Uptake and Metabolism of *N,N*-Dimethyl-2,2-diphenylacetamide in Resistant and Susceptible Plants

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The uptake and metabolism of *N,N*-dimethyl-2,2-diphenylacetamide (diphenamid) was studied in a plant species resistant to diphenamid (tomato) and a susceptible one (wheat). Plants were grown for 24, 48, and 120 hr in nutrient solution containing ^{14}C-diphenamid. On a per gram of fresh weight basis, intact tomato plants took up more herbicide than wheat plants during the 120-hr period. The percentage of diphenamid in extracts of wheat and tomato plants decreased from the 24 to 120-hr harvest. The quantity of diphenamid increased in extracts of wheat shoots but remained constant in tomato shoot extracts during the 120-hr period. Metabolites found in both plants included *N*-methyl-2,2-diphenylacetamide, 2,2-diphenylacetamide, and one unidentified compound. Extracts from whole tomato plants contained a glucose-diphenamid complex. Neither diphenamid nor its metabolites inhibited germination of wheat or tomato seeds, but 1.0 ppm of diphenamid inhibited elongation of wheat seedling roots and shoots.

Diphenamid (*N,N*-dimethyl-2,2-diphenylacetamide) is an herbicide applied preemergence and it is used to control a wide variety of annual grasses and broadleaf weeds in tomatoes, peppers, strawberries, and other crops. The mode of action of diphenamid has not been determined. Mann *et al.* (1965) found that the herbicide did not significantly inhibit protein synthesis in seedlings of barley or hypocotyls of Sesbania. Ashton *et al.* (1968) showed that diphenamid did not significantly affect the proteolytic activity of squash seedling cotyledons. On the basis of his studies with tomatoes, Lemin (1966) stated that the mechanism of resistance was due to *N*-demethylation of diphenamid. Golab *et al.* (1966) reported that the major metabolite in strawberries was also *N*-methyl-2,2-diphenylacetamide.

A short term preliminary study in this laboratory (Schultz and Tweedy, 1969) had shown that resistant and susceptible plants were able to *N*-demethylate diphenamid. This paper reports a study of the metabolism of diphenamid in resistant (tomato) and susceptible (wheat) plants.

MATERIALS AND METHODS

Plant Material. Wheat, *Triticum aestivum* (L.) variety Arthur, and tomato, *Lycopersicon esculentum* (Mill.) variety Pink Mozark x Tomboy, F_1, were used for these studies and they are susceptible and resistant to diphenamid, respectively. For degradation studies, the plants were propagated in sand in a growth chamber. Plants were watered every other day with half-strength Hoagland's nutrient solution.

For the germination and root tissue culture studies, the plants were germinated and grown in the dark on moistened filter paper in Petri dishes (60-mm diameter). The dishes were placed in an incubator at 24° C ± 1° C.

Chemicals. ^{14}C diphenamid (MMDA) (carbonyl labeled) having a specific activity of 10.70 μCi per mg was used in this study. The following ^{14}C-carbonyl-labeled compounds were also used: *N*-methyl-2,2-diphenylacetamide (MDA), specific activity, 15.04 μCi per mg; 2,2-diphenylacetamide (DA), specific activity, 15.34 μCi per mg and 2,2-diphenylacetic acid (DAA), specific activity, 3.65 μCi per mg. Each compound was judged to be radiochemically pure on the basis of thin-layer chromatography (tlc) and radioautography.

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Chromatography and Radioautography. Separation of MMDA and its degradation products on tlc was accomplished on 20- × 20-cm glass plates coated with a 0.25-mm layer of silica gel GF_{254} (Brinkmann Instrument, Inc., Westbury, N.Y.) or Eastman Chromagram sheets No. 6060 (Eastman Kodak Co., Rochester, N.Y.). The chromatograms were developed twice in benzene:diethylamine, (95 to 5) (System II) (Golab *et al.*, 1966). The position of MMDA and degradation products on these chromatograms was detected by their fluorescence-quenching under short wavelength ultraviolet light. The following R_f values were observed: MMDA, 0.77; MDA, 0.64; DA, 0.29; and DAA, 0.00.

Radioactive areas on the developed chromatograms were located by placing the plates in contact with No-Screen X-ray film (Eastman Kodak Co., Rochester, N.Y.). The exposure time before development was 7 days.

Estimation of Radioactivity. A liquid scintillation spectrometer was used to quantify the radioactivity. The scintillation fluid used for nonaqueous samples was 10 ml of a solution prepared according to Kallman and Furst (1951). Aqueous samples from ambient nutrient solutions or plant homogenates were counted in Bray's solution (Bray, 1960). The amount of quenching was determined by use of an internal standard (^{14}C-toluene) and radioactivity determinations are expressed as dpm.

Germination and Seedling Growth. Ten seeds of wheat or tomato were placed on filter paper in sterile Petri plates. To each dish was added 8 ml of distilled water containing 1, 10, or 100 μg per ml of MMDA, MDA, DA, or DAA. Only water was added to the control. Three dishes were used for each treatment and the experiment was replicated twice. The seeds were incubated at 24° C in the dark for 4 days for wheat and 7 days for tomato. After the respective time periods, the seedlings were removed, percent germination noted, and measurements made of the length of the roots and shoots.

Whole Plant Treatment and Analysis. Wheat seedlings (14 days old) and tomato seedlings (21 days old) were removed from sand cultures, rinsed free of sand, and placed in 100-ml beakers containing 50 ml of sterile half-strength Hoagland's solution. One-half microcurie of methanolic ^{14}C-diphenamid solution was added to each beaker. The beakers were placed on a gyratory shaker for 24, 48, or 120 hr. At the end of each of these time intervals, one-third of the plants were removed from the beakers. The roots were thoroughly rinsed with

distilled water and blotted dry with absorbent paper. The roots were excised from the shoots and both were weighed and either homogenized or frozen immediately.

The plant tissue was homogenized with a Virtis 45 homogenizer for 2 min in 25 to 30 ml of methanol. The resultant slurry was suction-filtered through Whatman No. 1 filter paper. The residue was washed thoroughly with methanol: acetone (1 to 1) until it was free of pigment. The filtrate and wash were combined and the volume determined. Portions (0.1 ml) were removed and radioassayed. The combined filtrate-wash was cleaned for tlc by eluting it under pressure through a 15.0 × 2.3 cm. (i.d.) glass column packed with 10 ml each of sodium sulfate, animal charcoal, and Florisil from top to bottom, respectively. The column was washed with 20 ml of methanol:acetone (1 to 1). The eluate and wash were combined and evaporated under a stream of dry air. The dried residue was dissolved in 2 ml of methanol for subsequent analysis by tlc. Approximately 10,000 dpm of each extract was used for tlc. The radioactive spots were located by radioautography and scraped into scintillation vials for radioassay.

Treatment of Excised Shoot and Subsequent Analysis. Shoots of wheat and tomatoes were excised under water to insure that the transpiration stream was not blocked by air bubbles. Sterile Hoagland's solution was added to the beakers displacing the water until the beakers contained approximately 50 ml of half-strength Hoagland's solution. The remainder of the procedure was the same as described above.

Treatment of Root Tissue Culture and Analysis. Seeds of wheat and tomato were surface sterilized and checked for microbial contamination by incubating in Petri dishes containing semi-solid potato dextrose agar (0.75%). The terminal 10 mm of the root tips was excised with a sterile scalpel and transferred to a 125 ml Erlenmeyer flask containing 50 ml of sterile Whites' medium (White, 1963). The cultures were incubated in the dark at 27° C ± 1° C, ^{14}C-MMDA was added to each flask (0.05 μCi, 1 μg per ml) and 14 days later the roots were removed and extracted as previously described. All tissue culture procedures were carried out under aseptic conditions in a transfer room.

Identification of Metabolites. To obtain sufficient quantities of the metabolites for identification, tomato plants were incubated in nutrient solution containing 10 ppm of pure nonlabeled MMDA and 0.1 ppm of ^{14}C-MMDA. After 5 days the plants were harvested and processed as described above. The extracts were subjected to tlc in System II and radioautography and then eluted from the thin-layer plates for identification by mass spectrometry and cochromatography. MMDA, MDA, Compound I (an unidentified compound, R_f 0.43), and DA were extracted from thin-layer plates with ethyl acetate. The spot which remained at the origin was polar and was therefore eluted with methanol. The polar spot was rechromatographed in benzene: formic acid (95 to 5) (System I) (Golab *et al.*, 1966). The spot which remained at the origin in System I was eluted with methanol, dried *in vacuo*, dissolved in 10 ml of 0.1*N* HCl, and heated at 70° C. After 4 hr, the mixture was extracted three times with 10-ml aliquots of chloroform. The chloroform extract was concentrated almost to dryness and spotted on thin-layer plates with authentic compounds; the plate was developed twice in System II, and a radioautograph was made. The solution remaining after chloroform extraction was placed under vacuum to remove residual chloroform, several aliquots were analyzed for glucose utilizing the Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.). Several aliquots were spotted on Eastman Kodak tlc sheets. The sheets had previously been dipped for 1 min in a solution containing 4 g of sodium bisulfide, 80 ml of water, 120 ml of ethanol, dried and activated for 15 min at 100° C. They were developed for a distance of 15 cm in ethyl acetate: methanol: acetic acid: water (60 to 15 to 15 to 10), dried, sprayed with aniline hydrogen phthalate, and heated at 85° C until spots appeared.

Sample Preparation for Analysis by Mass Spectrometry. Tlc plates were predeveloped in glass-distilled ethyl acetate. A sample containing approximately 2.4 × 10^6 dpm was streaked on each plate. Plates were developed twice in System II, dried, and exposed to X-ray film for 2 days. Areas on the tlc plates containing radioactivity were scraped separately into vials containing 5 ml of ethyl acetate. After 1 day, the ethyl acetate solution was filtered through glass wool, and the eluate was evaporated in a conical tube under a stream of nitrogen. The residue was dissolved in about 50 μl of ethyl acetate and quantitatively transferred to a quartz capillary in successive 10 μl portions, each of which was evaporated under vacuum before the next portion was added. The capillary was then placed in the direct-inlet probe system of the mass spectrometer (Model 270 GC-DF analytical mass spectrometer, Perkin-Elmer, Perkin-Elmer Corp., Norwalk, Conn.).

RESULTS

Seed Germination. None of the compounds tested inhibited the germination of tomato or wheat seeds.

Seedling Inhibition. The growth of wheat roots was severely inhibited by all three concentrations of MMDA (Figure 1). At 10 and 100 ppm herbicide concentrations the roots emerged and elongated to 25 mm, but further growth was inhibited.

Analysis of variance was made on the growth studies. At the 5% level, DA and DAA did not significantly inhibit growth of wheat roots at 1 or 10 ppm. MDA was stimulatory at 1 ppm and significantly inhibitory at 10 ppm. All compounds inhibited growth at 100 ppm at the 5% level. Wheat shoot growth was inhibited by MMDA at all concentrations, but to a lesser extent than were roots. MDA, DA, and DAA were inhibitory at 100 ppm only, and DA was the least inhibitory at this concentration.

Root and shoot growth of tomato was normal in the presence of 1.0 ppm of all compounds (Figure 1). At 10 ppm only MMDA was significantly inhibitory. All compounds inhibited growth of roots and shoots at 100 ppm. At this concentration, MDA was significantly less toxic than the other compounds for both roots and shoots.

Uptake of ^{14}C-MMDA from Nutrient Solution. The data in Table I show the amount of radioactivity recovered at the 24-, 48-, and 120-hr harvests in the homogenates of roots and shoots of intact plants and of excised shoots placed in nutrient solutions containing ^{14}C-MMDA. The amount of radioactivity found in wheat roots differed little between the 24-hr and the 48-hr harvest, whereas the activity increased in tomato roots at the 48-hr harvest. However, the radioactivity in roots of both species decreased by the 120-hr harvest. The decrease of radioactivity in roots at the 120-hr harvest may have been due to "leakage" of materials back into nutrient solution after an equilibrium had been reached, to a change in membrane permeability, or to export of the ^{14}C-label to the shoots in excess of that absorbed by the roots during this final period. It was not due to lack of ^{14}C-

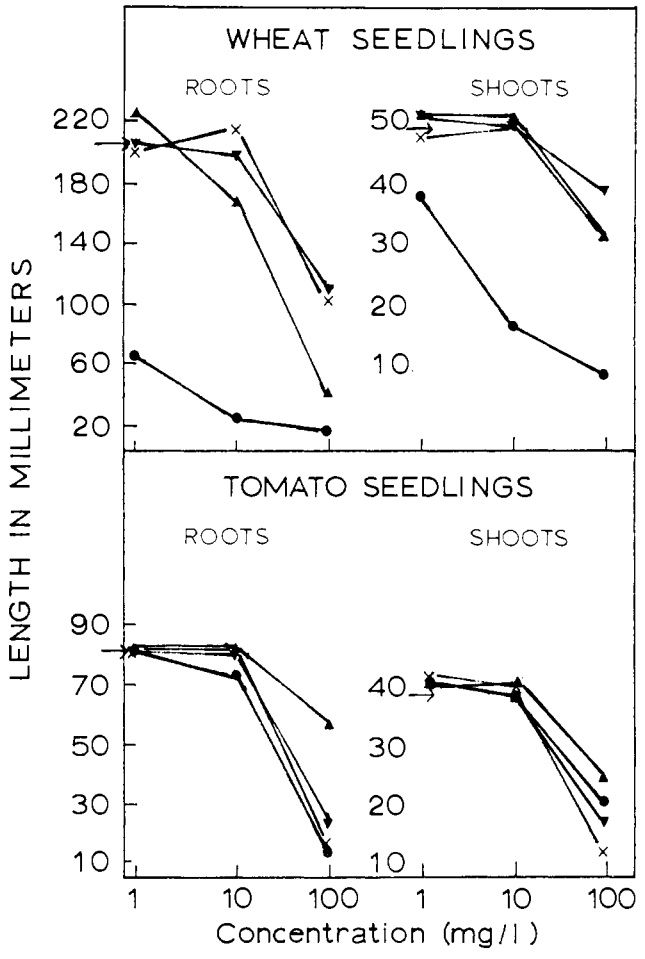


Figure 1. Effect of MMDA, MDA, DA, and DAA on growth of wheat and tomato seedlings roots and shoots. Arrow indicates water controls

MMDA in the nutrient solution, since a similar uptake pattern was observed in other experiments in which 10 ppm unlabeled MMDA along with 0.1 mg of ^{14}C-MMDA had been added to the nutrient solution (Schultz and Tweedy, 1969). Shoots of intact plants and excised shoots of both species accumulated increasing amounts of radioactivity over the 120-hr period.

Identification of Metabolites. MMDA, MDA, and DA were tentatively identified by cochromatography with authentic standards in System II. MMDA was also identified by its mass spectrum (M^+, *m/e* 239). The molecular ions of MDA and DA had such a low relative abundance that they were difficult to detect although high abundance fragment ions at *m/e* 165, 167, and 168 characteristic of these compounds were observed. Rechromatography of MDA and DA along with authentic standards in benzene:methanol (95 to 5) was also used. The following R_f values were observed: MDA, 0.38; DA, 0.24. An unidentified material (Compound I) was found in both wheat and tomato plants. It had an R_f of 0.43 in System II. We are presently attempting to identify this metabolite.

Metabolism of ^{14}C-MMDA in Wheat and Tomato Plants. The extracts from wheat roots contained 81%, 78%, and 76% of the total activity as MMDA after 24, 48, and 120 hr, respectively (Table II). Tomato root extracts contained 79, 77, 76% MMDA after 24, 48, and 120 hr. There was an increase in MDA in the roots of both species in the 48-hr harvest relative to the 24-hr harvest.

Table I. Uptake of ^{14}C-Diphenamid from Nutrient Solution by Wheat and Tomato Plants

Hours Following Treatment	dpm/g Fresh Weight[a] Wheat	Tomato × 1.21
Roots (Intact Plants)		
24	18,800	24,900
48	19,200	27,800
120	9,700	18,400
Shoots (Intact Plants)		
24	12,100	29,900
48	33,900	52,400
120	43,400	87,500
Excised Shoots		
24	29,300	27,300
48	46,800	50,700
120	91,200	80,500

[a] Average of duplicate analyses.

The shoots of intact plants of both species contained a lower percentage of MMDA at the 120-hr harvest than at the 24 and 48-hr harvest. However, the actual amount of MMDA and MDA (based on dpm/g fresh weight) increased in wheat over the 120-hr period, whereas the quantity decreased in tomato shoots from the 48-hr period to 120-hr harvest.

The percentages of MMDA and MDA decreased in excised shoots of wheat and tomato plants between the 24-hr and 120-hr harvest. Both species had increased percentages of DA between the 24-hr and the 120-hr harvest.

The quantities of MMDA and MDA increased in excised wheat shoots between the 24-hr and 120-hr harvests by 76 and 112%, respectively, but only by 29 and 18% in excised tomato shoots. Both species had an increased amount of Compound I and DA between the 24-hr and 120-hr harvests.

Rechromatography of the suspected DAA metabolite in System I showed that this compound was not DAA. In a preliminary experiment which consisted of overlayering the metabolite with concentrated HCl and then redeveloping the tlc plate in System II, the R_f of the radioactive spot changed from 0.00 to 0.64 corresponding to the R_f of MDA. Chloroform extracts of the hydrolyzate chromatographed with and without authentic MDA had an R_f value identical to that of MDA. When portions of the hydrolyzate were neutralized and subjected to the Glucostat reagent (which is specific for β-D-glucose), positive reactions were observed. When subjected to tlc, the nonradioactive portion of the hydrolyzate cochromatographed with glucose (R_f values: glucose–0.30, unknown–0.29, glucose plus unknown–0.29). It was concluded that the metabolite remaining at the origin in tlc solvent systems I and II was probably the *O*-glucoside of *N*-methyl-*N*-hydroxymethyldiphenylacetamide. McMahon and Sullivan (1965) found a metabolite in rat urine which they concluded was the *O*-glucuronide of *N*-methyl-*N*-hydroxymethyldiphenylacetamide. *N*-Hydroxymethyl compounds have been suggested as intermediates in *N*-demethylation reactions (Brodie *et al.*, 1958). The *N*-hydroxymethyl intermediate (or possibly an *N*-hydroxymethyl conjugate) could be converted to the *O*-glucoside, which in turn could be hydrolyzed to

Table II. Diphenamid Metabolism in Wheat and Tomato Plants

Compound	Percent Distribution and dpm of Total ^{14}C Activity Extracted at Hours Indicated[a] 24 Hr Wheat %	24 Hr Wheat dpm[b]	24 Hr Tomato %	24 Hr Tomato dpm	48 Hr Wheat %	48 Hr Wheat dpm	48 Hr Tomato %	48 Hr Tomato dpm	120 Hr Wheat %	120 Hr Wheat dpm	120 Hr Tomato %	120 Hr Tomato dpm
Intact Roots												
MMDA	81	15,200	79	19,700	78	15,000	77	21,400	76	7,400	76	14,000
MDA	9	1,700	12	3,000	11	2,200	15	4,100	18	1,700	8	1,500
Compound I	8	1,500	7	1,700	9	1,700	5	1,500	5	480	10	1,800
DA	0	...	1	240	0	...	...	...	0	...	0	...
MDAG	2	360	1	240	2	360	3	850	1	120	6	1,100
Intact Shoots												
MMDA	50	6,100	47	14,000	46	15,600	29	15,200	41	17,800	16	14,000
MDA	23	2,800	31	9,300	29	9,700	20	10,500	37	16,100	10	8,700
Compound I	25	3,000	5	1,500	21	7,000	9	4,700	18	7,900	16	14,000
DA	1	120	3	850	3	970	3	1,600	3	1,300	6	5,200
MDAG	1	120	14	4,200	1	360	38	19,800	1	480	52	45,500
Excised Shoots												
MMDA	48	14,000	55	15,000	30	14,000	42	21,300	27	24,700	25	19,400
MDA	16	4,700	20	5,400	10	4,700	12	6,100	11	10,000	8	6,400
Compound I	28	8,200	11	3,000	25	11,700	17	8,600	15	13,700	14	11,300
DA	5	1,500	2	600	11	5,100	3	1,600	27	24,700	6	4,800
MDAG	3	850	12	3,300	24	11,300	26	13,200	20	18,300	47	37,900

[a] Average of duplicate analyses. [b] Cpm/g fresh weight. Obtained by multiplying values in Table I by percents in Table II.

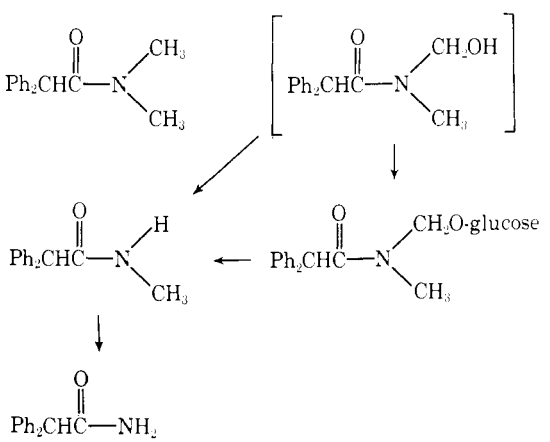
MDA. McMahon and Sullivan (1965) also suggested the presence of an *N*-glucuronide in the metabolism of MDA by rats. However, we did not find any glucoside formation in tomato plants treated with MDA (Schultz and Tweedy, 1969). The formation of a herbicide-glucose complex as a detoxication mechanism has been proposed by other workers (Colby, 1965; Ries *et al.*, 1968; Swanson *et al.*, 1966). The roots of wheat and tomato do not accumulate large amounts of the herbicide-glucose complex (MDAG) (Table II). In shoots of intact wheat plants extracted after 120 hr, only 1% of the extracted radioactivity was MDAG; whereas, in the shoots of intact tomato plants harvested at 120 hr, 53% of the extracted radioactivity was MDAG. MDAG was found in the extracts of excised shoots from both species (Table II).

^{14}C-MMDA Metabolism in Root Tissue Cultures. After 14 days 7.9×10^4 dpm (per g of fresh weight) was recovered from tomato roots. Of this amount, 6% was associated with Compound I, 7% with MDAG, and the remaining 87% with MMDA. A small amount of radioactivity (1.3×10^3 cpm per g fresh weight) was found in wheat root extracts. This radioactivity was probably due to equilibration with the radioactive solution since the roots were flaccid and presumably dead when removed from the nutrient solution. No metabolites were detected in these extracts or those from the controls.

DISCUSSION

The results of the tomato seedling inhibition study are opposed to those reported by Kesner and Ries (1967). They stated that the MDA metabolite inhibits the growth of tomato radicles at 1.0 ppm. In our study, MDA was not significantly different from the control, MMDA, DA, or DAA at 1.0 ppm, significantly less toxic than MMDA at 10 ppm, and significantly less toxic than MMDA, DA, or DAA at 100 ppm. Gentner (1969) also reported that the demethylated analogs of MMDA are less toxic than MMDA itself. Kesner and Ries (1967) reported that MDA is more toxic than MMDA to barnyardgrass seedlings at 1.0 ppm but not at 10 ppm. We found that wheat seedlings are severely inhibited by MMDA at 1.0, 10, and 100 ppm. MDA is inhibitory only at 10 and 100 ppm.

On the basis of this study we propose the following degradation scheme for MMDA in plants.



MDAG was formed in the excised shoots of both species (Table II), hence both species probably contain a glucosidyltransferase system which converts MMDA to a herbicide-glucose conjugate. Since intact wheat plants contained only a small amount of MDAG in comparison to tomatoes, it may be hypothesized that wheat roots contain a glucosidase which hydrolyzes the glucoside immediately or the herbicide may induce production of a glucosidase. Glucosidase production in tomatoes is probably minimal or absent, since intact and excised tomato shoots both contained large quantities of MDAG (Table II). Furthermore, if tomato contained an active glucosidase, a large buildup of MDA would be expected.

This study implies that the toxicity of MMDA to wheat is due to several interrelated factors. The parent compound is

highly toxic to wheat; the degradation product, MDA, is also toxic to wheat; and there is little glucoside accumulation in intact wheat plants. In tomatoes, however, MMDA is not toxic except at very high levels; MDA is also not toxic, and there is a large accumulation of glucoside in tomatoes. Selective uptake as far as quantity cannot be a factor in toxicity, since tomato plants take up more of the herbicide than wheat.

Although the identity of Compound I is still unknown we conclude that the resistance of tomato to MMDA is due primarily to the formation of a stable MMDA-glucose conjugate in tomato plants.

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