Absorption and Metabolism of Aminotriazole in Cotton

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Investigations of the absorption and metabolism of aminotriazole in cotton plants were undertaken prior to 1958 to determine if the material was translocated into cottonseed when used as a cotton defoliant. Experiments indicated that 3-amino-1,2,4-triazole was metabolized by the leaves and that the metabolites were translocated in small quantities to the seed. The metabolites remain unidentified, but are known to be water-soluble.

AMINOTRIAZOLE has been recognized as a physiologically active chemical since the discovery that it would inhibit chlorophyll synthesis in plants (*1*). The translocation and metabolism of 3-amino-1,2,4-triazole (3-ATA) in different plants have been studied by various workers including Rogers (*16*), Racusen (*15*), and Andersen (*2*). Racusen (*15*) first reported the presence of two metabolites in pinto beans. Massini (*8*) has identified one of the metabolites in beans as 3-amino-1,2,4-triazolyl alanine. Gentile and Frederick (*6*) presented evidence that one metabolite was a glucosamine adduct while Carter and Naylor (*4*) have shown ATA complexes with a glycine-serine derivative.

Pyfrom *et al.* (*14*) and Frederick and Gentile (*5*) have noted the effect of 3-ATA on various plant enzyme systems. Pyfrom and coworkers found a depression of catalase activity in barley seedlings, while Frederick and Gentile showed an inhibition of phosphorylase activity which could be reversed by the addition of manganese or iron.

The effects of 3-ATA on pigments of the cotton plant and its salts on cotton defoliation were reported (*9*, *10*).

The discovery of small quantities of spray residues of 3-ATA in cranberries has recently become a topic of national concern. Investigations of the absorption and metabolism of 3-ATA were initiated prior to the cranberry incident to determine if 3-ATA would enter the seed of cotton plants when it was used as a defoliant and regrowth inhibitor. This report, therefore, is concerned with the absorption and metabolism of 3-ATA in the cotton plant with special emphasis on translocation into the seed.

Materials and Methods

Cotton seeds of the variety Deltapine 15 were germinated and the plants grown in potting soil in the greenhouse. The plants were treated at the stages and in the manner described below with solutions of 3-ATA-5-C^{14}.

Seedling Cotton Experiment. Four young cotton seedlings in the two true-leaf stages of growth were painted with a small but uniform amount of a stock solution of 3-ATA-5-C^{14} which had a specific activity of 0.2×10^6 counts per minute per mg. One seedling was harvested at the end of the first, second, fourth, and eighth day after treatment. The plants were stored in the deep freeze prior to extraction and chromatography.

Mature Cotton Experiment. One third of a millicurie of 3-ATA-5-C^{14} was added to enough aminotriazole to make a total of 213 mg. of 3-ATA dissolved in 80 ml. of water. The solution had a specific activity of 0.2×10^6 c.p.m. per mg. and was sprayed at the equivalent rates of 1 and 2 pounds per acre.

Two lots of plants were used in the experiment. The first lot of plants had all of their bolls open at the time of spraying. Four plants were used for each application rate. The second lot consisted of plants which had all of their bolls closed at the time of spraying. These plants were selectively defruited at the time of flowering so that when they were sprayed they contained 10-, 20-, and 30-day unopened bolls. Six plants were used for each application rate.

The cotton was picked at maturity and ginned on a small knife-type gin. The seed was hulled in a small laboratory huller and the meats separated from the hulls by screening. The cottonseed meats, hulls and linters, and lint were monitored in a gas flow proportional counter.

The regrowth leaves from these plants were harvested and prepared for extraction and chromatography.

Single Leaf Experiment. A mature cotton leaf was excised from a field-grown plant and the basal end of the petiole was immersed in 1 ml. of the 0.2×10^6 c.p.m. per mg. stock solution. The leaf was kept in diffuse light in the laboratory and allowed to absorb the 3-ATA-5-C^{14}. Periodic distilled water additions were made as necessary to maintain the level of solution. The leaf was removed from the solution after 2 days and prepared for extraction and chromatography.

Analytical Procedures

Extraction. The leaves and seed from the treated plants and tissues were extracted with water in a Waring Blendor and passed through an IR 120 cation exchange column and subsequently prepared as described by Racusen (*15*) for chromatography.

Chromatography. The chromatographic standards and concentrated eluates from the exchange columns were spotted on five sheets of Whatman No. 1 filter paper. The eluates were spotted until each spot would monitor 1000 c.p.m. The papers were placed in five different solvent systems (all volume ratios): butyl alcohol–acetic acid–water (4:1:5 top layer); methanol–88% formic acid–water (80:15:5); isopropyl alcohol–ammonia–water (80:5:15); ethyl

Table I. R_f Values of 3-ATA Adducts Chromatographed in Butyl Alcohol–Acetic Acid–Water

Adducts	R_f
Organic acids	
Citric	0.40
Malic	0.44
Oxalic	0.05–0.52
Fumaric	0.85
Isocitric	0.45
Tartaric	0.33
Sugars	
Fructose-1,6-$(PO_4)_2$	0.28
Ribose	0.23
Xylose	0.18
Arabinose	0.17
Glucose	0.14
Mannose	0.16
Tyrosine	0.30
Metallic chlorides	
Cobalt	0.35
Copper	0.21
Iron	0.25
Lithium	0.48
Tin	0.23
Zinc	0.25

alcohol–butyl alcohol–water (4:1:1); and *n*-propyl alcohol–ethyl acetate–water (6:1:3). The 3-ATA-5-C^{14} chromatograms were either radioautographed or stripcounted to determine the R_f values and presence of metabolites.

Chromatographic standards were detected by the use of an alkaline nitroprusside reagent or phenol-chlorox or both as described by Block (*3*).

Standard Preparation. Addition Products. Aminotriazole was reacted in test tubes in an autoclave for 45 minutes with 1 to 2*M* mixtures of 14 amino acids, 8 sugars, 14 metallic chlorides, and 14 organic acids. The R_f values of the adducts formed in these mixtures are shown in Tables I and II and were compared with the metabolites of 3-ATA-5-C^{14}. In addition, 40 derivatives obtained from Amchem Products, Inc., were compared chromatographically to the metabolites.

Ring Substitution and Cleavage Products. Urazole (1,2,4-triazole-3,5-dione) was synthesized from urea by the action of hydrazine hydrochloride through an intermediate of hydrazodicarbonamide in accordance with the method of Pellizzari (*12*). Guanazole (3,5 - diimino - 1,2,4 - triazole) was synthesized by the action of hydrazine on dicyandiamide according to a method devised by Pellizzari (*13*). Aminoguanidine formate was produced by the action of formic acid on aminoguanidine bicarbonate. The formation of aminoguanidine formate is the first step in the production of 3-amino-(1H)-1,2,4-triazole described by Sjostedt (*17*).

Formyl hydrazine, 1,2,4-triazole, and formylaminoguanidine nitrate (supplied by the American Cyanamid Co.) were included in the chromatographic survey of possible substitution and cleavage products arising from the metabolism of 3-ATA.

Other chemicals which were tested as possible products of the cleavage of the triazole ring were cyanamide, urea, dicyandiamide, biuret, biurea, hydrazine, formamide, semicarbazide, ethyl formate, and hydrazodicarbonamide.

Table II. R_f Values of Postulated Metabolites

Material	Solvent Systems[a] I	II	III	IV	V
Aminotriazole	0.55	0.73	0.53	0.76	0.76
Metabolite *X*	0.13	0.37	0.12	0.09	0.32
Metabolite *Y*	0.23	0.61	0.18	0.20	0.52
Ring cleavage postulates					
Aminoguanidine bicarbonate	0.85	...	...	...	...
Aminoguanidine bicarbonate	0.39	...	0.07	0.12	0.24
(boiled with 1*N* NH_4OH)	...	...	0.15	0.27	0.46
	0.52	0.63	0.25	0.40	0.67
Aminoguanidine formate	0.85	...	...	...	...
Aminotriazole (peroxided)	0.33	0.38	0.31	...	0.49
Biurea	0.19	0.39	0.22	...	...
Dicyandiamide	0.55	...	...	...	...
Formylaminoguanidine NO_3	0.29	0.69	0.40	0.49	0.48
Formylhydrazine	0.45	...	...	...	...
Hydrazine sulfate	0.21	0.45	...	0.10	0.20
Semicarbazide	0.20	0.57	0.46	0.36	0.48
Urea	0.55	...	...	...	...
Ring substitution postulates					
Guanazole (impure)	0.23	0.38	0.17	0.13	0.24
	0.37	0.51	0.30	0.39	0.45
1,2,4-Triazole	0.71	...	...	...	...
Urazole	0.40	0.45	...	...	...
Salts and derivatives					
Furfuralamino-1,2,4-triazole	...	...	0.55	...	...
3-Glucosamino-1,2,4-triazole	0.21	...	0.31	...	...
HCl salt of aminotriazole	0.25	0.57	0.47	0.53	0.56
NO_3 salt of aminotriazole	0.26	...	0.54	...	...
PO_4 salt of aminotriazole	0.25	0.70	0.52	...	...
Reaction mixtures					
Citric acid, 3-ATA	0.40	...	...	...	...
Malic acid, 3-ATA	0.44	...	...	...	...
Oxalic acid, 3-ATA	0.32	...	...	...	...
Tartaric acid, 3-ATA	0.32	...	...	...	...
Arabinose, 3-ATA	0.17	...	...	...	...
Mannose, 3-ATA	0.16	0.41	0.31	0.29	0.39
Ribose, 3-ATA	0.23	0.51	0.36	0.34	0.43
Xylose, 3-ATA	0.18	...	...	...	...
Tyrosine, 3-ATA	0.30	...	...	...	...
Cupric chloride, 3-ATA	0.21	0.71	0.62	0.44	0.42
Ferric chloride, 3-ATA	0.25	0.68	0.47	0.51	0.44

[a] I. Butyl alcohol–acetic acid–water, 4:1:5 top layer. II. Methanol–88% formic acid–water, 80:15:5. III. Isopropyl alcohol–ammonia–water, 80:5:15. IV. Ethyl alcohol–butyl alcohol–water, 4:1:1. V. *n*-Propyl alcohol–ethyl acetate–water, 6:1:3.

Table III. Results of Greenhouse Trial with Aminotriazole-5-C^{14}

(Based on the radioactivity and expressed as p.p.m. aminotriazole equivalents[a])

Boll Age, Days	Treatment, Lb. of 3-ATA	Seed	Hulls	Lint	Burrs
Open	1	>0.13[b]	...	77.3	55.0
Open	2	>0.13	...	232.5	63.0
30	1	5.40	6.75	0.24	17.0
	2	11.9	9.65	0.16	32.3
20	1	43.4	23.3	1.29	18.5
	2	47.4	34.5	1.19	33.0
10	1	21.6	28.2	2.66	43.0
	2	51.9	43.7	2.63	36.6

[a] Since radioautograms indicated no unmetabolized 3-ATA-5C^{14} in the seed and chemical structures of metabolites are unknown, calculations are based on assumption that metabolites have same molecular weight as 3-ATA.

$$\text{P.p.m. 3-ATA equivs.} = \frac{\text{total obsd. c.p.m. in seed sample} \times 1{,}000{,}000}{\text{wt. of seed sample in mg.} \times \text{spec. activity of 3-ATA in c.p.m./mg.}}$$

[b] Lower limit of sensitivity.

Results

Seedling Cotton Experiment. The harvest of a seedling plant at the end of 1, 2, 4, and 8 days after treatment resulted in radioautograms (Figure 1) which clearly showed the presence of

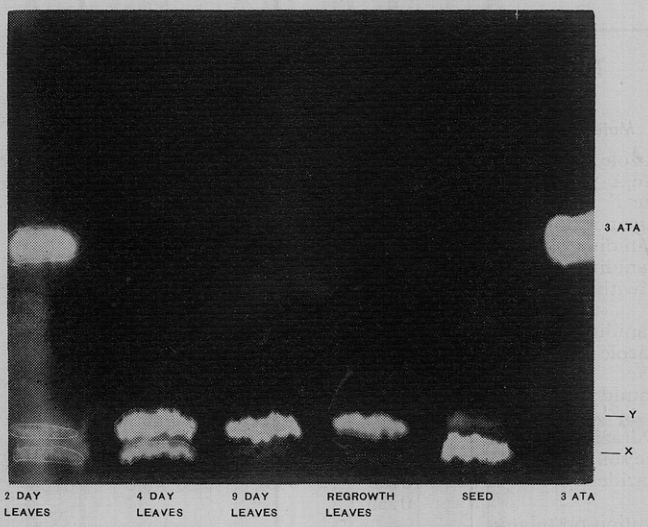

Figure 1. Radioautogram of chromatogram showing 3-amino-1,2,4-triazole-5-C^{14} and metabolites from various cotton tissues chromatographed in isopropyl alcohol–ammonia–water

two metabolites (*X* and *Y*) within 4 days after treatment. The radioautograms indicated that the *Y* metabolite was more abundant than metabolite *X* throughout the test period. Densitometer readings of the radioautograms indicated an average 4 to 1 ratio of *Y* to *X* within the young plants The radioautograms also indicated the disappearance of 3-ATA-5-C^{14} within the plants in about 4 days after treatment.

Mature Cotton Experiment. Data obtained from the greenhouse experiment indicated that there was no appreciable penetration of the radioactive carbon from 3-ATA into the kernels of the seed from bolls which were open at the time of spraying (Table III). The kernels of the seed from bolls which were closed at the time of spray application did, however, contain measurable amounts of the carbon-14.

The amounts of radioactivity which were translocated into the kernels were dependent upon the age of the kernels at time of treatment. The younger the seed, the more radioactivity found at the time of maturation of the seed.

Radioautograms produced from the extracted seed indicated that essentially all of the radioactivity was in the form of metabolites *X* and *Y*. Metabolite *X* was the predominant form in the seed (Figure 1).

Regrowth leaves harvested from the treated plants contained only a trace of 3-ATA, while metabolite *Y* was found to be the predominant form. The average R_f values of metabolite *X* and *Y* are given in Table II.

Single Leaf Experiment. The immersion of the cut petiole of a single leaf in the treatment solution resulted in the accumulation of 3-ATA-5-C^{14} within the leaf blade. At the end of the 2-day treatment period, the leaf had metabolized about one fifth of the 3-ATA absorbed. Strip-counts of the chromatograms indicated that there was a predominance of metabolite *X* over *Y* under these conditions.

Metabolite Identification. The reaction products, and ring substitution and cleavage products synthesized or obtained for the chromatographic identification of metabolities did not give R_f values comparable to metabolites *X* or *Y* in five solvent systems. It was therefore assumed that none of the products listed was either metabolite *X* or *Y*.

Discussion

It is believed that the 3-ATA sprayed on the external surface of the leaf is altered rapidly after entrance into the leaf. The results indicate that under normal conditions the *X* metabolite is formed rapidly and if not translocated, it becomes transformed to the *Y* metabolite. It appears that the *Y* metabolite is the final form and is not readily translocated within the plant. The *X* metabolite appears to move from the treated leaves to the apical and axillary meristem regions and in these rapidly growing tissues metabolite *X* becomes converted to metabolite *Y*. On the other hand, when metabolite *X* moves from the treated leaves to the storage tissues of the seed, it apparently does not undergo further metabolism. Hall (*7*) has shown that cottonseed from treated plants do not produce chlorotic seedlings. Apparently metabolite *X* was present either in insufficient quantities to produce chlorosis or was rapidly converted to metabolite *Y* during the germination and the *Y* metabolite was ineffective in producing chlorosis. Racusen (*15*) found that when compound *X* was isolated by his techniques of paper chromatography from bean tissue and tested on *Lemna minor*, it produced chlorosis and stunting but it was not as effective as 3-ATA in this response. He reported that compound *X* was the major product when bean leaves were infiltrated with 3-ATA by his reduced pressure techniques. Similar results were obtained in this experiment when the petiole of a single cotton leaf was immersed in 3-ATA-5-C^{14} stock solution. Based on the R_f values of metabolites *X* and *Y* in various solvent systems, they are believed to be identical with those found in soybeans by Palmer and Williams (*11*) and Racusen (*15*). It is believed that both metabolites *X* and *Y* contained the intact triazole ring. Further work is necessary to establish the identity of the two metabolites in cotton.

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