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INCORPORATION OF ^{14}C-QUERCETIN INTO AN ISOPROPYL ALCOHOL- AND ACID-INSOLUBLE FRACTION OF TOBACCO

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Abstract—In tobacco plants injected with randomly ^{14}C-labeled quercetin, approximately 20 per cent of the injected radioactivity was incorporated, within 15 min, into the isopropyl alcohol-insoluble fraction. "Lignin" prepared from the isopropyl alcohol-insoluble fraction with either 1 per cent HCl or by treatment with the standard 72 per cent H_2SO_4 procedure was found to contain 4 per cent of the injected radioactivity in the 1 per cent HCl-insoluble fraction and 10 per cent in the 72 per cent H_2SO_4-insoluble fraction. No radioactivity was detected in the aldehyde fraction from these "lignin" preparations after an alkaline nitrobenzene oxidation. Acidification of the oxidized alkaline mixture results in a loss of radioactivity, which indicates that ^{14}C-quercetin may be incorporated into "lignin".

INTRODUCTION

IN A PRELIMINARY study, Chorney *et al.*[1] reported that significant amounts of radioactivity appeared in the isopropyl alcohol-insoluble fraction of tobacco leaves infiltrated with randomly ^{14}C-labeled quercetin. The present study was undertaken to confirm this preliminary finding, and to study the distribution of radioactivity in the isopropyl alcohol-insoluble fraction.

RESULTS AND DISCUSSION

The radiochemical purity of the ^{14}C-quercetin (10 mc/g carbon) used in these experiments was maintained for about 30 days. Within 15 min after the injection of this randomly ^{14}C-labeled quercetin into a tobacco leaf, approximately 20 per cent of the injected radioactivity was incorporated into the isopropyl alcohol-insoluble fraction of the injected leaf (8 per cent) and stem (12 per cent). Approximately 40 per cent was found in the alcohol-soluble fraction. The other 40 per cent was distributed in the other plant parts. Extending the post injection time to 60 or 180 min did not alter the amount of ^{14}C-quercetin incorporated into stem isopropyl alcohol-insoluble fractions, but may have increased the amount that was incorporated into the leaf isopropyl alcohol-insoluble fraction (Table 1). The major radioactive compound

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[1] W. CHORNEY, E. MURPHY, S. WENDER and N. SCULLY, *Abstracts*, 133rd Meeting, American Chemical Society, p. 8A. San Francisco, Calif., April (1958).

in the alcohol-soluble fraction was identified as quercetin. Two other radioactive compounds in lesser concentrations co-chromatographed with phloroglucinol and 3,4-dihydroxybenzoic acid.

"Klason lignin"[2] prepared from the combined isopropyl alcohol-insoluble fraction of leaves or stems contained about half of the radioactivity of the alcohol-insoluble fraction, which represents about 10 per cent of the total radioactivity originally injected as ^{14}C-quercetin. After nitrobenzene oxidation, about 50 per cent of the activity present in the "Klason lignin" fraction disappeared on HCl acidification, presumably through evolution as a gaseous compound, probably carbon dioxide. No carbon-14 was detected in the aldehyde fraction. The method of Stone and Blundell[3] and of Onishi *et al.*[4] showed that aldehydes were produced. Alkaline nitrobenzene oxidation of leaf and stem "Klason lignin" from non-injected plants yielded 0·5 per cent vanillin and 0·2 per cent *p*-hydroxybenzaldehyde from leaf "lignin"; and 1·5 per cent vanillin, 0·9 per cent syringaldehyde, and 0·2 per cent *p*-hydroxybenzaldehyde from stem "lignin". When "Klason lignin" was carried through the oxidation and isolation procedure without the addition of nitrobenzene, no loss of radioactivity occurred on acidification and no radioactivity was detected in the aldehyde fraction.

TABLE 1. PERCENT INCORPORATION OF CARBON-14 INTO ISOPROPYL ALCOHOL INSOLUBLE FRACTION AFTER INJECTION OF 1·45 μc OF ^{14}C-QUERCETIN

Plant number	1	2	3
Metabolism time (min)	15	60	180
Injected leaf	7·9	15·0	12·5
Injected area	0·6	2·3	1·6
Stem	12·0	11·1	10·3

About 26 per cent of the ^{14}C activity was found in solution after alkaline nitrobenzene oxidation of randomly labeled "Klason lignin" isolated from tobacco plants grown in a $^{14}CO_2$ atmosphere. On acidification, 10 per cent of this activity was lost. A small amount of radioactivity was found in the aldehyde fraction (0·008 μc), which represents 0·12 per cent of the original ^{14}C present in this "lignin" preparation.

"HCl lignin" retained 4 per cent of the injected ^{14}C-quercetin. After alkaline nitrobenzene oxidation, only 5 per cent of the activity in this "lignin" preparation was accounted for, and 40 per cent of this remaining radioactivity was lost on acidification.

When ^{14}C-quercetin was subjected to the alkaline nitrobenzene procedure, 45 per cent of its radioactivity was lost on oxidation and the remainder on acidification. Neither degradation nor incorporation of quercetin was detected when the blank sample was carried through the lignin isolation procedure.

Because a loss of radioactivity occurs on acidification after alkaline nitrobenzene oxidation of "lignins" prepared from tobacco tissue injected with ^{14}C-quercetin, as well as from randomly labeled tobacco lignin and from ^{14}C-quercetin, it seems that quercetin, or its metabolic products, is rapidly incorporated into the "lignin". It is possible that the ^{14}C-quercetin is bound to the "lignin" structure or to other plant compounds and is not removed from them by the isolation procedure employed.

[2] Standard T-13m-54 of Technical Association of the Pulp and Paper Industry, July (1954).
[3] J. E. STONE and M. J. BLUNDELL, *Anal. Chem.* **23**, 771 (1951).
[4] I. ONISHI, N. NAGASAWA and K. YAMAMOTO, *J. Agr. Chem. Soc., Japan* **28**, 756 (1954).

EXPERIMENTAL

Preparation of ^{14}C-quercetin

Randomly labeled ^{14}C-rutin, 10 mc/g carbon was isolated from the leaves of buckwheat plants grown in a $^{14}CO_2$-enriched atmosphere.[5] Rutin (quercetin-3-rutinoside) was purified by chromatography on a cellulose column, using aqueous 80% sec-butyl alcohol as the developing and eluting solvent. ^{14}C-quercetin was prepared by hydrolyzing this purified rutin with 2% H_2SO_4. The quercetin (3,3′,4′,5,7-pentahydroxyflavone) was purified by cellulose column chromatography with aqueous 10% sec-butyl alcohol followed by aqueous 80% sec-butyl alcohol in which the quercetin was eluted. Fractions which contained only the ^{14}C-quercetin were combined, the solvent was removed by evaporation and the purified ^{14}C-quercetin was stored in a deep freeze at −20°. The purity of this preparation was checked periodically by radioautography of one-dimension paper chromatograms, which were run on S and S 589 Blue Ribbon paper. The two irrigating solvents used were 10 and 80% sec-butyl alcohols, respectively.

Infiltration of ^{14}C-quercetin

9-week-old greenhouse-grown tobacco plants, *Nicotiana tabacum* (c.v., one-sucker), were transferred to a controlled environment room 2 weeks prior to the experimental treatment. Just before injection of the ^{14}C-quercetin, all leaves below the eighth leaf from the terminal bud were removed. Each of three plants was injected into the basal portion of the petiole of the eighth leaf with 1·45 μc of purified ^{14}C-quercetin.[6] The injected leaf was allowed to metabolize the ^{14}C-quercetin for 15 min *on the plant*; after which it was removed and the plant was separated into the injected area, the injected leaf, other leaves, and the stem. Each portion was fixed in boiling 85% isopropyl alcohol. One plant (plant 1) was fixed immediately after removal of the injected leaf. Injected leaves removed from the other two plants (plants 2 and 3) were left for 45 or 165 min with their petioles immersed in H_2O before they were fixed in isopropyl alcohol. A blank sample was prepared by adding 1·45 μc of ^{14}C-quercetin to a leaf (8th) which had been fixed in isopropyl alcohol in order to establish changes in ^{14}C-quercetin that may have occurred during the chemical procedures employed following fixation.

Fixation and Extraction

After fixation, the plant material was extracted in a Soxhlet extractor for 15 hr, using fresh isopropyl alcohol. The insoluble white residue was designated *the isopropyl alcohol insoluble fraction.* After drying at 80° and weighing, this residue was ground in a Wiley mill to a particle size that will pass through a 60-mesh screen. The absolute activity was determined by solid counting of infinitely thick samples and comparing the counts to the counts from a randomly labeled plant standard, 1·687 μc/g.

"Lignin" Preparation

Leaf and stem lignin of two types were prepared from the combined alcohol-insoluble fractions; "HCl lignin", by the methods of MacDougall and DeLong,[7] and 72% H_2SO_4 "Klason lignin",[2] ^{14}C activity in these "lignins" was determined by solid counting. In addition, "Klason lignin" was prepared from randomly ^{14}C-labeled tobacco leaf tissue.[5] Portions of the above "lignins" and ^{14}C-quercetin were subjected to alkaline nitrobenzene oxidation[8] in order to determine ^{14}C that had been incorporated into the aldehyde fraction of "lignin". The distribution of ^{14}C radioactivity was determined in the fractions from the nitrobenzene oxidation procedure.

Aldehyde Determination

The aldehydes produced by alkaline nitrobenzene oxidation were separated by chromatography on Whatman No. 1 paper, using *n*-butyl alcohol saturated with 2% aqueous HN_4OH.[9] Then, locations were determined by examination under u.v. light (366 nm) after exposure to ammonia vapors. After the solutions were made alkaline with KOH, the individual aldehydes were extracted with ethyl alcohol and determined by measuring their absorbances at 354, 372 and 339 nm for vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde, respectively.

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[6] W. CHORNEY and N. J. SCULLY, Abstracts, *IX International Bot. Congress Montreal, Canada* **2**, 70 (*1959.*)
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