Biosynthesis of 3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline and 1-Methyl-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline in a Callus Culture of *Stizolobium hassjoo*

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DL-[β-^{14}C] 3,4-Dihydroxyphenylalanine (DOPA), [2-^{14}C]pyruvate and [2-^{14}C]acetate were administered to a callus culture of *S. hassjoo* and incorporation of the radioactivity into 3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (**I**) and 1-methyl-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (**II**) was examined. Incorporation of radioactivity from labelled DOPA into **I** and **II**, and from acetate into **I** were observed, while that from pyruvate into **I** and **II** and from acetate into **II** were hardly detected. The biosynthetic pathways of **I** and **II** were discussed.

Introduction

3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (**I**) and 1-methyl-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (**II**), non-protein imino acids, were detected in *Stizolobium hassjoo* [1], *Mucuna mutisiana* [2] and *M. deeringiana* [3], which belong to Lotoideae, a subfamily of Leguminosae. These imino acids, **I** and **II**, contain a β-phenethylamine residue, linked with one and two carbon unit, respectively. The origin of these carbon units is of biosynthetic interest. Synthetic mechanism of the isoquinoline ring is thought to start from phenylalanine, tyrosine or dopamine, which combines with an additional building block such as α-keto acid to generate an isoquinoline ring system [4, 5]. In our previous paper [1], we demonstrated that 3,4-dihydroxyphenylalanine (DOPA) could serve as a precursor both **I** and **II**.

This report describes the results obtained from tracer experiments using labelled putative precursors of **I** and **II** in a callus culture of *S. hassjoo,* and the biosynthetic pathway of **I** and **II** is also discussed.

Materials and Methods

Materials

A callus culture of *S. hassjoo* was obtained under the same condition as described previously [1]. Radiolabelled substrates used in this study were obtained from following sources: DL-[β-^{14}C]DOPA (54 mCi/mmol) and [2-^{14}C]pyruvate (8.2 mCi/mmol), NEN (Boston, USA); [2-^{14}C]acetate (45–55 mCi/mmol), CEA (Gif-sur-Yvette, France). ACS-II was purchased from The Radiochemical Centre Ltd (Amersham, England).

Feeding of radiolabelled compounds

31–33 Days old callus (0.92–2.5 g fresh wt) was incubated in 1.6–1.7 ml of the culture medium containing radiolabelled compounds (DOPA, 10 µCi; pyruvate, 50 µCi; acetate, 100 µCi) for 24 h at 27 °C in a flask with a centre well in which a small tube containing 0.5 ml scintillamine was placed to trap evolved carbondioxide. Amino acids were extracted succesively four times from the callus by refluxing in hot 80% ethanol. Extracts were concentrated with a rotary evaporator and spotted on thin-layer plates (Avicel, 10 × 10 cm), which developed ascendingly in phenol/water (27:10, w/v). Cellulose powder of each area corresponding to **I** and **II** on the chro-

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matograms was collected separately and eluted out with 80% ethanol. **I** and **II** were recrystallized four to six times with the synthetic samples from 10% acetic acid. Radioactivity was measured in ACS-II using a Beckman liquid scintillation spectrometer (model LSC-9000).

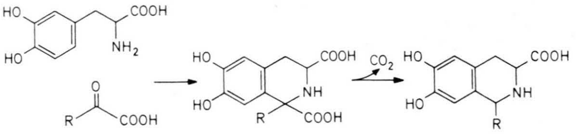
Results and Discussion

A callus culture of *S. hassjoo* at the late log phase, in which the highest accumulation of DOPA was observed during the growth cycle [6], was incubated with radiolabelled substrates as described previously [1]. After incubation, **I** and **II** were isolated from callus and the incorporation of radioactivity was examined. Table I shows that radiolabelled DOPA was practically utilized to the formation of **I** and **II**. About 10 and 0.6% of the radioactivity incorporated into the soluble fraction was found in **I** and **II**, respectively. The incorporation of radioactivity from DOPA into **I** and **II** was confirmed by co-crystallization with the authentic specimens until to show constant specific radioactivity as shown previously [1]. Incorporation of DOPA into tetrahydroisoquinoline alkaloids has been extensively investigated [7–12]. In all cases examined DOPA was predominantly incorporated into β-phenethylamine portion of the molecule. Battersby *et al.* [10] reported that 96% of the radioactive label from DOPA was located in the β-phenethylamine moiety of the aporphine alkaloid, glaucine. Similarly, Bhakuni and co-workers observed that 97% of the radioactivity of intact reticuline isolated from *Litsea glutinosa* fed with [β-^{14}C]-labelled DOPA was located in the "upper" half of the molecule [11]. Above results suggest the possibility that DOPA incorporated is exclusively utilized to the synthesis of 3,4-dihydroxy-β-phenethylamine unit of **I** and **II**.

Table I. Incorporation of radioactivity from labelled compounds administered as precursors of **I** and **II** in a callus culture of *S. hassjoo.*

Radiolabelled compound fed	% of radioactivitiy incorporated into			
	I		**II**	
[β-^{14}C]DOPA	9.97[a]	(3.98)[b]	0.592	(0.236)
[2-^{14}C]acetate	0.034	(0.0038)	0.0099	(0.0011)
[2-^{14}C]pyruvate	0.0075	(0.0046)	0.0038	(0.0023)

[a] % in the soluble fraction.
[b] % in total uptake.



R = H 3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (**I**)
R = CH_3 1-Methyl-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (**II**)

Scheme 1. Possible biosynthetic routes leading to 3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (**I**) and 1-methyl-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (**II**) in *S. hassjoo.*

On the basis of their results, it can be postulated that the tetrahydroisoquinolines, **I** and **II**, would be derived directly from DOPA and α-keto acid by the route shown in Scheme I. Synthesis of the tetrahydroisoquinoline ring system in **I** and **II** initiates to form C-N bridge between an aromatic ring carbon at C-6 and α-amino radical at C-2 of the alanyl residue of DOPA by the addition of oxogenous one or two carbon unit. In recent years the origin of C-1 and its substituent in tetrahydroisoquinoline alkaloids has been investigated in many laboratories [13–16]. Leete and Braunstein [15] showed that [3-^{14}C]pyruvate was incorporated specifically into the C-9 methyl portion of tetrahydroisoquinoline alkaloid, halonidine, in *Lophophora williamsii.* Stolle and Gröger [16] have similarly found that the two carbon unit (C-1 and C-10) of the β-carboline alkaloid, harmine, is formed from C-2 and C-3 of radiolabelled pyruvate in *Peganum harmala.*

The nature of the α-keto acid which contributes to generate C-1 and/or C-9 carbon block in **I** and **II** still remains obscure. To clarify this problem we administered [2-^{14}C]pyruvate or [2-^{14}C]acetate, which could be considered as putative precursors, to the callus and examined the incorporation of the radioactivity into **I** and **II**. Radioactive compound of another possible precursor, glyoxylate, was not available. *In vivo* incubation with pyruvate, very poor incorporation into both **I** and **II** was found (Table I). Only about 0.008 and 0.004% of the radioactivity of pyruvate incorporated into the soluble fraction were detected in **I** and **II**, respectively. Results of co-crystallization of labelled **I** and **II** with the authentic specimens (Table II) indicate that specific activities of **I** and **II** decreased to as low levels as those in the blank test (Table III) in which authentic **II** was recrystallized in the extract from the region of high radioactivity and of different R_f value of **II** on a thin-layer chromatogram of the extract of callus fed with [2-^{14}C]pyruvate. These results suggest that radioactivity of [2-^{14}C]pyruvate was hardly incorporated into **I** and **II**, and are not in agreement with those reported previously [15, 16]. Since it seemed reasonable that the two carbon unit (C-1 and C-9) in **II** and the one carbon unit (C-1) in **I** would be derived from acetic acid, [2-^{14}C]acetate was fed to the callus of *S. hassjoo* and the incorporation of radioactivity into **I** and **II** was investigated. Table I shows that the incorporation rate of radioactivity from labelled acetate into **I** was relatively high (0.034%), while that into **II** was low (0.0099%). The results of co-crystallization with authentic specimens also indicate that the incorporation of radioactivity from [2-^{14}C]acetate into **I** was significant but that into **II** was not (Table IV). Because of low yield of radioactive **I** after recrystallization, further oxidation of **I** to confirm the location of radioactivity in the molecule could not be carried out. The results obtained here suggest that the origin of C-1 in **I**, and C-1 and C-9 in **II** would be different from those in tetrahydroisoquinoline alkaloids such as anhalamine or anhalonidine [15, 16] although DOPA was confirmed to be the direct precursor of β-phenethylamine portion of **I** and **II** as well as in other tetrahydroisoquinolines. Failure of acetate and pyruvate to be incorporated into **II** is rationalized by suggesting that the callus of *S. hassjoo* contains no enzyme capable of utilizing both acetate and pyruvate directly for formation of two carbon units of **II**, *i. e.*, that neither acetate nor pyruvate can serve as the direct precursor of **II**. Battersby *et al.* [5] found that the administration of [1-^{14}C]acetate to the peyote cactus, *Lophophora williamsii* yielded radioactive pellotine, but they suggested that acetic acid was not a direct precursor of the two carbon unit. Incorporation of acetate into **I** was observed, suggesting that acetate could be the origin of the one carbon unit of **I** whether or not it is a direct precursor of **I**. But acetyl CoA derived from acetate seems not to be the direct precursor of **I**, because the incorporation of pyruvate, which could be easily converted to acetyl CoA, into **I** was not observed. The incorporation rate of DOPA into **II** was lower than that into **I**, indicating that the conversion of DOPA to **I** may be more active than that of the same precursor to **II** *via* a different route or that **I** may be synthesized from DOPA and acetate, and **II** may be synthesized from **I** by some subsequent reaction, *e.g.*, by methylation of C-1 portion of **I**. Further studies, however, should be required to clarify the synthetic mechanisms of the one and two carbon unit in **I** and **II**, respectively.

Table II. Recrystallization of radiolabelled **I** and **II** obtained after feeding of [2-^{14}C]pyruvate to a callus culture of *S. hassjoo*.

Compound recrystallized	No. of recrystallization	Weight [mg]	Specific activity [dpm/µmol]
I	1	46.0	172
	2	36.8	20.7
	3	28.4	5.1
	4	20.1	4.4
	5	13.1	2.7
	6	6.7	2.2
II	1	49.8	103
	2	39.2	5.8
	3	26.5	14.2
	4	16.0	2.9
	5	9.9	1.6
	6	2.9	1.1

Table III. Decrease in specific activity of **II** at blank test.

No. of recrystallization	Weight [mg]	Specific activity [dpm/µmol]
1	18.9	140
2	11.0	37.1
3	5.7	15.0
4	3.7	3.6

Table IV. Recrystallization of radiolabelled **I** and **II** obtained after feeding of [2-^{14}C]acetate to a callus culture of *S. hassjoo*.

Compound recrystallized	No. of recrystallization	Weight [mg]	Specific activity [dpm/µmol]
I	1	47.2	409
	2	38.4	80.5
	3	25.7	26.8
	4	13.9	19.0
	5	8.1	15.7
	6	2.1	15.6
II	1	40.9	93.2
	2	29.4	12.4
	3	14.5	8.29
	4	3.0	4.77

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