trimethoxy amphetamine (8) have been reported to exert moderate hypotensive action in anesthetized animals. Trimeglamide, which can be considered as an analog of azlactone, also exerts only a moderate and transient fall in blood pressure (11). The relative absence of cardiovascular action may be advantageous because these compounds may be more specific in their central effect.

The effect of these compounds on spontaneous motor activity, pentobarbital hypnosis, and conditioned avoidance response shows that all of the five compounds exerted a mild central nervous system depressant action. Compound III was more potent and, in addition to its effect on the three above-mentioned experiments, it also exerted a mild and transient hypotension. The effect on conditioned response is noteworthy because of the specific blockage of CAR without affecting escape response. Only compound I acted in a nonspecific way in blocking both CAR and escape response.

A study of the structure activity relationship has shown that substitution of R_1 and R_2 by trimethoxyphenyl group does not bring about marked change in pharmacological activity. Substitution of the methyl group at position R_2 and the 2,4,5-trimethoxyphenyl group at position R_1 prolongs the pentobarbital sleeping time. A study of compounds I and III has shown that trimethoxy groups at 2,3,4-position in R_1 substitution exert a hypotensive effect as opposed when trimethoxy groups are attached to 2,4,5-position. Substitution of 3,4,5-trimethoxyphenyl group at R_2 position does not bring about change in the biological activity in the molecule. Substitution of *p*-dimethylaminophenyl group at position R_1 with 3,4,5-trimethoxyphenyl group at position R_2 in the compound exerts a hypotensive effect with slight increase in spontaneous activity. These properties are not present when the substituent is the phenyl group at the R position.

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Incorporation of Proline-C^{14} into the Principal Alkaloids of *Datura stramonium* var. *tatula* and *Datura innoxia*

By GERALD SULLIVAN and MELVIN R. GIBSON

Proline-C^{14} made available to root cultures of *Datura stramonium* L. variety *tatula* Torrey and *Datura innoxia* Miller was incorporated into hyoscyamine and scopolamine. Root growth was affected in both species.

EXTENSIVE RESEARCH has been accomplished on the genus *Datura* in an attempt to elucidate the biosynthetic pathway or pathways to alkaloid production. Various amino acids have been used to gain clearer and more precise information concerning the production of alkaloids. James (1) reported significant increases in alkaloid content when *Atropa belladonna* L. leaves were supplied *l*-arginine and *l*-ornithine. Later, van Haga (2), working with sterile root cultures of *A. belladonna*, confirmed that additional amounts of arginine and ornithine yielded increased alkaloid production. However, these and other investigators (2–4) found proline to be ineffective in increasing the plant production of hyoscyamine. Work in this laboratory (5) on *D. stramonium* variety *tatula* in which glutamic acid was added to isolated root cultures caused an increase in growth of the roots and a small but statistically significant decrease in alkaloid content of the roots. In this instance it is possible that the glutamic acid entered the citric acid cycle, contributing to growth, but not the ornithine cycle, leaving unaffected tropane alkaloid production. Other work in this laboratory (6) on the same species showed that tested quantities of *l*-proline inhibited total alkaloid production in isolated root culture, but in one concentration appeared to change relative concentrations of hyoscyamine and scopolamine in favor of scopolamine. This latter work suggested that proline plays a dynamic role in the synthesis of

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tropane alkaloids. Cromwell (7) found that proline stimulated formation of hyoscyamine in *A. belladonna* roots when injected into the stem. In the same plant Schermeister *et al.* (8) found that proline was present in large amounts and accumulated in roots when the alkaloid concentration declined. These workers also found that neither arginine nor ornithine was present in detectable quantities and concluded that proline was a more direct precursor of the tropane alkaloids. Katz *et al.* (9) reported that *l*-proline-C^{14} was a precursor of hydroxyproline and ketoproline in the actinomycin peptides formed by *Streptomyces antibioticus*. Steward and Pollard (10) found that proline can be directly utilized in protein synthesis. When proline-C^{14} was supplied to growing tissue cultures of carrot root phloem, almost all of the radioactivity was found in the proline and hydroxyproline residues of the tissue protein, very little C^{14} being lost as CO_2. More recently, Miller and Kempner (11) found that proline-C^{14} was incorporated into the protein by *Candida utilis*. The ready incorporation of carbon from proline into carrot explants has been shown by Pollard and Steward (12), who demonstrated that radioactive carbon supplied as proline can be detected in the protein 15 minutes after its addition to the external solution.

From the work cited above it appeared obvious that further study was necessary to elucidate the role of proline in the biosynthesis of the tropane alkaloids. This is the purpose of this investigation. The study was limited to the two principal alkaloids of *Datura*, hyoscyamine and scopolamine. Of the two *Datura* species chosen for this investigation, each produces these alkaloids but differ in the relative quantity of each produced (13–16). *D. stramonium* L. variety *tatula* Torrey produces primarily hyoscyamine, whereas *D. innoxia* Miller produces primarily scopolamine.

EXPERIMENTAL

Growth and Processing of Root Cultures.—The methods and procedures followed in this portion of the investigation were those developed and utilized by French and Gibson (5). The seeds were obtained from plants grown in the Washington State University pharmacy greenhouse. The seeds of *D. stramonium* variety *tatula* and *D. innoxia* were sterilized with 3% hydrogen peroxide and shaken on a Kahn automatic shaker for 25 and 15 minutes, respectively. The sterilized seeds were placed in Petri dishes in the incubator for germination at 25 to 27° using sterile techniques. Upon germination, the young root radicle (1.0 to 1.5 cm. in length) was cut from the seed and placed in the appropriate nutrient solution. The root was allowed to grow for 7 days, then transferred to fresh nutrient solution and allowed to grow for 7 more days. The root was then removed and blotted dry on a paper towel, placed in a tared glass vial, and dried at 55 to 60° until constant weight was obtained.

The control root cultures were grown in White's nutrient solution (17); the experimental root cultures were grown in the same nutrient supplemented with: (*a*) 0.001 mg. of unlabeled *l*-proline per 50 ml. of nutrient solution, (*b*) 0.001 mg. of uniformly labeled *l*-proline-C^{14} [1] per 50 ml. of nutrient solution, and (*c*) 0.002 mg. of uniformly labeled *l*-proline-C^{14} per 50 ml. of nutrient solution.

Extraction and Separation of Alkaloids.—The extraction apparatus employed for this procedure was devised by French and Gibson (18). The powdered root was placed in the extractor and moistened with a few drops of a 1:1 solution of 10% ammonia and 95% alcohol. The moistened powder was allowed to stand for 1 hour. One milliliter of chloroform for every milligram of powdered root was poured into the solvent reservoir up to a maximum of 25 ml. The rate of flow of the solvent through the extractor was regulated to coincide with the evaporation of the chloroform on the warm counting planchet. The counting planchet was heated to a temperature of 58 to 60° by a hot plate. Upon completion of the extraction, the counting planchet was allowed to cool to room temperature and radioactivity determined.

The separation of the alkaloids was accomplished using thin-layer chromatography. The total alkaloid extract was subjected to five 200-μl. washings of chloroform. A 25-μl. portion of each chloroform washing was placed on a thin-layer plate. An 8 by 8 by $^1/_8$ in. glass plate with 0.5-mm. thickness of silica gel G [2] was employed. The thin-layer plate was then developed with the developing solvent of 15 ml. of diethylamine and 85 ml. of chloroform. The developing times varied between 55 and 60 minutes. At the conclusion of the developing period, the chromatographic plate was removed and placed in an air circulating oven for 30 minutes at 55 to 60°. The plate was removed from the oven and allowed to cool. It was then sprayed with iodoplatinic acid reagent (19). The alkaloids appeared as dark purple spots. The hyoscyamine and scopolamine spots were removed from the plate by removing all the silica gel from around the spots, using a spatula and a camel's hair brush, the alkaloid spots were scraped and brushed into separate counting planchets. The planchet containing the alkaloid on the silica gel was wetted with 1 drop of distilled water and stirred with a micro spatula until a uniform slurry was formed. The planchet was then placed under an infrared heating lamp and dried for 5 minutes. After cooling, the planchet was ready for counting.

Determination of Radioactivity of the Alkaloids.—The detection and recording of the activity of the radioactive alkaloids was accomplished with a windowless Nuclear-Chicago D-47 gas flow counter and a Nuclear-Chicago model 181B scaler. Nuclear-Chicago "Q-gas" (98.7% helium, 1.3% butane) was used. The planchet containing the radioactive alkaloid was inserted into the lead-shielded counting chamber and counted for a period of 30 minutes at 8 lb. pressure and 1300 v. The counts recorded by the scaler were converted and recorded as counts per

[1] Obtained from Nuclear-Chicago Corp., Des Plaines, Ill.
[2] E. Merck AG., Darmstadt, Germany (obtained from Brinkmann Instruments, Inc., Great Neck, Long Island, N. Y.).

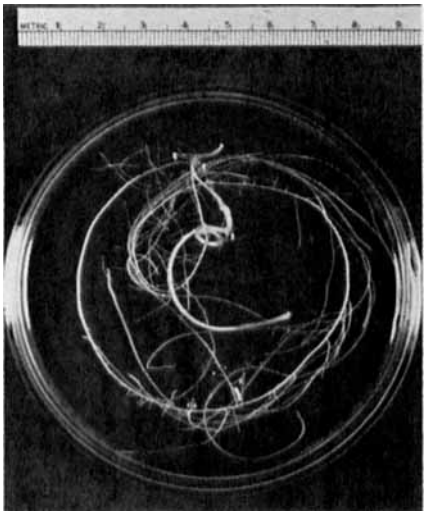

Fig. 1.—*D. stramonium* variety *tatula* root culture grown in control nutrient solution.

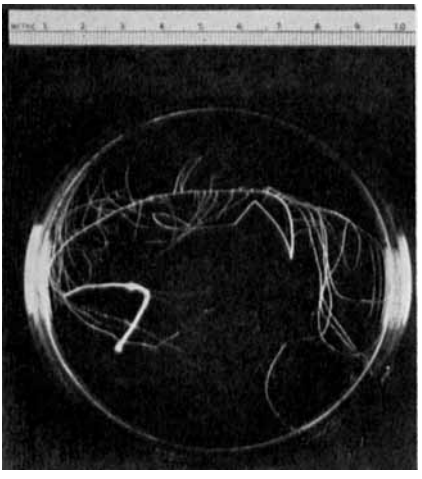

Fig. 2.—*D. innoxia* root culture grown in control nutrient solution.

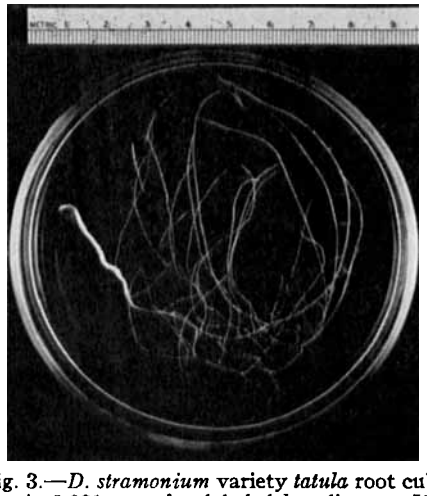

Fig. 3.—*D. stramonium* variety *tatula* root culture grown in 0.001 mg. of unlabeled *l*-proline per 50 ml. of solution.

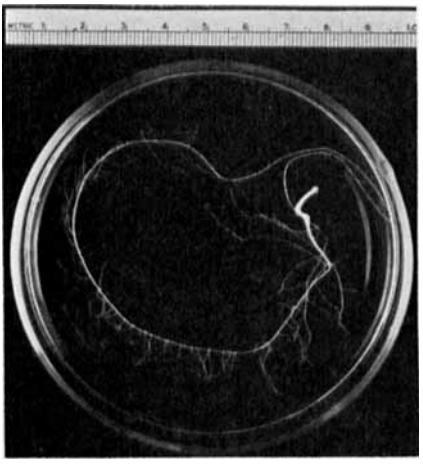

Fig. 4.—*D. innoxia* root culture grown in 0.001 mg. of unlabeled *l*-proline per 50 ml. of solution.

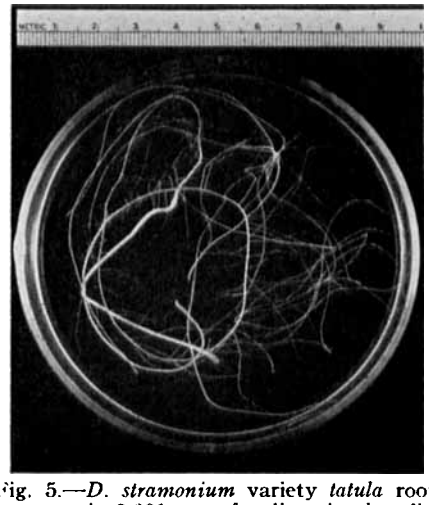

Fig. 5.—*D. stramonium* variety *tatula* root culture grown in 0.001 mg. of radioactive *l*-proline per 50 ml. of solution.

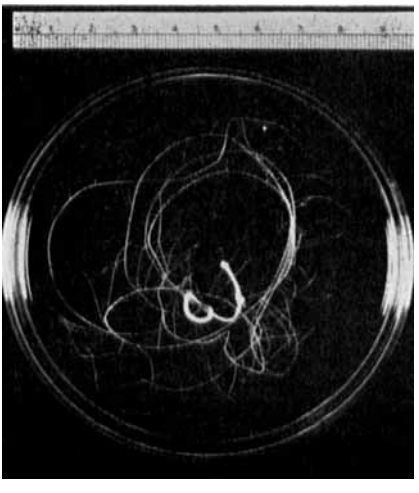

Fig. 6.—*D. innoxia* root culture grown in 0.001 mg. of radioactive *l*-proline per 50 ml. of solution.

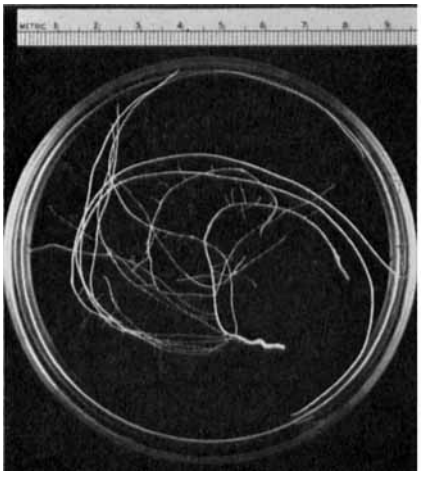

Fig. 7.—*D. stramonium* variety *tatula* root culture grown in 0.002 mg. of radioactive *l*-proline per 50 ml. of solution.

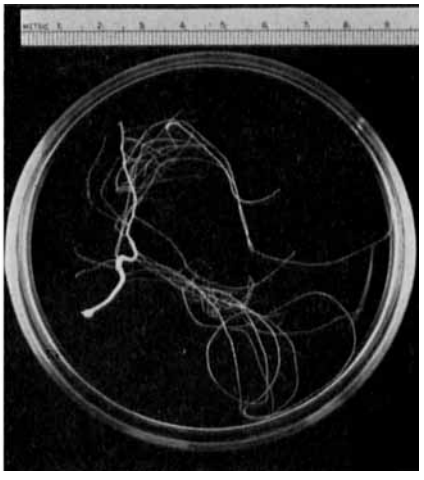

Fig. 8.—*D. innoxia* root culture grown in 0.002 mg. of radioactive *l*-proline per 50 ml. of solution.

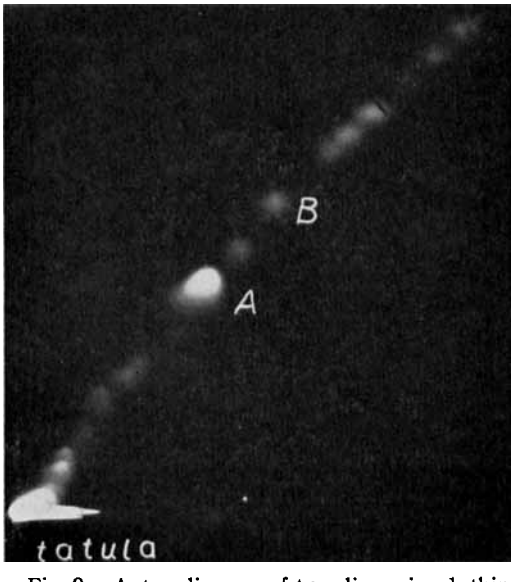

Fig. 9.—Autoradiogram of two-dimensional, thin-layer chromatographic separation of the components of an extract of a *D. stramonium* variety *tatula* root culture grown in a solution containing radioactive proline. Key: A, hyoscyamine; B, scopolamine.

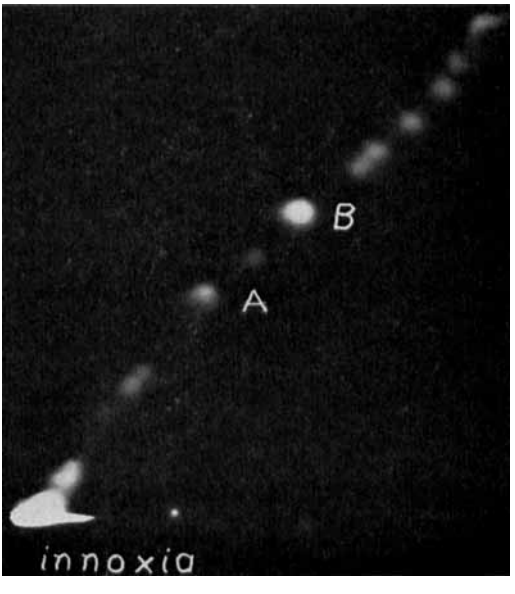

Fig. 10.—Autoradiogram of two-dimensional, thin-layer chromatographic separation of the components of an extract of a *D. innoxia* root culture grown in a solution containing radioactive proline. Key: A, hyoscyamine; B, scopolamine.

TABLE I.—DATA FOR DRY WEIGHT OF ROOTS OF *D. stramonium* VARIETY *tatula* AND *D. innoxia* GROWN IN CONTROL AND PROLINE-FORTIFIED NUTRIENT SOLUTIONS

	No. of Roots		Mean, mg.		Variance		95% Confidence Interval, mg.	
Nutrient Soln.	*D. stramonium tatula*	*D. innoxia*	*D. stramonium tatula*	*D. innoxia*	*D. stramonium tatula*	*D. innoxia*	*D. stramonium tatula*	*D. innoxia*
Control	102	107	14.6	9.2	52.84	14.19	16.0–13.2	9.9–9.5
Proline, 0.001 mg. 50 ml.	30	18	15.5	7.0	24.59	11.02	17.3–13.7	7.9–6.1
Proline,[a] 0.001 mg. 50 ml.	46	22	17.9	7.0	29.30	5.1	19.5–16.3	8.0–6.0
Proline,[a] 0.002 mg.	11	12	10.9	5.6	8.84	6.23	12.9–8.9	7.0–4.2

[a] Radioactive.

minute. The background count throughout this investigation ranged from 23 to 29 counts per minute.

Autoradiography was also employed in this investigation for the determination of the radioactivity of the individual alkaloids to amplify the direct-count data. X-ray exposure holders were constructed to conform to the size of the 8-in. square thin-layer chromatogram plates. All procedures were performed in a photography dark room. The developed thin-layer chromatogram was inserted into the X-ray exposure holder and 2 in. cut from the 8 by 10-in. Kodak medical X-ray, tinted, safety base, duplitized, no-screen film. The 8-in. square film was then placed directly on the thin-layer chromatogram and the X-ray exposure holder securely closed to prevent any light leakage. The sealed holder was weighted down and stored in a dark dry cabinet for an exposure period of 30 days. At the conclusion of the exposure period, the exposed film was removed from the holder and developed for 5 minutes in Kodak D19 developer solution. The resulting autoradiograms showed that all the alkaloids separated from the extract were radioactive.

A two-way autoradiogram was run for *D. stramonium* variety *tatula* and *D. innoxia* extracts of roots grown in 0.002 mg. of *l*-proline-C^{14} in 50 ml. of media for 2 weeks. The first and second developing phases were, respectively, 10:90 and 15:85 of diethylamine:chloroform. Exposure time was 5 months.

RESULTS

D. stramonium variety *tatula* and *D. innoxia* control roots grown in White's media are shown in Figs. 1 and 2. Roots grown in unlabeled *l*-proline are shown in Figs. 3 and 4, in *l*-proline-C^{14} in Figs. 5 and 6, and in double strength *l*-proline-C^{14} in Figs. 7 and 8. The autoradiograms of the root extracts are shown in Figs. 9 and 10.

The dry weight determinations indicate that *D. stramonium* variety *tatula* control roots had a mean dry weight of 14.6 mg.; roots grown in 0.001 mg. unlabeled proline, 15.5 mg.; roots grown in 0.001 mg. labeled proline, 17.9 mg.; and roots grown in 0.002 mg. labeled proline, 10.9 mg. *D. innoxia* control roots showed a mean dry weight of 9.2 mg.; roots grown in 0.001 mg. unlabeled proline, 7.0 mg.; roots grown in 0.001 mg. labeled proline, 7.0 mg.; and roots grown in 0.002 mg. labeled proline, 5.6 mg. These results are summarized in Table I.

Radioactivity determinations of isolated hyoscyamine and scopolamine of both species are shown in Tables II and III. It is obvious that the roots of both species were able to incorporate and metabolize the radioactive carbon of proline into hyoscyamine and scopolamine. It is also interesting to note that *D. stramonium* variety *tatula* which produces predominantly hyoscyamine incorporated more proline into this compound than into scopolamine. The ratio of radioactivity of hyoscyamine compared to scopolamine approaches 2:1. However, in *D. innoxia*, where the plant produces predominantly scopolamine, more proline is incorporated into this compound. The ratio of radioactivity is hyoscyamine compared to scopolamine approaches 1:2. This difference in proline incorporation in the two species is further borne out by the autoradiograms, Figs. 9 and 10.

DISCUSSION

From this study and a previous one (6), the conclusion that exogenous proline affects metabolism in the *Datura* species tested can be made. This effect is seen both in the habit of growth of the roots and total root growth. The response is associated in some unknown way with the concentration of the supplied exogenous proline, in this investigation made more complex by the degree of radioactivity of the supplied proline. The inhibitory action of

TABLE II.—DRY WEIGHT AND RADIOACTIVITY OF ALKALOIDS OF *D. stramonium* VARIETY *tatula* ROOTS GROWN IN 0.001 mg. LABELED PROLINE PER 50 ml. OF SOLUTION

Root No.	Root Wt., mg.	Counts Per Min.[a] Hyoscyamine	Hyoscine	Root No.	Root Wt., mg.	Counts Per Min.[a] Hyoscyamine	Hyoscine
1	10.0	12	17	24	15.8	31	15
2	11.9	16	19	25	17.0	21	8
3	19.6	14	16	26	21.5	27	7
4	11.4	25	8	27	27.1	11	6
5	31.1	22	30	28	20.3	21	13
6	30.2	12	5	29	20.6	39	10
7	21.6	33	25	30	23.0	33	20
8	9.2	30	14	31	24.1	32	20
9	23.7	28	42	32	15.9	40	13
10	8.5	10	18	33	14.8	32	15
11	18.9	18	15	34	19.9	36	17
12	15.8	34	23	35	14.5	34	12
13	12.3	27	9	36	18.8	18	24
14	19.6	13	3	37	23.9	41	18
15	15.4	31	3	38	13.0	26	15
16	11.0	29	29	39	16.8	22	21
17	24.8	41	21	40	19.8	50	16
18	14.2	8	0	41	19.3	41	19
19	18.3	21	17	42	16.0	18	3
20	21.1	28	9	43	26.2	25	24
21	13.2	13	9	44	12.8	39	13
22	20.2	25	13	45	18.9	26	12
23	10.2	12	0	46	14.8	20	7
Mean					17.9	26	15

[a] Half-hour counts.

TABLE III.—DRY WEIGHT AND RADIOACTIVITY OF *D. innoxia* ROOTS GROWN IN 0.001 mg. LABELED PROLINE PER 50 ml. OF SOLUTION

Root No.	Root Wt., mg.	Counts per Min.[a] Hyoscyamine	Hyoscine
1	3.7	2	6
2	6.6	7	7
3	9.2	5	8
4	3.2	0	0
5	8.8	9	16
6	7.7	3	15
7	10.0	9	23
8	8.6	8	13
9	5.9	2	4
10	4.2	2	7
11	6.5	0	2
12	4.9	2	5
13	5.5	1	9
14	11.3	7	13
15	7.5	6	16
16	5.8	0	0
17	9.3	7	23
18	3.5	3	2
19	8.9	6	16
20	8.9	12	18
21	6.4	0	0
22	7.5	1	9
Mean	7.0	4	10

[a] Half-hour counts.

amino acids on plants is not new (20, 21). Klein and Linser (22) found that in cut tobacco plants in proline-containing nutrient media the nicotine content increased in leaves and decreased in stems in some instances; whereas in others the media increased the nicotine content of both stems and leaves. The mechanism of these functions is unknown.

Some insight into the fate in plants of exogenous proline has been supplied by studies utilizing proline-C^{14}. The ready incorporation of carbon from proline in carrot explants has been shown by Pollard and Steward (12). They concluded from this study that the proline which enters the cell is either incorporated immediately into protein or, escaping this fate, is respired away. That the bulk of the proline appears as protein rather than as carbon dioxide suggests that protein synthesis, or at least the synthesis of proline-containing protein, occurs in the nonparticulate cytoplasm fraction before the proline ever gets to the principal site of the respiratory process—namely, the mitochondrial particles.

Thus, it can be anticipated that proline from an exogenous source is incorporated into the metabolic pool of *D. stramonium* variety *tatula* and *D. innoxia*. This is borne out by several observations. The dried roots of each are highly radioactive. The extract prepared as described in this paper is highly radioactive, but less so than the entire root. The hyoscyamine and scopolamine as well as other unidentified compounds are radioactive, separating out on the chromatogram as evidenced on the autoradiograms. The hypothesis that proline enters into the normal metabolism of the plant also is suggested by the fact that in the "hyoscyamine plant" (*D. stramonium* variety *tatula*) the greater radioactivity was in the hyoscyamine, whereas in the "scopolamine plant" (*D. innoxia*) the greater radioactivity was in the scopolamine.

SUMMARY

When *l*-proline-C^{14} was supplied to isolated roots of *D. stramonium* variety *tatula* and *D. innoxia*, growth was stimulated in the former using 0.001 mg. per 50 ml. of nutrient solution; however, growth was inhibited in the former at the 0.002-mg. concentration. Inhibition of growth was seen in *D. innoxia* at both levels of concentration.

The radioactive proline was picked up from the solution by the roots of both species and incorporated into hyoscyamine and scopolamine. The *D. stramonium* variety *tatula* roots which produce primarily hyoscyamine, compared to scopolamine, incorporated more radioactive proline into the principal alkaloid. *D. innoxia* roots which produce primarily scopolamine, compared to hyoscyamine, incorporated more radioactive proline into the principal alkaloid. There is evidence that the proline enters into the metabolism of these plants, possibly by way of protein. The work suggests that proline is an important compound utilized by these plants in the biosynthesis of the tropane alkaloids.

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