chlorophyll content according to the method of Arnon [17]. The chlorophyll content of the cell preparations used in this study varied from 40 to 80 μg of chlorophyll per ml of the assay medium.

Time-course and concentration studies with the two triazinones

The assaying medium for all metabolic studies contained 2 ml of the cell preparation in a 25 ml Erlenmeyer flask, 0.1 ml of radioactive substrate containing 1 μCi of radioactivity and 0.05 ml of the herbicide solution, making a final volume of 2.15 ml. The radioactive substrates used include $NaH^{14}CO_3$ (sp. act. 44.4 mCi/mM) for photosynthesis, L[U-^{14}C]leucine (sp. act. 290 mCi/mM) for protein synthesis, [2-^{14}C]uracil (sp. act. 55 mCi/mM) for ribonucleic acid (RNA) synthesis, and [1,2-^{14}C]acetic acid (sp. act. 56.2. mCi/mM) for lipid synthesis. All radioactive substrates were purchased from ICN, Chemical and Radioisotope Division, Irvine, California. Analytical grade standards (100% pure) of metribuzin and metamitron were generously provided by Dr. W. Draber, Forschungszentrum Wuppertal, Bayer AG (Bundesrepublik Deutschland). Metribuzin and metamitron were dissolved in methanol and made up to volume with distilled water so that the final methanol concentration was less than 1%. Herbicide concentrations of 0.1, 1, 10 and 100 μM were used in all assays. The Erlenmeyer flasks with the assay mixtures were sealed and placed in a shaking water bath at 25 °C. The flasks were illuminated from above with a combination of flourescent and incandescent lamps with 7.4 W/m² at the level of the flasks. The assay mixtures were incubated for 30, 60 and 120 min. At the end of each incubation period, samples were collected and treated accordingly for each metabolic process. A detailed description of the specific procedures followed for the treatment of the collected samples has been reported elsewhere [18]. The radioactivity of the treated samples was determined by radioassay with a

Beckman LS-250 liquid scintillation spectrometer having a counting efficiency of more than 90%. Photosynthesis was calculated as μmol CO_2/mg Chl · h, whereas protein, RNA and lipid synthesis were calculated as counts per minute (cpm) of the [^{14}C] from the respective radioactive substrates incorporated into the cells per 100 μg of chlorophyll. The results were also calculated as percent inhibition caused by each concentration of the two triazinone herbicides.

All assays were replicated three times, and the data were analyzed for variance in a completely randomized design with a factorial arrangement of the treatments. Duncan's Multiple Range Test was used to separate the treatment means.

Results and Discussion

The effects of the aminotriazinone herbicides metribuzin and metamitron on photosynthesis, protein, RNA and lipid synthesis of isolated leaf cells of *Abutilon theophrasti* are presented in Tables I through IV. Photosynthesis as measured by the fixation of $^{14}CO_2$ by the isolated leaf cells of *Abutilon theophrasti* was inhibited very strongly by metribuzin at 1, 10 and 100 μm (Table I). The respective inhibitory percentages (80, 91 and 90%) caused by these concentrations of metribuzin were observed as early as 30 min of incubation time and remained unchanged up to 120 min, the maximum exposure time examined in this study. Thus inhibition of photosynthesis by metribuzin was very rapid. Metribuzin at 0.1 μM did not inhibit photosynthesis significantly at any incubation period examined. In earlier investigations assaying the effect of metribuzin on the Hill reaction of photosynthesis in the presence of isolated spinach chloroplasts, the calculated pI_{50} values (negative logarithm of the concentration causing 50% inhibition) varied from 6.63 [4, 12] to 6.7 [5]. Thus, according to the results of those studies, metribuzin at 0.2 μM caused 50% inhibition of photosynthesis. In this study, metribuzin at 0.1 μM did not inhibit photosynthesis at any incubation time period examined. The lack of any inhibition of photosynthesis by the lowest concentration of metribuzin could be probably attributed to influences of biological barriers on the penetration of this herbicide that are more critical in studies using isolated plant cells as opposed to those using isolated chloroplasts.

Inhibition of photosynthesis of isolated leaf cells of *Abutilon theophrasti* by the two highest concentrations of metamitron (10 and 100 μM) was strong and rapid, reaching maximum levels as early as 30 min of incubation time. The percentage value of photosynthesis inhibition by metamitron at 100 μM was comparable to that caused by the same concentration of metribuzin (Table I). However, inhibition of photosynthesis by metamitron at 10 μM was lower than that caused by metribuzin at the same concentration, indicating that metamitron was a less potent inhibitor of photosynthesis than metribuzin at concentrations lower than 100 μM (Table I). These results are in agreement with findings of other studies reporting that metribuzin had higher pI_{50} values than metamitron and, therefore, it was a more potent inhibitor of photosynthesis than metamitron [4, 12, 13]. After 120 min of incubation, metamitron at 0.1 or 1 μM inhibited photosynthesis of leaf cells of *Abutilon theophrasti* significantly, but not very strongly, reaching values of only 20%. Thus, inhibition of photosynthesis by the two lowest concentration of metamitron appeared to be progressive in na-

Table I. The effect of metribuzin and metamitron on $^{14}CO_2$ fixation by isolated leaf cells of *Abutilon theophrasti*.

Herbicide concentration	Metribuzin		Metamitron	
[μM]	$^{14}CO_2$ Fixation [μmol CO_2/mg Chl/h]	Inhibition [%]	$^{14}CO_2$ Fixation [μmol CO_2/mg Chl/h]	Inhibition [%]
0	18.4 a	0	20.4 a	0
0.1	17.3 a	6	19.5 a	4
1	3.7 c	80	19.5 a	4
10	1.6 c	91	7.6 c	63
100	1.9 c	90	2.6 d	87

[a] Means within columns with similar letters are not significantly different at the 5% level by Duncan's Multiple Range Test.

Table II. The effect of metribuzin and metamitron on protein synthesis of isolated leaf cells of *Abutilon theophrasti* after a 2-h incubation period [a].

Herbicide concentration	Metribuzin		Metamitron	
	[^{14}C]Leucine incorporated	Inhibition[b]	[^{14}C]Leucine incorporated	Inhibition[b]
[μM]	[cpm/100 μg Chl]	(%)	[cpm/100 μg Chl]	(%)
0	75.937 a	0	73.720 a	0
0.1	80.430 a	− 6	76.587 a	− 4
1	84.294 a	−11	82.928 a	−10
10	75.500 a	1	73.907 a	0
100	73.329 a	4	72.029 a	2

[a] Means within columns with similar letters are not significantly different at the 5% level by Duncan's Multiple Range Test.
[b] A minus (−) sign in front of a percentage value indicates stimulation instead of inhibition.

ture, indicating that longer incubation periods may be needed for stronger inhibition to be obtained. The progressive character of the inhibition of photosynthesis by metamitron has been also reported in a recent study by van Oorschot and van Leeuwen [19]. They found that following treatment with metamitron, photosynthesis of susceptible plants such as beans (*Phaseolus vulgaris* L.) continued to decrease to a constant level, while photosynthesis of tolerant sugar beet (*Beta vulgaris* L.) plants was restored completely within a few hours as a result of rapid detoxication of this herbicide by sugar beets [19].

Protein synthesis of isolated leaf cells of *Abutilon theophrasti* was not affected by any herbicide, even at the maximum concentration of 100 μM and maximum exposure time of 120 min (Table II). Some stimulation of protein synthesis by the two lowest concentrations of both herbicides was observed at all incubation periods (Table II). However, these stimulations of protein synthesis were not statistically significant. Some of the most potent photosynthetic inhibitors that are used as herbicides such as the *s*-triazines and the substituted ureas have been reported to cause a slight inhibition of protein synthesis at

Table III. The effect of metribuzin and metamitron on RNA synthesis of isolated leaf cells of *Abutilon theophrasti* [a].

Incubation time	Metribuzin	[^{14}C]Uracil incorporated	Inhibition[b]	Metamitron	[^{14}C]Uracil incorporated	Inhibition
[min]	[μM]	(cpm/100 μg Chl]	[%]	[μM]	[cpm/100 μg Chl]	[%]
30	0	10.712 def	0	0	16.378 cd	0
	0.1	11.146 def	−4	0.1	15.438 cd	6
	1	8.910 efg	17	1	15.230 cd	7
	10	7.697 gh	28	10	10.825 e	34
	100	3.987 h	63	100	8.631 e	47
60	0	20.423 b	0	0	30.226 b	0
	0.1	19.458 b	5	0.1	29.016 bc	4
	1	12.866 cde	37	1	27.505 bc	9
	10	10.415 def	51	10	18.135 cd	40
	100	5.514 gh	73	100	12.997 de	57
120	0	37.845 a	0	0	58.467 a	0
	0.1	36.709 a	3	0.1	58.503 a	0
	1	15.895 bc	58	1	54.959 a	6
	10	14.002 cd	63	10	27.479 bc	53
	100	7.569 gh	80	100	18.125 cd	69

[a] Means within columns with similar letters are not significantly different at the 5% level by Duncan's Multiple Range Test.
[b] A minus (−) sign in front of a percentage value indicates stimulation instead of inhibition.

high concentrations [20, 22]. However, this effect of these compounds on protein synthesis has not been considered as playing an important role in the herbicidal action of these compounds. Similarly, the results of this study show that inhibition of protein synthesis is not involved in the herbicidal action of the aminotriazinone herbicides.

Significant inhibition of RNA synthesis as measured by [^{14}C]uracil incorporation into isolated cells of *Abutilon theophrasti* was observed with metribuzin at 10 and 100 μM at any incubation period and with 1 μM after 60 and 120 min of incubation (Table III). Metribuzin at 0.1 μM did not inhibit RNA synthesis significantly at any incubation period. Significant inhibition of RNA synthesis was also observed by the two highest concentrations of metamitron at any exposure time (Table III). Moreland and colleagues [21, 22] have pointed out that interference of herbicides with plant nucleic acid metabolism could be either a direct effect of the herbicides on the biosynthetic pathways involved in nucleic acid synthesis or an indirect effect caused by their primary action on cellular energy production. Inhibition of RNA synthesis by both metribuzin and metamitron was progressive and increased in magnitude as incubation time increased (Table III). Furthermore, significant inhibition of RNA synthesis was caused only by the concentrations of metribuzin and metamitron that caused significant inhibition of photosynthesis and the inhibition percentage values were lower than those observed for photosynthesis. Therefore, it could be suggested that the effect of these two aminotriazinones on RNA synthesis is not direct, but an indirect one expressed through their effect on photosynthesis which provides most of the cellular energy (ATP) needed for this biosynthetic reaction. However, a direct effect of these herbicides on RNA synthesis can not be excluded without further experimentation. The structural similarity of aminotriazinones and of their degradation products in plants to precursor bases of nucleic acids would seem to support the hypothesis of a direct effect of these chemicals on RNA synthesis. In particular, the similarity of the deaminated diketo metabolite of metribuzin (DADK) to thymine (2-methyl-2,4-dioxo-pyrimidine) is very close, and the possible transformation of this metabolite to its respecctive nucelotide could lead to disturbed nucleic acid synthesis in susceptible plants and needs to be examined. Studies based on the structural similarity of the *s*-triazines and of their metabolites to precursor bases of nucleic acids and designed to examine the interference of these chemicals with the nucleic acid metabolism of susceptible plants have been reported [23, 24, 25].

Lipid synthesis of isolated leaf cells of *Abutilon theoprasti* was inhibited strongly by the high concentrations of metribuzin (10 and 100 μM) as early as 30 min of incubation time (Table IV). Lipid synthesis of isolated cells of *Abutilon theophrasti* was not affected significantly by any concentration of metamitron at any incubation period examined (Table IV). Interference of herbicidal photosynthetic inhibitors with lipid synthesis has been also considered as an indirect effect caused by their primary effect on photosynthesis [22], and this may be the case for the observed inhibition of lipid synthesis by metribuzin. However, since inhibition of lipid synthesis by metribuzin at 10 and 100 μM was very strong and rapid, reaching high percentage inhibition values

Table IV. The effect of metribuzin and metamitron on lipid synthesis of isolated leaf cells of *Abutilon theophrasti* after a 30 min incubation period[a].

Herbicide concentration	Metribuzin		Metamitron	
[μM]	[^{14}C]Acetate incorporated [cpm/100 ∝g Chl]	Inhibition[b] [%]	[^{14}C]Acetate incorporated [cpm/100 μg Chl]	Inhibition [%]
0	149.370 a	0	148.300 a	0
0.1	150.929 a	−1	148.225 a	0
1	152.691 a	−2	145.060 a	2
10	32.409 c	78	139.746 a	6
100	21.408 c	86	124.831 a	16

[a] Means within columns with similar letters are not significantly different at the 5% level by Duncan's Multiple Range Test.
[b] A minus (−) sign in front of a percentage value indicates stimulation instead of inhibition.

comparable to those observed for photosynthesis, it can be suggested that inhibition of lipid synthesis by metribuzin may be independent of its effect on photosynthesis. The lack of any inhibition of lipid synthesis by metamitron would appear to support this suggestion. However, further experimental work is needed to support the validity of this suggestion.

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