The Origin of Carbons of Dihydrofuran Ring and C-6 in the Biosynthesis of Rotenone*

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For the investigation of rotenone biosynthesis, acetate-2- ^{14}C , mevalonic acid-2- ^{14}C lactone and methionine-methyl- ^{14}C were administered to *Derris elliptica* plants, respectively, and the distribution of carbon-14 in the labeled rotenone was determined by degradation. When mevalonic acid-2- ^{14}C lactone was incorporated into rotenone, the radioactivity was found equally in the carbons at both C-7' and C-8', indicating that these carbons are derived from the carbon-2 of mevalonic lactone. In the case of methionine-methyl- ^{14}C about 80% of the total radioactivity was found to enter two methoxyl groups. This result demonstrates that methionine is an efficient precursor of the methoxyl group. Furthermore, it is also suggested that methionine may be a precursor of the carbon at C-6.

It has been suggested that the biosynthesis of rotenone may be closely related to that of flavonoids and isoflavonoids.¹⁾ In recent studies on the incorporation of phenylalanine-2- ^{14}C and methionine-methyl- ^{14}C into rotenone, Crombie *et al.*²⁾ suggested that a migration of the aryl ring (ring A) might happen during the biosynthesis of rotenone (I in Fig. 1), and that this migration could take place in either a flavane or an isoflavone (or isoflavane) type intermediate. Aneja *et al.*³⁾ proposed a speculation on the origin of the isoprene unit and its modified form which are found in the natural non-terpenoid compounds such as marmersin (II)⁴⁾ and auroglucin (III). Birch *et al.*⁵⁾ also demonstrated experimentally that

isoprene units in auroglucin (III) are derived from mevalonic acid by the process related to the formation of terpenoids from the isoprene unit.

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1) H. Grisebach and W. D. Ollis, *Experientia*, **17**, 4 (1961).

2) L. Crombie and M. B. Thomas, *Chem. Comm.*, **1965**, 155; *J. Chem. Soc.*, (C) **1967**, 1769.

3) R. Aneja, S. K. Mukerjee and T. R. Seshadri, *Tetrahedron*, **4**, 256 (1958).

4) A. Chatterjee and S. S. Mitra, *J. Amer. Chem. Soc.*, **71**, 606 (1949).

5) A. J. Birch, J. Schofield and H. Smith, *Chem. and Ind.*, **1958**, 1321.

In this study, in order to obtain some informations of the origin of the isopropenyl dihydrofuran ring (ring E) and of the carbon at C-6 of rotenone, acetate-2-¹⁴C, mevalonic acid-2-¹⁴C lactone and methionine-methyl-¹⁴C were administered by means of a cotton wick inserted into the stem of *Derris elliptica* plants growing in soil. After air-dried stems and roots were ground to a powder, rotenone was extracted with chloroform and non-labeled rotenone was added as the carrier to the chloroform extracts. Then the radioactive rotenone obtained was purified and assayed. The results for the experiments of the incorporation of the three precursors into rotenone are shown in Table I. The labeled rotenone (I in Fig. 1) formed from mevalonic acid-2-¹⁴C lactone was oxidized by ozone in glacial acetic acid as indicated in Fig. 1. Formaldehyde (IV) generated by steam distillation of the above ozonide was converted into the dimedone derivative, and iodoform (V) was obtained by the treatment of the

FIG. 1.

TABLE I. INCORPORATION OF LABELED PRECURSORS

Precursor	Feeding time (days)	Total extracts (g)	Sp. act. of rotenone* (dpm/mmole)	Specific incorporation ratio (%)
Sodium acetate-2- ¹⁴ C (0.5 mCi)	8	1.27	51,020	0.064
Mevalonic acid-2- ¹⁴ C lactone (0.5 mCi)	15	1.81	46,750	0.007
Methionine-methyl- ¹⁴ C (0.2 mCi)	12	1.05	35,170	0.001

* Specific activity of rotenone purified after adding the carrier to the total extracts

TABLE II. DISTRIBUTION OF ¹⁴C IN ROTENONE DERIVED FROM MEVALONIC ACID-2-¹⁴C LACTONE

	Rotenone	Dimedone (C-7')*	Iodoform (C-8')*
Specific activity (dpm/mmole)	35,570 (1.00)**	16,010 (0.45)**	11,740 (0.33)**

* See Fig. 1

** Relative molar activity

residue of the steam distillation with NaOI. The results are shown in Table II. The values in brackets in Fig. 1 indicate the proportion of the activity in the compounds referred to rotenone as 1.00. The result indicates

that 78% of the activity of rotenone exists in the carbons at both C-7' and C-8' and that the activity is distributed equally between the C-7' and C-8' of rotenone. Therefore, it is suggested that dimethylallyl pyrophosphate derived from mevalonic lactone combines with the ring D before the formation of the dihydrofuran ring (ring E) and that both C-7' and C-8' were derived from C-2 of mevalonic acid. However, the mechanism for enzymatic formation of the end-ethylene of the isopropenyl group is still ambiguous from this result. A more extensive study is needed for elucidating whether the enzymatic

reaction proceeds specifically or non-specifically at the carbons of C-7' and C-8'

Aneja *et al.* also postulated that the dimethylallyl substituent attached to the benzene ring is generally oxidized to form either an epoxide or a glycol and then this epoxide or glycol undergoes transformation to form a substituted furan or dihydrofuran. In the case of rotenone, therefore, the isopropenyl dihydrofuran moiety may be formed through the corresponding epoxide (VII) or glycol (VIII) type intermediate derived from the

TABLE III. RADIOASSAY OF METHOXYL GROUP IN ROTENONE
(OCH₃: 15.74%)

Precursor	Rotenone (mg)	OCH ₃ (%)	Activity of CH ₃ I (dpm/mole)	Activity in OCH ₃ (%)
Methionine-methyl- ¹⁴ C ¹⁾	7.277	15.33	14,670	83.4
	6.881	15.39	14,450	82.1
	6.871	15.45	14,980	85.2
Sodium acetate-2- ¹⁴ C ²⁾	6.249	15.73	128	0.5
	6.893	15.92	179	0.7

Specific activity of rotenone: 1) 35,170 dpm/mole, 2) 51,020 dpm/mole

FIG. 2.

dimethylallyl adduct (VI) as shown in Fig. 2. However, since *o*-(2-alkenyl)-phenols are known to be easily converted to the dihydrofuran ring *via* the epoxidation with organic peracids *in vitro*,^{6,7)} it may be reasonable to say that the dihydrofuran ring in rotenone might be formed directly through an epoxy type compound in the enzymatic reaction rather than through a glycol. Deguelin (X) which is known as a minor constituent in *Derris* plants may be obtained instead of rotenone when the pyrone ring is formed by the cyclization. Furthermore, the end-ethylene moiety in rotenone is probably given by dehydration of the

6'-hydroxyl intermediate (IX) in the formation of the dihydrofuran ring from the epoxide.

The results of the measurements of carbon-14 activity in methoxyl groups of labeled rotenone obtained by the administration of methionine-methyl-¹⁴C and acetate-2-¹⁴C are shown in Table III. With methionine-methyl-¹⁴C, about 80% of the total radioactivity of rotenone was found in methoxyl groups, but with acetate-2-¹⁴C almost no activity was detected. Therefore, the S-methyl group of methionine obviously acted as an efficient precursor for the methoxyl groups of rotenone.

Furthermore, the labeled rotenone obtained from methionine methyl-¹⁴C was converted into both derritol (XI) which lost two carbons at C-6 and C-6a from rotenone and rotenol (XII) by refluxing with the mixture of zinc

6) S. W. Tinsley, *J. Org. Chem.*, **24**, 1197 (1959).

7) F. E. King, J. R. Housley and T. J. King, *J. Chem. Soc.*, **54**, 1392 (1954).

powder and ethanolic hydroxide solution. The results of radioassay for these products are presented in Table IV. In Fig. 3, the

TABLE IV. SPECIFIC ACTIVITY OF DERRITOL AND ROTENOL
(dpm/mmmole)

	Rotenone	Derritol	Rotenol
Methionine-methyl- ^{14}C	18,510 (1.00)	14,620 (0.79)	18,690 (1.05)
Sodium acetate-2- ^{14}C	4,372 (1.00)	4,067 (0.93)	4,196 (0.96)

The values in the brackets indicate relative molar activities

at C-6. Consequently, it may be concluded that methionine is also a precursor of the carbon at C-6. The reason why such a difference in incorporation between C-6 and methoxyl groups occurs is now under investigation.

EXPERIMENTAL

*Administration of labeled compounds to *Derris* plants.* An aqueous solution (0.5 ml) of each ^{14}C -labeled compound was administered to two-year-old *Derris elliptica* plants grown in a phytotron at 30°C by means of a cotton wick inserted into the main stem, and the

FIG. 3.

proportion of the radioactivity for these degradation products referred to rotenone as 1.00 is indicated in brackets. The difference in the activity between rotenone (I) and derritol (XII) means the sum of the activities of C-6 and C-6a. The value of rotenol was given only as a reference. Since Crombie *et al.*²¹ have demonstrated that the carbons at C-6a, C-12a and C-12 were derived from the carbons of the side chain of phenylalanine in a manner similar to the biosynthesis of isoflavone, it may be presumed that the methyl group of methionine can not be the origin of the carbon at C-6a.* Therefore, it is reasonable to say that most of the difference in activity between rotenone and derritol results from the carbon

plants were grown for 8~15 days under the same condition.

*Isolation of ^{14}C -labeled rotenone.*⁸⁾ After feeding labeled compounds, the plants were harvested. The stems and roots of the plants were dried at room temperature and ground to a powder in a glove box. This powder was then extracted with chloroform by a Soxhlet extractor for 48 hr. The extract was evaporated under reduced pressure and weighed. Non-labeled rotenone as the carrier (approximately 700 mg) was mixed to the above extract and about 5 times as much carbon tetrachloride as the mixture by weight was added to the mixture. The solution was allowed to stand overnight in a refrigerator. Crystals separated as the rotenone-carbon tetrachloride complex were filtered and washed with a small amount of cold carbon tetrachloride. The crude rotenone-carbon tetrachloride complex was dissolved in 90% ethanol

* After our oral presentation, almost the same conclusion on the origin of C-6 in rotenone biosynthesis was reported by Crombie *et al.* (*Chem. Comm.*, **1968**, 234).

8) S. Takei and S. Miyajima, *Oyo-konchu*, **3**, (1), 1 (1941).

(about 5 ml) and the solution was boiled for one or two minutes. After the solution was kept at 0°C overnight, crystals of the rotenone obtained were filtered and washed with cold 90% ethanol (3 ml). The labeled rotenone was recrystallized repeatedly from ethanol until constant specific radioactivity was obtained, mp 165~166°C (Table I).

Determination of radioactivity. Determination of radioactivity of labeled rotenone and the degraded products was carried out by one or both of the following two methods: (a) liquid scintillation counting by channel ratio technique (TEN. GSL-110 type), (b) dry combustion followed by the gas proportional counting reported previously.⁹⁾

Radioassay of methoxyl group. Determination of carbon-14 activity in the methoxyl group of labeled rotenone obtained from the incorporation of acetate-¹⁴C and methionine-methyl-¹⁴C was performed by the method reported in our recent paper.¹⁰⁾ Labeled methyl iodide generated by the decomposition of rotenone with Kirsten's reagent was introduced into an absorption tube. After the methyl iodide was weighed, the radioactivity of methyl iodide-¹⁴C was measured by either dry combustion followed by the gas proportional counting or liquid scintillation counting. The results are presented in Table III.

Degradation of the labeled rotenone. (A) The rotenone (270 mg) obtained from the incorporation of mevalonic acid-2-¹⁴C lactone was treated with ozone. Ozonized oxygen (33 mg/min) was passed through the solution in glacial acetic acid (15 ml) at room temperature for 7 min. Then water (15 ml) was added to the reaction mixture and the solution was steam-distilled after standing for 30 min. The distillate (30 ml) was added to an aqueous solution (100 ml) of dimedone (300 mg) and the solution was allowed to stand at 5°C for 72 hr. The formaldehyde dimedone (30 mg) produced

was recrystallized repeatedly from ethanol to a constant radioactivity, mp 198°C. Purification of formal-dimedone was also performed by thin layer chromatography on silica gel with ethanol-benzene-ethyl acetate (1:1:4).

After extraction of the residue of the steam-distillation with chloroform, the chloroform phase was washed with water and the solvent was removed. The residue was dissolved again in a mixture of water (1 ml) and dioxane (0.3 ml), and 3N aqueous sodium hydroxide solution (0.2 ml) and a slight excess of iodine in potassium iodide were added successively. The mixture was kept at 60°C for 10 min. After cooling, excess iodine was decomposed with 10% sodium hydroxide solution and the iodoform produced was collected by centrifugation and recrystallized from methanol-water. Yield 21 mg, mp 120°C. The activity of each compound is shown in Table II.

(B)¹¹⁾ The rotenone (530 mg) obtained from methionine-methyl-¹⁴C was refluxed with zinc powder (1200 mg) in ethanol (15 ml). To the boiling mixture, 20% aqueous potassium hydroxide solution (5 ml) was slowly added and allowed to boil for approximately 5.5 hr. After separation of zinc powder, the solution was acidified with diluted hydrochloric acid and extracted with ether. Then, the ether layer was extracted with 3% aqueous sodium hydroxide solution. The aqueous layer was acidified by addition of diluted hydrochloric acid again and extracted with ether. The ether solution was dried over anhydrous sodium sulfate and ether was removed. Derritol obtained was recrystallized from methanol or acetone-petroleum ether until constant radioactivity was obtained, mp 160~161°C.

From the ether layer containing alkali insoluble material in the above reaction mixture, a small amount of rotenol was obtained by recrystallization from methanol or acetone-petroleum ether, mp 117~119°C. The activities of derritol and rotenol are shown in Table IV.

9) M. Hamada and E. Kawano, *Anal. Chem.*, **38**, 943 (1966).

10) M. Chubachi, E. Kawano and M. Hamada, *ibid.*, **40**, 1183 (1968).

11) A. Butenandt, *Ann.*, **464**, 253 (1928).

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