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BIOSYNTHESIS OF FLAVONOIDS—XXXIV*.

OCCURRENCE OF THE "NIH-SHIFT" IN FLAVONOID BIOSYNTHESIS

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Abstract—The incorporation of *p*-tritio-cinnamic acid [3-^{14}C] into the flavonols kaempferol and quercetin in pea seedlings (*Pisum sativum*) and into the isoflavones formononetin and biochanin A in chana seedlings (*Cicer arietinum*) was investigated. The retention of tritium found was: kaempferol, 85 per cent; quercetin, 49 per cent; formononetin, 80 per cent; and biochanin A, 81 per cent. Degradation of the labelled formononetin to 3-nitro-5-tritio-4-methoxybenzoic acid proved that the tritium had migrated to a position *ortho* to the 4′-hydroxyl group. The results are discussed in terms of the chronological sequence of steps during flavonoid biosynthesis, in particular when hydroxylation in ring B occurs.

INTRODUCTION

A CONSEQUENCE of the hydroxylation of an aromatic substrate is an intramolecular migration of the group displaced by hydroxyl to an adjacent position on the aromatic ring. This phenomenon was extensively studied at the National Institutes of Health and was therefore named NIH-shift by Guroff *et al.*[1] The NIH-shift has so far almost exclusively been studied in bacterial and mammalian systems.[1,2] Recently Zenk has shown that when *p*-tritio-cinnamic acid is converted by young *Catalpa hybrida* leaf tissue to *p*-coumaric acid and *p*-hydroxybenzoic acid the tritium label is retained to 85 and 83 per cent, respectively, and migrates to the adjacent position.[3]

We have independently studied the occurrence of the NIH-shift in the biosynthesis of the flavonols quercetin and kaempferol in pea seedlings (*Pisum sativum*) and in the biosynthesis of the isoflavones biochanin A and formononetin in chana seedlings (*Cicer arietinum*) (Fig. 1).

RESULTS

p-*Tritio-cinnamic acid*-[3-^{14}C]

p-Tritio-cinnamic acid was prepared by the action of a purified phenylalanine-ammonia-lyase (E.C. 4.3.1.5) from potato discs[4] (95 units/mg protein) on *p*-tritio-L-phenylalanine (Nuclear Chicago). The acid was purified to radiopurity by paper chromatography with

* The series Biosynthesis of Flavonoids, and Biosynthesis of Isoflavonoids are now continued under this title. Biosynthesis of Flavonoids Part XVI: L. PATSCHKE and H. GRISEBACH, *Phytochem.* 7, 235 (1968); Biosynthesis of Isoflavones Part XVII: H. ZILG and H. GRISEBACH, *Phytochem.* 7, 1765 (1968).

† Address: Schänzlestr. 9–11.

[1] G. GUROFF, J. W. DALY, D. M. JERINA, J. RENSON, B. WITKOP and S. UDENFRIEND, *Science* **157**, 1524 (1967).

[2] J. W. DALY, D. M. JERINA and B. WITKOP, *Arch. Biochem. Biophys.*, in press.

[3] M. H. ZENK, *Z. Pflanzenphysiol.* **57**, 477 (1967).

[4] M. ZUCKER, *Plant Physiol.* **40**, 779 (1965); E. A. HAVIR and K. R. HANSON, *Biochemistry* **7**, 1896 (1968).

2·5% acetic acid and butanol/acetic acid/water (4:1:1) as solvents. Cinnamic acid-[3-^{14}C][5] was added as internal standard in the experiments.

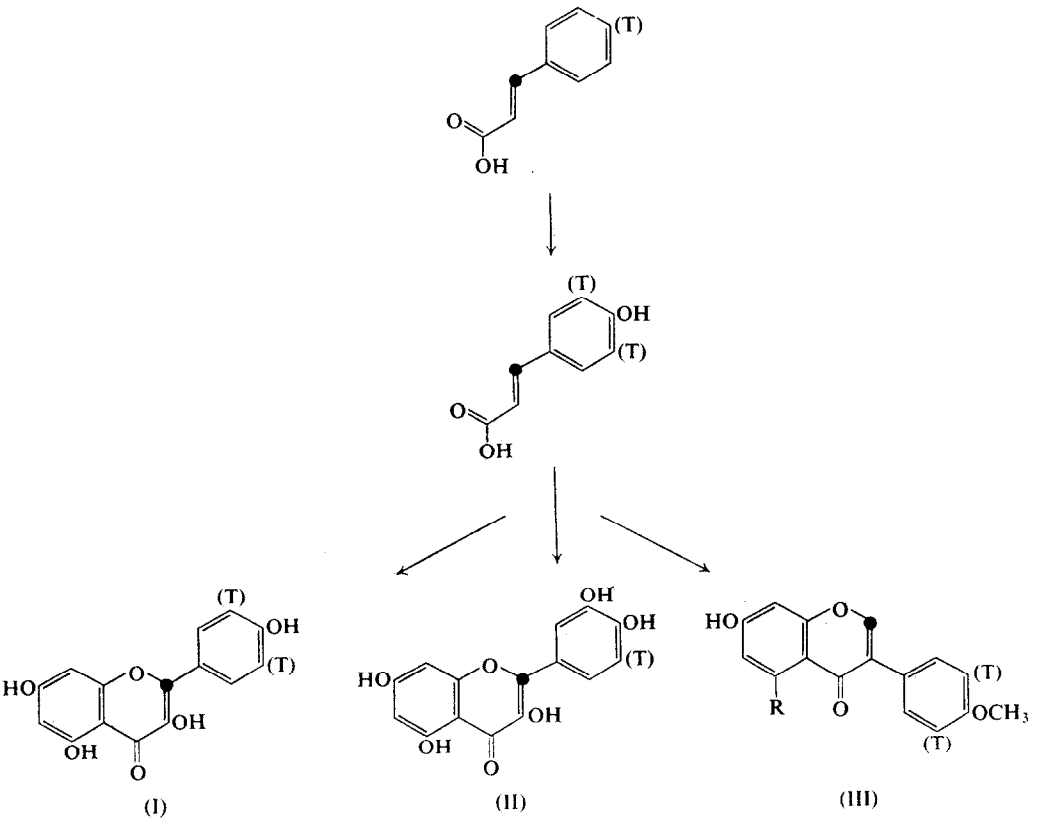

FIG. 1. INCORPORATION OF *p*-TRITIO-CINNAMIC ACID-[3-^{14}C] INTO KAEMPFEROL (I), QUERCETIN (II), FORMONONETIN (III, R=H) AND BIOCHANIN A (III, R=OH). ●=^{14}C.

Investigation of a Possible Exchange of Hydrogen in Ring B of the Flavonols during Hydrolysis

The major flavonoids of the pea plant are kaempferol 3-triglucoside and quercetin 3-triglucoside and their respective *p*-coumaric acid esters.[6] In the case of the isoflavone glycoside in *Cicer arietinum* it is possible to hydrolyse the glycosides with the glycosidases present in this plant by allowing a homogenate of the plants in water to stand for 6 hr.[7] No hydrolysis of the flavonol glycosides occurred under the same conditions with a homogenate from pea seedlings. With a mixture of 50 ml of water, 70 ml of methanol and 5·2 ml of conc. HCl hydrolysis was practically complete after 1 hr on the boiling water bath. In order to check the possibility of hydrogen exchange in ring B under the condition of hydrolysis quercetin and kaempferol were subjected to the same treatment in D_2O/methanol. The NMR spectrum proved that both substances failed to exchange in ring B, whereas protons at C-6 and C-8 in ring A exchanged to about 80 per cent under these conditions.

[5] S. H. BROWN and A. C. NEISH, *Can. J. Biochem. Physiol.* **33**, 948 (1955); H. C. BROWN and B. C. SUBBA RAO, *J. Am. Chem. Soc.* **80**, 5377 (1958).
[6] M. FURUYA and A. W. GALSTON, *Phytochem.* **4**, 285 (1965).
[7] W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **22b**, 627 (1967).

Incorporation of p-*Tritio-Cinnamic Acid*-[3-^{14}C] *into Kaempferol and Quercetin in Pea Seedlings*

Fifteen 11-day-old plants without roots were incubated for 21 hr under continuous illumination with 29·6 μc of *p*-tritio-cinnamic acid (7·22 μc/μmol) and 2·85 μc of cinnamic acid-[3-^{14}C] (0·695 μc/μmol) in 12 ml of 0·01 M sodium acetate buffer of pH 4·4. During this time 95 per cent of the radioactivity was taken up by the plants. The T/^{14}C ratio in the remaining solution was unchanged. The T/^{14}C ratios in the isolated and purified[8] flavonols is shown in Table 1. The retention of T in kaempferol was 85 per cent and in quercetin 49 per cent.

TABLE 1. INCORPORATION OF *p*-TRITIO-CINNAMIC ACID-[3-^{14}C] INTO FLAVONOLS IN *Pisum sativum*

Flavonol	Incorporation rate (%)*	Dilution*	T/^{14}C†	Retention of T (%)
Kaempferol	0·04	710	8·8	85
Quercetin	0·009	1850	5·1	49

* For ^{14}C.
† Initial T/^{14}C ratio 10·4.

Degradation experiments to localize the label via the sequence kaempferol tetramethyl ether → *p*-anisic acid → 3,5-diiodoanisic acid were very unsatisfactory. Tritium was therefor only located for the isoflavones (see below). The fact, however, that quercetin carries only about half of the amount of tritium compared with kaempferol is sufficient proof that the label in kaempferol is located adjacent to the 4′-hydroxyl group (see Discussion and Fig. 3).

Incorporation of p-*Tritio-Cinnamic Acid*-[3-^{14}C] *into Formononetin and Biochanin A in* Cicer arietinum

Ten 12-day-old plants without roots were incubated for 44 hr with an aqueous solution (17 ml) of 36·2 μc of *p*-tritio-cinnamic acid (8·025 μc/μmol) and 3·135 μc of cinnamic acid-[3-^{14}C] (0·695 μc/μmol). During this time 71 per cent of the radioactivity was taken up by the plants, and the T/^{14}C ratio in the solution increased from 11·5 to 12·3. This increase was very probably due to the formation of HTO since the aqueous phase still contained T but not ^{14}C after complete extraction of cinnamic acid with ether.

The isolation and purification of the isoflavones was carried out as described previously.[9] The results are shown in Table 2. The retention values for T are the same in both isoflavones.

For the localization of the label we had first planned to degrade biochanin A to *p*-methoxybenzoic acid (*p*-anisic acid)[10] and to convert this acid to the 3,5-diiodocompound with *N*-iodosuccinimide.[11] However, iodination of anisic acid with this reagent was unsatisfactory and gave several products.

[8] L. PATSCHKE and H. GRISEBACH, *loc. cit.*
[9] W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **22b**, 627 (1967).
[10] H. GRISEBACH and G. BRANDNER, *Z. Naturforsch.* **16b**, 2 (1961).
[11] G. GUROFF and A. ABRAMOWITZ, *Anal. Biochem.* **19**, 548 (1967).

By analogy with a method of King *et al.*,[12] formononetin was therefore degraded with conc. nitric acid to 3-nitroanisic acid identical with 3-nitroanisic acid obtained by direct nitration of anisic acid. When formononetin-[2-^{14}C-T] from the incubation experiment was subjected to this degradation the nitroanisic acid isolated contained 42 per cent of the tritium activity

TABLE 2. INCORPORATION OF *p*-TRITIO-CINNAMIC ACID-[3-^{14}C] INTO ISOFLAVONES IN *Cicer arietinum*

Isoflavone	Incorporation rate (%)*	Dilution*	T/^{14}C†	Retention of T (%)
Formononetin	0·64	47	9·2	80
Biochanin A	0·15	1130	9·35	81

* For ^{14}C.
† Initial T/^{14}C ratio 11·5.

in formononetin and no ^{14}C-activity. Theoretically 50 per cent of the T-activity should have been retained in the nitroanisic acid. Some exchange might have occurred during nitration, though nitroanisic acid itself did not exchange tritium under these conditions. That the nitroanisic acid did not contain ^{14}C was to be expected on the basis of our knowledge of the biosynthesis of isoflavones.[10]

DISCUSSION

From earlier results it was concluded that introduction of the 4′-hydroxyl group in flavonoids occurs at the cinnamic acid stage, i.e. *p*-coumaric acid is the direct precursor of flavonoids.[13] The present results are in agreement with this assumption, since the retention of T in kaempferol and in the isoflavones is about the same as in the hydroxylation of cinnamic to *p*-coumaric acid.[3] This hydroxylation has recently been found to be catalysed by the microsome fraction of apical buds of pea seedlings.[14] In collaboration with Conn and Russel[15] it could be shown that the retention of tritium in this system is from 82 to 93 per cent. Since the hydroxylation with the microsomal fraction requires NADPH and FH_4 as external reducing agents the enzyme must belong to the mixed function oxygenases.

However, the introduction of the 4′-hydroxyl group in flavonoids at a later stage cannot be excluded. It is rather unlikely on the basis of the present and earlier results[16] that hydroxylation occurs at the flavonol stage. From what is known about the mechanism of the NIH-shift,[1] hydroxylation of a flavonol in the 4′-position should lead to a low retention of the migrating hydrogen (tritium), because the positive charge in the cationic intermediate is delocalized by the contribution of a *p*-quinoid system (Fig. 2).

The loss of tritium through introduction of the 3′-hydroxyl group (retention in quercetin 49 per cent) is analogous to the reaction tyrosine → dopa (3,5-T-tyrosine → dopa, 55 per

[12] F. E. KING, M. F. GRUNDON and K. G. NEILL, *J. Chem. Soc.* 4583 (1952).
[13] H. GRISEBACH, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), Academic Press, London (1965).
[14] D. W. RUSSEL and E. E. CONN, *Arch. Biochem. Biophys.* **122**, 256 (1967).
[15] D. W. RUSSEL, E. E. CONN, A. SUTTER and H. GRISEBACH, *Arch. Biochem. Biophys.*, submitted for publication.
[16] L. PATSCHKE, W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **21b**, 45 (1966).

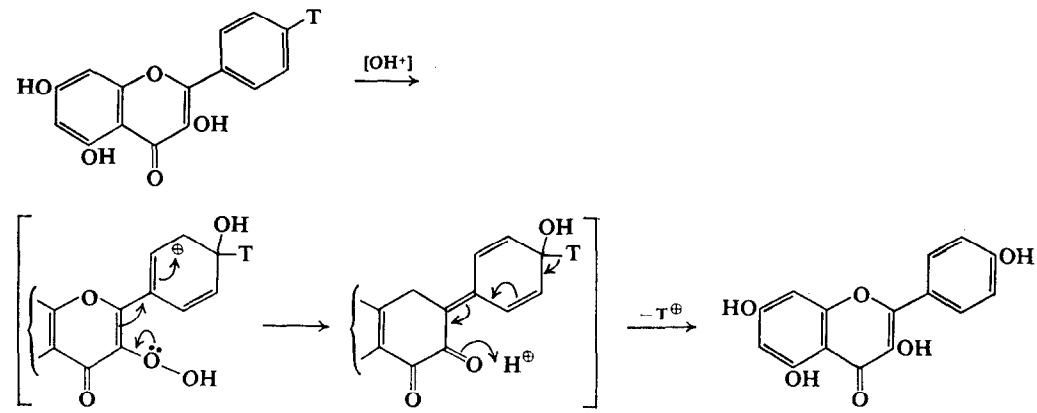

FIG. 2. REACTION SEQUENCE WHICH WOULD LEAD TO LOSS OF TRITIUM IF THE FLAVONOL WERE THE SUBSTRATE FOR HYDROXYLATION AT THE 4′-POSITION.

cent retention,[1,18] *N*-acetyl-3,5-T-tyramine → *N*-acetyl dopamine, 44 per cent retention[17]) and has been explained by formation of an *o*-quinoid structure as intermediate in the hydroxylation reaction (Fig. 3).

Arnheim and Zenk[19] have independently studied the NIH-shift in the biosynthesis of flavonoids in buckwheat. Their results are in complete agreement with our findings.

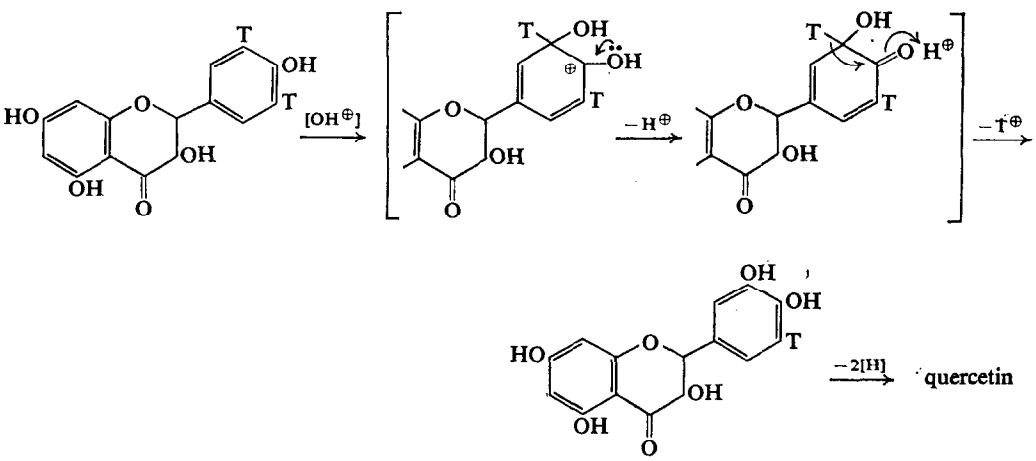

FIG. 3. PROPOSED REACTION SEQUENCE WHICH WOULD EXPLAIN LOSS OF TRITIUM AT C-3′ DURING HYDROXYLATION. HYDROXYLATION AT THIS POSITION IS ASSUMED TO TAKE PLACE WITH DIHYDROQUERCETIN AS SUBSTRATE.[8,16]

EXPERIMENTAL

Plants

The peas ("Palerbse") and chana seeds were a commercial variety. They were germinated for 24 hr in running water and for another 24 hr on moist filter paper and were then grown hydroponically in vermiculite.

[17] J. W. DALY, G. GUROFF, S. UDENFRIEND and B. WITKOP, *Arch. Biochem. Biophys.* **122**, 218 (1967).
[18] G. GUROFF, M. LEWITT, J. W. DALY and S. UDENFRIEND, *Biochem. Biophys. Res. Commun.* **25**, 253 (1966).
[19] N. ARNHEIM and M. H. ZENK, *Phytochem.* **8**, 107 (1969).

Isolation of Kaempferol and Quercetin[8]

The hydrolysis was modified as described in the text. After separation of kaempferol and quercetin by paper chromatography with 60 per cent acetic acid, the flavonols were further purified by additional chromatography in the same solvent and with butanol/acetic acid/water (4:1:1).

Degradation of Formononetin to 3-Nitroanisic Acid

(*a*) *Experiment with unlabelled material.* 100 mg of formononetin was heated with 3 ml of conc. HNO_3 for 45 min on the boiling water bath. The solution was then poured on ice and the light yellow crystals (13 mg) which precipitated were collected and dried. The product was purified by preparative TLC on silica gel G with toluene/ethylacetate/acetic acid (9:2:0·5). Two substances were present on the chromatogram: an u.v.-absorbing substance (dark blue, R_f 0·43) and a yellow compound (R_f 0·09). The compound with R_f 0·43 was identical in m.p. (190–192°),[20] u.v.-spectrum: λ_{max} (log ϵ) 235 nm (4·26), 317 nm (3·32), and chromatographic properties with 3-nitroanisic acid prepared by nitration of *p*-anisic acid with conc. HNO_3.

(*b*) *Degradation of the labelled formononetin.* 41 mg of formononetin (T-activity = 99,400 dpm) was degraded under the same conditions. Yield of the purified 3-nitroanisic acid 5·35 mg (17·5 per cent). T-activity = 7070 dpm.

Determination of Radioactivity

The determination of ^{14}C and T was carried out either with a Beckman scintillation spectrometer (CPM-100) or in the gas phase according to the method of Simon *et al.*[21] using a proportional counter with an anti-coincidence circuit (UNI-ZS, Fa. Berthold, Wildbad, Germany).

Acknowledgements—We thank Prof. B. Witkop, Bethesda, for the *p*-tritiophenylalanine and for valuable suggestions. The work was supported by Fonds der Chemischen Industrie.

[20] Beilsteins *Handbuch der Organischen Chemie*, Bd. X, 181.
[21] H. SIMON, H. DANIEL and J. F. KLEBE, *Angew. Chem.* **71**, 303 (1959).