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A PRELIMINARY STUDY OF THE METABOLIC FATE OF QUERCETIN AND RUTIN IN *NICOTIANA TABACUM*

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A PRELIMINARY STUDY OF THE METABOLIC FATE OF QUERCETIN AND RUTIN IN *NICOTIANA TABACUM*

CHAPTER I

INTRODUCTION

This study was undertaken for the purpose of learning whether quercetin and rutin are metabolized by green plants, and if so, some of the details of the process. The problem was part of a cooperative program established in 1954 by biochemists at the University of Oklahoma with a group of scientists at the Argonne National Laboratory, Argonne, Illinois (1). Rutin and quercetin are typical representatives of the flavonoid class of compounds and are constituents of various green plants (2). Rutin has been reported in concentrations varying from a few parts per million to concentrations of 6.4% in the fall leaves and 15.6% in the flowers of *Sophora japonica* (3) on a dry weight basis. The rutin concentration in a three-month old tobacco plant grown in soil under greenhouse conditions was determined on a fresh weight basis to be approximately 0.05% for the older leaves and approximately 0.012% for the younger leaves (4).

Although some suggestions have been made as to the physiological significance of the flavonoid pigments (5), no study has been made to determine whether these compounds may be assigned some specific role in

the overall plant metabolism. The following two reasons are generally offered: 1) flavonoid compounds appear to accumulate in plants in a manner like that of terminal metabolites and waste products, and 2) experimental methods for making such a study were not available.

The production of ^{14}C containing compounds and the development of techniques for detecting them provided a method for studying this problem. Thus, rutin and quercetin biosynthesized from $^{14}CO_2$ could be supplied to the plants and traced by the radioactive content to any of the substances into which they may have been transformed. The facilities for performing such experiments were available at the Argonne National Laboratory, where about ten years ago W. Chorney became interested in this study. Norbert J. Scully, John Skok, and Chorney had acquired considerable experience in growing plants in a $^{14}CO_2$ atmosphere (6); and since, buckwheat plants were known to contain a utilizable amount of rutin, these workers were able to obtain uniformly labeled ^{14}C rutin from buckwheat leaves. By hydrolysis of the rutin, ^{14}C quercetin could be made available.

The following is an outline of early experiments by Chorney (7), which provided the background for the research in this dissertation. Nicotiana tabacum was chosen as the experimental plant because the Argonne workers had experience in growing it, and because the isolation and identification of the major polyphenols of tobacco had been previously studied. No free quercetin was identified in the isolation studies. This suggested that quercetin might be rapidly metabolized in this plant. Further support for this suggestion was obtained when ^{14}C quercetin was supplied to tobacco in short-term experiments and no ^{14}C rutin could be isolated.

In the early experiments, a tobacco leaf on a plant was infiltrated

with ^{14}C quercetin, and after a defined metabolic time, the leaf was cut into pieces and dropped into boiling 85% isopropyl alcohol. Exhaustive extraction with 85% isopropyl alcohol then provided an alcohol soluble fraction and an alcohol insoluble fraction. A chromatographic and radioautographic survey indicated that, in addition to the infiltrated ^{14}C quercetin, radioactive 3,4-dihydroxybenzoic acid was present in the alcohol soluble fraction. The presence of radioactivity in a 72% sulfuric acid insoluble fraction indicated that quercetin might be involved in lignin formation.

This dissertation will present a description and discussion of the short-term experiments, based on Chorney's initial work, which traced the randomly labeled ^{14}C rutin and quercetin after injection of their ethanolic solutions into growing tobacco plants. The major part of this study is concerned with the characterization of the radioactivity located in the alcohol insoluble fraction. It is particularly concerned with that part which still remains insoluble after submitting the original insoluble fraction to acid hydrolysis.

CHAPTER II

PURIFICATION OF ^{14}C RUTIN AND ^{14}C QUERCETIN

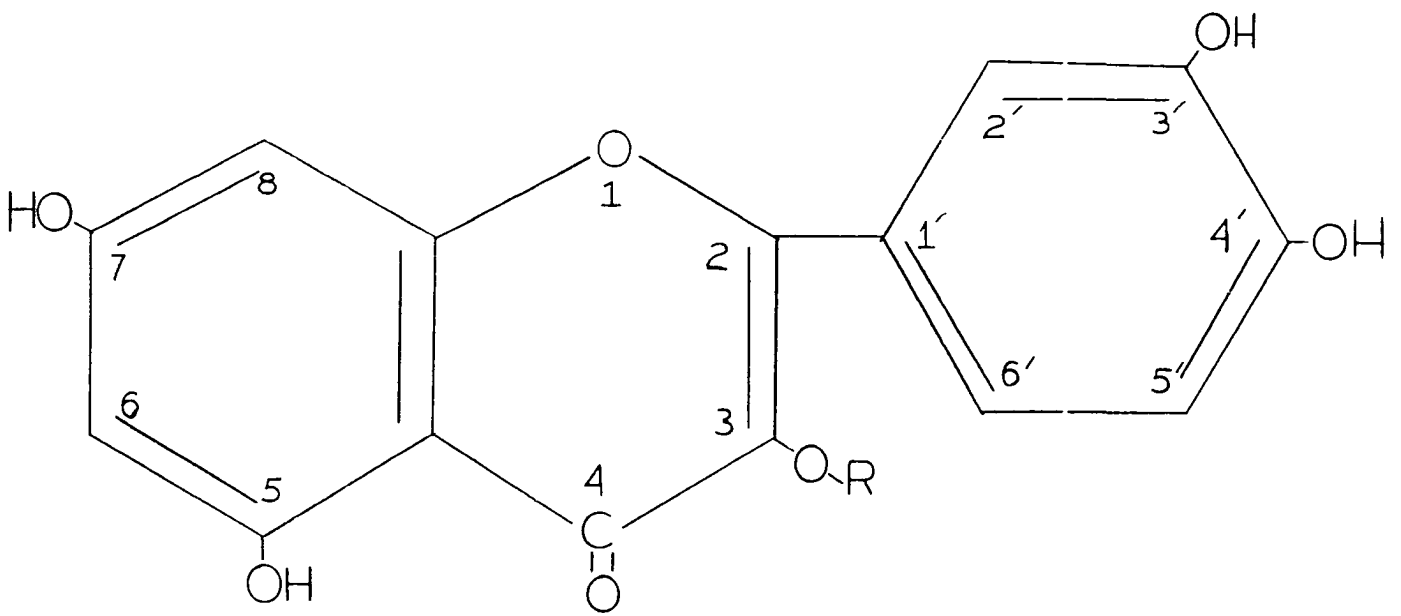
Introduction

Figure 1 shows the structural formulae of rutin and quercetin.

Recrystallized, randomly labeled rutin and quercetin from buckwheat plants were purified by adsorption chromatography on a cellulose column. The criterion of purity was the production by paper chromatographic methods of single radioactive spots or bands as detected by exposure to x-ray film. Two precautions were found to be necessary in order to maintain the chromatographic purity of ^{14}C rutin and quercetin. Radiodegradation could be held to a minimum if the compounds were stored in the solid state in a refrigerator freezing compartment. Storage as an ethanolic solution caused up to 25% degradation in as little as two weeks' time. Also, quercetin eluted from paper chromatograms was always found to be contaminated with radioimpurities. One chromatographic developing solvent, the water layer of chloroform-acetic acid-water (2:1:1 v/v), could not be used because it produced anomalous areas during chromatographic development. In the above cited observations, rutin was affected less than was quercetin. Rutin was stable for a longer period of time in solution and appeared to be unaffected during chromatography. Kaempferol and a kaempferol glycoside in the number three position gave indications of similar radiodegradation results.

FIGURE 1

STRUCTURAL FORMULAE OF RUTIN AND QUERCETIN



RUTIN: R = GLUCOSYL-RHAMNOSIDE

QUERCETIN: R = H

The aim of this phase of the study was to obtain ^{14}C rutin and quercetin of sufficient chromatographic and radioautographic purity for the metabolism studies. No attempt was made to determine the identity of all degradation products. However, the identity of some of the major ones have been tentatively suggested.

Chromatographic Survey of ^{14}C Flavonoid Compounds

Eight ethanolic solutions of ^{14}C flavonoid samples were analyzed to determine their radiopurity. The solutions, which had been stored in a refrigerator for about three years, were labeled as follows: $C^{14}R^2$ 220 mg., $C^{14}R^2$ pure, C^{14} Rutin, $C^{14}Q$, $C^{14}Q^2$ pure, C^{14} Quercetin, C^{14} kaempferol "glycoside", and C^{14} kaempferol, where R = rutin and Q = quercetin. Quercetin and kaempferol had been obtained by hydrolysis of their glycosides. Q^2C^{14} pure and kaempferol "glycoside" had been purified by paper chromatography. Each of the samples was spotted on a sheet of Schleicher and Schuell No. 589 blue ribbon chromatography paper until the radioactivity could be detected with a hand probe Geiger counter. The sheets were chromatographed two dimensionally with the aqueous layer of chloroform-acetic acid-water: 2:1:1 v/v ($C_2A_1W_1$) used for the first direction and with aqueous eighty percent secondary butyl alcohol (80% SBA) used for the second direction. The results, after exposing the chromatograms to x-ray film, are tabulated in Table 1.

Rutin and the kaempferol "glycoside" contained small concentrations of radioimpurities. The C^{14} Quercetin and $C^{14}Q$ contained an appreciable concentration of radioimpurities; while the eluted quercetin and kaempferol produced no film darkening from their R_f region and only darkened the film faintly in any one area.

TABLE 1[1]

CHROMATOGRAPHIC CHARACTERISTICS OF ^{14}C FLAVONOID SAMPLES

Sample	Rf value aq. layer $C_2A_1W_1$	Rf value 80% SBA	Film darkening intensity	Fluorescence U.V. light
$C^{14}R^2$ 220 mg.	0.73	0.36	Maximum	Purple
	0.77	0.50	Very faint	—
	0.77	0.00	Very faint	—
	0.92	0.95	Very faint	—
$C^{14}R^2$ pure	0.73	0.36	Maximum	Purple
	0.74	0.50	Very faint	—
	0.77	0.00	Faint	—
C^{14} Rutin	0.73	0.36	Maximum	Purple
$C^{14}Q$	0.28[2]	0.90[2]	Strong	—
	0.80	1.00	Faint	—
	0.90	1.00	Strong	—
$C^{14}Q^2$	0.70	0.89	Very faint	—
	0.80	0.98	Faint	—
	0.90	1.00	Faint	—
C^{14} Quercetin	0.27[2]	0.85[2]	Maximum	Faint yellow
	0.27	1.00	Faint	—
	0.73	0.36	Faint	—
	0.81	1.00	Very faint	—
	0.90	1.00	Very faint	—
C^{14} Kaempferol "glycoside"	0.73	0.50	Maximum	Purple
	0.75	0.00	Faint	—
C^{14} Kaempferol	0.00	0.00	Very faint	—
Ref. Rutin	0.73	0.36		Purple
Ref. Quercetin	0.28[2]	0.77[2]		Yellow

1 Darkening of film at origin occurred in all cases.

2 Tailing and spreading of spot occurred.

Purification of ^{14}C Quercetin

The sample designated $C^{14}Q$, with a specific activity of 10 μ C/milligram of carbon, was chosen to be purified further for use in the first metabolism experiment. To verify the complete degradation of sample $C^{14}Q^2$ pure and to determine the chromatographic behavior of the impurities in sample $C^{14}Q$ preparatory to purification, the samples were heavily streaked on 1.5 inch strips of the chromatographic paper and chromatographed with several solvent systems. The dried strips were passed through a gas flow strip counter to provide a quick observation of the extent of degradation, and the extent of separation produced by the solvent systems used. The strips were then exposed to x-ray film. After exposure, the strips were viewed under longwave length U.V. light (3660A°) and sprayed with a 1% ethanolic $AlCl_3$ solution followed by spraying with a diazotized para-nitroaniline and sodium acetate solution. The determined R_f values and colors produced are listed in Table 2.

Based on the results listed in Table 2, $C^{14}Q$ was purified by chromatography on a cellulose column using the following sequence of eluting solvents: aqueous 10% secondary butyl alcohol and aqueous 90% secondary butyl alcohol. The dry, packed column was washed with 250 ml. of water, 200 ml. of 50% ethanol and 200 ml. 90% secondary butyl alcohol. The cellulose was then washed with methanol until the secondary butyl alcohol had been removed. The column was then sucked dry by means of a water aspirator. About two to three inches of cellulose were dug out of the top of the column and the surface packed smooth again. To this surface was added, dropwise, a methanolic solution of the $C^{14}Q$ sample which contained a measured amount of radioactivity of 7.7×10^6 cpm. For this

TABLE 2

CHROMATOGRAPHIC BEHAVIOR OF $C^{14}Q^2$ PURE AND $C^{14}Q$

$C^{14}Q^2$ PURE

Solvent	R_f values	Fluorescence U.V. light	Chromogenic sprays 1% $AlCl_3$	Diazotized p-nitro-aniline
80% SBA	Origin	--	--	--
	0.99	Faint pale blue	--	Light orange
Aq. layer of $C_2A_1W_1$	Origin	--	--	--
	0.86	Faint pale blue	--	Yellow
H_2O	Origin	Faint pale blue	--	Faint yellow
	0.69	Pale blue	--	Yellow fades

$C^{14}Q$

80% SBA	0.77	Yellow	Bright yellow	Brown
	0.94	--	Faint yellow	Brown fades
	0.98	--	Faint yellow	Brown fades
Aq. layer of $C_2A_1W_1$	0.27	Yellow	Bright yellow	Brown
	0.30	--	Faint yellow	Tan fades
	0.44	--	--	Yellow fades
	0.61	--	--	Yellow fades
	0.68	--	--	Yellow fades
	0.85	Faint blue	--	Yellow fades
H_2O	Origin	Yellow	Bright yellow	Brown
	0.21	--	--	Tan fades
	0.50	--	--	Yellow fades
	0.80	Faint blue	--	Faint yellow fades
10% SBA	0.07	Yellow	Bright yellow	Brown
	0.13	--	Faint yellow	Faint yellow fades
	0.50	--	--	Faint yellow fades
	0.64	--	--	Faint yellow fades
	0.80	--	--	Faint yellow-green

determination and all subsequent radioactivity measurements, an end window, gas flow counter operating at a voltage of 2200 volts with an efficiency of 3.37% and an average background of 17 cpm with aluminum planchets was used. After charging the column, the methanol was evaporated by directing a current of air from a hair dryer down the tube of the column. To make certain the methanol had been removed, the column was dried by means of the water aspirator. Fresh, washed, dry cellulose was added to the top of the column, packed firmly and covered with glass wool.

The prepared column was inverted into a jar containing 10% SBA. The solvent was allowed to ascend the column until it reached the glass wool plug in the bottom. The column was then reverted to its original position and development continued downward. Table 3 lists the pertinent information of the fractions collected. Fraction numbers twenty-six through thirty-two were paper chromatographically analyzed for radioimpurities and for quercetin identification. They were spotted and chromatographed in 80% SBA, the aqueous layer of $C_2A_1W_1$ and 10% SBA. After drying, the papers were exposed to x-ray film, then examined under long wavelength ultraviolet light (3660A°) and sprayed with 1% ethanolic aluminum chloride solution. Table 4 lists the results of this analysis.

Fraction numbers twenty-seven through thirty-one were combined, evaporated to dryness and then recrystallized from hot ethanol and hot water. The fluffy bright yellow crystals produced were centrifuged and the mother liquor was decanted. The solid residue was washed with cold water, centrifuged again and dissolved in 5 ml. of ethanol. The solution was determined to contain 29 μ C of radioactivity. This prepared solution, after determining it was chromatographically pure, was used for the metabolism experiment described in Chapter 4.

TABLE 3

COLUMN PURIFICATION OF $C^{14}Q$

Fraction No.	Volume	Eluant color	cpm x 10^5 / vol.	Solvent
1	20 ml.	Light brown	6.33	10% SBA
2	"	Faint light brown	5.63	"
3	"	Clear	2.70	"
4	"	"	1.12	"
5	"	"	0.34	"
6	"	"	0.23	"
7	"	"	0.20	"
8	"	"	0.19	"
9	"	"	0.21	"
10	21 ml.	"	0.15	"
11	20 ml.	"	0.13	"
12	"	"	0.15	"
13	"	"	0.16	"
14	"	"	0.13	"
15	"	"	0.08	"
16	"	"	0.07	"
17	"	"	0.09	"
18	"	"	0.08	"
19	22 ml.	"	0.09	Started 90% SBA
20	20 ml.	"	0.10	"
21	"	"	0.08	"
22	"	"	0.08	"
23	"	"	0.08	"
24	"	"	0.13	"
25	"	"	0.16	"
26	10 ml.	Pale yellow	2.02	90% SBA
27	"	Yellow	12.10	"
28	11 ml.	Yellow	19.00	"
29	10 ml.	Light yellow	9.06	"
30	20 ml.	"	9.10	"
31	"	"	2.52	"
32	"	"	1.00	"
33	"	Clear	0.07	Started Methanol
34	"	"	0.45	"
35	"	"	0.22	"
36	"	"	0.15	"
37	"	"	0.14	Methanol
38	"	"	0.12	"
39	"	"	0.10	"
40	22 ml.	"	0.07	"
41	40 ml.	"	0.16	"
42	68 ml.	"	0.25	"
43	98 ml.	"	0.26	"

TABLE 4

ANALYSIS OF QUERCETIN FRACTIONS 26-32

Fraction No.	26	27	28	29	30	31	32	Q	Color with 1% $AlCl_3$
R_f values		0.77	0.77	0.77	0.77	0.77		0.77	Bright yellow
(80% SBA)	0.80	0.80	0.80	0.80	0.80	0.80	0.80		
		0.90	0.90						
R_f values	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
(aq. layer of $C_2A_1W_1$)		0.22	0.22	0.22	0.22	0.22		0.22	Bright yellow
		0.45	0.45						
		0.66	0.66						
		0.75	0.75	0.75	0.75	0.75			
R_f values	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	Bright yellow
(10% SBA)		0.52	0.52	0.52					
		0.71	0.71	0.71					

Purification of ^{14}C Rutin

Randomly labeled ^{14}C rutin with a specific activity of 10 μ C per milligram of carbon had been isolated from buckwheat by Chorney. He had developed a procedure for purifying this rutin by chromatography on a cellulose column. The procedure for preparing the column was the same as the one described for the purification of quercetin. Twenty-five milligrams of ^{14}C rutin dissolved in a minimum volume of methanol was dropped onto the top of a column of cellulose 41 cm. high and 3.5 cm. in diameter. The column was first subjected to ascending chromatography using 80% SBA. The column was then inverted and chromatography continued downward. The characteristics of the collected fractions are tabulated in Table 5.

Small aliquots of fractions one through ten were spotted and chromatographed with 80% SBA containing 0.1% aqueous 88% formic acid v/v (80% SBA, 0.1% F). The dried chromatogram was exposed to x-ray film, and sprayed with a 1% ethanolic aluminum chloride solution. After the papers had dried and the developed colors noted, the papers were sprayed with a diazotized para-nitroaniline solution. The results are tabulated in Table 6. Fractions five, six, seven, and combined fractions eight and nine were separately evaporated to dryness in vacuo. The yellow solids thus obtained were stored in a deepfreeze for subsequent tracer studies.

Some Observations Concerning Radioimpurities Mixed With ^{14}C Quercetin

The detection of radioactive substances on paper chromatograms by exposing them to x-ray film is a very sensitive method indeed. This inherent attribute may be of great advantage in metabolic studies by insuring the absence of impurities and enabling one to trace the resultant

TABLE 5

COLUMN PURIFICATION OF ^{14}C RUTIN

Fraction No.	Volume	Eluant color	cpm x 10^4 vol.	Solvent
1	20 ml.	Clear	3.88	80% SBA
2	20 ml.	"	2.63	"
3	20 ml.	"	1.57	"
4	23 ml.	Faint yellow	13.20	"
5	5 ml.	Yellow	31.10	"
6	8 ml.	"	574.00	"
7	18 ml.	"	452.00	"
8	22 ml.	Clear	3.62	"
9	20 ml.	"	1.36	"
10	22 ml.	"	1.01	Started H_2O
11	45 ml.	"	1.63	"
12	44 ml.	"	1.06	"
13	45 ml.	"	0.91	"
14	36 ml.	Faint yellow	1.90	H_2O
15	150 ml.	"	3.39	Started Methanol
16	194 ml.	Clear	0.54	H_2O & Methanol
17	160 ml.	"	1.31	Methanol
18	176 ml.	"	0.60	"

TABLE 6

ANALYSIS OF RUTIN FRACTIONS 1-10[1]

Fraction No.	Rf values	Chromogenic sprays: 1% $AlCl_3$	Chromogenic sprays: Diazotized p-nitro aniline
1	0.90	--	--
2	0.90	--	--
3	0.90	--	--
4	0.90	--	--
5	0.42	Yellow	Orange-yellow
	0.55(t)	--	--
	0.90(t)	--	--
6	0.42	Yellow	Orange-yellow
	0.55(t)	--	--
7	0.42	Yellow	Orange-yellow
8	0.42(t)	--	--
9	0.42(t)	--	--
10	0.42(t)	--	--
C^{14} rutin	0.00	--	--
10 μ C/gm carbon	0.42	Yellow	Orange-yellow
	0.55(t)	--	--
	0.90(t)	--	--
$C^{14}R^2$ 220 mg.	0.00	Yellow	Faint brown
	0.07	Dark yellow	Faint brown
	0.42	Yellow	Orange-yellow
	0.55(t)	Faint yellow	--
	0.90(t)	--	--

(t) trace darkening of x-ray film

[1] Chromatography solvent: 80% SBA, 0.1% Formic acid

metabolic products. Radioactivity, however, may also be a cause of degradation. Radioimpurities were detected in the flavonoid samples during purification. The increase in number of radioimpurities and their increase in concentration strongly suggested they were caused by radiodegradation and were not natural products isolated along with the desired flavonoid from the buckwheat plant and incompletely removed. Furthermore these impurities were not detectable in the samples studied immediately after their original purifications from the plant.

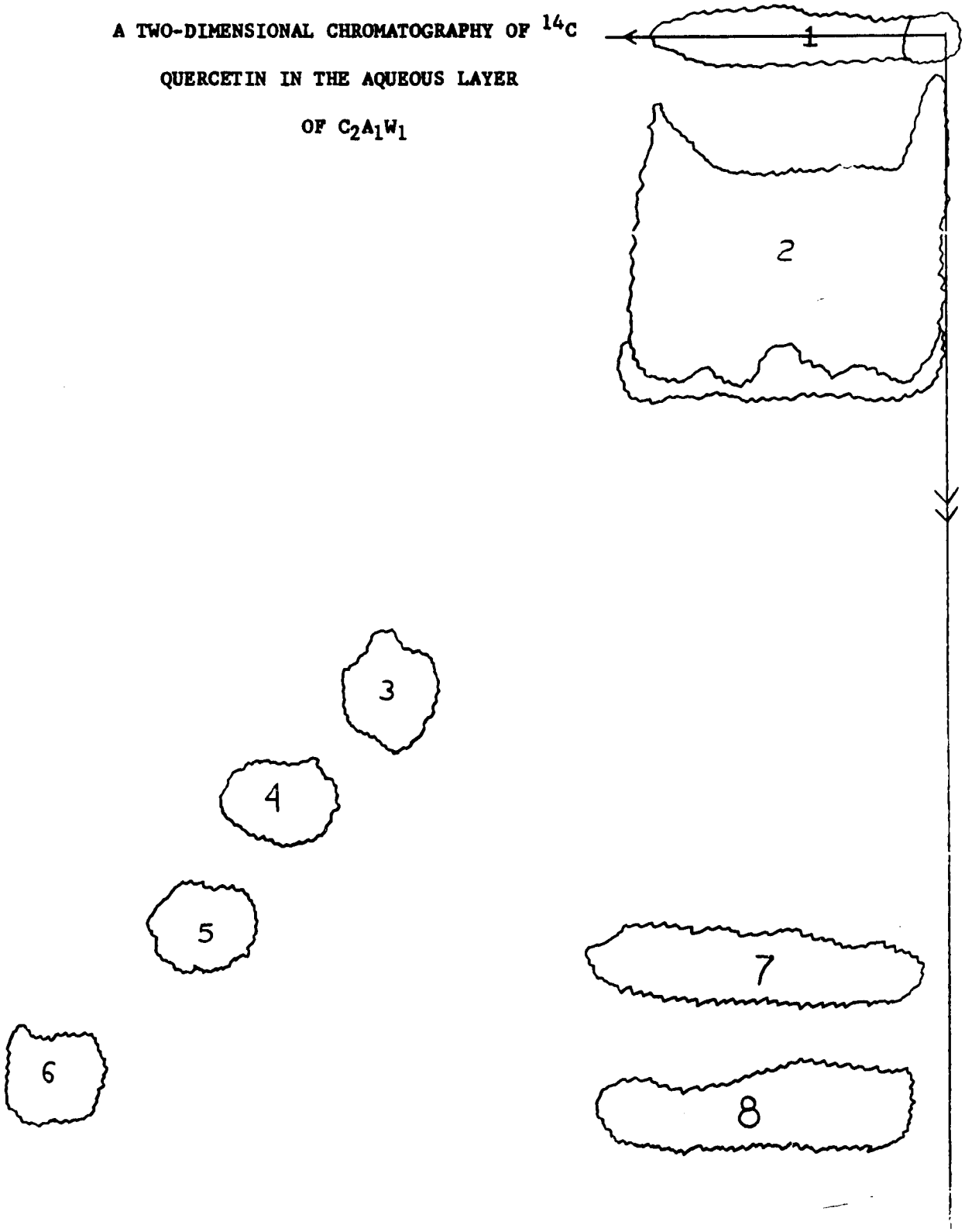
While radiodegradation could not be prevented, procedures were established which held the process to a minimal occurrence. Material stored in solution had to be purified before each experiment. Dry material could be stored for several weeks without appreciable radiodegradation occurring. Storage in a freezer also appeared to inhibit degradation. If purification was carried out by cellulose column chromatography, material could be obtained, which contained only barely detectable radioimpurities after heavy spotting. However, material purified by paper chromatography contained noticeably higher concentrations of radioimpurities. When rutin was studied in this manner, it gave indications of being a more stable compound than quercetin.

When purified ^{14}C quercetin was chromatographed in 80% SBA or aqueous 20% KCl, two faintly darkened areas were observed on the film, in addition to the quercetin area. These areas corresponded to areas having R_f values around 0.80 and 0.90 with 80% SBA and 0.27 and 0.44 with 20% KCl. The aqueous layer of chloroform, acetic acid, water (2:1:1 v/v) was employed as a chromatography solvent in the early experiments. With this solvent system even newly purified ^{14}C quercetin produced significant amounts of

radioimpurities. The number of these was not reproducible. This suggested the likelihood that varying concentrations of material were being spotted. However, the following work indicates that the solvent caused the anomalous spots. About 40 μg. representing about 0.058 μC of purified ^{14}C quercetin was spotted and chromatographed in two dimensions in this solvent. Figure 2 represents the developed x-ray film of this chromatogram. Area 2 produced maximum darkening and corresponds to quercetin. The others produced the same intensity of darkening. Although they were not very dark, they were easy to detect. Areas 7 and 8 definitely correspond to areas 5 and 6 but were separated after chromatography in the second direction. Either the solvent produced them or else it doesn't have the partitioning power to separate them in only one development. Area 1 could be due to irreversible adsorption on the paper with subsequent detection by the sensitive film. 20% KCl was substituted for this solvent in later studies in order to avoid these difficulties.

FIGURE 2

A TWO-DIMENSIONAL CHROMATOGRAPHY OF ^{14}C QUERCETIN IN THE AQUEOUS LAYER OF $C_2A_1W_1$



CHAPTER III

PROCEDURES

Injection

The best method of supplying a carbon containing compound to a living plant is still a controversial question. The controversy arises from the inability to determine whether the action of supplying or the chemical supplied are affecting the plant so that it reacts differently to the supplied compound than it would during the normal process of biosynthesis and metabolism. Also, for the purpose of this experiment, it was necessary for the plant to take up the material as quickly and as quantitatively as possible.

Probably the most suitable method known for combining these features is an injection method devised in the United States Department of Agriculture. This method was adapted by Chorney at the Argonne National Laboratory for injection into tobacco plants. The injection was made as detailed in the following description. The petiole near the stem of a growing plant was covered with a thin layer of Dow-Corning Silicone grease to prevent spreading and absorption of the applied solution. A few drops of an ethanolic solution of labeled rutin or quercetin was applied to the greased area from a 0.5 ml. graduated plunger syringe and a No. 27 hypodermic needle. The needle was then injected through this pool and into the petiole. The object was to puncture a vascular bundle. The needle was

removed, and the success of the injection could be determined by the decrease of the small pool. Before the solution was all drawn in, a few more drops were applied. This was continued until the desired amount of solution was drawn in, or until no more solution could be drawn in by that puncture. In such a case, a new puncture was made. The injected area was then washed with a few drops of the solution solvent. For the tobacco plant grown in controlled light and temperature rooms, as much as 0.5 ml. of solution could be taken up by the plant in about five minutes. (Figure 3 is a radioautograph of an injected leaf, after injection of ^{14}C rutin.)

Fixation and Extraction

After injection, the plant was allowed to metabolize for a defined length of time. Afterwards, the plant was cut into different fractions, each of which was cut into small pieces and immersed in boiling 85% isopropyl alcohol for a five-minute enzyme fixation period. The hot mixture was filtered through an appropriate size cellulose Soxhlet thimble and extraction was completed in a Soxhlet apparatus with fresh solvent for about fifteen hours. The injected plant had thus been divided into two main fractions. The combined extracts represented an alcohol soluble fraction and the residue represented an alcohol insoluble fraction.

Examination of Alcohol Soluble Fraction

This fraction was again divided; this time into an aqueous fraction and "tars" fraction. The isopropyl alcohol was evaporated from each extract on a steam bath by directing a stream of air perpendicular to the surface of the solution. Water was added to insure complete removal of the alcohol. The dark green solution had been changed into a clear yellow

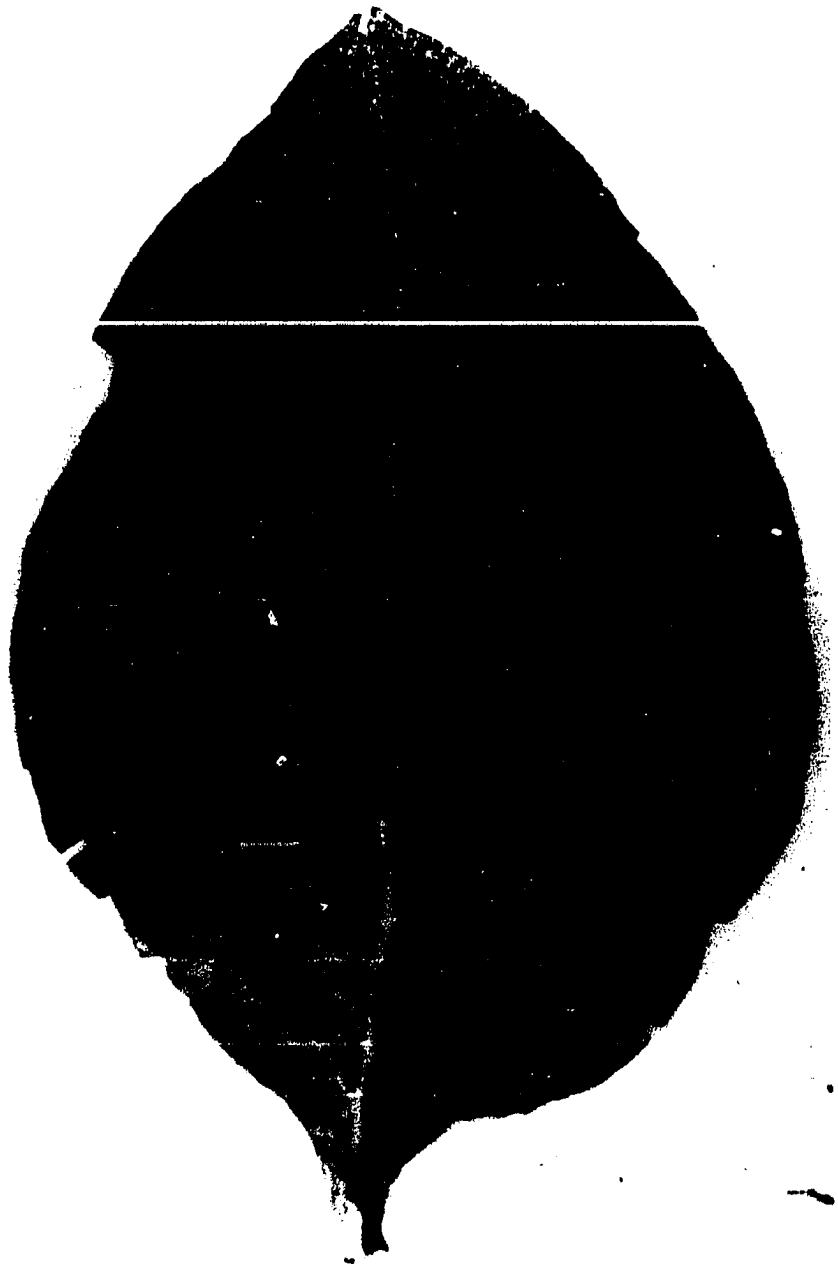


FIGURE 3

RADIOAUTOGRAPH OF A ^{14}C RUTIN INJECTED TOBACCO LEAF

Courtesy of William Chorney

solution as a supernatant above a black and gummy "tar" layer. The solution was decanted into a volumetric flask and diluted to the mark. The "tars" were dissolved in boiling isopropyl alcohol and benzene and stored in volumetric flasks. Aliquots were transferred from the flasks to aluminum planchets and evaporated to dryness over a hot water bath. The cpm were determined, and the total activity was calculated in microcuries for the individual fractions. An attempt was then made to characterize the radioactive substances in the alcoholic extracts, employing chromatographic and radioautographic techniques.

Examination of the Alcohol Insoluble Fraction

The white residues in the Soxhlet thimbles after extraction were dried overnight in a forced air oven vented to the outside atmosphere at a temperature of about 80°C. They were then weighed and ground in a Wiley mill to pass a sixty-mesh screen. The ground material was plated onto a plastic planchet with dimensions of 8 mm. diameter and 2 mm. deep. The cpm were determined. The absolute activity of the sample was determined by comparison of its cpm with the cpm of a uniformly labeled ^{14}C snapdragon standard of 3.61 C per gram of carbon or 1.687 C per gram of tissue, assuming infinitely thick samples. The samples were stored in the refrigerator when not being assayed.

The alcohol insoluble material prepared by this procedure was submitted to a lignin analysis. Two isolation procedures were followed for defining lignin in the alcohol insoluble fraction. The first utilized the studies made by MacDougall and DeLong (8, 9, 10) concerning the relative merits of extracting solvents in regards to the extraction of nitrogenous

material from plant tissue preparatory to lignin determinations. A mixture of the ground, dried, extracted tobacco and aqueous 1% HCl (150 ml. of 1% HCl per gram of material) was refluxed for four hours. Dow Corning anti-foam-A emulsion was added to decrease foaming. The mixture was filtered and washed with distilled water. The filtrate was saved for future study. The residue was oven dried. It was then transferred to a Soxhlet thimble and extracted for thirty hours with an ethanol-benzene solution, 1:2 v/v. The residue was then air dried, weighed and ground in a Wiley mill to pass a screen of mesh No. 40 and the radioactive content determined.

This material was oxidized by the alkaline nitrobenzene procedure of Stone and Blundell (11). A mixture of 0.1 gram of the ground material, 0.15 ml. of nitrobenzene, and 2.5 ml. of 2 N. sodium hydroxide was transferred to a stainless steel bomb of 5 ml. capacity. The bombs were secured at both ends by pressure screws whose threads had been coated with Dow Corning silicone grease. They were placed, horizontally, on a vertical movement mixing platform in an oven. The temperature was raised to 160° C over a one-hour period, after which the oxidation was allowed to proceed for three hours. The bombs were removed and allowed to cool overnight. The aldehydes were isolated from the oxidation mixture according to the procedure of Brown and Neish (12). The oxidation mixture was transferred to a separatory funnel, and the bomb was washed twice with 5 ml. of water and five times with 5 ml. of ether. Extraction was carried out and the ether was set aside. The aqueous mixture was extracted once more with 25 ml. of ether. The aqueous phase was acidified with concentrated HCl to a pH in the range 3-4 and allowed to stand from one to three hours. During this time, a flocculent solid precipitated. The mixture was adjusted to pH

6.4 with $NaHCO_3$ and extracted with two, 50 ml. portions of ether. The combined ether extracts were washed once with 25 ml. of water and then shaken with two 50 ml. portions of 2M $NaHSO_3$ to extract aldehydes. The combined bisulfite extracts were acidified with 25 ml. of 10 N H_2SO_4 and aerated under reduced pressure of 15 min. at room temperature and then on a hot water bath until the odor of sulfur dioxide was no longer detectable (about 25 min.). The aerated solution was extracted with two 50 ml. portions of ether, and the combined extracts were washed once with 25 ml. of water. The ether phase was transferred to 150 ml. beakers, and the ether was evaporated under a hood. The small aqueous residue was quantitatively transferred to a 2 ml. volumetric test tube with ethanol and diluted to volume.

The separation and identification of vanillin, syringaldehyde and parahydroxybenzaldehyde were accomplished by paper chromatography using n-butanol saturated with 2% aqueous NH_4OH (13) as the solvent system. The compounds were located by their ultraviolet fluorescence and by spraying the chromatograms with the ferric chloride-ferricyanide reagent (14) or with a saturated solution of 2,4-dinitrophenylhydrazine in 2N HCl.

The second method used for defining lignin was a 72% sulfuric acid procedure (15). One to two grams of ground, dried, alcohol extracted tobacco tissue were extracted in a Soxhlet extractor with 80 ml. of the ethanol-benzene solution for 17 hours. After extraction, the thimble was transferred to a small beaker and air dried under a hood overnight. The residue was then extracted in a Soxhlet extractor with about 80 ml. of water for six hours. To prevent foaming, an antifoam agent was added to the water. The thimble was oven dried at 85° C for 4-6 hrs. The dried

material was quantitatively transferred to a 125 ml. ground glass stoppered Erlenmeyer flask. The flask was set in an ice-water bath and 15 ml. of 72% sulfuric acid solution (specific gravity at $\frac{15.5^{o}}{15.5^{o}}$ C of 1.6389), which had been cooled to a temperature of 4-6° C was added to the flask with vigorous shaking during a one-minute period. During this period, the mixture became dark green in color. The flask was then stored in a refrigerator with a temperature range of 4-6° C, for about twenty-one hours, with occasionally vigorous shaking during the first five hours. During this time, the mixture became almost black in color and very viscous. After the first five hours, the viscosity decreased considerably. After twenty-one hours, the mixture was quantitatively transferred into a one-liter beaker with 560 ml. of water. A broken end stirring rod, a drop of antifoam agent were added to the beaker which was then covered with a watch glass. The mixture was brought to boiling. The boiling was continued for four hours on a sand bath under nearly a constant volume condition by occasionally adding boiling water. After overnight cooling, the hydrolysis mixture was centrifuged ten minutes per portion at 14,000 rpm in an angle centrifuge. The combined residues were washed until free of acid, transferred to platinum weighing dishes, oven dried at 85° C and a weight determination made. The radioactivity was determined and the preparation oxidized under alkaline conditions with nitrobenzene as described.

CHAPTER IV

RESULTS OF ^{14}C QUERCETIN INJECTION AND PREPARATION OF SAMPLE

Seeds of Nicotiana tabacum were allowed to germinate in flats in soil starting August 23, 1959. After germination they were transplanted to soil and again to quartz sand in glazed stone crocks and watered with a complete nutrient solution. About two weeks prior to injection, the plants were transferred to a controlled environment room. Injection took place on November 13, 1959. The plants were starting to form flower buds.

Two hundred and fifty μ l of the ethanolic solution of purified ^{14}C quercetin of specific activity 10 μ C/milligram of carbon was injected into each of three plants. This represents 1.45 μ C of activity as determined by counting and about 1 mg. of quercetin as determined by weighing. Before injection of the first plant, all the leaves below the eighth leaf out from the terminal bud were cut off and discarded. This eighth leaf was injected and left on the plant for a total of fifteen minutes. The plant was fractionated as follows: the injected leaf was cut from the plant with a razor blade and the injected area was separated from the rest of the leaf. The second leaf above was cut off, then the remaining five leaves and terminal bud were cut off, and finally the stem was cut away from the root and sectioned. Each fraction was weighed and fixed in boiling isopropyl alcohol as described. All the leaves of the second plant, except the eighth one out from the terminal bud were cut off.

This leaf was injected, and after fifteen minutes, it was cut off and transferred to water. The injected area was separated from the rest of the leaf under water and the leaf petiole left in the water for forty-five minutes. This gave a metabolism period of one hour. After this time, the injected leaf, the injected area and stem were fixed separately. The third plant was treated the same as the second except that after cutting off the injected leaf, it was allowed to metabolize for two hours and forty-five minutes or for a total of three hours. A blank was also worked up. For this, the eighth leaf out from the terminal bud of a plant was cut off, fixed and 250 μl of the ^{14}C quercetin solution added to the boiling mixture. Table 7 records weight determinations and the initial survey of the injected radioactivity.

The measurements indicate that about sixty percent of the injected activity can be accounted for. Chorney had performed an experiment (16), in which a plant he had injected was enclosed with a vibrating reed electrometer during a short term metabolism period and no radioactive gas was detected. Therefore, it was assumed that the injected radioactivity remains in the plant for the duration of these experiments. The discrepancies which show up in the determined radioactivity values most probably result from the method of measuring the activity in the alcohol insoluble fraction. In order to obtain more accurate values, the radioactivity in these fractions should be determined by combusting to CO_2. Also some of the unaccounted-for activity may have been present in the roots, since they were not assayed for activity.

TABLE 7

INITIAL SURVEY OF RADIOACTIVITY INJECTED AS ^{14}C QUERCETIN

Sample	1	2	3	4	$\frac{4}{1}$x100	5	$\frac{5}{1}$x100
Blank	1.45	20.2	1.25	1.70	118.0	0.00	0.0
15 min. inj. leaf	--	--	1.12	0.238	16.4	0.115	7.9
15 min. inj. area		--	0.07	0.041	2.8	0.091	0.6
15 min. 1st leaf above	1.45	--	1.02	0.000	0.0	--	--
15 min. 2nd leaf above		--	0.78	0.015	1.1	--	--
15 min. other leaves		--	0.90	0.005	0.3	--	--
15 min. stem		--	2.03	0.283	19.5	0.174	12.0
				Total	40.1		20.5
1 Hr. inj. leaf		19.6	1.28	0.324	22.3	0.218	15.0
1 Hr. inj. area	1.45	--	0.04	0.035	2.4	0.034	2.3
1 Hr. stem		57.1	1.84	0.225	15.5	0.161	11.1
				Total	40.2		28.4
3 Hr. inj. leaf		21.4	1.59	0.243	16.8	0.181	12.5
3 Hr. inj. area	1.45	--	0.054	0.032	2.3	0.023	1.6
3 Hr. stem		60.0	2.53	0.222	15.3	0.150	10.3
				Total	34.4		24.4

(-) Measurement was not determined

1 Theoretical amount of activity injected (μC)
2 Fresh weight (grams)
3 Weight after extraction and drying (grams)
4 Determined activity in alcohol soluble fraction (μC)
5 Determined activity in alcohol insoluble fraction (μC)

Chromatographic and Radioautographic Experiments on the Alcohol Soluble Fraction

As described previously, the isopropyl alcohol extracts were separated into a "tars" fraction and an aqueous fraction. After decantation of the aqueous fraction, the "tars" were taken up in benzene and isopropyl alcohol. Aliquots were streaked on strips of paper 1.5 inch wide and chromatographed with 20% KCl and 80% SBA. The dried chromatograms were exposed to x-ray film for 24 days and then sprayed with 1% $AlCl_3$ followed by diazotized para-nitroaniline and sodium acetate. The radioautograms of the 20% KCl chromatograms showed activity present at the origin in all cases. The chromatograms of the aqueous mixture also showed a much less intense band at the R_f area 0.41. The radioautograms of the 80% SBA chromatograms showed faint darkenings at the origin in all cases. Activity was also shown to be present in the R_f areas 0.62 and 0.78, the latter being the more intense. The blank produced similar radioautograms, with the areas other than quercetin being very faint. The results of this survey strongly indicated that the major compound which contained radioactivity was quercetin. Another compound, in lesser concentration, was tentatively identified as 3,4-dihydroxybenzoic acid by paper chromatographic comparison.

Three samples were purified further by chromatography on a cellulose column, in order to concentrate these radioactive substances. The first column was charged with the combined extracts of the one-hour injected leaf and injected area. The second column was charged with the combined extracts of the one-hour stem. Tables 8 and 9 describe the chromatographic procedure and tabulate the results obtained. At the bottom of the table

TABLE 8

COLUMN CHROMATOGRAPHY AND RESULTS OF THE ALCOHOL EXTRACTS OF THE 1 HR. INJECTED LEAF

Fraction	Volume (ml.)	Eluant color	cpm x 10^3 vol.	Solvent[1]	R_f values 80% SBA	R_f values 20% SBA
1	60	Green-black	0.0	1		
2	50	Yellow	0.0	1		
3	80	Pale yellow	0.0	1		
4	300	Pale yellow	0.0	1		
5	100	Colorless	0.0	1		
	Column sucked dry with aspirator					
6	10	Yellow	0.23	2		
7	16	Pale yellow	0.77	2	0.78	0.40
8	10	Colorless	1.20	2	0.79	0.40
9	10	Colorless	0.42	2		
10	10	Colorless	0.18	2		
	Started addition of solvent 3					
11	47	Colorless	0.56	2		
12	3	Colorless	0.006	2 & 3		
13	10	Green-black	3.70	3	0.71	0.00
14	10	Yellow	1.12	3	0.71	0.00
15	10	Pale yellow	0.46	3		
16	12	Colorless	0.17	3		
17	16	Colorless	0.24			
	Started addition of solvent 4					
18	22	Colorless	0.20	3		
19	18	Colorless	0.14	4		
20	102	Colorless	0.41	4		
21	102	Colorless	0.10	4		
	R_f Values of Reference Compounds					
Quercetin					0.77	0.00
3,4 Dihydroxybenzoic acid					0.78	0.38
Phloroglucinol					0.84	0.42
Phloroglucinol carboxylic acid					0.90	0.25

[1] Solvents: 1 - Benzene
2 - 5% SBA made 0.5% with 88% HCOOH
3 - 80% SBA made 0.5% with 88% HCOOH
4 - Methanol

TABLE 9

COLUMN CHROMATOGRAPHY AND RESULTS OF THE ALCOHOL EXTRACTS OF THE 1 HR. STEM

Fraction	Volume (ml.)	Eluant color	cpm x 10^3 vol.	Solvent[1]	Rf values 80% SBA	Rf values 20% SBA
1	10	Colorless	0.000	1		
2	11	Colorless	0.033	1		
3	13	Colorless	0.039	1		
4	14	Colorless	0.014	1		
5	10	Colorless	0.010	1		
	Column sucked dry with aspirator					
6	10	Yellow-brown	0.28	2		
7	11	Yellow-brown	0.41	2		
8	11	Yellow-brown	0.65	2	0.80	0.40
9	12.5	Pale yellow	0.87	2	0.80	0.42
10	12	Colorless	0.54	2	0.81	0.38
11	11	Colorless	0.15	2		
12	10	Colorless	0.01	2		
13	10	Colorless	0.01	2		
	Started addition of solvent 3					
14	60	Colorless	0.36	2		
15	10	Yellow	0.87	3	0.75	0.00
16	10	Pale yellow	0.50	3		
17	10	Colorless	0.28	3		
18	10	Colorless	0.09	3		
19	17.5	Colorless	0.07	3		
20	17	Colorless	0.08	3		
	Started addition of solvent 4					
21	68	Colorless	0.16	3 & 4		
22	46	Colorless	0.06	4		

Rf Values of Reference Compounds

Quercetin	0.77	0.00
3,4 Dihydroxybenzoic acid	0.78	0.38
Phloroglucinol	0.84	0.42
Phloroglucinol carboxylic acid	0.90	0.25

[1] Solvents: 1 - Benzene
2 - 5% SBA made 0.5% with 88% HCOOH
3 - 80% SBA made 0.5% with 88% HCOOH
4 - Methanol

are listed R_f values of reference compounds which were used for comparison with the R_f values of material from the injected plants.

As the table shows, quercetin was eluted off with the third solvent. This was confirmed by chromatographing fraction 15 from the leaf and injected area column, in two dimensions. Five percent isopropyl alcohol made to pH 3 with 88% formic acid (5% IFW) was used for the first direction and 80% SBA was used for the second direction. One darkened area was produced with R_f values of 0.00 and 0.79 respectively. This area became bright yellow when sprayed with 1% $AlCl_3$.

The 3,4-dihydroxybenzoic acid (DHB) was tentatively confirmed as a radioactive substance eluted with fraction 8. The confirmation was made by chromatographing fraction 8 with 5% IFW in the first direction and with butanol, acetic acid, water: 6:1:2 v/v (BAW) in the second direction. The R_f values for standard 3,4-DHB are 0.52 and 0.81 respectively and a purple color changing to brown was produced with diazotized para-nitroaniline spray reagent. Fraction 8 produced a radioactive spot with R_f values 0.45 and 0.81 respectively and gave the same color reaction. The first value was low because other non-radioactive materials are included in this fraction. Although indications for the presence of phloroglucinol were noticed, a thorough examination for the presence of phloroglucinol or its carboxylic acid in this fraction was not made.

Following this work, the blank leaf was examined. The aqueous and "tars" fractions were combined, and the organic solvent was evaporated off. The resulting mixture was diluted with water and extracted with benzene. The benzene extract was streaked and chromatographed with 20% KCl and 80% SBA. The origin was the only area darkend on the chromato-

gram developed with 20% KCl. 80% SBA produced three areas with R_f values of 0.82, 0.91 and 0.97. A considerable amount of tar-like material was present in this fraction which possibly might increase R_f values. The aqueous residue was fractionated further with a cellulose column. The fractions containing higher amounts of radioactivity were streaked and chromatographed in 20% KCl and 80% SBA. The results are described and tabulated in Table 10. This table shows that some of the radioimpurities which arise from ^{14}C quercetin are present in the blank. From a comparison of the film darkenings, they are much less concentrated than the quercetin but more concentrated than in the original solution. Further degradation probably occurred during fixation and extraction and possibly during the purification procedures. More work is necessary before the identity of each of these substances is determined, but for future reference, two may be tentatively identified as 3,4-dihydroxybenzoic acid and phloroglucinol.

Study of the Alcohol Insoluble Fraction

The aim of this series of experiments was to determine whether the unextracted radioactivity had become incorporated into lignin. The lignin was first characterized by oxidizing pre-extracted tissue with nitrobenzene under alkaline conditions and isolating characteristic aldehydes as described in Chapter 3. Ground samples from the blank, 15 min. injected leaf, 15 min. stem and 1 hr. injected leaf were studied initially. They were pre-extracted with 1% HCl and ethanol-benzene and oxidized with nitrobenzene. The radioactivity isolated in each of the three steps was determined and the percentage of the sample it represented was calculated. Also, a calculation was made to determine the radioactivity which would be

TABLE 10

COLUMN CHROMATOGRAPHY AND RESULTS OF THE ALCOHOL EXTRACTS OF THE BLANK LEAF

Fraction	Volume (ml.)	Eluant color	cpm x 10^3 vol.	Solvent[1]	R_f values 80% SBA	20% SBA
1	11.5	Yellow	4.19	1	0.17, 0.76	--
2	11.5	Yellow	5.75	1	0.81	0.40
3	10.5	Pale yellow	7.48	1	0.78, 0.85	0.40
4	9.5	Pale yellow	4.10	1	0.80, 0.85	0.45
5	10.0	Pale yellow	1.82	1	--	0.39
6	12.0	Colorless	0.89	1		
7	14.5	Colorless	0.61	1		
	Started addition of solvent 2					
8	41.0	Colorless	0.07	1 & 2		
9	8.0	Colorless	0.62	1 & 2		
10	6.5	Yellow	3.22	2	0.79	0.00
11	12.5	Pale yellow	1.52	2	0.77	0.00
12	11.5	Pale yellow	1.69	2		
	Started addition of solvent 3					
13	17.5	Colorless	0.74	2		
14	63.0	Colorless	1.01	2 & 3		
15	39.0	Colorless	0.31	3		

R_f Values of Reference Compounds

Purified ^{14}C quercetin used for injection	0.77, 0.83* 0.90*	0.00
Quercetin	0.77	0.00
3,4 Dihydroxybenzoic acid	0.78	0.38
Phloroglucinol	0.84	0.42
Phloroglucinol carboxylic acid	0.90	0.25

* trace darkening of film

[1] Solvents: 1 - 5% SBA made 0.5% with 88% HCOOH
2 - 80% SBA made 0.5% with 88% HCOOH
3 - Methanol

isolated from the total amount of the alcohol insoluble fraction of each of the four samples, during each of the three steps, in order to determine the percentage of the injected 1.45 μ C which would be isolated in this manner. Table 11 lists the results of this examination.

It can be seen from this table that the aldehyde fraction does not contain radioactivity. Eighty to eighty-five percent of the radioactivity contained in the alcohol insoluble fraction was extracted during the 1% HCl digestion. To obtain more information about the observation that 40-50% of the activity contained in the alkaline oxidation mixture was lost after acidification, a sample of standard ^{14}C quercetin was oxidized. The results of this are also included in Table 11. It was found to lose 46% of its activity upon acidification. Since no radioactivity was contained in the isolated aldehydes, but was carried through the acid digestion, it was decided that a method which isolates lignin in a total form should be used for characterizing the lignin. For this purpose, the standard sulfuric acid method described in Chapter 3 was used. The results will be combined with those from the plants injected with ^{14}C rutin and described in Chapter 6.

TABLE 11

RESULTS OF PRE-EXTRACTION AND NITROBENZENE OXIDATION OF THE ALCOHOL INSOLUBLE MATERIAL FROM ^{14}C QUERCETIN INJECTED TOBACCO

Pre-extraction Results

Sample	Weight (gm.)	Activity present (μC)	Activity isolated with 1% HCl (μC)	% of the activity in the alcohol insoluble fraction
Blank	1	0.0	0.0	0
15 min. inj. leaf	1	0.103	0.082	80
15 min. stem	1	0.086	0.074	86
1 hr. inj. leaf	1	0.170	0.190	112

Sample	Calculated activity from total alcohol insoluble (μC)	% of 1.45 μC	Activity isolated with EtOH benzene (μC)
Blank	0.0	0.0	0
15 min. inj.leaf	0.092	6.34	0
15 min. stem	0.150	10.4	0
1 hr. inj. leaf	0.243	16.8	0

Sample	Approximate weight after drying and grinding (gm.)
Blank	0.2
15 min. inj. leaf	0.2
15 min. stem	0.3
1 hr. inj. leaf	0.2

TABLE 11a

ALKALINE NITROBENZENE OXIDATION

Sample	Weight (gm.)	Activity present after oxidation (μC)	Activity isolated with 1st ether (μC)
Blank	0.1	0.0	0
15 min. inj. leaf	0.1	0.005	0
15 min. stem	0.1	0.003	0
1 hr. inj. leaf	0.1	0.011	0

Sample	Activity present after acidification (μC)	% lost on acidification
Blank	0.0	0
15 min. inj. leaf	0.003	39
15 min. stem	0.002	50
1 hr. inj. leaf	0.007	40

Sample	Activity present in aldehyde fraction (μC)	Activity isolated with other extractions (μC)
Blank	0	0
15 min. inj. leaf	0	0
15 min. stem	0	0
1 hr. inj. leaf	0	0

OXIDATION OF STANDARD ^{14}C QUERCETIN

Weight	Activity	Activity present in 1st ether	% of 0.135 μC	Activity present before acidification
10 mg.	0.135 μC	0.008 μC	0.67	0.074 μC

% of 0.135 μC	Activity present after acidification	Activity present in aldehyde fraction	Activity present in 2nd ether
54	0.040 μC	0+ μC	0 μC

CHAPTER V

RESULTS OF ^{14}C RUTIN INJECTION

Introduction

On March 25, 1960, three plants were injected with ^{14}C rutin with a specific activity of 10 μC/milligram of carbon. Fraction 7 from the purified rutin, as described in Chapter 2, was dissolved in 2 ml. of ethanol. The concentration was 25 μC per 1 ml. of solution or 5 μC per 0.2 ml., the amount desired for injection. The first plant injected was three months old from the start of germination. Flower buds were visible. All the leaves were cut off except numbers 10, 11, and 12 counting down from the terminal bud. 0.2 ml. of solution was injected into the petiole of the tenth leaf. The total time for injecting and washing was seven minutes. This leaf was then cut off, immersed in water; a small piece of the petiole was cut and the leaf transferred to a beaker of distilled water and allowed to metabolize for six hours. For future reference, this leaf will be referred to as RI-1-FP-IL. Then about 0.3 ml. of the solution was injected into the petiole of leaf number 11. The plant was allowed to metabolize for six hours. After this period, it was divided into flower buds, RI-1-FP-FB; stem from flower bud to just below number 12, RI-1-FP-TS; stem below leaf number 12 to root, RI-1-FP-BS; leaves number 11 and 12, RI-1-FP-OL; and root, RI-1-FP-R. Each fraction was fixed in boiling isopropyl alcohol.

The second plant injected was two months old from the start of germination. All the leaves below the tenth leaf out from the terminal bud were removed. 0.2 ml. of the ^{14}C rutin solution was injected into the petiole of this leaf. The plant was allowed to metabolize for six hours and divided into leaves and terminal bud, RI-1(+B)-L; stem, RI-1-(+B)-S; and root, RI-1-(+B)-R. The separate divisions were then fixed in boiling isopropyl alcohol.

The third plant injected was of the same age as the second plant, however, it had been growing in a boron deficient medium for fourteen days. It was treated in the same manner as the second plant. Its three reference labels for leaves, stem and roots are: (RI-1-(-B)-L, RI-1-(-B)-S, and RI-1(-B)-R.

A blank leaf was worked up as usual by fixing it in boiling isopropyl alcohol for five minutes and adding 0.2 ml. of rutin solution, RI-1-Bl. A boron deficient leaf was also fixed, but no rutin solution was added to the mixture, RI-1-(-B)-Bl.

After fixation and extraction, the radioactivity in the concentrated extracts were determined. Insufficient time prevented the determination of the radioactivity in the alcohol insoluble fraction. The alcohol extracts, after radioactivity determination, were streaked and chromatographed with water, and with 80% SBA made 0.1% with formic acid. The dried strips were exposed to x-ray film and the R_f values corresponding to darkened areas on the film listed with the initial radioactive survey in Table 12.

TABLE 12

INITIAL SURVEY OF RADIOACTIVITY INJECTED AS ^{14}C RUTIN

Sample	Activity injected (Theo. μC)	Fresh wt. (gm.)		Determined activity in alcohol solubles (μC)	% of the inj. 5 μC
Fraction 7					
RI-1-B1	5	14.3		6.60	135.00
RI-1-FP-T1		11.2		1.10	7.32
RI-1-FP-01		23.6		4.27	28.50
RI-1-FP-FB	15	0.9		0.10	0.67
RI-1-FP-TS		55.2		1.55	10.30
RI-1-FP-BS		137.2		0.92	6.14
RI-1-FP-R		129.7		0.11	0.68
			Total	8.05	53.61
RI-1-(+B)-L		40.5		3.38	67.60
RI-1-(+B)-S	5	21.2		0.048	0.96
RI-1-(+B)-R		17.2		0.022	0.44
			Total	3.450	69.00
RI-1-(-B)-L		32.0		3.23	64.60
RI-1-(-B)-S	5	17.6		0.042	0.84
RI-1-(-B)-R		13.6		0.027	0.54
			Total	3.299	65.98

TABLE 12a

INITIAL SURVEY OF RADIOACTIVITY INJECTED AS ^{14}C RUTIN

R_f Characteristics

Sample		H_2O	
Fraction 7	origin(+)	.28(+++)	.81(+)
RI-1-B1	origin(+)	.23(+++)	
RI-1-FP-IL	origin(+)	.32(++)	.80(+)
RI-1-FP-OL	origin(+)	.23(++)	
RI-1-FP-FB	origin(+)	.22(+)	
RI-1-FP-TS		.21(++)	
RI-1-FP-BS	origin(++)	.26(++)	
RI-1-FP-R	origin(++)		.69(+)
RI-1-(+B)-L		.18(++)	
RI-1-(+B)-S	origin(+)		
RI-1-(-B)-L		.22(++)	
RI-1-(-B)-S	origin(++)	.28(+)	

Sample		80% SBA	0.1% HCOOH	
Fraction 7			.46(+++)	
RI-1-B1			.46(+++)	
RI-1-FP-IL	origin(+)	.13(++)	.46(++)	
RI-1-FP-OL	origin(+)	.09(++)	.47(++)	.79(tailing +)
RI-1-FP-FB	origin(+)	.10(+)	.42(+)	
RI-1-FP-TS	origin(+)	.16(+)	.48(++)	.86(tailing +)
RI-1-FP-BS	origin(+)	.27(+)	.48(+++)	
		.31(+)		
RI-1-FP-R	origin(+)	.29(+)		
RI-1-(+B)-L	origin(+)	.10(+)	.44(+)	
RI-1-(+B)-S				.64(+)
RI-1-(-B)-L	origin(+)	.10(+)	.44(+)	
RI-1-(-B)-S				.70(+)

Intensity of film darkening
(+++) maximum
(++) dark
(+) faint

Study of the Alcohol Insoluble Fraction

In order to compare the results obtained from the ^{14}C quercetin injected with those from the ^{14}C rutin, five samples from the ^{14}C rutin injection were pre-extracted with 1% HCl and ethanol-benzene. This material was then oxidized with nitrobenzene and aldehydes isolated. The samples used were taken from RI-1-Bl, RI-1-(-B)-Bl, RI-1-FP-IL, RI-1-(+B)-L, and RI-1-(-B)-L. Only the RI-1-FP-IL sample was compared to the uniformly labeled snapdragon standard and found to have an absolute activity of 0.843. The results are tabulated in Table 13 along with an oxidation of standard ^{14}C rutin. This work had suggested the loss of activity on acidification of the oxidation mixture since more activity was lost than could be assumed to be caused by experimental loss alone. Therefore, a value was not determined for loss on acidification. However, as in the case of quercetin injection, no activity is present in the aldehyde fraction. One difference for the rutin injected plants is indicated in that sixty percent of the activity in the alcohol insoluble fraction is carried through the 1% HCl digestion.

TABLE 13

PRE-EXTRACTION AND NITROBENZENE OXIDATION OF ALCOHOL INSOLUBLE MATERIAL FROM ^{14}C RUTIN INJECTED TOBACCO

Sample	Weight (gm.)	Activity present (μC)	Activity isolated with 1% HCl (μC)	% of the activity in alcohol insolubles
RI-1-B1	0.7	--	0	0
RI-1-(-B)-RL	0.7	--	-	-
RI-1-FP-IL	1.4	1.18	0.48	40.7
RI-1-(+B)-L	2.7	--	0.34	-
RI-1-(-B)-L	2.3	--	0.38	-

Sample	Activity isolated with EtOH-Benzene (μC)	Approximate weight after drying and grinding (gm.)
RI-1-B1	0	0.2
RI-1-(-B)-B1	0	0.2
RI-1-FP-IL	0.018	0.25
RI-1-(+B)-L	0.026	0.7
RI-1-(-B)-L	0.004	0.6

ALKALINE NITROBENZENE OXIDATION

Sample	Weight (gm.)	Activity present after acidification (μC)	Activity present in isolated aldehydes (μC)
RI-1-B1	0.1	0.0	0
RI-1-(-B)-B1	0.1	0.0	0
RI-1-FP-IL	0.1	0.053	0
RI-1-(+B)-L	0.1	0.034	0
RI-1-(-B)-L	0.1	0.24	0

TABLE 13a

OXIDATION OF STANDARD ^{14}C RUTIN

Weight (mg.)	Activity (μC)	Activity present in 1st ether	% of 0.174 (μC)	Activity present before acidification (μC)
10	0.174	0.0153	8.8	0.167

% of 0.174 μC	Activity present before acidification (μC)	% lost on acidification
96	0.0835	50

Activity present in aldehyde fraction (μC)	Activity present in 2nd ether (μC)
0+	0

CHAPTER VI

STUDY OF 72% SULFURIC ACID LIGNIN FROM INJECTED TOBACCO PLANTS

Introduction

As described in the last two chapters, the aldehyde fraction from the nitrobenzene oxidation of pre-extracted plant material contained no radioactivity. The pre-extracted material was found to contain about 20% of the activity in the "alcohol insolubles" or about four percent of the injected activity in the case of the ^{14}C quercetin injected plants. In the case of the ^{14}C rutin injected plants, a single measurement shows that 60% of the activity located in the "alcohol insolubles" is carried through the pre-extraction treatment. These results established the fact that radioactivity was present in an acid insoluble fraction of the tobacco plant. However, the absence of activity in the aldehyde fraction left unsolved the problem of determining further what plant material may have incorporated the injected activity. To determine more definitely whether the injected activity was associated with lignin, it was decided to isolate the lignin in an undegraded form. This is still a major problem in lignin chemistry (17). No single method will isolate lignin or a lignin in a pure and unchanged form. Since for the purposes of this study radioactivity was being determined, the occurrence of changes would not interfere with the interpretation of results as long as they did not pro-

duce soluble degradation products. The most important objective was to obtain lignin as chemically pure as possible. The 72% sulfuric acid method described in Chapter 3 was used because its mode of action would seem to isolate such a lignin. With this treatment, that substance which is not hydrolyzed is defined as 72% sulfuric acid lignin or Klason lignin (18). It is realized that inherent in such a procedure there exists the possibility that during hydrolysis some polymerization or condensation may occur which would produce an acid insoluble substance. To gain some knowledge about this occurrence; and also, if possible, about any abnormalities that may have occurred in the plant with regards to its reaction towards the material being supplied to it because of the manner in which it was supplied, an alcohol insoluble sample from uniformly labeled tobacco tissue grown in a $^{14}CO_2$ atmosphere was carried through the procedure along with the injected samples. The compared step, which was particularly noted, was the acidification of the nitrobenzene oxidation mixture. This step previously had resulted in the loss of a gas which contained radioactivity. This step was investigated further by taking samples of two of the prepared Klason lignins and noting whether this gas was evolved when the reaction was carried out with the omission of the nitrobenzene.

72% Sulfuric Acid Lignin From Tobacco

Seventy-two percent sulfuric acid lignin was isolated from five samples of injected tobacco tissue and the uniformly labeled tobacco tissue. The first sample was taken from the blank from the ^{14}C quercetin injection. The second sample was taken from the combined, ground, alcohol insoluble material from the 15 min., 1 hr., and 3 hr. ^{14}C querce-

tin injected leaves. The third sample was taken from the combined, ground, alcohol insoluble material from the 15 min., 1 hr., and 3 hr. stems of the ^{14}C quercetin injected plants. The fourth sample was taken from RI-1-FP-OL. The fifth sample was taken from the combined, ground, alcohol insoluble material from RI-1-FP-BS and RI-1-FP-TS. The results of pre-extraction, isolation and oxidation are described and tabulated in Table 14.

It is quite apparent from this table that the isolated Klason lignin contains a significant amount of radioactivity. Approximately eleven percent of the radioactivity injected as ^{14}C quercetin was isolated with the Klason lignin. Of the activity not extractable with isopropyl alcohol, sixteen and thirty-four percent of it was isolated with the Klason lignin. These values should be considered as approximations because of the method used in their determination. The alcohol insoluble fractions were compared to a uniformly labeled snapdragon standard and the lignin was measured directly assuming infinite thinness. This latter value, especially is not too reliable as evidence by the detection of more than 100% of the activity when the material is put into a more soluble form during the nitrobenzene oxidation. The radioactivity in the alcohol insoluble fractions from the rutin injected plants was not determined, and the percent incorporation could not be determined for comparison purposes.

A further effort was made to characterize this isolated activity. One of the accepted criteria for wood lignin, as previously mentioned, is the production of certain phenolic aldehydes by an alkaline nitrobenzene oxidation. The lignin preparations examined thus far in the study have not produced these aldehydes with a radioactive content. Also, Klason lignin

TABLE 14

A STUDY OF THE 72% SULFURIC ACID LIGNIN FROM INJECTED TOBACCO PLANTS

Sample	Weight (gm.)	Activity present in alcohol insoluble[1]	Weight of lignin isolated (gm.)	% lignin in alcohol insoluble fraction	Activity in lignin[2] (μC)
Blank	1.02	0	0.0193	1.89	0
Combined inj. leaves	1.69	0.217	0.0652	3.86	0.036
Combined stems	2.33	0.190	0.1444	6.18	0.065
RI-1-FP-OL	2.12	--	0.0468	2.21	0.100
RI-1-FP-BS+TS	2.34	--	0.2892	12.40	0.102
Uniformly labeled tissue	1.02	--	0.0139	1.36	6.640

	% of activity in alcohol insoluble fraction	% of total activity injected[3]	Activity isolated with EtOH benzene extraction (μC)
Blank	--	--	0
Combined inj. leaves	16.5	6.4	0.015
Combined stems	34.2	5.33	0.014
RI-1-FP-OL	--	--	0.055
RI-1-FP-BS+TS	--	--	0.004
Uniformly labeled tissue	--	--	0.075

	Activity isolated with H_2O extraction (μC)	Activity isolated with H_2SO_4 (μC)
Blank	0	Not determined
Combined inj. leaves	0.015	"
Combined stems	0.007	"
RI-1-FP-OL	0.524	"
RI-1-FP-BS+TS	0.083	"
Uniformly labeled tissue	15.3	"

[1] Based on snapdragon standard

[2] Measured directly in planchet

[3] Calculated from activity determined to be present in "alcohol solubles" and "alcohol insolubles".

TABLE 14a

NITROBENZENE OXIDATION

Sample	Weight of lignin (mg.)	Calculated activity (μC)	Activity in 1st ether (μC)	% of activity in lignin
Blank	10	0	0	0
Combined inj. leaves	30	0.0166	0	0
Combined stems	30	0.0135	0	0
RI-1-FP-OL	30	0.0640	0	0.62
RI-1-FP-BS+TS	30	0.0160	0	2.5
Uniformly labeled tissue	10	4.78	0.0251	0.52

	Activity before acidification (μC)	% of activity in lignin	Activity after acidification (μC)
Blank	--	--	--
Combined inj. leaves	0.0332	200	0.0221
Combined stems	0.0128	95	0.0100
RI-1-FP-OL	0.0640	100	0.0522
RI-1-FP-BS+TS	0.0181	113	0.0128
Uniformly labeled tissue	1.23	25.6	1.11

	% loss on acidification	Activity in 2nd ether (μC)	Activity in aldehyde fraction (μC)
Blank	--	--	--
Combined inj. leaves	33.4	0	0
Combined stems	21.9	0	0
RI-1-FP-OL	18.5	0	0
RI-1-FP-BS+TS	29.3	0+	0+
Uniformly labeled tissue	9.75	0.0336	0.008

TABLE 14b

OXIDATION PROCEDURE WITHOUT NITROBENZENE

	Weight of lignin (mg.)	Calculated activity (μC)	Activity in 1st ether (μC)	% of activity in lignin	Activity before acidification (μC)
Combined inj. leaves	30	0.0135	0+	0+	0.0112
RI-1-FP-BS+TS	30	0.0160	0+	0+	0.0123

	% of activity in lignin	Activity after acidification (μC)	% loss on acidification	Activity in 2nd ether (μC)
Combined inj. leaves	83	0.0112	0	0
RI-1-FP-BS+TS	77	0.0124	0	0

	Activity in aldehyde fraction (μC)
Combined inj. leaves	0
RI-1-FP-BS+TS	0

isolated from uniformly labeled tobacco tissue produces less than one percent of this aldehyde fraction. The most notable characteristic of this isolated lignin is that it remains in the aqueous solution when oxidized and upon acidification evolves a gas which contains radioactivity. Since acid lignin is soluble in alkali, samples of the isolated lignin were carried through the oxidation procedure without adding nitrobenzene. The material was solubilized, but no radioactive gas was evolved on acidification, therefore, the material is being attacked by the nitrobenzene. Because this radioactive material cannot be characterized by accepted criteria, it may be a different type of material or may be a part of the acid lignin which is degraded differently by nitrobenzene.

The important question of whether tobacco plants normally form this material from quercetin and rutin or whether the plant reacted to the method of supply in an abnormal way to form the material cannot be answered. However, the results of the uniformly labeled material are similar to those obtained with the material from the injected plants, especially RI-1-FP-0L, and in particular that upon acidification a radioactive gas was evolved. This cannot be considered definite proof that the material formed from quercetin and rutin exists in plants, because other radioactive substances might be present in the uniformly labeled plants which produce the same result. But it does show that when acid lignin is isolated from normal tissue, a gas is evolved when the oxidation mixture of this material is acidified.

CHAPTER VII

A QUANTITATIVE DETERMINATION AND COMPARISON OF 72% SULFURIC ACID LIGNIN AND THE ALDEHYDES ISOLATED FROM THE ALKALINE NITROBENZENE OXIDATION OF THIS LIGNIN IN NORMAL AND BORON DEFICIENT TOBACCO LEAVES AND STEM.

Introduction

The determinations described in this chapter were carried out to supplement the study discussed in the previous chapters. A quantitative determination was made of tobacco 72% sulfuric acid lignin from non-injected plants. The purpose of this study was to determine what percentage of the alcohol insoluble fraction it represents and how this compares with the injected plants. The aldehydes: vanillin, syringaldehyde and para-hydroxybenzaldehyde, which were isolated from the nitrobenzene oxidation of this lignin, were quantitatively determined in order to see what percent of this lignin they represented, and to learn what importance they had in characterizing this lignin preparation. Also, the nitrogen present in the lignin preparation was determined in order to obtain some basic information about the composition of this isolated lignin.

Dr. John Skok and Dr. Wayne J. MacIlrath suggested that a part of the tobacco plants should be grown under boron deficient conditions and analyzed with the normally grown plants. The reason for this comparison was to check some reports that have been made which indicate that boron may be involved in the lignification process (19); and if so, to gain some

idea about what to investigate in future work along this line.

Procedures and Results

The same procedure described in Chapters 4 and 5 was followed for growing the tobacco plants. At the time of harvest, the plants were about seventy days old from the time of seeding and were starting to form flower buds. Those plants which had been put on a boron deficient medium, starting fourteen days previous to harvest, were producing black meristems and very brittle petioles, the symptoms of boron deficiency.

Six plants were selected for analysis: three from those grown on the normal media, and three from those grown on the boron deficient media. The six plants were each further divided into two parts. The leaves selected for analysis represented one part and the stem represented the other part. The procedure followed for analyzing the twelve samples was similar to the one described in Chapter 3. The initial alcohol extraction was facilitated by blending the plant material with cold isopropyl alcohol in a Waring blender. This was done because it had been decided to base the percentage lignin and percentage aldehydes on the alcohol insoluble material. The alcohol extracted material was filtered with a buchner funnel and the leaf residues blended and filtered twice more with aqueous 50% isopropyl alcohol. The stem residue was extracted only once more with 50% isopropyl alcohol. The filtered material was then dried at 85° C in the forced-air oven and ground to pass a 40-mesh screen. The plant material and growth media characterization, sample number, fresh weight, extraction procedure, approximate residue weight after extraction and drying, and residue color are tabulated in Table 15.

The 72% sulfuric acid procedure described in Chapter 3 was used to

TABLE 15

ALCOHOL EXTRACTION PROCEDURE AND RESIDUE CHARACTERISTIC OF NORMAL AND BORON DEFICIENT PLANT MATERIAL

	Sample No.	Fresh weight (gms.)	Isopropyl alcohol (ml.)	Extraction Procedure 1st 50% alcohol (ml.)	2nd 50% alcohol (ml.)
(-B) leaves	1	234	384	500	500
(-B) leaves	2	249	400	511	500
(-B) leaves	3	195	395	300	300
(+B) leaves	4	294	500	500	500
(+B) leaves	5	295	500	500	500
(+B) leaves	6	355	500	500	500
(-B) stems	7	58	250	200	--
(-B) stems	8	57	250	200	--
(-B) stems	9	63	250	200	--
(+B) stems	10	118	250	200	--
(+B) stems	11	120	250	200	--
(+B) stems	12	152	250	200	--

		Approx. wt after ext. (gms.)	Color of residue
(-B) leaves	1	17	dark brown
(-B) leaves	2	15	dark brown
(-B) leaves	3	12	dark brown
(+B) leaves	4	17	dark green brown
(+B) leaves	5	17	dark green brown
(+B) leaves	6	22	dark green brown
(-B) stems	7	2	brown
(-B) stems	8	2	brown
(-B) stems	9	2	brown
(+B) stems	10	2	light brown
(+B) stems	11	3	light brown
(+B) stems	12	3	light brown

make the lignin determination. Approximately one gram duplicates of each of the twelve samples were brought to constant weight and pre-extracted with water and ethanol-benzene. The lignin was isolated, centrifuged and brought to constant weight. The centrifugate was filtered through medium porosity sintered glass filters, washed, and brought to constant weight. The weights were combined and the percent lignin in the dry alcohol insoluble material calculated. A Kjeldahl nitrogen determination was made on the isolated lignin by Dr. Wayne J. MacIlrath. The weights, percent lignin, and percent nitrogen are tabulated in Table 16.

The methods of Stone and Blundell (11) and of Onishi, Nagasawa, and Yamamoto (20) were followed to determine the percent of vanillin, syringaldehyde, and para-hydroxybenzaldehyde obtained from the alkaline nitrobenzene oxidation of the sulfuric acid lignin. Spectrophotometer readings were made with a Beckman model DU quartz spectrophotometer. Using standard compounds, the absorption maxima were determined for measuring vanillin, syringaldehyde, and para-hydroxybenzaldehyde at the following wavelengths respectively: 354 mμ, 372 mμ, and 339 mμ. Standard concentration curves of the paper eluted compounds were determined for the concentration range 20μg to 100μg per 50 ml. of solution.

The percentage of each aldehyde present was determined in the following manner. Approximately 30 mg. samples of the isolated lignin were accurately weighed and oxidized with nitrobenzene as described in Chapter 3. The final ether extracts were evaporated under a hood and quantitatively transferred to 2 ml. volumetric test tubes with ethanol, and diluted to the volume mark. 500 l. aliquots were then spotted on Whatman No. 1 chromatography paper along with standard solutions, and an

TABLE 16

72% SULFURIC ACID LIGNIN ANALYSIS FROM NORMAL AND BORON DEFICIENT PLANT MATERIAL

Sample	Wt. of Sample Before drying	Wt. of Sample After drying	Dry wt. of lignin centrifuged	Dry wt. of lignin filtered	Total wt. of lignin	% lignin on dry wt. basis	% nitrogen
1-1	1.1081g	0.9627g	0.0578g	0.0010g	0.0588g	6.11	4.58
1-2	1.0317	0.8963	0.0603	0.0015	0.0618	6.90	
2-1	1.1124	0.9652	0.0522	0.0014	0.0536	5.55	
2-2	1.0589	0.9188	0.0482	0.0029	0.0511	5.56	3.92
3-1	1.0470	0.9228	0.0431	0.0020	0.0451	4.89	
3-2	1.0455	0.9219	0.0401	0.0019	0.0420	4.56	3.90
4-1	1.0754	0.9684	0.0253	0.0301	0.0554	5.72	
4-2	1.2597	1.1344	0.232	0.0238	0.0470	4.14	2.19
5-1	1.0678	0.9508	0.0233	0.0048	0.0281	3.00	
5-2	1.1336	1.0094	0.0239	0.088	0.0327	3.24	2.55
6-1	1.1606	1.0531	0.0294	0.0073	0.0367	3.48	
6-2	1.8284	1.6591	0.0385	0.0046	0.0431	2.60	2.50
7-1		1.0653	0.0788	0.0011	0.0799	7.50	
7-2		0.9510	0.0668	0.0011	0.0679	7.14	3.20
8-1		1.0080	0.0689	0.0011	0.0700	6.95	
8-2		0.3733	0.0264	0.0029	0.0293	7.85	2.73
9-2		1.0438	0.0833	0.0017	0.0850	8.14	2.40
10-1		1.1020	0.0469	0.0055	0.0524	4.76	
10-2		0.7359	0.0359	0.0040	0.0399	5.42	2.46
11-1		1.1093	0.0568	0.0069	0.0637	5.74	
11-2		1.1674	0.0613	0.0055	0.0668	5.72	1.92
12-1		1.0765	0.0680	0.0049	0.0729	6.77	
12-2		1.1178	0.0728	0.0070	0.0798	7.14	2.35

ethanolic blank. The papers were chromatographed in a chromatocab for about 16 hrs. with the n-butanol saturated with 2% aqueous NH_4OH irrigating solvent. The papers were then removed and allowed to dry overnight. The spots corresponding to the aldehydes sought for were made visible by exposing the papers to ammonia vapor under U.V. light. These and a blank were then circled and cut out. The circular pieces of paper were transferred to microsoxhlet extraction tubes and extracted for two hours with about 20 ml. of ethanol. The extracted papers were again viewed under U.V. light to make sure no fluorescence appeared. The ethanolic extracts were quantitatively transferred to 50 ml. volumetric flasks. The solutions were allowed to cool overnight. They were prepared for measurement in the Beckman DU spectrophotometer by adding 4 ml. of 0.2% ethanolic KOH and diluting to 50 ml. with ethanol. The solution was thoroughly mixed and allowed to stand for 15-30 min. Before measuring the optical density, three measurements compared to the eluted blank were made at the appropriate wavelength of each of the three aldehydes, and the average value recorded. The amount of aldehyde was read from the standard curve and its percent in the lignin calculated. Table 17 records the weight of lignin oxidized, the volume of nitrobenzene and NaOH used and the calculated percent of vanillin, syringaldehyde and para-hydroxybenzaldehyde isolated from the oxidation reaction.

Discussion

The determined percent of lignin in the normal leaves and stems is in agreement with the determined values for comparable plants listed in Table 14. The average values of 3.7 for the leaves and 6.1 for the stems from Table 16 against 3.9 and 6.2 from Table 14. It would seem reasonable

TABLE 17

DETERMINATION OF ALDEHYDES ISOLATED FROM NITROBENZENE OXIDATION

Sample	Weight lignin (mg.)	Volume of Nitrobenzene	Volume of NaOH(ml.)	Percent Vanillin	Percent Syringaldehyde
1	30	0.2	3	0.31	0.0
2	30	0.2	3	0.29	0.35
3	30	0.2	3	1.25	0.0
4	30	0.2	3	0.55	0.0
5	23	0.125	2	0.79	0.0
6	30	0.2	3	0.77	0.0
7	30	0.2	3	0.87	0.57
8	30	0.2	3	1.09	0.76
9	30	0.2	3	0.63	0.69
10	30	0.2	3	1.77	1.10
11	30	0.2	3	1.54	0.82
12	30	0.2	3	1.52	0.99

Sample	Percent Parahydroxybenzaldehyde
1	0.34
2	0.31
3	0.31
4	0.20
5	0.29
6	0.51
7	0.33
8	0.52
9	0.35
10	0.23
11	0.23
12	0.18

to assume from this, that the small amount of injected material incorporated with this lignin, represents a normal biochemical formation. Nothing can be said about chemical formation if the incorporation occurred during the isolation procedure, because of the small weight of material injected.

Table 17 shows that the aldehydes isolated from this lignin represents a very small percent and cannot be significantly used in this experiment to characterize the lignin. Roadhouse and MacDougall suggested in their report (21) that much of the original aldehyde producing material is present in the isolated lignin in a modified form which doesn't produce aldehydes. This occurs to a greater extent in young plants. A more thorough study of what happens to the aldehyde forming materials might be of significance in future injection experiments to show whether the injected material forms a substance which produces aldehydes in short-term experiments or in longer metabolism times or not at all. The uniform yield of para-hydroxybenzaldehyde is also in agreement with data reported by Roadhouse and MacDougall and suggests that it may be formed from other isolated materials. The fact that such exist is suggested by the large nitrogen content.

No significant conclusions can be made concerning the lignin content of the boron deficient plants since not enough plants were analyzed to give the data statistical validity. However, the information affords a chance for some postulates on which to devise further experiments. The largest difference in the lignin content occurs in the leaves. Approximately two-thirds more lignin was isolated from the boron deficient leaves. A very slight difference in favor of the boron deficient plants was noted in the stems. These results can be interpreted in two ways. 1) The boron

deficiency promoted an accumulation of lignin-like or acid insoluble substances. If the aldehyde isolation is used as a criterion, the former is indicated since a greater percentage of aldehydes is indicated in the lignin isolated from the normal plants. However, the accumulated material could be similar to the material formed from quercetin and rutin in which case no aldehydes would be formed and the latter interpretation would be more significant. The nitrogenous content of the lignin isolated from the boron deficient plants is higher which also indicates the presence of a larger amount of material having atypical lignin characteristics.

CHAPTER VII

SUMMARY

This study was initiated to determine whether rutin and quercetin could be assigned any specific function in the overall plant physiology of certain plants. Nicotiana tabacum plants were supplied with ^{14}C quercetin and ^{14}C rutin by an injection technique, and the fate of these compounds was traced by their radioactive content. The injected plants were allowed to metabolize for short-term periods. They were then fixed in boiling 85% isopropyl alcohol and separated into an alcohol soluble fraction and an alcohol insoluble fraction. The alcohol soluble fraction was cursorily examined by chromatographic and radioautographic techniques. The alcohol insoluble fraction was analyzed for lignin. The lignin content was defined by a 1% HCl hydrolysis and by a 72% sulfuric acid treatment and further characterized by an alkaline nitrobenzene oxidation.

In one experiment, approximately 1.45 μ C of quercetin, with a specific activity of 10 μ C/mg. of carbon was injected into sixty-day old plants with a fresh weight of about 100 gms. Of the injected activity 60 to 70% could be accounted for. Forty percent of the injected activity was located in the alcohol soluble fraction. In this fraction the presence of labeled quercetin, 3,4-dihydroxybenzoic acid and phloroglucinol was tentatively established. The formation of the latter two,

however, has not been definitely proven. Eleven percent of the injected activity was isolated with the 72% sulfuric acid lignin. This labeled material, isolated with the lignin fraction, is chemically attacked in a characteristic manner by the nitrobenzene oxidation. It was shown that upon acidification of the oxidation mixture, a definite amount of activity was evolved for the particular type of lignin. It amounts to 25 to 30% for 72% sulfuric acid lignin and 45 to 50% for 1% HCl lignin. ^{14}C quercetin was oxidized and found to lose 45% of the activity in the oxidation mixture upon acidification. The 72% sulfuric acid lignin isolated from uniformly labeled tobacco tissue was found to lose 10% of the activity in the oxidation mixture upon acidification. The isolated aldehydes, which are the accepted criteria for lignin, contained no radioactivity.

In another experiment, approximately 5 μC of rutin, with a specific activity of 10 μC/mg. of carbon was injected into sixty-day old plants and one ninety-day old plant. Rutin plus three unidentified radioactive substances were detected in the alcohol soluble fraction. Acidification, after oxidation of the isolated 72% sulfuric acid lignin, evolved 20 to 30% of the activity contained in the oxidation mixture.

A final combined analysis was made on the 72% sulfuric acid lignin isolated from young plants grown under normal conditions and under boron deficient conditions. The aldehyde and nitrogen percentages were determined and the results discussed.

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