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# A STUDY OF CERTAIN FLAVONOID COMPOUNDS IN THE LEAVES AND FLOWERS OF TOBACCO

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A STUDY OF CERTAIN FLAVONOID COMPOUNDS IN THE LEAVES

AND FLOWERS OF TOBACCO

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E.L.M.

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# A STUDY OF CERTAIN FLAVONOID COMPOUNDS IN THE LEAVES AND FLOWERS OF TOBACCO

## CHAPTER I

## INTRODUCTION

Because of the widespread occurrence of flavonoid and related polyphenolic compounds in the plant kingdom (1,2), botanists and plant physiologists have been interested in their significance in plant metabolism (3). Although extensive literature is available concerning the chemistry of flavonoids and their occurrence in plants, scientific facts in regard to their metabolic interrelationships and fate are still needed to clarify their physiological role in the plant (4). A cooperative program was therefore established in 1954 with a group of scientists at the Argonne National Laboratory, Lemont, Illinois, to study the metabolism of certain polyphenolic compounds in one-sucker tobacco, Nicotiana tabacum, using radioisotope techniques. According to a preliminary plan of research, the tobacco was to be grown in the greenhouse of the Argonne National Laboratory, dried, ground, packaged and shipped to this laboratory for isolation and identification studies. Early in 1954, the only flavonoid compounds previously reported in tobacco were rutin (5,6) and isoquercitrin (7,8). However, a preliminary study of the flavonoid compounds in tobacco, made at the Argonne National Laboratory in the

summer of 1954, indicated the presence of many compounds of polyphenolic nature. In order to provide background information for the planned tobacco plant infiltration study using radioactive flavonoid compounds to determine their metabolic changes in the plant, it was necessary to isolate and identify as many as possible of these flavonoid compounds. The details of the experimental studies undertaken on the isolation and identification of certain of these flavonoid compounds, are presented in Chapter II.

Tobacco plants were grown in an atmosphere of radioactive carbon dioxide, $C^{14}O_2$, in the growth chambers of the Argonne National Laboratory, to serve as a source for the isolation of the radioactive flavonoid compounds to be used for plant metabolism studies. After harvesting and preliminary processing of the tobacco plants, the radioactive tobacco extract was sent to this laboratory for the isolation of the radioactive flavonoid compounds. The experimental results and the procedure used for the isolation of the radioactive flavonoids are described in Chapter III.

Several methods have been published for the quantitative determination of rutin in plant materials. Naghski (9) reported a gravimetric method which was used by Badgett (10) to determine the rutin content of several varieties of Nicotiana rustica and Nicotiana glauca. Gage and Wender (11), and Turner (12) have published spectrophotometric methods for the quantitative determination of rutin which vary in experimental procedure, but both of which utilize the color developed by the rutin-aluminum complex. The gravimetric method is not specific for rutin in the presence of other flavonoid compounds. This method involves losses

through filtration and precipitation, and, besides being slow and tedious, cannot be applied to samples containing small amounts of rutin, less than 0.1%, since the rutin may fail to crystallize from solution. The spectrophotometric methods have the advantage of speed and simplicity; but the aluminum chloride complexing reagent is not specific for rutin and therefore determinations cannot be made in the presence of other flavonoid compounds. When the need arose for a method to determine rutin in the leaf tissue of young tobacco plants, an attempt was made to design a quantitative method which would combine the chromatographic isolation techniques for producing a pure rutin sample and the spectrophotometric method for speed and simplicity. The experimental details of this method of microanalysis for the rutin content of young tobacco plants, are presented in Chapter IV.

In the literature on the flavonoid compounds, great interest has been demonstrated in the activity of rutin (13) in animals placed under conditions of stress such as: x-ray treatment, vitamin deficient diets, frostbite, etc. Once a convenient method for the determination of rutin had been devised, the young tobacco plants growing in the greenhouse at the Argonne National Laboratory presented an opportunity to examine a plant placed under a condition of stress. Two such conditions were chosen: treatment with x-rays and the acceleration of growth induced by treatment with gibberellic acid. The experimental details of the treatment of the tobacco plants with x-rays and gibberellic acid and the results obtained, are presented in Chapters V and VI.

Previous research in this laboratory had indicated that for a given dry weight, the concentration of the flavonoid compounds seemed

much higher in the flowers of the tobacco plant than in the leaves. If this high flavonoid concentration could be associated with a functional part of the tobacco flower, some conclusions might be drawn as to possible functions of the flavonoid compounds in the tobacco plant. A preliminary study was, therefore, undertaken on the distribution of flavonoid compounds in the parts of the tobacco flower. The results of this investigation are presented in Chapter VII.

# CHAPTER II

## THE ISOLATION AND IDENTIFICATION OF CERTAIN FLAVONOID COMPOUNDS IN THE LEAVES OF TOBACCO

### Isolation From Tobacco Leaves

Extraction. Soxhlet extractors were used to extract 1800 grams of dried ground leaves of the one-sucker tobacco plant, Nicotiana tabacum, with 85% isopropyl alcohol. The alcoholic extracts were combined and reduced in volume to 100 milliliters. This reduced alcoholic tobacco leaf extract was poured into a liter beaker containing 50 grams of cellulose powder, Whatman ashless standard grade, and additional powdered cellulose added, while stirring, until the mixture no longer coalesced upon standing. The adsorbed extract-cellulose powder mixture was dried in vacuo to the point at which it would flow freely. This was accomplished by placing the beaker in a desiccator connected to a water aspirator.

Preliminary separation by column chromatography. A glass chromatographic column, 5 centimeters in diameter and 58 centimeters long, was packed to a depth of 40 centimeters with cellulose powder by adding approximately 10 grams at a time and tamping it firmly with a ramrod tipped with a plastic head. The dried cellulose powder-alcoholic mixture was then packed to a depth of 10 centimeters at the top of the column in a similar manner. Benzene was placed on the column as the first

developing solvent and a dark zone was observed to move rapidly down the column with the solvent front. The column eluate was successively collected in 500 milliliter fractions. Benzene development was continued until the dark material had been eluted from the column. The developing solvent was then changed to anhydrous ethyl acetate in preparation for the addition of the water-saturated ethyl acetate as the major developing solvent. Water-saturated ethyl acetate was passed onto the column until chromatographic testing of the eluate showed that the flavonoids had been eluted from the column. Chromatographic development of the column was concluded by first washing the column with 95% ethyl alcohol and then with water. The column fractions were then reduced in volume to 50 milliliters at reduced pressure.

In order to ascertain which column fractions contained flavonoid compounds, a chromatogram was prepared by spotting 0.2 milliliter of each concentrated fraction one-half inch apart on a line ruled off 3 inches below the top of a sheet of chromatographic paper, 52 by 58 centimeters, Schleicher and Schuell, No. 589, red ribbon. This chromatogram was developed in a chromatographic tank containing 15% acetic acid-water as a solvent system. After drying, the chromatogram was sprayed with 1% ethanolic aluminum chloride and observed under long wave ultraviolet light. The flavonoid spots were outlined with a pencil and their fluorescent colors recorded. The cellulose powder column fractionated the flavonoid compounds of tobacco leaves as shown in Table 1. The flavonoid aglycone-like material was eluted in the earlier fractions by the developing solvent, water-saturated ethyl acetate, and the flavonoid glycoside-like material moved off the column in later fractions.

TABLE 1

FRACTIONATION OF THE FLAVONOID COMPOUNDS BY CELLULOSE COLUMN

| Column Fraction | Developing Solvent | Volume ml. | Visible Color of Fraction | Fluorescent Color of Spots on 15% Acetic Acid Chromatogram |
|---|---|---|---|---|
| 0 | Benzene | 500 | Dk G | none |
| 1 | Anhyd. Ethyl Acetate | " | G | Y,Bl |
| 2 | Water Satd. Ethyl Acetate | " | YG | Y,Bl |
| 3 | " | " | " | Bl |
| 4 | " | " | Lt YG | " |
| 5 | " | " | " | " |
| 6 | " | " | YG | Y,Bl |
| 7 | " | " | " | " |
| 8 | " | " | " | " |
| 9 | " | " | C | Bl, BlG |
| 10 | " | " | " | " " |
| 11 | " | " | " | " " |
| 12 | " | " | " | " " |
| 13 | " | " | " | " " |
| 14 | 95% Ethanol | " | Dk G | Y, BlG |
| 15 | Water | " | Dk YG | BlG |

Y=yellow G=green Bl=blue Lt=light Dk=dark C=colorless

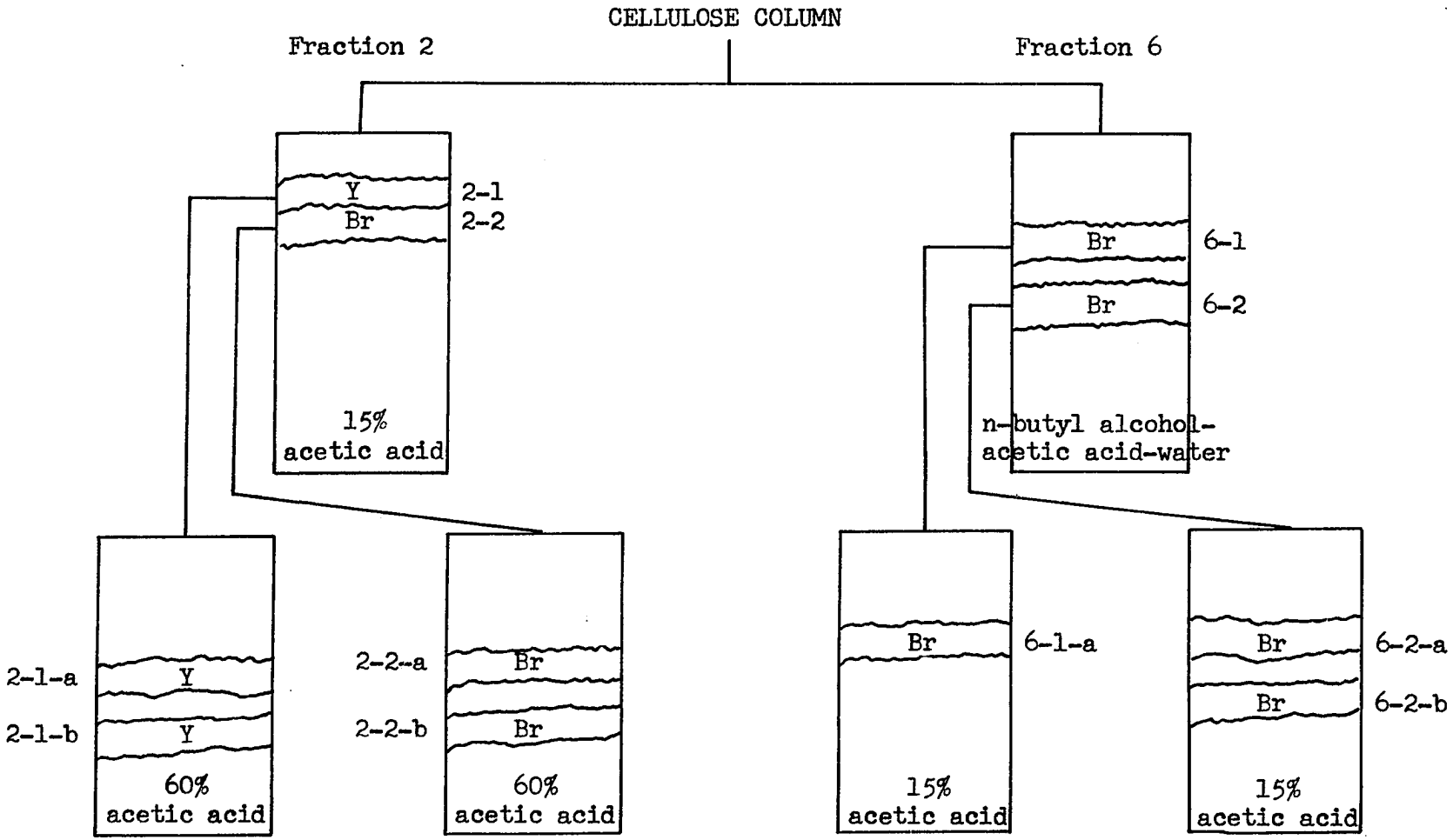
Purification by paper partition chromatography. The 15% acetic acid chromatogram indicated that the majority of the flavonoid aglycone-like material was present in column fraction 2. This fraction was streaked continuously along a line ruled 3 inches down from the top of a sheet of chromatographic paper, Whatman No. 3 MM, 18 1/4 by 22 1/2 inches. The chromatogram was developed in the 15% acetic acid-water solvent system and then dried. Observation under long wave ultraviolet light showed that the original material of the column fraction 2 had separated into yellow and brown zones (Figure 1). A 2 inch strip was cut lengthwise from one edge of the chromatogram and sprayed with 1% ethanolic aluminum chloride, dried, and observed under ultraviolet light. Both zones gave a positive test for the presence of flavonoid compounds. The yellow zone fluoresced yellow but with much greater intensity; the brown zone changed to a golden yellow. These zones will hereafter be designated with the prefix 2, since they were in column fraction 2. The number 2-1 and 2-2 will correspond to the order in which the various zones of fraction 2 appeared, moving downward from the origin on the 15% acetic acid chromatogram. The yellow and brown zones were in turn cut out of the 15% acetic acid chromatogram with scissors and each zone sewn across 3 inches below the top of separate sheets of new Whatman No. 3 MM chromatographic paper with a sewing machine. To insure that the solvent upon development would flow through this sewn strip which carried the flavonoid material, the area of the new sheet under the sewn strip was cut out and removed. These chromatograms were placed in a chromatographic tank and developed with the 60% acetic acid-water solvent system. After drying, the yellow zone, as observed under ultraviolet light, had

separated on its chromatogram into two yellow zones, called 2-1-a and 2-1-b (Figure 1). The brown fluorescent zone had separated on its chromatogram into two brown zones, called 2-2-a and 2-2-b. Again 2 inch strips were cut from the edge of each of the two chromatograms and sprayed with 1% ethanolic aluminum chloride and all zones gave a positive test for the presence of flavonoid material. These zones were then cut out of the chromatogram and eluted in a Soxhlet extractor with 95% ethyl alcohol. The alcoholic extract was reduced in volume to 10 milliliters and placed in a refrigerator to await further examination for purity and identity.

Column fraction 6 was shown by the original 15% acetic acid chromatogram to contain flavonoid glycoside-like substances. This fraction was streaked continuously with a pipette along a ruled line 3 inches from the top of a sheet of Whatman No. 3 MM chromatographic paper. The chromatogram was then developed in the n-butyl alcohol-acetic acid-water solvent system, 6:1:2 by volume. After drying, the chromatogram, as observed under ultraviolet light, revealed that this column fraction had been further fractionated into two brown zones, 6-1 and 6-2, and also into several blue zones. Again as a qualitative test for flavonoid compounds, a 2 inch strip was cut from the edge of the chromatogram and the strip sprayed with 1% ethanolic aluminum chloride. The two brown zones, 6-1 and 6-2, after spraying, now fluoresced yellow under ultraviolet light. Each zone, in turn, was cut out of the chromatogram and sewn across the top of separate new sheets of Whatman No. 3 MM chromatographic paper. The two chromatograms were next developed in the 15% acetic acid-water solvent system. After drying, the chromatogram

FIGURE 1

DIAGRAMS ILLUSTRATING THE PAPER CHROMATOGRAPHIC SEPARATION OF THE FLAVONOID COMPOUNDS IN TOBACCO LEAVES




Color in Ultraviolet Light Bl=Blue Br=Brown Y=Yellow

carrying flavonoid zone 6-1 showed under ultraviolet light a single brown zone, 6-1-a, and three blue zones which evidently had been covered up by the brown zone on the original n-butyl alcohol-acetic acid-water chromatogram. The chromatogram carrying the flavonoid zone, 6-2, now had two brown zones, called 6-2-a and 6-2-b (Figure 1). All zones turned from a brown to a yellow fluorescence under ultraviolet light after the test strips from the chromatograms had been sprayed with 1% ethanolic aluminum chloride. The unsprayed portion of the zones were cut out and eluted in Soxhlet extractors with 95% ethyl alcohol for 4 hours. The alcoholic extract was reduced to 10 milliliters and placed in the refrigerator to await further tests for purity and identity.

Identification of the Flavonoids From Tobacco Leaves

At this point each of the zones isolated from the alcoholic extract of tobacco leaves could be considered a chromatographically pure flavonoid compound in the solvent systems employed for their separation. In order to test their purity further as well as to characterize each isolated flavonoid compound for comparison with the available flavonoid literature for possible identification, the $R_f$ value (Table 2) was obtained for each flavonoid compound isolated from tobacco leaves in a series of solvent systems by the method of Gage, Douglass and Wender (14). Further, the color was recorded for each untreated compound in visible and ultraviolet light and with a series of chromogenic sprays which produce characteristic colors that can be used for qualitative identification of the flavonoid compounds (Table 3).

TABLE 2

CHROMATOGRAPHIC $R_f$ VALUES

| Compound | Solvent System | | | | |
|---|---|---|---|---|---|
| | 15% acetic acid | 60% acetic acid | n-butyl alcohol-acetic acid-water | benzene nitromethane water | 22% isopropyl alcohol-water |
| Standard Quercetin | 0.04 | 0.30 | 0.70 | - | - |
| 2-1-a | 0.03 | 0.29 | 0.69 | - | - |
| Standard Kaempferol | 0.05 | 0.39 | 0.84 | - | - |
| 2-1-b | 0.05 | 0.40 | 0.85 | - | - |
| 2-2-a | 0.03 | 0.43 | 0.83 | 0.15 | - |
| Demethylated 2-2-a | 0.04 | 0.27 | 0.69 | - | - |
| 2-2-b | 0.05 | 0.62 | 0.93 | 0.86 | - |
| Demethylated 2-2-b | 0.03 | 0.29 | 0.69 | - | - |
| Methylated 2-2-a | 0.15 | 0.80 | 0.86 | - | - |
| Methylated 2-2-b | 0.16 | 0.79 | 0.88 | - | - |
| Standard Pentamethyl Quercetin | 0.16 | 0.82 | 0.88 | - | - |

TABLE 2 CONTINUED

| Compound | Solvent System | | | | |
|---|---|---|---|---|---|
| | 15% acetic acid | 60% acetic acid | n-butyl alcohol-acetic acid-water | benzene nitromethane water | 22% isopropyl alcohol-water |
| 6-1-a | 0.56 | 0.69 | 0.52 | - | 0.61 |
| Aglycone, 6-1-a | 0.03 | 0.29 | 0.72 | - | - |
| 6-2-a | 0.34 | 0.72 | 0.66 | - | 0.44 |
| Aglycone, 6-2-a | 0.04 | 0.28 | 0.70 | - | - |
| 6-2-b | 0.53 | 0.75 | 0.67 | - | - |
| Aglycone, 6-2-b | 0.06 | 0.38 | 0.82 | - | - |
| Standard Rutin | 0.57 | 0.70 | 0.52 | - | 0.60 |
| Standard Isoquercitrin | 0.33 | 0.69 | 0.66 | - | 0.45 |

$R_f$ values were obtained on Schleicher and Schuell, No. 589, red ribbon, chromatographic paper.

TABLE 3

COLORS OF FLAVONOID COMPOUNDS FROM TOBACCO LEAVES

| Compound | Untreated | | Chromogenic Spray | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1% ethanolic aluminum chloride | | 1% ethanolic magnesium acetate | | Saturated ethanolic ammonium molybdate | | 1% ethanolic ferric chloride | |
| | V | UV | V | UV | V | UV | V | UV | V | UV |
| 2-1-a | Y | Y | Y | Y | Y | YG | Y | RBr | G | - |
| 2-1-b | Y | Y | Y | Y | Y | YG | Y | RBr | G | - |
| 2-2-a | Y | Br | Y | Y | Y | YG | Y | RBr | Br | - |
| 2-2-b | Y | Br | Y | Y | Y | YG | Y | RBr | Br | - |
| 6-1-a | Y | Br | Y | Y | - | - | Y | RBr | G | - |
| 6-2-a | Y | Br | Y | Y | - | - | Y | RBr | G | - |
| 6-2-b | Y | Br | Y | Y | - | - | NR | NR | G | - |

V=visible light Y=yellow G=green NR=no visible reaction

UV=ultraviolet light Br=brown R=red - = not determined

Compound 2-1-a.—The $R_f$ values and the yellow fluorescence in ultraviolet light of tobacco leaf compound 2-1-a before and after spraying with 1% ethanolic aluminum chloride agreed with the values from the literature for the flavonol, quercetin. Compound 2-1-a was spotted side by side with authentic quercetin on sheets of chromatographic paper. Separate chromatograms, each carrying compound 2-1-a and authentic quercetin, were run using each of the following solvent systems: 15% acetic acid; 60% acetic acid; and n-butyl alcohol-acetic acid-water. Tobacco leaf flavonoid compound 2-1-a compared with authentic quercetin in each of the three solvents. Compound 2-1-a was co-chromatographed two dimensionally with authentic quercetin by spotting a mixture of quercetin and 2-1-a in the upper corner of a sheet of chromatographic paper, 52 by 58 centimeters. This co-chromatogram was first developed in the solvent system n-butyl alcohol-acetic acid-water, and dried; then it was rotated 90 degrees and developed in a direction at a right angle with the first, this time using 60% acetic acid as the second developing solvent. After spraying with 1% ethanolic aluminum chloride, the two-dimensional co-chromatogram was observed under ultraviolet light. Quercetin did not separate from 2-1-a and only one single, well-defined spot was visible. Further, the ultraviolet absorption spectra were determined for 2-1-a and authentic quercetin on a Beckman spectrophotometer, model DU, in the region of 220 to 420 millimicrons. Both curves demonstrated similar maxima (Table 4). Therefore tobacco leaf flavonoid compound 2-1-a is identified as quercetin.

TABLE 4

ULTRAVIOLET ABSORPTION SPECTRA

| Compound | Maxima, millimicrons | |
|---|---|---|
| | Band I | Band II |
| Standard Quercetin | 375 | 258 |
| 2-1-a | 374 | 255 |
| Standard Kaempferol | 365 | 268 |
| 2-1-b | 365 | 264 |
| 2-2-a | 360 | 258 |
| Demethylated 2-2-a | 374 | 256 |
| 2-2-b | 358 | 255 |
| Demethylated 2-2-b | 375 | 256 |
| Standard Rutin | 361 | 258 |

Compound 2-1-b.—Tobacco leaf flavonoid 2-1-b also fluoresced yellow before and after spraying with 1% ethanolic aluminum chloride; and its $R_f$ values compared with the literature values for the flavonol, kaempferol. Therefore 2-1-b was chromatographed with authentic kaempferol in 15% and 60% acetic acid as well as in the n-butyl alcohol-acetic acid-water systems. Compound 2-1-b compared chromatographically in these three solvent systems with authentic kaempferol (Table 2). A mixture of 2-1-b and authentic kaempferol were co-chromatographed on a two-dimensional chromatogram in the solvent systems n-butyl alcohol-acetic acid-water and 60% acetic acid. Only one well-defined spot was visible under ultraviolet light after spraying the chromatogram with 1% ethanolic aluminum chloride. This indicates that the flavonoid compounds could not be separated by this solvent combination and were probably therefore identical. Tobacco leaf flavonoid 2-1-b is identified as kaempferol. Whether or not these two compounds, quercetin and kaempferol, occur as the aglycones in nature in the tabacco plant is still a question that needs further study. Since their glycosides also are present in tobacco leaves, the aglycones quercetin and kaempferol may possibly have been produced in the leaf drying process.

Compound 2-2-a.—The $R_f$ values of tobacco leaf compound 2-2-a suggested that it was a flavonoid aglycone. It gave a brown fluorescence before and a yellow fluorescence after spraying with 1% ethanolic aluminum chloride. This fact seemed to classify it as a flavone aglycone, as this group is characterized as having a brown fluorescence under ultraviolet light. It did not compare chromatographically with 11 of the more common members of the flavone group in more than two solvents. For

example, apigenin(4',5,7-trihydroxy flavone) and diosmetin (3',5,7-trihydroxy-4'-methoxy flavone) compare closely with 2-2-a in the solvent systems 60% acetic acid and n-butyl alcohol-acetic acid-water. However, when 2-2-a was refluxed with hydriodic acid, specific gravity 1.7, for 4 hours, a compound was obtained which fluoresced yellow before and after spraying with 1% ethanolic aluminum chloride. This yellow fluorescent compound compared chromatographically with authentic quercetin in three solvent systems (Table 2). The ultraviolet absorption spectrum of demethylated 2-2-a had maxima very similar to those of the absorption spectrum determined for authentic quercetin (Table 4). A mixture of demethlated 2-2-a and authentic quercetin was not separated when co-chromatographed on a two-dimensional chromatogram in the solvent systems n-butyl alcohol-acetic acid-water and 60% acetic acid.

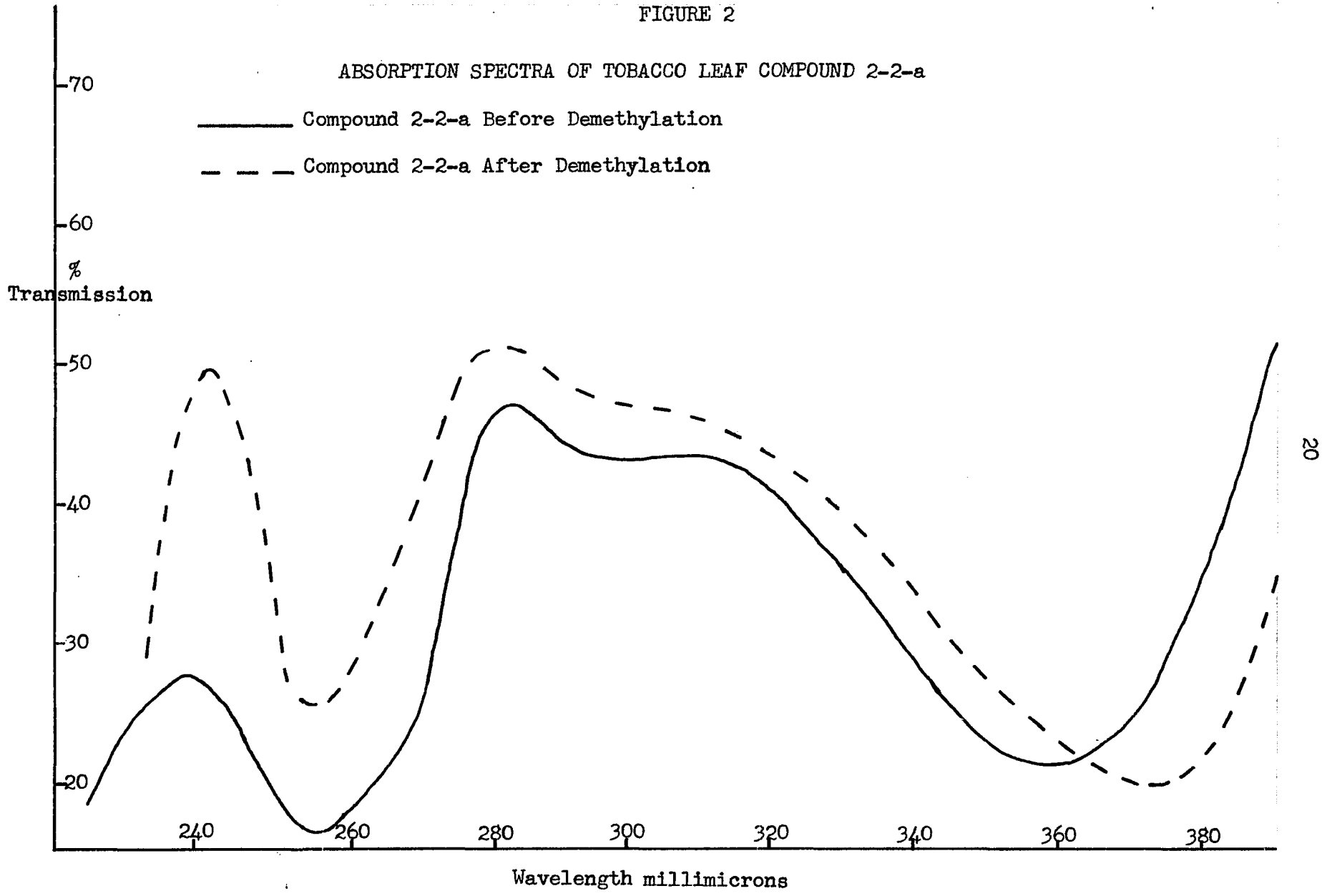
After refluxing 2-2-a with dimethyl sulfate and sodium carbonate in acetone for 6 hours, the product fluoresced blue under ultraviolet light, similar to authentic quercetin-3',4,3,5,7-pentamethyl ether and compared with it chromatographically in three solvent systems (Table 2).

A summation of this information indicates that 2-2-a is a methyl ether of quercetin. The $R_f$ value of 2-2-a bears the same relationship to the $R_f$ value of quercetin in the solvent system 60% acetic acid as do the monemethyl ethers of quercetin: rhamnetin (quercetin-3'methyl ether) and isorhamnetin (quercetin-7-methyl ether). This relationship further suggests that 2-2-a is a monomethyl ether of quercetin.

The tobacco leaf flavonoid compound 2-2-a fluoresces brown under ultraviolet light. The flavonol quercetin, obtained by demethylation of 2-2-a, fluoresces yellow. To explain this change in fluorescent color,

one needs to examine the special nature of the hydroxyl group at carbon number 3. The brightly fluorescent greenish-yellow color of the flavonols is attributed to the presence of a free hydroxyl group at the 3 position (15). If this group is substituted, as in the case of the flavonol-3-glycosides, the compound then absorbs ultraviolet radiation and appears as a dull brownish spot on a chromatogram. The flavones, lacking this hydroxyl group in the 3 position, also fluoresce brown in ultraviolet light. Substitution of a hydroxyl group in other positions on the quercetin nucleus seems not to produce this alteration of the fluorescent color. The quercetin-7-glycosides such as quererciméritrin (quercetin-7-glucoside), fluoresce yellow in ultraviolet light. The methyl ethers of quercetin such as rhamnetin, isorhamnetin and rhamnazin which are substituted in the 3' and 7 positions are yellow under ultraviolet light. Substitution of the hydroxyl group at the number 3 carbon of quercetin also has a hypsochromic effect on band I of its ultraviolet absorption spectrum. Band I for quercetin is at 375 millimicrons and shifts toward the shorter wavelengths when it is substituted with the glycoside group rutinose as in the case of Rutin (quercetin-3-rhamnoglucoside). With another glycoside of quercetin, quercitrin (quercetin-3-rhamnoside), band I for quercetin shifts from 375 when it is unsubstituted to 352 millimicrons when it is substituted at the 3 position with a rhamnose group. This is not the case if the sugar group is substituted at the number 7 carbon of quercetin, as with quercimeritrin, whose ultraviolet absorption spectrum shows band I at 375 millimicrons (identical with band I of quercetin). Upon demethylation, tobacco leaf flavonoid 2-2-a shows a similar spectral shift in that band I of its

FIGURE 2


ABSORPTION SPECTRA OF TOBACCO LEAF COMPOUND 2-2-a
Compound 2-2-a Before Demethylation
Compound 2-2-a After Demethylation
70
60
%
Transmission
50
40
30
20
240
260
280
300
320
340
360
380
Wavelength millimicrons

spectrum shifts from 360 millimicrons to 374 millimicrons for the product of the demethylation (Figure 2). The brown fluorescence of compound 2-2-a under ultraviolet light and the spectral shift of 14 millimicrons in its ultraviolet absorption spectrum suggest that in this compound the methyl group is substituted at the 3 position in the structure of tobacco leaf flavonoid 2-2-a. As further evidence for this position of substitution, 2-2-a gave a salmon pink color when its ethanolic solution was shaken with sodium amalgam and then acidified with hydrochloric acid. This is a test (16) for the blocking of the hydroxyl group at the 3 position in flavonols. Compound 2-2-a also gave a positive test when sprayed with saturated alcoholic ammonium molybdate. Assuming that the hydroxyl group at the number 3 carbon of the quercetin nucleus is blocked, this positive test indicates that the two ortho hydroxyl groups at position 3' and 4' are unsubstituted. This test is an adaptation by A.G. Kallianos of this laboratory of a microqualitative test used by Martini (17) for traces of molybdenum in which the test reagent was catechol (1,2-dihydroxy benzene). Investigation with flavonoid compounds of known structure indicate that this test is specific for ortho hydroxyl groups in flavonoid compounds if the hydroxyl group at carbon number 3 is blocked to prevent complexing of molybdenum across the hydroxyl group at the 3 position and the carbonyl group at the 4 position. This reagent has been shown not to complex the free hydroxyl group in the 5 position with the carbonyl group at the 4 position. Flavonoid structures were chosen with only the 5-hydroxyl group and the carbonyl group at the number 4 carbon open for reaction as in the case of kaempferol-3-rhamnoglucoside and apigenin. Both gave negative tests

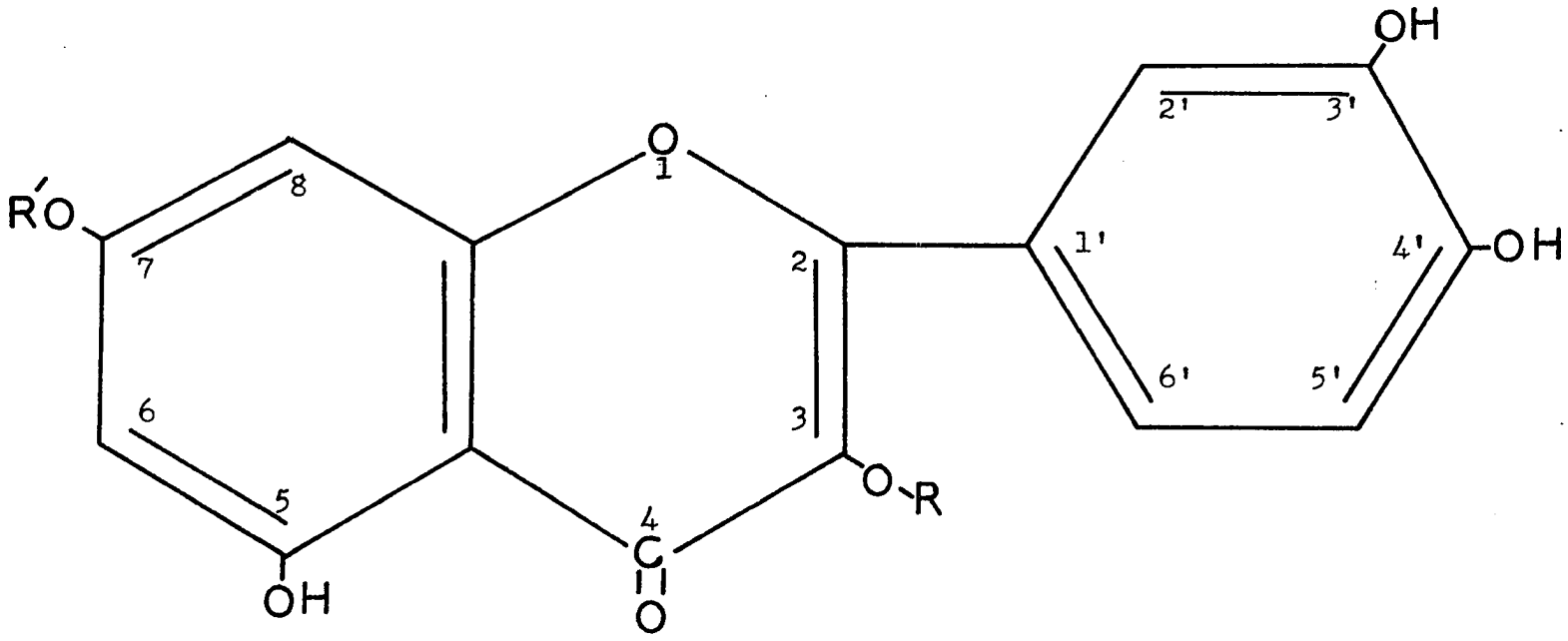
when sprayed with saturated ammonium molybdate. Since the brown fluorescence and the ultraviolet absorption specturm of 2-2-a are similar to those of isoquercitrin, 2-2-a was refluxed for four hours with 5% sulfuric acid. Chromatography in the 60% acetic acid solvent system showed no change in the $R_f$ value of compound 2-2-a. Authentic isoquercitrin was completely hydrolyzed by refluxing one hour with 5% sulfuric acid. Therefore, compound 2-2-a is not isoquercitrin.

The following structure is tentatively proposed for tobacco leaf compound 2-2-a: quercetin-3-methyl ether (Figure 3).

Compound 2-2-b.—Tobacco leaf compound 2-2-b also fluoresces brown under ultraviolet light. Upon refluxing 2-2-b with hydriodic acid, specific gravity 1.7, for four hours, a product was obtained which fluoresced yellow under ultraviolet and compared with authentic quercetin in three solvent systems (Table 2). The demethylated product of compound 2-2-b could not be separated from authentic quercetin when co-chromatographed on a two-dimensional chromatogram in the solvent systems n-butyl alcohol-acetic acid-water and 60% acetic acid. The product obtained from the demethylation of compound 2-2-b was also identical chromatographically in three solvent systems with the product of the demethylation of compound 2-2-a. The maxima of the ultraviolet absorption spectrum determined for the product of the demethylation of compound 2-2-b agreed with the maxima of the absorption spectrum determined for authentic quercetin (Table 4). Compound 2-2-b was refluxed in acetone with dimethyl sulfate for six hours and the product fluoresced blue under ultraviolet light and compared chromatographically in three solvent systems with the blue fluorescent pentamethyl ether prepared from authentic

FIGURE 3

PROPOSED STRUCTURES OF FLAVONOID COMPOUNDS FROM TOBACCO LEAVES




R = $CH_3$ R' = H Structure proposed for tobacco leaf compound 2-2-a

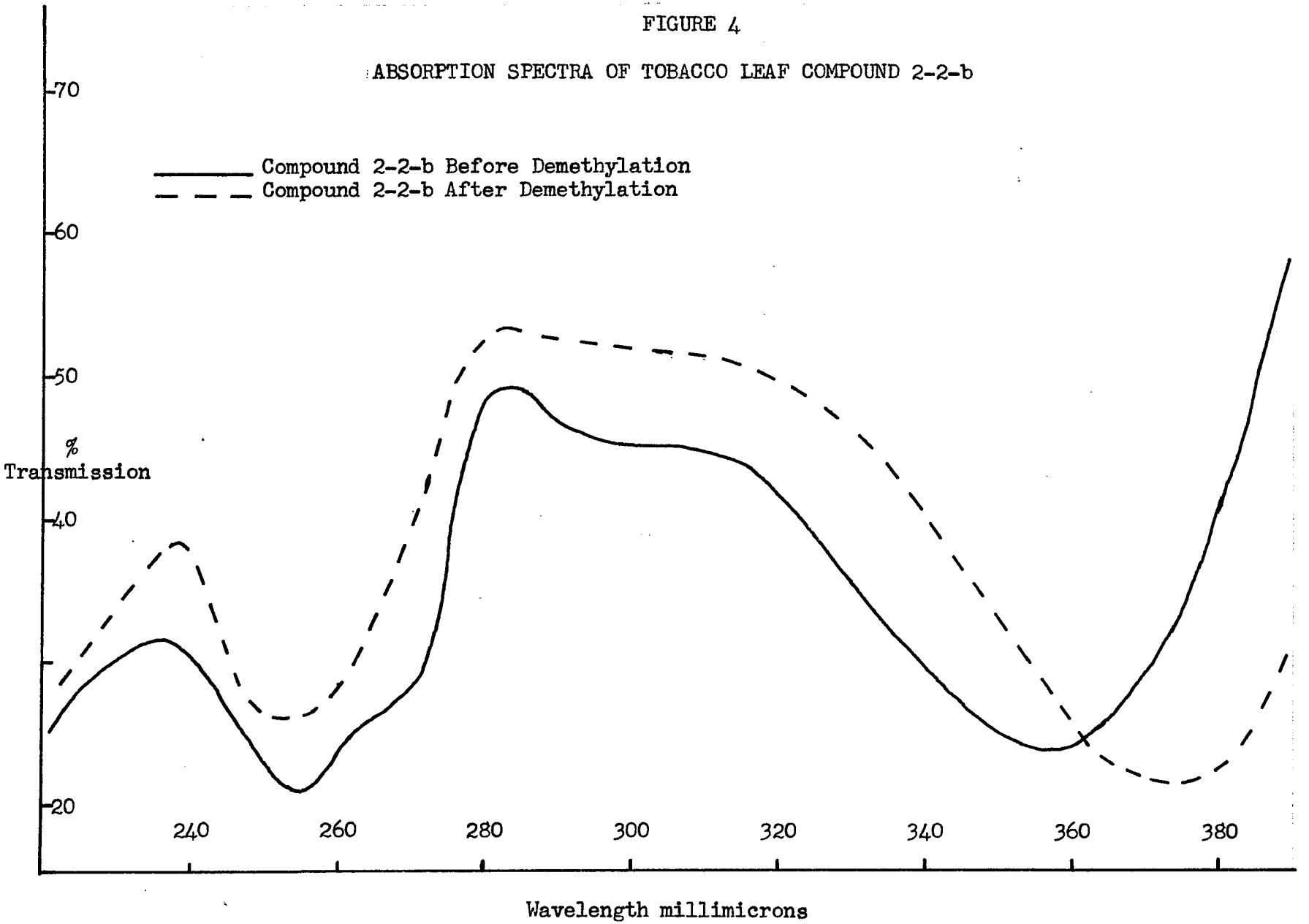
R = $CH_3$ R' = $CH_3$ Structure proposed for tobacco leaf compound 2-2-b

quercetin. The $R_f$ value of 2-2-b bears the same relationship to quercetin as that of a dimethyl ether of quercetin, rhamnazin (quercetin-3', 7-dimethyl ether). Therefore, compound 2-2-b may be a dimethyl ether of quercetin. The brown fluorescence of compound 2-2-b under ultraviolet light and the bathochromic shift of the maximum of band I of the ultraviolet absorption spectrum of 2-2-b from 258 millimicrons before demethylation, to 375 millimicrons for band I of the absorption spectrum of the product of demethylation of 2-2-b (Figure 4), again suggest that one of the methyl groups of compound 2-2-b is substituted at the 3 position. To support further this proposed position of substitution, an ethanolic solution of compound 2-2-b was shaken with sodium amalgam and upon acidification with hydrochloric acid, gave a salmon pink color as a positive test for the blocking of the hydroxyl group in the 3 position of a flavonol (17). Quercetin with a free hydroxyl group at the 3 position gives a green to brown color with this test. When compound 2-2-b is sprayed with 1% ammonium molybdate, it turns yellow in visible light and a red-brown in ultraviolet light to indicate that the ortho hydroxyl groups at 3' and 4' are unsubstituted. Since the hydroxyl group at the 5 position is very unreactive (18) and substitution here is rare in nature (19), the probability is greater that the second methyl group is substituted at the number 7 position. Therefore, the following structure is tentatively proposed for tobacco leaf compound 2-2-b: quercetin-3,7-dimethyl ether (Figure 3).

Compound 6-1-a.—The brown fluorescence and $R_f$ values (Table 2) of tobacco leaf flavonoid 6-1-a agreed with the literature values for rutin. Therefore, authentic rutin was chromatographed with 6-1-a.

FIGURE 4

ABSORPTION SPECTRA OF TOBACCO LEAF COMPOUND 2-2-b



Authentic rutin compared chromatographically with 6-1-a in the solvent systems 60% and 15% acetic acid and n-butyl alcohol-acetic acid-water (Table 2). The compound 6-1-a could not be separated from authentic rutin by two dimensional chromatography in the solvent systems n-butyl alcohol-acetic acid-water and 15% acetic acid. Upon crystallization 6-1-a melted at 194-96°C, uncorrected. This melting point was not lowered when crystals of authentic rutin were mixed with 6-1-a. Hydrolysis of 6-1-a in 5% sulfuric acid for four hours on a steam bath yielded a yellow fluorescent compound which compared chromatographically with authentic quercetin in three solvent systems (Table 2). The hydrolysis solution was neutralized with barium hydroxide and the solution deionized on an ion exchange column packed with an anion-cation exchange resin, MB-3. The deionized solution was reduced to 5 milliliters and chromatographed with authentic rhamnose and glucose in the solvent system, sec-butyl alcohol-ethyl alcohol-water (9:1:10 by volume). After spraying with aniline hydrogen oxalate to make the sugar spots visible, the sugars in the hydrolysate compared chromatographically with authentic rhamnose and glucose. Tobacco leaf flavonoid 6-1-a is therefore identified as rutin (quercetin-3-rhamnoglucoside). Rutin has previously been reported in tobacco leaves by Hasegawa (5), Neuberg and Kobel (20) and Couch and Krewson (6).

Compound 6-2-a.—The $R_f$ values of tobacco leaf flavonoid 2-2-a (Table 2) and the fact compound 6-2-a fluoresced brown under ultraviolet light agreed with the literature properties for isoquercitrin (quercetin-3-glucoside). Compound 6-2-a compared chromatographically with authentic isoquercitrin in the three solvent systems 15% and 60% acetic acid and

n-butyl alcohol-acetic acid-water. To distinguish between quercitrin and isoquercitrin, compound 6-2-a was chromatographed with authentic standard solutions of these compounds in the solvent system 22% isopropyl alcohol. Compound 6-2-a ($R_f$ = 0.45) compared chromatographically with isoquercitrin ($R_f$ = 0.45) and not with quercitrin ($R_f$ = 0.59) in this solvent system. Further, compound 6-2-a could not be separated from authentic isoquercitrin by two-dimensional chromatography in the solvent systems 15% acetic acid and 22% isopropyl alcohol. Upon hydrolysis with 5% sulfuric for 1 hour, compound 6-2-a yielded an aglycone which compared with authentic quercetin in three solvent systems (Table 2). The hydrolysate yielded a sugar which compared chromatographically in the solvent system sec-butyl alcohol-ethyl alcohol-water with authentic glucose. Tobacco leaf flavonoid 6-2-a is identified as isoquercitrin. It has been reported in tobacco leaves by Howard, Gage and Wender (8) and Kourilo (7).

Compound 6-2-b.—Tobacco flavonoid 6-2-b fluoresced brown under ultraviolet light and its $R_f$ values (Table 2) suggested that it was a flavonoid glycoside. Mr. Bradford of this laboratory had isolated a similar flavonoid glycoside from tobacco flowers and identified it as kaempferol-3-rhamnoglucoside. He had identified the aglycone, glycosides and the position of attachment of the glycoside group as well as established the relative glycoside-aglycone ratio per mole of the flavonoid glycoside. Tobacco leaf flavonoid 6-2-b compared chromatographically with this tobacco flower flavonoid in the three solvent systems 15% and 60% acetic acid and n-butyl alcohol-acetic acid-water. Furthermore, 6-2-b could not be separated from kaempferol-3-

rhamnoglucoside by two-dimensional chromatography in the n-butyl alcohol-acetic acid-water and 15% acetic acid solvent systems. Upon hydrolysis with 5% sulfuric acid, compound 6-2-b yielded two sugars which compared chromatographically with standard rhamnose and glucose in the sec-butyl alcohol-ethyl alcohol-water system. Tobacco leaf flavonoid 6-2-b is identified as kaempferol-3-rhamnoglucoside. Kaempferol-3-rhamnoglucoside has been reported in Calystegia japonica chois by Hukuti (21).

# CHAPTER III

## THE ISOLATION OF ACTIVE FLAVONOID COMPOUNDS FROM THE LEAVES OF TOBACCO

### The Initial Processing of Radioactive Tobacco Leaves

In connection with the plant metabolism studies, one-sucker tobacco plants, Nicotiana tabacum, were grown in an atmosphere of carbon-14 labeled carbon dioxide in the growth chamber of the Argonne National Laboratory. Immediately after harvesting, the plants were placed in a deep freeze. The plants were harvested and stored in separate packages, according to the number of days of growing time in the radioactive atmosphere, and according to whether the tissues were those of leaves, roots, or stems. The leaves were further subdivided according to their size and relative position on the plant. Each package was processed separately. The frozen leaves were partially crushed in their plastic bags and dropped into a beaker of boiling 85% isopropyl alcohol, and the solution was allowed to boil for three minutes to inactivate the enzyme systems. This alcoholic mixture was then reduced to fine particle size in a Waring Blendor. This tobacco leaf blend was decanted into a Soxhlet thimble. The thimble was placed in a Soxhlet extractor and extracted with 85% isopropyl alcohol until no visible color remained in the extract. This alcoholic extract was then added to the filtered alcoholic extract obtained by decanting into the Soxhlet thimble. The whole

extract was then reduced in volume on a hot plate to approximately 100 milliliters. This reduced alcoholic tobacco leaf extract was poured into a pint wide-mouth Mason jar, and Whatman standard grade ashless cellulose powder added, while stirring, until the particles no longer coalesced upon standing. The jar was placed in an oven and the tobacco leaf extract-cellulose powder mixture dried at 50°C until the powder would flow readily. When all packages had been processed in this manner, they were stored for further investigation.

Isolation of the Active Tobacco Leaf Flavonoid Compounds

Preliminary Separation by Column Chromatography. The jar containing the dry tobacco leaf extract-cellulose powder mixture, which represented 411 grams of main stem tobacco leaves from plants exposed to $C^{14}O_2$ for 43 days, package no. 11, was opened and the contents packed with a ramrod on a chromatographic column containing Whatman standard grade cellulose powder. The column had been prepared in the same manner as that previously described in Chapter II. Benzene was placed on the column as the first solvent, and 500 milliliter fractions of column eluate were collected. When the majority of the dark tar-like material and chlorophylls had been removed by the benzene, the solvent was changed to anhydrous ethyl acetate. As soon as the column eluate could be identified as ethyl acetate to indicate that essentially all the benzene had been washed off the column, the major developing solvent, water-saturated ethyl acetate was started onto the column and continued until chromatography of each fraction on 2 inch strips of chromatographic paper in the 15% acetic acid-water solvent system indicated that the majority of the flavonoid compounds had been eluted from the column.

The column was washed first with 500 milliliters 95% ethyl alcohol and then with 500 milliliters of distilled water. The column fractions were reduced in volume; placed in 100 milliliter volumetric flasks and stored in the refrigerator. A chromatogram was prepared by spotting 50 microliters of each column fraction 1 inch apart on a line ruled across 3 inches below the top of a sheet of chromatographic paper, Schleicher and Schuell, No. 589, red ribbon, 52 by 58 centimeters. The chromatogram was developed in the solvent system 15% acetic acid-water, dried and sprayed with 1% ethanolic aluminum chloride. This chromatogram, when observed under ultraviolet light, indicated which column fractions contained flavonoid compounds (Figure 5). In order to establish the relative activity of the column fractions, 100 microliters of each 100 milliliter column fraction was micro-pipetted into an aluminum planchet and the activity determined in a Nuclear Model D47 gas flow counter (Table 5).

Isolation by Paper Partition Chromatography. Observation of the 15% acetic acid chromatogram of the column fractions under ultraviolet light indicated that the methylated quercetin compounds were in column fractions 1,2,3, and 4. These fractions were added together and their combined volume reduced to 25 milliliters. The combined fraction was then streaked continuously across 3 inches down from the top of 8 sheets of Whatman No. 3 MM chromatographic paper. These chromatograms were then developed in the 15% acetic acid-water system. After drying, a single brown fluorescent zone could be seen under ultraviolet light, near the origin on these chromatograms. When a 2 inch strip, cut from the edge of one of the chromatograms from the 15% acetic acid-water

FIGURE 5

FRACTIONATION OF THE RADIOACTIVE FLAVONOID COMPOUNDS BY A CELLULOSE COLUMN



Developing Solvent: 80% Secondary Butyl Alcohol

Chromogenic Spray: 1% Ethanolic Aluminum Chloride

TABLE 5

FRACTIONATION OF ACTIVE FLAVONOID COMPOUNDS BY CELLULOSE COLUMN

| Fraction | Volume ml. | Solvent | Visible Color | Activity* counts/min. |
|---|---|---|---|---|
| 0 | 500 | Benzene | Dk G | 10,444 |
| 1 | " | Anhydrous Ethyl Acetate | " " | 7,737 |
| 2 | " | " | G | 620 |
| 3 | " | Water-Saturated Ethyl Acetate | Lt G | 1,928 |
| 4 | " | " | " | 1,461 |
| 5 | " | " | " | 2,556 |
| 6 | " | " | " | 2,772 |
| 7 | " | " | Dk G | 13,451 |
| 8 | " | " | Lt G | 9,203 |
| 9 | " | " | " | 1,096 |
| 10 | " | " | " | 1,400 |
| 11 | " | " | YG | 1,382 |
| 12 | " | " | " | 1,016 |
| 13 | " | " | " | 567 |
| 14 | " | " | " | 808 |
| 15 | " | " | " | 4,992 |
| 16 | " | " | " | 3,105 |
| 17 | " | " | " | 967 |
| 18 | " | 95% Ethanol | Dk G | 29,171 |
| 19 | " | Water | YG | 6,824 |

Y=yellow G=green Dk=dark Lt=light
* 10 microliter aliquot of 100 milliliter column fraction

system, was sprayed with 1% ethanolic aluminum chloride, a zone corresponding to the brown zone now fluoresced yellow to indicate the presence of flavonoid compounds. Furthermore, when this test strip was exposed to x-ray film in an x-ray exposure holder for one week and developed, a darkened zone on the x-ray film coincided with the flavonoid zone on the chromatogram to indicate the presence of activity in this zone.

While observing the chromatogram under ultraviolet light, the single brown fluorescent zone near the origin was cut out of each chromatogram with scissors. This zone was then sewn 3 inches below the top across a new sheet of Whatman No. 3 MM chromatographic paper, and the 8 chromatograms with the sewn strips were developed in the solvent system 60% acetic acid-water. Observation of the dried chromatogram under ultraviolet light now showed two brown fluorescent zones. Each zone was cut out of the chromatogram and eluted with 95% ethyl alcohol in a Soxhlet extractor for four hours. Each of the extracts was then reduced in volume and stored in a 10 milliliter volumetric flask in the refrigerator. A spot of the extract from the top zone could not be separated from tobacco leaf flavonoid compound 2-2-a by two-dimensional chromatography in the solvent systems n-butyl alcohol-acetic acid-water and 60% acetic acid-water. Compound 2-2-a is the tobacco leaf compound for which the structure, quercetin-3-methyl ether, had been proposed in Chapter II. A radioautogram of this two-dimensional chromatogram was made by placing it together with a sheet of Eastman x-ray film, 14 by 17 inches, in a lead backed x-ray holder. The x-ray film was removed after 3 weeks exposure to the chromatogram and, upon development, the x-ray

film showed only one darkened spot. This spot coincided with the spot on the chromatogram that represented tobacco leaf 2-2-a and the radioactive top brown fluorescent zone. Therefore, 2-2-a and the top zone were probably identical, and furthermore, the top zone was apparently radiopure as well as chromatographically pure. A 100 microliter aliquot was taken of the 10 milliliters of extract of the top zone and its activity measured in a gas flow counter. The aliquot gave an activity measurement of 1036 counts per minute. This represents 0.00009% of the total activity in the alcohol soluble fraction of the radioactive tobacco leaves.

A spot of extract from the bottom brown fluorescent zone could not be separated from tobacco leaf flavonoid 2-2-b on a two-dimensional chromatogram developed in the n-butyl alcohol-acetic acid-water and 60% acetic acid-water solvent systems. Compound 2-2-b is the tobacco leaf compound for which the structure, quercetin-3,7-dimethyl ether, was proposed in Chapter II. A radioautogram made of this two-dimensional chromatogram showed only one darkened spot. This radioautogram spot coincided with the spot on the chromatogram that represented 2-2-b and the bottom brown fluorescent zone. Therefore, the bottom zone was probably identical with 2-2-b and apparently radiopure. A 100 microliter aliquot was then taken of the 10 milliliters of extract of the bottom brown fluorescent zone and the activity measured in a gas flow counter. The aliquot gave an activity measurement of 1821 counts per minute. This represented 0.00017% of the total activity in the alcohol soluble fraction of the radioactive tobacco leaves.

The aluminum chloride sprayed chromatogram of the column

fractions when observed under ultraviolet light showed that the flavonol glycosides, rutin and kaempferol-3-rhamnoglucoside, were in column fractions 11 through 16. These column fractions were combined and reduced in volume to 40 milliliters. This combined fraction was streaked continuously across, 3 inches down from the top, of 16 sheets of Whatman No. 3 MM chromatographic paper. The chromatograms were developed in the n-butyl alcohol-acetic acid-water solvent system and dried. When these chromatograms were observed under ultraviolet light, the original streaked column fractions had now been separated by this solvent system into two brown fluorescent zones and several blue zones. The bottom brown fluorescent zone appeared to be the result of a clean cut separation, free from other fluorescent compounds. The top brown fluorescent zone, however, was partly obscured by an overlapping blue zone. The brown fluorescent zones were cut out of the chromatograms and sewn across the top of new sheets of Whatman No. 3 MM chromatographic paper and developed in the 15% acetic acid-water solvent system. After drying, the top brown fluorescent zone which had separated from the blue zone in this solvent system, was cut out of the chromatogram and eluted in a Soxhlet extractor for 4 hours with 95% ethyl alcohol. The extract was reduced to 10 milliliters in volume and stored in a 10 milliliter volumetric flask in the refrigerator. A spot of extract representing this top zone could not be separated from authentic rutin on a two-dimensional chromatogram developed in the solvent systems n-butyl alcohol-acetic acid-water and 15% acetic acid-water. A radioautogram was made of this two-dimensional chromatogram. After development, the x-ray film had only one darkened spot to indicate that the radioactive tobacco leaf rutin was

apparently radiopure. This darkened spot also coincided with the flavonoid spot on the two-dimensional chromatogram which represented the radioactive tobacco leaf rutin.

A quantitative determination of the radioactive tobacco leaf rutin was made by the method of Gage and Wender (11). A 1 milliliter aliquot of the radioactive rutin was diluted to 10 milliliters in a volumetric flask with 1% ethanolic aluminum chloride. The optical density of the solution was 0.236 as determined at 405 millimicrons on a model DU Beckman spectrophotometer, using a 1 centimeter quartz cell. The wave length, 405 millimicrons, had been chosed from a maximum in the ultraviolet absorption spectrum of rutin which was determined on this same instrument for a solution of authentic rutin at a concentration of 50 micrograms of rutin in 10 milliliters of 1% ethanolic aluminum chloride. The optical density of this solution of radioactive rutin was compared to a standard curve which was constructed by determining the optical density of solutions of authentic rutin containing 5,25,50 and 100 micrograms, each of which was in 10 milliliters of 1% ethanolic aluminum chloride. An optical density of 0.236 was equivalent to 45.7 micrograms of rutin for the 1 milliliter aliquot of 10 milliliters active rutin or 457 micrograms for the total radioactive rutin isolated from the radioactive tobacco leaves.

An activity of 804 counts per minute was obtained for a 100 microliter aliquot of the 10 milliliters of radioactive tobacco leaf rutin as measured in a gas flow counter having an efficiency of 41%. This was equivalent to 80,000 counts per minute for the total active tobacco rutin or 201,000 disintegrations per minute corrected for the efficiency of the

gas flow counter. Calculated on the basis of rutin being 53.11% carbon, and using the conversion factor, $2.2 \times 10^6$ disintegrations per minute as equivalent to 1 microcurie, the rutin isolated from the radioactive tobacco leaves had a specific activity of 376.4 microcuries per gram carbon.

Although the bottom brown fluorescent zone appeared to contain only one compound after chromatography in the n-butyl alcohol-acetic acid-water system, because of the required radiopurity the zones were cut out of the chromatograms and sewn across the tops of new sheets of Whatman No. 3 MM chromatographic paper and developed in the 15% acetic acid-water solvent system. After drying, the brown zones were cut out of the chromatograms and eluted with 95% ethyl alcohol in Soxhlet extractors for 4 hours. The extract was reduced in volume and diluted to 10 milliliters in a volumetric flask and stored in the refrigerator.

A spot of the extract representing this brown zone could not be separated from authentic kaempferol-3-rhamnoglucoside on a two-dimensional chromatogram in the solvent systems n-butyl alcohol-acetic acid-water and 15% acetic acid water. A radioautogram was made of this two-dimensional chromatogram. After development, only one darkened spot was visible on the x-ray film. This darkened spot coincided with the spot on the two-dimensional chromatogram which represented the radioactive kaempferol-3-rhamnoglucoside. A 100 microliter aliquot was taken of the radioactive kaempferol-3-rhamnoglucoside and its activity determined in a gas flow counter. The aliquot registered 15,023 counts per minute. Thus the radioactive kaempferol-3-rhamnoglucoside represented 0.0014% of the total radioactivity in the alcohol soluble fraction of

the active tobacco leaves.

# CHAPTER IV

## A QUANTITATIVE METHOD FOR THE MICROANALYSIS OF RUTIN IN THE LEAVES OF TOBACCO

### Isolation of Rutin From Tobacco Leaves

This method of quantitative determination of rutin was designed especially to be applied to fresh green plant tissue in starting weights of an order of 1 gram, so that a small tobacco leaf may be removed from the plant and analyzed for rutin.

Extraction. The leaf was removed from the tobacco plant, weighed in a tared Soxhlet thimble, and dropped immediately into a Soxhlet extractor filled with 85% isopropyl alcohol which had previously been brought to reflux temperature. This procedure quickly inactivated the enzyme systems of the tobacco leaf tissue and at the same time initiated the extraction process. The leaf tissue was extracted for 4 hours. The leaf extract was removed and the extractor reservoir refilled with 85% isopropyl alcohol and the extraction continued an additional 4 hours. The combined leaf extract was reduced in volume and diluted to 25 milliliters in a volumetric flask. This volume was sufficient for starting leaf tissue weights of from 1 to 50 grams.

Isolation by Paper Partition Chromatography. A measured volume of the tobacco leaf extract, representing 0.3 gram of starting fresh leaf weight, was continuously streaked with a pipette across 3 inches

down from the top of a sheet of chromatographic paper, Schleicher and Schuell, No. 589, Red Ribbon, 52 by 58 centimeters. This chromatogram was placed in a chromatographic tank and developed with benzene, reagent grade, as a solvent system. The tar-like materials and chlorophylls were moved down and off the chromatogram by the benzene solvent system while the flavonoid substances had not moved from where they were streaked at the origin. The dried chromatogram was returned to the chromatographic tank and developed in the solvent system 80% sec-butyl alcohol. The chromatogram was permitted to develop until the solvent front reached the bottom edge of the chromatogram. When the dried chromatogram was observed under ultraviolet light, rutin appeared as a brown fluorescent zone overlapped by a blue zone approximately one-half way down from the origin. As an aid in accurately locating the rutin zone for the cutting process, the chromatogram was observed not only in ultraviolet light but also in visible light where the rutin zone appeared yellow against the white paper background. If at this point the cut was made too high (toward the origin) above the rutin zone, a blue zone would be included which would not separate in the next solvent system; and if the cut was made too low (toward the solvent front) below the rutin zone, the kaempferol-3-rhamnoglucoside zone would be included with the rutin zone. The rutin zone could not be sprayed with 1% ethanolic aluminum chloride at this point in the separation procedure even though it would have been an excellent aid in locating the rutin zone, because it was found that the aluminum-rutin complex would not chromatograph as a sharply defined zone in either of the solvent systems, 15% tert-butyl alcohol or distilled water.

When the rutin zone had been located, it was cut out of the chromatogram and sewn across the top of a new sheet of chromatographic paper and the chromatogram developed in the 15% tert-butyl alcohol-water solvent system. When this chromatogram was dried and observed under ultraviolet light, the brown fluorescent rutin zone appeared about one-half way down from the origin and three blue zones, which had been separated from the rutin zone, appeared near the solvent front. The 15% tert-butyl alcohol promotes a more sharply defined rutin zone; but if this isolation scheme were expanded to include one or more of the blue zones, distilled water would be used as a solvent system to replace the 15% tert-butyl alcohol system. The dried chromatogram was sprayed with 1% ethanolic aluminum chloride, and the rutin zone now appeared as a yellow fluorescent zone under ultraviolet light. Since as little as 0.25 micrograms of aluminum chloride sprayed rutin can be detected under ultraviolet light, the 1% ethanolic aluminum chloride spray aids not only in a better quantitative recovery in the cutting process, but also renders the rutin, as a aluminum complex, more soluble in water than the uncomplexed rutin is in 95% ethyl alcohol. These factors promote a more quantitative elution of the rutin from the chromatographic paper.

The Spectrophotometric Quantitative Determination of Rutin. The aluminum chloride sprayed chromatograms were observed under ultraviolet light, and the yellow fluorescent rutin zone was cut out of the chromatogram. The zone was then folded in an accordion-like manner and placed in an 8 inch pyrex test tube. A 25 milliliter volume of distilled water was pipetted into the test tube and the tube placed in a hot water bath for 15 minutes. The test tube was removed from the hot water bath and

allowed to equilibrate until it reached room temperature. A prior attempt to extract this yellow fluorescent rutin zone in a Soxhlet extractor with distilled water led to a partial destruction of the rutin-aluminum complex by the prolonged high temperature.

The water extract of the aluminum-rutin complex was then poured into a 1 centimeter quartz cell and the optical density of the solution determined in a model DU Beckman spectrophotometer, at a wave length of 415 millimicrons. This wave length had been previously chosen from a maximum in the ultraviolet absorption spectrum determined for a solution concentration of 100 micrograms of rutin in 25 milliliters of distilled water. This general spectrophotometric procedure is basically the method used by Gage and Wender (11), who determined rutin quantitatively at this same wave length.

In order to prepare a standard curve to which the optical densities of the unknown samples of rutin could be compared, 25,100,200, 300 and 500 microgram aliquots of authentic rutin were streaked on individual sheets of chromatographic paper. The same isolation procedure was followed for each chromatogram as was used in the chromatographic isolation of rutin of unknown concentration from the tobacco leaf extract. After spraying the chromatograms of the known concentrations of rutin with 1% aluminum chloride, each of the zones was cut out, folded, and placed in 8 inch pyrex test tubes. Again 25 milliliters of distilled water was pipetted into each and the test tube placed in a hot water bath for 15 minutes. After equilibration, the optical density was determined for each aluminum-rutin complex solution in the Beckman spectrophotometer. The optical density was plotted vs. rutin concentration and a straight

line drawn connecting the points representing the rutin samples of known concentration. By referring the optical densities of the solutions of isolated tobacco leaf rutin to this standard rutin curve, the concentration could be read directly from the curve for the streaked aliquot and the total rutin content could be calculated for the tobacco leaf tissue per gram green fresh weight.

$$\text{Concentration (micrograms of rutin per gram fresh leaf tissue)} = \frac{\text{Concentration (micrograms of rutin in streaked aliquot)} \times \text{Volume (isolated rutin, ml.)}}{\text{Weight (fresh leaf tissue, g.)} \times \text{Volume (streaked aliquot, ml.)}}$$

# CHAPTER V

## THE QUALITATIVE AND QUANTITATIVE EFFECT OF GIBBERELLIC ACID ON THE FLAVONOID COMPOUNDS OF TOBACCO LEAVES

Six young tobacco plants which had been grown for 65 days in soil culture under greenhouse conditions of high nitrogen and an 18 hour photoperiod, were divided into two lots of three each. Each of the three plants of one lot was treated with 50 micrograms of gibberellic acid in 50% ethyl alcohol by pipetting the solution onto the surface, near the base of the midrib, of the most recently expanded leaf of the terminal bud. Each of the tobacco plants of the other lot was treated in the same manner except that the 50% ethyl alcohol solution contained no gibberellic acid. As a reference base, the leaf, 2 nodes below the treated leaf, was marked with a tag and will be called the "tagged leaf."

At the end of 9 days the treated plants began to show the first signs of gibberellic acid treatment as compared to the three control plants (Figure 6). In the gibberellic acid treated plants, the plant stem was smaller in diameter; the internode length was noticeably longer; the leaf color was a lighter shade of green; and an average of 4 additional leaves had been elaborated by the terminal bud. The differences between the control and treated tobacco plants as to physical characteristics 22 days after the initial treatment, were similar to those already noted, except perhaps magnified to a greater degree. Similar




FIGURE 6. GIBBERELLIC ACID TREATED TOBACCC PLANT

growth stimulation has been reported in other types of plants treated with gibberellic acid by Stow and Yamaki (22).

At the end of 22 days after treatment, one control and one treated tobacco plant were harvested. Leaves were chosen by their position on the stem so as to give a cross section view physiologically of the treated and control tobacco plants. Corresponding leaves were taken from both control and treated plants. Using the tagged leaf as a base reference point, the tobacco leaves were harvested in this order: (1) 2 nodes below the tagged leaf, (2) the tagged leaf, (3) 2 nodes above the tagged leaf (the leaf treated with gibberellic acid), (4) the two leaves from the 6th and 7th node above the tagged leaf, and (5) the terminal bud with 8 small leaves. Each leaf or group of leaves was harvested separately, placed in a tared Soxhlet thimble and weighed. The thimble was dropped immediately into a Soxhlet extractor which had been previously brought to reflux temperature and extracted with 85% isopropyl alcohol for a total of 8 hours. The alcoholic leaf extract was reduced in volume, placed in a 25 milliliter volumetric flask and diluted to the mark with 85% isopropyl alcohol.

An aliquot of the alcoholic leaf extract was streaked across the top of a sheet of chromatographic paper, Schleicher and Schuell, No. 589, red ribbon. This streaked chromatogram was developed in the 80% secondary butyl alcohol-water solvent system. While observing this dried chromatogram under ultraviolet light, the rutin zone was cut out with scissors. The rutin zone was then sewn with a sewing machine across the top of a new sheet of chromatographic paper and the chromatogram developed in the 15% tert-butyl alcohol-water solvent system. After drying, the

chromatogram was sprayed with 1% ethanolic aluminum chloride. The yellow fluorescent rutin zone was cut out, folded and placed in an 8" pyrex test tube. Then 25 milliliters of distilled water was pipetted into the test tube and the tube placed in a hot water bath for 15 minutes. The test tube was removed and allowed to equilibrate until the solution had reached room temperature.

The optical density was then determined at a wave length of 415 millimicrons on a Beckman spectrophotometer and compared with a standard curve for the rutin concentration. Each determination of the rutin concentration in the leaves in the other positions on the gibberellic acid treated and control tobacco plants, was made in a similar manner (Tables 6 and 7).

A graph representing the distribution of rutin in the gibberellic acid treated and control tobacco plants was plotted (Figure 7). In the control plant, the rutin concentration appears high in the older lower leaves on the tobacco plant, dropping to a low in the fully expanded median leaves, rising in the newly expanding leaves just below the terminal bud and finally falling slightly in the terminal bud and in the very small leaves just in the process of elaboration from the terminal bud. In the gibberellic acid treated plant (Figure 7), the pattern of the rutin concentration appears to be about the same in the lower and median leaves; but a significant increase occurs in the concentration of rutin in the very small leaves and the terminal bud of the gibberellic acid treated tobacco plant.

As another technique for demonstrating the distribution of the flavonoid compounds in the control plant, a chromatogram was prepared by

TABLE 6

RUTIN DISTRIBUTION IN THE GIBBERELLIC ACID TREATED TOBACCO PLANT

| Position Number | Leaf Position | Weight of Leaf Tissue grams | Volume Extract ml. | Volume Streaked ml. | Optical Density | Rutin in Volume Streaked micrograms | Micrograms Rutin per Gram Leaf Tissue | % Rutin by Weight |
|---|---|---|---|---|---|---|---|---|
| 1 | 2 nodes below tagged leaf | 7.28 | 25 | 1 | 0.078 | 108 | 371 | 0.037 |
| 2 | tagged leaf | 7.00 | 25 | 1 | 0.024 | 53 | 189 | 0.019 |
| 3 | 2 nodes above tagged leaf | 6.77 | 25 | 1 | 0.018 | 48 | 177 | 0.018 |
| 4 | two leaves 6 and 7 nodes above tagged leaf | 3.57 | 25 | 2 | 0.022 | 51 | 178 | 0.018 |
| 5 | terminal bud with 8 small leaves | 1.70 | 25 | 4 | 0.087 | 116 | 426 | 0.043 |

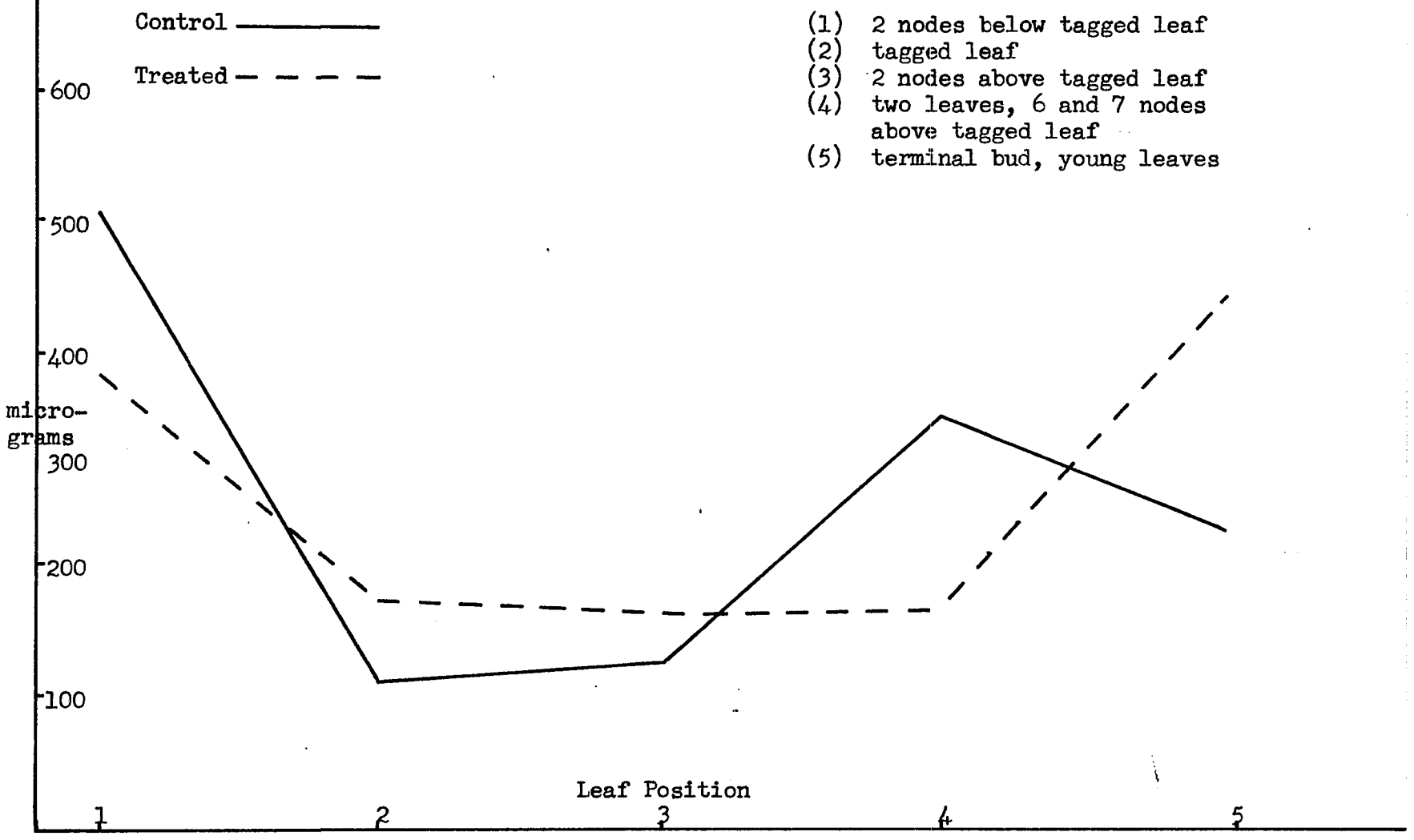
TABLE 7

RUTIN DISTRIBUTION IN THE CONTROL PLANT FOR GIBBERELLIC ACID TREATED TOBACCO PLANTS

| Position Number | Leaf Position | Weight of Leaf Tissue grams | Volume Extract ml. | Volume Streaked ml. | Optical Density | Rutin in Volume Streaked micrograms | Micrograms Rutin per Gram Leaf Tissue | % Rutin by Weight |
|---|---|---|---|---|---|---|---|---|
| 1 | 2 nodes below tagged leaf | 5.05 | 25 | 2 | 0.173 | 202 | 501 | 0.050 |
| 2 | tagged leaf | 6.04 | 25 | 2 | 0.028 | 58 | 120 | 0.012 |
| 3 | 2 nodes above tagged leaf | 7.59 | 25 | 2 | 0.052 | 83 | 135 | 0.014 |
| 4 | two leaves 6 and 7 nodes above tagged leaf | 6.61 | 25 | 2 | 0.148 | 176 | 333 | 0.033 |
| 5 | terminal bud and 8 small leaves | 3.38 | 25 | 3 | 0.071 | 98 | 240 | 0.024 |

FIGURE 7

RUTIN DISTRIBUTION IN GIBBERELLIC ACID TREATED TOBACCO PLANTS
Micrograms Rutin per Gram Fresh Leaf Tissue



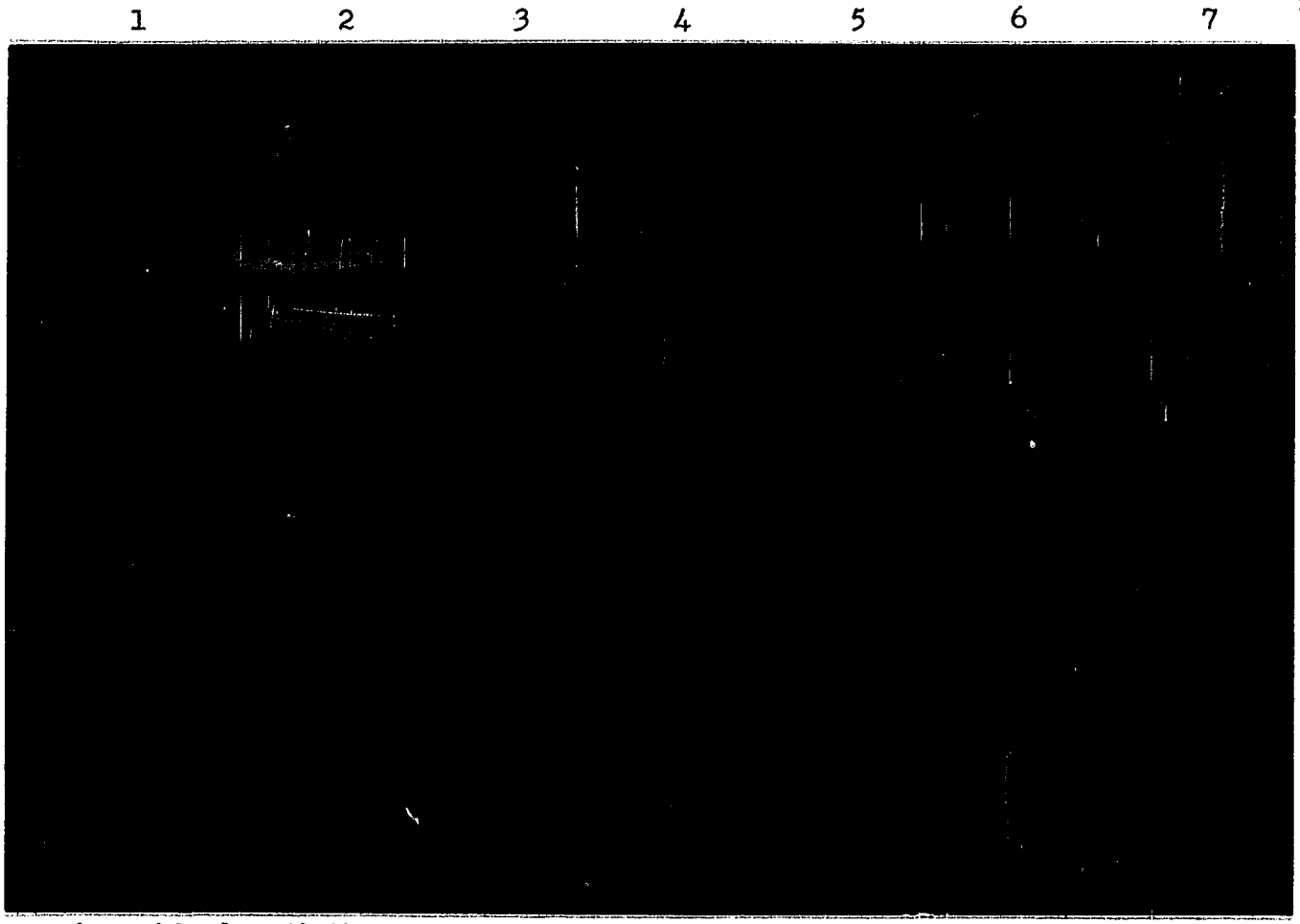
streaking each of the tobacco leaf extracts side by side in 3 inch long streaks across the top of a sheet of chromatographic paper in an order corresponding to their position on the tobacco stem. This chromatogram was developed in the 80% sec-butyl alcohol-water solvent system, dried and sprayed with 1% ethanolic aluminum chloride. Upon examination of the chromatogram under ultraviolet light, rutin appeared to have the same relative distribution as indicated by the quantitative data (Figure 8). Again the blue fluorescent group "X" appears only in the very young leaves and terminal bud of the control plant. The chlorogenic-like group seems to remain relatively constant in concentration from leaf position to leaf position on the tobacco stem.

For a qualitative portrayal of the effect on some of the flavonoid compounds in the tobacco plant by gibberellic acid treatment, each of the tobacco leaf extracts was spotted on a two-dimensional chromatogram. These extracts were the same extracts from which aliquots were taken for the quantitative rutin determinations in the leaf positions already described. The chromatograms were developed in the 80% sec-butyl alcohol-water and 15% tert-butyl alcohol-water solvent systems. After spraying with 1% ethanolic aluminum chloride, the chromatograms were observed under ultraviolet light and the flavonoid compounds outlined in pencil and their relative apparent concentrations noted (Table 8). For the purposes of comparison, each two-dimensional chromatogram was spotted with tobacco leaf extract equivalent to 0.3 grams fresh leaf weight.

Treatment with gibberellic acid seems to cause little or no qualitative differences in the flavonoid compounds, as identified in this laboratory, in the leaves of tobacco plants. Rutin, kaempferol-3-

FIGURE 8

THE DISTRIBUTION OF THE FLAVONOID COMPOUNDS IN THE CONTROL TOBACCO PLANT




1. Chlorogenic Acid 3. Sixth and Seventh Nodes Above Tagged Leaf 5. Tagged Leaf 7. Rutin

2. Terminal Bud and Young Leaves 4. Two Nodes Above Tagged Leaf 6. Two Nodes Below Tagged Leaf

rhamnoglucoside and scopoletin rhamnoglucoside follow a similar pattern of distribution as to leaf position on the tobacco stem as already noted above in the quantitative determination of rutin. The compound, scopoletin rhamnoglucoside, was first isolated from tobacco leaves and identified by Mr. C. H. Yang of this laboratory. Scopoletin has been reported in tobacco leaves as the aglycone by Best (23). However, in this study of green fresh leaf tissue only a trace of what might have been scopoletin was visible on some of the chromatograms. A possible explanation of this observation might be that the major part of the scopoletin, like quercetin and kaempferol, occurs as the rhamnoglucoside in tobacco leaves and the free scopoletin may have originated by hydrolysis of the glycoside in the isolation process.

A blue fluorescent compound, chlorogenic acid, has been reported in the dried leaves of tobacco, Pennsylvania Seed Filler, by Roberts and Wood (24). However, by the chromatographic techniques used in this laboratory at least 2 very similar blue fluorescent compounds have been isolated. Neither of these compounds as yet gives a clear cut identification with authentic chlorogenic acid from the commercial source, coffee beans. Hence this group will be referred to as chlorogenic acid-like. The concentration of the chlorogenic acid-like group and the methylated quercetin compounds apparantly remained relatively constant from leaf position to leaf position on the tobacco stem. The most notable qualitative difference in the flavonoid, or related compounds, possibly induced by the gibberellic acid treatment of the tobacco plant, is the appearance of an unidentified blue fluorescent group of compounds in the older base leaves of the tobacco plant. This group of blue fluorescent

TABLE 8

FLAVONOID COMPOUNDS IN GIBBERELLIC ACID TREATED TOBACCO PLANTS

| Leaf Position | | Flavonoid Compound Present | | | | | |
|---|---|---|---|---|---|---|---|
| | | Rutin | Kaempferol-3-rhamnoglucoside | Chlorogenic Acid-Like | Scopoletin Glycoside | Methylated Quercetin | Blue Fluorescent Group "X" |
| Control | | | | | | | |
| 1 | 2 nodes below tagged leaf | XXX | XX | XX | XX | X | 0 |
| 2 | tagged leaf | XX | X | XX | XX | X | 0 |
| 3 | 2 nodes above tagged | XX | X | XX | 0 | X | 0 |
| 4 | 6,7 nodes above tagged | XXX | XX | XX | XX | X | X |
| 5 | terminal bud | XX | X | XX | X | X | XXX |
| Gibberellic Acid Treated | | | | | | | |
| 1 | 2 nodes below tagged leaf | XXX | XX | XX | XX | X | XX |
| 2 | tagged leaf | XX | X | XX | X | X | X |
| 3 | 2 nodes above tagged | XX | X | XX | 0 | X | 0 |
| 4 | 6,7 nodes above tagged | XX | X | XX | X | X | XX |
| 5 | terminal bud | XXX | XX | XX | XX | X | XXX |

Relative Concentration XXX = Heavy XX = Intermediate X = Trace 0 = Not Visible

compounds, which we shall call group "X," had appeared near the origin of the two-dimensional chromatogram of only the younger leaves in the control plants (Figure 9). This observation has been confirmed by examination of the two-dimensional chromatograms of not only the control plants used in the x-ray and gibberellic acid studies, but also in a chromatographic study of the young leaves of tobacco plants grown under different temperatures now in progress at the Argonne National Laboratory. Until the present time, the physiological effect of gibberellic acid was thought to be expressed at and above the point of application of the gibberellic acid. However in a study of the pinto bean plant using radioactive gibberellic acid, Dr. N. J. Scully and Mr. Ronald Watanabe of the Argonne National Laboratory have obtained a radioautogram of a pinto bean plant treated with radioactive gibberellic acid in which the lower stem and roots of the bean plant, below the point of application, darkened the x-ray film. Of course, the darkening agent may not still be in the form of gibberellic acid. However, it is an interesting correlation with the observation that in the tobacco plants, a possible physiological effect of gibberellic acid treatment has also been expressed at a point below the site of application. The blue fluorescent group "X", which was normally associated with the young leaves in the control and treated plants, had now appeared in the older base leaves of the tobacco plant. The result of this investigation was encouraging enough to warrant its repetition using a larger plant population to reduce the possibility of individual plant variation. Further, the association of this blue fluorescent group "X" with the actively metabolizing younger leaves and its activity demonstrated in a tobacco plant placed under

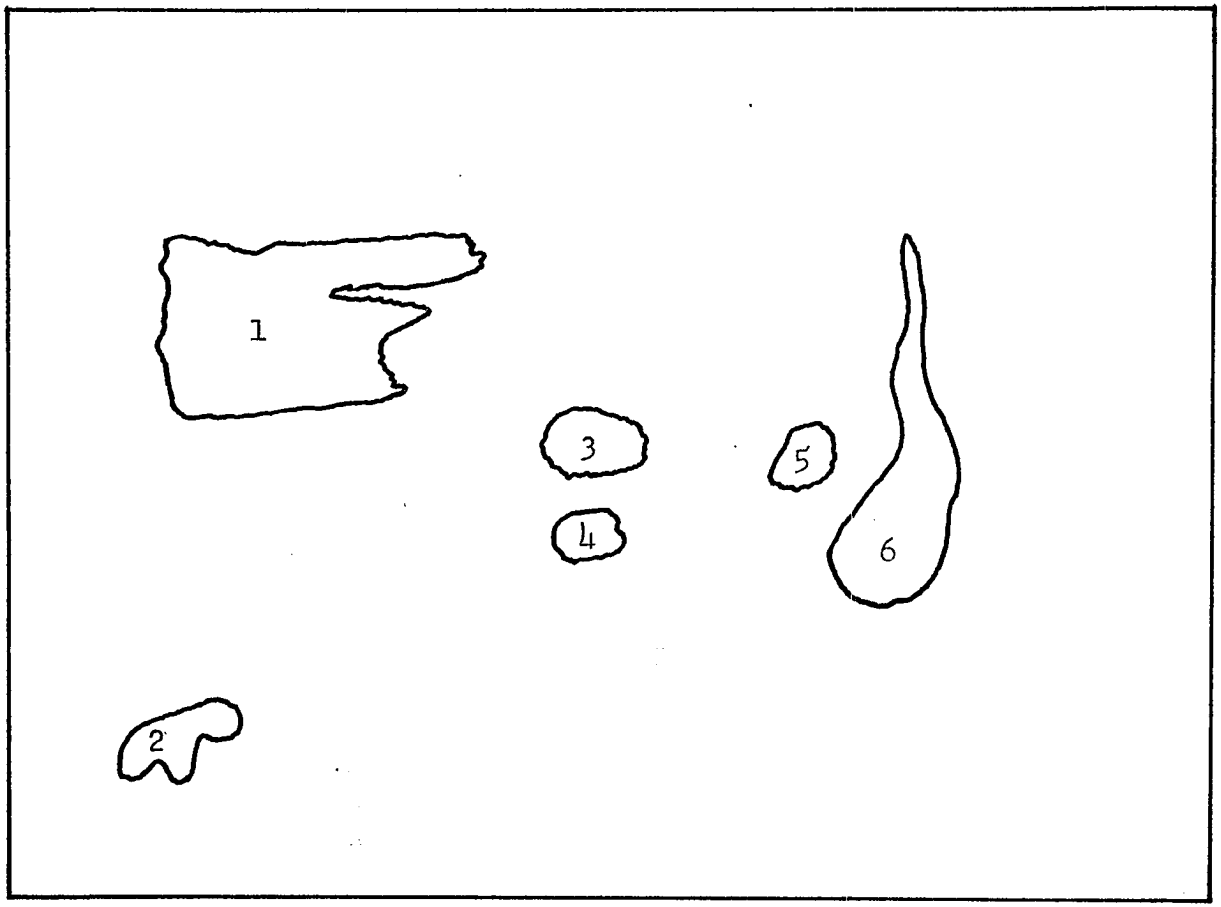

1
2
3
4
5
6

FIGURE 9

TWO-DIMENSIONAL CHROMATOGRAM OF TERMINAL BUD AND YOUNG LEAVES OF CONTROL TOBACCO PLANT



1. Blue Fluorescent Group "X"
2. Methylated Quercetin Group
3. Quercetin-3-Rhamnoglucoside
4. Kaempferol-3-Rhamnoglucoside
5. Scopoletin Rhamnoglucoside
6. Chlorogenic Acid-Like Group

a condition of stress by the gibberellic acid treatment, should justify further attempts at isolation and identification of one or more members of this group.

# CHAPTER VI

## THE QUANTITATIVE AND QUALITATIVE EFFECT OF X-RAY TREATMENT ON THE FLAVONOID COMPOUNDS OF TOBACCO

A group of eight young tobacco plants which had been grown for 65 days in soil culture under greenhouse conditions, were divided into four lots of two each. The experimental plan called for the tobacco plants to receive a variable dose of x-rays designed such that at least one plant would receive a lethal dose. The investigation could then be conducted on tobacco plants which had received a sublethal dose of x-rays. The pots containing the tobacco plants were set on previously calibrated isodose lines 22 and 27 inches from the x-ray window. The roots of all plants were shielded with a wall of lead bricks thus exposing the leaves and stems just at soil level. However, since the leaves of the tobacco plant are in rosette form and overlapped the edges of the pot, each leaf of the tobacco plant received a variable dose of x-rays. The dose to be received by each plant was calculated by placing the Victoreen r-Meter thimble at the terminal bud of the tobacco plant. In one lot, each of two plants received 54.5 roentgens per minute for 18 minutes and 20 seconds for a total x-ray dose of 5000 roentgens. In a third lot of two plants, each received 54.5 roentgens per minute for 183 minutes and 30 seconds for a total x-ray dose of 10,000 roentgens. After treatment, the tobacco plants were returned to the greenhouse.

After several days of observation, it became obvious that the different x-ray doses received by the three lots of tobacco plants were of such latitude as to produce three distinct levels of physiological response to the x-ray treatment. In the tobacco plants which received an x-ray dose of 1000 roentgens, the terminal bud continued to grow and elaborate leaves; but these leaves were more narrow than those of the control plants with mosaic surfaces and curled edges (Figure 10). In the plants which received an x-ray dose of 5000 roentgens, the terminal bud was severely stunted; but it continued to grow, producing very narrow strap leaves with mosaic surfaces. Lateral buds were released in this plant; but they failed to develop normally (Figure 11). The plants which received an x-ray dose of 10,000 roentgens produced rapidly developing laterals; but little or no further growth of the terminal bud (Figure 12). This varied level of response made comparison of the treated plants with the controls difficult at any level except 1000 roentgens; but a comparison of the major leaf groups such as base leaves, young leaves, or lateral leaves was deemed interesting enough to justify analysis of the plants at these other radiation levels.

The tobacco plants were harvested at the end of 31 days. Leaves for rutin analysis or chromatographic comparison were chosen by their position on the tobacco plant stem in a manner such that a comparison could be made between leaves in different stages of metabolic activity. Each leaf to be analyzed was cut from the tobacco stem, placed in a tared Soxhlet thimble and weighed. The thimble was dropped immediately into a Soxhlet extractor which had previously been brought to reflux temperature, and extracted with 85% isopropyl alcohol for a total of 8 hours. The

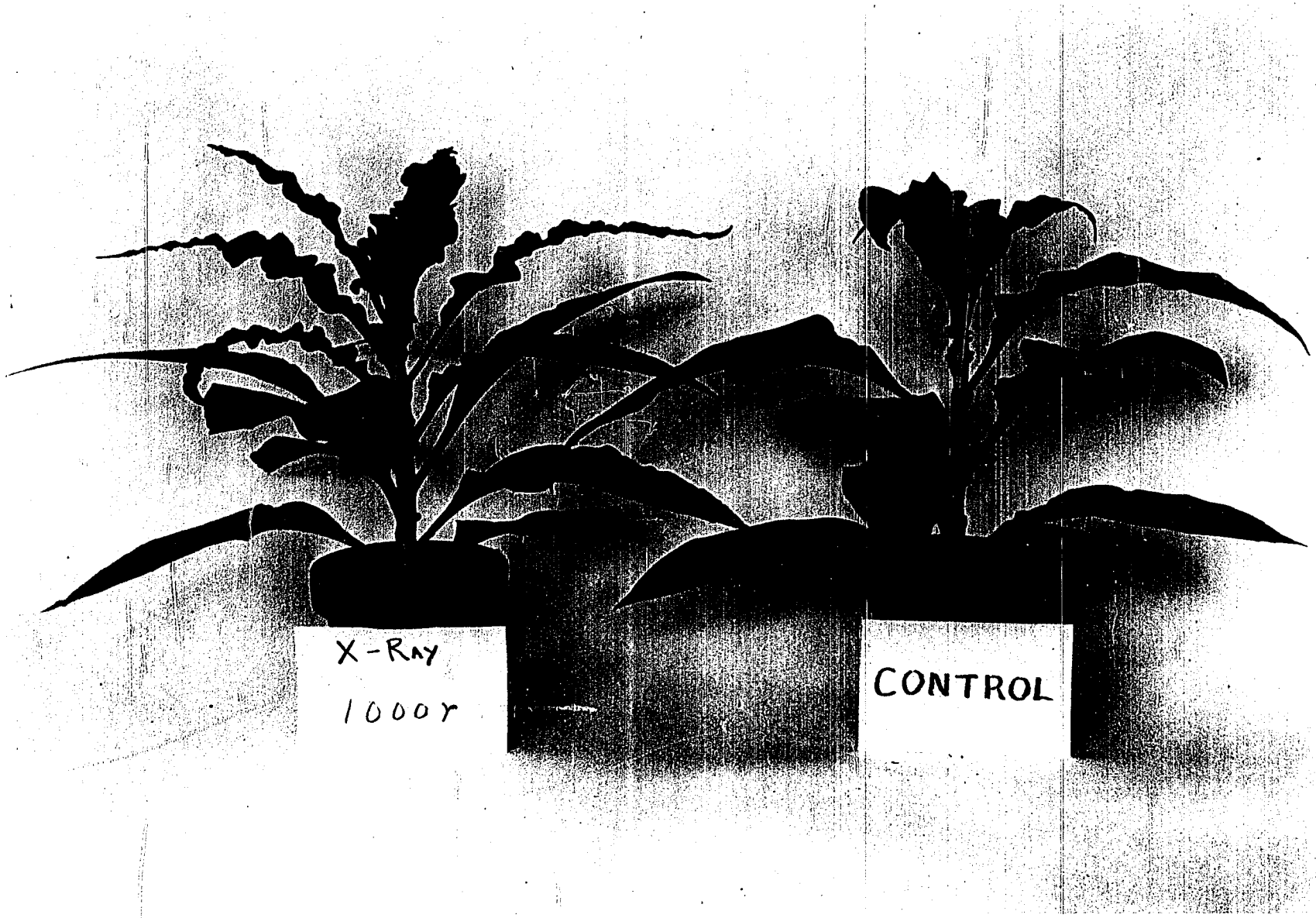



FIGURE 10. X-RAY TREATED TOBACCO PLANT, 1,000 ROENTGENS

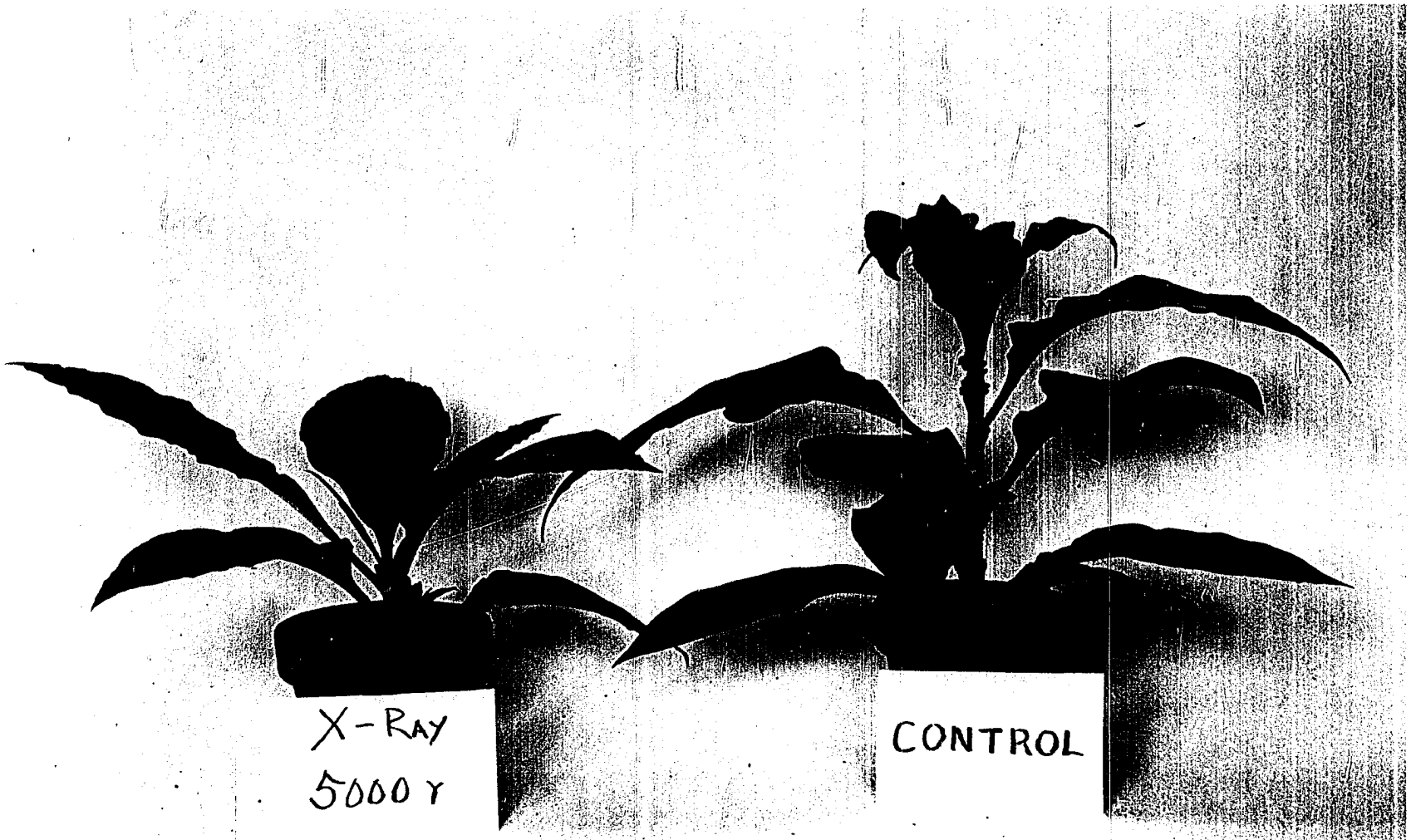



FIGURE 11. X-RAY TREATED TOBACCO PLANT, 5,000 ROENTGENS

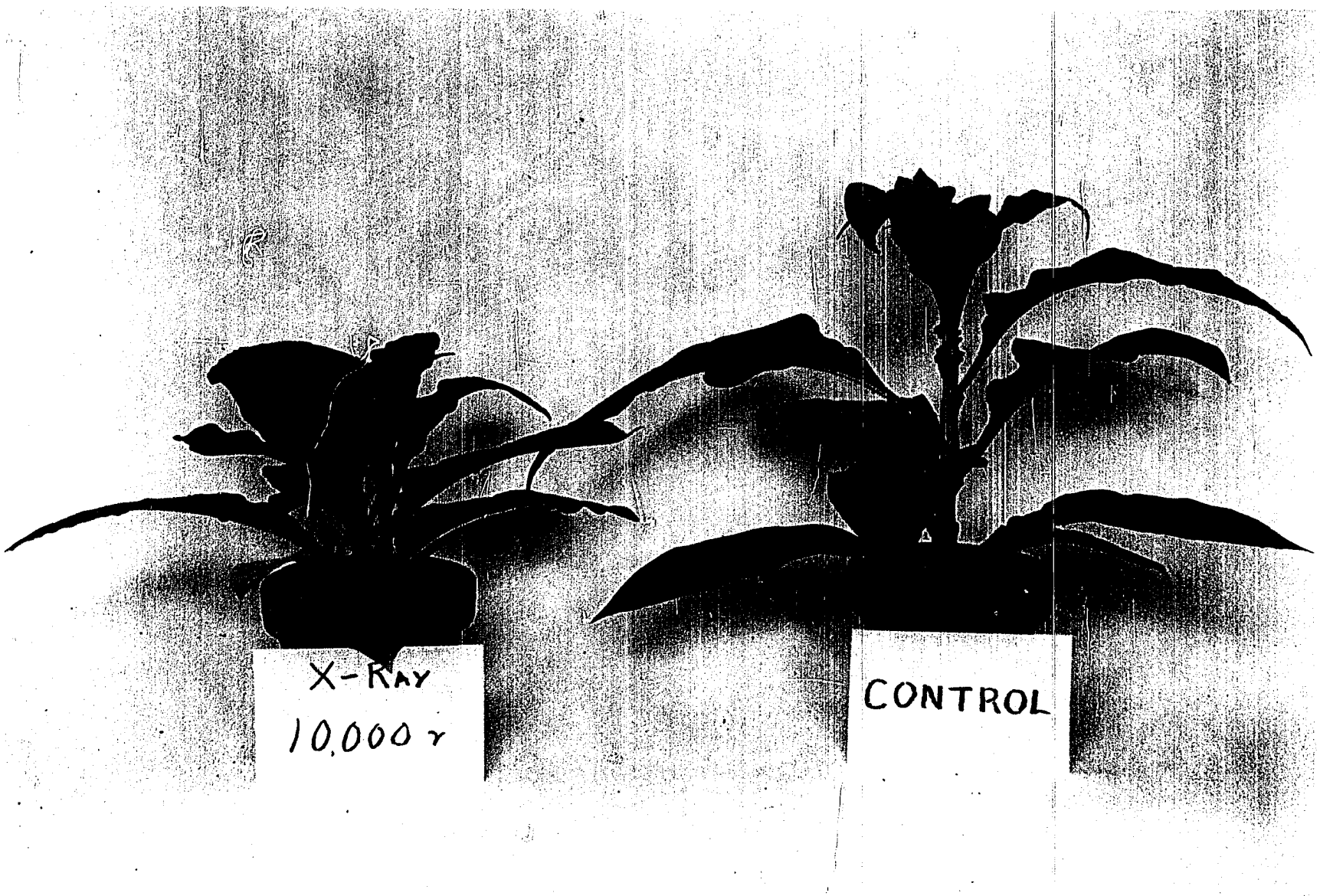



FIGURE 12. X-RAY TREATED TOBACCO PLANT, 10,000 ROENTGENS

alcoholic extract was reduced in volume; placed in a 25 milliliter volumetric flask and diluted to the mark with 95% isopropyl alcohol. The leaves chosen from the other positions on the tobacco plant were treated in a similar manner.

For the analysis of rutin in the various leaf positions, a measured aliquot of each tobacco leaf extract to be analyzed was streaked across 3 inches below the top of a sheet of chromatographic paper, Schleicher and Schuell, No. 589, red ribbon, 52 by 58 centimeters. This chromatogram was developed in the 80% sec-butyl alcohol-water solvent system until the solvent front had reached the bottom edge of the chromatogram. The rutin zone was cut out of the dried chromatogram and sewn with a sewing machine across the top of a new sheet of chromatographic paper. This chromatogram with the sewn rutin zone was then developed in the 15% tert-butyl alcohol solvent system. After spraying the dried chromatogram with 1% ethanolic aluminum chloride, the rutin zone was cut out, folded in accordion fashion and placed in an 8 inch pyrex test tube. The tube was placed in a hot water bath for 15 minutes, removed, and allowed to equilibrate for 2 hours until it reached room temperature. Each tobacco leaf extract to be analyzed for rutin was treated in a similar manner.

The optical density of these solutions of the aluminum-rutin complex was determined in a Beckman spectrophotometer at a wave length of 415 millimicrons. The rutin concentration of each solution was then obtained by comparing its optical density to a standard curve. The total rutin concentration was then calculated by the method outlined in Chapter IV for each tobacco leaf or group of leaves analyzed (Table 9).

A bar graph was constructed by plotting the rutin concentrations of the three leaf positions, base, young, and lateral leaves for the three different levels of x-ray treatment (Figure 13). A line graph was constructed by plotting the rutin concentration for the control and the tobacco plant treated with 1000 roentgens vs. the position of the leaf on the tobacco stem (Figure 14). The rutin concentration in the control plant exhibited a distribution similar to the control plant used in the gibberellic acid study (Figure 7). The low rutin concentration in the intermediate leaves, No. 3 (Figure 14), can possibly be correlated with the general disruption of their metabolism by the x-ray treatment, since all these leaves showed comparatively heavy damage. The intermediate leaves, No. 2 (Figure 14), showing only a slight mosiac leaf surface, have about the same rutin concentration as the corresponding leaves on the control plant. This suggests that the rutin concentration in these leaves is inversely proportional to the extent of the x-ray damage. However, this is evidently not the case in the young leaves. Here the rutin concentration falls, as expected in the young leaves of of the control plant, but rises in the young leaves of the tobacco plant receiving 1000 roentgens of x-ray. This may be another case in which flavonoid activity is demonstrated in an organism placed under stress. This result is repeated in the case of the tobacco plant treated with 10,000 roentgens of x-ray, but is contradicted in the case of the 5,000 roentgen treatment (Figure 13). It is, however, difficult to draw any comparison in the case of the 5,000 and 10,000 roentgen treated tobacco plants since they received a near lethal dose of x-rays as indicated by the stunting of the terminal bud and the release of the lateral buds.

TABLE 9

RUTIN DISTRIBUTION IN X-RAY TREATED TOBACCO PLANTS

| No. | Treatment Dose roentgen | Leaf Position | Leaf Weight grams | Volume Extract ml. | Volume Streaked ml. | Optical Density | Total Rutin micrograms | Micrograms Rutin per Gram Fresh Tissue | % Rutin by Weight |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Control | Base leaf nodes 4,5 | 9.49 | 25 | 4 | 1.270 | 1,300 | 856 | 0.086 |
| 2 | " | Intermediate nodes 6,7 | 6.08 | 25 | 7 | 0.708 | 746 | 438 | 0.044 |
| 3 | " | Intermediate nodes 8,9,10 | 9.81 | 25 | 3 | 1.040 | 1,038 | 882 | 0.088 |
| 4 | " | Young leaves nodes 14-21 | 1.38 | 25 | 15 | 0.173 | 201 | 243 | 0.024 |
| 1 | 1,000 | Base leaf nodes 4,5 | 6.89 | 25 | 5 | 0.792 | 830 | 602 | 0.060 |
| 2 | " | Intermediate nodes 6,7 | 2.98 | 25 | 10 | 0.412 | 438 | 368 | 0.037 |
| 3 | " | Intermediate nodes 8,9,10 | 5.47 | 25 | 7 | 0.368 | 394 | 257 | 0.026 |
| 4 | " | Terminal bud and nodes 14-21 | 2.20 | 25 | 10 | 0.471 | 495 | 563 | 0.056 |

TABLE 9 CONTINUED

| No. | Treatment Dose roentgen | Leaf Position | Leaf Weight grams | Volume Extract ml. | Volume Streaked ml. | Optical Density | Total Rutin micrograms | Micrograms Rutin per Gram Fresh Tissue | % Rutin by Weight |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 5,000 | Base Leaf node 4 | 9.53 | 25 | 5 | 0.278 | 304 | 159 | 0.016 |
| 2 | " | Intermediate node 5 | 5.17 | 25 | 7 | 0.097 | 126 | 87 | 0.009 |
| 3 | " | Terminal Bud | 2.87 | 25 | 10 | 0.222 | 250 | 44 | 0.004 |
| 4 | " | Lateral Away From Source | 5.11 | 25 | 7 | 0.422 | 448 | 313 | 0.031 |
| 5 | " | Lateral Toward Source | 8.39 | 25 | 5 | 0.348 | 375 | 223 | 0.022 |
| 1 | 10,000 | Base Leaf node 4 | 6.91 | 25 | 5 | 0.215 | 243 | 176 | 0.018 |
| 2 | " | Terminal Bud | 2.07 | 25 | 10 | 0.327 | 354 | 428 | 0.043 |
| 3 | " | Lateral | 6.75 | 25 | 5 | 0.328 | 355 | 263 | 0.026 |

FIGURE 13

RUTIN DISTRIBUTION IN X-RAY TREATED TOBACCO PLANTS

Micrograms Rutin per Gram Fresh Leaf Tissue

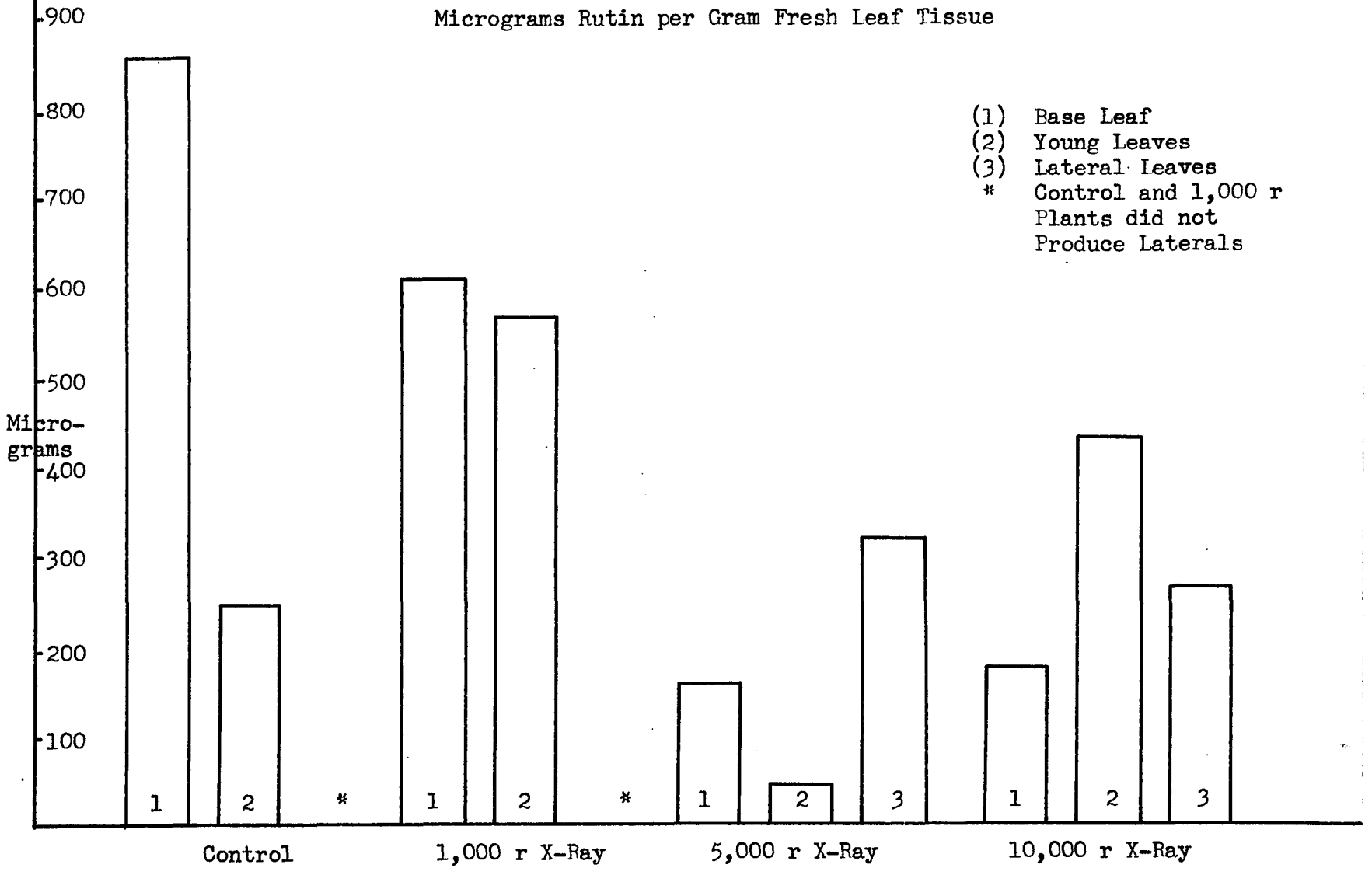
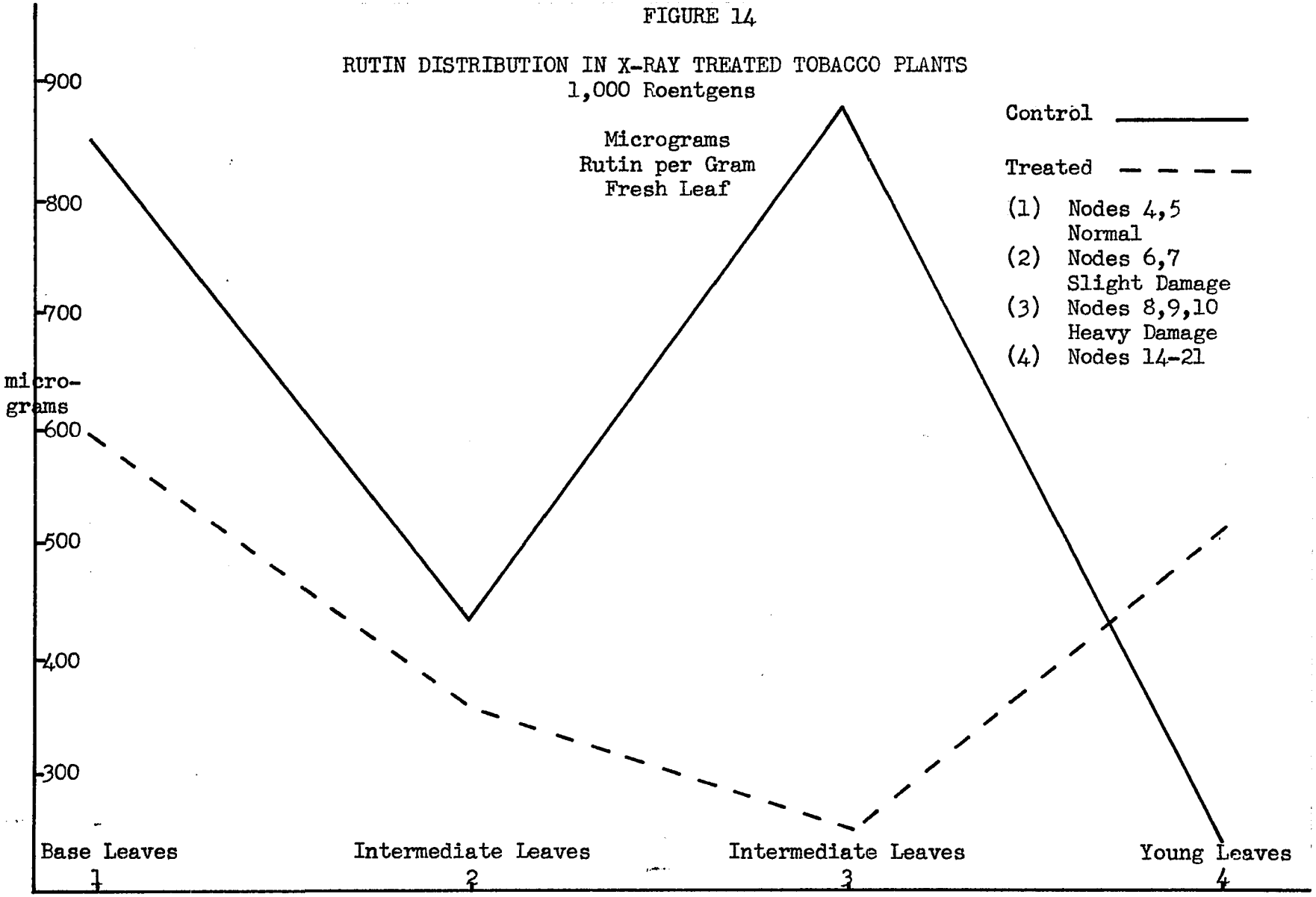


FIGURE 14

RUTIN DISTRIBUTION IN X-RAY TREATED TOBACCO PLANTS
1,000 Roentgens



For a qualitative comparison of the tobacco leaves from the various positions on the tobacco stem, an aliquot of each tobacco leaf extract, equivalent to 0.3 gram of fresh leaf tissue, was spotted on a two-dimensional chromatogram. This chromatogram was then developed in the 80% sec-butyl alcohol-water and 15% tert-butyl alcohol-water solvent systems. After spraying the dried chromatograms with 1% ethanolic aluminum chloride, these chromatograms were then observed under ultra-violet light and the fluorescent spots outlined with a pencil and an estimate made as to their relative concentration (Table 10). Apparently no notable qualitative changes are induced in the flavonoid compounds of tobacco leaves by x-ray treatment of the plant. No alteration of the distribution of the blue fluorescent group "X" occurred as in the case of gibberellic acid treatment. The occurrence and apparent concentration of the rhamnoglucosides of quercetin, kaempferol and scopoletin are not changed by x-ray treatment. However, other important quantitative and qualitative changes may have occurred as a result of the x-ray treatment which were too subtle to be noted by observation of a two-dimensional chromatogram of the crude plant extract. The extensive isolation and identification necessary to obtain more accurate results were beyond the scope of this investigation and especially were not justified by the small plant population treated.

TABLE 10

FLAVONOID COMPOUNDS IN X-RAY TREATED TOBACCO PLANTS

| No. | Treatment Dose roentgens | Leaf Position | Rutin | Kaempferol-3-rhamnoglucoside | Chlorogenic Acid-Like | Scopoletin Glycoside | Methylated Quercetin | Blue Fluorescent Group "X" |
|---|---|---|---|---|---|---|---|---|
| 1 | Control | Base Leaf | XXX | XX | XX | XX | X | 0 |
| 2 | " | Intermediate | XXX | XX | XX | XX | X | X |
| 3 | " | Intermediate | XXX | XX | XX | XX | X | X |
| 4 | " | Young Leaves | XX | X | XX | XX | X | XX |
| 1 | 1,000 | Base Leaf | XXX | XX | XX | XX | X | 0 |
| 2 | " | Intermediate | XX | X | XX | XX | X | 0 |
| 3 | " | Intermediate | XX | X | XX | XX | X | X |
| 4 | " | Young Leaves | XXX | XX | XX | XX | X | XX |
| 1 | 5,000 | Base Leaf | XX | X | XX | XX | X | 0 |
| 2 | " | Intermediate | X | X | XX | XX | X | X |
| 3 | " | Young Leaves | X | X | XX | XX | X | XX |
| 4 | " | Lateral | XX | X | XX | XX | X | XX |
| 5 | " | Lateral | XX | X | XX | XX | X | XX |
| 1 | 10,000 | Base Leaf | XX | X | XX | XX | X | 0 |
| 2 | " | Young Leaves | XX | X | XX | XX | X | XX |
| 3 | " | Lateral | XX | X | XX | XX | X | XX |

Relative Concentration XXX - Heavy XX - Intermediate X - Trace 0 - Not visible

## CHAPTER VII

## THE DISTRIBUTION OF THE FLAVONOID COMPOUNDS IN THE PARTS OF THE TOBACCO FLOWER

In order to determine if any difference exists in the parts of the tobacco flower as to the flavonoid compounds present, 126 fully open tobacco flowers which had just reached full color development were harvested. The flowers were sectioned with a razor blade into the following parts: the corolla, which included corolla lobes and tube; the stamen, which included the anther and filament; the pistil, which included the stigma, style and ovary; and the calyx with the calyx lobes. Each of the four flower parts was processed separately. As the flower parts were sectioned, they were dropped immediately into 200 milliliters of boiling 85% isopropyl alcohol to inactivate the enzyme systems and initiate the extraction process. After 1 hour of extraction, the solids were filtered off, and the alcoholic extract was reduced to 25 milliliters in volume.

A two-dimensional chromatogram was prepared for each of the 4 flower parts by spotting an aliquot of the reduced alcoholic extract in one corner of a sheet of chromatographic paper, Schleicher and Schuell, No. 589, blue ribbon, 58 by 58 centimeters. These 4 chromatograms were then developed in 80% sec-butyl alcohol-water and 15% tert-butyl alcohol-water solvent systems. After drying, the chromatograms were sprayed

with 1% ethanolic aluminum chloride and observed under ultraviolet light. The fluorescent spots were outlined in pencil and tentatively identified, in those cases where a spot agreed with the fluorescent color and position on the chromatogram with that previously established for an authentic flavonoid compound. A comparison of the two-dimensional chromatograms of the 4 parts of the tobacco flowers was made. The presence or absence of a flavonoid compound was noted as well as its apparent relative concentration (Table 11).

The corolla seemed to contain most of the flavonol glycosides, rutin and kaempferol-3-rhamnoglucoside, found in the tobacco flower, with traces of both of these glycosides occurring in the calyx and stamen. A qualitative difference is noticeable with the pistil in that these flavonol glycosides appear to be absent on the two-dimensional chromatogram of the pistil. Furthermore, the pistil contains principally compounds with with a blue fluorescence, of which, the chlorogenic acid-like compounds, do not appear on the two-dimensional chromatogram of the stamen.

As a further comparison of the distribution of the flavonoid compounds, a chromatogram was prepared by streaking each extract of the tobacco flower parts side by side across the top of a sheet of chromatographic paper. The chromatogram was developed in the 80% sec-butyl alcohol-water solvent system, dried and sprayed with 1% ethanolic aluminum chloride. When observed under ultraviolet light, the chromatogram (Figure 15) showed a distribution similar to that indicated by the two-dimensional chromatograms.

This association of the flavonol glycosides with the male part

TABLE 11

THE DISTRIBUTION OF THE FLAVONOID COMPOUNDS IN THE TOBACCO FLOWER

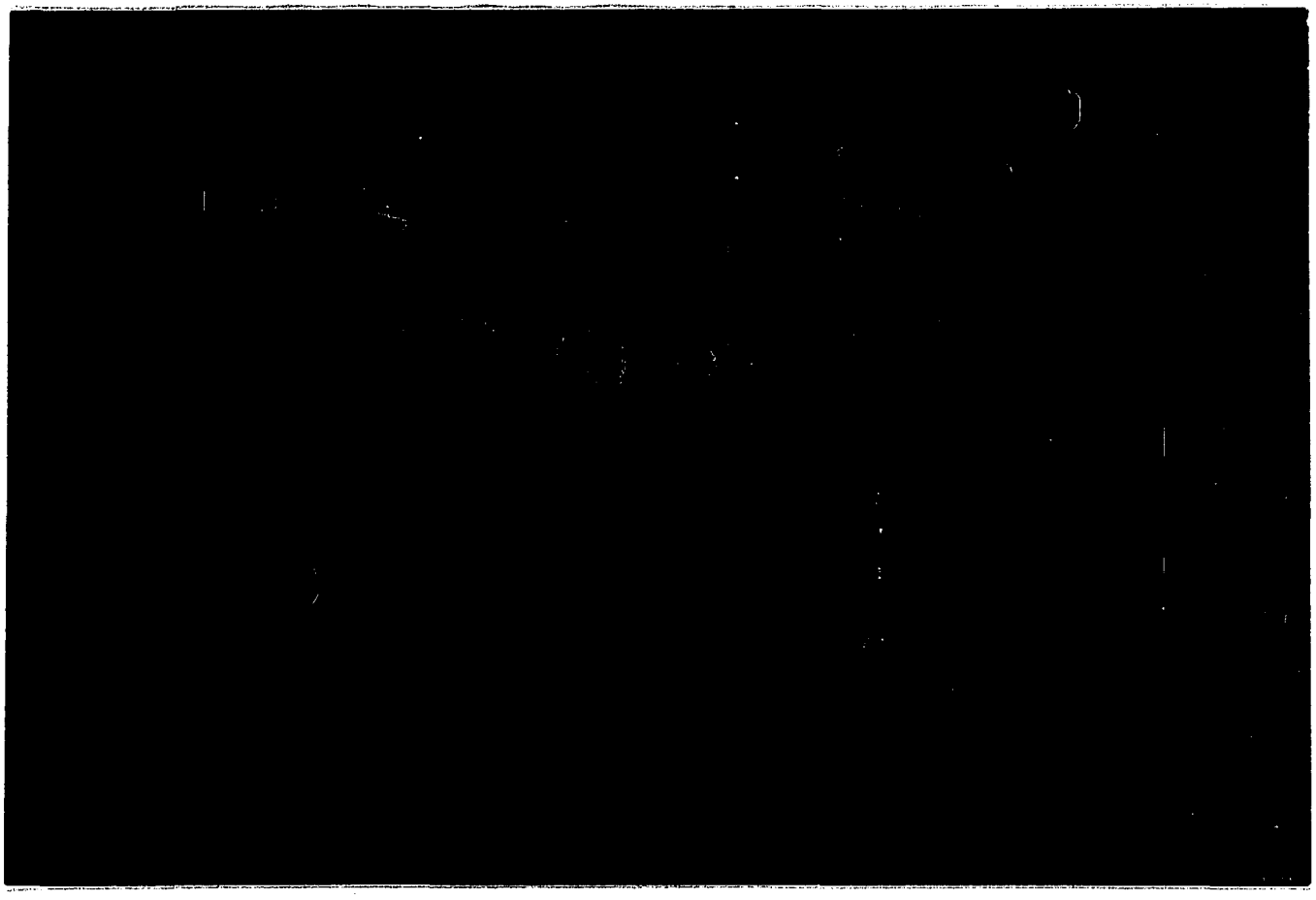
| Flavonoid Compound | Calyx | Corolla | Stamen | Pistil |
|---|---|---|---|---|
| Rutin | XX | XXX | X | 0 |
| Kaempferol-3-rhamnoglucoside | XX | XXX | X | 0 |
| Scopoletin Rhamnoglucoside | -- | -- | 0 | X |
| Chlorogenic Acid-Like Compounds | -- | -- | 0 | X |
| Blue Fluorescent Group "X" | XX | XX | XX | XX |

Relative Concentration XXX=Heavy XX=Intermediate X=Trace 0=Not Visible --=Not Determined

FIGURE 15

THE DISTRIBUTION OF THE FLAVONOID COMPOUNDS IN THE PARTS OF THE TOBACCO FLOWER

| Chlorogenic Acid | Pistil | Stamen | Corolla | Calyx | Kaempferol Glycoside | Rutin |
|---|---|---|---|---|---|---|



and the related blue fluorescent compounds with the female part of the tobacco flower, immediately suggests that the flavonol glycosides play some role in the reproductive process. Kuhn and Low (25) have reported that the inability of two varieties of Forsythia to cross-pollinate is associated with the presence of rutin in the pollen of one and quercitrin (quercetin-3-rhamnoside) in the other. Moewus (26) has suggested a role for peonin and isorhamnetin-3,4-diglucoside in the sexual processes of the green alga, Chlamydomonas eugametos.

In further investigations to follow the development of the tobacco flower pistil through the stages of tobacco fruit, seed and seedling, the flavonol glycosides, rutin and kaempferol-3-rhamnoglucoside, were not resolved again by chromatographic techniques until the tobacco seedling had entered a period of rapid development 39 days after the tobacco seed was sown. Prior to this point, the blue fluorescent compounds seemed to predominate. For example, a two-dimensional chromatogram of the alcoholic extract of the immature tobacco seed scraped from the tobacco fruit, showed only blue fluorescent compounds, principally the chlorogenic acid-like compounds. The flavonol glycosides were either absent or in a concentration not detectable by the chromatographic methods used. In the case of rutin this was 0.25 micrograms.

## SUMMARY

A paper chromatographic technique was developed for the isolation and identification of flavonoid compounds from the dried leaves of one-sucker tobacco, Nicotiana tabacum. The following flavonoid compounds were isolated in chromatographically pure form and identified as: rutin, isoquercitrin, kaempferol-3-rhamnoglucoside, quercetin, and kaempferol. In addition, two different methyl ethers of quercetin were isolated which have never before been reported in a natural product. These two methyl ethers have been characterized by color tests, chromatographic $R_f$ values, ultraviolet absorption spectra as well as methylation and demethylation reactions. A tentative structure has been proposed for these methyl ethers: quercetin-3-methyl ether and quercetin-3,7-dimethyl ether.

The paper chromatographic technique of isolation has been applied to tobacco plants grown at the Argonne National Laboratory in an atmosphere of radioactive carbon dioxide and the following radioactive flavonoid compounds were isolated in chromatographically pure form: rutin, kaempferol-3-rhamnoglucoside, quercetin-3-methyl ether and quercetin-3,7-dimethyl ether. A quantitative determination was made on the radioactive rutin, and the specific activity was calculated.

A method for the quantitative determination of rutin in fresh tobacco leaf tissue was developed utilizing chromatographic techniques

and ultraviolet spectrophotometry. This quantitative method was then used to measure the effect of gibberellic acid treatment on the rutin content of young tobacco plants. A significant increase occurred in the rutin content of the young leaves and terminal bud of the treated plant as compared to a control plant. This suggests that flavonoid activity in these tissues was possibly induced by the gibberellic acid treatment. An application of this quantitative method to young tobacco plants treated with a sublethal dose of x-rays also shows a significant increase in the rutin content of the young leaves and terminal bud of the treated plant as compared to a control plant.

The parts of the tobacco flower were examined chromatographically and the male part was found to contain the brown fluorescent flavonol glycosides, rutin and kaempferol-3-rhamnoglucoside, whereas the female part of the flower was shown to contain predominantly blue fluorescent compounds.

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