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53. **Shoji Shibata and Izumi Imaseki**: Phytochemical Investigation on Cultivation of Medicinal Plants. X.* On the Alkaloid Biogenesis in Ephedra. (2).[1]

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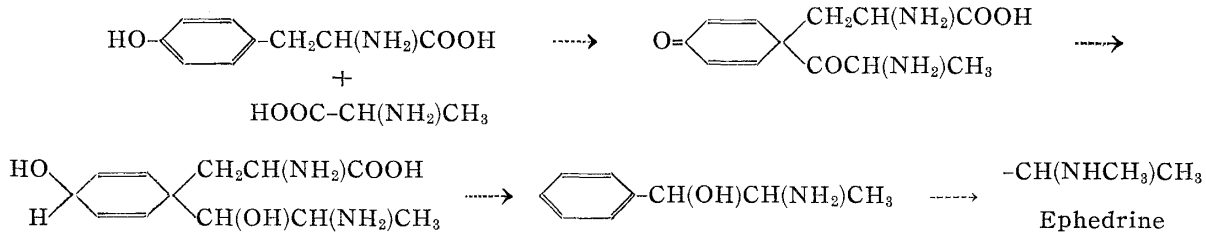
Using ^{15}N-labeled ammonium sulfate as a tracer, we confirmed previously that the formation of *l*-ephedrine can take place in the aerial part of Ephedra plant.[1]

The present communication is concerned with further investigation on the pathway of the biosynthesis of ephedrine.

Numerous works have been reported concerning the biosynthesis and metabolic change of epinephrine (Adrenaline) whose structure is closely related to ephedrine. Using ^{14}C as a tracer element, Gurin *et al.*[2] proposed that phenylalanine would take part as a precursor of epinephrine and denied the theory forwarded by Rosenmund and Dornsaft[3] that the condensation of benzaldehyde and sarcosine might occur in animal body to give epinephrine.

In analogy to the hypothesis which has been advanced by Burn[4] who claimed that epinephrine may be formed by the condensation of dihydroxyphenylalanine (DOPA) and glycine or sarcosine, Robinson[5] suggested, though he noted as being purely speculative, that a similar scheme might be applied to the formation of ephedrine, starting with tyrosine and alanine.



An additional contribution to studies on the biosyntheses of analogous alkaloids has been made by Marion *et al.*[6] by the use of ^{14}C as a tracer element in proving that hordenine in barley is formed from tyrosine and tyramine.

Regarding the above-mentioned works, it seems necessary to give a direct evidence that alanine or phenylalanine takes part as a precursor of ephedrine.

Thus the present experiment was designed and Ephedra plant was fed ^{15}N-labeled alanine or phenylalanine. After a certain period of hydroponic cultivation, ^{15}N-concentration in ephedrine isolated from the plant material was determined by mass-spectrometry.

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* Part IX. This Bulletin, **3**, 328(1955).

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1) Part(1). S. Shibata, I. Imaseki : This Bulletin, **1**, 285(1953).
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4) J. H. Burn : Sir Jessy Boot Foundation Lecture, Nottingham Univ., p. 1. (1950).
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Experimental

Syntheses of ^{15}N-Labeled Amino Acids—DL-^{15}N-Alanine was prepared by the method of Kendall and McKenzie[7] using $^{15}NH_4Cl$ derived from $(^{15}NH_4)_2SO_4$(6.93 atom% excess ^{15}N) as a source of ^{15}N. DL-^{15}N-Alanine thus obtained gives m.p. 289~290°; 6.924 atom% excess ^{15}N.

DL-^{15}N-Phenylalanine was prepared by the method of Dakin[8] and Schoenheimer *et al.*[9] ^{15}N was introduced at the final step of the reaction by amination of phenylpyruvic acid with $^{15}NH_3$ (10.16 atom% excess ^{15}N). The product gives m.p. 263°; 9.965 atom% excess ^{15}N.

Plant Material—The plant material employed for this experiment was *Ephedra distachya* L. grown in the Experimental Field for Medicinal Plants attached to the Tokyo University Forestry Experimental Station at Tojo, Chiba Pref.

The plant was removed from ground on May 17, 1954, for the experiment using ^{15}N-alanine (Experiment A), and on September 29, 1955, for the experiment using ^{15}N-phenylalanine (Experiment B).

The hydroponic cultivation of the plant started on the following day when the tracer compound was administered in the culture solution. The excised aerial portion of the plant was used for the Experiment A just the same as indicated in the previous work[1] in which $(^{15}NH_4)_2SO_4$ was used as the N source. Experiment B was carried out employing both the detached aerial portion (Expt. B-1) and the whole plant (Expt. B-2).

The plant was cultivated in a room, avoiding direct sunlight for 1 week after administration of the ^{15}N-amino acids.

The components of the solution employed for the cultivation are given in Table I.

Table I.

Expt.	A Aerial Part (Fresh wt. 223.8 g.)	B-1 Aerial Part (Fresh wt. 200.0 g.)	B-2 Whole Plant (Fresh wt. 305.0 g.)
	DL-^{15}N-Alanine (6.924 Atom% excess ^{15}N)	DL-^{15}N-Phenylalanine (9.965 Atom% excess ^{15}N)	DL-^{15}N-Phenylalanine
N	500 mg./L.(0.036 M.)	500 mg./L.(0.036 M.)	100 mg./L.(0.007 M.)
P_2O_5		5.0 mg./L.(KH_2PO_4)	
K_2O		5.0 mg./L.(KCl)	
MgO		4.0 mg./L.($MgSO_4 \cdot 7H_2O$)	
CaO		4.0 mg./L.($CaCl_2$)	
Fe_2O_3		Trace ($FeCl_3$)	

pH of the solution was adjusted to 6.6.

The plant material was removed from the solution on the 7th day of cultivation, weighed, and dried at 55~65° to constant weight.

The isolation of *l*-ephedrine from the grassy portion of the plant material was achieved as described in the previous peper.[1] *l*-Ephedrine which was isolated in the form of its hydrochloride melted at 217°, giving a single spot of Rf 0.81±0.01 on a paper chromatogram developed with BuOH:HOAc:H_2O(4:1:2).

The content of total-N, protein-N and non-protein-N in the plant materials were also determined by the usual method,[1] while the ^{15}N-concentration in each fraction was estimated with the Consolidated Mass Spectrometer, Model 21-103 A.

Results

The temperature range recorded during the cultivation was 17.5~22.0° for Experiment A and 18.0~28.5° for Experiment B.

The growth of plant observed during the experimental cultivation A and B-1 was slight, while it was much evident in the case of Experiment B-2.

At the end of cultivation, the weight of the plant materials, the amount, and pH of the culture solution used, and free and bound ammonia remaining in the solution were estimated as given in Table II.

On examination by paper chromatography, no amino acids other than that administered could be detected in the media employed.

Table III shows the results of analyses of the concentration of ^{15}N in ephedrine isolated and

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8) H. D. Dakin : J. Biol. Chem., **82**, 439(1929).
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the calculated amount of newly formed ephedrine.

The amount of protein-N and total-N which were accumulated or newly absorbed during the experiment are given in Table IV.

Table II.

Expt. No.	Plant materials: Grassy stem Fresh (g.)	Grassy stem Dried (g.)	Woody stem Fresh (g.)	Woody stem Dried (g.)	Residual culture solution: Amt. remaining (cc.)	pH	Free NH_3* (mg.)	Bound NH_3** (mg.)	Amt. of culture soln. supplied (cc.)
A	182.5	54.5	41.3	10.5	174	7.2	1.9	4.6	500
B-1	140.0	49.2	38.5	18.0	350	6.8	3.0	5.3	500
B-2	158.0	61.8	—	—	740	6.8	2.8	5.2	1000

* NH_3 steam-distilled out at the original pH.

** NH_3 steam-distilled out after addition of 0.1*N* NaOH.

Table III.

Expt. No.	Tracer compd. given*	Ephedra alkaloid accumulated: %	Ephedra alkaloid accumulated: mg.**	*l*-Ephedrine formed: ^{15}N%excess	*l*-Ephedrine formed: mg.	Ephedrine formed/Ephedra alkaloid accumulated %	Ephedrine-N formed/Total-N absorbed %
A	DL-^{15}N-Alanine	0.297±0.016	161.5	0.006±0.005	±	—	—
B-1	DL-^{15}N-Phenylalanine	0.205±0.006***	100.9	0.012±0.003	0.12	0.12	0.03
B-2	〃	0.199±0.009***	123.9	0.019±0.001	0.24	0.20	0.08

* Concentration of ^{15}N : 6.924 atom%-excess in alanine; 9.965 atom%-excess in phenylalanine.

** Content (mg.) of alkaloid in the whole grassy portion of the plant materials : Expt. A : 54.5 g.; Expt. B-1 : 49.2 g.; Expt. B-2 : 61.8 g.

*** Content of alkaloid in the plant material at the start of the experiment (Expt. B) was 0.200±0.006%.

Table IV.

Expt.		Accumulated N: %	Accumulated N: mg.	Accumulated N: Ratio	Absorbed N: ^{15}N %excess	Absorbed N: mg.	Absorbed N: Ratio	Absorbed N per plant %	Absorbed N/Accumulated N %
A	Total-N	3.62±0.02	1972.9	100	0.255±0.004	72.7	100	0.133	3.69
	Protein-N	2.37±0.05	1291.7	65	0.134±0.001	25.0	34	0.046	1.94
	Non-protein-N	1.25	681.2	35	—	47.7	66	0.088	7.00
B-1*	Total-N	2.75±0.01	1353.0	100	0.295±0.003	40.1	100	0.082	2.96
	Protein-N	1.90±0.03	934.8	69	0.141±0.003	13.2	33	0.027	1.41
	Non-protein-N	0.85	418.2	31	—	26.9	67	0.055	6.44
B-2*	Total-N	2.71±0.03	1674.8	100	0.142±0.002	23.9	100	0.039	1.43
	Protein-N	2.03±0.07	1254.5	75	0.022±0.001	2.8	12	0.005	0.22
	Non-protein-N	0.68	420.2	25	—	21.8	88	0.034	5.03

* Content of accumulated N in the plant material at the start of experiment (Expt. B) : Total-N 2.58±0.06%; protein-N 2.11±0.01%; non-protein-N 0.47%.

Discussion

The results set out in Tables II to IV indicate that ^{15}N in phenylalanine is incorporated into ephedrine formed during one-week cultivation of Ephedra plant, whereas it is not the case for ^{15}N-alanine.

It should be noted, however, that the amount of ^{15}N fixed in ephedrine derived from ^{15}N-phenylalanine is much less than that obtained by the administration of ^{15}N in the form of ammonium sulfate.[1] This would reasonably be understood by the fact that ammonium ion is much readily absorbed by a plant than organic amino acids.

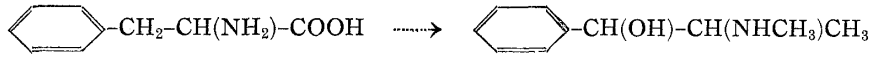
Comparison of the content of ^{15}N fixed in the protein and the non-alkaloidal fraction of Ephedra plant fed $(^{15}NH_4)_2SO_4$, ^{15}N-alanine, and ^{15}N-phenylalanine (500 p.p.m. N each) showed that the highest rate of absorption of N is obtained in the plant

material treated with ammonium sulfate, and the lowest value is given in that fed phenylalanine. The ratio of the ^{15}N-absorption in the protein fraction of the plant materials treated with alanine and phenylalanine (1.7 : 1.0) almost corresponds to the ratio of content of these amino acids constituting proteins of the plant material (1.6: 1.0), which was estimated approximately by the area of spots developed by ninhydrin on a paper chromatogram[10] and by retention paper chromatography.[11]

Since the decomposition of the administered amino acids might occur in the solution, liberating ammonia which might be re-utilized to form alkaloid, free and bound ammonia (ammonium salt and amide) in the residual culture solution were estimated (Table II). The result showed that the presence of ammonia in the culture solution is so slight that it cannot give any significant influence to the biosynthesis of an alkaloid.

According to Schoenheimer's experiment,[12] the possibility of direct interchange of ^{15}N of amino acids administered with ^{14}N of ephedrine present in the plant could be ruled out.

Thus it should not be unreasonable to conclude that phenylalanine takes part as a precursor of ephedrine whereas alanine is not incorporated into ephedrine.



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Summary

By the hydroponic cultivation of Ephedra plant using ^{15}N-labeled amino acids as the nitrogen source, it has been shown that phenylalanine takes part as a precursor in the biosynthesis of *l*-ephedrine.

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