Abspaltung von Guanin, Adenin, Uracil, 5-Bromuracil und 5-Joduracil wurde bei 260 und 280 mμ, von Cytosin bei 265 und 280 mμ, von Naphthoimidazol bei 230 und 240 mμ, von 5.6-Dimethylbenzimidazol bei 245 und 260 mμ, von 6-Azauracil bei 250 und 280 mμ und von 8-Azaguanin bei 255 und 275 mμ verfolgt. Eine Inkubationsdauer von 60 Min. reicht im allgemeinen vollständig, um einen deutlichen Effekt zu erhalten. Bei längerer Inkubation (3 Stdn.) überlagern sich z. B. bei Thymidin andere, langsamer verlaufende Reaktionen.

Wir danken Herrn Dr. D. GRÜNBERGER für die Überlassung von 8-Azaguanosin, Herrn Dr. H. DELLWEG für die Isolierung von α-Ribazolphosphat aus Vitamin B_{12} und Herrn Dr. K. GAURI für die Synthese von α-Thymidin nach HOFFER [19].

The Role of General Metabolites in the Biosynthesis of Natural Products

I. The Terpene Marrubiin

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The biosynthesis of the diterpene marrubiin has been studied using some labelled possible precursors: [2-^{14}C] mevalonate, [1-^{14}C] acetate, [2-^{14}C] acetate, [2-^{14}C] pyruvate, [2,3-^{14}C] succinate, [1,4-^{14}C] succinate, [5-^{14}C] ketoglutarate and [1,5-^{14}C] citrate. Metabolism times of 24, 36, 48, 72 and 96 hours were used. The labelled compounds were supplied in solution to flowering white horehound (*Marrubium vulgare* L.), cut at the base of the stem.

The incorporation results indicate that marrubiin undergoes a rapid turnover with a biological half life of about 24 hours. The rate of up-take of ^{14}C from the precursors is in the following order: [2-^{14}C] mevalonate, [2-^{14}C] acetate, [1-^{14}C] acetate, [2-^{14}C] pyruvate and [2,3-^{14}C] succinate. Incorporation of ^{14}C from the other compounds is negligible. If [2-^{14}C] mevalonate is used, labelled marrubiin is formed 10—100 times more active than with the other precursors.

The results suggest that all the precursors are degradated to acetic units through the Krebs cycle before their utilization, with the exception of mevalonate.

Some samples of marrubiin (I) have been degradated chemically to the corresponding keto-derivative (II), in order to determine the distribution of radioactivity between the decahydronaphthalenic and furanic parts. The results are in good agreement with the distribution of the radioactivity in the molecule, as expected from the terpenoid structure of marrubiin.

Marrubiin is the bitter principle of the white horehound *(Marrubium vulgare* L; fam. Labiatae/Marrubiae), a plant that grows in dry areas. It also occurs widely in other higher plants.

The structure of this compound was elucidated only in 1953 bz COCKER and al. [1] on the basis of spectroscopic and degradation studies. Though confirmation of the direct chemical synthesis is still lacking, marrubiin has been assigned the formula I, corresponding to a furanoid diterpene. The function of substances of this type in plants is still unknown, and little is known of the mechanism of their formation in plant tissues.

The general scheme of the biosynthesis of natural products with terpenoid and steroid structures is linked with the theory formulated by RUZICKA [2] under the name of the biogenetic law of active isoprene.

Whereas the literature contains a considerable amount of information on the biosynthesis of the animal sterols [3], it contains much less on the biosynthesis of typical sterols [3] and terpenes [4] of higher plants.

In the preliminary investigation into the mechanism of formation of marrubiin [5], the incorporation of some simple precursors such as [1-^{14}C] acetate,

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[1,4-^{14}C] succinate and [2-^{14}C] mevalonate at one or two indicative stages of the metabolism was studied.

It was decided to extend this study by analysing the kinetics of the incorporation of these and other possible precursors. The substances studied were [2-^{14}C] mevalonate, [1-^{14}C] acetate, [2-^{14}C] acetate, [1,4-^{14}C] succinate, [2,3-^{14}C] succinate, [5-^{14}C] ketoglutarate, [1,5-^{14}C] citrate and [2-^{14}C] pyruvate.

The stages of metabolism were 24, 36, 48, 72, 96 hours.

The choice of mevalonate was based on its importance as a necessary stage in the biosynthesis of terpenes [4], and the consequent need to check that it is also used in the formation of marrubiin.

The two acetates, labelled on the methyl and on the carboxyl group respectively, were used to verify the fundamental role of active acetate.

The other substances used were pyruvate and anions of bicarboxylic and tricarboxylic acids, chosen from among the most important components of the Krebs cycle.

It seemed important to study the role of the Krebs cycle in the biosynthesis of molecules of plant origin. It leads to the formation of CO_2 and molecules containing from 3 to 6 carbon atoms. The formed CO_2 could be utilized in the synthesis of natural products by carboxylation reactions.

Similar investigations have been carried out on a phytosterol [6] and on a long straight-chain paraffin [7], which are among the principal components of wistaria flowers *(Wistaria Sinensis)*.

Materials and Methods

a) Cultivation of the plant and administration of the labelled compounds

The radioactive compounds were supplied by the Radiochemical Centre at Amersham (UK), and were diluted with a high-purity carrier (Merck).

The white horehound plants (*Marrubium vulgare* L) were used for these experiments in June and July, i. e. during their flowering period, when the terpene anabolism is most active.

The plants were cut above the roots and incubated at 25 °C with solutions consisting of distilled water, labelled compounds, and adenosine triphosphate (ATP) as a source of energy and of phosphate.

The solution was prepared by dissolution of 100 mg of ATP and the quantity of radioactive compound shown in Table 1 in 250 ml of water.

N	Precursor	Amount administered [mM]	Radioactivity [μC]	Specific activity [μC/mM]
1	[1-^{14}C] acetate	0.50	100	200
2	[2-^{14}C] acetate	0.50	100	200
3	[1.4-^{14}C] succinate	0.50	25	50
4	[2.3-^{14}C] succinate	0.50	25	50
5	[5-^{14}C] ketoglutarate	0.50	25	50
6	[1.5-^{14}C] citrate	0.25	25	100
7	[2-^{14}C] pyruvate	0.50	25	50
8	[2-^{14}C] mevalonate	0.50	25	50

Table I. Possible precursors used in marrubiin biosynthesis.

Each parent solution was divided between 5 containers, in which the stems were immersed, the average overall weight of the plant tissue being 300 g.

The metabolism times were calculated from two hours after administration, since this is the time required for the complete absorption of the solution by the tissue.

50 to 100 ml of distilled water were added roughly every 10 hours.

After the predetermined metabolism time (24, 36, 48, 72 and 96 hours), the plants were dried at 60 °C until all moisture had been eliminated.

b) Extraction and purification of marrubiin

The leaves, flowers, and stems were shredded, pulverized, and extracted for 36 hours with diethyl ether. The dark green ether extract was decolorized with vegetable charcoal, and the colourless liquid, when dried in a rotary evaporator, gave a yellow crystalline residue. The residue was taken up in a little ether and left for a few hours to remove traces of chlorophyll and fats because the marrubiin is much less soluble in ether than the other ones. The process was repeated several times.

The crude marrubiin was crystallized several times from ethyl alcohol until its radioactivity was constant. The yield varied between 200 and 250 mg.

The chemical purity of the product was assessed by means of its melting point (158–160 °C), and for some samples by infrared spectroscopy and elementary analysis.

The radiochemical purity of each sample was checked by thin-layer chromatography with a 7:3 v/v mixture of ethyl acetate and cyclohexane as the eluent.

c) Degradation of marrubiin

The marrubiin (I) was degraded to the corresponding keto derivative (II) (Fig. 3) in order to find the

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distribution of the radioactivity between the decahydronaphthalene portion and the furan ring. The degradation was carried out by ozonolysis of the anhydrotetrahydromarrubiin as described in the literature [1].

d) Radioactivity measurements

The radioactivity was measured with a liquid scintillation counter supplied by SELO. The scintillators used were 2,5-diphenyloxazole (PPO) as the primary scintillator and 1,4-bis-2(5-phenyloxazolyl)benzene (POPOP) as the secondary scintillator in toluene solution. The efficiency of the instrument was found to be about 70% in the measurement of standard samples of ^{14}C hexadecane under the same experimental conditions.

Results and Discussion

Table 2 shows the results obtained in the study of the biosynthesis of marrubiin.

The kinetics of the incorporation of the various precursors are shown in Figs. 1 and 2.

The incorporation of labelled compounds decreases in the following order: [2-^{14}C] mevalonate, the two acetates, [2-^{14}C] pyruvate and [2,3-^{14}C] succinate. The amounts of [1,4-^{14}C] succinate, [5-^{14}C] ketoglutarate and [1,5-^{14}C] citrate incorporated were so small that these cannot be regarded as significant precursors for the "in vivo" formation of marrubiin.

N	Precursor	Metabolism time [hours]	Specific activity of marrubiin [$\mu C/mM \cdot 10^{-3}$]	Specific * Incorporation [$\cdot 10^{-4}$]	Total * Incorporation [$\cdot 10^{-4}$]
1	[1-^{14}C] acetate	24	27.49	1.38	10.0
		36	42.00	2.10	15.0
		48	36.60	1.83	13.5
		72	21.00	1.05	7.5
		96	22.00	1.10	8.0
2	[2-^{14}C] acetate	24	32.60	1.63	12.0
		36	51.00	2.55	19.0
		48	39.60	1.98	14.5
		72	15.00	0.75	5.5
		96	15.57	0.78	6.0
3	[1.4-^{14}C] succinate	24	0.45	0.09	0.6
		36	—	—	—
		48	1.64	0.33	2.5
		72	1.19	0.24	1.8
		96	1.50	0.30	2.0
4	[2.3-^{14}C] succinate	24	3.00	0.60	4.5
		36	3.43	0.69	5.0
		48	4.50	0.90	6.5
		72	1.50	0.30	2.0
		96	1.19	0.24	1.8
5	[5-^{14}C] ketoglutarate	24	1.51	0.30	2.2
		36	1.05	0.21	1.5
		48	1.65	0.33	2.4
		72	1.20	0.24	1.8
		96	0.60	0.12	0.9
6	[1.5-^{14}C] citrate	24	0.90	0.09	1.4
		36	1.35	0.13	2.0
		48	2.26	0.22	3.4
		72	1.81	0.18	2.7
		96	1.51	0.15	2.2
7	[2-^{14}C] pyruvate	24	2.54	0.50	3.8
		36	4.50	0.90	6.8
		48	8.59	1.72	12.8
		72	4.63	0.92	7.0
		96	3.00	0.60	4.5
8	[2-^{14}C] mevalonate	24	70.00	14.00	84.0
		36	49.00	9.80	58.1
		48	36.00	7.20	43.5
		72	18.70	3.74	22.5
		96	15.90	3.18	19.5

Table II. Incorporation data of some precursors in marrubiin at various metabolism times. * The specific incorporation is the ratio between the specific activity of the marrubiin and the specific activity of the precursors. The total incorporation is the ratio between the total activity of the marrubiin and the total activity of the precursors.

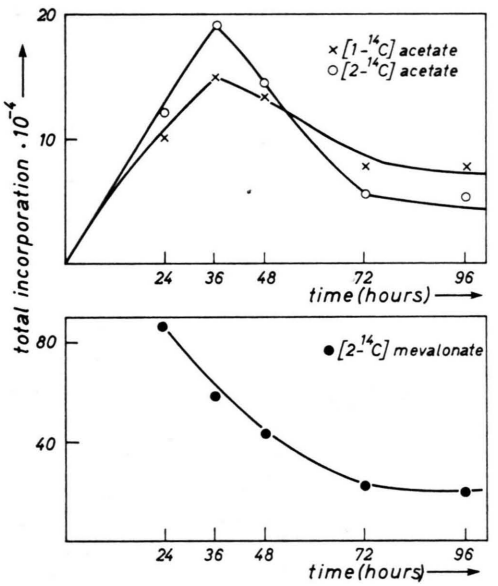

Fig. 1. Incorporation, at various time intervals, of some precursors in the terpene marrubiin.

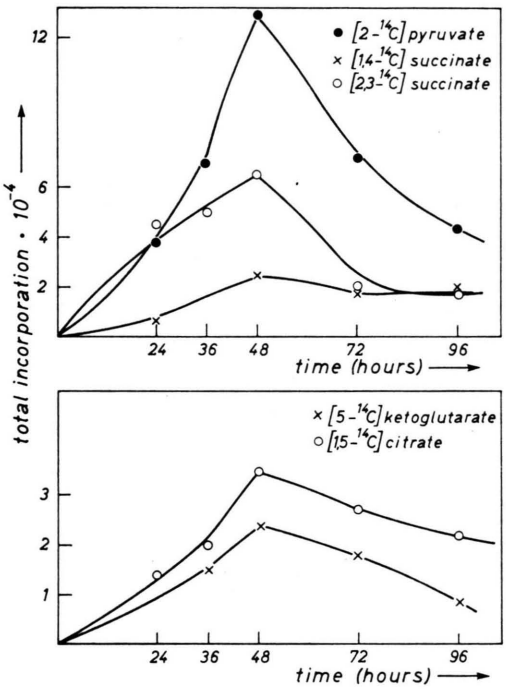

Fig. 2. Incorporation, at various time intervals, of some precursors in the terpene marrubiin.

The maximum incorporation of nearly all the compounds occurred between the 36th and the 48th hour of metabolism, the only exception being mevalonate for which the maximum must have occurred before the 24th hour.

After the 48th hour, there is a considerable loss of activity in the marrubiin, with a tendency towards levelling between the 72nd and 96th hour.

It can be seen from the graphs that there is a fairly rapid turnover of marrubiin, from which it can be calculated that the biological half-life is about 24 hours.

The analysis of these curves (apart from that of mevalonate) shows that the early, ascending part of the curve probably corresponds to the following two phenomena: 1. the assimilation of the radioactive compound by mechanical transport processes, enabling it to reach the cell location at which the biosynthesis occurs; and 2. the sequence of metabolic reactions leading to the conversion of the precursor into marrubiin.

Since the precursors used are metabolites similar to one another, the rate of physiological assimilation should show a similar behaviour in every case; consequently, the qualitative differences in the ascending parts of the curves are actually due to the series of reactions leading to the synthesis of the compound.

There may also be competitive reaction sequences, in which the precursor is converted by different metabolic paths into marrubiin.

The descending part of each curve is due to the exhaustion of the radioactive compound and to the dilution of this and of the labelled marrubiin by the corresponding endogenous compounds.

The amount of mevalonate incorporated is much greater than that of the other precursors, owing to its acknowledged specificity in the biosynthesis of terpenoid substances. The specificity of mevalonate and its biogenetic proximity to marrubiin are shown by its rapid utilization by the organism for the formation of the terpene.

It is certain that all or nearly all of the mevalonate was consumed during the first 24 hours after administration, and that after the 24th hour, owing to the rapid biological exchange, the activity of the marrubiin decreases and tends towards a fairly constant value between the 72nd and 96th hour.

The relatively high incorporation of mevalonate provides further confirmation of the terpenoid structure of marrubiin.

The course of incorporation of the two acetates, labelled on the methyl and on the carboxyl group respectively, is roughly similar, with a maximum around the 36th hour of metabolism. The activity of

the marrubiin is lower and the incorporation is slower than in the case of mevalonate, since after undergoing the condensation to form acetoacetyl coenzyme A, it is converted by a complex route into β-hydroxymethyl-glutaryl coenzyme A, and thence into mevalonate.

The incorporations of the two acetates confirm that the formation of the terpen occurs via mevalonate for condensation head by tail and partial decarboxylation. Also the slight differences in the uptake of the two acetates are in agreement with the oversaid pathway.

Pyruvate also exhibits good incorporation. Though this compound enters into various metabolic cycles, it may be assumed that it undergoes oxidative decarboxylation to active acetate.

Analysis of the behaviour of the succinate, ketoglutarate and citrate, bearing in mind the Krebs cycle, leads to the following general conclusions:

The succinate may be converted into acetyl CoA, and thence by the usual route into the terpene. Before conversion into acetyl CoA, the succinate must be oxidized to oxalacetate (Krebs cycle), and the subsequent transformation follows two paths. In the first, it undergoes double decarboxylation outside the cycle itself. In this case, however, the marrubiin biosynthesized from [1,4-^{14}C] succinate should not be active, whereas activity, though only slight, is actually observed. The second route follows the cycle as far as citrate, and the reverse reaction of this symmetrical molecule can lead to the formation of oxalsuccinate and labelled acetyl CoA.

[2,3-^{14}C] succinate is converted by these two routes into acetyl CoA without loss of its two radioactive atoms. The kinetic of the incorporation of this compound into marrubiin is similar to those of acetates and pyruvate, though the amount incorporated is smaller.

[5-^{14}C] ketoglutarate and [1,5-^{14}C] citrate are utilized in the Krebs cycle in the same manner as [1,4-^{14}C] succinate. In fact, the specific activity of the marrubiin biosynthesized from these three compounds is of the same order however, insignificant.

In other experiments we have tested the incorporation also of $H^{14}CO_3^{\ominus}$ in marrubiin but it was very low. The specific incorporation was $0.061 \cdot 10^{-4}$ and $0.091 \cdot 10^{-4}$ and the total incorporation $0.091 \cdot 10^{-4}$ and $0.136 \cdot 10^{-4}$ respectively at the 24th and 48th hour of metabolism. In this case the order of incorporation was smaller by a factor of about 10 than in the experiments with [1,4-^{14}C] succinate, [5-^{14}C] ketoglutarate, [-,5-^{14}C] citrate. This fact however, does not exclude also a partecipation of $^{14}CO_2$ derived from the same compounds in the Krebs cycle, to the radioactivity of marrubiin.

The results of chemical degradation of the most active samples of marrubiin are respresented in Table III. In every case the specific activity per mg

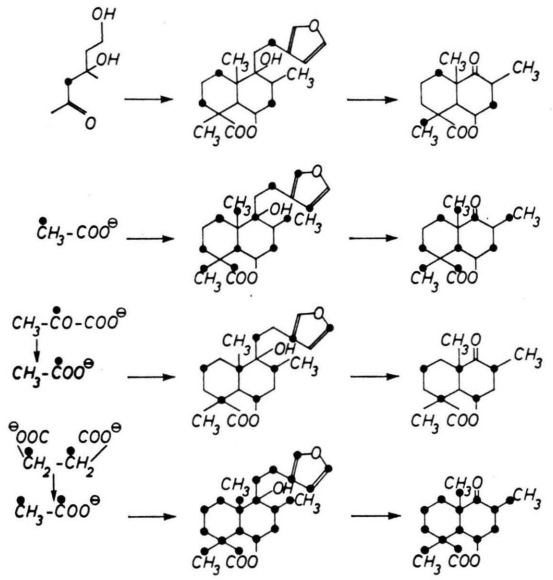

Fig. 3. Localization of the ^{14}C atoms in the marrubiin (I), biosynthesized from the various precursors and in ketolactone (II).

N	Precursor	α* of marrubiin carbon [μc/mg C · 10^{-4}]	α* of ketolactone carbon [μc/mg C · 10^{-4}]	α* of marrubiin [μc/mM · 10^{-2}]	α* of ketolact. [μc/mM · 10^{-2}]	Remaining activity in ketolactone found [%]	calculated [%]
1	[1-^{14}C] acetate	1.12	1.01	2.65	1.66	62	62.5
2	[2-^{14}C] acetate	0.50	0.47	1.17	0.76	65	75
3	[2,3-^{14}C] succinate	0.12	0.12	0.30	0.21	70	70
4	[2-^{14}C] pyruvate	0.12	0.10	0.30	0.18	60	62.5
5	[2-^{14}C] mevalonate	1.58	1.58	3.78	2.61	70	75

Table III. Distribution of ^{14}C in marrubiin (I) and in ketolactone (II). * α = specific activity.

of carbon is of the same order in the marrubiin and in the degradation products, demonstrating (at least for the precursors used by us) the common biogenetic origin of the side chain attached to C_6, including the furan system, and of the decahydronaphthalene system.

The distribution of radioactivity between the decahydronaphthalenic and furanic parts is in good agreement with the suggestion that all the metabolites, with the exception of mevalonate, are degraded to acetate through the Krebs cycle before their utilization.

In Fig. 3 it is shown the localization of the ^{14}C atoms in the marrubiin (I) derived from the various precursors and in ketolactone (II). These schemes are delined conforming to the terpenoid structure of marrubiin. The found values of radioactivity remained in decahydronaphthalenic part agree with the calculated ones.

It seems from these results that marrubiin cannot be obtained with a high specific activity and in high radiochemical yields by the administration of precursors, generally used in the biosynthesis of various natural organic products, to cut plants.

The same phenomenon has also been observed in other biosynthetic studies on terpenes [8], and is one of the reasons for the difficulty experienced in the study of the mechanism of formation of these compounds, since the biogenetic paths can be studied only by laborious degradation of labelled compounds having a certain activity.

The low incorporations could be explained by competition with other terpenoid substances present in the plant of *Marrubium vulgare* [9], or by mechanical prevention of the labelled compounds from reaching the cell location at which the biosynthesis takes place.

These "compartmentalizations" of synthetic mechanisms, which are probably relatively complex in higher plants, have been suggested by other Authors [10], and can be elucidated by means of parallel studies on extracellular and subcellular enzymatic preparations.

The Authors wish to thank Dr. Luigi Contursi for his collaboration in the first part of the present study.

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