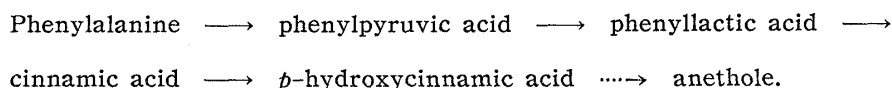**143. Ko Kaneko :** Biogenetic Studies on Natural Products. V.[1]
Biosynthesis of Anethole by *Foeniculum vulgare.* (2).

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In the preceding paper,[1] it was suggested that the phenylpropane skeleton of phenyl-alanine was incorporated *in vivo* into anethole molecule without rearrangement of its side chain.   Therefore, it has been suggested that the sequence of anethole biosynthesis from phenylalanine might be as follows :

Phenylalanine $\longrightarrow$ phenylpyruvic acid $\longrightarrow$ phenyllactic acid $\longrightarrow$

cinnamic acid $\longrightarrow$ *p*-hydroxycinnamic acid $\cdots\rightarrow$ anethole.

On the other hand, Neish, *et al.*[2] suggested that, in salvia plant, phenylalanine, phenylpyruvic acid, phenyllactic acid, and tyrosine are good precursors of plant phenolic acids, such as *p*-hydroxycinnamic acid, caffeic acid, etc., and the process of the change of *p*-hydroxycinnamic acid to caffeic acid to sinapic acid is the main pathway of lignin formation.

Direct evidence suggested that such a pathway was also present in Umbelliferae plants. Moreover, phenylpyruvic acid, cinnamic acid, and *p*-hydroxycinnamic acid were present in the acid fraction of Foeniculum plants.

The only literature pertaining to the biogenesis of these acids is a note by Geissman and Swain[3] in which they reported that caffeic acid can be synthesized from phenylala-nine by buckwheat.

As for biosynthesis of anethole from phenylalanine in a plant, there are two possible ways; the one is to prove the pathway from phenylalanine by the cell-free enzyme system of the plant and the other is to detect it by the use of ^{14}C-labeled hypothetical inter-mediate from above results.

In the present work, anethole biosynthesis was investigated by the cell-free enzyme system obtained from *Foeniculum vulgare.*

Experimental

Cultivation of Plant—The root of *Foeniculum vulgare* M. was grown in a greenhouse after being cut down in summer, and used in autumn and winter.

Extraction and Isolation of Enzyme System—The plants were harvested when grown to 20 cm. in height in the greenhouse. 100 g. of the plant was minced into small pieces and homogenized in a large-scale Waring blender at 0° to 5°, with 100 cc. of isotonic 0.3M sucrose solution, which contained 0.03M of nicotinamide, 0.001M of $MgCl_2$, and 0.5M of NaF.   The plant brei was centrifuged at 0° for 20 min.   The supernatant solution contained relatively large amount of *p*-hydroxycinnamic acid. *p*-Hydroxycinnamic acid was extracted from this deproteinized supernatant with Et_2O, which was washed twice with satd. $NaHCO_3$ solution. $NaHCO_3$ solution was acidified with dil. HCl solution and reëxtracted with Et_2O.   *p*-Hydroxycinnamic acid was obtained after evaporation of Et_2O and recrystallized from H_2O, melting at 208°.   The mixed m.p. with an authentic sample of synthesized *p*-hydroxycinnamic acid, described later, did not show any depression and its Rf value on paper chromatogram agreed with that of the authentic sample.

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1) Part IV. This Bulletin, **8**, 611(1960).
2) D. R. McCalla, A. C. Neish : Can. J. Biochem. Physiol., **37**, 537(1959).
3) T. A. Geissman, T. Swain : Chem. & Ind. (London), **1957**, 987.

	BuOH satd. with 1.5N NH$_4$OH	BuOH-EtOH (50:10) satd. with 1.5N NH$_4$OH
Authentic p-hydroxycinnamic acid (synthesized)	0.268	0.388
Crystals extracted from the plant	0.267	0.390
Cinnamic acid (synthesized)	0.512	0.565

3×40 cm. sheet of Toyo Roshi No. 51-A filter paper used.

120 cc. of the supernatant solution of the homogenate was precipitated with (NH$_4$)$_2$SO$_4$(solid) to 100% saturation at 0°. This precipitated protein was collected by centrifugation in the cold, taken up in a minimum amount of 0.02M phosphate buffer (pH 7.0), and exhaustively dialysed against the same buffer at 0°. Anethole synthesis from phenylalanine[2-^{14}C] is expected by this protein fraction and its lyophilised product.

Bioassay of Enzymatic Synthesis of Anethole and p-Hydroxycinnamic Acid—The incubation mixture for the assay of the anethole biosynthesis contained 0.01M adenosine triphosphate (ATP), 0.005M MgCl$_2$, 0.01M tris(aminomethane) buffer (pH 7.0), 0.02M NaH$_2$PO$_4$, 0.02M dl-phenylalanine (containing 3 mc of dl-phenylalanine[2-^{14}C]), 0.02M dl-methionine, 0.02M 2-oxoglutarate, 2 mg. of diphosphopyridine dinucleotide (DPH$^+$), 2 mg. of nicotinamide, and 2 mg. of glutathione. Total volume of the reaction mixture was 3.0 cc. and the mixture was set in a large Thunberg tube, eliminating the air and replaced with N$_2$ or H$_2$. The Thunberg tube was shaken in Warburg bath at 30° for 3~4 hr.

The reaction mixture was acidified with 10% HCl, 20 mg. of pure anethole added, and extracted with Et$_2$O for 5 hr. The Et$_2$O solution was extracted with a satd. NaHCO$_3$ solution, Et$_2$O was evaporated to a small volume, and purified by silica gel chromatostrip, as described previously.[1] The anethole fraction was extracted with Et$_2$O.

The bicarbonate solution was acidified with 10% HCl and reëxtracted for 5 hr. with Et$_2$O. This Et$_2$O extract was evaporated to a small volume, 20 mg. of p-hydroxycinnamic acid added, and submitted to paper chromatography, applied in a narrow band to a 12×38 cm. sheet of Toyo Roshi No. 51-A filter paper. This chromatogram was developed with BuOH satd. with 1.5N NH$_4$OH. The phenolic acid bands were detected by spraying bromophenol blue solution. The Rf values of p-hydroxycinnamic acid and cinnamic acid were as described above. The spot of p-hydroxycinnamic acid was eluted with Et$_2$O using a small Soxhlet extractor.

Measurement of Radioactivity—Radioactive samples were burnt and its radioactivity measured as described previously.[1]

Preparation of Phenylpyruvic Acid, p-Hydroxyphenylpyruvic Acid, Cinnamic Acid, and p-Hydroxycinnamic Acid—Phenylpyruvic acid was prepared extemporaneously from $α$-acetamidocinnamic acid,[4] obtained by the method given in the literature.[5] The crystals of phenylpyruvic acid melted at 152° with decomposition.

p-Hydroxyphenylpyruvic acid, m.p. 218~219°(decomp.), was prepared by a method similar to that for phenylpyruvic acid, from p-hydroxybenzaldehyde instead of benzaldehyde.[6]

p-Hydroxycinnamic acid, m.p. 208°, was prepared by the method of Furst, et al.[7] A mixture of 12.2 g. of p-hydroxybenzaldehyde and 10.4 g. of dry malonic acid was dissolved in 5 cc. of dry pyridine containing 5 drops of piperidine. The solution was placed in an incubator at 37° for 7 days and poured into a mixture of 18% H$_2$SO$_4$ and crushed ice. The crystals were collected by filtration and recrystallized from EtOH and H$_2$O.

Cinnamic acid, m.p. 134~135°, was prepared by the same method as for p-hydroxycinnamic acid synthesis, using benzaldehyde instead of p-hydroxybenzaldehyde.

Result

The cell-free enzyme system from *Foenicum vulgare* was found to synthesize anethole from phenylalanine[2-^{14}C]. The enzyme reaction was ten times more rapid in H$_2$ or N$_2$ gas phase than in air.

The optimum pH of this reaction was 7.0 and the synthetic activity sharply dropped in either acid or alkaline condition, as shown in Fig. 1.

The activity of anethole synthesis by this enzyme system required ATP, nicotinamide, DPN$^+$, suitable concentration of phosphate, and respiratory substrates. If the reaction mixture did not contain ATP, DPN$^+$, and nicotinamide, the synthetic activity lowered.

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4) Org. Syntheses, Coll. Vol. 2, 519(1948).
5) *Ibid.*, 1 (1948).
6) J. A. Saul, V. M. Trikojus : Biochem. J., **42**, 80(1948).
7) A. Furst, H. A. Harper, R. J. Seiwald, M. D. Morris, R. A. Neuè : Arch. Biochem. Biophys., **13**, 190(1951).
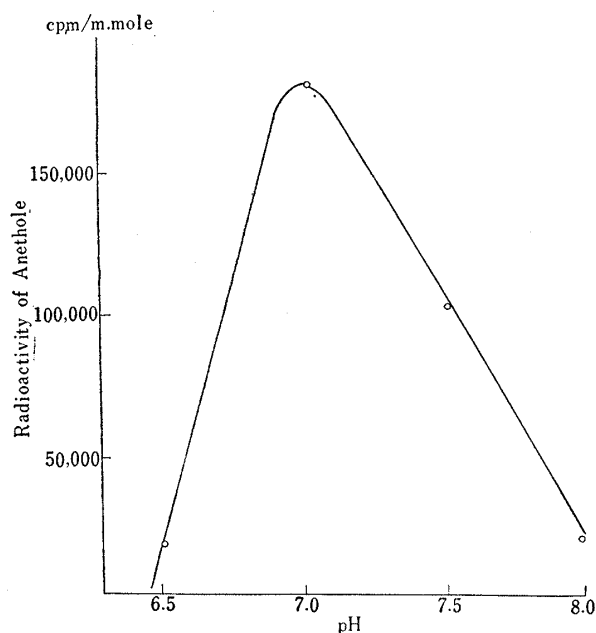
Fig. 1.   Effect of pH on the
Anethole Biosynthesis

Assay conditions as in the text,
incubated at 30° for 3 hr. Each
incubation tube contained 150 mg.
of lyophilized enzyme.

Effect of Respiratory Substrate—The synthetic activity was stimulated by the addition of a member of tricarboxylic acid cycle (Table I). The most suitable substrate was 2-oxoglutarate. The evidence presented indicates that the synthetic activity depends on the active hydrogen derived from TCA cycle, based on the fact that synthetic activity was inhibited by malonate (Table II). Therefore, it appears that the members of TCA cycle act as a hydrogen donor for the synthetic process.   The synthetic activity was activated by ascorbate, and it therefore seems probable that ascorbate maintains a reductive state for the enzyme system, same as in the case of cytochrome reduction.[8]

TABLE I.   Effect of the Members of Tricarboxylic Acid Cycle

Added	Concn. (mole)	Specific activities of anethole (cpm/mM)
None		887
2-Oxoglutarate	0.02	7,921
Malate	0.02	3,028
Citrate	0.02	3,294
l-Hydrogenglutamate	0.02	1,720

Assay condition as indicated in the text, incubated at 30° for 3 hr.
Each incubation tube contained 150 mg. of lyophilized enzyme.

TABLE II.   Effect of Ascorbic Acid and Malonic Acid for Anethole Biosynthesis

Added	Concn. (mole)	Radioactivity of anethole (cpm/mM)	(Ratio)
None		7,956	100
Malonate	0.01	5,268	66
None		7,527	100
Ascorbate	0.02	19,747	262
	0.01	16,933	213

Assay conditions as in the text, incubated at 30° for 3 hr. Each incubation
tube contained 150 mg. of lyophilized enzyme.

Effect of Phosphate—From the fact that the enzymatic synthesis of anethole was activated by addition of ATP, Mg^{2+}, and members of TCA cycle, it is suggested that the synthetic process had a reductive and free energy-requiring processes.   Its free energy was possibly supplied from TCA cycle.   The synthetic pathway was affected by the concentration of phosphate, as shown in Table III.

8) H. Lundegrardh : Physiol. Plantarum, **7**, 375(1954).

Table III. Effect of the Concentration of Phosphate

Phosphate concn. (mole)	Radioactivity of anethole (cpm/mM)
0.01	17,204
0.0033	10,036
0.0011	13,410

All other conditions as in the text, except the concentration of phosphate; incubated at 30° for 4 hr. Each incubation tube contained 150 mg. of lyophilized enzyme.

Inhibitory Effect of Several Phenolic Acids—In biogenetic studies, Shemin and Lynen established the so-called dilution or inhibition method to detect the biosynthetic process by use of labeled and non-labeled precursor.[9,10]

Table IV demonstrates the inhibitive effect upon anethole biosynthesis by the cell-free enzyme system.   The incorporation of phenylalanine[2-^{14}C] into anethole was inhibited by the addition of p-hydroxycinnamic, cinnamic, and phenylpyruvic acid.   The inhibition of p-hydroxyphenylpyruvic acid seems to indicate that p-hydroxycinnamic acid was produced from p-hydroxyphenylpyruvic acid in the same enzyme system.

Table. IV. Inhibitory Effect of Hypothetical Precursors on Anethole Synthesis

Compd. added	Concn. (mole)	Radioactivity of anethole (cpm/mM)	(Ratio)
None		7,969	100
Phenylpyruvic Acid	0.01	2,305	28.9
p-Hydroxyphenylpyruvic Acid	0.01	5,531	69.4
Cinnamic Acid	0.01	2,023	24.3
p-Hydroxycinnamic Acid	0.01	1,773	22.2

Assay conditions as in the text, but 0.01M of ascorbic acid added; incubated for 3 hr. Enzyme system used was freshly preparated by the method described in the experimental section without lyophilization.

Phenylalanine[2-^{14}C] was converted into p-hydroxycinnamic acid at a faster rate than into anethole by the same enzyme reaction, as shown in Table V.   Therefore, in anethole synthesis from phenylalanine, it seems that cinnamic and p-hydroxycinnamic acid exists as intermediates.

Table V. Comparative Studies on the Synthesis of Anethole and p-Hydroxycinnamic Acid from Phenylalanine[2-^{14}C]

	Radioactivity (cpm/mM)	
	Anethole	p-Hydroxycinnamic Acid
Fresh enzyme	8,810	17,907
Boiled enzyme	0	0

Assay conditions as in Table IV.

Discussion

Several experiments were carried out in order to establish whether anethole could be synthesized from phenylalanine by Foeniculum enzyme system.

In Foeniculum enzyme system, anethole was synthesized from phenylananine more efficiently in anaerobic than aerobic condition, in the presence of a member of TCA cycle, ATP, DPN$^+$, and Mg^{2+}.   In view of above results, it was assumed that TCA cycle supplied hydrogen molecule for anethole synthesis, same as in the fatty acid synthesis in the liver of a rat.[11]   However, there were a few differences in the two synthetic processes;

 9) D. Shemin : Rapport 3ème Congrès International de Biochimie, 170(1955).
10) F. Lynen : Proc. Internl. Symposium of Enzyme Chemistry, Tokyo and Kyoto, 57(1958), Maruzen, Tokyo.
11) P. Hele, G. Popjàk, M. Lauryssens : Biochem. J., **65**, 348(1957).

the synthesis of fatty acid in the liver was activated by addition of malonate but the synthesis of anethole was obviously inhibited by malonate.

When Foeniculum plant was cultivated in a greenhouse in winter, the plant grows rapidly, and the content of fennel oil is low, but the content of p-hydroxycinnamic acid is rather great. By the inhibition method, it seems that p-hydroxycinnamic, cinnamic, and phenylpyruvic acids are present as the powerful intermediates from phenylalanine to anethole. This fact is in good agreement with accumulation of a large amout of p-hydroxycinnamic acid in the plant and with the recent studies on the synthesis of lignin and flavone.[2,3]

Summary

The cell-free enzyme system of Foeniculum plant synthesized anethole from phenylalanine on addition of ATP, DPN$^+$, Mg^{2+}, and 2-oxoglutarate in anaerobic condition. In this enzyme reaction, p-hydroxycinnamic, cinnamic, and phenylpyruvic acids seem to be a powerful intermediate of the anethole biosynthesis, which was detected by the inhibition method.

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144. Shigehiko Sugasawa[*1] and Yoshio Deguchi[*2] : Synthesis of β-Carboline Derivatives. III. A Synthesis of Dimethoxybenzindoloquinolizine.

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In the first paper of this series,[1] Sugasawa, Terashima, and Kanaoka described a new synthesis for hexahydrobenzindolo[3,2-h]quinolizine, which appeared to have opened a new general route for synthesis of β-carboline derivatives. Syntheses of tetrahydrobenzindoloquinolizines from isoquinolyl- and quinolyl-indoles provided additional examples.

In this paper will be described a synthesis of 2,3-dimethoxy-5,6,8,9,14,14b-hexahydrobenz[a]indolo[3,2-h]quinolizine (XIII) as a more probable prototype of a fundamental skeleton not yet met in the vegetable kingdom reported in the previous paper.[2]

Thus, ethyl 2-indolecarboxylate was condensed with 3,4-dimethoxyphenethylamine to yield the amide (I) and this was cyclized to give 1-(2-indolyl)-6,7-dimethoxy-3,4-dihydroisoquinoline (II), from which tetrahydro derivatives (III and III') were prepared by the conventional method. Since the gramine formation of (II) did not proceed neatly, being accompanied by a side reaction, this was dehydrogenated first to give (IV), which then yielded

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*2 Present address : Osaka Laboratories, Fujisawa Pharmaceutical Industries, Ltd., Kashima-cho, Higashiyodogawa-ku, Osaka (出口義雄).
1) Part I. S. Sugasawa, M. Terashima, Y. Kanaoka : This Bulletin, 4, 16(1956).
2) Part II. S. Sugasawa, S. Takano : Ibid., 7, 417(1959).