THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

STUDIES OF FLAVONOID COMPOUNDS FROM THE SWEET ORANGE AND THE GRAPEFRUIT

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Norman, Oklahoma

1961

STUDIES OF FLAVONOID COMPOUNDS FROM THE

SWEET ORANGE AND THE GRAPEFRUIT

APPROVED BY

DISSERTATION COMMITTEE

ACKNOWLEDGMENT

The author wishes to express his deep appreciation to Dr. Simon H. Wender for suggesting this problem, for providing a research assistantship from funds made available to him, and for his counsel, encouragement, and patience during the course of this research. He would also like to express his gratitude to Sunkist Growers, Inc., for providing funds which made possible these investigations.

The author also wishes to thank the faculty and staff of the Department of Chemistry for their guidance, cooperation, and assistance.

Finally, he wishes to express his appreciation to his fellow graduate students, whose fellowship and cooperation will long be remembered.

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STUDIES OF FLAVONOID COMPOUNDS FROM THE SWEET ORANGE AND THE GRAPEFRUIT

CHAPTER I

INTRODUCTION

The first isolation of a flavonoid compound from citrus fruit was recorded in 1828 by Lebreton (1), who obtained from a tincture of macerated oranges a crystalline substance which he called hesperidin. Subsequent investigation over many years finally established that hesperidin is a flavanone glycoside, the 7-rhamnoglucoside of hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone, Figure 1), and that it is the principal flavonoid of the sweet orange, Citrus sinensis. Naringin, the predominant flavonoid of the grapefruit, Citrus paradisi, was discovered in 1857 by De Vry (2) in flowers of the shaddock, Citrus grandis, of which the grapefruit is thought to be a mutant. Naringin is the 7-rhamnoglucoside of naringenin (4′,5,7-trihydroxyflavanone, Figure 1). Eriocitrin, the 7-rhamnoglucoside of eriodictyol (3′,4′,5,7-tetrahydroxyflavanone, Figure 1), and disomin, the 7-rhamnoglucoside of the flavone diosmetin (3′,5,7-trihydroxy-4′-methoxyflavone, Figure 2), together with hesperidin have been established as the most abundant flavonoid glycosides of the lemon, Citrus limon (3,4,5,6).

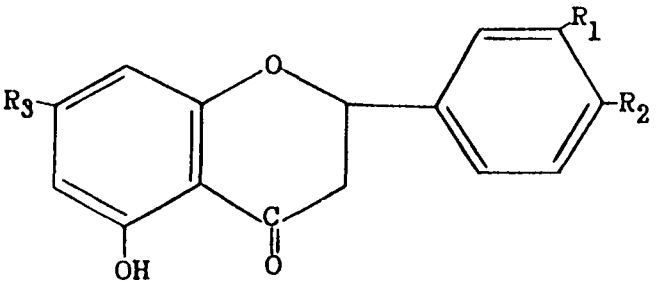
Investigations on other citrus fruits, such as the sour orange,

Citrus aurantium, the tangerine, Citrus reticulata, and the trifoliate orange, Poncirus trifoliata, have resulted in the identification of a number of other citrus flavonoid compounds. Although this work has been adequately reviewed (7,8,9), certain of these compounds should be mentioned here. These include: the flavanone glycosides neohesperidin, a 7-rhamnoglucoside of hesperetin which differs from hesperidin only in position of the linkage between rhamnose and glucose (10,11,12) and poncirin, a 7-rhamnoglucoside of isosakuranetin (5,7-dihydroxy-4'-methoxyflavanone, Figure 1) (13,14,15,16); the flavone glycoside rhoifolin, the 7-rhamnoglucoside of apigenin (4',5,7-trihydroxyflavone, Figure 2) (17); and certain highly methoxylated flavones, such as auranetin (3,4',6,7,8-pentamethoxyflavone, Figure 2) (18) and nobiletin (3',4',5,6,7,8-hexamethoxyflavone, Figure 2) (19), which have not been reported as occurring in any other natural source.

The flavonoid compounds found in citrus fruits are of considerable interest. Much of this interest was originally aroused by reports which indicated that citrus flavonoids might be physiologically active. In a series of papers in 1936, Szent-Györgyi and associates (20,21,22) reported that an extract from lemon juice, called "citrin", was effective in decreasing capillary permeability and in increasing the lowered resistance of minute blood vessels to bleeding in patients with certain pathological conditions. The active fraction of this lemon juice extract, which these workers called "vitamin P" because of a vitamin-like activity which they attributed to it, was reported to consist mainly of flavonoid compounds. These reports greatly increased interest in flavonoids from citrus as well as those from other sources, and stimulated commercial production of

FIGURE I

FLAVANONES FOUND IN CITRUS FRUITS



Hesperidin - R_1= OH, R_2= OCH_3, R_3= Rhamnoglucosyl

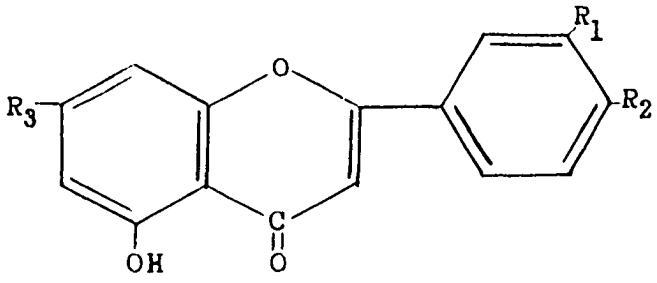
Naringin - R_1= H, R_2= OH, R_3= Rhamnoglucosyl

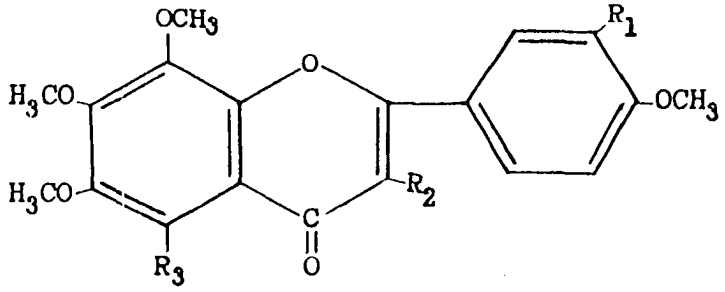
Eriocitrin - R_1= OH, R_2= OH, R_3= Rhamnoglucosyl

Poncirin - R_1= H, R_2= OCH_3, R_3= Rhamnoglucosyl

FIGURE 2

FLAVONES FOUND IN CITRUS FRUITS





Rhoifolin - R_1= H, R_2= OH, R_3= Rhamnoglucosyl

Diosmin - R_1= OH, R_2= OCH_3, R_3= Rhamnoglucosyl

Auranetin - R_1= H, R_2= OCH_3, R_3= H

Nobiletin - R_1= OCH_3, R_2= H, R_3= OCH_3

flavonoids from citrus waste. Commercially produced citrus flavonoid preparations, particularly those from oranges and lemons, have been employed in many investigations designed to elucidate the possible physiological role of flavonoids. The results of most of these investigations, which have often been conflicting and uncertain, have been extensively reviewed (23,24,25). Because of the failure of these investigations to establish the identity of a substance of vitamin nature in flavonoid fractions supposedly having "vitamin P" activity (indeed, there was even considerable negative evidence regarding any physiological activity at all in such fractions) the term "vitamin P" fell into disfavor. However, interest in flavonoids from citrus and other sources continued, and the term "bioflavonoid" was applied to those which appeared possible to be biologically active (26). Reports have continued to suggest that the bioflavonoids may have some important pharmacological properties, indicating that they may be involved in such functions as maintenance of the integrity of the capillary tree, inhibition of the oxidation of epinephrine, and physiological action of vitamin C.

The flavonoids present in citrus fruits are of technological and economic interest to the expanding citrus processing industry, which now utilizes more than half of the annual orange, grapefruit, and lemon crop of the United States. An essential feature of this industry is the profitable utilization of the waste material from processing for manufacture of by-products. This waste, which amounts to more than half the weight of citrus fruits, is mainly composed of the peel and a fraction known as rag and pulp, which consists of fragments of the fruit core and segment walls. From the brightly colored epidermal layer of the peel, the flavedo, large

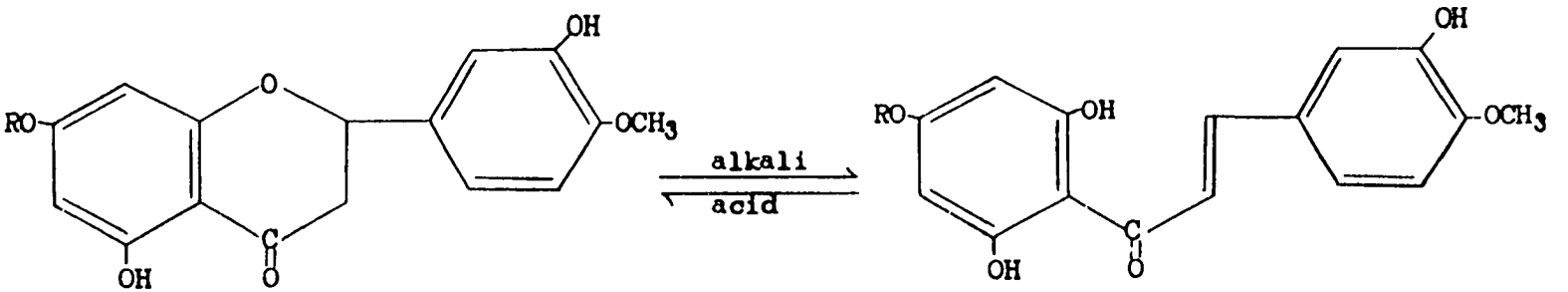
quantities of essential oils are produced. The white, spongy, parenchymatous layer of the peel, the albedo, and the rag and pulp are used for manufacture of pectin and cattle feed. The albedo, rag, and pulp also contain almost 90% and 75-80% respectively of the total flavonoid glycosides of the grapefruit and orange (27,28). Two and one-half to 4% of the dry weight of these fruits consists of flavonoid glycosides, with the lemon appearing similar in this respect. Thus, profitable recovery of these compounds from citrus wastes is of commercial interest.

There has been a relatively small but steady commercial production of several citrus flavonoid preparations, principally fractions similar to the original "citrin" from the lemon, and hesperidin fractions from the orange, during the last twenty five years. These products have been employed mostly for investigational use, both laboratory and clinical, and for use in pharmaceuticals (23,24,25). In recent years there has also been commercial production of naringin from grapefruit wastes. Naringin products have apparently found little use clinically or in pharmaceuticals but are used in the food industry to enhance the piquant flavor of beverages, confections, and marmalade made from sweet oranges (29). Hesperidin and naringin have also been recommended as coupling agents in the preparation of acid azo dyes and wood stains, and as starting materials for production of fine chemicals (27,28).

Processes for production of hesperidin and naringin are based on conversion of the flavanone glycosides to the corresponding chalcones. In alkaline medium the γ-pyrone ring of flavanones opens to produce yellow colored, highly soluble chalcones, which may be reconverted to the less soluble flavanones by acidifying the medium (Figure 3). In a typical

FIGURE 3

HESPERIDIN AND HESPERIDIN CHALCONE



Hesperidin

Hesperidin Chalcone

procedure, used by Sunkist Growers, Inc. for the production of hesperidin products (29), ground orange peel is heavily limed to give a pH of about 11. The flavanone glycosides present, which consist mostly of hesperidin, are converted to the corresponding chalcones, and the extract containing these compounds is then squeezed from the peel residue by powerful presses. The exudate is filtered, pumped to a glass-lined tank, and brought to pH 6 with hydrochloric acid. The acidulated solution is heated and allowed to stand overnight, during which time the chalcones are reconverted to flavanones, which crystallize out within a few hours at room temperature. The crystalline material is filtered, and may be recrystallized to obtain more highly purified hesperidin. Naringin is produced in a similar process.

Flavonoid compounds may affect the general quality and acceptability of processed citrus products, such as juice and canned segments. Naringin is extremely bitter, one part in 50,000 being detectable by taste, and is responsible for much of the bitterness of the grapefruit. Since the peel, segment walls, and core of the grapefruit contain most of the naringin, great care must be exercised to minimize extraction of this compound from these nonedible portions of the fruit during processing, or excessively bitter products may result. Debittering of grapefruit products by enzymatic hydrolysis of the naringin to the aglycone naringenin or the monoglucoside of naringenin, called prunin, neither of which is bitter, has been suggested (30).

Unlike naringin, hesperidin is tasteless, but it is very insoluble in most solvents, with only one part being soluble in 50,000 parts of water at 21°C. Hence, if excessive amounts of hesperidin are extracted

into orange or lemon products during processing, crystalline masses of this glycoside are likely to form in the finished product. Also, excessive hesperidin may precipitate as a scale on processing equipment, necessitating costly shutdowns of processing plant operations.

The flavonoid compounds of citrus fruits are also of interest because of their possible contribution to the nutritional value of fresh fruit. The presence of flavonoids in oranges has been exploited in advertisements urging consumption of fresh oranges.

The occurrence of flavonoid compounds in citrus fruits invites conjecture regarding the possible function and biosynthesis of these compounds. It has been generally observed (12,27,28,31) that the absolute flavonoid content per fruit increases during the growth of citrus fruits up to a certain equatorial diameter (about 2 in. for grapefruit and oranges) and then becomes approximately constant; the increase in size and weight of the growing fruit then results in a decrease of the percentage of flavonoids with maturity. The percentage of total solids which consists of flavonoids in fruit in the early stages of development is very high. Workers in Florida (27,28) reported up to 75% naringin on a dry weight basis in grapefruit one-half inch in diameter, and up to 35% hesperidin in sweet oranges of that size. The extraordinarily high concentration of flavonoids found in small citrus fruits appears to indicate that these compounds may have an important metabolic role in citrus, particularly in early development of the fruit. However, such a metabolic role has not been demonstrated for flavonoids in citrus or other plants (32,33,34), and the actual function remains obscure. And, although some progress has been made regarding the biosynthesis of flavonoids in plants (35,36,37), the major questions

regarding flavonoid biosynthesis in citrus remain unanswered.

Regardless of the interest in the flavonoids of citrus fruits, and the many investigations of the content of these compounds in various species and varieties of fruit, the knowledge of the individual flavonoid content of the various fruits is still rather meager. Investigations have generally succeeded only in identifying the flavonoids present in largest quantity in a given fruit. The sweet orange, C. sinensis, may be presented as an example. Hesperidin has long been known as the principal flavonoid of this fruit, and flavonoid preparations from this source have received much attention and study because of the possible physiological activity of such substances. Nevertheless, no flavonoid other than hesperidin had previously been unequivocally identified in this fruit or flavonoid-rich preparations from it, even though the presence of such compounds was suspected (22,38,39). Paper and column chromatographic studies in this laboratory have indicated that most hesperidin samples, including even supposedly pure research ones, have varied in composition, containing other flavanone glycosides, and often non-flavanone materials. A similar situation has apparently existed with other citrus fruits and flavonoid-rich preparations produced from them, although recent studies by Horowitz (3,4,5,6) have served to clarify the situation with regard to the flavonoid content of the lemon.

The general lack of chromatographic purity of flavonoid samples from citrus, and the absence of unequivocal elucidations of the flavonoid contents of most citrus fruits are probably attributable to the extreme difficulty in separating closely related flavonoid glycosides, particularly the flavanone glycosides which predominate in the orange and grapefruit. These

compounds have a remarkable tendency to form complex crystals with other similar glycosides, which greatly affect their solubility and other physical properties, thus making their purification by classical methods, such as solvent partition and recrystallization, very difficult. This is especially true when one glycoside must be separated from a closely related one which is present in much greater quantity. Also, lack of unequivocal methods for identification of small quantities of pure flavanone aglycones has impeded identification work.

The development of satisfactory procedures by which components of complex flavonoid mixtures from citrus fruits could be separated and purified, and application of these methods in studies to elucidate further the flavonoid content of oranges and grapefruit were the objectives of this thesis research. The accomplishment of these objectives could be expected to be of use for several reasons. More complete determination of the flavonoids of the orange could yield information concerning the variations in flavonoid composition which might be expected in hesperidin fractions prepared from it, and thus ultimately assist in explaining some of the conflicting results obtained in laboratory and clinical tests in which commercial hesperidin samples have been employed (22,23,24). Also, methods of separation and purification of the citrus flavonoids would make possible the preparation of highly purified samples of individual flavonoids which could be used in physiological studies. And, additional information concerning the flavonoids of oranges and grapefruit would be of value in studies on the biosynthesis and role in plant metabolism of flavonoids.

This work was initiated because of the interest of Sunkist Growers, Inc., in the flavonoid content of the orange, and of hesperidin fractions

which this corporation produces from this fruit. In Chapter II the isolation, purification, and identification of closely related flavanone glycosides from the sweet orange, C. sinensis, are described. A flavonoid fraction isolated in this laboratory from fresh orange peel, and hesperidin samples provided by Sunkist Growers were examined, with column and paper chromatographic procedures being successfully employed for these studies. When some success was achieved in the study of the orange flavanones, application of similar methods was attempted for examination of a commercial naringin product prepared by Sunkist Growers from the grapefruit, C. paradisi. In Chapter III the isolation, purification, and identification of some flavonoids from this source are described.

CHAPTER II

STUDIES OF SOME FLAVONOID COMPOUNDS FROM THE SWEET ORANGE

Isolation of a Flavonoid-Rich Fraction from Fresh Orange Peel

Two cases of ripe Sunkist Valencia Senator oranges, grown by the Francis Citrus Association, Tustin, California, and kindly supplied by Sunkist Growers, Inc., Ontario, California, were washed by dipping in "Vel" solution, scrubbing briefly, and rinsing thoroughly in tap and finally distilled water. The peels (flavedo and albedo) were carefully removed and ground with isopropyl alcohol in a laboratory wet grinder and extractor (40). The ground peel was then extracted twice with approximately 6 l. of isopropyl alcohol and once with approximately 6 l. of 85% isopropyl alcohol - 15% water at boiling temperature for 5 hr. per extraction. The filtered extracts obtained, which were orange-red in color, were combined and reduced in volume *in vacuo* to about 800 ml. A heavy precipitate which formed was filtered, and this precipitate was then twice suspended in and filtered from hot methyl alcohol. Chromatographic study of the methyl alcohol washings showed that they contained the same components as the precipitate, plus other substances which did not appear to be flavonoids. The washings were not studied further. The precipitate, designated Precipitate 2, was thoroughly washed with hot acetone, which

removed all of its orange-yellow color. A powdery, light-weight tan material remained, which in appearance and on paper chromatograms was very similar to Sunkist hesperidin products. This material gave a deep red-purple color on reduction with magnesium and hydrochloric acid, and a red-orange color when dissolved in concentrated sulfuric acid; both reactions are typical of flavanones.

Preliminary Chromatographic Studies of Flavonoids from Oranges

Paper Chromatographic Studies

Previous work in this laboratory had indicated that paper chromatographic methods were quite effective for separating many closely related flavonoids, particularly flavones and flavonols and their glycosides. The flavonoid-rich fraction isolated from fresh orange peel, Precipitate 2, and hesperidin preparations produced from oranges by Sunkist Growers were, therefore, subjected to paper chromatography in order to determine the feasibility of these methods for separation of flavonoid mixtures from oranges and also to obtain information regarding the complexity of the various samples.

Samples provided by Sunkist were: Product No. 168, Hesperidin Complex, a commercial product said to contain 63% hesperidin plus other unknown flavonoids; Product No. 165, Hesperidin Purified, a commercial product containing 80% minimum hesperidin; Sample No. D-2775, Crude Hesperidin; and Sample No. A6209, Hesperidin (Purified), a highly purified research sample of hesperidin, which was the purest hesperidin available. Methyl alcohol solutions of these preparations and Precipitate 2 were spotted and streaked heavily on Whatman 3 MM and No. 1 papers. The

chromatograms were developed in a number of solvent systems, including n-butyl alcohol:acetic acid:water (6:1:2, v/v/v, hereafter referred to as BAW), 15% acetic acid:water, 60% acetic acid:water,benzene: nitromethane: water (3:2:5, v/v/v, hereafter called BNW), n-butyl alcohol:pyridine: benzene:water (5:3:1:3, v/v/v/v, called BAPBW), solvents containing dimethyl sulfoxide (in which hesperidin is very soluble), and solvents containing ligroin (41). The chromatograms revealed that all the samples contained more than one component, although the quantities of all but the major component, presumably hesperidin, were very low. The complexity appeared to vary directly with the stated purity of the sample, with Precipitate 2 appearing similar to the Hesperidin Complex. Most of the trace components appeared to be very similar to the major component, and were therefore, thought to be flavanone glycosides. Sample No. A6209 appeared to be very pure, but when chromatograms of heavy concentrations of this substance were sprayed with 1% alcoholic aluminum chloride and viewed under very intense U.V. light (3660Å, Blak-Ray, Ultra-Violet Prod. Inc., San Gabriel, California), traces of impurities were visible.

These studies indicated that the use of paper chromatography for separation of these orange flavonoid fractions was not feasible. The R_f values of the trace components were very similar to those of the major component in all solvent systems, making separation difficult. Also, the very low concentrations of the trace components in relation to the concentration of the major component, hesperidin, made the obtaining of sufficient quantities of the unknown substances for identification a prohibitive task. Hence, it was necessary to turn to another method for separation of the flavonoid fractions from oranges.

Pilot Column Chromatographic Studies

Column chromatography, employing the synthetic hydrous magnesium silicate, Magnesol (Food Machinery and Chemical Corp., Westvaco Chemical Division, New York), as adsorbant, had also been used successfully in this laboratory for separation of some mixtures of flavonoids (42,43). Therefore, the various Sunkist hesperidin samples and Precipitate 2 were chromatographed on a number of pilot columns employing this adsorbant. These pilot studies indicated that by means of chromatography on Magnesol columns closely related flavanone glycosides from oranges could be separated, and sufficient quantities of at least some of the glycosides present in minor quantity could be prepared in pure form for identification studies.

Best results were obtained when the column was packed, under 5 lb. air pressure, with Magnesol slurried in methyl alcohol. The column was then washed thoroughly with methyl alcohol to remove some yellow colored material from the Magnesol. A methyl alcohol solution of the flavanone mixture was placed carefully on the column, and when this solution had been adsorbed on the Magnesol, but before the top of the column had become dry, a small wash portion of methyl alcohol was added. When this had moved into the Magnesol, but before the surface of the adsorbant had become dry, a plug of glass wool was placed in the column above the adsorbant to protect the surface from damage during solvent filling. The column was then filled with the eluting solvent, water-saturated ethyl acetate. Movement of the flavanone glycosides on the column was slow, but good separation was attained if the column was long enough. Precipitate 2 was selected for the first large scale studies employing this technique.

Studies on the Flavanone Glycosides of Precipitate 2 (Flavonoid-Rich Fraction from Fresh Orange Peel)

Chromatographic Fractionation of Precipitate 2

A 5.5 x 100 cm. chromatographic column was prepared by packing with a slurry of Magnesol (dry cleaning grade) in methyl alcohol under 5 lb. air pressure. The packed column, depth 58 cm., was washed thoroughly with pure methyl alcohol. (All solvents used in this work were redistilled to guard against the possibility of concentration of trace solvent impurities when fractions were reduced in volume.) Three grams of Precipitate 2, the flavanone glycoside material which had been isolated from fresh orange peel, were heated in methyl alcohol for 30 min., and a small quantity of fluffy brownish material which would not go into solution was removed by filtration. The clear yellow filtrate was reduced *in vacuo* to about one liter, cooled, and placed on the Magnesol column. The material was adsorbed on the top of the column, producing a zone about 10 cm. in depth which was slightly yellow in visible light and bright blue-green in ultraviolet light. Upon addition of the developing solvent, water-saturated ethyl acetate, the color of the adsorbed material changed to yellow-green in U.V. light. Separation of components became visible after the leading edge of the material had moved about half-way down the column.

In Figure 4A, the zones which appeared during elution of this column are shown, with the designation given to them. Two or three blue fluorescing zones, which were very close together and hard to distinguish from one another, were the first visible substances to move off the column. The chromatographic behaviour and color reactions of these substances, which

were present in very low quantity, indicated that they were not flavonoids, so they were not studied further. A narrow zone, colorless in visible and yellow-green in U.V. light, moved off the column next and was designated fraction Ppt. 2D-4. The quantity of this fraction was also very low. The next zone moving down the column was a wide, bright yellow-green fluorescing one, which was colorless in visible light and which appeared to contain the major component of the original flavanone glycoside material. Before this zone moved off the column another green fluorescing zone appeared to be moving out of its leading edge. Therefore, this slightly faster moving zone plus the first portion of the wide zone from which it was emerging were collected as fraction Ppt. 2D-5.

Above the wide, bright yellow-green fluorescing zone on the column there appeared to be some pale green fluorescing material, colorless in visible light. This merged with a zone near the top of the column which was yellow in visible light and yellow-green in U.V. light. Clumps of crystalline material, presumably hesperidin which had precipitated during elution, were interspersed within these zones on the upper portions of the column. As these zones appeared to be very strongly adsorbed on the Magnesol, the column was extruded after elution of all the preceding zones was complete, and the Magnesol on which the upper yellow-green fluorescing zone, designated fraction Ppt. 2D-8, was adsorbed, was cut out and saved for further study.

Further Fractionation of Fraction Ppt. 2D-5

Of the fractions obtained from Precipitate 2, fraction Ppt. 2D-5 appeared most likely to contain a flavanone glycoside (or glycosides)

other than hesperidin in sufficient quantity for identification study. This fraction was, therefore, subjected to further investigation.

On evaporation of the water-saturated ethyl acetate solvent, a considerable quantity of white crystalline material formed in fraction Ppt. 2D-5, and this precipitate was dissolved in warm methyl alcohol. This methyl alcohol solution was then chromatographed through a Magnesol column 5 x 110 cm., packed to a depth of 60 cm. Two blue fluorescent zones which appeared to be identical with the blue fluorescent zones obtained in the original column chromatography of Precipitate 2 were the first to move off the column. A very narrow green fluorescing zone, which paper chromatograms revealed to be identical with the original fraction Ppt. 2D-4 was eluted next. Subsequent investigations of fraction Ppt. 2D-4 revealed that this fraction could be separated into two components on further passage through smaller Magnesol columns. The two components were present in very small quantity, and complete separation of one from the other was extremely difficult, even on repeated passage through Magnesol columns. Therefore, sufficient quantities of these components in pure enough form for identification studies were not obtained. R_f values for these fractions in BAW solvent on Whatman No. 1 paper were about 0.75 and 0.65, compared to a value of 0.45 for hesperidin. This gave credence to the possibility that these substances might have been flavanone monoglycosides.

Following the first blue and green fluorescing zones on the column was a wide yellow-green fluorescing zone, colorless in visible light, which slowly moved down the column. When this zone had moved about one-half way down the column it separated into three yellow-green fluorescing zones, designated according to the order in which they moved off the

column as fractions Ppt. 2D-55,56 and 57 (Figure 4B). Paper chromatography of these fractions indicated that complete separation had not yet been obtained, but a concentration of one compound in each fraction had been achieved. By repeated passage through appropriate sizes of Magnesol columns further purification of these fractions was achieved, and eventually chromatographically pure compounds were obtained, as described below.

Purification and Tentative Identification of Fraction Ppt. 2D-55

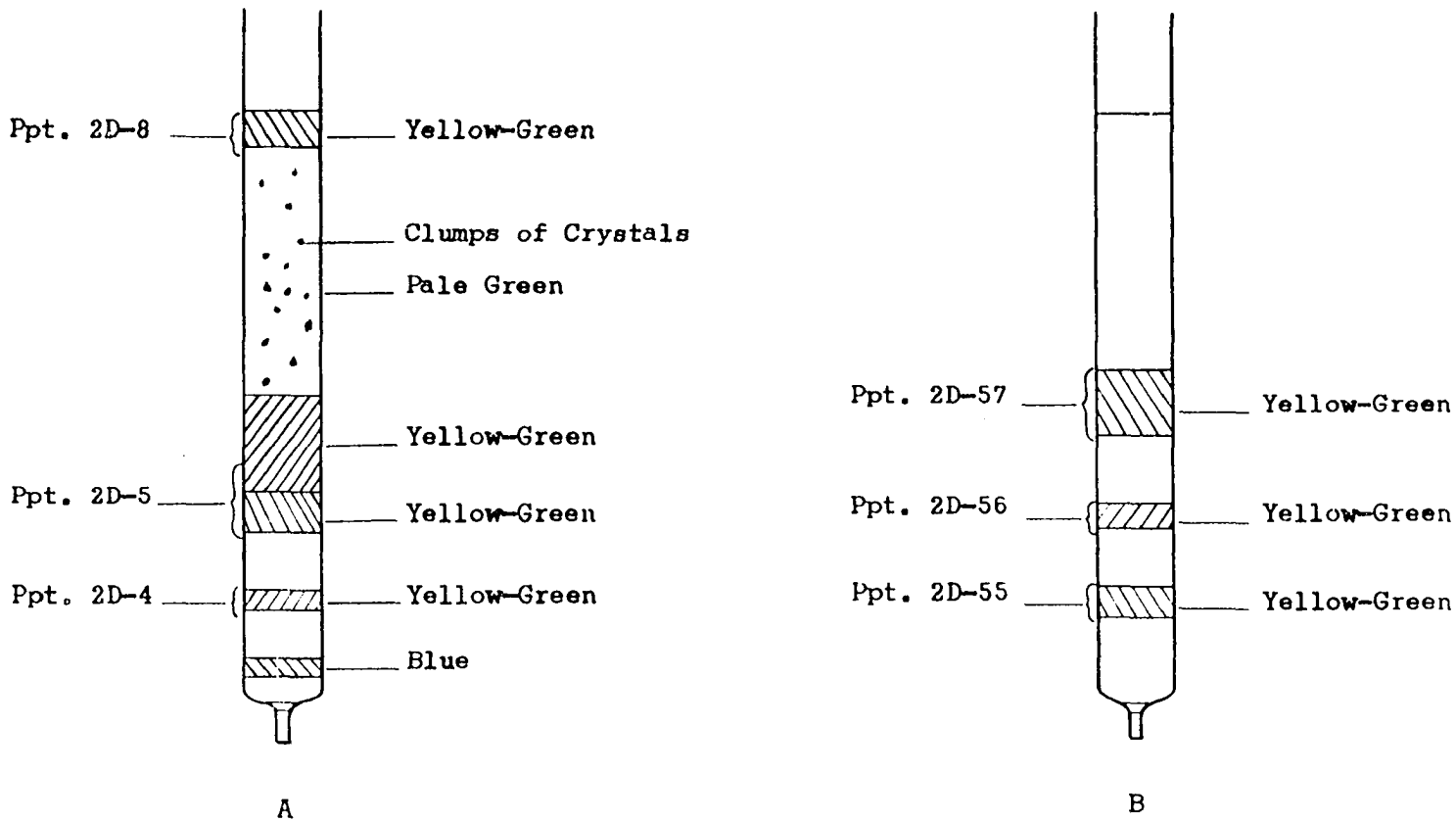
Fraction Ppt. 2D-55 was reduced to dryness *in vacuo*, and the residue was dissolved in methyl alcohol. This solution was chromatographed on a Magnesol column 4.5 cm. in diameter which was packed to a depth of 37 cm. Two green fluorescing zones, which paper chromatography revealed to be traces of fractions Ppt. 2D-56 and 57, trailed the major green fluorescing zone off the column. This major zone was subjected to similar rechromatographing procedures three more times, employing Magnesol columns 4.5 x 24 cm., 3.5 x 55 cm., and 3.5 x 55 cm. Each rechromatographing further purified the major fraction, until on the last column no further separation of impurities could be detected. Paper chromatograms indicated a high degree of purity of the resulting fraction Ppt. 2D-55.

Upon reduction of the ethyl acetate solution of purified fraction Ppt. 2D-55, a white flocculent material precipitated. This was recrystallized from 95% ethyl alcohol as white crystalline aggregates that appeared to be made up of minute needles which melted at 207-208° on a Fischer melting point block. These crystals were considerably more soluble than hesperidin crystals. The ultraviolet absorption spectrum of this compound

FIGURE 4

COLUMN CHROMATOGRAPHIC FRACTIONATION OF
FLAVANONES FROM ORANGE PEEL

Fluorescence in Ultraviolet Light



in 95% ethyl alcohol was typical of the spectra given by flavanone glycosides, with maxima at 283 and 331 mμ and minima at 247 and 315 mμ , log ϵ = 4.31 at 283 mμ . This spectrum served definitely to confirm that Ppt. 2D-55 was a flavanone glycoside, but could give no further aid in establishing the identity of the molecule since all flavanone glycosides and all flavanone aglycones give essentially the same U. V. spectra (Figure 5).

The R_f values of fraction Ppt. 2D-55 in BAW (0.59), in 15% acetic acid (0.79), and in distilled water (0.51), using Whatman No. 1 paper and descending chromatography, differed slightly, but significantly, from those of reference samples of naringin (0.53, 0.80, 0.64) and hesperidin (0.47, 0.75, 0.51, Table 1). (The reference hesperidin sample was Sunkist Sample No. A6209, which previously had been shown not to be chromatographically pure, but which was the best hesperidin sample available. The unavailability of pure reference samples was a problem which plagued much of this work.)

Five milligrams of purified Ppt. 2D-55 were hydrolyzed by refluxing with 20 ml. of 3% hydrochloric acid for 4 hours. The aglycone produced was obtained from the aqueous hydrolysis mixture by extracting three times with ethyl acetate. The aqueous solution remaining after the aglycone had been extracted was deionized by passing it through columns of Amberlite IR 45 anion exchange resin and Dowex 50 X 2 cation exchange resin. The deionized solution, containing the sugars from Ppt. 2D-55, was reduced in vacuo to about 5 ml. This concentrate was chromatographed against glucose, galactose, and rhamnose standards in the BAPBW solvent system. When the developed chromatogram was air dried for 2 hr., sprayed

FIGURE 5

ULTRAVIOLET ABSORPTION SPECTRA OF A
FLAVANONE GLYCOSIDE AND AGLYCONE

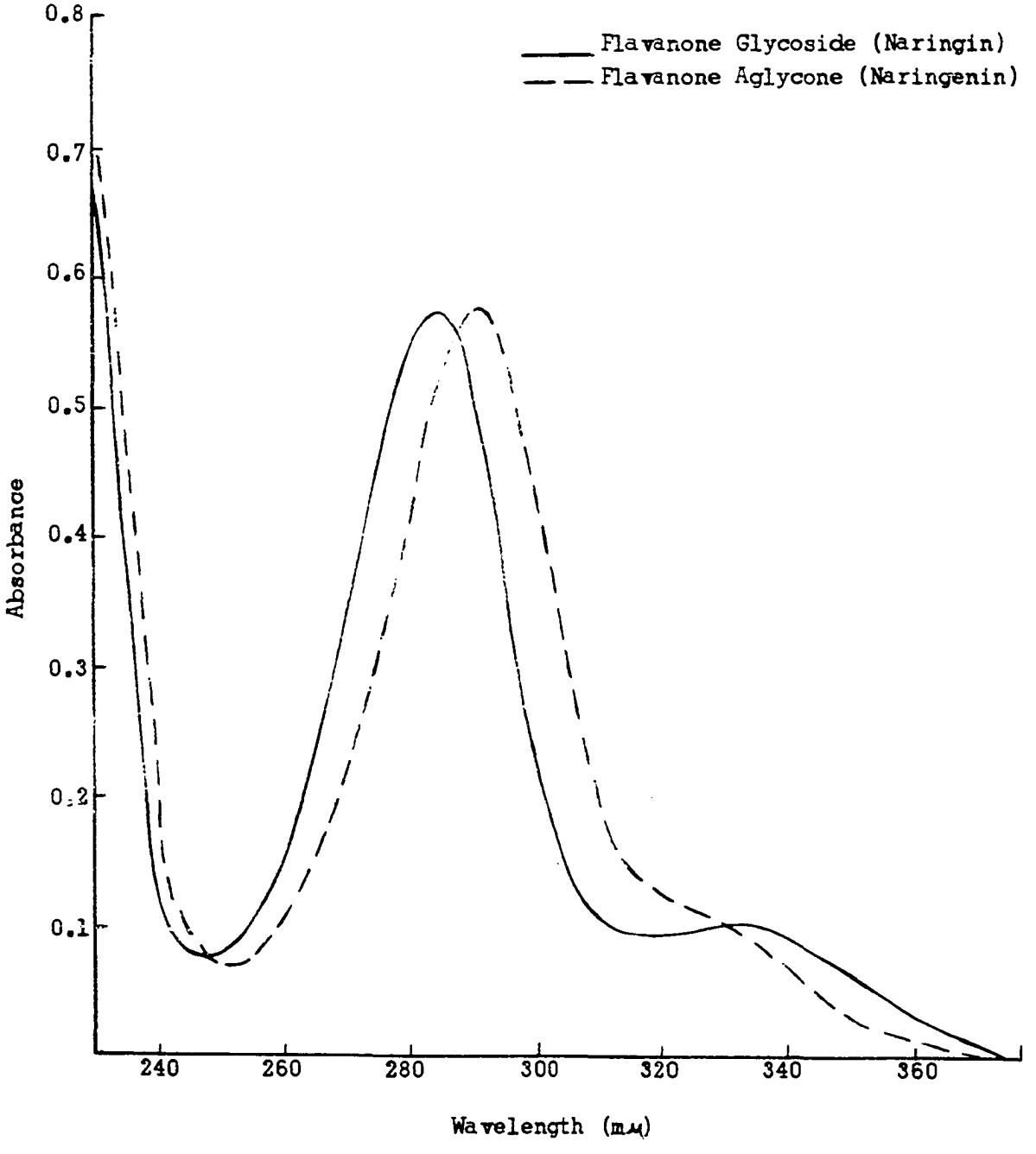


TABLE 1

R_f VALUES OF REFERENCE FLAVANONE GLYCOSIDES AND GLYCOSIDES ISOLATED FROM ORANGES

Glycoside	Distilled Water	15% Acetic Acid	n-Butyl Alcohol: Acetic Acid: Water (6:1:2)
Naringin	0.64	0.80	0.53
Hesperidin (A6209)	0.51	0.75	0.47
Ppt. 2D-55	0.51	0.79	0.59
Ppt. 2D-56	0.63	0.80	0.52
Ppt. 2D-57	0.50	0.75	0.45

with o-aminobiphenyl spray reagent (44), air dryed for 5 min. more, and heated in an oven at 110°C. for 5 min., the sugar solution from Ppt. 2D-55 was found to give spots corresponding to rhamnose and glucose.

The ethyl acetate extract of the hydrolysis mixture was chromatographed against hesperetin, naringenin, eriodictyol, homoeriodictyol (4',5,7 trihydroxy-3'-methoxyflavanone), and isosakuranetin reference standards. The R_f values for the aglycone from Ppt. 2D-55 in BAW (0.94), in 60% acetic acid (0.83), and NBW (0.97) agreed most closely with those for the reference isosakuranetin, kindly supplied by Dr. Hasegawa of Japan. Hence Ppt. 2D-55 was tentatively identified as a rhamnoglucoside of isosakuranetin. However, the very similar fluorescence and R_f values of the flavanone aglycones (Table 2) made unequivocal identification of these compounds by paper chromatography very difficult. Therefore, R_f values alone were not considered adequate evidence to establish with certainty the identity of the aglycone of fraction Ppt. 2D-55. The small quantity of sample available precluded confirmation of identification by classical methods such as determination of melting point and making of derivatives. It was necessary, therefore, to develop a method for positively identifying very small quantities of flavanone aglycones.

Development of a Method for Positive Identification of Very Small Quantities of Flavanone Aglycones

A method for positively identifying very small quantities of flavanone aglycones was developed by adapting to semi-micro scale the classical aqueous potassium hydroxide degradation of flavanones. The products of such a degradation were phloroglucinol and a substituted cinnamic acid; for instance, hesperidin yielded phloroglucinol and isoferulic acid, while

TABLE 2

R_f VALUES OF REFERENCE FLAVANONE AGLYCONES AND AGLYCONES OF GLYCOSIDES FROM ORANGES

Aglycone	Nitromethane: Benzene Water (2:3:5)	n-Butyl Alcohol: Acetic Acid: Water (6:1:2)	60% Acetic Acid
Isosakuranetin	0.97	0.94	0.83
Hesperetin	0.92	0.92	0.79
Naringenin	0.82	0.93	0.77
Eriodictyol	0.36	0.89	0.69
Homoeriodictyol	0.93	0.92	0.80
Ppt. 2D-55	0.97	0.94	0.83
Ppt. 2D-56	0.82	0.93	0.76
Ppt. 2D-57	0.92	0.92	0.78

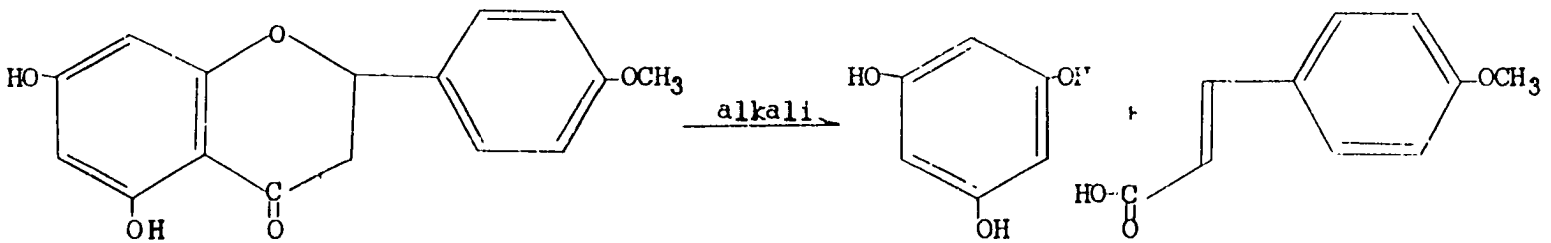
isosakuranetin yeilded phloroglucinol and p-methoxycinnamic acid (Figure 6). Paper chromatographic studies showed that such degradation products could be differentiated and positively identified on the basis of their R_f values in different solvent systems, of their fluorescence under ultraviolet light in air and when exposed to ammonia vapor, and of their color reactions with chromogenic spray reagents (Tables 3 and 4). Hence, if a small quantity of a flavanone aglycone was properly degraded, the degradation products could be positively identified by paper chromatography, and the identity of the original molecule could thus be established with certainty.

It was found that degradation of flavanones was satisfactorily accomplished by mixing the aglycone with a small quantity of 30% aqueous potassium hydroxide (usually about 2-3 ml. of alkali per milligram of aglycone) and refluxing for 2.5 hr. The degradation mixture was then made acidic (pH about 4) with dilute sulfuric acid, and extracted 3 times with ethyl ether. The ether extract was washed twice with small quantities of water, reduced in volume and chromatographed in comparison with standard substituted cinnamic acids and phloroglucinol.

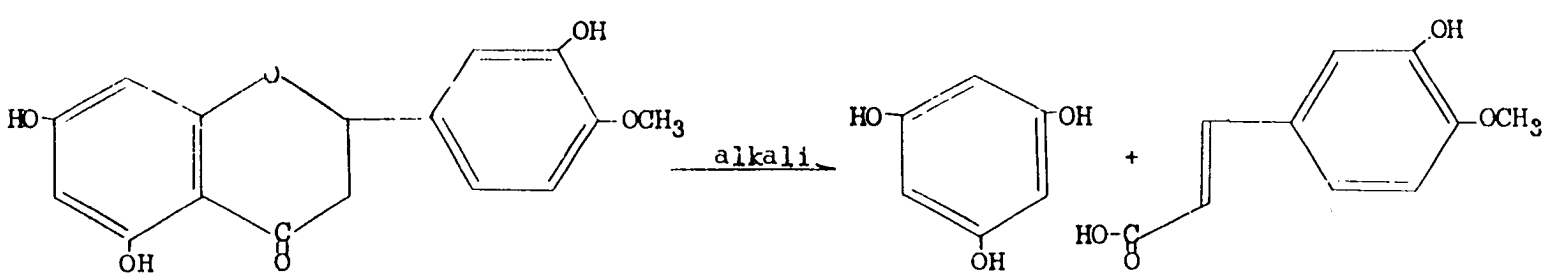
Most of the substituted cinnamic acids required for reference standards in these studies were available in relatively pure form commercially. However, it was necessary to synthesize the o-, m- and p-methoxycinnamic acids required for comparison with degradation products. This was accomplished by condensing the appropriate aldehydes with malonic acid according to the method of Vorsatz (45). The o- and m-methoxycinnamic acids were of interest because of the possibility that fraction Ppt. 2D-55 might have been a glycoside of the aglycone citronetin. Citronetin, which had

FIGURE 6

DEGRADATION OF ISOSAKURANETIN AND HESPERETIN



Isosakuranetin Phloroglucinol p-Methoxycinnamic Acid



Hesperetin Phloroglucinol Isoferulic Acid

TABLE 3

R_f VALUES OF REFERENCE AROMATIC ACIDS AND PHLOROGLUCINOL

Compound	Solvent System* 1	2	3	4
o-Coumaric Acid	0.88	0.47	0.34	0.85
p-Coumaric Acid	0.86	0.44	0.26	0.79
Ferulic Acid	0.81	0.31	0.25	0.92
Isoferulic Acid	0.83	0.40	0.29	0.90
Phloroglucinol	0.70	0.79	---	---
o-Methoxycinnamic Acid	---	0.61	0.38	---
m-Methoxycinnamic Acid	---	0.59	0.34	---
p-Methoxycinnamic Acid	---	0.54	0.27	---
Anisic Acid	---	0.46	0.24	---
3,4 Dimethoxycinnamic Acid	---	0.44	0.28	---
3,4 Dimethoxybenzoic Acid	---	0.37	0.21	---

*Solvent System: 1. n-Butyl Alcohol: Acetic Acid: Water (6:1:2), descending chromatography.
2. n-Butyl Alcohol: Ethyl Alcohol: Ammonia-Ammonium Carbonate Buffer (40:11:19), ascending chromatography.
3. n-Butyl Alcohol: Nitromethane: Ammonia-Ammonium Carbonate Buffer (2 parts buffer-saturated alcohol, 1 part nitromethane), ascending chromatography.
4. Chloroform: Acetic Acid: Water (2:1:1), ascending chromatography.

TABLE 4

FLUORESCENCE AND COLOR REACTIONS OF REFERENCE AROMATIC ACIDS AND PHLOROGLUCINOL

Compound	Fluorescence		Color Reactions	
	Ultraviolet Light	Ultraviolet Light in NH_3 Fumes	Diazotized Sulfanilic Acid (49)	Diazotized p-Nitro-aniline (50)
o-Coumaric Acid	blue white	yellow green	orange	wine
p-Coumaric Acid	negative	bright blue	red pink	gray purple
Ferulic Acid	blue	sky blue	purple	gray purple
Isoferulic Acid	blue	tan or cream	orange pink	dark purple
Phloroglucinol	negative	blue	yellow brown	brown
o-Methoxycinnamic Acid	blue	fades	----	----
m-Methoxycinnamic Acid	blue	fades	----	----
p-Methoxycinnamic Acid	deep purple (faint)	fades	----	----
Anisic Acid	negative	negative	----	----
3,4 Dimethoxycinnamic Acid	blue	fades	----	----
3,4 Dimethoxybenzoic Acid	negative	negative	----	----

been reported as a constituent of the Ponderosa lemon, was first referred to as 5,7-dihydroxy-2'-methoxyflavanone (46), but was later described by other authors (47) as being 5,7-dihydroxy-3'-methoxyflavanone.

An n-butyl alcohol: ethyl alcohol: ammonia-ammonium carbonate buffer (40:11:19 v/v/v, buffer 1.5 N with respect to both ammonia and ammonium carbonate) solvent system (henceforth called BEAAC) (48) and a modification of this system prepared by mixing one part nitromethane with two parts of n-butyl alcohol saturated with ammoni-ammonium carbonate buffer (called BNAAC) were found to be very useful solvent systems for identification of the cinnamic acids produced by degradation. These systems gave small, concentrated spots with no tailing, and therefore permitted easier location of weak fluorescing spots and gave more clearly defined spots with indicator spray reagents, particularly for very low concentrations of acids. R_f values in these solvent systems, plus fluorescence, permitted differentiation between o-, m-, and p-methoxycinnamic acids. A chloroform: acetic acid: water (2:1:1, lower layer) system, called CAW, was also used for chromatography of the hydroxy substituted cinnamic acids. These three solvent systems gave best results using ascending chromatography and Whatman No. 1 paper. BAW, employed with descending technique, was found to be a good solvent for chromatography of phloroglucinol and a fair solvent for the hydroxycinnamic acids.

Diazotized sulfanilic acid (49) and diazotized p-nitroaniline (50) spray reagents were employed with chromatograms of the cinnamic acids containing free hydroxyl groups, and phloroglucinol, not only to assist in locating the substances on the chromatogram, but also to produce colors which were often characteristic and useful in identification. For chroma-

tograms of the cinnamic acids possessing only methoxy substituents, and which, therefore, would not couple with the diazotized reagents used, buffered methyl red (0.1% alcoholic methyl red - 0.167 M phospate buffer, 1:2) and 0.4% 2,6-dichlorophenolindophenol in 95% ethyl alcohol were found useful as spray reagents for locating the acid spots, particularly those of low fluorescence, on the chromatograms. With the former spray, acids appeared as pink spots on a yellow background, while with the latter they appeared as red spots on a blue background. The sensitivity of these two sprays appeared to be about equal.

The procedures described above were successfully employed for degradation of one milligram samples of the flavanone aglycones hesperetin, homoeriodictyol, isosakuranetin, and naringenin and for identification of their degradation products. Satisfactory results were not obtained with eriodictyol, probably because the caffeic acid which should have been produced on degradation of this compound was destroyed by the alkaline degradation conditions.

Identification of Fraction Ppt. 2D-55 (Isosakuranetin-7-Rhamnoglucoside)

By employing the method for positively identifying very small quantities of flavanone aglycones described above, the identity of the aglycone of Ppt. 2D-55 was definitely established. The aglycone obtained by hydrolysis of 5 mg. of fraction Ppt. 2D-55 and evaporation of the solvent from the ethyl acetate extract of the hydrolysis mixture was degraded by refluxing for 2.5 hr. with 8 ml. of 30% aqueous potassium hydroxide. The ether extract of the degradation mixture was chromatographed with o-, m-, and p-methoxycinnamic acids, with 3,4-

dimethoxycinnamic, anisic, and 3,4-dimethoxybenzoic acids, and with phloroglucinol in the BEAAC and BNAAC solvent systems, and with phloroglucinol only in BAW. Papers were examined under ultraviolet light and under ultraviolet light while exposed to ammonia. Finally the papers were sprayed with buffered methyl red and 2,6-dichlorophenol-indophenol reagents to locate definitely the spots produced by acids with low intensity or no fluorescence. Spots corresponding to phloroglucinol and p-methoxycinnamic acid in R_f values and fluorescence were found in the degradation extract, thus confirming that the aglycone of fraction Ppt. 2D-55 was isosakuranetin.

The molecule of fraction Ppt. 2D-55 consisted, therefore, of the aglycone isosakuranetin plus rhamnose and glucose. The similarity of this compound to hesperidin and naringin in R_f values and fluorescence indicated that it was probably a rhamnoglucoside. The major peak of the ultraviolet absorption spectrum of Ppt. 2D-55 was shifted from 283 mμ in 95% ethyl alcohol to 306 mμ in 0.04 M alcoholic aluminum chloride, indicating that the 5-OH group of the isosakuranetin molecule was open and available for chelation. Since the 4'-position contained a methoxy group, the sugar was undoubtedly attached to the 7-position.

As a final check of structure the product obtained by complete methylation of fraction Ppt. 2D-55 was compared with that obtained from authentic naringin. Isosakuranetin-7-rhamnoglucoside and the well known compound naringin would be expected to give the same products on complete methylation. Five milligrams each of fraction Ppt. 2D-55 and naringin were completely methylated by refluxing for 6 hr. with 15 ml. of anhydrous acetone, 1 ml. of dimethylsulfate, and 3 g. of anhydrous

potassium carbonate. Upon filtration and reduction of the acetone filtrate by boiling, white crystalline material, M.P. 203-205° (block), precipitated out in each case. When the crystals produced by complete methylation of the two starting materials were mixed, no depression in the melting point was observed, indicating that they were identical.

Purification and Identification of Fraction Ppt. 2D-56 (Naringin)

Fraction Ppt. 2D-56 was purified, employing essentially the same procedure as described for fraction Ppt. 2D-55 above, by rechromatographing it successively on two Magnesol columns with dimensions of 3.8 x 40 cm. and 4.5 x 45 cm. Traces of fractions Ppt. 2D-55 and 57 were removed on both columns, but paper chromatograph indicated the major fraction from the second column was pure. When this fraction was reduced to dryness *in vacuo*, a white solid with a slight tinge of yellow color was deposited. This was easily soluble in ethyl acetate, ethyl alcohol, and methyl alcohol. The ultraviolet absorption spectrum of this compound was typical of that for flavanone glycosides, with maxima at 283 and 330 mμ and minima at 245 and 316 mμ, $\log \epsilon = 4.37$ at 283 mμ. R_f values for purified fraction Ppt. 2D-56 in BAW (0.52), 15% acetic acid (0.80), and in distilled water (0.63) checked with those for authentic naringin. PPt. 2D-56, like authentic naringin, was found to have a bitter taste.

Five milligrams of fraction Ppt. 2D-56 were hydrolyzed by refluxing it for 3 hr. with 20 ml. of 3% hydrochloric acid, and the aglycone and sugar fractions were separated by ethyl acetate extraction as already described for fraction Ppt. 2D-55. The aglycone was chromatographed with reference aglycones in BAW, in 60% acetic acid, and in

NBW solvent systems, and the R_f values obtained (0.93, 0.76, and 0.82 respectively) checked with those for authentic naringenin in all solvents.

The aglycone of fraction Ppt. 2D-56 was degraded by refluxing it with 30% potassium hydroxide for 2.5 hrs. and the degradation products were chromatographed with o-coumaric, p-coumaric, ferulic, and isoferulic acids and phloroglucinol in the BAW, BEAAC, BNAAC, and CAW solvent systems. Papers were examined under ultraviolet light in air and in ammonia fumes. The chromatograms were sprayed with diazotized sulfanilic acid and diazotized p-nitroaniline spray reagents. The degraded aglycone of fraction Ppt. 2D-56 produced spots on these chromatograms which agreed in R_f, fluorescence, and colors produced by reaction with diazotized reagents with those for phloroglucinol and p-coumaric acid (Tables 3 and 4). Therefore, the aglycone of fraction Ppt. 2D-56 was confirmed as naringenin.

The solution containing the sugars which were hydrolyzed from Ppt. 2D-56 was deionized by ion exchange resins and chromatographed as described above for the sugars of fraction Ppt. 2D-55. In this manner the sugars were identified as rhamnose and glucose.

Five milligrams of fraction Ppt. 2D-56 were completely methylated by refluxing for 6 hr. with 15 ml. of anhydrous acetone, 1 ml. of dimethyl sulfate, and 3 g. of anhydrous potassium carbonate. Upon filtration and reduction by boiling of the acetone filtrate, white crystalline material, m.p. 203-205° (block) fell out. When this material was mixed with completely methylated authentic naringin, no depression of melting point was obtained.

Purification and Identification of Fraction Ppt. 2D-57 (Hesperidin)

White needle crystals which had formed in the original fraction Ppt. 2D-57 were dissolved in hot methyl alcohol and rechromatographed on a 3.8 x 35 cm. Magnesol column. The major component from this column was rechromatographed twice more on columns of the same dimensions with no separation of trace materials being detected on the last column. A white precipitate, made up of minute needle crystals, formed in the receiving flask immediately after the fraction had moved off the column. This compound melted at 262-64° (block) and was found to be very insoluble in acetone, ethyl acetate, and water, very slightly soluble in ethyl alcohol, and slightly soluble in methyl alcohol. An ethyl alcohol solution of fraction Ppt. 2D-57 gave a typical flavanone glycoside ultraviolet absorption spectrum, with Maxima at 284 and 331 mμ , and minima at 247 and 316 mμ , log ϵ = 4.36 at 284 mμ . The R_f values of 0.45 in BAW, 0.75 in 15% acetic acid, and 0.50 in distilled water were very close to those of the purest available reference hesperidin, Sunkist sample A6209. Fraction Ppt. 2D-57 appeared to be chromatographically pure.

Fraction Ppt. 2D-57 was somewhat difficult to hydrolyze with 3% hydrochloric acid, possibly due to the great insolubility of the glycoside, and it was found that hydrolysis could be accomplished most efficiently by refluxing the glycoside with a mixture of two parts sulfuric acid, sixty five parts acetic acid, and thirty eight parts water for 4 hr. The hydrolysis mixture was then extracted with ethyl acetate to remove the aglycone, and the aqueous fraction was saved for sugar analysis. The aglycone solution was chromatographed with standard flavanone aglycones in

the BAW, 60% acetic acid,and NBW solvent systems, and the R_f values (0.92, 0.78, and 0.92) were found to be very close to those of both hesperetin and homoeriodictyol (Table 2).

The aglycone of fraction Ppt. 2D-57 was degraded by refluxing with 30% aqueous potassium hydroxide, and the ether extract of the acidified degradation mixture was chromatographed with o-coumaric, p-coumaric, ferulic, and isoferulic acids and phloroglucinol in the BAW, BEAAC, BNAAC and CAW solvents. The chromatograms were examined under ultraviolet light in air and when exposed to ammonia fumes, and after spraying with diazotized sulfanilic acid and diazotized p-nitroaniline reagents. In this way, phloroglucinol and isoferulic acid were found to be the degradation products of the aglycone of fraction Ppt. 2D-57, and the aglycone was therefore identified as hesperetin.

The aqueous hydrolysis mixture of fraction Ppt. 2D-57, from which the aglycone had been extracted, was deionized by ion exchange resins and chromatographed with standards as previously described for fraction Ppt. 2D-55. By this means the sugars of Ppt. 2D-57 were identified as rhamnose and glucose.

On the basis of its melting point, R_f values, aglycone, and sugars, fraction Ppt. 2D-57 was identified as pure hesperidin.

Further Examination of Fraction Ppt. 2D-8

The Magnesol on which fraction Ppt. 2D-8, the uppermost zone from the original precipitate 2 column, was adsorbed was slurried with methyl alcohol and placed on top of a very short Magnesol column. After this had packed, the column was washed thoroughly with 50% methyl alcohol-50% water,

which appeared to remove most of the fluorescent material. The yellow-colored eluate was reduced almost to dryness in vacuo and dissolved in pure methyl alcohol. A Magnesol column 5 x 25 cm. was prepared by packing from a methyl alcohol slurry under 5 lb. pressure, and the methyl alcohol solution of Ppt. 2D-8 was placed on it. The column was eluted successively with pure methyl alcohol, 50% aqueous methyl alcohol, 25% aqueous methyl alcohol, and water. The fraction eluted with 50% methyl alcohol, designated Ppt. 2D-82, appeared to contain the largest quantity of material, and was chosen for further study.

Paper chromatographic studies of Ppt. 2D-82 revealed that it contained some hesperidin, and probably traces of isosakuranetin-7-rhamnoglucoside and naringin. However, it also appeared to contain other substances which had lower R_f values in BAW than hesperidin. The R_f values and fluorescence of the spots produced by these substances chromatographed in BAW were: 0.32, orange-yellow; 0.27, pale green; 0.15, green; 0.10 orange-yellow: 0.06, pale green.

Since the substances with R_f values in BAW of 0.32 or less were very tightly adsorbed on Magnesol, further chromatography on this adsorbant appeared to offer little hope for separation of these materials. Therefore, a number of pilot columns were run in an attempt to find an adsorbant which would be more suitable for this purpose. Of the adsorbants tried, only barium carbonate offered any hope for success. Therefore a 2.5 x 15 cm. column was prepared by packing with a slurry of barium carbonate (Mallinckrodt Analytical Reagent) in methyl alcohol. A very tight, slow column was obtained, due to the small particle size of the barium carbonate. A small portion of Ppt. 2D-82 was placed on this column,

and the column was eluted with pure methyl alcohol. A number of zones appeared on the column, but there was much tailing and the zones were difficult to distinguish. Eight fractions were collected, somewhat arbitrarily, during several days of elution. Paper chromatograms of these fractions indicated that a crude separation had been attained, with the first fractions from the column containing the substances with higher R_f values in BAW, and the later fractions containing the shorter running substances. However, no fraction containing only one compound was obtained.

Further experiments with larger and longer barium carbonate columns and larger quantities of fraction Ppt. 2D-82 were not successful, because of the great amount of time required (often a month or more) for elution of material from the columns, and because of the tendency of the small barium carbonate particles to "leak" during the elution, thus resulting in channeling of the column, which destroyed its effectiveness. As a result of these difficulties, and because of time limitations, further work was not done on fraction Ppt. 2D-82. Some of the substances found in this fraction may have been decomposition products formed as a result of the somewhat alkaline conditions produced when the Magnesol columns were washed with water. However, it appears likely that at least some of these substances might be unknown flavanone glycosides, possibly triglycosides, which remain to be isolated and identified.

Studies on Hesperidin Preparations Provided by Sunkist Growers

From the hesperidin samples and products provided by the Sunkist Growers, Inc., Research Department, Ontario, California, Sample No. A6209 (Purified Hesperidin) and Product No. 168 (Hesperidin Complex) were

chosen for further study. Both these preparations were produced from the peel and pulp of oranges used in the citrus processing industry. As previously noted, Hesperidin Complex was a commercial product which would be expected to contain other flavonoids in addition to hesperidin. Sample No. A6209 was a highly purified research sample of hesperidin. It could be safely assumed that any impurities found in this material would be present, probably in greater quantity, in all the other preparations. Hesperidin Complex and A6209 were subjected to the procedures developed in the studies on the flavonoid fraction isolated from fresh orange peel in this laboratory, Precipitate 2.

When Hesperidin Complex, Product No. 168, was chromatographed on a Magnesol column as previously described, essentially the same elution pattern was obtained as that given by Precipitate 2 (see Figure 4A). Further chromatographing of the fraction from this product which appeared to correspond to fraction Ppt. 2D-5 gave fractions apparently corresponding to Ppt. 2D-55, 56 and 57. By employing the procedures for identification of flavanone glycosides presented previously, each of these fractions was identified, and the presence of isosakuranetin-7-rhamnoglucoside, naringin, and hesperidin in the Hesperidin Complex was established.

Purified Hesperidin, Sample No. A6209, when chromatographed on a Magnesol column, gave the same general pattern of separation as was obtained with Precipitate 2 and Hesperidin Complex, except that the blue fluorescing substances which moved off the column first when these preparations were chromatographed were absent in the highly purified hesperidin. When the fraction from this preparation corresponding to Ppt.

2D-5 was chromatographed further, only two fractions were obtained. Examination of these fractions by previously described methods indicated that they corresponded to the crude Ppt. 2D-55 and 57 fractions, containing mostly isosakuranetin-7-rhamnoglucoside and hesperidin. Further very careful chromatographic fractionation of these fractions finally produced small quantities of a fraction which appeared to be identical with Ppt. 2D-56, and which was eventually confirmed as naringin. Obviously, naringin was present in much smaller quantity in Purified Hesperidin (A6209) than in Hesperidin Complex (Product No. 168) or Precipitate 2.

Conclusion

The experimental results obtained in the studies described above indicate that the flavanone glycoside content of the fruit of the sweet orange, Citrus sinensis, and of the so-called hesperidin fractions produced from it, is relatively complex. These investigations revealed the presence of two flavanone glycosides, isosakuranetin-7-rhamnoglucoside and naringin, which have never before been reported as constituents of the sweet orange, and indicated the probable presence of several others. Hesperidin appeared to be the major flavanone glycoside, while the others appeared to be present in very small quantities.

As noted previously, hesperidin has long been known as the principal flavanone glycoside of the sweet orange. The only other flavonoid ever reported as being present in this fruit was another flavanone glycoside, eriodictyol glucoside (22); however, no supporting chemical evidence was ever presented for this claim. This compound was not found in the studies reported here, although it is possible that it might be present in some

of the fractions not investigated.

A 7-rhamnoglucoside of isosakuranetin was first reported in blooms of the trifoliate orange, Poncirus trifoliata, by Hattori et al. (13), who called this compound "poncirin". It has since been found in the leaves and fruit of P. trifoliata (14, 15) which is often grown as an ornamental shrub in Japan, and is used as a rootstock for commercial citrus varieties in the United States and elsewhere. Since no sample of poncirin was available, the isosakuranetin-7-rhamnoglucoside isolated from the sweet orange in these studies could not be compared directly with it in order to determine if they were identical.

Naringin is, as mentioned previously, the principal flavanone glycoside of the grapefruit, Citrus paradisi. It has also been reported in several other citrus fruits, including the shaddock (Citrus grandis) (2), the sour orange (Citrus aurantium) (17), and the trifoliate orange (P. trifoliata) (14,15), but never in the sweet orange (C. sinensis).

Column chromatography as employed in these investigations, with Magnesol as adsorbant, proved to be a good method for separation of some closely related flavanone glycosides, even when one compound was present in much greater quantity than the other constituents of the mixture. This method was successfully employed for preparation of chromatographically pure isosakuranetin-7-rhamnoglucoside, naringin, and hesperidin. Although somewhat tedious, the method could be employed to obtain chromatographically pure samples of flavanone glycosides, such as hesperidin, for use in biological studies. Such studies with pure compounds would probably aid in removing some of the confusion regarding possible physiological action of flavanones.

CHAPTER III

STUDIES OF SOME FLAVONOID COMPOUNDS FROM THE GRAPEFRUIT

Studies on Flavonoid Glycosides from Sunkist Naringin, Product No. 610E2

The successful application of the previously described chromatographic methods for isolation, purification, and identification of closely related flavanone glycosides to study of some flavonoid compounds from the sweet orange prompted a similar study of some flavonoids from the grapefruit. A commercial naringin product, Sunkist Product No. 610E2, produced from the peel and pulp of grapefruit used in the citrus processing industry, was employed for these studies.

Chromatographic Fractionation of Sunkist Naringin

A 6 x 100 cm. chromatographic column was prepared by packing to a depth of 72 cm. with Magnesol (dry cleaning grade) slurried in methyl alcohol, under 5 lb. air pressure. Three and one half grams of naringin, Sunkist Product No. 610E2, were heated in methyl alcohol for 30 min., and some insoluble brown material was filtered out. The clear yellow filtrate was reduced to about 600 ml. *in vacuo*, cooled, and placed on the Magnesol column. The column was washed with a small quantity of pure methyl alcohol and eluted with water-saturated ethyl acetate.

A group of faintly visible zones, mostly blue fluorescing, but including a few which fluoresced brown and pale green in ultraviolet light, were the first visible substances to move off the column. Although a few of these substances may have been flavonoids, the quantity was so small and it was so difficult to distinguish between the zones that no attempt was made to separate them, and they were not studied further.

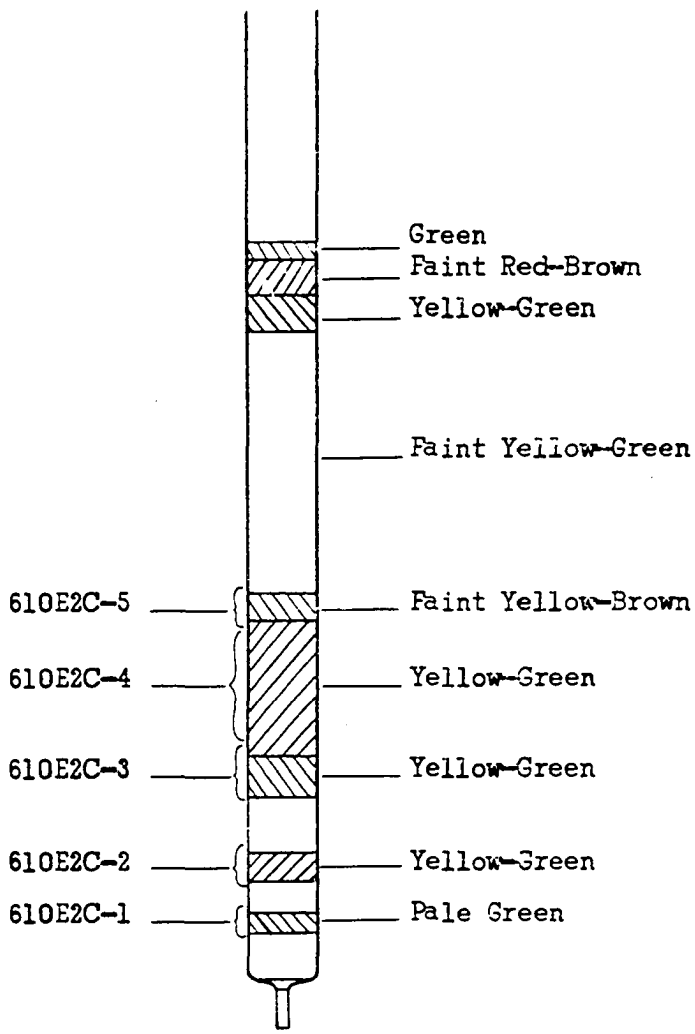
In Figure 7 the remaining zones which appeared during elution of this column are shown with the designations given to them. A narrow zone, colorless in visible and yellow-green in ultraviolet light, moved off the column next and was designated fraction 610E2C-1. The quantity of this fraction was also very low. The next zone moving down the column was a bright yellow-green one, collected as fraction 610E2C-2. This was followed by a wide, bright yellow-green fluorescing zone which appeared to be the major component of the original naringin sample. As this zone moved down the column another yellow-green fluorescing zone began separating from its leading edge, but did not completely separate before elution of the major zone began. Hence, the slightly faster moving zone, plus the first portion of the wide zone from which it was emerging (fraction 610E2C-4) were collected together as fraction 610E2C-3.

In the trailing edge of the major zone, 610E2C-4, there appeared to be a small, faint yellow-brown fluorescing zone. This faded as it moved down the column, but the eluate thought to contain it was collected as fraction 610E2C-5. Above this zone on the column there were several faintly visible zones, as shown by Figure 7, but these were so tightly adsorbed on the column, and the quantities appeared so low, that no attempt was made to elute them.

FIGURE 7

COLUMN CHROMATOGRAPHIC FRACTIONATION
OF FLAVONOIDS FROM GRAPEFRUIT

Fluorescence in Ultraviolet Light



Paper chromatographic study of the fractions obtained above indicated that fractions 610E2C-2,3 and 5 were most likely to contain flavonoids other than naringin in sufficient quantity for identification studies. Therefore, these fractions were subjected to further investigations.

Further Purification and Characterization of Fraction 610E2C-2

Fraction 610E2C-2 was reduced to dryness *in vacuo* and the residue was dissolved in methyl alcohol. This solution was chromatographed on a Magnesol column 4 cm. in diameter which was packed to a depth of 54 cm. Two green fluorescing zones, which paper chromatography revealed to be traces of fractions 610E2C-1 and 3 respectively, preceded and trailed the major green fluorescing zone off the column. Also, a faint green fluorescing zone moved out of the major zone, 610E2C-2, moving between it and 610 E2C-1. The major zone was rechromatographed three more times on Magnesol columns of 4 x 64, 4.5 x 21, and 4.5 x 30 cm., which removed all traces of 610E2C-1 and 3 from this fraction. However, it still appeared to contain traces of the pale green fluorescing zone which moved just ahead of it on the column. As a result of losses on the columns during this extended chromatography, the quantity of the still impure fraction 610E2C-2 was reduced below that needed for identification study. Therefore, more of this substance was obtained by chromatographic fractionation of Sunkist naringin as described above, and it was subjected to further purification. However, no sample of fraction 610E2C-2 was ever obtained which did not appear to contain a trace of the substance moving slightly ahead of it on columns. Therefore, unequivocal identification of this fraction was not possible.

The R_f values of fraction 610E2C-2 in BAW (0.67), 15% acetic acid (0.81), and distilled water (0.59) differed from those of any available reference flavanone glycoside (Table 5). When the solvent was evaporated from fraction 610E2C-2 a white solid with a tinge of yellow color was obtained. This substance gave an ultraviolet absorption spectrum which was typical for flavanone glycosides (max. 283 and 331 mμ).

A portion of fraction 610E2C-2 was hydrolyzed by refluxing it with 3% hydrochloric acid for 3 hr., and the aglycone was extracted from the hydrolysis mixture with ethyl acetate and compared chromatographically with reference flavanone aglycones. The aglycone checked with authentic naringenin in R_f values in BAW, 60% acetic acid, and NBW solvent systems.

The deionized aqueous hydrolysis mixture was chromatographed with reference sugars, and thus found to contain rhamnose and glucose, apparently in about equal quantities. Therefore, fraction 610E2C-2 appeared to be a rhamnoglucoside of naringenin, but not the common naringenin-7-rhamnoglucoside, naringin. However, the admitted impurity of the fraction 610E2C-2 prevented unequivocal confirmation of this conclusion.

Purification and Identification of Fraction 610E2C-3 (Isosakuranetin-7-Rhamnoglucoside)

Fraction 610E2C-3 was reduced to dryness *in vacuo*, dissolved in methyl alcohol, and rechromatographed on a 4 x 61 cm. Magnesol column. A trace of fraction 610E2C-2 preceded the major green fluorescing zone off the column, while a relatively wide zone, which proved to be identical with fraction 610E2C-4, naringin, followed it. The major zone, fraction 610E2C-3, was then purified further by successive rechroma-

TABLE 5

R_f VALUES OF REFERENCE FLAVONOID GLYCOSIDES AND FLAVONOID GLYCOSIDES FROM GRAPEFRUIT

Glycoside	Distilled Water	15% Acetic Acid	n-Butyl Alcohol: Acetic Acid: Water (6:1:2)
Naringin	0.64	0.80	0.53
Prunin (Naringenin-7-Glucoside)	0.45	0.72	0.70
Isosakuranetin-7-Rhamnoglucoside (Ppt. 2D-55)	0.51	0.79	0.59
Rhoifolin	0.15	0.54	0.50
610E2C-2	0.59	0.81	0.67
610E2C-3	0.59	0.82	0.62
610E2C-5	0.15	0.54	0.50

tography on three more Magnesol columns with dimensions of 4 x 64, 4 x 72, and 4.5 x 31 cm. Complete separation from traces of fraction 610E2C-4, naringin, was difficult, but only one zone was visible on the last column through which the purified fraction 610E2C-3 was passed, and paper chromatograms indicated that the eluted fraction was pure.

Purified fraction 610E2C-3 was found to crystallize as small clumps of needles, the melting point of which varied with the solvent and conditions of crystallization. The highest melting crystals, from ethyl alcohol, had a melting point of 209-210°. The ultraviolet absorption spectrum of this compound was typical of that for flavanone glycosides, with maxima at 283 and 331 mμ and minima at 247 and 312 mμ . R_f values for purified fraction 610E2C-3 in BAW (0.62), 15% acetic acid (0.82), and distilled water (0.59) did not agree with those of any reference flavanone glycoside available.

A small portion of fraction 610E2C-3 was hydrolysed by refluxing for 4 hr. with 3% hydrochloric acid, and the resulting aglycone was obtained from the aqueous hydrolysis mixture by extracting three times with ethyl acetate. This aglycone was chromatographed with reference flavanone aglycones in BAW, 60% acetic acid, and in NBW solvent systems, and the R_f values obtained (0.94, 0.83, and 0.97) agreed with those for reference isosakuranetin. The aglycone was then degraded by refluxing with 30% potassium hydroxide for 2.5 hr., and the degradation products were identified by paper chromatography, as described in Chapter II. These degradation products were found to be p-methoxycinnamic acid and phloroglucinol, thus confirming the aglycone of fraction 610E2C-3 as isosakuranetin.

The aqueous portion of the hydrolysis mixture from fraction 610E2C-3

was deionized by ion exchange resins, reduced *in vacuo*, and chromatographed against standard sugar solutions. The sugars were found to be rhamnose and glucose.

The position of sugar attachment to the aglycone, isosakuranetin, was established by spectrophotometric studies. The major peak of the ultraviolet absorption spectrum of 610E2C-3 was shifted from 283 mμ in 95% ethyl alcohol to 305 mμ in 0.04 M alcoholic aluminum chloride, indicating that the 5-OH group of the isosakuranetin was open. Since the 4' position contained a methoxy group, the sugar was necessarily attached at the 7-position. Studies in this laboratory with flavanones of established structure revealed that if there was a free -OH group at the 7-position, the major peak of the ultraviolet absorption spectrum was shifted to 326-327 mμ when solid anhydrous sodium acetate was added to an absolute ethyl alcohol solution of a flavanone, and the solution was allowed to stand for 5 min. before reading of the spectrum. If the 7-OH group was covered, however, as by glycosidation, no shift would occur under these conditions. (Horowitz (5) also has recently noted this phenomenon.) The spectrum of Fraction 610E2C-3 was not affected when sodium acetate was added to an alcoholic solution of it, thus providing added evidence that this compound was a 7-glycoside.

Fraction 610E2C-3 was, then, apparently a 7-rhamnoglucoside of isosakuranetin. However, it was somewhat different from the isosakuranetin-7-rhamnoglucoside isolated from the orange (fraction Ppt. 2D-5S of Chapter II) in R_f values, and the melting point of these compounds was greatly depressed when they were mixed. It seems probable that the difference may be in the position of attachment of the rhamnose to the glucose in the

sugar moiety. If this is the case, the relationship between the two isosakuranetin-7-rhamnoglucosides would be analogous to that between hesperidin and neohesperidin.

Purification and Identification of Fraction 610E2C-5 (Rhoifolin)

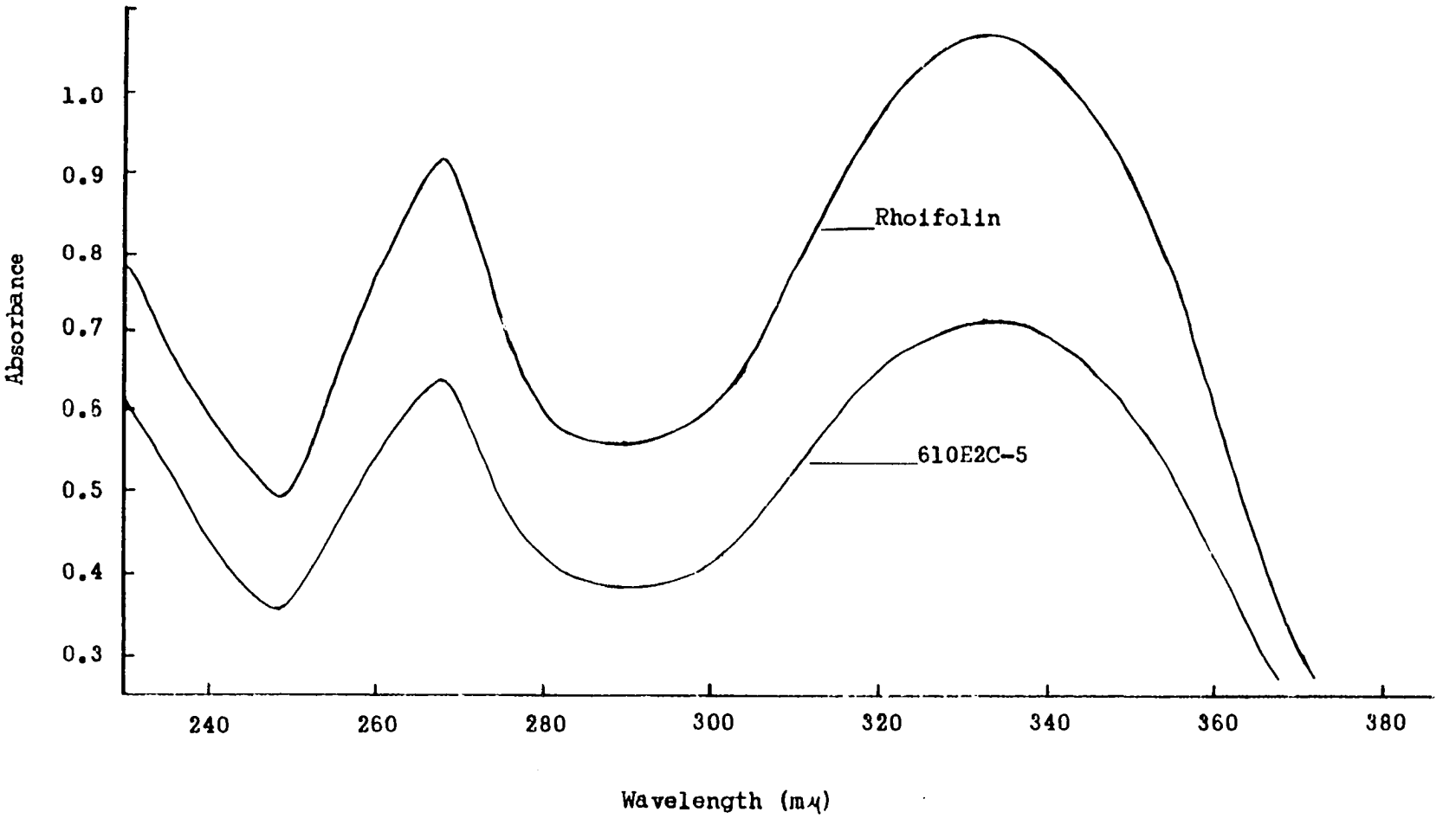
Paper chromatography of fraction 610E2C-5, the fraction which was thought to contain a faint yellow-brown zone which appeared on the original 610E2C column, revealed that it contained a substance which might be a flavone glycoside. 610E2C-5 was, therefore, subjected to further chromatography on a 4 x 62 cm. Magnesol column. A wide green fluorescing zone, which proved to be naringin, preceded a yellow-brown fluorescing zone down the column. This yellow-brown fluorescing zone was rechromatographed two more times, employing successively 4 x 60 and 4 x 50 cm. Magnesol columns. Paper chromatography of the resulting fraction 610E2C-5 indicated that a pure compound had been obtained.

Purified fraction 610E2C-5 produced spots on paper chromatograms which were brown in ultraviolet light, and yellow in ultraviolet light when exposed to ammonia vapor or after spraying with alcoholic aluminum chloride; these color reactions were typical for flavones. The Rf values for this fraction in BAW (0.50), 15% acetic acid (0.54), and distilled water (0.15) were identical with those given by authentic co-chromatographed rhoifolin (apigenin-7-rhamnoglucoside, see Figure 2 and Table 5). The ultraviolet absorption spectrum of fraction 610E2C-5 had maxima at 268 and 333 mμ and minima at 248 and 292 mμ, and was found to be identical with the spectrum produced by authentic rhoifolin (Figure 8).

A portion of purified fraction 610E2C-5 was hydrolyzed by reflux-

FIGURE 8

ULTRAVIOLET ABSORPTION SPECTRA OF
RHOIFOLIN AND FRACTION 610E2C-5



ing with 3% hydrochloric acid for 3 hr. The aglycone was extracted from the aqueous hydrolysis mixture with ethyl acetate, and chromatographed against reference flavone aglycones. The R_f's for the aglycone of fraction 610E2C-5 in BAW (0.89), in 60% acetic (0.66), and in NBW (0.48) were found to correspond with those of the co-chromatographed apigenin.

The aqueous solution remaining after the aglycone of fraction 610E2C-5 was extracted from the hydrolysis mixture was deionized by ion exchange resins, reduced in vacuo to about 5 ml., and chromatographed against reference glucose, rhamnose, and apiose solutions. The reference sugars included apiose (tetrahydroxyisovaleraldehyde) in order to be certain that 610E2C-5 was not the 7-apioglucoside of apigenin, called apiin, which was very similar to rhoifolin. The unknown and reference sugar samples were chromatographed in BAPBW and BAW solvent systems and sprayed with o-aminobiphenyl and aniline-hydrogen oxalate (51) chromogenic reagents. The solution containing the sugars hydrolyzed from fraction 610E2C-5 gave spots which corresponded to glucose and rhamnose in R_f values and color reactions. Therefore, fraction 610E2C-5 was confirmed as apigenin-7-rhamnoglucoside (rhoifolin).

Studies on Flavonoid Aglycones Produced by Hydrolysis of Sunkist Naringin, Product No. 610E2

The investigations described above indicated that the commercial naringin product, Sunkist Product No. 610E2, contained several flavanone glycosides other than naringin, plus the flavone glycoside rhoifolin. Column chromatography of this substance on Magnesol columns also indicated that it might contain other flavonoids, probably glycosides, in such small quantities that accumulation of sufficient amounts of them for study would

be very difficult employing the methods used in the glycoside studies described above. Therefore, Sunkist naringin was hydrolyzed, and studies were initiated to determine if the resulting aglycone mixture could be fractionated in such a manner as to remove the bulk of the naringenin produced from other aglycones which might be present, thus permitting study of these aglycones by paper chromatography or other methods.

Hydrolysis and Chromatographic Fractionation of Sunkist Naringin

Eighteen grams of naringin, Sunkist Product No. 610E2, were mixed with 2 l. of 3% hydrochloric acid and the mixture was refluxed for 4 hr. The aqueous hydrolysis mixture was extracted three times with equal volumes of ethyl acetate. The combined ethyl acetate extracts were reduced *in vacuo* to dryness and dissolved in methyl alcohol. Paper chromatography of this methyl alcohol solution, designated fraction 610E2HB, showed that complete hydrolysis had been obtained. These chromatograms indicated that the bulk of the hydrolysis product consisted of naringenin, but very faint spots revealed the presence also of other aglycones.

The methyl alcohol solution of fraction 610E2HB was placed on a 6 x 65 cm. Magnesol column prepared as previously described, and the column was eluted with water-saturated ethyl acetate. A wide blue to blue-green fluorescing zone was the first to move down the column. This was collected in two fractions, designated 610E2HB-1 and 2. This zone was followed by an area which appeared to consist of zones which fluoresced in various shades of brown, yellow-brown, yellow-green, and blue. These zones overlapped and were very diffuse, and were therefore collected together as fraction 610E2HB-3. Other diffuse zones of various fluorescence remain-

ed on the column, but were not eluted for study at this time.

Paper chromatographic examination of the above fractions indicated that fractions 610E2HB-1 and 2 consisted mostly of naringenin, and these fractions also gave faint spots which corresponded to isosakuranetin. Fraction 610E2HB-3 produced a spot corresponding to naringenin, but also gave other spots which appeared to be flavonoid aglycones. Of the spots other than naringenin, those which were present in greatest concentration fluoresced brown and yellow in ultraviolet light, and therefore appeared possibly to be flavone and flavonol aglycones. Fraction 610E2HB-3 was subjected to further purification in order to try to isolate and identify some of these brown and yellow fluorescing substances.

Further Fractionation of Fraction 610E2HB-3

Examination of several solvent combinations on chromatostrips 52 coated with silicic acid indicated that some separation of flavone and flavonol aglycones could be attained on this adsorbant, using eluting solvents consisting of varying percentages of acetone in benzene. Therefore, separation of the components of fraction 610E2HB-3 was attempted by chromatography on silicic acid.

A silicic acid column was packed, under 5 lb. air pressure, from a slurry of silicic acid (Mallinckrodt analytical reagent, 100 mesh) in 25% acetone–75% benzene. Fraction 610E2HB-3 was reduced to dryness *in vacuo*, dissolved in acetone, and enough benzene was added to give a solution of 25% acetone–75% benzene. This solution was then placed on the prepared column (dimensions 6 x 71 cm.), and the column was eluted with 25% acetone in benzene under atmospheric pressure. A wide, brown fluorescing

zone moved off the column first upon elution, followed by a blue fluorescing zone, and other faintly fluorescing diffuse zones. The brown fluorescing zone was collected as two fractions 610E2HB-31 and 32. Paper chromatography of these fractions showed that a concentration of the aglycones other than naringenin had been obtained in fraction 610E2HB-32.

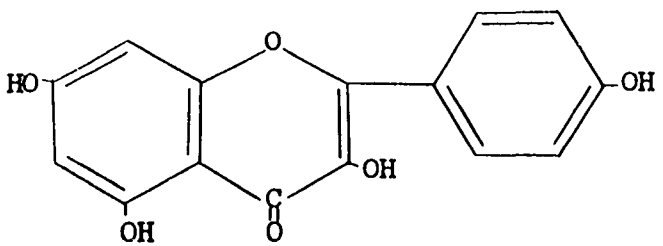
Fraction 610E2HB-32 was therefore reduced to dryness, dissolved in acetone, and enough benzene was added to give a solution of 16% acetone-84% benzene. This was chromatographed on a 4.5 x 32 cm. silicic acid column packed as described above from a slurry of silicic acid in 16% acetone-84% benzene, and the column was eluted with this same solvent. The fractions collected from this column, and the zones which these fractions included were: 610E2HB-321, all of the eluate collected before appearance of the first plainly visible zone, possible including a faint yellow-brown fluorescing zone; 610E2HB-322, a yellow-brown fluorescing zone; and 610E2 HB-322, a red-brown fluorescing zone. Paper chromatography revealed that 610E2HB-321 contained mostly naringenin, that HB-322 contained a small amount of naringenin, plus two other substances which resembled apigenin and the flavonol aglycone kaempferol (3,4′5,7-tetrahydroxyflavone, Figure 9) in fluorescence and R_f values, and that 610E2HB-323 contained mostly a substance resembling apigenin. Fraction 610E2HB-322 was chosen for further study.

Identification of Kaempferol and Apigenin from Fraction 610E2HB-322

Fraction 610E2HB-322 was rechromatographed through a 2 x 40 cm. silicic acid column, as described for fraction 610E2HB-32 above, employing 16% acetone in benzene as solvent. A yellow-brown fluorescing zone

FIGURE 9

KAEMPERFOL



was the first visible substance to move off this column, and was collected as fraction 610E2HB-3222. This was followed by a red-brown fluorescing zone, fraction 610E2HB-3224.

On paper chromatograms fraction 610E2HB-3222 produced a spot which was pale yellow in visible light and fluoresced yellow in ultraviolet light, bright yellow in ultraviolet light when exposed to ammonia fumes, and yellow-green in ultraviolet light after spraying with alcoholic aluminum chloride. The fluorescence and the R_f values of this compound in BAW (0.88), 60% acetic acid (0.56), and NBW (0.50) solvent systems (Table 6) were identical with those of co-chromatographed authentic kaempferol. The ultraviolet absorption spectrum of fraction 610E2HB-3222 had maxima at 268 and 366 $m\mu$, which corresponded with the absorption maxima for authentic kaempferol. Thus the presence of kaempferol in Sunkist naringin, and therefore in the grapefruit, was established. It appeared probable that this compound was present in the original naringin product as a glycoside, but that the quantity was too small for isolation in the original glycoside studies previously described.

Fraction 610E2HB-3224 produced a spot on paper chromatograms which fluoresced brown in ultraviolet light, and which fluoresced yellow in ultraviolet light when exposed to ammonia fumes and after spraying with aluminum chloride. The fluorescence and R_f values in BAW (0.89), 60% acetic acid (0.66), and NBW (0.48) solvent systems corresponded with those of co-chromatographed authentic apigenin. The ultraviolet absorption spectrum was identical with that of authentic apigenin, with maxima at 269 and 332 $m\mu$, confirming fraction 610E2HB-3224 as apigenin. The presence of apigenin in hydrolyzed Sunkist naringin was to be expected,

TABLE 6

R_f VALUES OF REFERENCE FLAVONOID AGLYCONES AND AGLYCONES OF GLYCOSIDES FROM GRAPEFRUIT

Aglycone	Nitromethane: Benzene Water (2:3:5)	n-Butyl Alcohol: Acetic Acid: Water (6:1:2)	60% Acetic Acid
Naringenin	0.82	0.93	0.77
Isosakuranetin	0.97	0.94	0.83
Apigenin	0.48	0.89	0.66
Kaempferol	0.50	0.88	0.56
610E2C--2	0.82	0.93	0.77
610E2C--3	0.97	0.94	0.83
610E2C--5	0.48	0.89	0.66
610E2HB--3222	0.50	0.88	0.56
610E2HB--3224	0.48	0.89	0.66

since its 7-rhamnoglucoside, rhoifolin, had already been identified in the unhydrolyzed product during the previously described glycoside studies.

Conclusion

The investigations described above indicate that the flavonoid content of the grapefruit, Citrus paradisi, is probably much more complex than has heretofore been suspected. Very little has previously been known concerning the flavonoids of this fruit; until very recently naringin, the principal flavanone glycoside, was the only flavonoid which had been identified from this source. Horowitz and Gentili (53) have recently reported isolation of an isosakuranetin-7-rhamnoglucoside from grapefruit. The studies described here on flavonoid glycosides from the grapefruit also established the presence in this fruit of isosakuranetin-7-rhamnoglucoside, as well as another flavanone glycoside that is apparently a rhamnoglucoside of naringenin, but which differs from naringin. The probable presence of other flavanone glycosides was indicated, and the presence of the flavone glycoside rhoifolin was established. Studies on the flavonoid aglycones obtained by hydrolysis of glycosides from the grapefruit established the presence of the flavonol kaempferol, which probably occurs in the fruit as a glycoside. Indications were that all of these compounds occurred in much smaller quantity than naringin, the principal glycoside. The quantity of kaempferol, and hence of any glycoside from which this aglycone might have been hydrolyzed, appeared particularly low.

The 7-rhamnoglucosides of isosakuranetin and naringenin, which differ only in the methylation of one hydroxyl group, the 4′-group, have been found to exist together in two other sources - in the trifoliate orange

by Hattori et al. (14,15) and in the sweet orange as reported in this thesis research. Hence, it appears logical that an analagous situation should be found to exist in the grapefruit.

The question regarding why the isosakuranetin-7-rhamnoglucosides isolated from the orange and the grapefruit are different cannot be answered on the basis of the evidence now available. It appears that this difference is most probably in the point of attachment of the rhamnose to the glucose in the sugar moiety. The basis of this hypothesis is an analogous situation which exists in the case of hesperidin and neohesperidin (11).

The occurrence of naringenin, apigenin, and kaempferol in the grapefruit should be of interest in studies on the biosynthesis of flavonoids. These three flavonoids have identical patterns of hydroxylation in the benzene rings, and differ only in the level of oxidation of the heterocyclic ring. Thus it would appear that these compounds might all be produced from a common precursor, which would support the theory that interconversion between the various classes of flavonoids occurs in the plant cell.

The chromatographic methods developed in the studies of the flavanone glycosides of the sweet orange proved to be useful also in the studies of the grapefruit flavonoids. Column chromatography, employing silicic acid as adsorbant, also proved useful in the studies on aglycones produced by hydrolysis of the flavonoid glycosides from grapefruit. It appears that by further refinement of technique, employing both Magnesol and silicic acid columns, methods for complete separations of flavone, flavonol, and flavanone aglycones could be developed.

CHAPTER IV

SUMMARY

The flavonoid compounds found in citrus fruits have received considerable attention, principally because of their possible physiological activity. However, little was known of the individual flavonoid content of the various fruits, previous investigations having usually succeeded only in identifying the one or two flavonoids present in greatest quantity in a particular fruit. This situation was attributed to extreme difficulty in separating closely related flavonoid glycosides.

Flavonoid preparations from the sweet orange, Citrus sinensis, were subjected to studies by chromatographic techniques for the purpose of finding a method of separating closely related flavonoid glycosides. Column chromatography, employing Magnesol as adsorbant and water-saturated ethyl acetate as eluting solvent, proved to be a good method for separation of some closely related flavanone glycosides; even glycosides present only in trace quantities were isolated by means of this procedure. By repeated passage through appropriate sizes of Magnesol columns, the isolated fractions were purified, and eventually chromatographically pure compounds were obtained.

Identification of pure flavanone glycosides obtained by column chromatography was achieved by a combination of semi-micro and paper chromatographic techniques. A method for positive identification of very

small quantities of flavanone aglycones obtained by hydrolysis of purified glycosides was developed by adapting to semi-micro scale the aqueous potassium hydroxide degradation of flavanones, and identifying the degradation products by paper chromatography. Employing these methods, two flavanone glycosides never before found in the sweet orange, isosakuranetin-7-rhamnoglucoside and naringin, were identified. Hesperidin, the principal flavanone glycoside of the sweet orange, was prepared in chromatographically pure form.

The chromatographic procedures developed in studies on the sweet orange flavonoids were applied to investigation of a flavonoid preparation from the grapefruit, Citrus paradisi. Isosakuranetin-7-rhamnoglucoside and another flavanone glycoside which appeared to be a rhamnoglucoside of naringenin, but differed from the principal glycoside of the grapefruit, naringin (naringenin-7-rhamnoglucoside), were isolated. A flavone glycoside, rhoifolin (apigenin-7-rhamnoglucoside) was also identified in the grapefruit flavonoid preparation.

The isosakuranetin-7-rhamnoglucosides isolated from the sweet orange and grapefruit differed slightly, the difference between the two compounds possibly being in position of the linkage of rhamnose to glucose in the sugar moiety of the molecule.

The flavonoid aglycones obtained by hydrolysis of the grapefruit flavonoid preparation were fractionated by chromatography, first on Magnesol and then on silicic acid columns. Apigenin and the flavonol aglycone kaempferol were isolated and identified in this manner. It is likely that kaempferol exists in the grapefruit as a glycoside, but the quantity is so small that it was not detected in the previous glycoside studies.

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