culent yellow precipitate was filtered off. The filtrate was extracted with four 50-ml. portions of benzene until no more color was removed. The benzene extract was then used to dissolve the precipitate, and the resulting solution was dehydrated by azeotropic distillation.

The crude chalcone solution in dry benzene was passed onto a column of Magnesol, prewashed with anhydrous benzene. The chalcone developed as a bright yellow zone near the top of the column. Under ultraviolet light, this zone exhibited dark brown fluorescence. The first 125 ml. of eluant was colorless, and, after evaporation of the benzene, yielded 1.6 g. of unreacted VI. A sharp, distinct separation between this ketone and the unreacted V which followed in the next 75 ml. of eluant was not accomplished. The unreacted, radioactive veratraldehyde was recovered from the eluant.

The chalcone, by this time, had developed as a zone below the dark impurities at the top of the column and also below a narrow pale yellow zone just under the dark impurities. After washing with a total of two columns of anhydrous benzene, the Magnesol was extruded from the top of the column, and then cut with a stainless steel spatula into the three zones. The top zone contained unknown impurities. The second zone contained tetramethyleriodictyol. It was eluted with anhydrous acetone and combined with the crude tetramethyleriodictyol obtained in a later reaction. The third zone contained the purified chalcone and was eluted with the anhydrous acetone. After removal of the solvent, VII weighed 2.3 g. (30%).

3′,4′,5,7-Tetramethyleriodictyol-2-C^{14} (VIII). A mixture of 2.3 g. of VII dissolved in 300 ml. of 95% ethanol, and 11 ml. of concentrated hydrochloric acid in 30 ml. of distilled water was refluxed for 20 hr. Then 500 ml. of distilled water was added. The resulting bright yellow precipitate was filtered off without suction, and the filtrate was extracted three times with a total of 150 ml. of benzene. This benzene was then used to dissolve the precipitate. The solution was filtered to remove the drops of water present and dried by azeotropic distillation.

The flavanone solution was then passed onto a column of Magnesol, prewashed with anhydrous benzene. The flavanone was adsorbed tightly onto the adsorbent and appeared as an ivory-colored zone in visible light and as a dull gray in ultraviolet light. The chalcone developed just below VIII. About 250 ml. of anhydrous benzene was needed to wash the unreacted VII from the column. The chalcone was recovered from this eluate and twice recycled through the same ring-closure reaction and chromatographic separation. The Magnesol, which now contained only the desired flavanone and a small amount of impurities on the top surface, was washed with 300 ml. of anhydrous acetone. This removed VIII very readily. The flavanone solution was combined with additional fractions of VIII from subsequent runs. The solvent was removed from the combined solutions and VIII was obtained as a pale yellow solid which weighed 1.5 g., a 66% yield of the chalcone.

3′,4′,5,7-Tetramethylquercetin-2-C^{14} (IX). The methylated eriodictyol was converted by means of *n*-butyl nitrite and hydrolysis, adapted from a procedure by Row and Seshadri,[8] into the corresponding tetramethylquercetin. A total of 0.45 g. of IX was obtained.

Quercetin-2-C^{14} (X). The tetramethylquercetin was dried, made into a paste with acetic anhydride, and demethylated, using hydriodic acid, sp. gr. 1.70. The yield of X was 0.3 g. The over-all conversion of labeled potassium cyanide into quercetin was 3.5%. Previous runs with unlabeled material had given an 8% yield. In the C-14 synthesis, however, a 29% recovery of intermediates, based on the labeled potassium cyanide, was obtained.

The labeled quercetin was compared on paper chromatograms with authentic synthetic quercetin and with natural quercetin obtained by hydrolysis of buckwheat rutin. The labeled product showed only one spot and gave the same R_f value as the two standards in every solvent system tried. The R_f values obtained in *n*-butyl alcohol–acetic acid-water (6:1:2, by vol.) and in 60% aqueous acetic acid with S&S #589, red ribbon paper, were 0.72 and 0.34, respectively.

In order to show the position of the labeled carbon atom, 1.3 mg. of X was diluted with 68.5 mg. of unlabeled quercetin for various analyses. The diluted quercetin had a calculated specific activity of 0.00055 mc./mM. The diluted quercetin was completely methylated with dimethyl sulfate to produce white-needle crystals of pentamethylquercetin (XI), m.p. 147°. This melting point was not depressed when a mixed melting point was taken with an authentic sample of unlabeled pentamethylquercetin. The sample of XI had a specific activity of 0.0005 mc./mM.

Seventy milligrams of XI was degraded, using 15 ml. of a solution containing 4 g. of potassium hydroxide in 45 ml. of absolute ethanol, and refluxing for 8 hr. After the ethanol was distilled off, the resulting solid was dissolved in 10 ml. of water, and concentrated hydrochloric acid was added to make the solution acid. The acidic solution was extracted with three 10-ml. portions of ethyl ether. The ether solution was extracted with two 10-ml. portions of 5% sodium bicarbonate solution. The ketone fragment, which remained in the ether, was obtained as an oil which did not contain any detectable radioactivity. The bicarbonate solution, containing III, was acidified. About 10 mg. of III was collected; m.p. 179°; specific activity 0.0005 mc./mM.

The sample of veratric acid obtained by degradation was then decarboxylated to produce radioactive carbon dioxide and a residue containing no detectable radioactivity.

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Two New Flavonol Glycosides in Commercial Xanthorhamnin

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Commercially available xanthorhamnin (rhamnetin-3-rhamninoside), supposedly pure, has been separated by mass paper chromatography into pure xanthorhamnin plus two other flavonol glycosides apparently new to the literature. All three of these glycosides contain 2 moles of rhamnose to 1 mole of galactose to 1 mole of flavonol aglycone. The carbohydrate attachment is through the number three position on each flavonol. The aglycones have been identified as rhamnazin (3′,7-dimethyl ether of quercetin), rhamnetin (7-methyl ether of

quercetin), and quercetin (3,3′,4′,5,7-pentahydroxyflavone).

Xanthorhamnin commonly is isolated from Persian or Avignon berries. Previous workers[1] have reported the presence of quercetin, rhamnazin, and rhamnetin in a hydrolyzate of the flavonoid portion of these berries, but have been unable to separate in pure form and identify the glycosides of quercetin and rhamnazin present in the berries before hydrolysis.

The anomalous behavior of xanthorhamnin in the determination of its acid dissociation constants has been reported earlier.[2] This behavior has now been accounted for by the discovery that this material was a mixture of the three closely related flavonol glycosides above, even though the commercially available xanthorhamnin had been recrystallized several times and had the expected neutralization equivalent within experimental error. Using the procedure of the previous paper[2] and the method of Simms[3] to interpret the data, the apparent acid dissociation constants of the pure xanthorhamnin have been determined to be pK'_1 8.69, pK'_2 11.28, and pK'_3 12.22.

EXPERIMENTAL

Paper chromatographic separation of the commercial xanthorhamnin. A methyl alcohol solution containing 300 mg. of commercial xanthorhamnin (S. B. Penick and Co., N. Y.) was applied as bands to 36 sheets of Whatman 3MM chromatography paper, 18 1/4 × 22 1/2 in., prewashed with a 2% hydrochloric acid solution and then with distilled water. The chromatography chamber was first allowed to equilibrate for 10 hr. in the presence of 2 l. of distilled water and of 200 ml. of the organic layer of the solvent system *n*-butyl alcohol-chloroform-acetic acid-water (4:4:1:1, by vol.). The solvent trays were then filled with the organic layer of the solvent system; the sheets were next allowed to develop for approximately 10 hr., then removed and allowed to air dry. Three major zones resulted and could be detected by ultraviolet "black-light": Fraction I (R_f 0.68); Fraction II (R_f 0.55); and Fraction III (R_f 0.37). Each zone was cut out separately and eluted with methyl alcohol. The eluates were concentrated to 100 ml., and a large quantity of ethyl ether was added. Fractions I and II each yielded an amorphous yellow precipitate. Each was recrystallized as a yellow crystalline compound from 50% ethyl alcohol - 50% isopropyl alcohol. Fraction III did not crystallize under these conditions, but remained as a brownish oil. The 300-mg. sample yielded 20 mg. of Fraction I, 80 mg. of Fraction II, and about 10 mg. of Fraction III.

Fraction I. Hydrolysis with 2% sulfuric acid yielded the aglycone rhamnazin, and the sugars rhamnose and galactose. The aglycone had m.p. 214°, and its acetyl derivative, m.p. 154°, which check well with the literature.[4] All melting points were determined on a Fisher-Johns melting point block. This aglycone co-chromatographed with authentic rhamnazin in all four different solvent systems tried, and no lowering of the melting point was observed on mixing the aglycone from Fraction I with authentic rhamnazin. Demethylation of the aglycone with hydriodic acid produced quercetin.

The sugars rhamnose and galactose were identified by means of paper chromatography, using known standards for comparison on Whatman No. 1 paper in the solvent system *n*-butyl alcohol-pyridine-water (2:1:1.5, by vol.). Spray reagents consisted of solutions of aniline hydrogen oxalate and of naphthoresorcinol. Co-chromatography of each sugar with an authentic sample of L-rhamnose and D-galactose was carried out in the system above as well as in *n*-butyl alcohol–acetic acid–water (4:1:5, by vol.) and phenol-saturated with water solvent systems.

For determination of moles of sugar to moles of aglycone in the rhamnazin glycoside, 10 mg. of Fraction I, m.p. 187°, was completely hydrolyzed in 2% sulfuric acid for 2 hr. After cooling the mixture, the aglycone was collected on a weighed sintered glass filter and dried *in vacuo* over phosphorus pentoxide at 80° to constant weight. Three moles of sugar to 1 mole of rhamnazin were found.

For determination of the ratio of rhamnose to galactose in the glycoside, 1.5 mg. of the glycoside was hydrolyzed, and the sugar solution was separated from the aglycone, as described above. The sugar solution was then streak chromatographed on Whatman 3MM chromatography paper in the *n*-butyl alcohol-pyridine-water system, cut out, and eluted with distilled water into a 5-ml. volumetric flask and made up to volume. Each of these steps was performed as quantitatively as possible. A 1-ml. aliquot was then reacted with the anthrone reagent according to the method of Yemm and Willis,[5] with the exception that the anthrone reagent was made by dissolving the crystalline anthrone in 85% sulfuric acid instead of in 70% sulfuric acid. The optical density was determined at 625 mμ on the Beckman spectrophotometer. Simultaneously with the unknown, samples containing known microgram amounts of the authentic sugars were carried through each step of the procedure. Standard curves, one for each sugar, were obtained by plotting optical density against sugar concentration. Both rhamnose and galactose yielded a straight line relationship at concentrations from 0–80 micrograms. The sugars were found to be present in the ratio of 2 moles of rhamnose to 1 mole of galactose.

Methylation of the rhamnazin glycoside with dimethyl sulfate, followed by hydrolysis, and subsequent recrystallization from ethyl alcohol according to the method of Shimokoriyama[6] yielded the tetramethoxy quercetin, m.p. 195–196°, indicating that sugar is attached only to the number three position in the glycoside.

Thus, the new glycoside has a trisaccharide containing 2 moles of rhamnose and 1 mole of galactose attached to the number three position of rhamnazin.

Fraction II (*pure xanthorhamnin*). Hydrolysis yielded the aglycone rhamnetin, m.p. 293°; acetyl derivative 187°,[4] and the sugars rhamnose and galactose. Studies similar to those on Fraction I above confirmed these identities of the aglycone and sugars. There were 2 moles of rhamnose to 1 mole of galactose to 1 mole of rhamnetin, and the sugar was attached at the number three position.

This glycoside appears to be pure xanthorhamnin, corresponding qualitatively and quantitatively in aglycone and sugar content to the flavonoid in the literature by that name, although the m.p. 195° of the pure xanthorhamnin is higher than the previously reported value, 160°.[7]

Fraction III. Hydrolysis yielded the sugars rhamnose and

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galactose and an aglycone identified as quercetin by previously described procedures.[8]

This flavonol glycoside, probably new to the literature, has a trisaccharide containing 2 moles of rhamnose and 1 mole of galactose attached to the number three position of the aglycone quercetin (1 mole).

TABLE I

R_f VALUES OF FLAVONOL GLYCOSIDES AND AGLYCONES

Compound	Solvent System: *n*-Butyl Alcohol–Acetic Acid–Water, 4:1:5, by vol.	Acetic Acid–Water 15:85, by vol.	Acetic Acid–Water 2:3, by vol.
Fraction I	0.54	0.70	0.86
Fraction II	0.51	0.68	0.84
Fraction III	0.45	0.62	0.78
Aglycone from I	0.82	0.10	0.60
Aglycone from II	0.80	0.10	0.52
Aglycone from III	0.77	0.10	0.41

TABLE II

COLOR REACTIONS OF FLAVONOL GLYCOSIDES AND AGLYCONES[a]

Compound	Spray Reagents: Benedict's Solution V.	Benedict's Solution U.V.	1% Alcoholic Aluminum Chloride V.	1% Alcoholic Aluminum Chloride U.V.	Basic Lead Acetate V.	Basic Lead Acetate U.V.
Fraction I	Y	Y	Y	Y	Y	YGr
Fraction II	Y	OBr	Y	Y	Y	OBr
Fraction III	Y	OBr	Y	Y	Y	OBr
Aglycone from I	Y	YBr	Y	YGr	Y	Y
Aglycone from II	Y	O	Y	YGr	Y	O
Aglycone from III	Y	O	Y	YGr	Y	O

[a] V. = Visible light, U.V. = Ultraviolet light, Y = Yellow, Br = Brown, Gr = Green, O = Orange.

Materials and apparatus for acid dissociation constant determination. The purified xanthorhamnin prepared as described above, was dried for several weeks at *ca.* 1 mm. in a vacuum desiccator containing separate vessels of phosphorus pentoxide and potassium hydroxide pellets. A sample of 59.19 mg. of the xanthorhamnin required 0.0180 ml. of 3.8472*N* sodium hydroxide (corrected for blank) to attain the first end point in the titration curve, giving an observed molecular weight of 855. The pentahydrate, $C_{34}H_{42}O_{20}\cdot 5H_2O$ would require 860.76.

Carbonate-free sodium hydroxide solutions were standardized against potassium hydrogen phthalate. Oxygen-free nitrogen passed through suitable traps to remove acidic and basic gases was swept over the titration solutions. Beckman *p*H 7.00 buffer was used to standardize the *p*H meter, and the glass electrode system was calibrated with Sörenson glycine-sodium hydroxide buffer standards.[9] Meter readings agreed with the buffer values within 0.05 *p*H unit.

The titrations were performed in 10-ml. beakers fitted with covers having holes for admitting the electrodes, buret, and nitrogen, and were placed on the door of a Beckman model G *p*H meter. The meter was operated with the door open, using Beckman 290-E and 270 electrodes, and shielding was provided by surrounding the electrode system with aluminum foil. The solutions were stirred magnetically with a borosilicate glass-encased stirring bar, except while readings were being taken. The standard base was measured with a calibrated Gilmont micro buret-pipet. The experiments were performed in an air-conditioned room at 24° ± 0.5°; no other temperature control was attempted.

Procedure for pK′ determination. Using a microbalance, 59.19 mg. of the xanthorhamnin was weighed out. This was dissolved in 4.00 ml. of water. The solution was placed on the *p*H meter and swept with nitrogen, with stirring, until the meter reading was constant. The buret was then inserted, and increments of 3.8472*N* sodium hydroxide were added so as to produce *p*H increments of about 0.1. A blank of 4.00 ml. of water was titrated in the same way. Meter readings were corrected to the Sörenson buffer values. The *p*H range studied was 7 to 12, with the results shown by representative data in Table III.

TABLE III

XANTHORHAMNIN *p*K′ DETERMINATION USING 3.8472*N* SODIUM HYDROXIDE

Base, ml.	*p*H	Base, ml.	*p*H	Water: Base, ml.	Water: *p*H
0.0012	7.49	0.0201	10.43	0.0001	9.83
0.0025	7.80	0.0217	10.67	0.0002	10.18
0.0042	8.09	0.0235	10.85	0.0003	10.38
0.0065	8.39	0.0248	10.95	0.0004	10.50
0.0095	8.74	0.0280	11.16	0.0006	10.71
0.0125	9.10	0.0300	11.26	0.0008	10.85
0.0152	9.47	0.0325	11.40	0.0010	10.96
0.0165	9.70	0.0350	11.51	0.0013	11.07
0.0174	9.87	0.0380	11.64	0.0017	11.19
0.0183	10.08	0.0410	11.72	0.0022	11.30
0.0192	10.25			0.0028	11.40
				0.0035	11.50
				0.0045	11.62
				0.0057	11.72
				0.0070	11.82

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