Role of Ascorbic Acid Oxidase in the Hyoscyamine–Scopolamine Ratio in *Datura innoxia*

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Isolated root cultures of *Datura innoxia* Miller to which benzoin anti-oxime (Cupron) was added showed a change in the habit of growth but no change in the weight of the roots nor in the hyoscyamine and scopolamine content, indicating that ascorbic acid oxidase activity was not involved in alkaloid biosynthesis. An improved method for the germination of *D. innoxia* seeds was developed.

ASCORBIC ACID OXIDASE, which is widespread in higher plants (1–3), has been implicated as a terminal oxidase in cellular respiration (4–7). Ascorbic acid itself is required for the rapid synthesis of large amounts of collagen and may be involved in the conversion of proline to hydroxyproline (8, 9). There is some question as to whether proline is hydroxylated before or after incorporation into collagen (9–11). The use of ^{18}O has shown that the hydroxyl oxygen of hydroxyproline is incorporated directly from the atmosphere (12, 13); ascorbic acid may act directly by generating free hydroxyl radicals (8); a direct effect of ascorbic acid on the collagen-synthesizing fibroblasts has also been suggested (14).

Proline is also involved in the biosynthesis of alkaloids in *Datura* species (15). Proline-^{14}C was incorporated into both hyoscyamine and scopolamine and other alkaloidal constituents of both *D. innoxia* Miller and *D. stramonium* L. variety *tatula* Torrey. However, hydroxyproline is apparently not a direct precursor of scopolamine or of the other alkaloids of *D. innoxia* (16). Scopolamine is apparently synthesized from hyoscyamine (17).

The present study was made to determine the possible relationship between ascorbic acid oxidase and tropane alkaloid biosynthesis in *D. innoxia*, since both the enzyme and the alkaloids appear to be related to proline–hydroxyproline metabolism. Ascorbic acid oxidase was inhibited by benzoin anti-oxime (C_6H_5-$CHOHC{:}NOHC_6H_5$)[1] since under physiological conditions it chelates only cupric ion (18).

EXPERIMENTAL

Growth and Processing of Root Cultures.—The methods used in this investigation for the growth and processing of root cultures were modified from those developed in this laboratory by French and Gibson (19) and Sullivan and Gibson (15).

Previous work in this laboratory was hindered by the low germination rate of *D. innoxia* as well as other species of *Datura*. When the seeds were incubated on damp filter paper, only 1 to 2% sprouted. Other workers have used various treatments to hasten the germination of *D. innoxia* (20, 21). Trials at the beginning of this investigation showed that when the seeds were presoaked in sterile White's nutrient solution (22), the germination rate increased to over 30%; also, the rate of injury to the radicles was less than when mechanical methods were used to weaken the seed coat. White's nutrient solution as used throughout this work was sterilized in an autoclave.

Seeds of *D. innoxia* were sterilized by shaking for 15 min. with 3% hydrogen peroxide. Then the seeds were placed on sterile filter papers in Petri dishes. The seeds were covered with 20 ml. of White's nutrient solution and placed in an incubator at 26–27°. After 3 days, the seeds were removed from the incubator; aseptically transferred one seed at a time, using forceps, to new sterile Petri dishes; and moistened with 5 ml. of sterile, demineralized, distilled water. The seeds usually started to germinate after the fourth day. The radicles were severed from the seed when they were 1 to 1.5 cm. long and placed in 50 ml. of nutrient solution. At the end of the first week the roots were transferred to fresh nutrient solution. After 2 weeks the roots were removed from the nutrient solution and dried at 55° to a constant weight.

Control roots were cultured in White's nutrient solution; the test roots were cultured in White's nutrient solution containing 10^{-3} *M* of benzoin anti-oxime.

Extraction and Separation of Alkaloids.—The procedure used for the extraction of the alkaloids from the dried root cultures was based on earlier work by French and Gibson (19), except that the root extract was allowed to drip directly onto a thin-layer chromatography plate rather than chromatographic paper strips.

Preliminary tests showed that it was necessary to combine roots in groups of four to seven, weighing about 25 mg., in order to obtain a concentration of hyoscyamine and scopolamine that could be measured spectrophotometrically. However, in computing the alkaloid contents, the weight of the crushed root samples was converted to a base of exactly 25 mg. These roots were weighed and then crushed together with a mortar and pestle. The crushed root powder was placed on top of a

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[1] Marketed as Cupron by Eastman Organic Chemicals, Rochester, N. Y.

TABLE I.—DRY WEIGHT OF CONTROL ROOTS OF *D. innoxia*

Root No.	Root Wt., mg.	Root No.	Root Wt., mg.	Root No.	Root Wt., mg.
1	4.3	45	5.3	89	3.9
2	6.2	46	2.7	90	7.6
3	3.4	47	2.8	91	7.9
4	4.7	48	6.5	92	5.6
5	5.9	49	7.8	93	4.8
6	5.1	50	2.2	94	5.1
7	6.0	51	6.1	95	4.7
8	4.7	52	6.5	96	3.7
9	5.5	53	3.7	97	4.2
10	7.5	54	5.6	98	4.3
11	7.7	55	4.4	99	6.3
12	4.6	56	7.4	100	7.5
13	6.0	57	2.8	101	1.5
14	7.7	58	3.6	102	7.2
15	5.0	59	7.3	103	7.0
16	8.4	60	8.7	104	6.1
17	3.4	61	2.9	105	5.6
18	3.9	62	7.5	106	4.3
19	9.8	63	2.5	107	4.6
20	8.2	64	6.3	108	8.8
21	4.3	65	3.8	109	1.6
22	4.1	66	1.8	110	5.7
23	2.9	67	2.1	111	4.7
24	6.1	68	2.9	112	4.8
25	5.9	69	2.4	113	8.5
26	5.9	70	11.0	114	7.8
27	7.6	71	7.8	115	6.8
28	5.4	72	5.8	116	4.6
29	11.0	73	6.3	117	6.2
30	4.4	74	5.5	118	4.1
31	10.1	75	5.7	119	7.4
32	8.5	76	5.9	120	4.5
33	3.8	77	8.2	121	9.5
34	10.7	78	6.0	122	8.1
35	7.4	79	5.8	123	7.0
36	3.2	80	9.1	124	5.1
37	2.4	81	5.2	125	5.0
38	6.6	82	4.0	126	2.7
39	5.8	83	3.0	127	9.4
40	1.5	84	5.7	128	3.7
41	3.2	85	5.0	129	3.1
42	5.9	86	4.9	130	11.5
43	2.1	87	6.8	131	5.2
44	5.9	88	5.5		
		Mean	5.6		
95% Confidence interval			5.4–5.8		

cotton pledget in an extractor cut from the lower end of a 50-ml. needle-valve buret, the extractor having a capacity of about 10 ml. The root powder was moistened with a 1:1 mixture of 10% ammonium hydroxide and 95% ethanol and allowed to stand for 1 hr. Then 2 ml. of chloroform was added to the extractor and 1 ml. of chloroform for each milligram of root powder was added to the reservoir above the extractor. The extract was allowed to drip directly onto a thin-layer chromatography plate, adjusting the stopcock of the extractor so that each drop dried completely before the next drop fell onto the plate. A jet of cool air was directed at the spot to facilitate drying.

The procedure used for the separation of alkaloids was developed by Sullivan and Gibson (15). The alkaloids of the root extract were separated on a 0.5-mm. thick matrix of Silica Gel G.[2] The root extract was placed 1.5 cm. from the bottom of the 8 by 8 in. plate and 3.0 cm. from the left edge of the plate. The chromatogram was developed in two directions, 15 cm. in each direction, using 10 ml. of diethylamine and 90 ml. of chloroform as the developing solvent for the first phase, and 15 ml. of diethylamine and 85 ml. of chloroform for the second phase. Both phases were developed in an incubator at 30°. Between phases and after the second phase the plates were dried at 55° for 15 min. in a Freas circulating dry air oven and then allowed to cool for 30 min. Preliminary chromatograms indicated the location on the plates of hyoscyamine and scopolamine. After cooling, the probable areas of location of hyoscyamine and scopolamine were covered for protection; then the rest of the plate was sprayed with iodoplatinic acid solution (23) to verify the position of the alkaloids. The hyoscamine and scopolamine spots were removed from the plate with a razor blade and a camel's hair brush.

The alkaloids were extracted from the Silica Gel G in an apparatus developed by Gibson in this laboratory (unpublished work). The extraction was set up in the following manner. Two circles of Whatman No. 2 filter paper, 6 mm. in diameter, were inserted into the bottom of the barrel of a 1-ml. hypodermic syringe. The alkaloid-containing silica gel was put on top of the filter paper. A small pledget of cotton was put on top of the silica gel, and the column was tamped down. A 2.5 in. long 19-gauge needle was attached to the syringe and the entire apparatus was inserted through the hole of a rubber stopper plugging

TABLE II.—DRY WEIGHT OF ROOTS OF *D. innoxia* GROWN IN 10^{-8} *M* BENZOIN ANTI-OXIME IN WHITE'S NUTRIENT SOLUTION

Root No.	Root Wt., mg.	Root No.	Root Wt., mg.	Root No.	Root Wt., mg.
1	1.8	29	9.6	57	10.4
2	2.8	30	8.7	58	7.3
3	4.0	31	12.0	59	12.0
4	2.8	32	7.8	60	8.2
5	4.6	33	9.0	61	7.7
6	4.2	34	6.2	62	25.5
7	2.8	35	4.9	63	4.0
8	1.7	36	7.4	64	4.4
9	4.8	37	4.2	65	8.2
10	1.4	38	4.7	66	5.9
11	5.6	39	5.3	67	6.3
12	4.0	40	10.8	68	7.5
13	5.4	41	9.2	69	3.3
14	2.5	42	9.3	70	4.3
15	3.5	43	3.4	71	7.1
16	5.2	44	5.2	72	5.4
17	1.3	45	9.8	73	6.6
18	9.3	46	8.3	74	3.9
19	4.8	47	7.4	75	7.4
20	8.0	48	8.8	76	5.3
21	8.4	49	7.4	77	3.3
22	7.5	50	7.4	78	5.5
23	7.0	51	8.5	79	3.1
24	11.7	52	5.0	80	9.4
25	7.3	53	8.3	81	6.0
26	6.3	54	9.4	82	6.7
27	6.3	55	9.7		
28	5.7	56	8.3		
		Mean	6.6		
95% Confidence interval			5.9–7.3		

[2] The Silica Gel G is a product of E. Merck AG., Darmstadt, Germany, and is distributed by Brinkmann Instruments, Inc., Great Neck, L. I., N. Y.

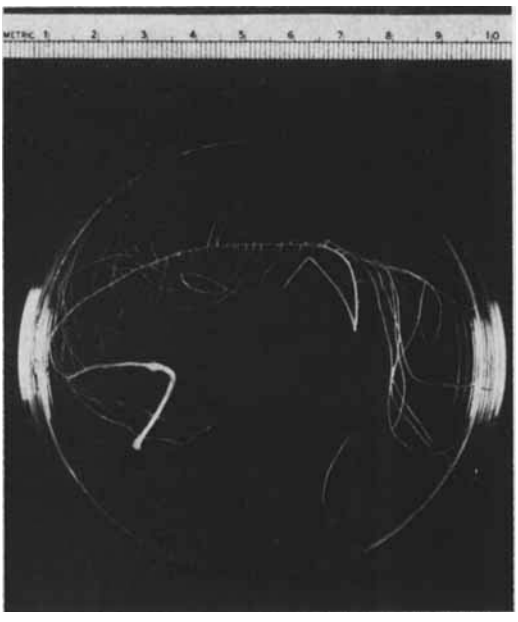

Fig. 1.—*D. innoxia* control root grown in White's nutrient solution.

the top of a small bell jar. One milliliter of absolute methanol was allowed to be in contact with the silica gel column for 30 min. Then 9 ml. of methanol was added to a reservoir above the extractor consisting of a 10-ml. hypodermic syringe attached to a 19-gauge needle inserted through a cork in the top of the 1-ml. syringe. By creating a vacuum in the bell jar, the extract was drawn into a 3-in. evaporating dish. The extract was evaporated to dryness over a water bath adjusted to approximately 65°.

Assay of Alkaloids.—The alkaloids hyoscyamine and scopolamine were quantitatively assayed spectrophotometrically using the Vitali-Morin reaction in a technique developed by French (24). Two-tenths of 1 ml. of fuming nitric acid was added to the dried methanol extract of silica gel containing hyoscyamine or scopolamine. The acid was then evaporated to dryness for 3 min. on a boiling water bath. The residue was redissolved in 4 ml. of acetone and made up to 25 ml. with successive 3-ml. washings of acetone. To determine the amount of alkaloid in each sample, 0.1 ml. of 3% w/v potassium hydroxide in absolute methanol was added to each 25-ml. flask of acetone solution of alkaloid. Each sample was measured against a blank prepared from 25 ml. of acetone and 0.1 ml. of 3% potassium hydroxide in methanol. The transmittance at 560 mμ with a 1-cm. light path was measured in a Beckman DU spectrophotometer, 3 min. after the addition of 3% potassium hydroxide in methanol for scopolamine, 7 min. for hyoscyamine.

RESULTS

The dry weights of both the control roots and the benzoin anti-oxime treated roots are summarized in Tables I and II. The confidence intervals indicate that there is no significant difference at the 5% level in dry weight between the control roots and the benzoin anti-oxime treated roots. However, benzoin anti-oxime does change the habit of growth of roots, as shown in Figs. 1 and 2.

To determine alkaloid content standard curves were prepared by plotting on semilog paper the transmittance values against known concentrations of pure hyoscyamine and scopolamine dissolved in 50% ethanol. The straight line curves then obtained showed adherence to Beer's law. The k values for hyoscyamine and scopolamine were computed from the standard curves and the Bouger-Beer relationship (25). The concentrations of alkaloid in milligrams were computed from these k values. The results of the alkaloidal assays of the control roots and benzoin anti-oxime treated roots are summarized in Tables III and IV. The overlapping confidence intervals indicate that there is no significant difference at the 5% level in alkaloid content between the control roots and the benzoin anti-oxime-treated roots.

DISCUSSION

The results obtained in this investigation seem to indicate that ascorbic acid oxidase is not related to hyoscyamine and scopolamine biosynthesis, nor is any other copper-containing enzyme.

At least five enzymes could serve as terminal oxidases in plants: polyphenol oxidase, lactase, ascorbic acid oxidase, cytochrome oxidase, and peroxidase (4). The first three, being copper-containing enzymes, are apparently eliminated from participation in alkaloid biosynthesis by the results of this study. The last two are iron-containing enzymes, which still may be considered, since they were not inactivated in this study. However,

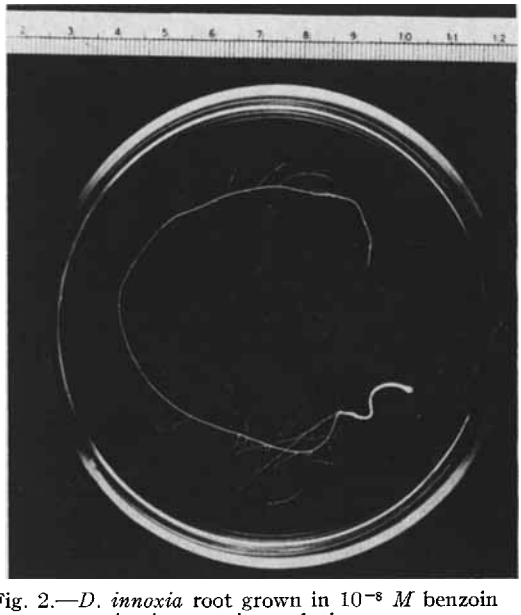

Fig. 2.—*D. innoxia* root grown in 10^{-8} *M* benzoin anti-oxime nutrient solution.

TABLE III.—ALKALOID CONTENT OF CONTROL ROOTS OF *D. innoxia*

Sample	Hyoscyamine[a]	Scopolamine[a]
1	0.00853	0.0220
2	0.00160	0.0188
3	0.00559	0.0195
4	0.00321	0.0141
5	0.00207	0.0127
6	0.00231	0.0145
7	0.00750	0.0102
8	0.0102	0.0182
9	0.00830	0.0210
10	0.00208	0.0153
11	0.00490	0.0141
12	0.00644	0.0225
13	0.0158	0.0238
14	0.0150	0.0274
15	0.0151	0.0155
16	0.0120	0.0183
17	0.0151	0.0226
18	0.00388	0.0191
19	0.00979	0.0213
Mean	0.00786	0.0185
95% Confidence interval	0.00577–0.00995	0.0164–0.0206

[a] Milligrams per 25 mg. crushed root sample.

TABLE IV.—ALKALOID CONTENT OF ROOTS OF *D. innoxia* GROWN IN 10^{-8} *M* BENZOIN ANTI-OXIME IN WHITE'S NUTRIENT SOLUTION

Sample	Hyoscyamine[a]	Scopolamine[a]
1	0.0125	0.0241
2	0.00964	0.0121
3	0.0113	0.0194
4	0.0154	0.0187
5	0.0101	0.0188
6	0.00402	0.0106
7	0.00660	0.0106
8	0.0114	0.0136
9	0.00846	0.00956
10	0.00358	0.0120
11	0.0133	0.0253
Mean	0.00966	0.0159
95% Confidence interval	0.00715–0.0122	0.0121–0.0196

[a] Milligrams per 25 mg. crushed root sample.

copper has also been suggested to be involved in cytochrome oxidase activity (26–28), but this has been disputed (29).

The effect on the habit of growth appears to be due to a growth inhibiting effect on the secondary roots compensated for, however, in total weight by greater primary root elongation. Another chelating agent, EDTA, has been shown to suppress the elongation of wheat roots by suppressing cell multiplication (30).

SUMMARY

The benzoin anti-oxime, at a concentration of 10^{-8} *M*, changes the habit of growth of isolated 2-week-old root cultures of *D. innoxia* but has no significant effect on the dry weight of the roots. At this concentration, the benzoin anti-oxime also has no significant effect on the amount of the hyoscyamine or scopolamine found in the roots, and these results indicate the lack of ascorbic acid oxidase involvement in the oxidation of hyoscyamine to scopolamine under the conditions of this study.

It was also concluded that the low germination rate of *D. innoxia* could be improved by presoaking the seeds in White's nutrient solution. The use of nutritive solutions to stimulate the germination of species of *Datura* is suggested.

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