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AMINOTRIAZOLYLALANINE: A METABOLIC PRODUCT OF AMINOTRIAZOLE FROM PLANTS

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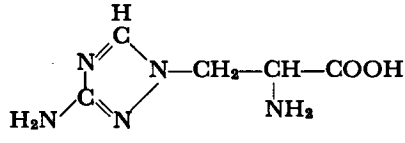
ABSTRACT

β (3-amino-1,2,4-triazolyl-1-) α-alanine is the major metabolic product of 3-amino-1,2,4-triazole in bean and tomato plants. Its preparation from aminotriazole-treated bean plants is described. The metabolite is translocated through both phloem and xylem systems; it is quite stable in the plant. The formation of ATX results in detoxication and is probably the cause of recovery of plants treated with sublethal doses. The relation of the substance to various metabolites of aminotriazole described earlier is discussed.

INTRODUCTION

In the course of an investigation into the uptake and translocation of ^{14}C-labeled 3-amino-1,2,4-triazole (AT) [1]) by plants (MASSINI 1958) it had been noted that extracts of treated plants contained two radioactive compounds besides AT itself. One of these compounds, called ATX, had been isolated from such extracts. An investigation of its chemical properties revealed that it was an alanilyl derivative of AT (MASSINI 1959b). BRAUN (1963) investigated the structure of ATX by means of X-ray diffraction and found that it is β (3-amino-1, 2,4-triazolyl-1-) α-alanine



ATX is optically active: $[\alpha]_D$ $-43°$ in water and $-10°$ in 1 N HCl. A comparison of the shift of optical rotation upon acidification between ATX and similar natural amino acids makes it probable that ATX is the l-form.

Fig. 1 shows the electron density pattern of a crystal of ATX, projected along its c-axis.

In this paper the preparation of ATX and the results of a study on the possible significance of its formation in the plant are reported and discussed.

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[1]) Abbreviations: AT, 3-amino-1,2,4-triazole; ATX, β(3-amino-1,2,4-triazolyl-1-) α-alanine.

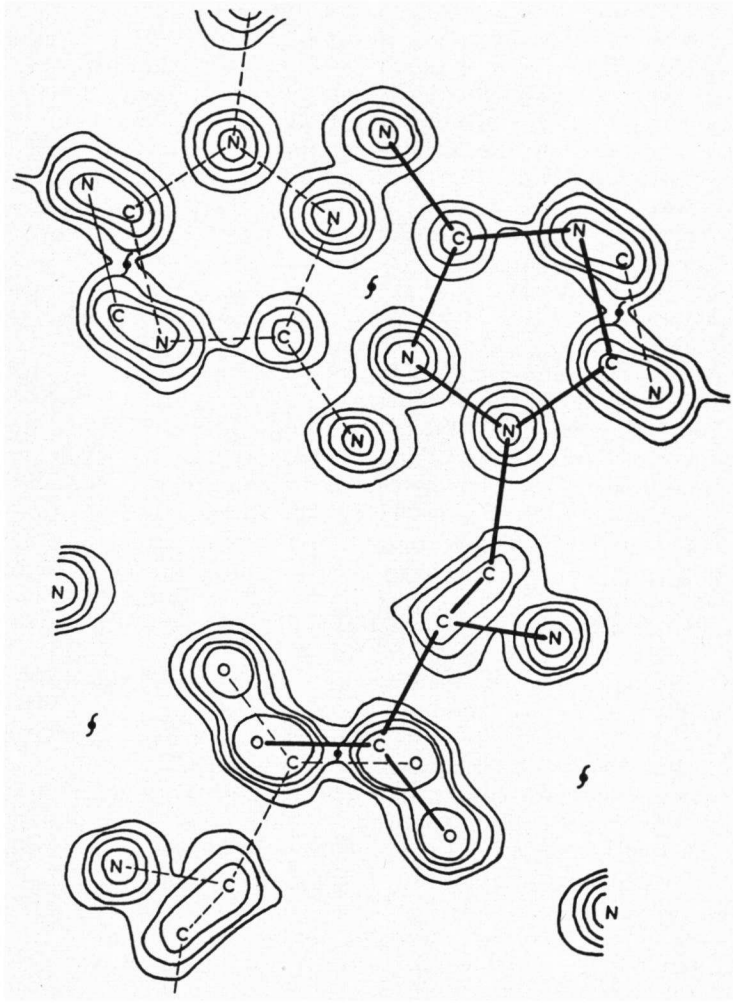

Fig. 1. Electron density pattern of a crystal of ATX. Projection along c-axis of part of the unit cell. Atoms connected by broken lines are at a height of $^1/_2$ of the c-axis above the equivalent atoms of the molecule indicated by full lines. $\S$ = twofold screw axis. (Courtesy P. B. Braun 1963).

Methods

Preparation of ATX: 20 seedlings of French dwarf bean were placed two by two with their cut-off stems in 50 ml of a 0.05 % solution of AT in water. The plants were allowed to take up the solution for 1 week (room temperature, light). They were then frozen in liquid nitrogen, ground and extracted with 200 ml of boiling 70 % ethanol for 1 minute. The homogenate was strained through nylon cloth and the residue extracted twice with 100 ml of water at 50° for 1 hour. The

collected extracts were centrifuged for 1 hour at 500 × g, the supernatant concentrated in vacuo, adjusted to pH 2.0 with HCl and centrifuged during 1 hour at 12000 × g. The supernatant was chromatographed on a column (1.2 × 20 cm) of the cation exchange resin "Amberlite CG 120" (Rohm and Haas Co, Philadelphia, USA). The resin was used in the NH_4^+-form and buffered with ammonium formate buffer of pH 2.1 (0.5 N formic acid + 0.01 N NH_4OH). The column was eluted at a rate of 15 ml/h with the same buffer to which 0.5 N NH_4OH was added gradually, in order to achieve a continuous rise of the pH. One droplet of each fraction was spotted on filter paper and sprayed with the H-acid reagent of RACUSEN (1958). The fractions which contained ATX gave a red spot. ATX was eluted when pH 3.3 was reached.

The fractions containing ATX were desalted by adsorption on a column of CG 120 in the H^+-form and elution with 0.5 N NH_4OH. The eluate was concentrated to about 10 ml and the ATX precipitated by addition of 1 ml of 1 M $AgNO_3$, which forms an insoluble addition compound with ATX. The collected precipitate was suspended in water and decomposed with H_2S. The precipitate of Ag_2S was centrifuged off and the supernatant decolorized with charcoal. The pH of the acid solution was adjusted to 6, the solution concentrated as far as possible and the ATX precipitated by addition of 10 volumes of acetone. This procedure yielded usually about 50 mg of ATX in the form of white clusters of fine needles.

The crystals thus obtained were not suitable for X-ray diffraction. For this purpose use was made of the higher solubility of the HCl salt of ATX: ATX was dissolved in dilute HCl and precipitated slowly by addition of just enough ammonia to raise the pH to 7. The crystals were washed with ice-cold water and acetone and dried.

Paper chromatography: In order to facilitate the comparison of ATX with metabolites of AT found by other investigators AT and ATX were chromatographed on strips of Whatman nr. 1 paper by the ascending method, at 25°, in various solvents (see table 1). ATX was detected on the papers by the H-acid reagent of RACUSEN (1958) which forms a red azo dye with AT and its derivatives, and by ninhydrin (0.2 % in 96 % ethanol).

The technique for application of the radioactive solution to plants and for radioactive assay has been described in a former paper (MASSINI 1958).

Autoradiography of the plants: A procedure adapted from YAMAGUCHI and CRAFTS (1958) was followed. The plants were laid out flat, pressed loosely between blotting paper in a herbarium press and frozen immediately by pouring liquid nitrogen over the press. The press with the frozen plants was transferred to a desiccator placed in a deep freeze box and freeze-dried at — 7° by evacuating the desiccator for 3 to 5 days. The dry plants were mounted on blotting paper, covered with a sheet of "Mylar" film, 6 μ thick (Dupont de Nemours) and pressed strongly at room temperature between a sheet of plywood and sponge rubber for one night (with the foil on the side of the ply-

wood). Finally a sheet of X-ray film ("Osray", Gevaert) was exposed to the preparation for several weeks in a black cardboard folder.

Results

Formation of ATX: When [^{14}C] AT was applied to a leaf of a tomato or bean seedling and the plant (without the treated leaf) extracted after 24 hrs, most of the activity was in the form of ATX; a small part of the activity was AT, and varying amounts were found in an unknown compound with an R_F value lower than ATX in solvent nr. 13 (see table 1). When plants were allowed to take up a solution of [^{14}C] AT by the roots or by the cut-off stem during 24 hours, AT was more radioactive than ATX. However, the absolute amount of ATX formed was higher in stem-treated plants because of the rapid uptake of the chemical by the transpiration stream.

When extracts or homogenates of tomato plants were incubated with AT no ATX was formed. Neither could ATX be detected in bean plants which were not treated with AT.

Table 1

R_{AT} values of ATX and of several unknown metabolites of AT described earlier*.

Solvent nr.	Solvent system	ATX	unknown	lit.
1	methanol-formic a.-w. 80:15:5	.39	.30	Racusen "X"
2	n. butanol-acetic a.-w. 27:7:17	.55	.29	(1958)
3	i. propanol-NH_4OH-w. 6:2:2	.75	.47	
4	pyridine-w. 8:2	.20	.33	
5	phenol-w. 8:2	.76	.73	
6	n. butanol-propionic a.-w. 2:1:1.4	.42	.35	Carter & Naylor "I"
7	phenol-w. 72:28	.76	.68	(1959)
3	i. propanol-NH_4OH-w. 6:2:2	.75	.75	Herret & Linck "II"
8	n. butanol-ethanol-w. 52.5:32:15.5	.09	.12	(1961)
9	n. butanol-ethanol-w. 1:4:1	.14	.26	Miller & Hall "Y"
10	n. propanol-ethylacetate-w. 6:1:3	.31	.68	(1961)
11	n. butanol-acetic a.-w. 4:1:5 (top layer)	.33	.42	
1	methanol-formic a.-w. 80:15:5	.39	.84	
12	i. propanol-NH_4OH-w. 80:5:15	.21	.34	
11	n. butanol-acetic a.-w. 4:1:5 (top layer)	.33	.24	Miller & Hall "X"
1	methanol-formic a.-w. 80:5:15	.39	.51	(1961)
12	i. propanol-NH_4OH-w. 80:5:15	.21	.23	
9	n. butanol-ethanol-w. 1:4:1	.14	.12	
10	n. propanol-ethylacetate-w. 6::13	.31	.42	
13	t. butanol-formic a.-w. 70:15:15	.27	—	Massini (1959a)
14	α-picoline-w. 6:4	.57	—	

*) $R_{AT} = \frac{\text{distance travelled by the metabolite}}{\text{distance travelled by AT}}$

Since CARTER and NAYLOR (1959) found that the label of serine was incorporated into a derivative of AT when bean plants where treated with AT and [^{14}C] serine, the formation of ATX from AT and [^{14}C] serine was studied. Bean plants were allowed to take up a solution containing 0.05 % AT, not labeled, and 4.10^{-5} M [^{14}C]-serine (about 10 μC) through the cut-off stems. After 8 days the plants were extracted and the extract chromatographed on paper after addition of carrier ATX and serine. About 20 % of the activity of the extract was found in a spot which coincided exactly with the spot of ATX in three solvents; serine contained about the same amount of activity. The most active spot had R_F values of 0.3 in solvents nr. 13 and 14 and 0.5 in solvent nr. 5.

Translocation and action of ATX: ATX did not induce chlorosis in tomato seedlings when applied to the primary leaves in amounts of up to 100 μg per plant. With AT the seedlings developed chlorotic zones with as little as 0.3 μg per plant.

In order to study the translocation of ATX, a batch labeled with ^{14}C was prepared with a yield of 66 mg of [^{14}C] ATX, specific activity 0.04 mc/mmole. Bean plants were treated with this preparation by spreading out 9 μg in aqueous solution on a primary leaf. After 24 hrs the plants were cut in parts and the parts extracted. The area of application was discarded. Only 1.6 % of the amount applied had been taken up; most of it was translocated out of the treated leaf and had accumulated in the stem, the top and the newly formed roots.

In bean plants which had been treated with [^{14}C] ATX by the leaf injection method (BIDDULPH 1491) the distribution of the activity was quite comparable to the one found in similar experiments with AT (MASSINI, 1958). Between 10 and 20 % of the amount taken up was translocated to the stem, the roots and the top.

When bean plants were placed with the roots in a solution containing [^{14}C] ATX the substance was retained strongly by the roots, the stem and the veins of the primary leaves. The substance was again accumulated strongly in the top. No appreciable redistribution took place when plants treated in this way were kept on water for three days before they were analysed. This contrasts with the behaviour of AT which was translocated rapidly to the top in a similar experiment (MASSINI, 1958).

In conclusion it can be stated that ATX is taken up at a moderate rate by leaves of French dwarf bean and that it is translocated to the growing zones in a manner typical of phloem-translocated substances, but that it is less mobile than AT. The strong adsorption in the stem is probably due to its basic groups (CHARLES 1953).

In the extracts of plants treated with [^{14}C] ATX for some days, only one substance could be detected by paper chromatography; its spot coincided with ATX. The place of AT was not radioactive. It seems, then, that the transformation of AT to ATX is irreversible.

The translocation and fate of AT and of ATX in tomato plants was studied in an experiment of longer duration: 1 or 2 μg of [^{14}C] AT was applied to the primary leaves of 44 very young seedlings. At the

beginning of the experiment and after 1 to 28 days a sample of the plants was removed. Some of the plants were freeze-dried, combusted and assayed for ^{14}C in the form of $BaCO_3$. From other plants autoradiograms were prepared. From the last lot (28 days) the chlorotic zones were cut out and extracted. The extracts were chromatographed in three solvents.

After 3 days all the plants began to develop chlorotic zones in the newly formed parts. After 14 days many plants began to recover and at the end of the 4 weeks period almost all the plants had recovered. Many of these plants displayed the green-white-green pattern characteristic of recovery from AT-chlorosis: the oldest parts which had formed before application of the herbicide and the youngest ones which had formed after recovery were green; parts of intermediate age were

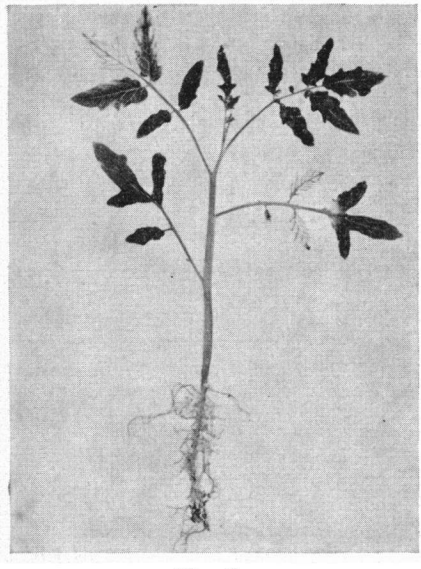

Fig. 2.

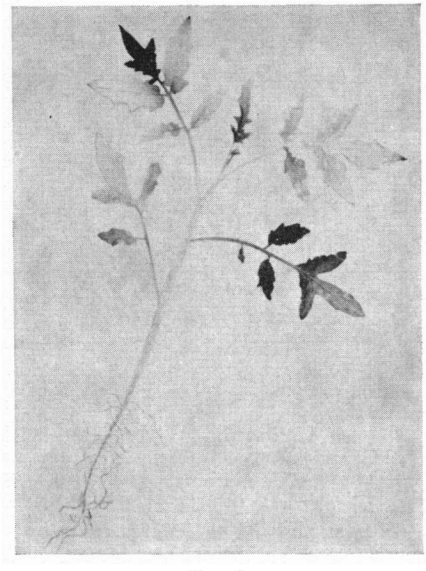

Fig. 3.

Fig. 2. Tomato seedling, treated with 2 μg of [^{14}C] AT, after 4 weeks. The chlorotic parts appear white in the picture.

Fig. 3. Autoradiogram of the tomato seedling shown in fig. 2. Exposure 8 weeks. Note the accumulation of radio-activity in the chlorotic parts.

chlorotic. Fig. 2 shows such a plant. Fig. 3, the autoradiogram of the same plant, reveals that the radioactivity is concentrated strongly in the chlorotic parts although some activity had migrated into the newly formed parts.

All the plants which had been combusted contained the same amount of radioactivity. Hence we can conclude that AT is not metabolized to CO_2.

In the extracts of the chlorotic zones about 60 % of the activity was found in a spot coinciding with ATX and the rest in a zone with an R_F of 0.3 and 0.5 in solvents 13 and 14 respectively and which coincided with the ATX spot in solvent 5. The AT spot was free of activity.

Discussion

ATX is the main product of metabolism of AT in French dwarf bean and in tomato plants. The transformation is not reversible, and ATX is only very slowly metabolized further.

The substance is not phytotoxic; it is taken up and translocated through the plant, apparently by both the phloem and the xylem system, but it is distinctly less mobile than AT itself.

It thus seems probable that AT is distributed in the plant as such and displays its phytotoxic action without being transformed. Concomitantly with its action it is partly transformed—presumably by coupling with serine—to ATX and thereby detoxicated. This reaction is probably responsible for the recovery which can be observed in plants which have been treated with a sublethal dose of AT and display the characteristic green-white-green pattern.

Table 2

Color reactions of AT, ATX and of several metabolites of AT described earlier.

test	AT	ATX	Racusen X	Carter & Naylor 1	Herret & Linck's II
ninhydrin	—	purple	purple	blue-green	blue-green
H-acid	red	red	orange-red	red	orange-pink
nitroprusside	blue	—		green	
p-dimethylamino-benzaldehyde	yellow	yellow	yellow	yellow	

The relation of this reaction to the general intermediate metabolism of the plants is not known. It seems not to be related to the biosynthesis of the normal hetero-cyclic amino acids histidine and tryptophan since ATX differs from them in that its aliphatic chain is connected to a nitrogen atom of the ring.

However, three natural amino acids have been described which contain a heterocyclic ring system connected with a nitrogen atom to the β-position of alanine: β (uracil-3) alanine (willardiine (Gmelin, 1959)), β (pyridon-4-1-yl) alanine (leucenol, (Adams & Johnson 1949)) and β (pyrazol-1-yl) alanine (Noe & Fowden 1960). The last one resembles ATX most; furthermore the authors present evidence that the pyrazole ring is incorporated as such into the amino acid; the same seems to be the case with ATX.

Several unknown metabolites of AT have been described hitherto by different authors but none of them has been characterized sufficiently for identification. In table 1, R_{AT}, the relative mobility of

ATX (with AT as a standard substance), is compared with R_{AT} of some metabolites which seem to resemble ATX. Table 2 lists the color reactions of AT, ATX and these substances.

ROGERS (1957) found a compound which had an R_f value of 0.4 to 0.5 in "liquefied phenol". On account of coincidence of R_f values in one solvent he concluded that the compound is identical with a "glucose adduct" of AT.

RACUSEN (1958) described a substance "X" which has much in common with ATX. His report that X induces chlorosis in Lemna minor in high concentration does not exclude identity with ATX, which has been found to be non-toxic to tomatoes. However, the R_{AT} values of the two compounds differ rather markedly. Also there is a certain discrepancy in the paper electrophoretic mobility: X has an isoelectric point at pH 7 whereas ATX does not move between pH 3 and 7, in accordance with its pK values of 2.1 and 7.9 (see ref. 13 and 2).

CARTER and NAYLOR (1959, 1961) describe a metabolite "1" which is labeled when the plants are treated with [^{14}C] AT or with AT + [^{14}C] glycine or [^{14}C] serine. In view of the structure of ATX and its presumed biosynthesis, comparison between ATX and "1" seems especially interesting. However, although the R_{AT} values of "1" seem to exclude identity of the two substances it resembles Racusen's X more than ATX in its paper electrophoretic behaviour. "1" had no toxic effects on bean plants.

A metabolite which resembles ATX very much in paper chromatography has been decribed by HERRET and LINCK (1961). It is non-toxic to thistle.

Finally, MILLER and HALL (1961) describe two metabolites of AT which are formed in cotton plants. On the basis of the R_{AT} values their compound "X" could be identical with ATX.

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