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degree of

DOCTOR OF PHILOSOPHY

BY

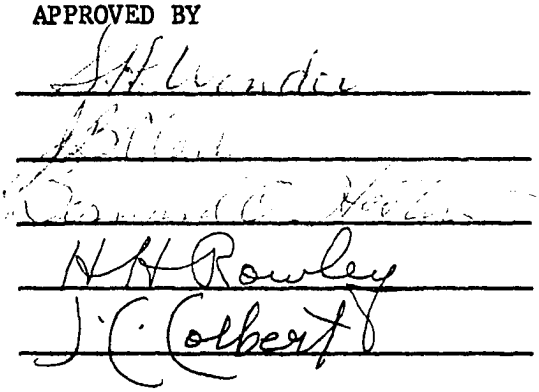
HUGH DOUGLAS BRAYMER

Norman, Oklahoma

1960

THE METABOLIC FATE OF SOME PHENOLIC COMPOUNDS IN THE RAT

APPROVED BY



DISSERTATION COMMITTEE

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CHAPTER I

INTRODUCTION

Coumarin and flavonoid compounds are widely distributed in nature. The consequent intake of certain of these compounds in foods, cigarette smoke (1), and some pharmaceuticals leads to an interest in the metabolism of these compounds.

Many investigators have concerned themselves with the metabolism of flavonoid compounds and with their physiological properties; many different properties ranging from radiation protection to birth control (2) have been attributed to them.

Reports on the metabolism of flavonoid compounds often have been contradictory. Garino (3) and Fukuda (4) have reported that these compounds are excreted unchanged in the urine. In contrast, Murray et al. (5) and Petrakis et al. (6) have reported some of the breakdown products found in the urine of animals given flavonoids.

The metabolism of quercetin is particularly interesting because no one has been able to account for what happens to the portion of the molecule that contains the phloroglucinol nucleus. Kallianos (7) reported the presence of radioactive phloroglucinol and phloroglucinol carboxylic acid in the animal stomach after the animal had

been given random labeled quercetin by stomach tube. On the other hand, Masri et al. (8) stated these compounds were products of the procedure rather than of the animal.

The only other report of finding a part of the phloroglucinol nucleus from a flavonoid compound was by Watanabe (9) in Japan. In that case, rabbits were given (/) catechin, and δ-(3, 4-dihydroxyphenyl)-γ-valerolactone was isolated from the urine and identified. Watanabe also postulated a metabolic path by which the molecule could be produced.

One of the primary purposes of this research has been to help clarify the confusion regarding the metabolism of quercetin. It was hoped that this clarification could be aided by identifying the quercetin breakdown products in the urine. This research also made use of a synthetic diet which should help clarify previous studies such as those of Murray et al. which used a natural diet. While the synthetic diet is almost devoid of phenolic-type compounds, the natural diet has considerable amounts of these compounds which appear in the urine even of the control animals.

Another endeavor of this research has been to expand the studies of Kallianos (10) on the effect of gastric mucin preparations. It was hoped that the additional studies would prove whether the breakdown was enzymatic as well as whether the breakdown was operative in the live animal.

Of related interest to the research for this paper was the identification of the quercetin methyl ethers isolated from tobacco. Dunlap (11) had found compounds similar to the quercetin methyl ethers

in commercial rutin, and commercial rutin has been utilized by many researchers in physiological experiments. Also, the identification was of interest because the covering of certain of the hydroxyl groups of quercetin appears to modify the metabolism of these compounds in the animal.

Relatively little research has been performed previously on the urinary metabolites of coumarins. Thus, one of the primary aims of this author's work has been to identify the urinary metabolites of esculetin, esculin, and scopoletin. This includes the metabolites that are present only in small amounts. Williams has studied the formation of glucuronides and sulfates of esculin, coumarins, and some hydroxy coumarins (12), (13), (14); however, it is hoped that the research reported in this paper will help clarify the metabolic fate of the hydroxy substituted coumarin compounds.

CHAPTER II

THE ISOLATION AND IDENTIFICATION OF THE URINARY METABOLITES OF SCOPOLETIN IN THE ALBINO RAT

Synthesis of Scopoletin (7-hydroxy-6-methoxycoumarin)

To 25 g. of esculin dissolved in 500 ml. of absolute ethanol were added 12.8 g. of sodium bicarbonate and 13.0 g. of benzyl chloride. After the reaction mixture had been refluxed for 48 hours on a steam bath, the solution was allowed to cool, and then one liter of water was added. The resulting precipitate was filtered and dried, melting point 182° C. (15). The yield for this step was not calculated because of the difficulty encountered in drying the precipitate completely.

The 7-benzyl esculin (30 g.) was then hydrolyzed using 500 ml. of 7% sulfuric acid-water with heating on a steam bath for six hours. The hydrolysate was then cooled in the refrigerator (4° C.). The resulting white precipitate was recrystallized by solution in methanol and addition of water until cloudiness resulted. Precipitation was completed with cooling. The resulting white precipitate, after drying, melted at 192° C. The melting point reported in the literature for 7-benzyl esculetin is 192-193° C. (16). The overall yield for the first two steps in the synthesis is 72%.

The 7-benzyl esculetin (14.2 g.) was dissolved in 300 ml.

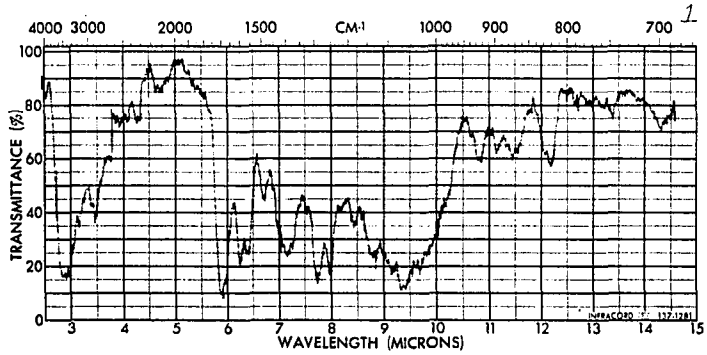
of dry acetone, and 21.8 g. of anhydrous potassium carbonate and 14.6 g. of dimethyl sulfate were then added. After the reaction mixture was refluxed for eight hours on a steam bath, it was filtered to remove the inorganic salts, and then the filtrate was evaporated at room temperature until only 25 ml. remained. Water was added until the 7-benzyl-6-methyl ether of esculetin precipitated out. The precipitate was filtered, and then recrystallized from a methanol-water mixture. The crystals, when dry, gave a melting point of 123° C. (literature, 123° C. [16]). The yield for this step was 92%.

The 7-benzyl-6-methyl esculetin ether (13.8 g.) was dissolved in 150 ml. of glacial acetic acid plus 75 ml. of concentrated hydrochloric acid. After this debenzylation mixture was heated for four hours on a steam bath, the solvent was removed at reduced pressure. The resulting precipitate was dissolved in methanol and decolorized with charcoal; the solution was allowed to evaporate slowly to produce light tan crystals. The melting point of these crystals was 205° C. compared to literature values of scopoletin ranging from 204° C. (16) to 207° C. (17). The compound proved identical with authentic scopoletin by paper chromatographic comparison. Infrared absorption curves of the synthetic scopoletin and intermediates in its synthesis are in Figure 1.

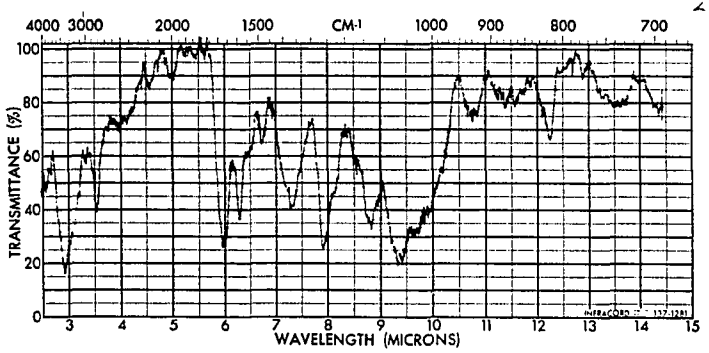
The paper chromatogram of this scopoletin preparation, when developed in butanol-ethanol-ammonium carbonate buffer (40:11:19 v/v) (18), revealed the presence of trace amounts of two other blue fluorescing compounds. Because the scopoletin was to be used for metabolism studies, it was necessary to eliminate these trace materials. Recrystallizations from several solvents were tried without success.

FIGURE 1

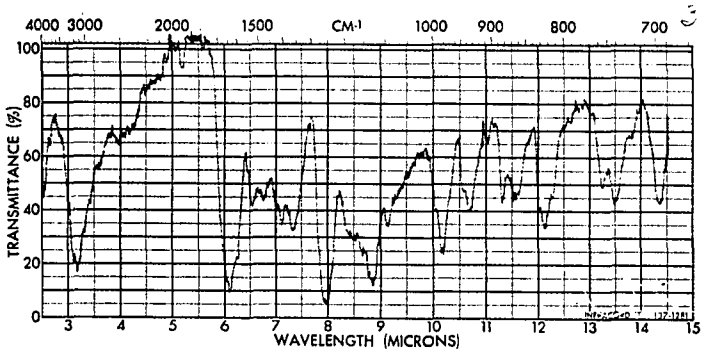
INFRARED CURVES OF SCOPOLETIN AND OF THE INTERMEDIATES IN ITS SYNTHESIS



Esculin



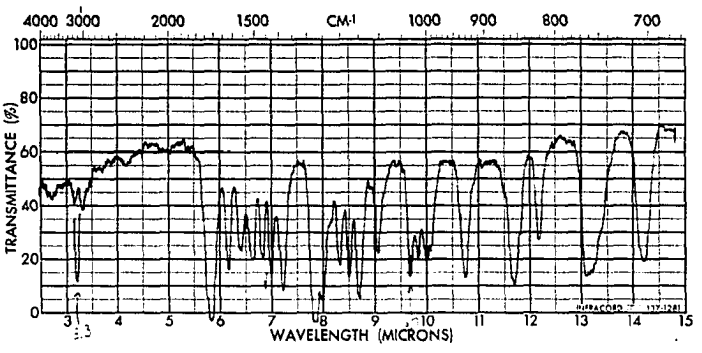
7-Benzyl Ether of Esculin



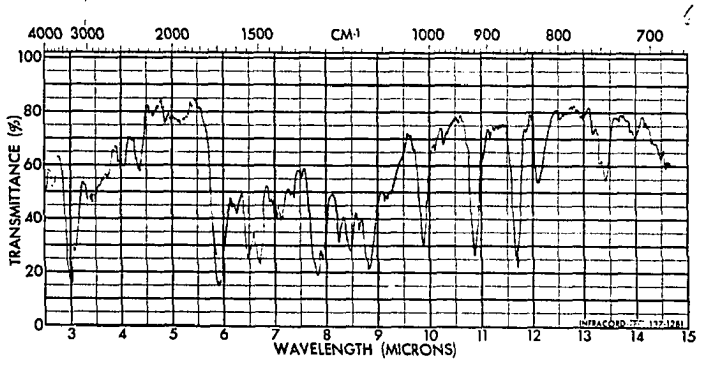
7-Benzyl Ether of Esculetin

FIGURE 1 (Continued)

INFRARED CURVES OF SCOPOLETIN AND OF THE INTERMEDIATES IN ITS SYNTHESIS



7-Benzyl Ether of Scopoletin



Scopoletin

The method that was found to work successfully was to dissolve the scopoletin in the least possible amount of a saturated solution of sodium carbonate. The solution was then extracted several times with chloroform (100 ml. each) and then slowly acidified with hydrochloric acid. Acidification brought about the precipitation of fine white needles of scopoletin. These crystals did not contain any blue fluorescing substances other than scopoletin when examined by paper chromatography in the aforementioned solvent system. The yield for the debenzylation step was 82%.

The overall yield for the complete synthesis was 55%. The method of Seshadri (19) utilizes a slightly different approach for the synthesis of scopoletin; it necessitates separation of isomers and gives a reported yield of only 26%. In our laboratory, however, the yield by the Seshadri procedure was considerably less than 26%.

Direct methylation of esculetin and a total synthesis starting with guaiacol (o-methoxy phenol) were also attempted, but both methods were found to be unsatisfactory for synthesizing scopoletin.

Isolation and Identification of Urinary Metabolites of Albino Rats Fed Scopoletin

Three female albino rats (Sprague-Dawley), weighing 250-300 g., were fed a vitamin B complete test diet (Nutritional Biochemicals Corporation) for a period of three weeks. The urine of these rats was collected under toluene and then spotted on S & S #589 red ribbon chromatographic paper (11 1/2" x 12"). Each was then developed in the first direction in chloroform-acetic acid-water (2:1:1 v/v, lower layer) and then in the second direction in 20% potassium chloride-water. The paper

chromatogram was then observed under long wavelength ultraviolet light and found to be free of blue fluorescing compounds.

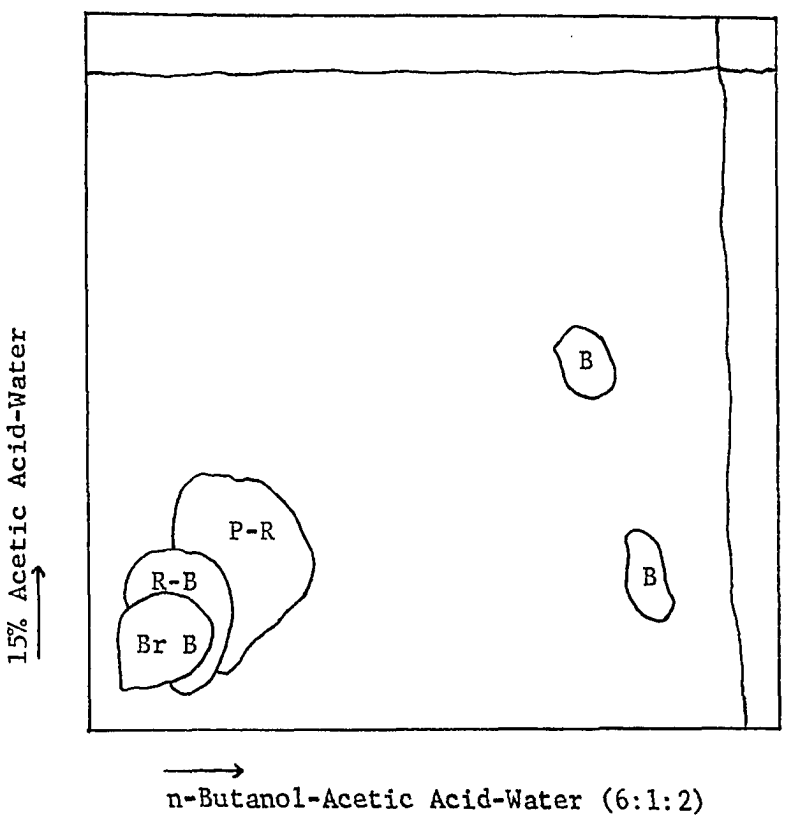
The same animals were then fed the same diet as previously, but this time 1% scopoletin had been added to it. The urine from the rats to which scopoletin had been fed was treated in the same manner as the control urine. When the chromatogram of the urine from the rats was checked under ultraviolet light, it was found to contain at least five spots that fluoresced blue (Figure 2).

The chromatographic separation of these fluorescent compounds was accomplished by streaking the urine on S & S #589 red ribbon chromatography paper (7 1/2" x 21") and then developing the chromatogram in n-butanol-pyridine-benzene-water (5:3:1:3 v/v, upper layer). Five distinct zones resulted, as shown in Figure 3. Zone 1 fluoresced bright blue under ultraviolet light and its color was enhanced slightly by ammonia vapor. Zone 2 was not visible under ultraviolet light but gave a red color when sprayed with diazotized sulfanilic acid (20). Zone 3 fluoresced blue under ultraviolet light and appeared to be composed of two zones moving with almost the same R_f values. Zone 4 fluoresced pale yellow under ultraviolet light. Zone 5 was bright blue fluorescent under ultraviolet light, and its color was enhanced by ammonia.

Zone 1 was further purified by cutting the zone out of 12 chromatograms. These were then separately sewn onto clean sheets of S & S #589 red ribbon chromatography paper and developed again in n-butanol-acetic acid-water (6:1:2 v/v). The desired zone was then cut out and eluted with 75% ethanol-water. The combined eluates were

FIGURE 2

TWO-DIMENSIONAL CHROMATOGRAM OF URINE FROM RAT FED DIET CONTAINING 1% SCOPOLETIN



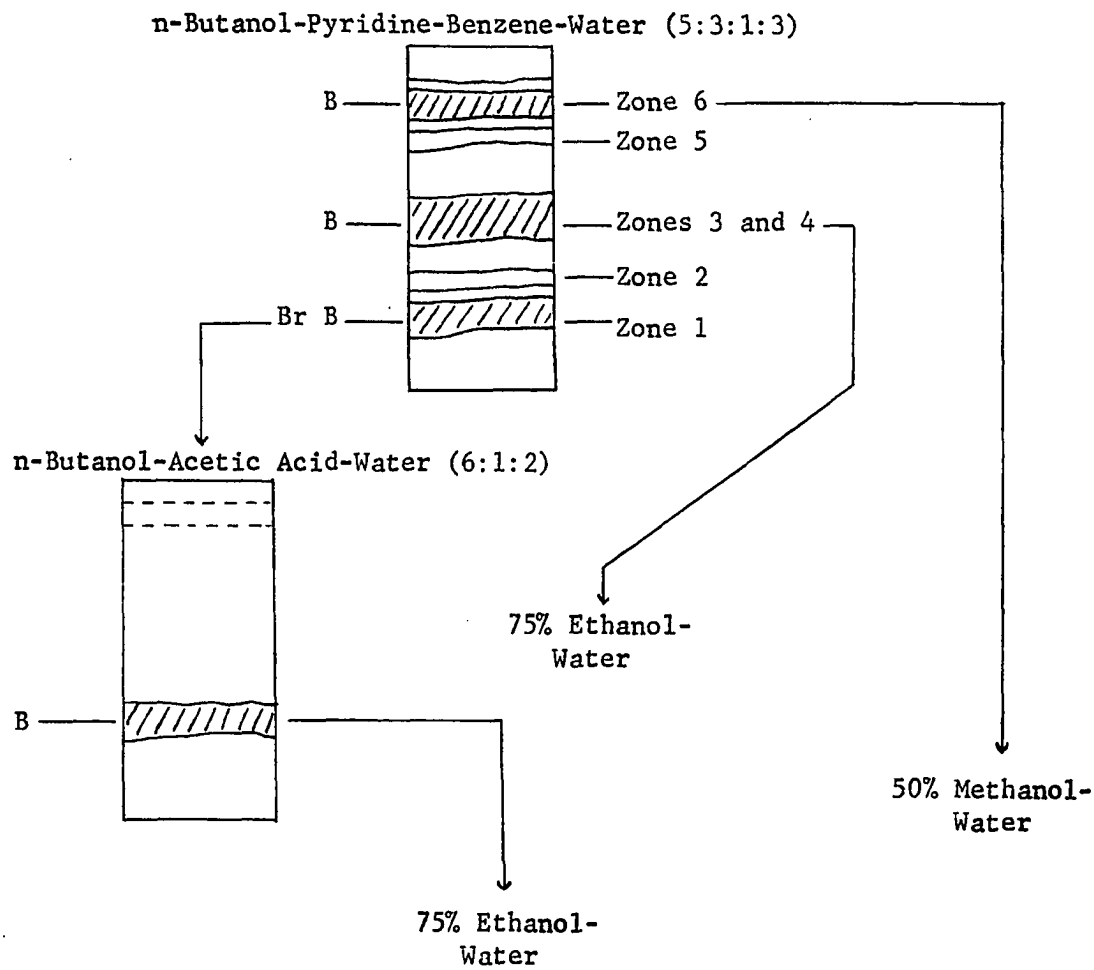
Colors Above Are in Ultraviolet Light

Br - Bright
B - Blue
R - Rose
P - Purple

FIGURE 3

CHROMATOGRAPHIC SEPARATION OF THE URINE OF RAT FED SCOPOLETIN

(Colors Are in Ultraviolet Light)



B - Blue
Br - Bright

reduced in volume *in vacuo*. This concentrate was then compared by ascending chromatography in five different solvent systems with authentic scopoletin and found to be identical.

A procedure which would completely separate the two zones present in zone 3 was not found. The zone was therefore cut out and eluted with the 75% ethanol-water. The eluate was reduced in volume (10 ml.) and then hydrolyzed with 6N hydrochloric acid (100 ml.) at room temperature for several hours. The hydrolysate was extracted twice with 25 ml. portions of water-saturated ethyl acetate. The organic extracts were then concentrated to approximately 2 ml. and spotted on six sheets of S & S #589 red ribbon chromatography paper along with standards of scopoletin and esculetin (6, 7-dihydroxycoumarin). The papers were next developed in six different solvent systems: chloroform-acetic acid-water (2:1:1 v/v, lower layer), n-butanol-acetic acid-water (6:1:2 v/v), 15% acetic acid-water, 20% potassium chloride-water, n-butanol-pyridine-dioxane-water (70:20:5:5 v/v), and benzene-propionic acid-water (2:1:1 v/v, upper layer). The hydrolyzed zone 3 was found to contain both scopoletin and esculetin (Table 1). When a paper containing the original zone 3 was developed in n-butanol-pyridine-benzene-water (5:3:1:3 v/v, organic phase) and then sprayed, first with 3N hydrochloric acid and secondly with a solution of 100 mg. barium chloride in 50 ml. of 70% ethanol, followed by a solution of sodium rhodizonate (10 mg. in 20 ml. of 50% ethanol), the top part of the zone gave the positive test for sulfate (21). The lower portion of the zone gave a positive test with p-dimethylaminobenzaldehyde, a reagent for hippuric acid derivatives (22). Zone 3 would appear to be

TABLE 1

R_f VALUES OF COMPOUNDS ISOLATED FROM URINE OF ANIMALS FED SCOPOLETIN

Compound	20% Potassium Chloride-Water	Chloroform-Acetic Acid-Water (2:1:1)	n-Butanol-Acetic Acid-Water (6:1:2)	15% Acetic Acid-Water
Scopoletin	0.14	0.89	0.92	0.53
Esculetin	0.26	0.33	0.88	---
Zone 6	0.30	0.01	0.09	0.20
Zone 3	0.26	0.03	0.11	---

a mixture of scopoletin and esculetin sulfates. However, the positive test for hippuric acid indicates that the scopoletin or esculetin may also be conjugated with glycine. The other possibility is that one of the coumarins may be conjugated with sulfate and the other with glycine.

Zone 6 was immediately eluted from 20 sheets after the first chromatographic separation with 50% methanol-water. Two-fifths of the total eluate (50 ml.) was then made 6N with hydrochloric acid and heated for 20 minutes at the boiling point. The hydrolysate was then extracted three times with 25 ml. portions of water-saturated ethyl acetate. The extract was concentrated and compared chromatographically in five solvent systems with unhydrolyzed zone 6 and scopoletin. These chromatograms indicated the presence of some unhydrolyzed material in addition to a considerable amount of scopoletin (Table 1).

Since the amount of acid necessary to hydrolyze glucuronides will also destroy the glucuronic acid, it was necessary to use a specific enzyme in order to establish that zone 6 is a glucuronide of scopoletin.

The zone 6 eluate was taken to dryness <u>in vacuo</u> and then dissolved in 25 ml. of phosphate buffer at a pH of 6. Fifty milligrams of β-glucuronidase (Nutritional Biochemicals Corporation) was added and the mixture was incubated at 37° C. for 24 hours. A blank made up in the same way except that no enzyme was added was incubated at the same time. These enzymatic hydrolysates were then extracted three times with 15 ml. portions of ethyl acetate. The organic extracts were concentrated and chromatographed. These chromatograms, when developed in chloroform-acetic acid-water (2:1:1), revealed that the sample containing the enzyme was hydrolyzed to scopoletin and some

esculetin. The blank was unchanged. This indicates that zone 6 is a mixture of the glucuronides of scopoletin and esculetin.

CHAPTER III

THE ISOLATION AND IDENTIFICATION OF THE URINARY METABOLITES OF ESCULETIN AND ESCULIN

Purification of Esculin

Two grams of esculin (Nutritional Biochemicals Corporation) were dissolved in a small amount of methanol and adsorbed on Magnesol (Food Machinery and Chemical Corporation, New York) packed in a chromatographic column under five pounds pressure using water-saturated ethyl acetate as the slurrying liquid. On development of the column with water-saturated ethyl acetate, the esculetin that occurred as an impurity was moved rapidly through the column. The esculin and another blue fluorescing glycoside were then eluted separately with methanol. Observation of progress during the elution was made with long wavelength ultraviolet light.

The methanol eluate fraction containing the purified esculin was then evaporated slowly at room temperature. When most of the material had precipitated out of solution, the solid (262 mg.) was filtered off and dried *in vacuo*. The purity of this material was checked by dissolving a small amount of the solid in methanol and spotting it on S & S #589 red ribbon chromatography paper. The paper was then developed in n-butanol-acetic acid-water (6:1:2 v/v). This solvent system had previously been found to give the best reso-

lution of esculetin, esculin and of the other glycoside that is present as a trace impurity. The new chromatogram revealed no fluorescent spot other than that of esculin. The column separation, in addition to the slow crystallization, resulted in chromatographically pure esculin.

Purification of Esculetin

The impure esculetin was obtained by hydrolysis of esculin. This was accomplished as follows: 10.2 g. of esculin (Nutritional Biochemicals Corporation) was dissolved in 360 ml. of 7% sulfuric acid and heated on a steam bath for six hours. The resulting fine needle crystals were filtered off after the hydrolysate had been cooled in the refrigerator. The precipitate was washed with a small amount of water and then with a small amount of ether. The washed precipitate was then recrystallized from 95% ethanol after decolorization with charcoal. The resulting precipitate weighed 4.2 g. By chromatography, this solid esculetin preparation was found to contain a small amount of unhydrolyzed esculin.

A portion of the esculetin preparation (2 g.) was dissolved in methanol and adsorbed on a column that had been packed under five pounds of pressure using a slurry of Magnesol in ethyl acetate. The column was then developed using water-saturated ethyl acetate as the solvent. The first two fractions coming off the column (each approximately 125 ml.) were not saved because they contained impurities as revealed by examination under ultraviolet light after chromatography in the n-butanol-acetic acid-water (6:1:2 v/v) solvent system. The

remaining fractions that came off the column with water-saturated ethyl acetate were combined and reduced in volume. The resulting precipitate was then recrystallized from 95% ethanol; 300 mg. of chromatographically pure esculetin were obtained.

Synthesis and Purification of 6-Hydroxy-7-Methoxycoumarin from Esculetin

Three grams of the impure esculetin were dissolved in 300 ml. of methanol which contained 1.34 g. of sodium hydroxide. The solution was mechanically stirred while 4.25 g. of dimethylsulfate were slowly added and then refluxed for six hours on a steam bath.

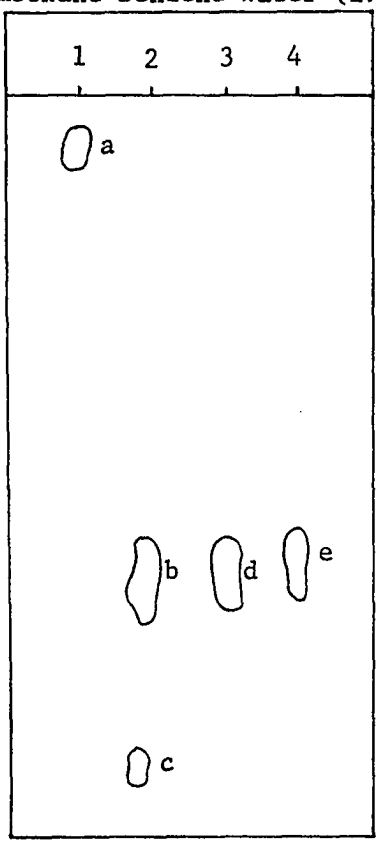
After cooling, the reaction mixture was neutralized with 1N sodium hydroxide and reduced in volume in vacuo to about 100 ml. This solution was extracted with three portions of chloroform; this was extracted with three portions of 10% sodium carbonate (150 ml. each time); the carbonate solution was neutralized with hydrochloric acid and extracted three times with nitromethane (150 ml. each time). The chromatogram of the various fractions when developed in nitromethane-benzene-water (2:3:5 v/v, organic phase) is shown in Figure 4.

The 7-methyl ether of esculetin was purified using a column containing Magnesol packed in a slurry of chloroform. Two nitromethane extracts containing the reaction products were chromatographed using nitromethane as the developing solvent. The first zone to come off the column fluoresced pale blue in long wavelength ultraviolet light. Moving a little behind on the Magnesol was a bright blue zone. These two zones were collected separately and reduced in volume at room temperature until crystallization took place. The pale blue zone

FIGURE 4

CHROMATOGRAM OF THE VARIOUS FRACTIONS OBTAINED IN THE SYNTHESIS OF THE 7-METHYL ETHER OF ESCULETIN

Nitromethane-Benzene-Water (2:3:5)



1. Aqueous phase after extraction with chloroform.
2. Chloroform phase after extraction with sodium carbonate.
3. Neutralized sodium carbonate phase after extraction with nitromethane.
4. Nitromethane extract.

a. R_f = 0.14, blue-green when exposed to ammonia under ultraviolet light (esculetin).

b. R_f = 0.85, blue under ultraviolet light.

c. R_f = 0.91, blue under ultraviolet light (6, 7-dimethoxycoumarin).

d. R_f = 0.83, blue turning to reddish-purple when exposed to ammonia under ultraviolet light (7-methoxy-6-hydroxycoumarin).

e. R_f = 0.82, blue color brightens when exposed to ammonia under ultraviolet light (scopoletin).

gave crystals that melted at 188° C. as compared to a literature value of 184° C. (23) for the 7-methyl ether of esculetin. This compound also showed the characteristic fluorescence of esculetin derivatives that have the 7-hydroxy group substituted. The bright blue zone gave crystals that melted at 208° C. and were chromatographically identical in fluorescence and R_f values with authentic scopoletin. The 7-methyl ether of esculetin has R_f values on paper very similar to those of scopoletin, but it fluoresces a reddish-purple color rather than the bright blue when exposed to ammonia vapor.

Administration[a] of Esculin and Esculetin to the Rats

Male rats of the Holtzman strain, weighing 300-350 g., four to six months of age, were kept on a vitamin B complete test diet (Nutritional Biochemicals Corporation) for at least ten days prior to the experiments. The esculetin or esculin, in about 2-5 ml. water suspensions, was administered by stomach tube to animals which had been lightly anesthetized with ether. Three rats received 40 mg. of esculin; three received 10 mg. of esculin; three received 40 mg. of esculetin; and three received 10 mg. of esculetin. Four rats served as controls. Each rat was kept in a separate metabolism cage, and the urine from each animal was collected under toluene for 24 hours. During the collection period the rats were deprived of food but had access to water.

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[a]Rats were fed and urine collected by P. L. Petrakis, University of Oklahoma Medical School.

Isolation[b] and Identification of Urinary Metabolites

The urine collected from the individual rat fed esculetin and esculin was filtered and the volume of urine adjusted to 50 ml. in volumetric flasks. The urine from each individual rat was then streaked on S & S #589 red ribbon paper (7 1/2" x 23"), and the paper was developed in the n-butanol-acetic acid-water (6:1:2 v/v) solvent system. From the urine of animals fed 40 mg. of esculin, five major blue fluorescing zones could be seen under long wavelength ultraviolet light (Figure 5). These were called B-1, B-2, B-3, B-4, and B-5. The R_f value for each in n-butanol-acetic acid-water (6:1:2 v/v) is 0.81, 0.76, 0.40, 0.12, and 0.09, respectively. A two-dimensional chromatogram of the urine is seen in Figure 6.

The part of the chromatograms containing the somewhat overlapping zones B-1 and B-2 was cut out, sewn onto a new sheet of S & S paper, and developed in 15% acetic acid-water. The development was stopped when both zones had moved across the bottom sewn line. After drying, the chromatograms were developed in the nitromethane-benzene-water (3:2:5 v/v, organic phase) solvent system. This gave satisfactory separation of zone B-1 from zone B-2. Each zone was cut out of the chromatogram, sewn onto a new sheet of paper, and then developed in 15% acetic acid-water for 16 to 18 hours.

The zones containing B-1 and B-2 were cut out of the chromatogram and sewn onto fresh sheets of Whatman #1 paper which had been pre-washed with the n-butanol-acetic acid-water (6:1:2 v/v)

---

[b] Isolation and part of the identification was done in cooperation with Dr. C. H. Yang.

FIGURE 5

CHROMATOGRAPHIC SEPARATION OF URINARY METABOLITES OF ESCULIN

(Colors Are Under Ultraviolet Light)

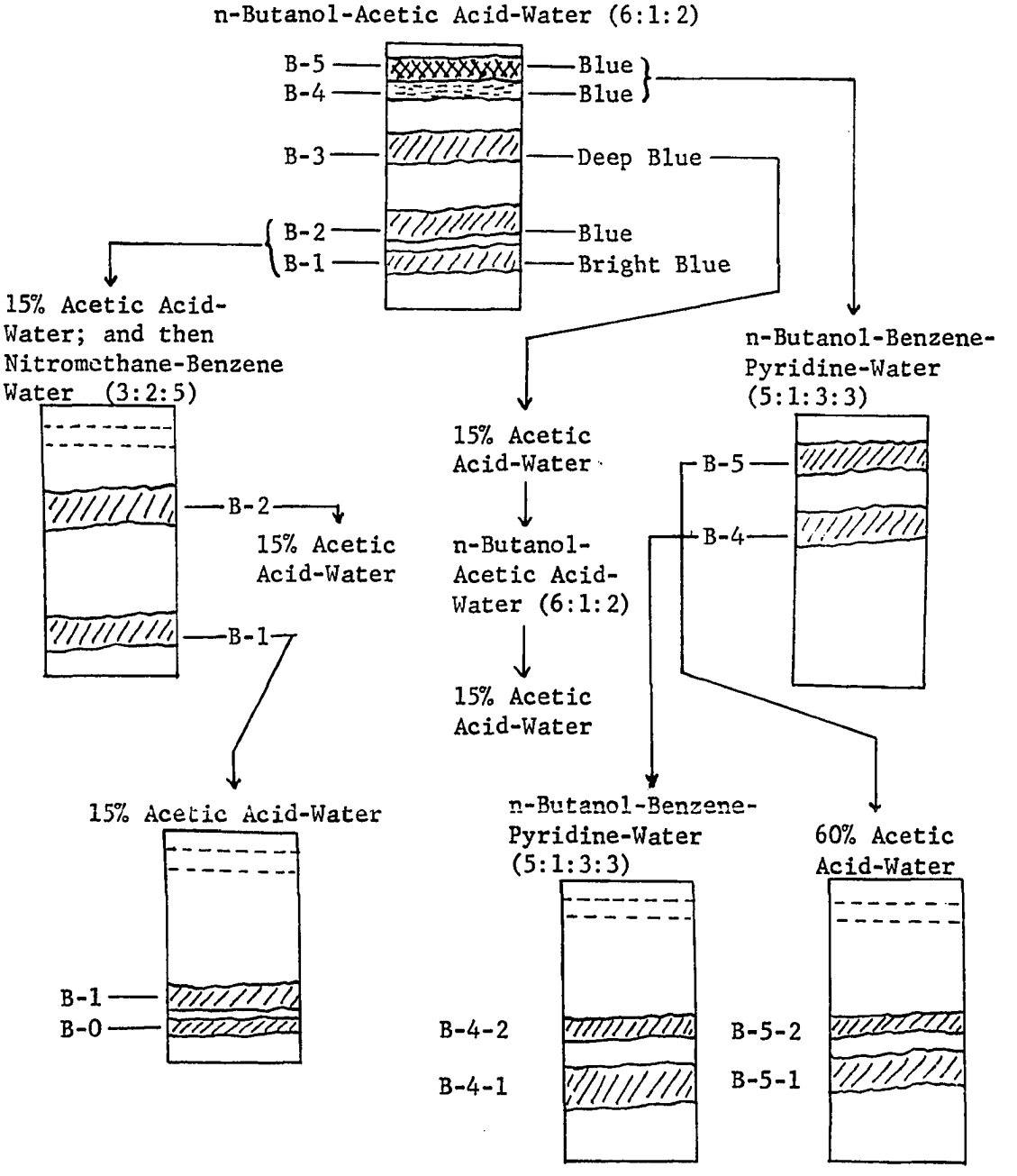
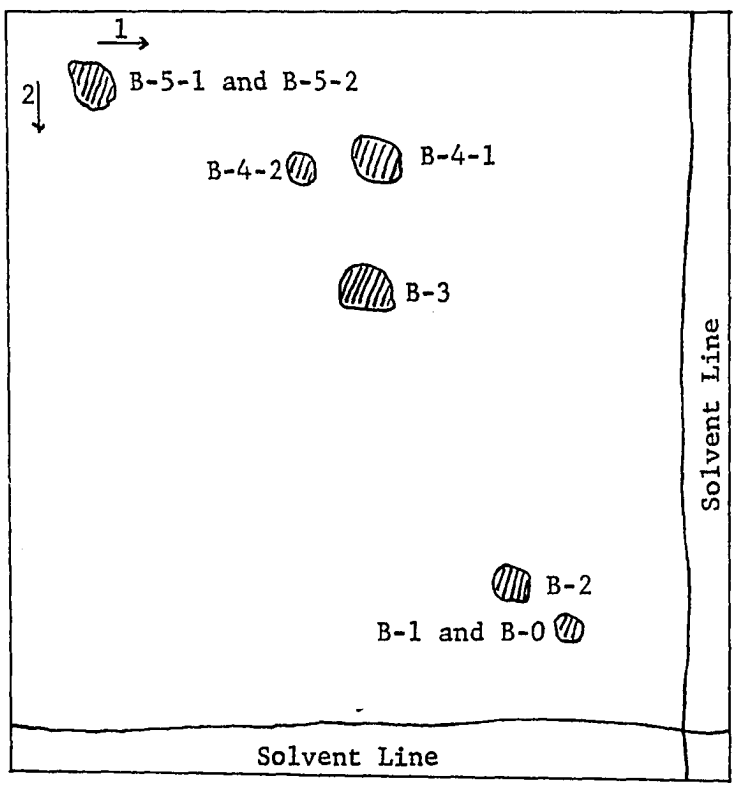


FIGURE 6

TWO-DIMENSIONAL CHROMATOGRAM OF 100 λ OF URINE

FROM A RAT FED 40 MG. ESCULIN



1. n-Butanol-Pyridine-Benzene-Water (5:3:1:3)
2. n-Butanol-Acetic Acid-Water (6:1:2)

B-0 = 7-Mechoxy-6-Hydroxycoumarin
B-1 = Scopoletin
B-2 = Esculetin
B-3 = Esculin
B-4-1 = Conjugated Compound
B-4-2 = Conjugated Compound
B-5-1 = Conjugated Compound
B-5-2 = Conjugated Compound

system. Final development of B-2 was carried out in the n-butanol-acetic acid-water (6:1:2 v/v) system. This brought B-2 into a narrow, even zone. The use of Whatman #1 paper in the last step development was to facilitate the elution of B-2 from the paper. This elution was carried out with 70% ethanol-water.

Zone B-2 was identified as esculetin by chromatographic comparison in five different solvent systems and by mixed chromatography in two dimensions (Table 2).

When zone B-1 was developed in the 15% acetic acid-water for 16-18 hours, a trace of another compound pulled away from B-1. This new zone, called zone B-0, was eluted from the paper with 70% ethanol-water. It was found by chromatographic comparison to be identical with synthetic 7-methoxy-6-hydroxycoumarin (Table 2).

The final purification step of zone B-1 consisted of its development in n-butanol-acetic acid-water (6:1:2 v/v) on washed chromatography paper as described above. The pure B-1 was eluted with 50% ethanol-water. One-dimensional chromatographic comparison and two-dimensional mixed chromatography of B-1 with authentic scopoletin proved that B-1 was identical with scopoletin (Table 2).

The B-3 zone from the first n-butanol-acetic acid-water (6:1:2 v/v) system development was cut out and sewn onto a new sheet of S & S #589 paper and developed in 15% acetic acid-water system. The B-3 zone was again cut out and sewn on S & S #589 paper and developed in n-butanol-acetic acid-water (6:1:2 v/v) for 24 hours. Final development of zone B-3 was carried out on pre-washed Whatman #1 paper in 15% acetic acid-water, and the

TABLE 2

R_f VALUES OF COMPOUNDS ISOLATED FROM URINE OF ANIMALS FED ESCULIN AND ESCULETIN

Compound	20% Potassium Chloride-Water	Chloroform-Acetic Acid-Water (2:1:1)	n-Butanol-Acetic Acid-Water (6:1:2)	Ethanol-n-Butanol-Ammonium Carbonate (40:11:19)
Esculetin	0.26	0.33	0.88	0.48
Esculin	---	0.06	0.31	0.13
Scopoletin	0.14	0.89	0.92	0.46
6-Hydroxy-7-Methoxycoumarin	0.15	0.92	0.87	---
B-0	0.13	0.93	0.88	---
B-1	0.15	0.88	0.92	0.47
B-2	0.24	0.34	0.89	0.47
B-3	---	0.08	0.32	0.15
B-4-1	0.40	0.02	0.08	0.13
B-4-2	0.39	0.02	0.07	0.11
B-6-1	0.36	0.02	0.10	0.05
B-6-2	0.36	0.02	0.06	0.11

compound B-3 was eluted from the paper with 50% ethanol-water. B-3 was found to be identical with esculin by one-dimensional comparison and two-dimensional mixed chromatography (Table 2).

The two purplish-blue zones which stayed near the origin in the first separation by n-butanol-acetic acid-water (6:1:2 v/v) were cut out as one zone and sewn onto a fresh sheet of S & S paper and developed in n-butanol-benzene-pyridine-water (5:1:3:3 v/v, organic phase) for 24 hours. This system pulled the B-4 zone completely away from the B-5 zone in that the latter moved very little in this solvent system. The zone containing B-4 was cut out of the paper, sewn onto another fresh sheet of S & S paper and again developed in the n-butanol-benzene-pyridine-water system for 24 hours. By this development the B-4 zone was separated into two zones. The one with the higher R_f value seemed to be the major part of B-4. These two zones were designated as B-4-1 (larger R_f value) and B-4-2 (smaller R_f value). When urine from each rat fed only 10 mg. of esculin plus the basal ration was analyzed in the same manner as above, the B-4-2 zone was not found on the chromatogram. With the above exception, all other compounds found in the urine of the rats fed 40 mg. of esculin were also found to be present in the case of an animal administered only 10 mg. of esculin. The zones B-4-1 and B-4-2 were separately cut out of the chromatogram, sewn on fresh sheets of Whatman #1 paper (washed) and developed in the n-butanol-benzene-pyridine-water system. Each zone was then eluted with ethanol-pyridine (8:1).

Compounds B-4-1 and B-4-2 were hydrolyzed with 6N hydro-

chloric acid at room temperature for three hours. The hydrolysates were then extracted with ethyl acetate, and the extract was concentrated in vacuo. These extracts were then compared by ascending paper chromatography with esculetin, scopoletin and 7-methoxy-6-hydroxycoumarin. The extract from hydrolyzed compound B-4-1 showed the presence of esculetin. The extract from hydrolyzed compound B-4-2 contained 7-methoxy-6-hydroxycoumarin and a small amount of scopoletin.

Compounds B-4-1 and B-4-2 were spotted on S & S #589 paper very heavily and then developed in the 15% acetic acid-water system. The paper was then sprayed using the modified method of Burma described in Chapter II. Both compounds B-4-1 and B-4-2 gave positive results, indicating that they are conjugated sulfates. B-4-1 is therefore a sulfate of esculetin and B-4-2 is 7-methoxy-6-hydroxycoumarin sulfate.

The upper blue zone, B-5, which was separated from B-4 in the first n-butanol-benzene-pyridine-water development, did not move in any other system tried except in 60% acetic acid-water. Consequently, this zone was cut out, sewn on S & S paper and developed in the 60% acetic acid-water system. This development resulted in separation of B-5 into two blue zones designated B-5-1 (larger R_f value) and B-5-2 (smaller R_f value). The B-5-2 zone was missing from the urine of rats fed only 10 mg. esculin.

The B-5-1 and B-5-2 zones were then further purified by cutting out the zones, sewing them onto washed Whatman #1 paper, and developing them in 60% acetic acid-water. They were then eluted with a 50:50 solution of ethanol and acetic acid.

After B-5-1 and B-5-2 were hydrolyzed with 5N hydrochloric acid for four hours at reflux temperature, the hydrolysates were extracted with ethyl acetate as described in Chapter II for the scopoletin glucuronide. By paper chromatography, both extracts were found to contain esculetin.

Compounds B-5-1 and B-5-2 were also treated with β-glucuronidase in much the same manner as the scopoletin glucuronide. In this case, however, 15 ml. of phosphate buffer and 10 mg. of the enzyme were used. The extraction and chromatography were carried out as described in Chapter II. The compound B-5-1 was hydrolyzed by the enzyme to give esculetin and thus is established as an esculetin glucuronide. However, compound B-5-2 was unchanged. Therefore, B-5-2 may not be a glucuronide. Since there is a possibility that it is a very stable glucuronide, no final conclusion can be made on this point at present.

CHAPTER IV

THE ISOLATION AND IDENTIFICATION OF THE URINARY METABOLITES OF QUERCETIN AND SOME RELATED COMPOUNDS

Administration of Quercetin to the Animals

Four female albino rats were placed in metabolism cages (Acme Company) and fed Nutritional Biochemicals Synthetic Complete Test Diet. The urine collected under toluene after 10 days will later be referred to as the normal urine. At the end of two weeks on the synthetic diet, the animals were placed on a diet containing 3% quercetin and 97% of the above test diet. The urine was then collected for a period of two weeks under toluene.

Extraction of the Urine

The urine from the rats was collected every 48 hours and then extracted in continuous liquid-liquid extractors using a steam bath for heating. The samples were extracted first with ether and then with ethyl acetate. The size of the sample extracted varied somewhat but was approximately 125 ml. of urine for each 250-300 ml. of extracting solvent. The extracts were concentrated at room temperature by evaporation to a volume of approximately 20 ml. Except when in use, these extracts were kept under refrigeration.

Two-Dimensional Chromatography of the Urine Extracts

The ether extract of the rats' normal urine was heavily spotted on S & S #589 chromatography paper, red ribbon. A two-dimensional chromatogram was developed, the first dimension in chloroform-acetic acid-water (2:1:1 v/v, organic phase), and the second dimension in 20% potassium chloride-water (23). The chromatogram was then sprayed with diazotized sulfanilic acid followed by 20% sodium carbonate (20). Except for three spots which were barely visible, there was almost no reaction at all on the paper. The same procedure was followed for the urine extracts of the animals fed quercetin (Figure 7). Since the urine was extracted on the basis of two day lots, there were seven chromatograms, the urine of the first and second days on through the urine of the thirteenth and fourteenth days.

As the length of time the animals were on the quercetin diet increased, there was a noticeable increase in phenolic-type compounds in the urine. In the urine collected the first and second days there were only small amounts of two compounds (Figure 7) that reacted with the diazotized sulfanilic acid spray to produce visible colored products; these are designated Q-2 and Q-5. There was an increase in the amounts of these in the urine of the third and fourth days, and Q-3 appeared at that time. In the extract of the urine collected on the fifth and sixth days, there were, in addition to the compounds above, compounds Q-1, Q-4, Q-6, Q-7 and Q-8. The isolation and identification of the individual compounds follows.

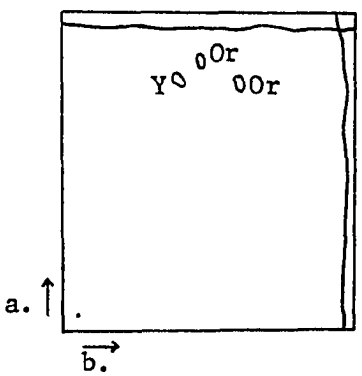
Isolation and Identification of Individual Urinary Metabolites

The ether extract of the urine was streaked on twenty sheets

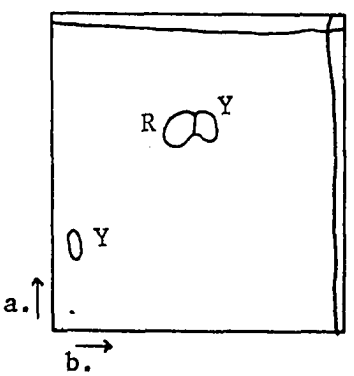
FIGURE 7

TWO-DIMENSIONAL CHROMATOGRAMS OF RAT URINE EXTRACTS

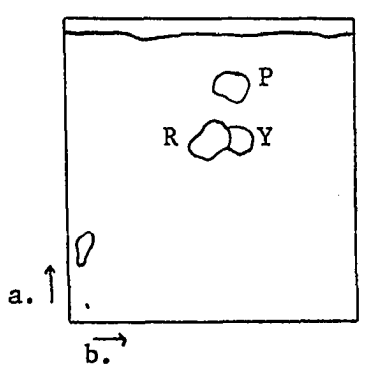
Colors Are in Visible Light after Spraying
with Diazotized Sulfanilic Acid Reagent



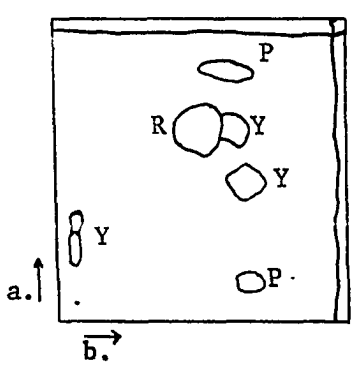
Ether Extract of Rat Urine After 10 Days on Synthetic Diet



Ether Extract of Rat Urine (Quercetin Diet) 1 and 2 Days



Ether Extract of Rat Urine (Quercetin Diet) 3 and 4 Days



Ether Extract of Rat Urine (Quercetin Diet) 5 and 6 Days

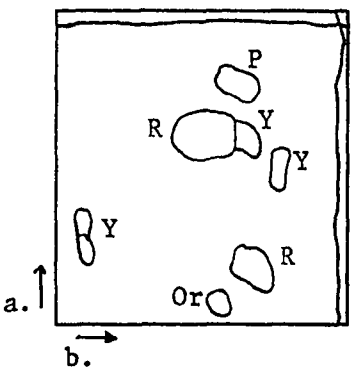
Y = Yellow
Or = Orange
R = Red
P = Pink

a. Chloroform-Acetic Acid-Water (3:2:5)
b. 20% Potassium Chloride-Water

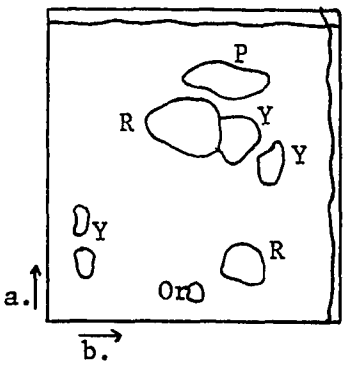
FIGURE 7 (Continued)

TWO-DIMENSIONAL CHROMATOGRAMS OF RAT URINE EXTRACTS

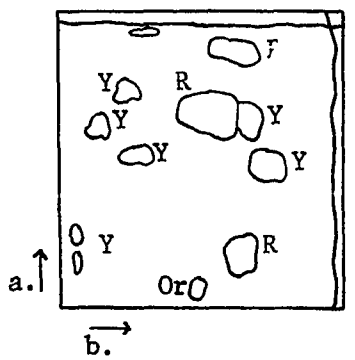
Colors Are in Visible Light after Spraying
with Diazotized Sulfanilic Acid Reagent



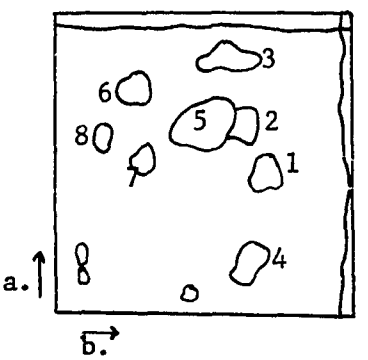
Ether Extract of Rat Urine (Quercetin Diet) 7 and 8 Days



Ether Extract of Rat Urine (Quercetin Diet) 9 and 10 Days



Ether Extract of Rat Urine (Quercetin Diet) 11 and 12 Days



Ether Extract of Rat Urine (Quercetin Diet) 13 and 14 Days

Y = Yellow
Or = Orange
R = Red
P = Pink

1 = Q-1
2 = Q-2
3 = Q-3
4 = Q-4

5 = Q-5
6 = Q-6
7 = Q-7
8 = Q-8

(7 1/2" x 23") of S & S #589 red ribbon paper and developed in the 20% potassium chloride-water system. After development, a 1 to 1 1/2 cm. guide strip was cut off the side of each paper. These strips were then sprayed with diazotized sulfanilic acid-sodium carbonate reagent (24). This revealed the presence of four zones as illustrated in Figure 8. The zone moving the farthest in the system was cut out of the paper and sewn onto a fresh sheet of S & S #589 paper and then developed in the chloroform-acetic acid-water (2:1:1 v/v, organic phase) system (Figure 8). A guide strip from that paper was sprayed with the diazotized sulfanilic acid reagent. After spraying, one could see three zones in visible light: the farthest moving one was pink after spraying; the middle one was yellow; and the slowest moving one was yellow-brown. The farthest moving zone was cut out of the papers and eluted with 75% ethanol-water; so was the middle zone. The eluates were concentrated in vacuo, spotted on S & S #589 paper and developed two-dimensionally in 20% potassium chloride-water and chloroform-acetic acid-water. The farthest moving zone was found by R_f values to be compound Q-3, and the middle zone was compound Q-1.

Compounds Q-1 and Q-3 were compared by paper chromatography to a number of aromatic acids that one might suspect to be present. Q-3 was found to be identical with homovanillic acid (4-hydroxy-3-methoxy-phenylacetic acid) (Tables 3 and 4), which had been reported as a urinary metabolic product by DeEds et al. (24). Q-1 was found by paper chromatography to be identical with m-hydroxyphenylacetic acid (Tables 3 and 4), also reported by DeEds et al. (24).

When Q-3 was developed in chloroform-acetic acid-water, the

FIGURE 8

SEPARATION OF COMPOUNDS Q-1 AND Q-3

Colors Are Under Visible Light Except
Where Otherwise Designated

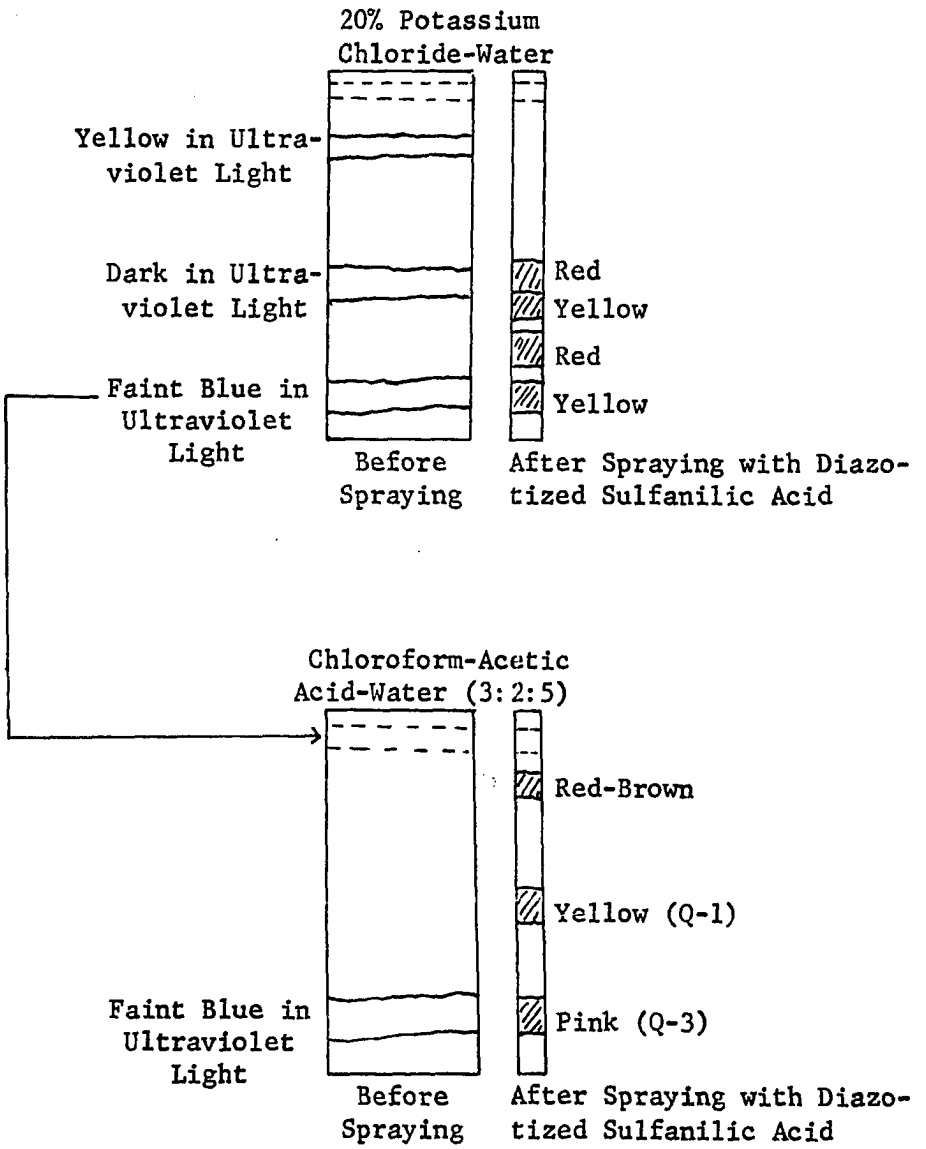


TABLE 3

R_f VALUES OF REFERENCE AROMATIC ACIDS

Compound	Solvent System* 1	2	3	4	5	6	7	8
Homovanillic Acid	0.77	0.85	0.87	0.79	0.51	0.71	---	---
m-Hydroxyphenylacetic Acid	0.80	0.73	0.96	0.74	0.66	0.48	---	---
3, 4-Dihydroxyphenylacetic Acid	0.72	0.25	0.82	0.72	0.54	0.16	---	---
m-Hydroxyphenylpropionic Acid	0.53	0.76	0.96	0.77	---	---	0.15	0.40
m-Hydroxycinnamic Acid	0.21	0.76	0.91	0.46	---	---	0.21	0.44
Vanillic Acid	0.34	0.87	0.92	0.66	---	---	0.07	0.25

*Solvent System:
1. 20% Potassium Chloride-Water
2. Chloroform-Acetic Acid-Water (2:1:1 v/v, lower layer)
3. n-Butanol-Acetic Acid-Water (6:1:2 v/v)
4. 15% Acetic Acid-Water
5. Isopropanol-Ammonium Hydroxide-Water (8:1:1 v/v)
6. Benzene-Propionic Acid-Water (2:1:1 v/v)
7. n-Butanol-Ammonium Carbonate Buffer (11:19) (18) Saturated with Chloroform
8. n-Butanol-Ethanol-Ammonium Carbonate Buffer (11:40:19)

TABLE 4

COLOR REACTION OF REFERENCE AROMATIC ACIDS

Compound	Color Developing Agent			
	Diazotized Sulfanilic Acid (20)	Diazotized p-Nitro-aniline (25)	Ammonium Molybdate (26)	Diazotized Benzidine (27)
Homovanillic Acid	Violet	Grey	No Color	-----
m-Hydroxyphenylacetic Acid	Yellow	Purple	No Color	-----
3, 4-Dihydroxyphenylacetic Acid	Pink-Brown	Brown	Yellow	-----
m-Hydroxyphenylpropionic Acid	Yellow	Purple	No Color	-----
m-Hydroxycinnamic Acid	Yellow	Purple	No Color	-----
Vanillic Acid	Orange	-----	No Color	Brown

front half of the spot was pink while the trailing half was orange. This is the same behavior that is exhibited when homovanillic and homoisovanillic acids are mixed. It was never possible to pull these completely apart for unequivocal proof, but all chromatograms studied indicated that homoisovanillic acid was also present in the urine. All chromatographic comparisons were run side by side on the same sheet of chromatographic paper to minimize the number of variable conditions.

To separate the remainder of the compounds, it was found advantageous to develop the streaked chromatograms in the chloroform-acetic acid-water (2:1:1 v/v, organic phase) solvent system first. The separation achieved by this solvent system is shown in Figure 9.

The slowest moving zone in this system (designated CAW-1) was cut out and eluted with 75% ethanol-water. When spotted on S & S #589 paper and developed two-dimensionally in chloroform-acetic acid-water and then in 20% potassium chloride water, CAW-1 was found to be identical with compound Q-4. This compound was compared to various phenolic acids and found to be identical with 3, 4-dihydroxyphenyl-acetic acid (Tables 3 and 4). This compound was reported as present in the urine by DeEds (24). The failure of Petrakis (6) to find this compound was probably because his sodium carbonate extraction brought about oxidation of the compound.

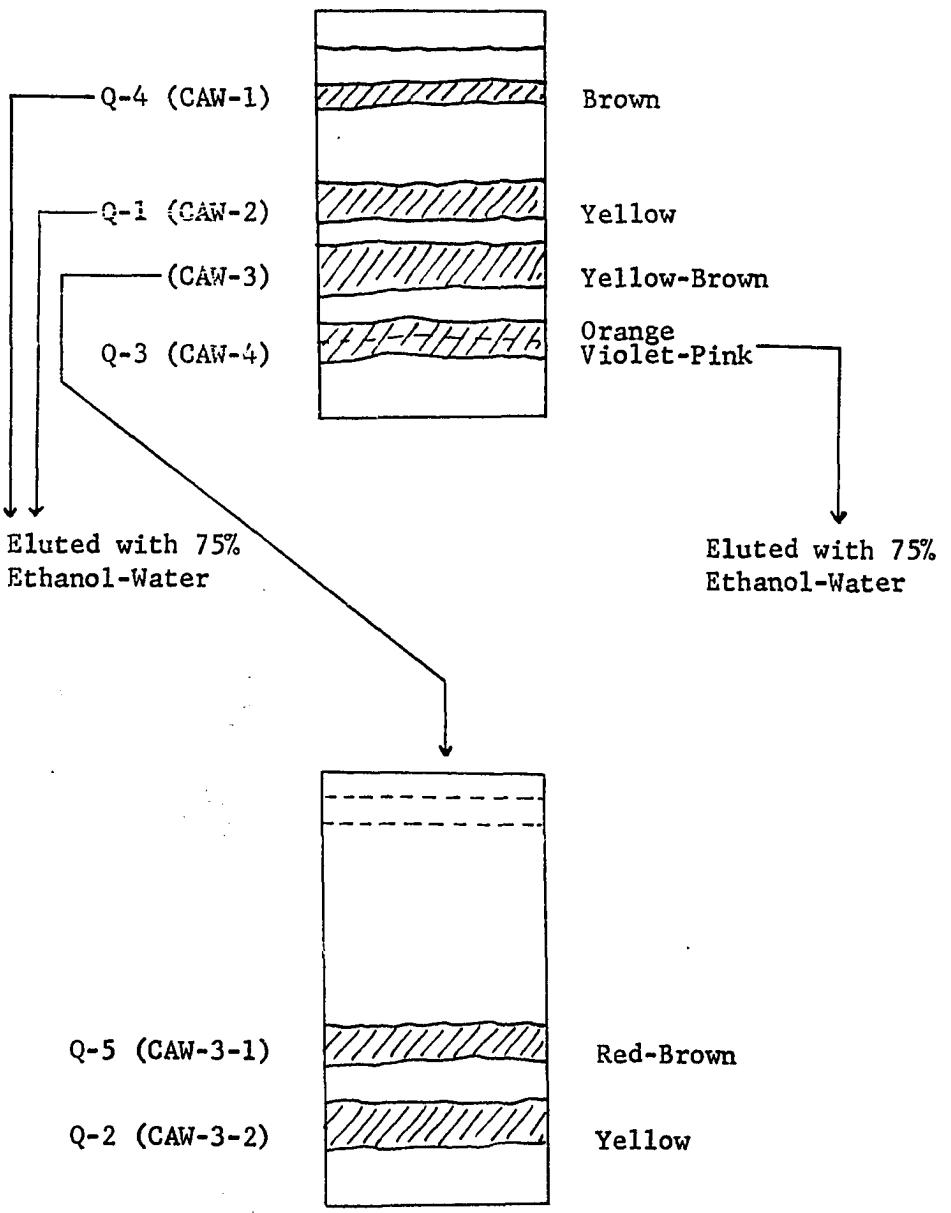
The zone designated CAW-2 in Figure 9 was also eluted with 75% ethanol-water. Paper chromatographic comparison proved that it was the same as Q-1, whose identification has already been discussed.

The zone designated CAW-3 was sewn on fresh sheets of S & S

FIGURE 9

CHROMATOGRAPHIC SEPARATION OF COMPOUNDS Q-1, Q-2, Q-3, Q-4 AND Q-5

Colors in Visible Light when Sprayed with
Diazotized Sulfanilic Acid Reagent



#589 paper and developed in the 20% potassium chloride-water. This system resolved the CAW-3 zone into two zones, designated CAW-3-1 and CAW-3-2 (Figure 9). These zones were then eluted with absolute ethanol in order to minimize the amount of salt eluted.

The zone CAW-3-2 was spotted on an 11 1/2" x 12" sheet of S & S #589 paper and developed two-dimensionally in chloroform-acetic acid-water and in 20% potassium chloride-water. This established that CAW-3-2 was identical with compound Q-2. On comparison with various phenolic acids, and by mixed paper chromatography, Q-2 was found to be identical with m-hydroxyphenylpropionic acid (Tables 3 and 4). The standard m-hydroxyphenylpropionic acid was prepared by catalytic reduction of m-hydroxycinnamic acid using 10% palladium on powdered charcoal. The m-hydroxycinnamic acid was prepared from m-hydroxybenzaldehyde using the Doebner modification of the Perkin reaction (28).

Zone CAW-3-1, when developed two-dimensionally as described before, was found to be identical with compound Q-5. Since this compound, as well as Q-2, were excreted by the rats beginning with the first day, they were of considerable interest. Compound CAW-3-1 was compared chromatographically to numerous phenolic compounds and was found not to be identical with any of those tested. The chromatographic behavior of this compound is shown in Table 5. The compound was very similar to 3, 4-dihydroxyphenylacetic acid in its color reactions with the diazotized sprays. Chromatograms of the compound were sprayed with three reagents used for catechol type compounds: saturated aqueous solution of ammonium molybdate (26), 2% aqueous solution of phosphomolybdic acid followed by ammonia vapor (29), and 0.8% cobalt nitrate

TABLE 5

CHROMATOGRAPHIC BEHAVIOR OF COMPOUND Q-5

R_f Values

Solvent System	Q-5	Compound G	Compound H
20% Potassium Chloride-Water	0.48	0.56	0.58
Chloroform-Acetic Acid-Water (2:1:1)	0.81	0.92	0.68
n-Butanol-Acetic Acid-Water (6:1:2)	0.94	0.90	0.76
15% Acetic Acid-Water	0.69	0.78	0.77
Isopropanol-Ammonium Hydroxide-Water (8:1:1)	decomposed	---	---
Benzene-Propionic Acid-Water (2:1:1)	0.58	0.89	0.44

Color Reactions

Color Developing Agent	Q-5	Compound G	Compound H
Diazotized Sulfanilic Acid Reagent	Pink Changing to Brown	Yellow	Pink
Diazotized p-Nitro Aniline Reagent	Brown	---	---
Ammonium Molybdate Reagent	Yellow	No Color	Yellow
Ferric Chloride Reagent	Green	---	---
Phosphomolybdic Acid Reagent	Blue	---	---
Potassium Permanganate	Brown	---	---
Silver Nitrate	Black	---	---
Cobalt Nitrate	Blue	---	---

hexahydrate followed by a 40% solution of sodium hydroxide (30) (Table 5). All of these reagents gave positive test results that Q-5 was an ortho-dihydroxy compound. The ultraviolet absorption spectrum of Q-5 was almost identical with the spectra of 3, 4-dihydroxyethylbenzene and δ-(3, 4-dihydroxyphenyl) - γ-valerolactone (9). This helps to establish that Q-5 is a 1, 2, 4 trisubstituted phenolic compound with adjacent hydroxy groups.

The infrared absorption spectrum of Q-5 indicated a free hydroxy group at 3500 cm^{-1} and a carbonyl peak at 1700 cm^{-1} that is very likely a lactone carbonyl. This peak is at a lower wave number than is usual, but is in good agreement with the lactone carbonyl in 6, 7-dihydroxycoumarin which occurs at 1670 cm^{-1}. The infrared spectrum also helps to substantiate the 1, 2, 4 trisubstitution. Bellamy (31) lists absorptions in the following regions for this type substitution: 1225-1175 cm^{-1}, 1125-1090 cm^{-1}, two bands between 1070-1000 cm^{-1}, and one band 1175-1125 cm^{-1}. Q-5 has the following absorptions: 1190 cm^{-1}, 1110 cm^{-1}, 1040 cm^{-1}, 1000 cm^{-1}, and 1150 cm^{-1}.

Compound Q-5 was heated at steam bath temperature in a 6N solution of hydrochloric acid for one hour. At the end of this time the solution was extracted with ether, the extract concentrated and spotted on a sheet of S & S #589 paper. The paper was developed in chloroform-acetic acid-water and sprayed with the diazotized sulfanilic acid reagent. This treatment revealed that the compound was unchanged by acid hydrolysis.

The compound was then subjected to basic saponification by heating at steam bath temperature in a 1N alcoholic sodium hydroxide

solution for 20 minutes. The solution was then neutralized with hydrochloric acid and extracted with ether. Both the ether extract and the original acid hydrolysate were treated chromatographically, and the compound was recovered unchanged.

Compound Q-5 was methylated with diazomethane, and the infrared absorption curve of the product was taken on a Model 137 Perkin Elmer Infracord. The spectrum indicated that there was probably a hydroxy group still present that had not been methylated by the diazomethane. This indicated that a hydroxy group likely was present on the non-aromatic portion of the molecule. The absorption peak due to the carbonyl was unchanged by the methylation.

An alkaline fusion of this compound was also carried out. The compound was dissolved in 1N sodium hydroxide and heated in an oven at 110° C. for 24 hours. The residue was dissolved in water, neutralized with hydrochloric acid and extracted with ether. The extract was then developed two-dimensionally in chloroform-acetic acid-water and in 20% potassium chloride-water. The paper, after spraying with the diazotized sulfanilic acid reagent, revealed the presence of a compound that appears to be 3, 4-dihydroxybenzoic acid. This will have to be repeated on a larger amount of Q-5 in order to prove this identity unequivocally.

Compound Q-5 was compared by paper chromatography to the lactone isolated by Dr. Watanabe (9) from the urine of rabbits fed (+) catechin. These lactones are compounds G, δ-(3-hydroxyphenyl)-γ-valerolactone, and compound H, δ-(3, 4-dihydroxyphenyl)-γ-valerolactone, both kindly supplied by Dr. Watanabe of Japan. The

R_f values of the above are found in Table 5. Compound Q-5 is very similar in chromatographic behavior and color reaction to the compound H, but it was not identical.

Compounds Q-6, Q-7 and Q-8 were not isolated from this urine because of insufficient urine material. In order to isolate these compounds, ten female albino rats were treated in the same manner as described previously; the extractions and related procedures were the same. The isolation procedure, however, was considerably different.

The ether concentrate (50 ml.) was extracted three times with 50 ml. of a saturated solution of sodium bicarbonate. The bicarbonate extract (150 ml.) was then re-extracted with two 50 ml. portions of ether. The bicarbonate solution was adjusted to a pH of approximately four, and then it was extracted with three 50 ml. portions of ethyl acetate. This gave a resolution of the urine into an ether extract which contained the non-acidic compounds and an ethyl acetate fraction which contained the acidic compounds. Compound Q-5 and the unchanged quercetin were in the non-acidic fraction. The acidic fraction was checked by two dimensional chromatography and found to contain compounds Q-1, Q-2, Q-3, Q-4, Q-6, Q-7, and Q-8.

The ether extract (non-acidic) was adsorbed on a Magnesol column that was packed in methanol. The column was then developed with water-saturated ethyl acetate, and seven 500 ml. fractions were collected. The fractions were reduced in volume to 15 ml. each, spotted on S & S #589 paper and developed in chloroform-acetic acid-water. Compound Q-5 was present in fractions one and two.

The ethyl acetate extract was adsorbed on a Magnesol column

that was packed in methanol. The column was developed with chloroform-water saturated ethyl acetate (2:1). There were eight fractions taken of the eluate; each of these was reduced in volume to 15 ml., spotted on S & S #589 paper and developed in chloroform-acetic acid-water. Fractions one through three contained most of the acids with the exception of 3, 4-dihydroxyphenylacetic acid; fraction four contained all of the acids, and fractions five and six contained mainly 3, 4-dihydroxyphenylacetic acid.

Fractions one through three were combined and streaked on 15 sheets of S & S #589 paper (7 1/2" x 23"). These were then developed in 15% acetic acid-water. In order to locate each zone, a guide strip was cut off and sprayed with diazotized sulfanilic acid reagent. An orange zone with an R_f value of approximately 0.6 was cut out of the papers and eluted with methanol. The eluate was reduced in volume to 5 ml. and spotted on S & S #589 paper along with various other phenolic acids. This compound was found to be impure at this state; therefore, it was streaked on five sheets of S & S #589 paper and developed in chloroform-acetic acid-water (2:1:1 v/v, organic phase). The same procedure of location and elution as described above was used to elute the zone. It was then spotted on S & S #589 paper along with various other phenolic acids, and found to be identical with vanillic acid (Tables 3 and 4). It was distinguished from isovanillic acid by the method of Hergert and Goldschmid (27).

Two-dimensional chromatograms of the urine had indicated that compounds Q-7 and Q-8 were in the same position as m-hydroxybenzoic acid and m-hydroxycinnamic acid. The guide band from the

paper run in 15% acetic acid-water had a yellow zone after spraying at 0.5 (approximately). This zone was cut out and eluted with methanol.

When spotted on S & S #589 paper and developed two-dimensionally in 20% potassium chloride-water and chloroform-acetic acid-water, this zone was found to contain four spots. Two of these compounds had previously been proved to be m-hydroxyphenylacetic acid and m-hydroxyphenylpropionic acid. Mixed paper chromatography indicated that m-hydroxybenzoic acid was one of the other two. Mixed paper chromatography also indicated that the other spot was m-hydroxycinnamic acid. This compound, Q-8, was separated from the other three by chromatography, first in 20% potassium chloride-water, and then in 15% acetic acid-water. The R_f values and color reactions indicated that the unknown compound is m-hydroxycinnamic acid (Tables 3 and 4).

Feeding of Tetramethyl Quercetin to the Animals

One female albino rat was placed in a metabolism cage (Acme Company) and fed Nutritional Biochemicals Synthetic Complete Test Diet. The animal was maintained on this diet for five days. At the end of that time the animal was put on a diet that contained 1% 3', 4', 5, 7-tetramethyl quercetin and 99% of the above test diet. The urine from the animal was collected under toluene for 72 hours. At the end of that time the urine was acidified to pH 5 and extracted four times with 50 ml. portions of ether.

Chromatography of Urine Extract

The above extract was concentrated to 3-5 ml. and spotted on S & S #589 paper. The paper was then developed two-dimensionally in

chloroform-acetic acid-water and in 20% potassium chloride-water. When examined under ultraviolet light (long wavelength), there were two fluorescent compounds visible. One of these corresponded to the original tetramethyl quercetin, and the other was apparently also a flavonoid. Since this animal was in a metabolism cage of such design that it was practically impossible to mix the food with the urine, this established that the flavonoid was absorbed by the animal intact.

Administration of 3, 4-Dihydroxyphenylacetic Acid to the Rat

The procedures and amounts used here were identical with those used for the studies with tetramethyl quercetin. Chromatography of the urine revealed only homovanillic acid and unchanged 3, 4-dihydroxyphenylacetic acid. DeEds et al. (32) stated that administration of 3, 4-dihydroxyphenylacetic increased the amount of m-hydroxyphenylacetic acid excreted by animals fed a normal diet. They therefore attributed the increase in m-hydroxyphenylacetic acid to be due to the prior formation of 3, 4-dihydroxyphenylacetic acid from the quercetin. From the experiments performed here, it seems very unlikely that this is the case.

CHAPTER V

EXPERIMENTS ON THE FATE OF FLAVONOID COMPOUNDS WITH GASTRIC MUCIN AND IN THE RAT STOMACH

Breakdown of Various Flavonoids by Gastric Mucin

Kallianos (10) reported that gastric mucin (Nutritional Biochemicals) had the ability to break down quercetin to 3, 4-dihydroxybenzoic acid, phloroglucinol and phloroglucinol carboxylic acid.

The first experiment of this author was to check the effectiveness of this preparation on various flavonoids. The compounds tried were: quercetin (3, 5, 7, 3', 4'-pentahydroxyflavone), morin (3, 5, 7, 2', 4'-pentahydroxyflavone), myricetin (3, 5, 7, 3', 4', 5'-hexahydroxyflavone), robinetin (3, 7, 3', 4', 5'-pentahydroxyflavone), tricin (5, 7, 4'-trihydroxy-3', 5'-dimethoxyflavone), flavone, flavonol (3-hydroxyflavone), rutin (3-rhamnoglucoside of quercetin), and quercitrin (3-rhamnoside of quercetin). The procedure used was as follows: 0.5 g. of gastric mucin was homogenized with 5 ml. of citrate buffer (pH 2.5). To this homogenate was added 10-15 mg. of the compound to be tested. Blanks were made up for each compound. These were identical to the sample except that the gastric mucin was left out.

The blanks and samples were incubated at 41° C. for 72 hours. At the end of this time the solutions were extracted with 100 ml. of ether and chromatographed two-dimensionally in 20% potassium chloride-water and chloroform-acetic acid-water (2:1:1 v/v, organic phase). All papers, with the exception of those of flavone and flavonol, were sprayed with diazotized sulfanilic acid, followed by sodium carbonate (20). The blank and sample papers of flavone and flavonol were sprayed with a buffered solution of methyl red (18).

Quercetin, morin, rutin, quercitrin, robinetin, flavone, and flavonol were all changed during the experiment. The myricetin and tricin were unaffected by the treatment.

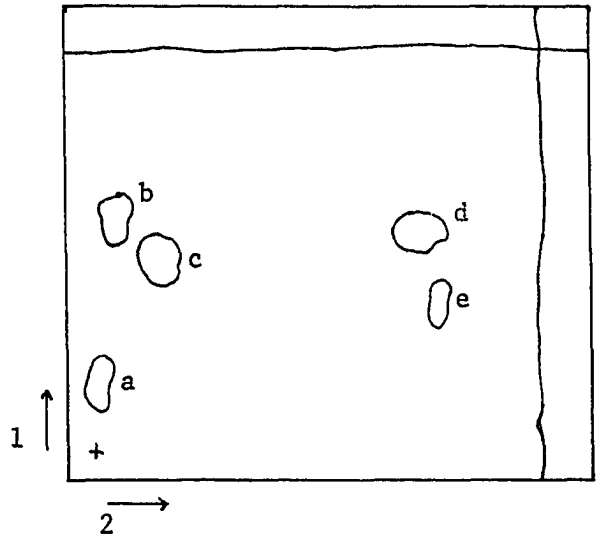
Chromatographic Identification of Quercetin Breakdown Products

The two-dimensional chromatogram of the breakdown products of quercetin with the gastric mucin preparation is found in Figure 10. The area labeled "c" had R_f values and color reaction identical to 3, 4-dihydroxybenzoic acid, as reported by Kallianos (33), who had also reported that phloroglucinol and phloroglucinol carboxylic acid were the other products. Neither of these latter two compounds, however, were found. There were two compounds, instead, which appeared somewhat similar (Figure 10, areas labeled "a" and "b") to phloroglucinol and phloroglucinol carboxylic acid. Whereas phloroglucinol and phloroglucinol carboxylic acid fluoresce blue in ultraviolet light when exposed to ammonia, neither of these compounds demonstrated this property.

The area labeled "a" had R_f values and color reactions very similar to 4, 6-dihydroxybenzalcoumaran-3-one. The former compound could not be eluted from paper with any solvent tried; consequently,

FIGURE 10

TWO-DIMENSIONAL CHROMATOGRAM OF QUERCETIN BREAKDOWN PRODUCTS WITH GASTRIC MUCIN



1. 20% Potassium Chloride-Water
2. Chloroform-Acetic Acid-Water (2:3:5)

a. Yellow with Diazotized Sulfanilic Acid
b. Yellow with Diazotized Sulfanilic Acid
c. Red with Diazotized Sulfanilic Acid
d. Yellow with Diazotized Sulfanilic Acid
e. Orange with Diazotized Sulfanilic Acid

side by side comparison was not accomplished. Sodium hydroxide degraded the known coumaranone to phloroglucinol, phloroglucinol carboxylic acid, and phloroglucinol carboxaldehyde. For comparison on the unknown, it was not possible to obtain a sufficient amount of material in area "a" to perform the degradation and obtain identifiable products.

Attempted Fractionation of Gastric Mucin

Fractionation of gastric mucin to obtain a highly active fraction was tried, using the method of Kallianos (33), which was adapted from the work of Richmond (34). One gram of gastric mucin was homogenized with 50 ml. of citrate buffer (pH 2.5). This was then passed through a column of Amberlite ion exchange resin IRC-50. The first fraction that came through contained most of the activity. A rough check of activity was made by taking the fractions from the columns and adding 25 mg. of quercetin. These were then incubated for 72 hours at 41° C. along with a standard that contained 500 mg. of gastric mucin and 25 mg. of quercetin. At the end of the incubation period, the samples were all extracted with 100 ml. of ether. The extracts were concentrated to a very small volume, and the total volume of each was spotted on separate sheets of 11" x 12" S & S #589 red ribbon paper. The papers were developed two-dimensionally in 20% potassium chloride-water and chloroform-acetic acid-water. After development, the papers were sprayed with the diazotized sulfanilic acid reagent. The area occupied by the individual breakdown products was taken as a measure of the activity. The fraction eluted off the column did not show any

increase in activity over the original gastric mucin.

Twenty-two milliliters of a solution of gastric mucin, made up as described above, was fractionated by Dr. M. R. Shetlar using a Spinco continuous electrophoresis apparatus. This fractionation resulted in 22 fractions. Not any of the fractions contained an appreciable amount of protein. Fractions 19 and 20 were high in bound carbohydrate material. Each fraction was checked for quercetin degradation activity by adding 10 mg. of quercetin and letting the mixture incubate for 72 hours at 41° C. The only fractions showing any activity were numbers 12 through 16, and the amount of breakdown in these four fractions was not appreciable.

Fractionation of the material was then tried by dialysis. Twenty-five grams of gastric mucin was homogenized with 125 ml. of distilled water. This was poured into a 1 5/8" by 16" sausage casing and tied shut. The casing was suspended in a beaker containing one liter of distilled water, and was stirred by a magnetic stirrer under refrigeration for 48 hours. At the end of this time, the water was replaced by a liter of fresh water.

The dialysate (2 l.) was lyophilized. The total weight of material that was dialyzed out of the bag was 1.9 grams. A portion of this (0.6 g.) was dissolved in 25 ml. of citrate buffer (pH 2.5), and 25 mg. of quercetin was added. Two controls were set up. One contained 0.6 g. of the lyophilized material and 25 ml. of buffer. The other contained 25 mg. of quercetin and 25 ml. of the buffer. The three solutions were then incubated at 41° C. for 72 hours, and were then extracted with 50 ml. volumes of ether. The lyophilized dialysate plus quercetin

gave about the same amount of breakdown as did the original gastric mucin; however, there were two additional spots on the chromatogram of the products.

These two additional spots were also found to be present in the lyophilized dialysate to which quercetin had not been added. The solution which contained only quercetin and buffer gave a barely detectable amount of breakdown. The compounds produced by the buffer and quercetin alone were identical with the products from gastric mucin treatment. The area of spots did not indicate that the dialysis had concentrated the material appreciably.

Since the paper chromatographic method of determining the activity was only a rough approximation, it became necessary to find a more quantitative method. The most practical method to measure the amount and rate of breakdown involved the manometric technique with a Warburg apparatus. In order for this technique to be successful, the reaction had to use oxygen or else carbon dioxide had to be respired. Therefore, it was necessary to know whether the reaction was oxygen dependent or not.

The oxygen dependence was determined by using a small flask that was so constructed that nitrogen could be used to flush oxygen out of the system. In this experiment, 0.5 g. of gastric mucin was homogenized in 10 ml. of citrate buffer and 25 mg. of quercetin was added. The flask was then flushed with nitrogen for 30 minutes, sealed and incubated at 41° C. for 72 hours. The breakdown products were assayed by using paper chromatography. In this experiment, there was no detectable breakdown. A similar experiment was carried out where an antioxidant

was added (ascorbic acid) rather than the nitrogen atmosphere. That also resulted in no detectable breakdown.

Since the reaction required oxygen, the manometric technique should have been applicable. The experiment with the Warburg was set up as follows: four Warburg flasks were used for the reaction and one as a thermobar. Each reaction flask held 2.6 ml. of citrate buffer containing 0.2 g. of gastric mucin. In the right side bulb was placed 0.2 mg. quercetin in 0.2 ml. of buffer. The center well of two flasks contained 0.2 ml. of 1N sodium hydroxide. In the other two flasks, the center well contained 0.2 ml. of water. The apparatus was given 15 minutes to equilibrate at the reaction temperature (37^{o} C.). Readings of the manometers were taken periodically for three hours. The amount of oxygen uptake in that period of time was found to be insignificant. This information coupled with the activity of the dialysate made it appear very unlikely that the catalyst is enzymatic in nature.

Since the manometric technique was impractical in measuring such a slow reaction, it was necessary to seek another method. Thus the following method was employed: 20 g. of gastric mucin was homogenized in 1000 ml. of the citrate buffer (containing 0.1 g. of p-chloromercuribenzoate). This solution was filtered through a celite mat which was necessary in order to eradicate as much turbidity as possible. A standard curve was prepared by adding 1 ml. of 95% ethanol and 1 ml. of 1% aluminum chloride in 95% ethanol to 9 ml. of gastric mucin solution. This was used as the blank in a Beckman DU spectrophotometer. For the standards, varying amounts of quercetin were added in the above mentioned 1 ml. of 95% ethanol.

For the rate study, 2.5 mg. of quercetin were added to 250 ml. of the gastric mucin solution and incubated at 41° C. Another 250 ml. of gastric mucin minus the quercetin were incubated to use as the spectrophotometer blank. At five hour intervals, a 10 ml. sample was drawn from both flasks, and 1 ml. of 1% aluminum chloride in 95% ethanol was added. The optical density was then measured. The amount of quercetin left after the various periods of time is shown in Table 6.

At the end of 92 hours the remaining solution was extracted with ether and spotted on S & S #589 paper and chromatographed two dimensionally in 20% potassium chloride-water and chloroform-acetic acid-water (2:1:1 v/v, organic phase).

After spraying with diazotized sulfanilic acid reagent (20), the chromatograms did not contain any of the breakdown products found before. There was only one compound that was visible on the paper after spraying. That compound turned blue in visible light when exposed to acid fumes (hydrochloric) and red when exposed to ammonia fumes. That type of color change is typical of anthocyanin pigments. Evidently the filtering of the gastric mucin solution removed either the catalyst or some co-factor necessary for the breakdown.

Activity of Gastric Juice

Gastric juice of a patient (Rose) at the Veterans Administration Hospital in Oklahoma City, Oklahoma, was obtained by pumping his stomach. One half of this gastric juice was dialyzed and then lyophilized, and the other half was directly lyophilized.

The two fractions were each dissolved in 25 ml. of citrate buffer (pH 2.5), and 25 mg. of quercetin were added to each fraction.

TABLE 6

RATE OF QUERCETIN DISAPPEARANCE WITH THE GASTRIC MUCIN PREPARATION

Time (Hours)	Mg. Quercetin/10 ml.
0	0.107
5	0.114
10	0.085
15	0.066
20	0.058
25	0.053
30	0.043
34	0.037
42	0.031
48	0.025
57	0.019
66	0.014
72	0.011
92	0.003

A blank containing 25 ml. of buffer and 25 mg. of quercetin was also prepared. After the three samples had then incubated for 24 hours at 41° C. utilizing magnetic stirring, they were each extracted with 100 ml. of ether. The ether extracts were reduced in volume and spotted on Whatman #1 paper. Neither fraction showed any breakdown at all of the quercetin.

Experiments Involving the Tied-Off Rat Stomach

Four white female rats of the Sprague-Dawley strain, each approximately 400 g. in weight, were used for this experiment. Each animal's abdominal cavity was opened surgically, the stomach was tied off with surgical gut just below the pylorus, and the abdominal cavity was then closed with surgical staples.

Quercetin (10 mg.) suspended in distilled water was then given to the animals by means of stomach tubes. At the end of 4, 7, 11, and 24 hours, the animals were sacrificed and their stomachs removed. The content of each stomach was extracted with 200 ml. portions of ether. These extracts were then reduced in volume and spotted on Whatman #1 paper. The papers were developed two-dimensionally in 20% potassium chloride-water and chloroform-acetic acid-water (2:1:1 v/v, organic phase). They were examined under ultraviolet light and sprayed with diazotized sulfanilic acid reagent. Quercetin was the only compound found on these papers.

CHAPTER VI

THE ISOLATION AND IDENTIFICATION OF THE METHYL ETHERS OF QUERCETIN IN TOBACCO FLOWERS

Isolation of Quercetin Methyl Ethers[c]

Samples, each containing 100 g. of powdered, oven-dried flowers from tobacco plants, Nicotiana tabacum, One-sucker variety, grown in the greenhouse at Argonne National Laboratory during 1958, were extracted with 500 ml. portions of the following solvents in the order named: n-pentane, benzene, chloroform, ethyl acetate, and acetone. Every 500 ml. extract was concentrated in vacuo to 5 ml. and studied by paper chromatography. The flavonol ethers were primarily in the chloroform fraction, although at least two such compounds were present in small amounts in the ethyl acetate extract.

Each 5 ml. chloroform concentrate was streaked onto eight sheets of Whatman #3MM chromatography paper (approximately 7" x 22 1/2"), and the chromatograms were developed in 15% acetic acid-water for about 24 hours. The upper part of each chromatogram containing the methylated flavonol compounds, which moved only a relatively short distance in this solvent, was cut out and sewn onto a new sheet of S & S #589 red ribbon chromatography

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[c]The isolation and separation of the compound and its demethylation were carried out by Dr. C. H. Yang and is included here for completeness.

paper. Each sheet was then developed in n-butanol-acetic acid-water (6:1:2 v/v). After drying, the papers were viewed under long wavelength ultraviolet light (3660 A^{o}). A dark brown zone was seen near the solvent front; it was poorly separated from some blue-fluorescing material. The broad, dark brown zone containing the mixture of suspected methylated flavonols was cut from each chromatogram, eluted with methanol, and then subjected to further extended chromatography, first in 15% acetic acid for 36-48 hours, then on fresh sheets in 60% acetic acid-water. The latter effected separation of the quercetin dimethyl ether from a trace amount of a brown fluorescing zone which had the same mobility as authentic quercetin-3-methyl ether on paper chromatograms (Table 7). The yield of this latter compound from the 1958 tobacco flowers was insufficient to confirm its identity. After elution of the brown fluorescing zone containing the quercetin dimethyl ether, the methanol eluates were subjected to further chromatography on S & S #589 paper using four different solvent systems in this order: 15% acetic acid-water, ethyl acetate-formic acid-water (10:2:3 v/v, upper layer), n-butanol-acetic acid-water (6:1:2 v/v), and finally 60% acetic acid-water. The quercetin dimethyl ether zone of each chromatogram was then pure enough for identification studies.

Identification of Quercetin-3, 3'-Dimethyl Ether

On paper chromatograms the quercetin dimethyl ether exhibited a dark brown fluorescence under ultraviolet light. After the compound had been sprayed with a 1% solution of aluminum chloride in ethanol, it gave a yellow fluorescence. Flavone aglycones such as apigenin (4', 5, 7-trihydroxyflavone), flavonol glycosides such as isoquercitrin (quercetin-3-glucoside), and certain 3-methyl ethers of flavonols such as

TABLE 7

R_f VALUES OF METHYLATED COMPOUNDS
(Descending Chromatograms)

Compound	15% Acetic Acid-Water	60% Acetic Acid-Water	n-Butanol-Acetic Acid-Water (6:1:2 v/v)	Nitromethane-Benzene-Water (2:3:5 v/v upper layer)
Quercetin-3-methyl ether	0.18	0.65	0.88	0.13
Quercetin-3, 7-dimethyl ether	0.19	0.73	0.89	0.85
Quercetin-3-methyl ether (from tobacco)	0.17	0.63	0.87	0.13
Quercetin-3, 3'-dimethyl ether (from tobacco)	0.19	0.72	0.88	0.85
Quercetin	0.07	0.40	0.71	0.04

quercetin-3-methyl ether and quercetin-3, 7-dimethyl ether exhibit this fluorescence behavior.

After the isolated tobacco quercetin dimethyl ether was refluxed with hydroiodic acid, specific gravity 1.7, for four hours, a product was obtained which proved to be quercetin (3, 3', 4', 5, 7-pentahydroxyflavone). Identity was established by comparison of color tests, fluorescence, and co-chromatography with authentic quercetin in five different solvent systems.

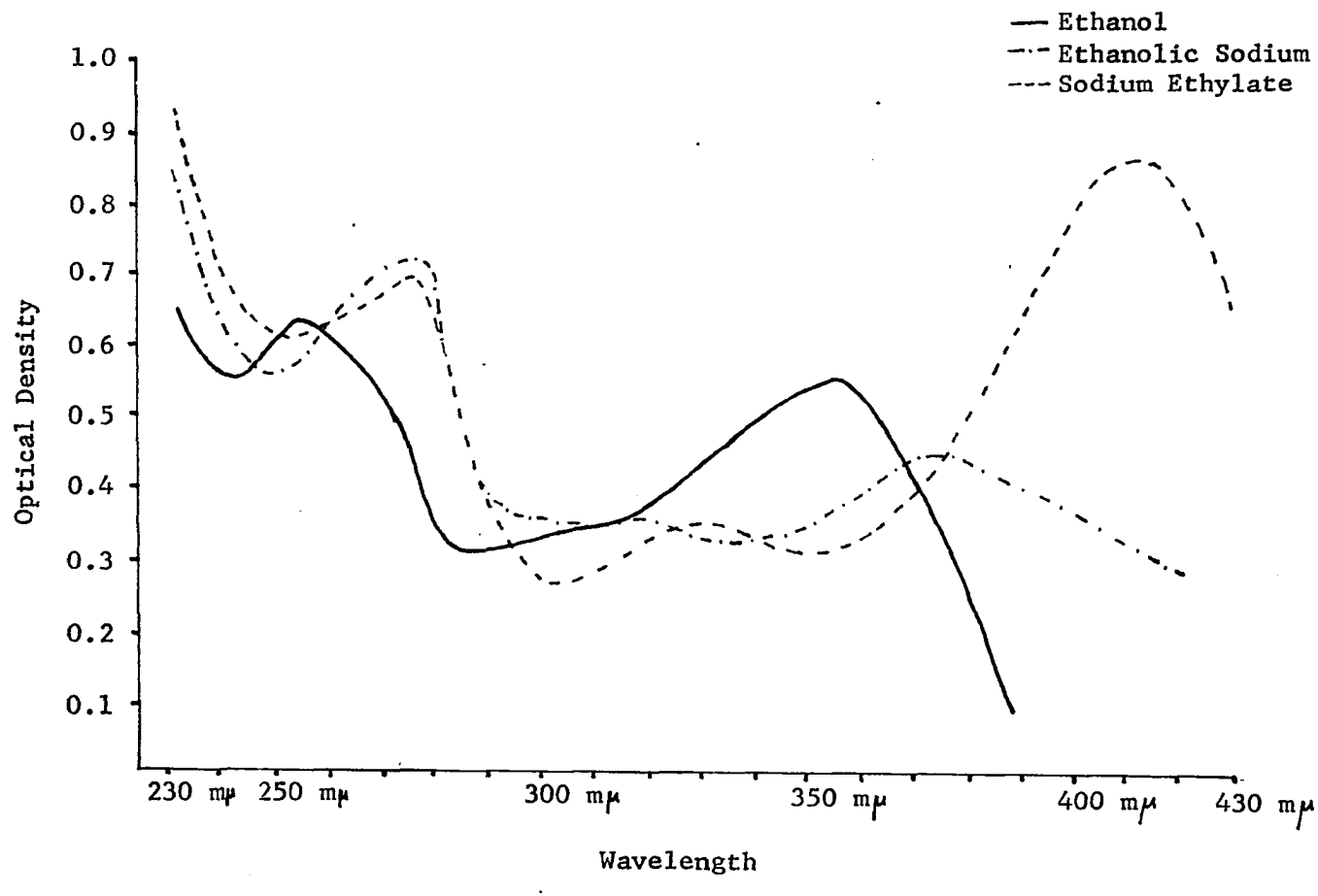
An attempt was made to hydrolyze the quercetin dimethyl ether from tobacco by heating in 7% sulfuric acid solution for 12 hours on a steam bath. No sugar was found on paper chromatograms of the reaction mixture nor was there any significant change observed in the unknown compound, although a trace of some non-flavonol material could be located on the chromatogram. The method of Jurd and Horowitz (35) was used to help establish that the hydroxy on carbon 3 is covered. When one adds sodium ethylate to the anhydrous ethanol solution of the unknown, if one or both of the hydroxyl groups in the 3 or 4' positions are covered, then there is a shift in the long wavelength band. The suspected dimethyl compound shifted from 358 mμ to 410 mμ indicating that one or both hydroxyl groups are covered (Figure 11).

These tests indicated that the unknown compound was not a flavone nor a glycoside of quercetin.

After the tobacco quercetin dimethyl ether was refluxed with dimethyl sulfate and sodium carbonate in acetone for six hours, the product fluoresced blue under ultraviolet light and was identified as quercetin-3, 3', 4', 5, 7-pentamethyl ether by chromatographic comparison

FIGURE 11

SPECTRAL SHIFTS OF QUERCETIN 3, 3'-DIMETHYL ETHER



with an authentic sample. Thus, the unknown was definitely a methyl ether of quercetin with at least one methoxy group at the 3-position.

Mixtures of quercetin-3-methyl ether and quercetin-3, 7-dimethyl ether, which were synthesized and purified as described in later paragraphs, could be readily separated by paper chromatography using the solvent system nitromethane-benzene-water (2:3:5 v/v, upper layer) with R_f values of 0.13 and 0.85 respectively. The naturally occurring compound had an R_f value of 0.83 in this solvent system, thus indicating the likelihood of its being a dimethyl and not a monomethyl quercetin-3-methyl ether.

The fluorescence was quenched by the addition of acetic anhydride to the solid compound by the method of Kuhn and Low (36), indicating a free 5-hydroxy group on the quercetin. The reaction of the isolated compound with alcoholic ferric chloride solution likewise indicated a free phenolic group at the 5-position.

The addition of anhydrous sodium acetate to the solution of the tobacco quercetin dimethyl ether by the method of Jurd and Horowitz (35) caused a shift in the short wavelength band of its ultraviolet absorption spectrum (Figure 11) from 254 mμ to 275 mμ . This indicates that the 7-hydroxy position of the tobacco quercetin dimethyl ether is open.

The long wavelength band of the ultraviolet absorption spectrum of the tobacco unknown did not shift when the ethanol solution was saturated with boric acid and anhydrous sodium acetate (37). Thus, at least one hydroxyl of the ortho-dihydroxy group (3', 4') of quercetin was blocked.

Degradation of the isolated tobacco quercetin dimethyl ether was carried out by dissolving 1 mg. of the unknown in 30 ml. of a 2N solution of sodium hydroxide in a mixture of 50% ethanol-water and evaporating the solution to dryness in an oven at 120° C. The residue was dissolved in water, acidified with hydrochloric to a pH of 2, and extracted four times with 20 ml. portions of ether. The ether solution was concentrated to 1 ml. and studied by paper chromatography.

Vanillic acid (4-hydroxy-3-methoxybenzoic acid) was proved by paper chromatography in four different solvent systems (Table 8) and distinguished from isovanillic acid by the method of Hergert and Goldschmid (27). Thus, the 4'-position of the tobacco quercetin dimethyl ether has a free phenolic group, whereas the 3'-position has a methoxy group on it. The structure of the isolated tobacco compound is therefore quercetin 3, 3'-dimethyl ether.

Preparation of Pure Quercetin-3-Methyl Ether and Quercetin-3, 7-Dimethyl Ether

These two compounds were synthesized by the method reported by Pankajamani and Seshadri (38) for quercetin-3, 7-dimethyl ether. On paper chromatographic examination, the resulting methylated quercetin precipitate appeared to be a complicated mixture containing probably five or more different derivatives of quercetin. Using methyl alcohol as the suspending medium, the precipitate was absorbed onto Magnesol (Food Machinery and Chemical Corporation, New York). The column was developed with a solvent system containing two parts of water-saturated ethyl acetate and one part nitromethane. The faster-moving brown-fluorescing material, with some traces of blue-fluorescing impurities, moved rapidly off

TABLE 8

CHROMATOGRAPHIC COMPARISON OF DEGRADATION PRODUCT TO VANILLIC ACID

Solvent System	Degradation Product	Vanillic Acid
n-Butanol-Ethanol-Ammonium Carbonate Buffer (20:11:19)	0.22	0.22
Chloroform-Acetic Acid-Water (2:1:1)	0.95	0.96
n-Butanol-Acetic Acid-Water (6:1:2)	0.93	0.96
20% Potassium Chloride-Water	0.47	0.51

the column, leaving the major portion of blue-fluorescing substances on the column. The eluates containing the brown-fluorescing mixture were then further purified by extended paper chromatography using, in order, the solvent systems 60% acetic acid-water, 15% acetic acid-water, and nitromethane-benzene-water (2:3:5 v/v, upper layer) for purification of the quercetin-3-methyl ether. To obtain pure quercetin-3, 7-dimethyl ether, the 60% acetic acid, nitromethane-benzene-water, and finally 60% acetic acid systems were used. Each of these compounds was eluted from its final chromatogram with 50% methyl alcohol-water. The purified quercetin-3-methyl ether checked in every respect (fluorescence, R_f values, color tests, and spectral studies) with authentic quercetin-3-methyl ether, which was kindly furnished by Dr. R. M. Horowitz, U.S.D.A. Fruit and Vegetable Laboratory, Pasadena, California. The 3-methyl ether had an absorption peak shift from 257 mμ to 272 mμ when treated with anhydrous sodium acetate. The shift on treatment with sodium ethylate was from 359 mμ to 410 mμ. The identity of the purified synthetic quercetin-3, 7-dimethyl quercetin was checked using similar procedures to those described above for the determination of the structure of the tobacco quercetin-3, 3'-dimethyl ether isolated from tobacco. Kuhn and Low's test showed the presence of a free 5-hydroxyl group. Spectral procedures of Jurd and Horowitz resulted in a shift of the absorption peak from 358 mμ to 404 mμ when treated with sodium ethylate and indicated the covering of either 3 or 4' hydroxy. Treatment with anhydrous sodium acetate did not result in a shift and thus indicated a covered 7 hydroxy group. Jurd's spectral test for a free dihydroxy group was positive, and on degradation the compound produced 3, 4-dihydroxybenzoic acid. The

synthetic compound was thereby established to be the 3, 7-dimethyl ether of quercetin.

CHAPTER VII

SUMMARY

The synthesis of scopoletin from esculin via benzylation as described in this dissertation is the first method reported that will give an overall yield high enough to make the synthesis of large amounts of this compound practical.

The solution of the synthesis problem made possible the study of the urinary metabolites of scopoletin. The metabolites identified from rats fed scopoletin were esculetin, unchanged scopoletin, scopoletin glucuronide and scopoletin sulfate. The finding of esculetin as one of the metabolites was quite unexpected since demethylation of phenolic ethers in biological systems has not been previously proved.

The investigation as to the urinary metabolites produced when animals are fed esculin and esculetin resulted in the identification thus far of scopoletin, 6-hydroxy-7-methoxycoumarin, esculetin sulfate, 6-hydroxy-7-methoxycoumarin sulfate and esculetin glucuronide.

Administration of quercetin to rats resulted in the excretion of numerous phenolic breakdown products. The compounds identified were 3, 4-dihydroxyphenylacetic acid, m-hydroxyphenylacetic acid, m-hydroxycinnamic acid, m-hydroxyphenylpropionic acid, vanillic acid, homovanillic acid, and tentatively, m-hydroxybenzoic acid. A neutral compound was isolated and characterized as an o-dihydroxy lactone; it may be the

key to what happens to the phloroglucinol portion of the molecule. The absence of m-hydroxyphenylacetic acid when the animal was fed 3, 4-dihydroxyphenylacetic acid created doubt as to the validity of DeEds theory that the m-hydroxyphenylacetic was produced from the 3, 4-dihydroxyphenylacetic acid. Further experimentation is needed, however, before one draws any definite conclusion on this point.

The appearance of 5, 7, 3', 4'-tetramethylquercetin in the urine of an animal that was fed this compound is proof of absorption of the intact molecule in at least that one instance. The absence of phenolic acids in the above case led to the assumption that to have breakdown, certain of the hydroxy groups of quercetin derivatives must be free.

The experiments with gastric mucin strongly indicated that the breakdown which took place was not an enzymatic reaction. The breakdown was much too slow and the active fraction was dialyzable. The experiment with the tied-off animal stomachs proved rather conclusively that quercetin breakdown did not occur in the stomach under the experimental conditions used.

Two quercetin methyl ethers isolated from tobacco were characterized. One of them was identified conclusively as the 3, 3'-dimethyl ether of quercetin. The other was tentatively identified as the 3-methyl ether of quercetin.

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