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11-4

78. Izumi Imaseki : Phytochemical Investigation on Cultivation of Medicinal Plants. XIII.[1] On the Alkaloid Biogenesis in Datura. (3).[2]

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Numerous workers have reported that the alkaloid formation in Datura plant would take place in the roots and not in the leaves. However, as described in the previous paper,[3] Shibata and Imaseki observed a small increase of alkaloid in the scion of Datura grafted upon tomato stock. Therefore, it should be concluded that although Datura alkaloids are formed principally in the roots, some possibility of alkaloid formation in the leaves cannot entirely be ruled out. A similar conclusion has also been given by some workers. Cromwell[4] recongnized that the excised leaves of *Atropa belladonna* cultivated in the dark would respond to the biogenesis of alkaloid; similarly James[5] pointed out that the alkaloid formation would take place in the aerial part of *A. belladonna*. As has been shown in our previous works, a tracer technique using ^{15}N is the most valuable for the experiment on alkaloid biogenesis. The present study has been planned to use distilled water (Experiment I) or $(^{15}NH_4)_2SO_4$ solution (Experiment II) as the medium of cultivation of detached leaves. After cultivation, the amount of nitrogen and content of the alkaloid and saccharides in the leaves were determined. At the same time, investigations were made to see whether ^{15}N had been incorporated in the hyoscyamine and scopolamine isolated from these plant material.

Experimental

Material and Method

Experiment I—*Datura Tatula* L. was sown in the beginning of April, 1955, in the Medicinal Plant Field attached to this Institute and the plants growing in unified state were selected. On August 1, in the stage of flower-bud formation, 84 mature leaves (4.94±0.1 g. in fresh weight), as well as the top younger and the bottom older leaves, were picked at random from 3 plants about 99.3±0.4 cm. in height, and every three leaves were stood in their petioles in a vessel, filled with distilled water, and kept in the dark. These were divided into 4 groups, each consisting of 7 vessels. One group was tested as a control and the leaves of each group were collected at the end of 2, 4, and 6 days. The leaf materials, after being collected, were rapidly dried, weighed, and pulverized to prepare samples for determinations of alkaloid, total N, protein N, total saccharide, monosaccharide, oligosaccharide, and polysaccharide. The room temperature range recorded was 20~30° and the humidity was 75±9% during the cultivation.

Experiment II—**(A)** The Datura plants were cultivated in 1956 under the same conditions as given in Experiment I. On July 12, 130 mature leaves (3.64±0.18 g. in fresh weight) in the stage of growing were collected. These were divided into 2 groups and one group of 25 leaves was dried immediately as a control. The remaining 105 leaves were dipped by their petioles into a medium containing $(^{15}NH_4)_2SO_4$ during 3 days and then they were left in distilled water for succeeding 3 days in the dark. The medium for cultivation consisted of $(^{15}NH_4)_2SO_4$ 0.015 mol./L. (N : 425 p.p.m., 12.592 atom% ^{15}N excess), and sucrose 0.005 mol./L.; pH 6.2.

The leaves were removed from the solution on the 6th day of cultivation and weighed. The leaf material, whose petiole was cut off to avoid mechanical contamination of $(^{15}NH_4)_2SO_4$, was dried and submitted to the determination of the amount of alkaloid, total N, protein-N, amino-N, amide-

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1) S. Shibata, I. Imaseki, M. Yamazaki : This Bulletin, **5**, 71(1957).
2) Part (2). I. Imaseki : *Ibid.*, **3**, 329(1955).
3) S. Shibata, I. Imaseki : J. Pharm. Soc. Japan, **73**, 797(1953).
4) B. T. Cromwell : Biochem. J. (London), **31**, 551(1937).
5) W. O. James : New Phytologist, **48**, 172(1949); cf. "The Alkaloid," Academic Press, Vol. 1, 53 (1950).

N, and N of alcohol-soluble portion by the methods given below. The ^{15}N-concentration in each fraction was estimated with the Consolidated Mass Spectrometer, Model 21-103 A. The room temperature range recorded during the cultivation was 22~28° and humidity was 82~85%. The amount of the solution absorbed was 770 cc. for 0~1 day, 550 cc. for 1~2 days, 530 cc. for 2~3 days, 425 cc. for 3~4 days, 215 cc. for 4~5 days, and 185 cc. for 5~6 days ($^{15}NH_4^+$ 1850 cc., H_2O 825 cc., total 2675 cc.).

(B) An experiment was repeated under the same conditions. Twenty-one young leaves were cultivated during 4 days in the medium containing $(^{15}NH_4)_2SO_4$, 0.015 mol./L. (pH 6.2) alone, and afterward in distilled water for 3 days. The collected leaves were dried and pulverized. The pulverized material was extracted to isolate total alkaloid and ^{15}N concentration of the alkaloid was estimated.

Procedure—(1) Alkaloid: The determination was made as described in the previous paper.[3]

(2) Total N: Determined by the micro-Kjeldahl method using the leaf material.

(3) Protein-N: The protein fraction given here is that of leaf material precipitated by 10% CCl_3COOH and was measured by the micro-Kjeldahl method.

(4) Ammonia-N: Estimated by the Folin's method.[6]

(5) Amide-N: The amide-N given here is that obtained by deduction of the amount of ammonia-N from the nitrogen estimated by the Delwiche's method.[7]

(6) Amino-N: The water-soluble portion of leaf material was estimated by the Van Slyke method.

(7) N in EtOH-soluble Portion: This is N in the fraction extractable with EtOH.

(8) Measurment of ^{15}N Concentration: The determinations of alkaloid N, total N, protein-N, ammonia-N, amide-N, and N in EtOH-soluble portion were as described in the previous paper.[3] For the amino-N, 1 g. of leaf material was extracted with hot water; the aqueous extracts was then adjusted with H_2SO_4 to 2~3%, and heated at 100° for 2.5 hrs. to hydrolyze the amide. The solution was basified with K_2CO_3 and ammonia was removed by suction. The non-volatile portion was passed through an Ambrerlite IR-112 column which absorbed amino acid and other cationic substances. The amino acid was removed from the Amberlite column by elution with 2*N* AcONa and was used for ^{15}N-determination.

(9) Monosaccharide: The leaf material was extracted with 80% EtOH for 1.5 hrs. and EtOH was evaporated to leave an aqueous layer, which was then treated with $CdSO_4$ reagent ($3\,CdSO_4 \cdot 8\,H_2O$ (26.2 g.) and *N* H_2SO_4 (132 cc.) in 1000 cc. H_2O), heated at 100° for 3 mins., and filtered. The amount of saccharide in the filtrate was estimated as glucose by the micro-Bertrand method.

(10) Oligosaccharide: The above saccharide solution was then adjusted with HCl to 2% solution and used for the estimation of oligosaccharide by the above method. The amount of oligosaccharide was given as the difference of the result obtained by this fraction and the amount of monosaccharide.

(11) Polysaccharide: The leaf material was extracted with water, heating at 100° for 1.5 hrs. The filtered solution was used for estimation of polysaccharide by the foregoing methods (9 and 10). The amount of polysaccharide was derived by the deduction of the amount of mono- and oligosaccharides from this estimated result.

(12) Paper Partition Chromatography: The alkaloid fraction was separated by developing with a solvent mixture of BuOH : AcOH : H_2O (4 : 1 : 5). The spot was detected with the Dragendorff and ninhydrin reagents.

Results

Experiment I—The results obtained by the present experiment are shown in Table I and Figs. 1 and 2. The amounts of alkaloid, protein-N, and mono- and polysaccharide in each sample which was picked up on 2nd, 4th, and 6th days of cultivation were recognized as different statistically. However, in comparision with the control, total N and polysaccaride showed no remarkable difference by the different cultivation period. Thus the starvation experiment on leaves detached from the plants showed an increase in alkaloid, whereas there was no marked difference between the rate of formation of hyoscyamine and that of scopolamine.

By the paper chromatography of the alkaloid fraction only two spots corresponding to hyoscyamine (Rf 0.78 ± 0.01) and scopolamine (0.69 ± 0.02) were detected by Dragendorff's reagent, while a slight spot appeared at Rf 0.33 ± 0.01 by ninhydrin reagent. No marked quantitative difference was shown in the spots developed on the paper chromatograms by each experimental group.

Experiment II. (A) and (B)—It was observed that leaf color turned yellowish green. At the end of these experiments, change of color was similar as observed on the 3rd or 4th day of Experiment I. Weight of leaf material was 3.64 ± 0.11 g. when fresh (0.45 g. of dry material) at the beginning and was 2.46 ± 0.10 g. in fresh weight (0.46 g. of dry material) at the end of Experiment II-A.

6) O. Folin, *et al.*: J. Biol. Chem. **11**, 523(1912).
7) C. C. Delwiche: *Ibid.*, **189**, 167(1951).

Table I. Experiment I
Starvation Experiment in Detached Datura Leaves (mg./10 leaves)

		0 day			2nd day			4th day			6th day		
		%	mg.	ratio	%	mg.	ratio	%	mg.	ratio	%	mg.	ratio
Alkaloid	Total alk.	0.233±0.010	15.8	100	0.256±0.012	16.1	100	0.356±0.006	22.0	100	0.394±0.03	24.6	100
	Hyoscyamine	0.182	12.3	78	0.200	12.6	78	0.282	17.4	79	0.307	19.1	78
	Scopolamine	0.046	3.1	20	0.051	3.2	20	0.068	4.2	19	0.079	4.9	20
	Other bases	0.002	0.1	1	0.003	0.2	1	0.003	0.2	1	0.008	0.5	2
Nitrogen	Total N	4.36 ±0.08	295.2	100	4.99 ±0.04	300.8	100	4.84 ±0.02	299.1	100	4.82 ±0.02	300.3	100
	Protein-N	3.78 ±0.06	255.9	87	3.21 ±0.03	201.6	67	1.99 ±0.05	123.0	41	1.86 ±0.06	115.9	39
	Nonprotein-N	0.58	39.3	13	1.58	99.2	33	2.85	176.1	59	2.96	184.4	61
Saccharide	Total sacch.	4.24 ±0.07	287.0	100	2.87 ±0.08	180.2	100	2.79 ±0.05	172.4	100	1.47 ±0.09	91.6	100
	Mono- 〃	0.84 ±0.06	56.9	20	0.06 ±0.02	3.8	2	—	—	—	—	—	—
	Oligo- 〃	2.38 ±0.07	161.1	56	1.77 ±0.08	111.2	62	1.71 ±0.08	105.7	61	0.48 ±0.06	29.9	33
	Poly- 〃	1.02 ±0.07	69.1	24	1.04 ±0.08	65.3	36	1.08 ±0.05	66.7	39	0.99 ±0.09	61.7	67
Fresh weight*		4.94±0.10			4.83±0.20			3.91±0.14			2.69±0.10		
Dry weight*		0.68			0.63			0.62			0.62		
Leaf color		green			slightly yellow			yellow			brown		

* g./leaf

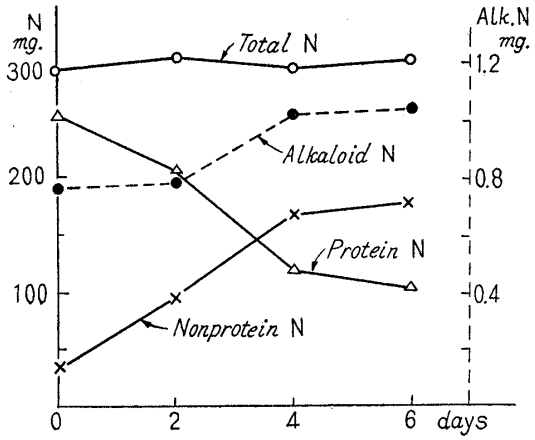

Fig. 1. Variation of N Content in Experiment I (mg./10 leaves)

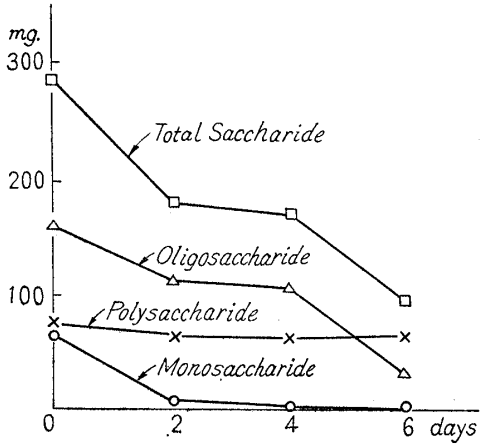

Fig. 2. Variation of Saccharide Content in Experiment I (mg./10 leaves)

Table II. Experiment II
Alkaloid Biogeneses in Detached Dutura Leaves by Assimilation of $^{15}NH_3$-N

Experiment II-(A) mg./10 leaves, $(^{15}NH_4)_2SO_4$=12.592 atom% ^{15}N excess

	Control			Treatment				Formed		
	%	mg.	ratio	%	mg.	ratio	^{15}N % excess	mg.	ratio	Formation ratio*%
Total Alkaloid	0.384±0.002	17.3	100	0.397±0.005	18.1	100	—	0.089	100	0.49
Hyoscyamine	0.210	9.4	54	0.218	9.9	55	0.060±0.001	0.047	53	0.48
Scopolamine	0.161	7.2	42	0.159	7.3	40	0.073±0.003	0.042	47	0.58
Other bases	0.009	0.4	2	0.016	0.9	4	—	—	—	

Experiment II-(B)

Total Alkaloid : 0.529±0.004% (16.3 mg.), ^{15}N% excess : 0.138±0.003%
Amount formed, 0.179 mg., Formation ratio*, 1.10%

* The ratio of the alkaloid formed by ^{15}N-feeding to the total amount of alkaloid in the leaves.

Table III. Experiment II-(A)
Nitrogen Metabolism in Detached Datura Leaves by Assimilation of $^{15}NH_3$-N
$(^{15}NH_4)_2SO_4$=12.592 atom % ^{15}N excess, mg./10 leaves

	Control			Treatment				Formed		Formation ratio[a] %	Increasing ratio[b] %
	%	mg.	ratio	%	mg.	ratio	^{15}N% excess	mg.	ratio		
Total N	6.18±0.01	278.2	100	7.18±0.03	327.8	100	1.847±0.005	48.1	100	14.7	+15.1
Protein-N	4.88±0.04	219.8	79	2.62±0.05	119.6	37	0.475±0.003	4.5	9	3.8	—45.6
Amino-N	0.24±0.02	10.8	4	1.44±0.04	65.7	20	1.765±0.005	9.2	19	14.0	+83.5
N in alcohol soluble portion	0.84±0.02	37.8	14	1.51±0.03	68.9	21	2.093±0.005	11.4	24	16.6	+45.2
Amide-N	0.07±0.02	3.2	1	0.87±0.04	39.7	12	4.340±0.007	13.7	28	34.5	+92.0
Ammonia-N	0.07±0.02	3.2	1	1.03±0.04	47.1	14	3.341±0.007	12.5	26	26.6	+93.3

a) The ratio of N incorporated by ^{15}N-feeding to the total amount of N in the leaves.
b) The ratio of N increased after ^{15}N-feeding to the total amount of N in the leaves.

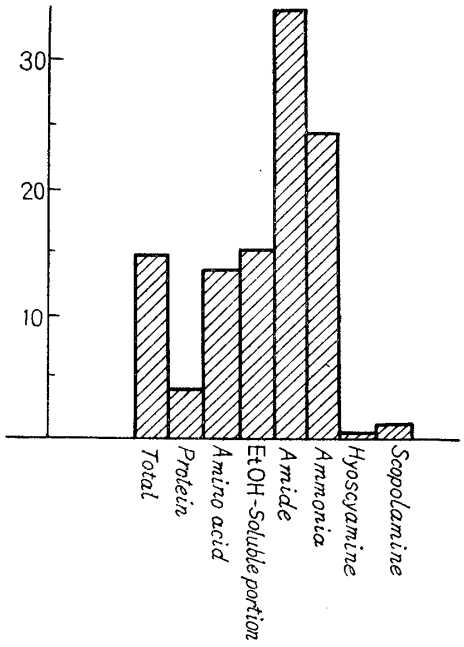

Fig. 3. Incorporation Rate of ^{15}N in Experiment II-(A)
$(^{15}NH_4)_2SO_4$: 12.592 atom% ^{15}N excess=100

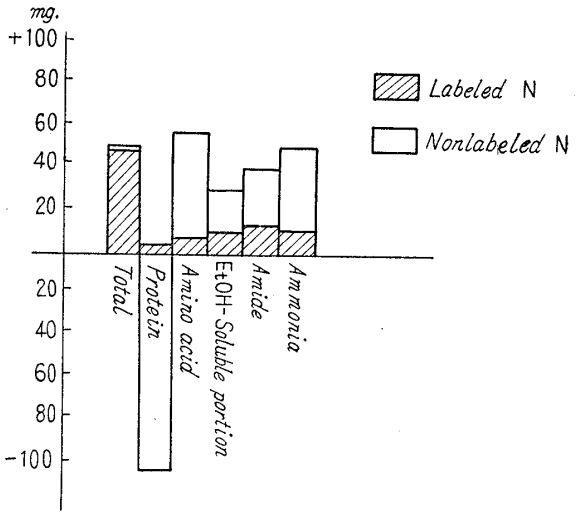

Fig. 4. Vicissitude of Nitrogen Content in 10 leaves, in Experiment II-(A)

Tables II and III, and Figs. 3 and 4 give the results of analyses of alkaloid and nitrogen. ^{15}N was incorporated into every N fraction of the leaf material.

The paper partition chromatography of alkaloid fraction gave the same result as in Experiment I.

Discussion

In Experiment I, the detached leaves of Datura which were dipped in distilled water by their petioles and placed in darkness showed an increase of alkaloid, especially at the later part of the experimental period. However, no marked difference between the rate of formation of hyoscyamine and scopolamine was shown. Although total nitrogen did not vary during the experiment, protein was decomposed rapidly to about 55% after six days, which might be transformed into other non-protein N fraction. In regard to saccharide, mono- and oligo-saccharides decreased, while the amount of polysaccharide remained unchanged. Under the conditions of Experiment I, protein and saccharides of the leaf were consumed rapidly, while the alkaloid was synthesized, though the amount was not so large.

In Experiment II-(A), although the increase of alkaloid in the detached leaves hardly appeared by the usual assay method, it was evidently proved by mass spectrometry.

as given in Table Ⅲ and Fig. 3, that $^{15}NH_3$–N was incorporated into alkaloid nitrogen during the experimental period. The rate of formation of hyoscyamine and scopolamine was almost equal. Consequently, it has been confirmed that Datura leaf has an ability to produce the alkaloid. The amount of total nitrogen which increased during the experiment corresponded to the amount of ^{15}N absorbed, while the amount of protein–N decreased about 46% after 6 days' cultivation as the decomposition surpassed biosynthesis, which was shown by the small incorporation of $^{15}NH_3$–N. Amino acid, amide, ammonia, and N of ethanol-soluble portion were chiefly derived from ordinary N sources in the leaf material, while the small incorporation of $^{15}NH_3$–N in these fractions was also proved (Table Ⅲ, Fig. 4). The ratio of the alkaloid formed from $^{15}NH_3$–N to the amount of ^{15}N-labeled total N absorbed in the leaves was 1.2~1.7% in the intact plant[2] and about 0.2% in the detached leaves as given by the present experiment. Perhaps the alkaloid formation in the detached leaves is not efficient as that taking place in the intact plants.

In the experiment with the medium containing $^{15}NH_3$–N alone (Experiment II–(B)), the rate of alkaloid formation was little more than that by Experiment II–(A) (Table II) using the medium containing $(^{15}NH_4)_2SO_4$ and sucrose. The results of the present study involving above three experiments have apparently shown that the formation of alkaloid can also take place in the leaves of Datura plants.

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Summary

The detached leaves of *Datura Tatula* L. was cultivated with their petioles in distilled water or $(^{15}NH_4)_2SO_4$ solution. ^{15}N was proved to be incorporated into the alkaloid isolated from the leaves. By these results, it could be concluded that the alkaloid can be biosynthesized in the leaves of Datura plant.

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