are separated and analyzed in a larger quantity than by any other method hitherto used when the molasses examined was mixed with sugars, proteins, or rubberized substances. The tryptophan content of molasses has never been mentioned in any literature. The result of paper partition chromatography by the one- and two-dimensional methods and recovery rates will be dealt with on another occasion.

Summary

Nineteen kinds of amino acid contained in beet molasses from Hokkaido were quantitatively separated and analyzed by the new method in which ion exchanger and paper partition chromatography were applied, when the amino acids were mixed with sugars, proteins and other inorganic substances.

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104. Shoji Shibata, Izumi Imaseki, and Mikio Yamazaki: Phytochemical Investigation on Cultivation of Medicinal Plants. XIV*. On the Alkaloid Biogenesis in Ephedra. (4).**

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Earlier papers of this series introduced some new findings on the biosynthesis of ephedrine in Ephedra. ^{15}N-Labeled amino group of phenylalanine, but not alanine, was proved to be incorporated into *l*-ephedrine by one week's cultivation of Ephedra in the Houghland solution added with ^{15}N-labeled amino acids.[1] Therefore, it is quite likely that phenylalanine serves as a precursor of ephedrine. The preceding work also provided an evidence that ^{14}C-methyl group of methionine is transferred into N-methyl group of ephedrine in intact Ephedra plant.[2]

The present experiment has been designed to obtain a precise evidence for the origin of methyl group in the γ-position of the side chain in ephedrine molecule, and ^{14}C-labeled formate was administered to Ephedra plant to investigate the localization of radioactivity in *l*-ephedrine molecule isolated after ten days' cultivation.

Experimental

Plant Material—*Ephedra distachya* L. grown in the Experimental Field for Medicinal Plants attached to Tokyo University Forestry Experimental Station at Tojo, Chiba Pref., was lifted from the ground on May 22, 1957. The hydroponic cultivation of the plant started from the following day employing the Houghland solution added with ^{14}C-labeled formate.

The components of the solution are as follows: KNO_3, $5\times10^{-3}\,M$; KH_2PO_4, $1\times10^{-3}\,M$; $Ca(NO_3)_2$·

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* Part XIII. This Bulletin, **5**, 447(1957).
** (3). *Ibid.*, **5**, 71(1957).
*** Hongo, Tokyo (柴田承二, 山崎幹夫).
**** Kamimeguro 8-Chome, Meguro-ku, Tokyo (今関和泉).
1) S. Shibata, I. Imaseki: This Bulletin, **4**, 277(1956).
2) S. Shibata, I. Imaseki, M. Yamazaki: *Ibid.*, **5**, 71(1957).

$4H_2O$, $5\times10^{-3}M$; $MgSO_4\cdot7H_2O$, $2\times10^{-3}M$; $H^{14}COONa$, $3\times10^{-4}M$ (0.5 mc.); minor elements, trace.

The plant (1.6 kg.) was divided into two bottles, each of which was filled with 2 L. of the medium, and cultivated for 10 days as indicated in the preceding paper.[2] The temperature range recorded during the cultivation was 18~22°.

The plant material was removed from the solution on the 10th day of cultivation and the grassy portion (dry weight : 122.5 g.) was used for extraction of *l*-ephedrine. The procedure of extraction of *l*-ephedrine was indicated in the previous paper.[1] Yield of *l*-ephedrine : 0.17%.

Degradation of *l*-Ephedrine—*l*-Ephedrine hydrochloride (250 mg.) isolated from the plant material was heated with 85% H_2SO_4 (2.5 g.) on a boiling bath for 12 hrs. to give methylamine and methyl benzyl ketone. *d*-Tartrate of methylamine (m.p. 176°) and semicarbazone of methyl benzyl ketone (m.p. 190°) were prepared for determination of radioactivity.

Methyl benzyl ketone was oxidized with alkaline $KMnO_4$ to give BzOH (m.p. 122.5°)(yield : 43%) and AcOH.

BzOH (35 mg.) was decomposed by the Schmidt reaction[3,4] treating with conc. H_2SO_4 (0.12 g.), benzene (0.2 cc.), and NaN_3 (60 mg.) at 40° for 1 hr. in giving aniline (characterized as benzoate, m.p. 162°; yield, 23%) and CO_2 (precipitated as $BaCO_3$; yield, 35%).

AcOH derived from methyl benzyl ketone as mentioned above was degraded by the Schmidt reaction[3,5] using conc. H_2SO_4 (0.12 g.) and NaN_3 (60 mg.) at 60° for 2 hrs. to give methylamine (chloroplatinate, m.p. 230°(decomp.)) and CO_2 ($BaCO_3$, 28 mg.).

A modified apparatus used for the Schmidt reaction is shown in Fig. 1.

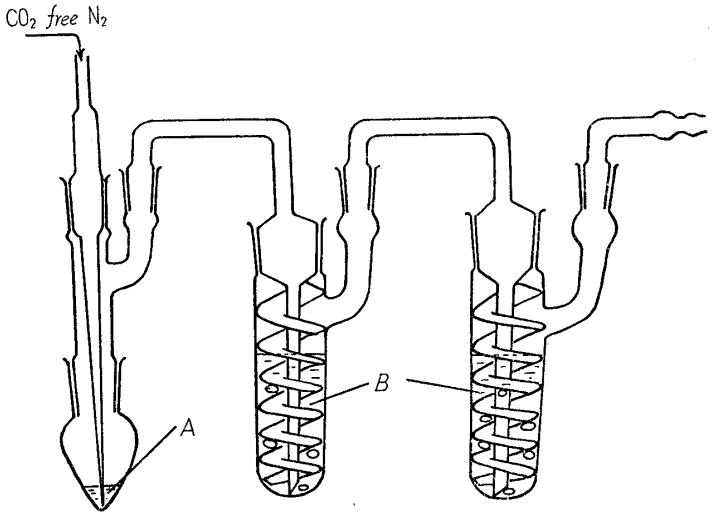


Fig. 1.

Schmidt Reaction Apparatus

A : Reaction flask B : Absorption vessel

The sample (AcOH and BzOH were used in the present study) is placed in the flask A, and mixed with NaN_3 and conc. H_2SO_4 under ice-cooling. The flask A is connected with the vessel B containing satd. $Ba(OH)_2$ solution. The flask is heated slowly in a water bath (40~60°) in N_2 (CO_2-free) stream. CO_2 evolved is absorbed in vessel B to form $BaCO_3$. When AcOH is used as the sample, the vessel B is replaced, after the reaction finished, with another vessel B filled with dil. HCl. The reaction mixture in the flask A is made alkaline and then heated in N_2-stream to absorb CH_3NH_2 in dil. HCl in B. In the case of BzOH, the Schmidt reaction mixture in the flask A is made alkaline and aniline is taken up in ether.

Results and Discussion

l-Ephedrine isolated from the plant after 10 days' hydroponic cultivation using ^{14}C-formate as a source of C_1-fragment was degraded as shown in Chart 1.

The radioactivity of each degradation fragment was measured with a "Q"-gas flow counter (Nuclear Instrument and Chemical Corp.) attached to the Scaler (Kaken Model 32) to present the localization of incorporation of C_1-fragment :

3) H. Wolff : Org. Reactions, **III**, 307(1946).
4) L. H. Briggs, J. W. Lytteleton : J. Chem. Soc., **1943**, 421.
5) C. Schuerch, E. H. Huntress : J. Am. Chem. Soc., **71**, 2233(1949).

Chart 1.

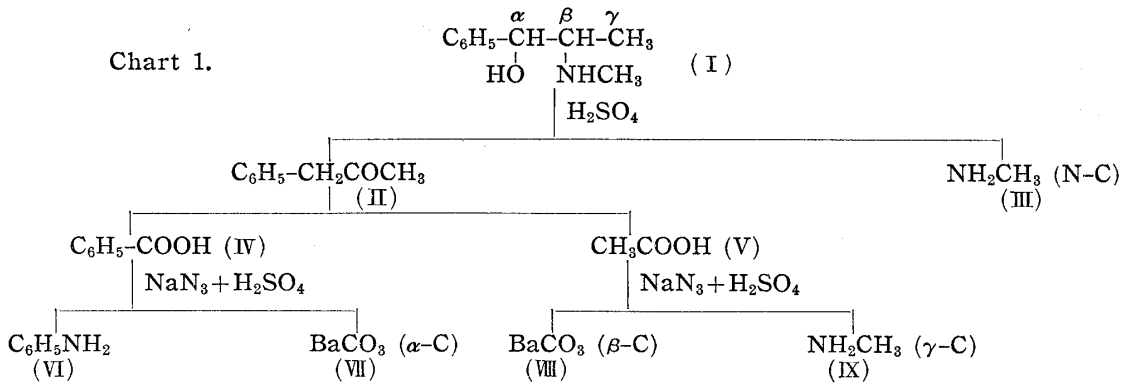


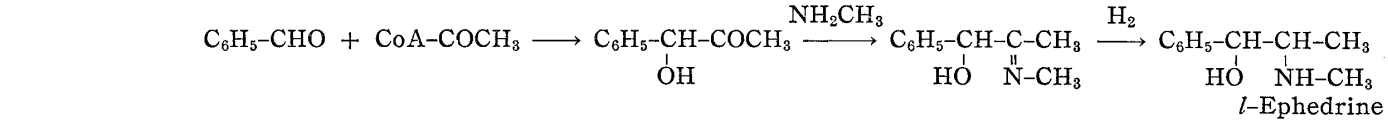
Table I.

	Found c.p.m./mM	Corrected[a] c.p.m./mM	Ratio of Incorporation[b] (%)	Form of compd. used for estimation of radioactivity
l-Ephedrine (I)	8,400	8,400	100	Hydrochloride, m.p. 216°
Methylamine (N-C) (III)	3,000	3,500	42	*d*-Tartrate, m.p. 176°
Benzyl methyl ketone (II)	4,200	4,900	58	Semicarbazone, m.p. 190°
Benzoic acid (IV)	1,000	1,200	14	m.p. 112.5°
Aniline	1,200	1,400	17	Benzoate, m.p. 162°
Methylamine (γ-C) (IX)	2,600	3,100	37	Chloroplatinate, m.p. 230°
$BaCO_3$ (α-C) (VII)	0	0	0	
$BaCO_3$ (β-C) (VIII)	300	300	4	

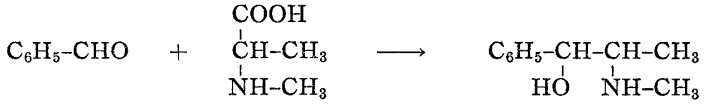
a) Correction was made on the basis of the radioactivity of *l*-ephedrine.
b) Radioactivity(%) of the degradation fragment calculated on the basis of activity of *l*-ephedrine.

The present experimental results suggest some considerations on the scheme of biosynthesis of *l*-ephedrine in Ephedra. Hilderbrandt and Klavehn[6] presented a practical asymmetric synthesis of *l*-ephedrine involving the biological reaction of benzaldehyde in fermenting sugar solution in the presence of yeast.

The mechanism for ketol formation which is involved in the above reaction was studied thoroughly by Smith and Hendlin[7] who represented it as follows :



Akabori and Momotani[8] reported that *dl*-pseudoephedrine was formed by the condensation of benzaldehyde and N-methylalanine on boiling with pyridine, and they suggested that such a reaction would be involved in the biosynthesis of ephedrine and also in that of epinephrine.



The above mechanisms may be considered as plausible for the biosynthesis of ephedrine in the plant.

On the other hand, however, several evidences have been presented for the alternative scheme of biosynthesis in which phenylalanine is responsible as the precursor of ephedrine. By the isotopic labeling method, the incorporation of phenylalanine

6) G. Hildebrandt, W. Klavehn : U.S. Pat. 1,956,950 (C. A., **28**, 4072(1934)); D. R. P. 548,459 (C. A., **26**, 3623(1932)). cf. C. Neuberg, J. Hirsch : Biochem. Zeitschr., **115**, 282(1921).
7) P. F. Smith, D. Hendlin : J. Bacteriol., **65**, 440(1953).
8) S. Akabori, K. Momotani : Proc. Imp. Acad. (Tokyo), **17**, 506(1941).

was confirmed by Gurin and Delluva[9] in the biosynthesis of epinephrine, and that of tyrosine in the case of hordenine formation was shown by Marion, *et al.*[10]

$$C_6H_5\text{-}CH_2\text{-}{}^{14}CH(NH_2)\text{-}{}^{14}COOH \xrightarrow{\text{adrenal gland}} (3,4)\,(OH)_2C_6H_3\text{-}CH(HO)\text{-}{}^{14}CH_2(NHCH_3)$$ Epinephrine

$$(p)HO\text{-}C_6H_4\text{-}CH_2\text{-}{}^{14}CH(NH_2)\text{-}COOH \xrightarrow{\text{sprouting barley}} (p)\text{-}HO\text{-}C_6H_4\text{-}CH_2\text{-}{}^{14}CH_2[N(CH_3)_2]$$ Hordenine

As reported in our previous paper,[1] phenylalanine labeled with ^{15}N was found to be incorporated into *l*-ephedrine in Ephedra, whereas it was not the case with ^{15}N-labeled alanine.

As ephedrine is regarded to be a homolog of epinephrine and hordenine, it seems quite plausible that phenylalanine would take part as a precursor in the biosynthesis of ephedrine.

Once this concept has been accepted, the origin of methyl group in the γ-position of side chain in the ephedrine molecule should be considered. It is noted that ephedrine and other Ephedra bases differ from epinephrine and hordenine in possessing the C_6–C_3 unit in their constructions, while the latter contains the C_6–C_2 unit.

The direct conversion of the carboxyl of phenylalanine into γ-methyl group of ephedrine may be plausible at first glance, it seems not to be acceptable from biochemical point of view.

As indicated in the biogenesis of epinephrine and hordenine, phenylalanine is readily decarboxylated in the first step of biosynthesis of ephedrine, and the coupling of C_1-fragment occurs at the same position by the aid of appropriate biological factors.

The present experiment resulted in the incorporation of ^{14}C-formate in almost the same extent in N-methyl and C-methyl (the γ-methyl) of ephedrine.

As the specific activity localized in both methyls in a remarkably high degree, the above hypothesis of C_1-fragment incorporation can conclusively been adopted.

The formation of hydroxyl at the carbon atom adjacent to the benzene ring of ephedrine would occur at the appropriate stage of biosynthesis for which no evidence has yet been provided.

$$C_6H_5\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH \longrightarrow C_6H_5\text{-}CH_2\text{-}CH_2(NH_2) \leftarrow {}^{14}C \longrightarrow C_6H_5\text{-}CH(HO)\text{-}CH(NH\text{-}{}^{14}CH_3)\text{-}CH_3$$

The authors wish to thank Prof. S. Watanabe of the Tokyo University Forestry Experimental Station in Chiba Pref. for supplying Ephedra used for the present study. The expenses of this work were supported by the Grant in Aid for Scientific Research provided by the Ministry of Education to which the authors' thanks are due.

Summary

It was proved that formate labeled with ^{14}C is incorporated into γ-methyl and N-methyl groups in *l*-ephedrine molecule in *Ephedra distachya*. The mechanism of biosynthesis of ephedrine coupling with the C_1-fragment incorporation is discussed.

(Received August 10, 1957)

9) S. Gurin, A. M. Delluva : J. Biol. Chem., **170**, 545(1947).
10) E. Leete, L. Marion : Can. J. Chem., **31**, 126(1953).