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STUDIES ON FLAVONOID AND ANTHOCYANIN PIGMENTS

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STUDIES ON FLAVONOID AND ANTHOCYANIN PIGMENTS

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STUDIES ON FLAVONOID AND ANTHOCYANIN PIGMENTS

CHAPTER I

INTRODUCTION

The flavonoid pigments are generally considered to belong to the group of pigments comprising the anthocyanins, anthoxanthins, and xanthones which make up a large majority of the red, blue, and yellow water soluble pigments of plants. The implication that all these compounds are water soluble is somewhat misleading since many of the flavonoid pigments are not soluble in water, but are soluble in organic solvents.

The flavones and xanthones are derivatives of 1,4 (or gamma) pyrone (Figure 1). Thus, the basic structure of xanthone is dibenzo-1,4-pyrone (Figure 2) and that of flavone is 2-phenyl-benzo-1,4-pyrone (Figure 3). The xanthones are orange-colored pigments which occur rarely in plants.

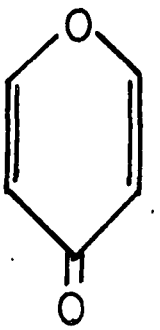
The anthocyanins are responsible for the red and blue pigmentation of flowers, of many fruits, and to some extent of other plant tissues. They are all derivatives of 2-phenyl benzopyrylium salts (Figure 4) and all, with the exception of a few nitrogen containing derivatives, are hydroxy derivatives existing in the plant as the glycosides.

The flavonoid pigments or anthoxanthins, which are generally yellow or orange in color, are found in flowers, fruits, leaves, bark, and roots, and appear to be generally distributed in plant tissues.

Flavone, the basic structure of flavonoid pigments, is found in nature as the yellow deposit on the stems, leaves, and seed capsules of plants in the genus Primula.[28]

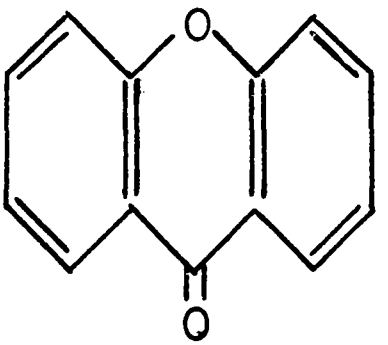
The numbering of the 2-phenyl-benzo-1,4-pyrone nucleus in Figure 5 is the generally accepted scheme today; whereas, that illustrated in Figure 6 was often employed in the earlier literature.

If a hydroxyl group is substituted at position 3, the compound is known as a flavonol (Figure 7). Addition of two hydrogens across the double bond between carbon atoms number 2 and 3 results in a flavanone (Figure 8). If the side chain phenyl group is attached to the 3 position instead of to the 2, then the resulting compound has the structure of an iso-flavone (Figure 9). Thus, the flavonoid pigments may be divided into the four sub-classes: flavones, flavonols, flavanones, and isoflavones. All four classes may have hydroxyl groups substituted on the benzopyrone nucleus and on the side chain phenyl group. These hydroxyl groups in turn may have a sugar residue or a methyl group substituted for the hydrogen. Compounds with a sugar residue attached are designated as glycosides, while those without one are known as aglycones. Rutin (Figure 10), a 3-rutinoside of 3,5,7,3',4' pentahydroxyflavone would be called a flavonol glycoside; whereas, quercetin (Figure 11), 3,5,7,3',4' pentahydroxyflavone would be referred to as a flavonol aglycone. The flavonoid pigments may occur in nature either as the glycoside or as the aglycone.



1,4-Pyrone

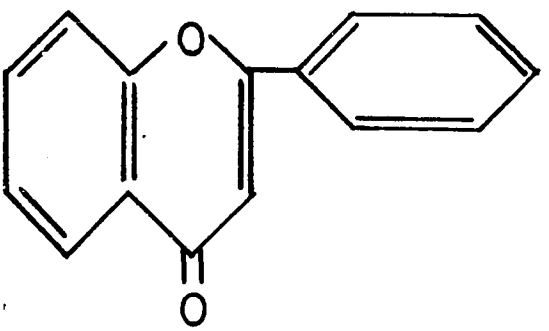
Figure 1



Xanthone

Dibenzo-pyrone

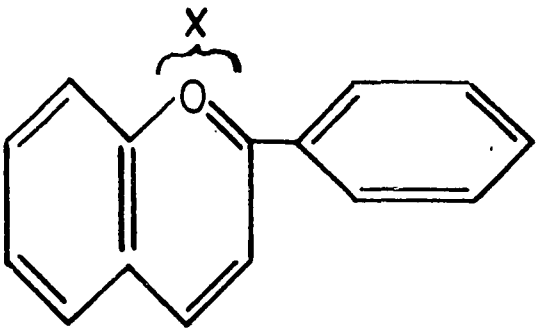
Figure 2



Flavone

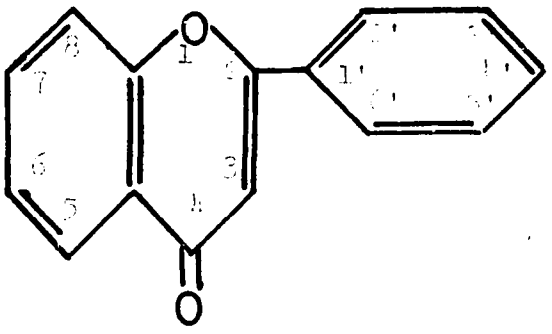
2-Phenyl-benzo-pyrone

Figure 3



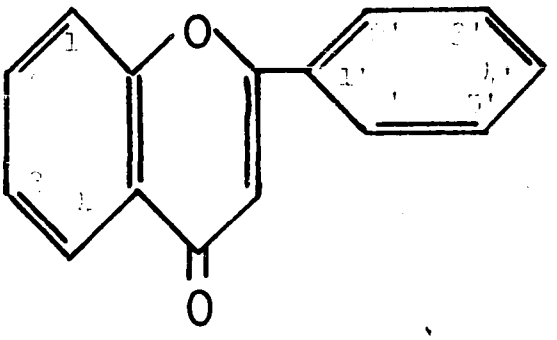
2-Phenyl-benzo-pyrylium salt

Figure 4



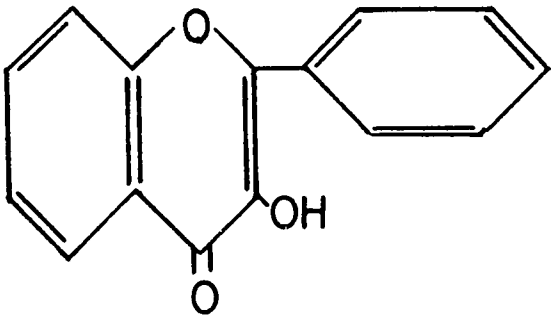
Numbering System Used in Present Literature

Figure 5



Numbering System Used in Earlier Literature

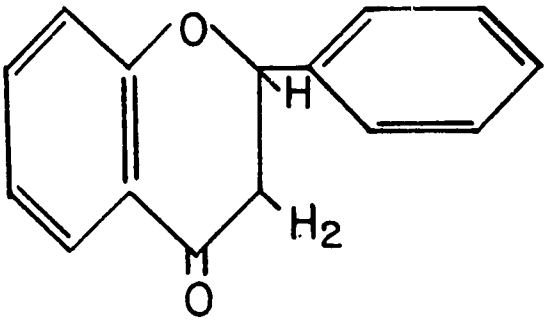
Figure 6



3-Hydroxy-2-phenyl-benzopyrone

Flavonol

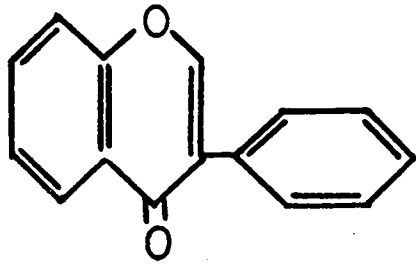
Figure 7



2,3-Dihydroflavone

Flavanone

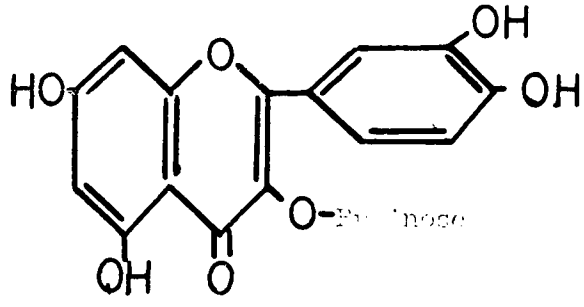
Figure 8



3-Phenyl-benzopyrone

Iso-flavone

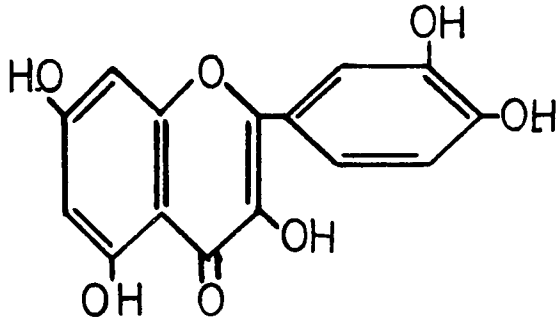
Figure 9



Flavonol Glycoside

Rutin

Figure 10



Flavonol Aglycone

Quercetin

Figure 11

The anthocyanin pigments are quite easily detected by the red or blue coloration that they give to the plant material. Occasionally, leaf tissue containing anthocyanins may appear red-orange or brown due to the background effect of associated flavonoid and carotenoid pigments.

A number of reagents for use in the detection of the flavonoid pigments has been reported in the literature. Since most of them are not specific in their action, it is usually desirable to apply several of these tests before drawing any conclusions. Most flavonoid compounds show an intense yellow coloration in basic or in concentrated acid solution. Reduction of the pigments in an aqueous solution results in colored products. Silver nitrate is reduced by some of the flavonoid compounds. Treatment with metals will often precipitate the pigments as colored salts or as "lakes". Many give the characteristic "phenol test" with ferric chloride solution. Some of the pigments are fluorescent in ultraviolet light or form fluorescent complexes when treated with certain reagents such as aluminum chloride solution, boric acid-citric acid reagent, Benedict's solution, and sodium carbonate solution.[8, 20, 37]

One of the most significant advances in the last ten years in the field of the anthocyanin and flavonoid pigments has been the development of improved methods for the detection, separation, and characterization of these compounds.[7, 8, 10, 11, 19] The major impetus along these lines has been produced by the application of chromatographic techniques. Chromatographic techniques have been utilized in this research to resolve a commercial flavonoid preparation, xanthorhamnin, which was presumably a pure compound, into its major component glycosides. The commercial

mixture was separated into its three components; studies were made to ascertain their structure; and sufficient material was prepared in order that physical constants could be determined on the pure compounds. These experiments and their results are described in Chapter II of this thesis.

In Chapter III, the results of studies undertaken to determine the action of tyrosinase and the hydrolases, emulsin and alpha glucosidase, on certain flavonoid compounds are described. The ability or inability of the enzymes to act on these compounds was determined using chromatographic techniques. The hydrolysis of all the flavonoid glucosides tested by emulsin indicated that the attachment of the glucose moiety to the flavonoid aglycone was of a beta configuration. Tyrosinase was found to be inactive on several flavonoid compounds containing a monohydroxy phenyl side ring, suggesting that tyrosinase was likely not responsible for the conversion of the monohydroxy compounds to the corresponding dihydroxy compounds in vivo.

The last three chapters are concerned with studies which were undertaken in the hope that they might give some clue as to the function, if any, of the anthocyanin and flavonoid pigments in plants. The role of the flavonoid and anthocyanin pigments in plants is still open to speculation. Moewus' reports[25, 26] of the effect of certain flavonoids on sexual processes in the green alga, Chlamydomonas eugametos, have not been confirmed.[34] It has been suggested that the anthocyanin pigments function in certain plants by screening out the deleterious ultraviolet light rays from sun light.[13] The experiments reported in Chapter V were

designed to test this hypothesis utilizing the rate of $C^{14}O_2$ fixation in the anthocyanin-free and anthocyanin-containing sections of the Coleus leaf. The results seem to indicate that the anthocyanin pigments offer no immediate protection against ultraviolet light, if the CO_2 fixation is considered as the criterion.

Many workers have suggested that the anthocyanin pigments may function in enzyme systems for oxidation-reduction reactions. Studies reported in Chapter VI were undertaken to determine if a flavonoid-protein complex might exist in plants. The enzyme ascorbic acid oxidase was investigated with negative results. Studies on protein preparations from spinach and corn yielded more encouraging results in that a flavonoid-like material was found associated with the plant protein. This flavonoid-like substance could not be separated from the protein even on prolonged dialysis. The role of the flavonoid-protein complex in the plant, if any, remains for elucidation by future investigators.

Some workers are of the opinion that the anthocyanin and flavonoid pigments in plants are eliminated waste products, formed merely by accident, which are glycosized and thus made water soluble. The experimental data in Chapter IV are concerned with the method of detoxification in the plant. The results indicate that the plant did detoxify phenol, at least in part, to form a phenylglucoside. One might, therefore, consider other phenol glycosides such as the flavonoids and anthocyanins as possible detoxification products.

CHAPTER II

PREPARATION OF CHROMATOGRAPHICALLY PURE XANTHORHAMNIN FROM, AND STUDIES ON THE COMPONENT GLYCOSIDES OF COMMERCIAL XANTHORHAMNIN

Introduction

In 1952 Howard and Wender[16] determined the acid dissociation constants of rutin, the 3-rutinoside of 3,5,7,3',4' pentahydroxyflavone, and xanthorhamnin, the 3-rhamninoside of 7-methyl-3,5,7,3',4'-pentahydroxyflavone. It was possible to demonstrate four dissociable acid groups up to a pH of 12.5 for rutin, while only two were found for xanthorhamnin. This is somewhat surprising, as xanthorhamnin has three potentially acid dissociable groups. Since then, it was observed that the commercial xanthorhamnin was not a pure compound but a mixture, as it could be partially resolved, paper chromatographically[42], into three components by the n-butyl alcohol-acetic acid-water (4:1:5 by volume) solvent system.[10] Studies were therefore undertaken to prepare chromatographically pure xanthorhamnin in order that the experiments reported previously[16] could be repeated in the absence of the contaminating components, and the identity of the contaminating flavonoids ascertained.

Preliminary attempts to purify the xanthorhamnin by either solvent fractionation or by column chromatography using Magnesol[18] were

unsuccessful. The technique of mass or thick-paper chromatography was therefore resorted to in order to separate the commercial xanthorhamnin into its components.

In the following paragraphs, details of the materials, methods, and experimental procedures utilized in this phase of the research are described.

Materials and Methods

Chromatographic Paper and Its Preparation for Mass Paper Chromatography

Whatman 3 MM filter paper, 18" x 22", was selected as the chromatographic paper since its thickness permitted resolution of a larger quantity of material per sheet of paper than regular Whatman #1.

It was necessary to pre-wash the papers with a 2% hydrochloric acid solution in order to remove material in the paper, probably trace metals, that was found to cause the nearly irreversible adsorption of the flavonoid glycosides. The washing was accomplished by placing the papers in the chromatograph troughs in the usual manner for development and then filling the troughs with approximately 125 ml. of 2% hydrochloric acid solution. The acid solution moved down the paper and dripped off the end of the paper. When all of the acid solution had moved onto the paper, the troughs were filled with distilled water which also moved down the paper and dripped off the end. The procedure was repeated with distilled water, after which the papers were removed and allowed to air dry. The dried papers were placed in the chromatograph troughs and

washed with distilled water again before a final drying to insure the complete removal of the hydrochloric acid, as failure to do so was found to result in the hydrolysis of the flavonoid glycosides.

Application of Material to the Paper Chromatograms

A methanol solution of the commercial xanthorhamnin was applied to the paper by means of a capillary tube in three narrow bands ten centimeters long at a distance of seven centimeters from the bottom edge of the paper. Appropriate spaces were left between bands and from the side of the paper such that after development the individual flavonoid bands could be cut out; one end of the paper strip was cut to a point, and the other end placed in an eluting solvent without loss of the material.

Chromatographic Solvent System for the Resolution of Commercial Xanthorhamnin into Its Components

Although it was possible to show that the commercial xanthorhamnin was not a pure compound by the n-butyl alcohol-acetic acid-water (4:1:5% by volume) solvent system, the resolution was not sufficiently good to permit the preparation of the pure components by mass chromatography. None of the usual solvent systems[10] used for resolution of flavonoid compounds was found satisfactory, and other systems were tried. A system composed of n-butyl alcohol-chloroform-acetic acid-water (4:4:1:1 by volume) was found to give a satisfactory resolution of the mixture. The mixture was resolved on Whatman #1 filter paper into three major components with R_F values of 0.37, 0.55, and 0.68. Similarly, a

satisfactory resolution could be obtained with the thicker Whatman 3 MM filter paper.

A detailed description of the procedure for the solvent system will be given here as it was essential that special care be taken in its utilization. Two liters of boiling, distilled water and 200 ml. of the organic phase of the solvent system were placed in the bottom of the chromatograph chamber, the chromatograph papers were placed in the racks, the chamber lid was closed, and the system allowed to equilibrate for ten hours before filling the solvent trays with the organic layer of the solvent system. After the chromatograms had developed for approximately ten hours, they were removed and allowed to air dry.

Location of the Flavonoid Bands and Their Elution from the Paper

The resolved components of the commercial xanthorhamnin could be located by their brown fluorescence in ultraviolet light, 3660 Å maximum. It was, therefore, possible to place the developed chromatogram under ultraviolet light and, with a pair of scissors, cut out the individual flavonoid bands. The elution of the flavonoid material from the paper strips was carried out as previously described by Ice and Wender.[19] Absolute methyl alcohol was selected as the eluting solvent because of the solubility of the xanthorhamnin components in it, and its high volatility which facilitated the subsequent concentration of the eluants from the paper.

Preparation of the Acetyl Derivative of the Flavonoid Aglycone

Three to five milligrams of the flavonoid aglycone were dissolved

in three milliliters of acetic anhydride containing a trace of sulfuric acid (1 drop of concentrated sulfuric acid to fifty milliliters of acetic anhydride) and the mixture refluxed on a water bath for one hour. After cooling, seven milliliters of water were added and the mixture was allowed to stand overnight in the refrigerator to hydrolyze the unreacted acetic anhydride. The acetyl derivative, which precipitated, was separated by centrifugation, and then purified by recrystallization from 95% ethyl alcohol.

Demethylation of Flavonoid Aglycones

A small sample, as little as one milligram, was mixed with a drop of acetic anhydride giving the mixture a paste-like consistency. Two milliliters of 85% hydriodic acid were added, and the mixture was refluxed for two hours. Eight to ten milliliters of water were then added, and, after cooling, the precipitated aglycone was separated by centrifugation. The precipitated aglycone was taken up in 95% ethyl alcohol and recrystallized, or the alcohol solution was used directly for paper chromatographic studies. Failure to bring about any change in the molecule by the above treatment was assumed to indicate that the aglycone did not contain a methyl group.

Identification of Sugars Present in the Flavonoid Glycosides

A two to three milligram sample of the flavonoid glycoside was dissolved in two milliliters of 2% sulfuric acid and refluxed for two hours. The solution was cooled and the precipitated aglycone was removed by centrifugation. The resulting supernatant solution was neutra-

lized with barium hydroxide and, after removal of the precipitated barium sulfate by centrifugation, this solution was concentrated to near dryness. The concentrate was then spotted on Whatman #1 filter paper, and the chromatograms were developed in the solvent system composed of n-butyl alcohol-pyridine-water (2:1:1.5% by volume).[30] Solutions of aniline hydrogen oxalate and napthoresorcinol were used as spray reagents to locate the position of the sugars.[30] Controls of glucose, mannose, galactose, ribose, rhamnose, and arabinose were run simultaneously. Once the sugars were tentatively identified, additional runs were made in the n-butyl alcohol-acetic acid-water (4:4:1 by volume) and the phenol-saturated with water solvent systems. A compound which co-chromatographed with an authentic sample in the three solvent systems was considered to be identical with the authentic sample.

Determination of Moles of Sugar to Moles of Aglycone in the Flavonoid Glycosides

An estimation of the moles of sugar to moles of aglycone may be made by completely hydrolyzing a known amount of the glycoside, quantitatively collecting the aglycone formed, and weighing it.[19] If the sugars have been identified, and the approximate molecular weight of the flavonoid is known, then one can calculate the ratio of moles of sugar to moles of aglycone.

Approximately ten mg. of the flavonoid glycoside was weighed to the nearest 0.1 mg. and then hydrolyzed by boiling in 2% sulfuric acid for two hours. After cooling the mixture, the aglycone was collected on

a weighed sintered glass filter and dried *in vacuo* over phosphorus pentoxide at 80° C to constant weight.

Determination of the Position of Sugar Residues in Quercetin Derivatives

Complete methylation of a quercetin-7-glucoside or quercetin-3-glucoside, followed by acid hydrolysis, yield tetramethoxy quercetin derivatives with very different melting points. The tetramethoxy quercetin derivative of the 3-glucoside has a melting point of 195° C, whereas, that of the 7-glucoside has a melting point of 285° C.[3] Since the aglycone components of the commercial xanthorhamnin were all quercetin or its methoxy derivatives, the preparation of a tetramethoxy derivative was of value in determining the position of the sugar residues. The preparation of the tetramethoxy derivative of a 3-5 mg. sample of the purified flavonoid glycosides from commercial xanthorhamnin was accomplished by methylation with dimethyl sulfate followed by hydrolysis, and subsequent recrystallization from ethyl alcohol according to the method of Shimokoriyama.[36]

Determination of the Ratio of Two Sugars Present in a Flavonoid Glycoside

Approximately 1.5 mg. of the glycoside was hydrolyzed, and the sugar solution separated from the aglycone as previously described. The solution containing the sugars was then applied as a ten centimeter band and a two centimeter band to Whatman 3 MM filter paper and developed in the n-butyl alcohol-pyridine-water solvent system previously described. The position of the sugar bands was located by spraying the portion of

the chromatogram containing the two centimeter band with the aniline-oxalate spray reagent. Simultaneously, samples containing 100, 200, 300, and 400 micrograms of the identified sugars were applied in a similar manner to the Whatman 3 MM filter paper and also developed in the same manner. The areas containing the sugars from the ten centimeter bands were cut out and eluted with distilled water into a five milliliter volumetric flask and made up to volume. A one milliliter aliquot was then reacted with the anthrone reagent for sugars according to the method of Yemm and Willis[45], with the exception that the anthrone reagent was made up by dissolving the crystalline anthrone in 85% sulfuric acid instead of 70% sulfuric acid. The optical density of the solutions was determined at 625 mu on the Beckman Spectrophotometer. A standard curve was obtained by plotting optical density against sugar concentration. Both rhamnose and galactose yielded a straight line relationship at concentrations from 0 to 80 micrograms. The concentration of the sugars in the hydrolyzed solution could readily be determined using the standard curve, and the ratio of the two sugars could then be calculated in moles.

Experimental

Separation of Commercial Xanthorhamnin into Its Component Glycosides

In a typical experimental run, 300 mg. of the commercial xanthorhamnin was applied as bands to 36 sheets of Whatman 3 MM filter paper and separated in the n-butyl alcohol-chloroform-acetic acid-water solvent system. The three major bands, R_F = 0.68, 0.55, and 0.37, were cut out and eluted with methyl alcohol. The methyl alcohol eluates were concen-

trated to a small volume, 10 ml., and a large quantity of ether was added. Fraction I and Fraction II, R_F = 0.68 and 0.55 respectively, each yielded an amorphous yellow precipitate. Fraction III, R_F = 0.37, yielded a brown oil. Recrystallization of Fraction I and Fraction II from 50% ethyl alcohol-50% iso-propyl alcohol yielded yellow crystalline compounds. Fraction III, however, consistently came out as a brown oil. R_F values and color reactions of the three components are recorded in Tables 1 and 2. The 300 mg. sample yielded 20 mg. of Fraction I, 80 mg. of Fraction II, and about 10 mg. of Fraction III.

Characterization of Fraction I

Fraction I had a melting point of 187° C. uncorrected. Hydrolysis yielded an aglycone with a melting point of 214° C. The aglycone yielded an acetyl derivative with a melting point of 153-154° C. Treatment of the aglycone with hydriodic acid yielded an aglycone which co-chromatographed with quercetin. Rhamnazin, the 3',7-dimethoxy quercetin, has a melting point of 214° C. and yields an acetyl derivative of 155° C.[23] The aglycone from Fraction I co-chromatographed with authentic rhamnazin, and no lowering of melting point was observed on admixing of the two on subsequent determination of the melting point. The aglycone would appear to be identical with rhamnazin.

The sugars present after hydrolysis were identified by means of paper chromatography as rhamnose and ga lactose. The sugars were found to be present in the ratio of two moles of rhamnose to 1 mole of galactose. There were 3 moles of sugar to one mole of aglycone.

Table 1

R_F Values of the Flavonoid Components

of Commercial Xanthorhamnin

Compound	Solvent System		
	n-butyl alcohol: acetic acid: water 4:1:5 by volume	acetic acid: water 15:85 by volume	acetic acid: water 40:60 by volume
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 Fraction I	0.82	0.10	0.60
Aglycone from Fraction II	0.80	0.10	0.52
Aglycone from Fraction III	0.77	0.10	0.41

Treatment of the glycoside with dimethyl sulfate and subsequent hydrolysis yielded a methoxy derivative with a melting point of 195-196° C., indicating that the sugars were attached to the number three hydroxyl group.

Characterization of Fraction II

Fraction II melted at 195° C. Hydrolysis yielded an aglycone with a melting point of 293° C. The aglycone yielded an acetyl derivative with a melting point of 177° C. Treatment of the aglycone with hydriodic acid yielded an aglycone which co-chromatographed with quercetin. Rhamnetin, 7-methoxyquercetin, has a melting point of 293° C. and yields an acetyl derivative with a melting point of 178° C.

The sugars, galactose and rhamnose, were identified in the hydrolyzed solution. There were 2 moles of rhamnose to 1 mole of galactose. A ratio of 3 moles of sugar to 1 mole of aglycone was found.

Treatment of the glycoside with dimethyl sulfate and subsequent hydrolysis yielded a methoxy derivative with a melting point of 194-195° C., indicating that the sugars are attached to the number three hydroxyl group.

Partial Characterization of Fraction III

Fraction III consistently yielded an oil and, therefore, no melting point could be obtained. Hydrolysis of the oil yielded an aglycone with a melting point of 312-313° C. which co-chromatographed with quercetin. The aglycone yielded an acetyl derivative with a melting point of 192-193° C. Treatment of the aglycone with hydriodic acid

Table 2

Color Reaction of Flavonoid Components of Commercial Xanthorhamnin

Compound	Color Reactions					
	Spray Reagent					
	Benedict's Solution		1% alcoholic		basic lead acetate	
	V.	U.V.	V.	U.V.	V.	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 Fraction I	Y	YGR	Y	YGR	Y	Y
Aglycone from Fraction II	Y	O	Y	YGR	Y	O
Aglycone from Fraction III	Y	O	Y	YGR	Y	O

V. = Visible Light
U.V. = Ultraviolet Light
Y = Yellow
BR = Brown
GR = Green
O = Orange

failed to change the chromatographic characteristics of the molecule indicating that the treatment had no effect and that the aglycone probably had no methoxy group. Quercetin has a melting point of 314° C. and yields an acetyl derivative with a melting point of 193-194° C.[21] The aglycone of the glycoside in Fraction III is apparently identical with quercetin.

Glactose and rhamnose were found in the hydrolyzed solution of Fraction III. There were two moles of rhamnose to one of galactose.

Treatment of the oil with dimethyl sulfate and subsequent hydrolysis yielded a methoxy derivative with a melting point of 194-195° C. indicating that the sugars are attached to the number three hydroxyl group.

Discussion

The data on Fraction I indicate that it is a glycoside of rhamnazin with a trisaccharide containing two moles of rhamnose and one of galactose attached to the number three hydroxyl group.

Fraction II appears to be a derivative of rhamnetin with a trisaccharide containing two moles of rhamnose and one mole of galactose attached to the number three hydroxyl group. The pure compound in Fraction II appears to be identical with xanthorhamnin. However, xanthorhamnin is reported to have a melting point of 167° C.[4], whereas, Fraction II has a melting point of 195° C. The higher melting point of Fraction II found for xanthorhamnin is probably due to the fact that it was a much purer sample of xanthorhamnin than had been obtained previously.

The data on Fraction III indicate that the compound is a glycoside of quercetin with the sugars attached to the number three hydroxyl group. There are two moles of rhamnose to one mole of galactose corresponding to Fractions I and II, although it was not possible to ascertain the number of moles of sugar to moles of aglycone with the sample obtained.

Thus, the supposedly pure commercial xanthorhamnin has been fractionated by the above described procedures into three fractions. Each fraction is a flavonol glycoside, containing two moles of rhamnose to one mole of galactose. The carbohydrate attachment is through the number three position on each flavonol. The flavonol aglycones have been found to be rhamnazin, rhamnetin, and quercetin, respectively. The glycoside of the rhamnetin appears to be the pure xanthorhamnin.

CHAPTER III

ACTION OF ENZYMES ON FLAVONOID COMPOUNDS. I. ENZYMATIC HYDROLYSIS STUDIES ON CERTAIN FLAVONOID GLYCOSIDES

Introduction

Experimental evidence sufficient to resolve the question as to whether the glucosyl unit is attached alpha or beta onto quercetin (3,3',4',5,7-pentahydroxyflavone) has not been previously recorded for isoquercitrin (quercetin 3-glucoside) and quercimeritrin (quercetin 7-glucoside). The present study was, therefore, undertaken to aid in the establishment of the alpha or beta union of glucose to the aglucone in these two quercetin glucosides as well as in a quercetin glucoside of unknown structure from apricots[44], and in hesperetin 7-glucoside and naringenin 7-glucoside. The quercetin glucoside from apricots had been shown to be a quercetin 3-glucoside, but was not identical with isoquercitrin (quercetin 3-glucoside) in that it could be separated from isoquercitrin by means of paper chromatography.

Materials and Methods

Preparation of the Alpha-Glucosidase

Forty milligrams of takadiastase (Parke, Davis and Co.) were dissolved in 0.125 M citrate buffer, pH 3, and spotted as a band at the center of a Whatman 3 MM paper strip, 46 x 10 cm. The paper strip,

saturated with the citrate buffer of pH 3, was placed between two 38 x 20 cm. glass plates. The ends of the paper strips were allowed to dip into troughs containing the citrate buffer. The system was allowed to equilibrate for 45 minutes, after which 110 V. direct current was applied for 40 hours. The paper was then removed from between the glass plates, and 2 cm. wide strips were cut parallel to the point of application of the enzyme solution (origin) toward the side of the paper which had been attached to the positive pole. Each 2 cm. wide strip was eluted with citrate buffer until 0.5 ml. of eluant from each was obtained. These eluants were divided into two portions. To one portion was added an equal volume of 0.5 M citrate buffer of pH 7, containing maltose (16 mg./ml.). To the second portion was added an equal volume of the pH 7 citrate buffer containing cellobiose (16 mg./ml.). The solutions were mixed by shaking and allowed to incubate at room temperature for 6 hours. Aliquots of the solutions were spotted on Whatman No. 1 paper and developed for 12 hours with a n-butyl alcohol-pyridine-water system (2:1:1.5 by volume, upper layer removed and one more part of pyridine added). The glucose, if present, could then be located by spraying with an aniline-hydrogen oxalate solution and identification indicated by comparing the spots developed with those of glucose, maltose, and cellobiose.[30]

A fraction, no. 1, eluted from the strip cut from the filter paper extending 0 to 2 cm. from the origin, apparently contained substances interfering with clearcut analysis of results, and was eventually discarded. A fraction, no. 2, eluted from the strip cut from the paper extending 2 to 4 cm. from the origin, hydrolyzed both maltose and cello-

biose. A fraction, no. 3, eluted from the strip cut from the paper extending 4 to 6 cm. from the origin, hydrolyzed maltose, but not cellobiose. Additional fractions eluted from 2 cm. strips cut from the paper in the region 6 to 12 cm. from the origin also were capable of catalyzing hydrolysis of maltose, but not of cellobiose. There was a marked decrease, however, in the activity. Many repetitions of this experiment gave essentially the same results. Similar experiments were run on the beta-glucoside salicin and on methyl-alpha-D-glucoside. The fraction no. 2 hydrolyzed both of these glucosides; whereas, fractions no. 3 catalyzed hydrolysis of methyl-alpha-D-glucoside but not of salicin. Therefore, the fraction no. 3, eluted from the strip cut from the paper extending 4 to 6 cm. from the origin on the positive charged side, was selected as the enzyme solution to be used for studies on the flavonoid glucosides.

The Emulsin Preparation

Tests similar to the above, but using an emulsin preparation (Nutritional Biochemical Corp., Cleveland, Ohio) were performed on cellobiose, salicin, methyl-alpha-D-glucoside and maltose. The emulsin behaved as expected by catalyzing the hydrolysis of the two beta-glucsides, but not of the alpha-glucosides.

Sources of Flavonoid Glycosides

The isoquercitrin (quercetin 3-glucoside) was prepared by the partial hydrolysis of rutin[3] and was chromatographically identical with isoquercitrin from grapes. The hesperitin 7-glucoside and naringenin

7-glucoside were prepared as described by Fox et al.,[9] from hesperidin and naringin respectively. The quercimeritrin (quercetin 7-glucoside) was obtained through the courtesy of Dr. T. R. Seshadri, Delhi, India. The unknown quercetin glucoside from apricots was a sample that was prepared by Williams and Wender.[44]

Experimental

Enzymatic Hydrolysis of Isoquercitrin

A preparation of alpha-glucosidase was obtained as described above, and its pH was adjusted to approximately 5 by the addition of an equal volume of 0.5 M citrate buffer of pH 7. A few crystals of authentic isoquercitrin were then added to the solution, and the mixture agitated to dissolve the flavonoid glucoside. The mixture was allowed to incubate at room temperature for 6 hours.

For the emulsin studies, a few crystals of the isoquercitrin were added to 0.5 ml. of a citrate buffer of pH 5 and brought into solution by shaking and heating. To the mixture was added approximately 3 mg. of the solid emulsin, and the mixture shaken and allowed to incubate at room temperature for 6 hours. Individual controls on each of the citrate buffers plus isoquercitrin, but containing no enzyme, were run at the same time.

The presence or absence of the aglycone quercetin and/or the original, unhydrolyzed isoquercitrin were ascertained at the end of the incubation period by paper chromatography, using a 15% acetic acid-water system. No hydrolysis of the isoquercitrin could be detected with the

controls and with the alpha-glucosidase preparation, but practically complete hydrolysis of the isoquercitrin was observed with the emulsin under the experimental conditions used.

Enzymatic Hydrolysis of Other Flavonoid Glucosides

By the method described above for isoquercitrin, the flavonoid glucosides; hesperetin 7-glucoside, naringenin 7-glucoside, quercimeritrin; and the unknown quercetin glucoside from apricots were investigated and found to be hydrolyzed only by emulsin. Neither emulsin nor the alpha-glucosidase had any effect on quercitrin (quercetin 3-rhamnoside) or rutin (quercetin 3-rhamnoglucoside).

Discussion

Both isoquercitrin and the quercetin 3-glucoside from apricots are hydrolyzed by emulsin and not by alpha-glucosidase proving that their differences in chromatographic characteristics can not be attributed to the attachment of the sugar residue to the aglycone.

Hesperidin, from which the hesperetin 7-glucoside had been prepared, has been shown by Zemplén and co-workers[46] to be a beta-rhamnoglucoside. Apparently, the partial hydrolysis of the hesperidin with formic acid to obtain the hesperetin 7-glucoside did not alter the glucosyl attachment to the aglycone in this particular case. If this were also to apply to the partial hydrolysis of rutin (quercetin 3-rhamnoglucoside)[47] and to naringin (naringenin 7-rhamnoglucoside), then from the results reported there, these two rhamnoglucosides could be considered to have the glucosyl unit attached beta onto the aglycone.

Experimentally, rutin was not hydrolyzed with emulsin or the alpha-glucosidase.

ACTION OF ENZYMES ON FLAVONOID COMPOUNDS. II. ACTION OF TYROSINASE ON FLAVONOID COMPOUNDS WITH A MONO-HYDROXY SIDE-PHENYL RING

Introduction

Tyrosinase catalyzes the oxidation of both polyphenols and monophenols.[29] The action of tyrosinase on polyphenols refers to the oxidation of an ortho dihydric phenol to the ortho quinone; whereas, the action on monophenols is the introduction of an orthophenolic group, thus oxidizing a monophenol to an ortho dihydric phenol. The action on monophenols (monophenolase activity) is that which is of interest in these studies.

Since the monophenolase activity of tyrosinase has been shown to be relatively non-specific, studies were made to determine if tyrosinase is capable of introducing an orthohydroxyl group into the side ring. Such information would be of interest in proposing metabolic pathways of synthesis of various flavonoid compounds.

Paper chromatographic techniques permit the determination of whether or not an ortho phenolic group was introduced into such flavonoid compounds by the action of tyrosinase since the chromatographic characteristics[7, 10] of the ortho dihydric phenolic compound are usually markedly different from that of the monophenolic compounds. For example, kaempferol, 3,5,7,3', tetrahydroxyflavone, has an R_F value of 0.48 in the 60% acetic acid-40% water solvent system; whereas, the ortho dihy-

dric phenol, quercetin (3,5,7,3',4', pentahydroxyflavone) has an R_F value of 0.40 in the same solvent system. Therefore, if one allows the tyrosinase to act on the kaempferol under suitable conditions, and then studies the flavonoid in the mixture using the above chromatographic system, one should be able to detect any conversion of kaempferol to quercetin, provided the quercetin itself was not further changed chemically.

Materials and Methods

Tyrosinase Preparations

Potato tyrosinase was prepared by utilizing a portion of the method described by Kubowitz.[22] One kilogram of fresh potato peels was ground up in a Waring blendor containing 500 ml. of distilled water. The minced material was filtered through cheese cloth, the volume determined, and sufficient acetone was added to make the mixture 37% by volume acetone. The precipitate was separated by centrifugation and extracted with 100 ml. of distilled water. Ammonium sulfate was added to the extract to make it 68% saturated with respect to ammonium sulfate. The precipitate was separated by centrifugation and extracted with 25 ml. of distilled water. The extract was stored in the refrigerator until ready for use. No determination of the units of tyrosinase activity was made, but the preparation was capable of oxidizing tyrosine very rapidly to the dopa quinone, as evidenced by the rapid formation of the red color when tyrosine was added to the enzyme solution.

Mushroom tyrosinase was obtained from Nutritional Biochemical Corp. It had tyrosinase activity of 500 units/ml.

Experimental

Incubation of Flavonoids in the Presence of Tyrosinase

To 100 microliters of the tyrosinase, either the commercial or that prepared from potato peels, was added 100 microliters of 0.05 M phosphate buffer, pH. 5, containing ascorbic acid at a concentration of 1 mg./ml. A few crystals of the flavonoid were then added to the mixture, and the mixture was stirred briefly with a small glass stirring rod. A small stream of air from a capillary tube was allowed to bubble through the mixture. After incubation at room temperature for three hours, the mixture was concentrated to near-dryness and then extracted with methyl alcohol. The methyl alcohol extract was applied to a paper chromatogram, and the chromatogram was developed in 60% acetic acid-40% water or in the n-butyl alcohol-propionic acid-water solvent system. The flavonoid compounds after development were located on the paper chromatograms by observing them under ultraviolet light or spraying the papers with Benedict's solution. The flavonoids, kaempferol (3,5,7,3'-tetrahydroxyflavone), apigenin (5,7,3'-trihydroxyflavone), rhoifolin (the 7-rhamnoglucoside of apigenin), naringenin (5,7,4' trihydroxyflavanone) were treated in the above manner. In the case of each of the above flavonoid compounds, there was only the original compound on the chromatograms. No evidence for the presence of a new compound could be detected.

Similar experiments were performed in which the incubation procedure was varied. Ascorbic acid was omitted in some experiments. Catechol was added in others. None of these variations resulted in any action of the tyrosinase on the flavonoids used.

Discussion

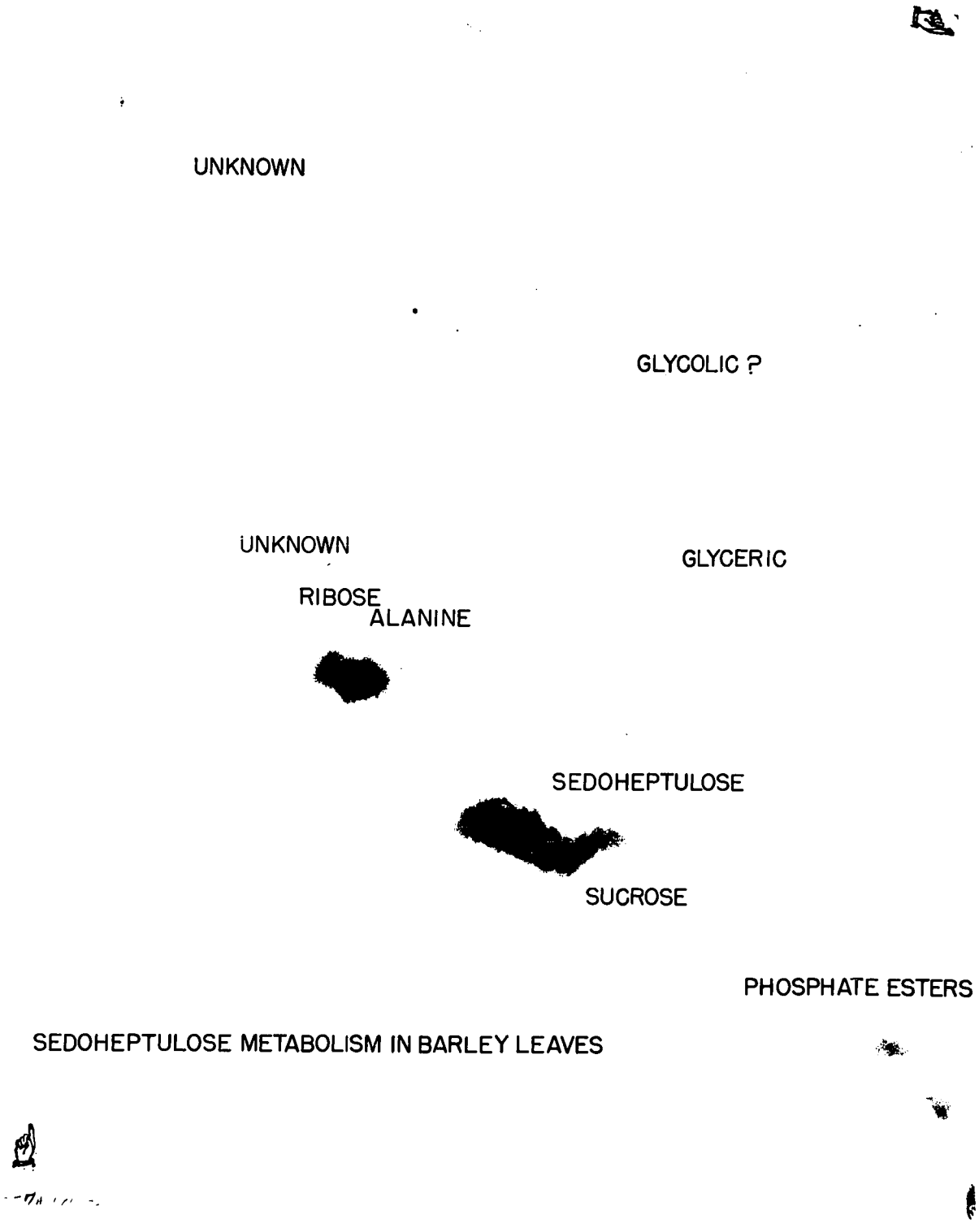
Under the conditions studied, tyrosinase was not able to introduce a second phenolic group into the side phenyl ring of a flavonoid with a monophenolic side phenyl ring. One might attribute this inactivity to the ability of the flavonoid to complex the copper which has been shown to be the prosthetic group of the tyrosinase molecule.[22] However, this explanation seems unsatisfactory since the enzyme preparation still was able to act on tyrosine even in the presence of the flavonoid compounds used in these studies.

CHAPTER IV

CHARACTERIZATION OF PHENYLGLUCOSIDE FORMED FROM BIOSYNTHESIZED SEDOHEPTULOSE-C^{14} AND GLUCOSE-C^{14} IN PLANT LEAVES

Introduction

In studies by Tolbert and Zill[41] on the metabolism of sedoheptulose-C^{14} in plant leaves, an unidentified compound which was formed in considerable quantities, and which contained C^{14} was observed on radioautograms of developed paper chromatograms of the extracts of leaves fed sedoheptulose-C^{14}. The compound referred to in this case is the one labeled "UNKNOWN" in the upper left-hand ocrner of the chromatogram shown in Figure 12. The compound had an R_F value of 0.80 in the water-saturated phenol solvent system and 0.65 in the n-butyl alcohol-propionic acid-water sovent system.[5] The compound was formed in either the light or the dark. Even in etiolated seedlings the compound was formed in considerable quantities. The percentage of the C^{14} in the unknown compound of the total C^{14} activity remaining in a leaf fed sedoheptulose-C^{14}, increased with time, after feeding. Subsequent studies carried out by Tolbert[39] showed that leaves fed glucose-C^{14} (biosynthesized) likewise formed the compound. After the unknown was separated from other plant constituents by two dimensional chromatography, and its position on the paper located by making radioautograms, it could be eluted with distilled water and investigated chemically. If the eluate was treated with the



SEDOHEPTULOSE METABOLISM IN BARLEY LEAVES

Figure 12

Chromatogram of C^{14} Products of Sedoheptulose-C^{14}

Metabolism in Barley Leaves

crude plant enzyme, Polidase S, and rechromatographed, the C^{14} activity was no longer in the area of the unknown, but rather all the C^{14} activity was in the positions normally occupied by glucose on the paper chromatograms. Co-chromatography of the C^{14} activity after Polidase S treatment with 50 ug. of carrier glucose, which was detected by the aniline-trichloracetic acid spray test, indicated that the radioactivity was present as glucose. No radioactive material other than glucose could be detected on the paper chromatogram, indicating that the moieties of the molecule other than glucose were not readily labeled by the sugar fed to the plant. Treatment of the eluates with Dowex 50 (H^+) at 100° C for one hour likewise resulted in hydrolysis of the compound to glucose-C^{14}. Double labeling experiments with P^{32} phosphate and glucose-C^{14} indicated that the compound contained no phosphorus. Since both the biosynthesized glucose-C^{14} and sedoheptulose-C^{14} were prepared according to the method of Tolbert and Zill,[40] which utilized chromatographic separation of the sugars by the water saturated phenol solvent system, the possibility exists that traces of phenol remain in the sugar solutions, and that the compound was phenylglucoside resulting from detoxification of the contaminating phenol. Charcoal is known to adsorb phenolic substances. Charcoal-treated glucose-C^{14} solution still formed the unknown in considerable quantities. Charcoal-treated sedoheptulose-C^{14} yielded only traces of the unknown compound, but due to the low specific activity of the sedoheptulose-C^{14} solutions used, the results were not conclusive.

The available experimental data at this point suggested several possible explanations for the presence of the compound on the chromato-

grams. The fact that only intermediates of hexose metabolism were present on the chromatograms suggested that the compound might be functioning in hexose metabolism. Its rapid formation, even in starved etiolated plant leaves, suggested that it might be functioning in glycoside metabolism. Another possibility mentioned previously was that the compound might be a detoxification product of phenol. Experiments were, therefore, undertaken to ascertain the identity of the unknown compound.

Materials and Methods

C^{14} Labeled Sugar Solutions

The uniformly labeled sedoheptulose-C^{14} and glucose-C^{14} solutions were prepared by biosynthesis by Drs. N. E. Tolbert and L. P. Zill.[40] The specific activity of the two sugars was 3.7 microcuries/mg. The sedoheptulose-C^{14} solution contained 1.4 mg./ml. and the glucose-C^{14}, 0.7 mg./ml. The glucose 1-C^{14} and glucose 6-C^{14}, prepared by organic synthesis, were obtained from the National Bureau of Standards. The specific activity of the glucose 1-C^{14} was 1.3 microcuries/mg., and that of the glucose 6-C^{14}, 2.2 microcuries/mg. Solutions were made up by dissolving 5 mg. of the sugar in 5 ml. of distilled water.

Killing Procedure for Leaf Tissue

The leaf was placed in a mortar, and liquid nitrogen poured over the leaf. The frozen leaf was then ground into a fine powder with a pestle, suspended in distilled water, poured into a thick-walled centrifuge tube (15 ml.), quickly placed into a boiling water bath,

and allowed to remain there for five minutes. The procedure is considered sufficiently rapid to prevent appreciable enzymatic action before the heat inactivation becomes effective.

Separation of the Unknown from the Other Plant Constituents

The boiling suspension of the leaf tissue obtained by the killing procedure above was centrifuged, and the supernatant solution was poured off and saved. The residue was discarded. The supernatant solution was concentrated to a small volume, 100 microliters, by placing the tubes containing the solution in a beaker of warm water, 60 - 80° C, and blowing a stream of air into the tubes. Aliquots of the concentrated extract equivalent to 25 - 100 mg. of the original plant tissue were separated by two dimensional chromatography using Whatman #1 filter paper (18 x 22 inches). The chromatograms were developed first, in the short direction, with water-saturated phenol and then with the n-butyl alcohol-propionic acid-water solvent system.[5] A dye solution containing an orange dye, Tropeolin OOO, and a red dye, Crocein Scarlet, was applied to the left of the origin of the paper chromatograms before development. The movement of the dyes served as visual aids for control of the development of the chromatogram, and their R_F values served as references in locating the radioactive components on the chromatograms. The unknown moved slightly faster than the orange dye in both solvent systems.

The two dimensional paper chromatographic system described above separated the unknown from the major C^{14} containing products of the sedoheptulose-C^{14} and glucose-C^{14} metabolism, although traces of other compounds are present in the area occupied by the unknown.

The exact location of the unknown was obtained by pressing the developed and dried paper chromatogram directly against sensitive x-ray film (Eastman-Kodak "No-Screen") and allowing it to stand for at least one month. The location of the C^{14} activity could then be ascertained by the position of the blackened areas on the x-ray film after development.

Counting Method

The relative amount of radioactivity in a given area or spot on the chromatogram was determined with an end window Geiger-Müller tube.

Synthesis of Phenylglucoside

Phenylglucoside was synthesized according to the method described by Helferich and Hillebrecht.[14]

Experimental

Formation of the Unknown from Biosynthesized Glucose-C^{14} Vs Synthesized Glucose 6-C^{14} and Glucose 1-C^{14}

Etiolated Thatcher wheat leaves or green leaves six days old were cut off at the base and immediately placed into a solution of one of the three sugars. Each leaf was fed the equivalent of 100 microliters of one of the three sugar solutions. When all the sugar solution had been taken up by the leaf, distilled water was added to the tube holding the leaf so that the leaf had a continual supply of water. It took approximately six hours for the sugar solution to be taken up, and then the leaf was allowed to stand for an additional ten hours. At the end

of this time, 16 hours from initial feeding, the leaf was killed, and the activity in the unknown determined as described previously. The results are shown in Table 3.

Effect of Addition of Phenol on the Formation of the Unknown from Glucose 6-C^{14}

Etiolated Thatcher wheat leaves were fed the equivalent of 100 microliters of the glucose 6-C^{14} solution plus 60, or 300 micrograms of phenol as described above, and the activity in the unknown determined. In another experiment, the unmetabolized glucose 6-C^{14} equivalent to 150 microliters from previous experiments, was eluted off a two-dimensional paper chromatogram and fed to an etiolated Thatcher wheat leaf as above, and the activity in the unknown determined. The results are recorded in Table 4.

Effect of Ether Extraction of Biosynthesized Glucose-C^{14} Solution on the Formation of the Unknown

Two hundred microliters of the biosynthesized glucose-C^{14} solution were acidified with hydrochloric acid and extracted several times with diethyl ether. It was then divided into two portions. One portion was fed to one etiolated leaf, and the other portion was fed to a second leaf after the addition of 60 micrograms of phenol. A control of the untreated solution was run at the same time. A similar experiment was run using green wheat leaves. The results are recorded in Table 5.

Table 3

FORMATION OF UNKNOWN IN LEAVES FROM BIOSYNTHESIZED GLUCOSE-C^{14} VERSUS SYNTHESIZED GLUCOSE 6-C^{14} AND GLUCOSE 1-C^{14}

Sugar Solution	Counts/sec. in Unknown Area	
	Etiolated Leaves	Green Leaves
Biosynthesized Glucose-C^{14}	3.5	3.0
Glucose 6-C^{14}	trace	trace
Glucose 1-C^{14}	trace	trace

Table 4

EFFECT OF ADDITION OF PHENOL ON FORMATION OF UNKNOWN IN LEAVES FROM GLUCOSE 6-C^{14} AND GLUCOSE 1-C^{14}

Sugar Solution	Counts/sec. in Unknown Area	
	Etiolated leaves	Green leaves
Glucose 6-C^{14} 60 ug. phenol	7.0	4.5
Glucose 6-C^{14} 300 ug. phenol	0.0	---
Glucose 6-C^{14} Eluted from Chromatogram	25	35
Glucose 6-C^{14} No phenol	trace	trace

Table 5

EFFECT OF ETHER EXTRACTION OF BIOSYNTHESIZED GLUCOSE-C^{14} SOLUTION ON THE FORMATION OF THE UNKNOWN IN LEAVES

Uniformly Labeled Glucose-C^{14} Solution	Etiolated Leaves	Green Leaves
Untreated	3.5	4.0
Ether Extracted	trace	trace
Ether Extracted 60 ug. phenol	6.5	7.0

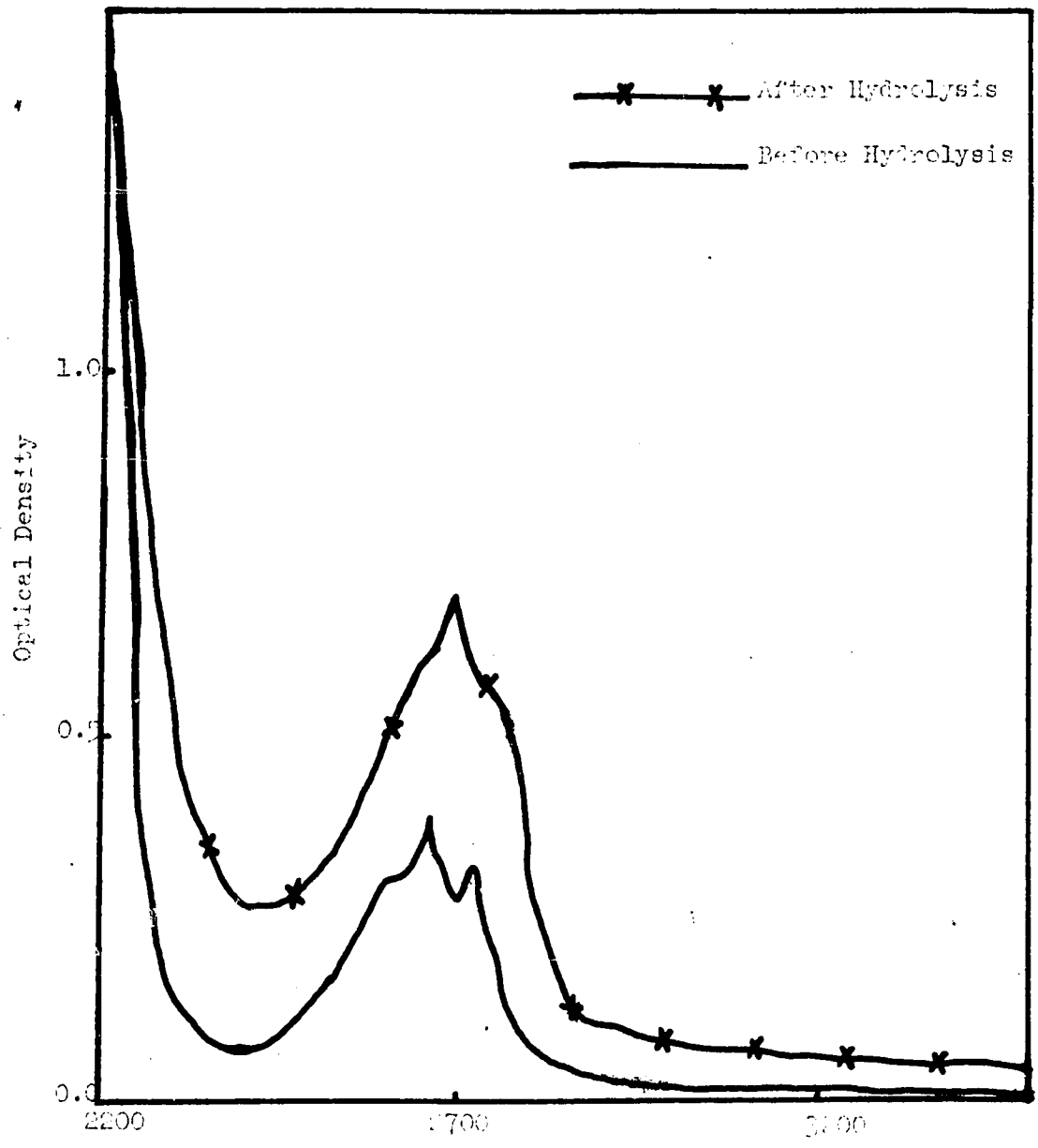


Figure 12

Absorption Spectra of Anthemis Phenylglucoside

Before and After Acid Hydrolysis

Figure 14

Absorption Spectra of Solubilized Hydrolysate

Action of Emulsin on the Unknown

An area containing the unknown, approximately 4 counts/second of C^{14} activity, was eluted into a test tube, and a few crystals of emulsin were added to the solution. After incubation at room temperature for six hours, the solution was applied to a paper chromatogram and developed in the usual two solvent systems. Since the radioactivity appeared in the glucose area, it was eluted and chromatographed with authentic glucose. The radioactivity co-chromatographed with glucose.

Determination of Absorption Spectrum of Unknown Before and After Hydrolysis

Since the paper chromatograms on which the unknown is separated from the other constituents are contaminated with phenol, it is necessary to remove the contaminating phenol before any absorption spectrum of the unknown can be determined in the ultraviolet region of the spectrum. The separation of the phenol can best be accomplished by rechromatographing the unknown compound in a non-phenolic system. Therefore, the unknown compound was eluted from several chromatograms and rechromatographed in a solvent system composed of ethanol: 15% acetic acid (8:1 by volume) solvent system. The traces of phenol moved with the front, whereas, the unknown had an R_F of 0.8. The unknown was located by its radioactivity and was eluted with distilled water into a Beckman cuvette. The absorption spectrum from 220 to 300 mμ of the unknown was determined using the eluate from a control chromatogram as a blank.

The solution was then made 2 N with hydrochloric acid and placed in a 5 ml. flask with a water-cooled reflux condenser attached. The mixture was heated on a steam bath for two hours and the absorption spectrum was determined. The two spectra are shown in Figure 14.

Similarly, some authentic phenylglucoside was developed in the above chromatographic system, and the absorption spectrum of the eluate determined. The eluate was treated in the same manner as the unknown and, its absorption spectrum was determined. The spectra of the phenylglucoside before and after acid hydrolysis are shown in Figure 13. The spectrum of the unknown compound (Figure 14) both before and after hydrolysis appears essentially identical with that of phenylglucoside before and after hydrolysis.

Co-Chromatography of the Unknown with Phenylglucoside

Portions of unknown solution containing three to four counts/second of C^{14} activity were spotted on the origin of each of three paper chromatograms. To each was added sufficient authentic phenylglucoside such that the phenylglucoside could be readily detected. This was accomplished by placing the chromatogram over a calcium tungstate screen and irradiating the chromatogram with ultraviolet light (2537 Å. maximum). The phenylglucoside appeared as a blue-violet spot on the chromatogram and as a dark shadow on the screen. The chromatograms were developed in the following solvent systems: n-butyl alcohol-propionic acid-water;[5] acetic acid-water, 15:85 by volume; and ethyl alcohol-15% acetic acid, 8:1 by volume. Radioautograms were prepared of the developed chromato-

grams. In all the above solvent systems, the unknown and authentic phenylglucoside co-chromatographed.

Discussion

As shown in Table 3, only a trace of activity was observed in the area occupied by the unknown when synthesized glucose 6-C^{14} was fed to either etiolated or green plant leaves; whereas, the biosynthesized glucose-C^{14}, isolated from chromatograms developed in water-saturated phenol, formed appreciable quantities, although in smaller amounts than reported by Tolbert and Zill.[40] The traces of C^{14} activity in the unknown area obtained when glucose 6-C^{14} was fed to the plant could conceivably be due to traces of phenylalanine, leucine, or isoleucine, which have approximately the same R_F values in the solvent systems used as does the unknown.

The results in Table 4 show that the unknown was formed when phenol was added to the glucose 6-C^{14} solution or when the glucose 6-C^{14} was eluted off paper chromatograms which have been exposed to phenol. At very high phenol concentrations, 300 micrograms, the metabolism of the plant was apparently completely inhibited.

The results in Table 5 show that extraction of the biosynthesized sugar solutions with diethyl ether results in the failure of the plant leaf to form any of the unknown compound. Such experimental results can best be interpreted to mean that some contaminant was removed by the ether extraction. Addition of small quantities of phenol to the sugar solutions results in the formation of the unknown compound, indi-

cating that it was probably a phenol contaminant removed by the ether extraction.

The above indirect evidence that the unknown was phenylglucoside was confirmed by the determination of the absorption spectrum before and after hydrolysis of the unknown. The two absorption spectra were essentially identical to those obtained for phenylglucoside treated in the same manner. The unknown also co-chromatographed with authentic phenylglucoside. Hydrolysis of the unknown by emulsin indicates that it is the beta form of phenylglucoside that was obtained.

The observation that smaller amounts of the unknown were formed in the experiments reported here than in those reported by Tolbert and Zill a year previously may be explained on the basis that the phenol was slowly subliming from the paper chromatograms on which the sugars were stored, or undergoing changes in the dilute sugar solutions after elution from the papers. Consequently, the concentration of the contaminating phenol was probably lower in the sugar solutions used in these experiments than in those reported previously.

The fact that the unknown is phenylglucoside suggests that the phenylglucoside may be a detoxification product of phenol in the plant since it was formed when either glucose-C^{14} or sedoheptulose-C^{14} was fed to the plant. Sedoheptulose-C^{14} apparently could not be used directly for detoxification, but rather had to be converted by the alternate glucose metabolism pathway to glucose-C^{14} which could then be used in the detoxification process. It is of interest that the mechanism of detoxification of phenol in the plant may be different from that in the ani-

mal body where phenol is converted to either phenol sulfate or the phenyl glucuronide before excretion.[1] It is possible, however, that the latter products are also formed in the plants since an exhaustive study to ascertain their presence has not been made.

It would be of interest to know whether the flavonoid and anthocyanin aglycones are also converted to the glycoside form when fed to a plant. Such experiments would help establish the plausibility of the concept that the flavonoid and anthocyanin glycosides are detoxification products in the plant.

CHAPTER V

EFFECT OF LIGHT QUALITY ON PHOTOSYNTHESIS IN ANTHOCYANIN-CONTAINING LEAF TISSUE. I. DEMONSTRATION OF FILTER EFFECT OF ANTHOCYANINS BY MEANS OF $C^{14}O_2$ FIXATION AND CHLOROPHYLL FLUORESCENCE

Introduction

There are some conflicting reports in the literature as to whether the rate of photosynthesis is affected by anthocyanin pigments in the leaves of certain plants. Sen reported a more intense photosynthesis in the red varieties of Eranthemum, although the chlorophyll content was greater in the varieties with green leaves.[35] Gabrielsen observed a filter effect of the anthocyanins in the red leaves of Corybis maxima var. purpurea and Prunus ceracifera var. pessate.[12] The red leaves assimilated CO_2 more effectively in violet blue light than in yellow green light; the difference was attributed to the greater absorption of the yellow green light by the red anthocyanin pigments. Though these two reports seem to differ, it is quite possible that the higher rate of CO_2 fixation observed by Sen was due to differences other than the anthocyanin pigment content since he used different varieties of plants in his experiments.

In order to test the filter effect theory reported by Gabrielsen, modern tracer techniques and the intensity of chlorophyll fluorescence

have now been utilized in the investigations reported here to measure the rate of photosynthesis in both anthocyanin-containing leaf tissue and anthocyanin-free tissue. A variety of Coleus was selected as the experimental plant as it had random sections with and without visual anthocyanins. Photographs of the upper and lower surface of a Coleus leaf are shown in Figure 15 and Figure 16. The anthocyanin pigments, as can be seen in the photographs, are located primarily in the upper epidermis of the leaf. With such a leaf, it is therefore possible to study the rate of photosynthesis in anthocyanin-containing tissue and anthocyanin-free tissues which had essentially the same previous environmental treatment and which can be exposed to the same light intensities when measuring the rate of photosynthesis.

If the anthocyanin does exert a filter effect, the rate of photosynthesis, at least at relatively low light intensity, should be markedly decreased in the anthocyanin-containing section of the leaf as compared with the anthocyanin-free section of the leaf, when the leaf is exposed to light in a region of the spectrum where the anthocyanins absorb very strongly. On the other hand, in a region where the anthocyanin pigments absorb only slightly, there should be little or no difference in the rate of CO_2 fixation in the two sections of the leaf.

Preliminary studies on the anthocyanin pigments in the Coleus leaf used in these studies showed that a dilute aqueous hydrochloric acid extract of the leaf had a maximum absorption at 5200 A and absorbed very little above 6450 A. Although the absorption of the pigments in the leaf itself may not be identical to that of the hydrochloric acid ex-

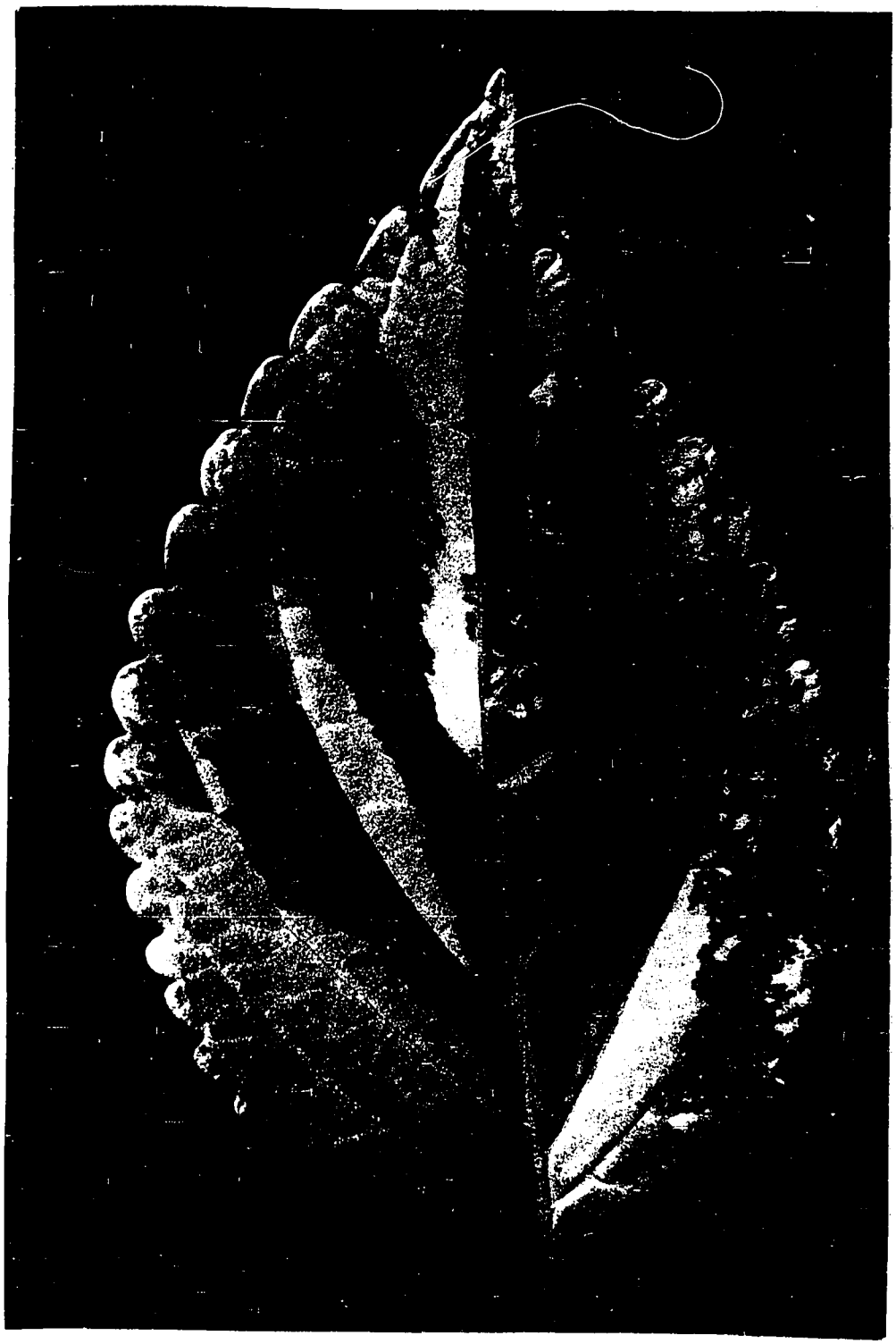


Figure 15

Photograph of Upper Surface of Coleus Leaf

Figure 16

Photograph of Lower Surface of *Coleus* Leaf

tract, it apparently is very similar as a difference spectrum, obtained by subtracting the spectrum of the anthocyanin-free section of the leaf from the anthocyanin section of the leaf, had an absorption maximum at 5250 Å. Therefore, if one uses green light (4650-5450 Å), the CO_2 fixation rate should be much lower in the anthocyanin-containing section than in the anthocyanin-free section if there is a filter effect; whereas, in red light, 6450 Å or greater, the rate should be nearly equal in both sections.

It has been shown that the action spectrum as determined by the intensity of chlorophyll fluorescence is essentially the same as that determined by CO_2 fixation.[38] Therefore, the intensity of the chlorophyll fluorescence might also be considered as a measure of photosynthetic ability. Accordingly, one would expect the intensity of the chlorophyll fluorescence to be lower in the anthocyanin section of the leaf when irradiated with green light.

Materials and Methods

Light Sources

Red light of wave lengths greater than 6450 Å was obtained by allowing the light from a G. E. reflector flood lamp to pass through a red filter, Corning 2030, from an otherwise light-tight box. A water bath, 5 cm. thick, was placed in front of the filter to prevent any heating effects from the light source. The photosynthesis chamber was usually placed about 20 cm. from the lamp (8 cm. from the red filter).

Green light was obtained by using a filter system similar to the one described by Withrow et al.[43] The light from a G. E. photoflood lamp was allowed to pass through three separate filter systems composed of a 5 cm. thick water bath, 5 cm. thick solution of copper sulphate (200 g./liter) and 2.5 cm. of picric acid solution (0.3 g./liter). The system is essentially the same as Withrow's and therefore should have cut off points at 4650 Å. and 5600 Å. The light passing through the filter system described appeared green to the eye.

Preparation of $NaHC^{14}O_3$ Solution

The $NaHC^{14}O_3$ solution was prepared by treating a known amount of $BaC^{14}O_3$ with an excess of lactic acid and collecting the $C^{14}O_2$ in a 0.1 N sodium hydroxide solution. The apparatus consisted of two flasks, one with a side glass sac, connected by a tube with ground glass joints and an outlet to permit partial evacuation. A weighed amount of $BaC^{14}O_3$, 15-20 mg., with a specific activity of 0.0893 millicuries/mg., was placed in the flask with the side sac. Approximately 1 ml. of 3% lactic acid was then placed in the side glass sac by means of a curved pipette. In the second flask was placed approximately 1 ml. of 0.1 N sodium hydroxide. The two flasks were connected by means of the glass tube and a slight vacuum pulled on the system. The system was then sealed off, the flask with the side sac rotated so that the acid was poured into the $BaC^{14}O_3$, thus releasing the $C^{14}O_2$. The closed apparatus was allowed to stand at room temperature for one day, the system unsealed, and then the flask, containing the $NaHC^{14}O_3$ solution was closed with a ground glass

jointed cap. The $NaHC^{14}O_3$ solution was stored in the deep freeze and removed only when used and then immediately returned to the deep freeze.

Photosynthesis Chamber

The photosynthesis chamber used was a modification of those described by Burris et al.[6] A chamber with inside dimensions, 9.0 x 9.0 x 2.0 cm. was made from 3/8 in. lucite with a removeable top, which had been machined so that it fit tightly. A notch was cut on the inside of the midpoint of both sides about 2 cm. from the top in order that a small porcelain boat could be placed in the notches. The top had an inlet tube, directly over the boat, made by sealing into the top a 5/8 in. lucite tube. A rubber serum vial cap was placed over the inlet tube making the chamber, for practical purposes, air tight.

The peicle of the leaf was placed in a small tube made from the bottom 3 cm. of a 3 ml. conical centrifuge tube, and this tube was held in place by placing a layer of glass beads about 2 cm. deep in the bottom of the chamber. A heavy piece of black paper was placed on the back side of the chamber when it was desired to irradiate the leaf from only one direction.

The procedure for generating $C^{14}O_2$ in the photosynthesis chamber consisted of placing an aliquot of standard $NaHC^{14}O_3$ solution in the porcelain boat. The cover was then placed on the chamber, and a small amount of 25% lactic acid was introduced through the serum cap into the boat by means of a hypodermic needle and syringe. The $C^{14}O_2$ was immediately released and the rate of $C^{14}O_2$ fixation was determined as described later.

Determination of Chlorophyll

Total chlorophyll was determined essentially according to the method of Arnon.[2] A leaf section containing no large veins was weighed to the nearest milligram (30-100 mg. sample) and then ground in a mortar in 85% acetone (3.5 ml.). The extract was decanted into a centrifuge tube and the mortar and pestle washed with 85% acetone. The insoluble material was centrifuged down, the volume noted, and the supernatant solution poured directly into a Beckman cuvette. A solution of 85% acetone was used as a blank and the optical density determined at 6450 and 6630 Å. Chlorophyll was then calculated by the following equation:

$$C = (20.2\ D_{6450} + 8.02\ D_{6630}) \times \frac{V}{W}$$

C = mg. chlorophyll/g. of plant tissue

D_{6450} = optical density at 6450 Å.

D_{6630} = optical density at 6630 Å.

V = volume in ml. of extract

W = weight in grams of plant tissue

Determination of the $C^{14}O_2$ Fixed

After a leaf had been allowed to fix $C^{14}O_2$ in the photosynthesis chamber for a given period of time, the leaf was removed from the chamber, and a section of the leaf cut out and weighed on a torsion balance to the nearest milligram. The weighed leaf section was then frozen under liquid nitrogen, ground by means of a mortar and pestle and extracted with a few milliliters of distilled water. The extract was

poured quickly into a thick walled centrifuge tube, the mortar and pestle were rinsed with distilled water, and the rinsings were added to the centrifuge tube. The centrifuge tube containing the extract was immediately plunged into boiling water and allowed to stand in it for five minutes. The solid material was centrifuged down and the total volume was noted. A 25 microliter aliquot of the extract was then plated out on a metal planchlet, and the C^{14} activity determined in a Geiger-Müller shielded counting chamber. The C^{14} fixed was then calculated on a fresh weight basis, and by utilizing a previous chlorophyll analysis, on a chlorophyll basis.

Experimental

Determination of $C^{14}O_2$ Fixation in the Two Sections of The *Coleus* Leaf in Red and Green Light

A *Coleus* leaf was removed from the plant and placed in a small test tube of water. A sample of both the anthocyanin-free and anthocyanin-containing section of the *Coleus* leaf was cut out and a chlorophyll determination made. The remainder of the leaf was then placed in the chamber and allowed to photosynthesize for a period of ten minutes in either red or green light before the $C^{14}O_2$ was released. $C^{14}O_2$ fixation was then allowed to proceed for a period of five or ten minutes. At the end of this time, the leaf was removed and the amount of $C^{14}O_2$ that had been fixed was determined as described above. The results are recorded in Table 6.

Determination of Intensity of Chlorophyll Fluorescence in the Two Sections of the *Coleus* Leaf in Green Light

The *Coleus* leaf was irradiated with green light from the upper surface, and a long time exposure photograph taken of the leaf with a Corning 2030 filter over the lens of the camera (Figure 17). The filter removed the green light with the result that the major portion of the light entering the camera was due to the chlorophyll fluorescence. An exposure time of two hours was necessary to get a satisfactory negative. Similarly the chlorophyll fluorescence was determined when the lower surface of the leaf was irradiated with green light (Figure 18).

Discussion

The total amount of $C^{14}O_2$ fixed in five and ten minute intervals in both red and green light in the two sections of the leaves recorded in Table 6 shows quite conclusively that the rate of CO_2 fixation on a chlorophyll basis was markedly lower in the anthocyanin section of the leaf in green light, but there was little or no difference in the fixation rate between the two sections in red light.

Figures 15 and 16 are photographs of the upper and lower surfaces of the *Coleus* leaf as they appear in visible light. Figures 17 and 18 are photographs of the leaf taken with a special filter (which permits the passage of the red chlorophyll fluorescence, but not green light) over the lens of the camera. The photograph shown in Figure 17 was taken when the top surface of the leaf was exposed to green light. Figure 18 is a photograph taken in a similar manner when the lower surface of the leaf is exposed to green light.

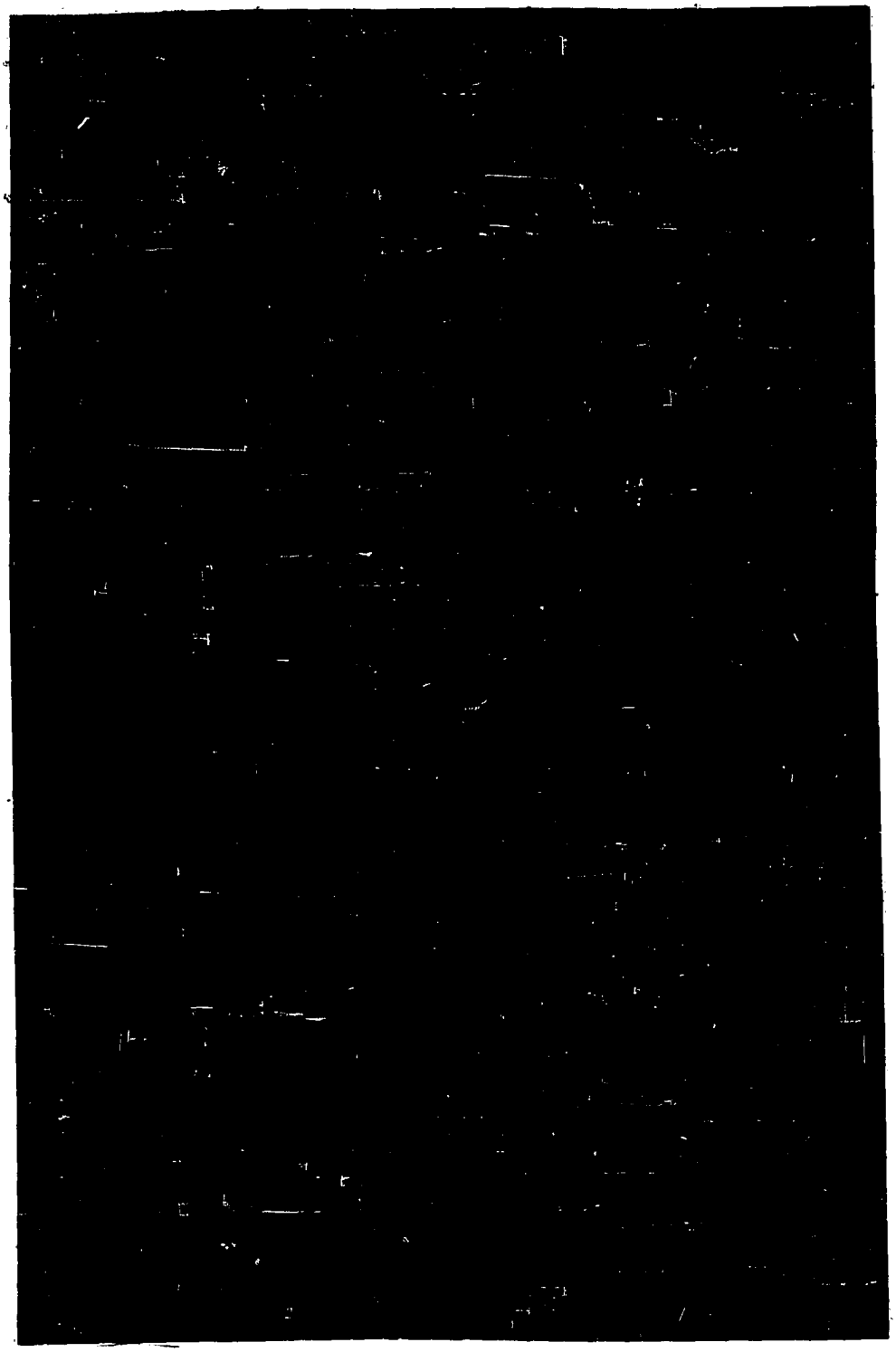


Figure 17

Photograph of the Chlorophyll Fluorescence of Coleus Leaf with Top Surface Exposed to Green Light

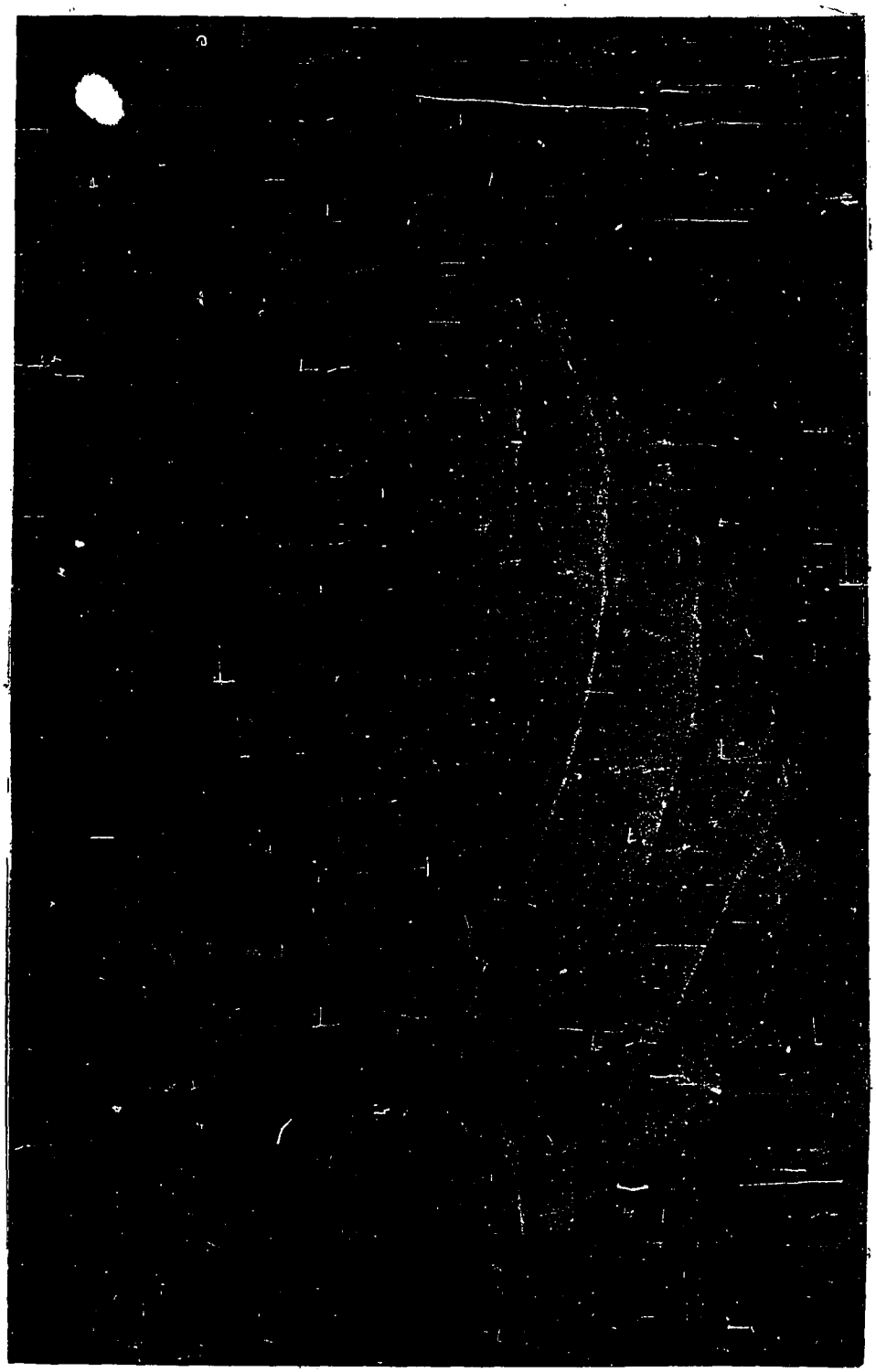


Figure 18

Photograph of Chlorophyll Fluorescence of Coleus Leaf with the Lower Surface Exposed to Green Light

Table 6

EFFECT OF LIGHT QUALITY ON THE RATIO OF $C^{14}O_2$ FIXED IN THE ANTHOCYANIN-CONTAINING TO THE ANTHOCYANIN-FREE SECTION OF THE COLEUS LEAF*

Quality of Light	Time of P.S.	Section of leaf	Counts/sec. 100 mg. Plant tissue	µg. of Chlorophyll/ Gram plant Tissue	Counts/sec. 100 mg. Chlorophyll	Ratio*
red	10 min.	anthocyanin	1775	412	430	.97
		chlorophyll	1520	345	441	
red	5 min.	anthocyanin	1191	485	246	1.14
		chlorophyll	1176	546	215	
green	10 min.	anthocyanin	81	261	31	.14
		chlorophyll	243	112	217	
green	5 min.	anthocyanin	575	554	104	.37
		chlorophyll	1268	450	282	

* $\frac{\text{Counts fixed per 100 µg. of chlorophyll in anthocyanin-containing section}}{\text{Counts fixed per 100 µg. of chlorophyll in anthocyanin-free section}}$

A careful study of the photographs shows that the chlorophyll fluorescence was nearly the same in both sections of the leaf when irradiated on the lower surface of the leaf. When irradiated on the top surface, however, the chlorophyll fluorescence was much higher in the anthocyanin-free section of the leaf. One explanation for these observations is that when irradiated from the lower surface, the anthocyanins are unable to filter out the light before it gets to the chlorophyll; whereas, when irradiated from the top surface, the anthocyanins can remove a large percentage of the light by absorption before it gets to the chlorophyll. Thus the filter effect of the anthocyanins was confirmed by these chlorophyll fluorescence studies in the two sections.

EFFECT OF LIGHT QUALITY ON PHOTOSYNTHESIS IN ANTHOCYANIN-CONTAINING LEAF TISSUE II. EFFECT OF ULTRAVIOLET LIGHT

Introduction

No function for the anthocyanin pigments in plants has been demonstrated to our knowledge. Among the functions often attributed to the anthocyanins is that they filter out deleterious ultraviolet rays from sunlight. The investigations reported in this section were intended to test this hypothesis.

If the anthocyanin pigments do have a protective effect, then anthocyanin-containing leaf tissue should be more resistant to injury from ultraviolet irradiation. Photosynthesis has been shown to be markedly diminished by ultraviolet irradiation.[15] Therefore, one would expect ultraviolet irradiation to be less effective in lowering the rate of photosynthesis in anthocyanin-containing tissue. A variety of Coleus, used in previous studies to show the filter effect of anthocyanin pigments, is an ideal plant for such a study, since it contains both anthocyanin-free and anthocyanin-containing leaf tissue. Previous studies have shown that the rate of photosynthesis in red light in both types of tissue within the same leaf is directly proportional to the chlorophyll content. Therefore, it should be possible to study the effect of ultraviolet light on photosynthesis in both types of tissue under the same conditions and determine whether or not there is any protective action.

Materials and Methods

Ultraviolet Light Sources

A General Electric 15 watt germicidal lamp, 2537 Å. wavelength maximum, was used as the ultraviolet light source.

Irradiation of the Leaf with Ultraviolet Light

The experimental leaf was cut from the Coleus plant, and its stem placed in a small test tube filled with water. Samples of 30-70 mg. of both types of leaf tissue were cut out, and a chlorophyll analysis was performed according to the method of Arnon.[2] The remainder of the leaf, with its stem still in water, was placed at a distance of 3 cm. from the lamp tube of the germicidal lamp. The time of exposure to the lamp's irradiation varied from five to thiry minutes.

Experimental

Determination of $C^{14}O_2$ Fixation Rate After Ultraviolet Exposure

The leaf was allowed to stand for ten minutes in air in red light in order for equilibration to occur and then was allowed to photosynthesize for ten minutes in the presence of $C^{14}O_2$. After the ten minute photosynthesis period with $C^{14}O_2$, the leaf was removed and a sample from the two sections cut out, weighed, frozen in liquid nitrogen, extracted as described in the previous section, and the total $C^{14}O_2$ fixed determined. The results of these experiments are recorded in Table 7.

A second group of experiments was run in which the leaf was

Table 7

EFFECT OF ULTRAVIOLET LIGHT, 2537 Å MAXIMUM, ON $C^{14}O_2$ FIXATION IN THE ANTHOCYANIN-CONTAINING AND ANTHOCYANIN-FREE SECTIONS OF THE COLEUS LEAF*

Time Exposed to U.V.	Section of leaf	Counts/sec. 100 mg. Plant tissue	µg. of Chlorophyll/ Gram Plant tissue	Counts/sec. 100 mg. Chlorophyll	Ratio a
7 min.	anthocyanin	1875	427	439	0.77
	chlorophyll	2260	397	569	
15 min.	anthocyanin	2201	610	394	0.57
	chlorophyll	2306	336	686	
30 min.	anthocyanin	366	563	66	0.50
	chlorophyll	492	371	133	

* Leaf allowed to fix $C^{14}O_2$ for 10 minutes in red light.

a. $\frac{\text{Counts fixed per 100 µg. of chlorophyll in anthocyanin-containing section}}{\text{Counts fixed per 100 µg. of chlorophyll in anthocyanin-free section}}$

Table 8

EFFECT OF ULTRAVIOLET LIGHT, 2537 Å. MAXIMUM, ON $C^{14}O_2$ FIXATION IN THE ANTHOCYANIN-CONTAINING AND ANTHOCYANIN-FREE SECTIONS OF THE COLEUS LEAF*

Time Exposed to U.V.	Section of leaf	Counts/sec. 100 mg. Plant tissue	µg. of Chlorophyll/ Gram Plant tissue	Counts/sec. 100 mg. Chlorophyll	Ratio[a]
7 min.	anthocyanin	3886	558	661	0.93
	chlorophyll	3845	539	713	
15 min.	anthocyanin	2921	539	542	0.83
	chlorophyll	2771	413	671	
30 min.	anthocyanin	2026	502	403	0.90
	chlorophyll	1989	448	444	

* Leaf allowed to stand for 16 hours after exposure to ultraviolet light.

[a] $\frac{\text{Counts fixed per 100 µg. of chlorophyll in anthocyanin-containing section}}{\text{Counts fixed per 100 µg. of chlorophyll in anthocyanin-free section}}$

allowed to stand overnight, approximately 18 hours, before the $C^{14}O_2$ fixation rate was determined. The results of these experiments are recorded in Table 8.

Aliquots of the extracts equivalent to less than 25 mg. of leaf tissue were chromatographed on Whatman #1 filter paper (18 in. x 22 in.). The chromatograms were developed first (in the short direction) with the water-saturated phenol solvent system and then in the butyl alcohol-propionic acid-water solvent system. A photograph of a chromatogram of $C^{14}O_2$ fixation products in the anthocyanin section of the leaf after ultraviolet light (2537 Å) treatment is shown in Figure 19. The chromatogram of anthocyanin-free section of the leaf was essentially identical and is, therefore, not shown. Figure 20 is a photograph of a chromatogram of $C^{14}O_2$ fixation pattern in an untreated Coleus leaf. The key compounds are labeled on the chromatograms.

Discussion

The results shown in Table 7 indicate that the ultraviolet light, 2537 Å. maximum, has a marked effect on the ratio of $C^{14}O_2$ fixation in the anthocyanin-section to the anthocyanin-free section of the Coleus. The results recorded in Table 8 show that if the irradiated leaf was allowed to stand overnight, the ratio of the $C^{14}O_2$ fixation in the two sections approaches unity. However, there still seems to be a trend in the direction of a lower fixation rate in the anthocyanin section on a chlorophyll basis than on the anthocyanin-free section of the leaf. The results certainly do not seem to indicate any protective action on the part of the anthocyanin pigments.

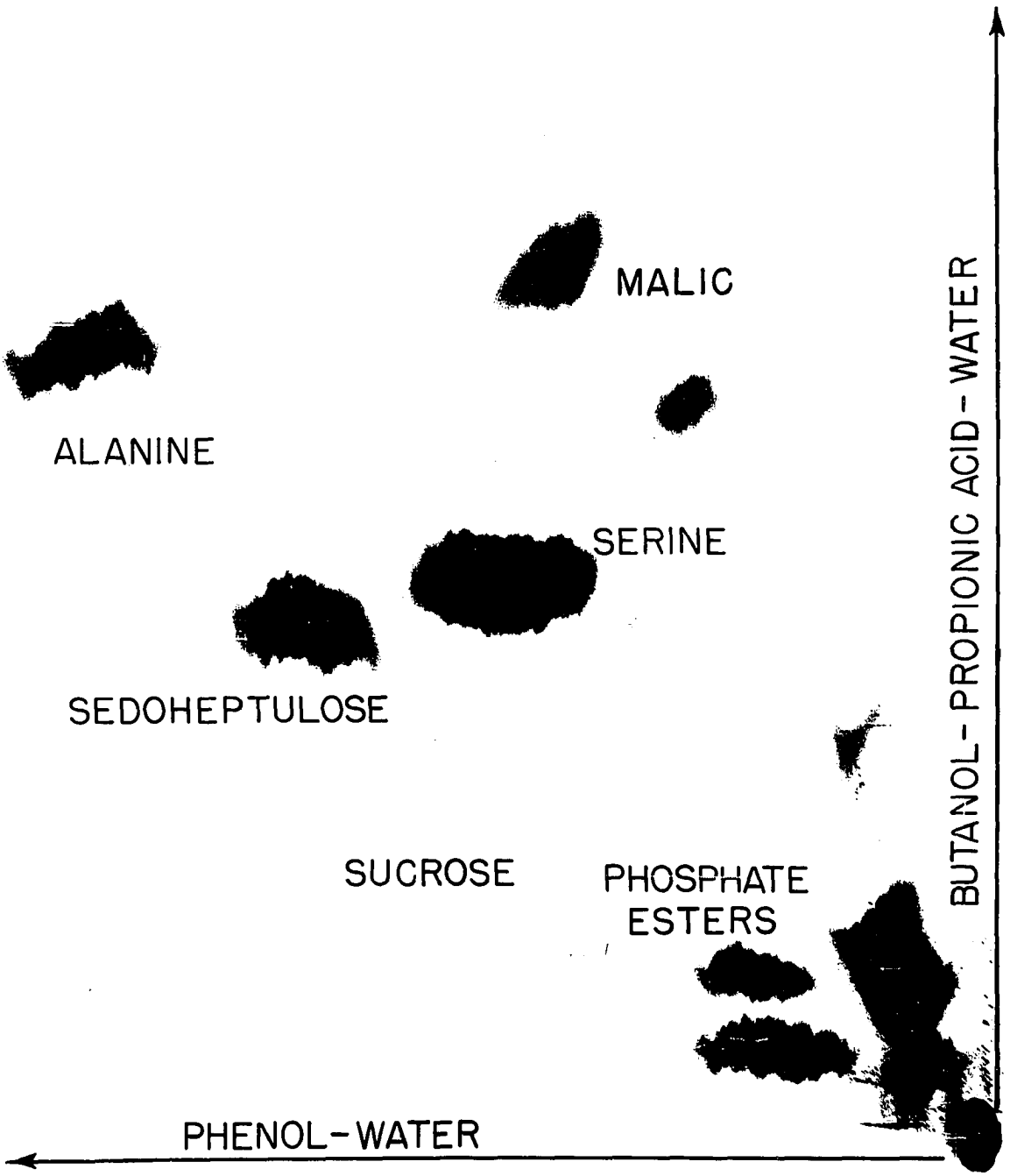


Figure 19

Chromatogram of $C^{14}O_2$ Products in Ultraviolet-Irradiated Coleus Leaf After 10 Minutes Photosynthesis

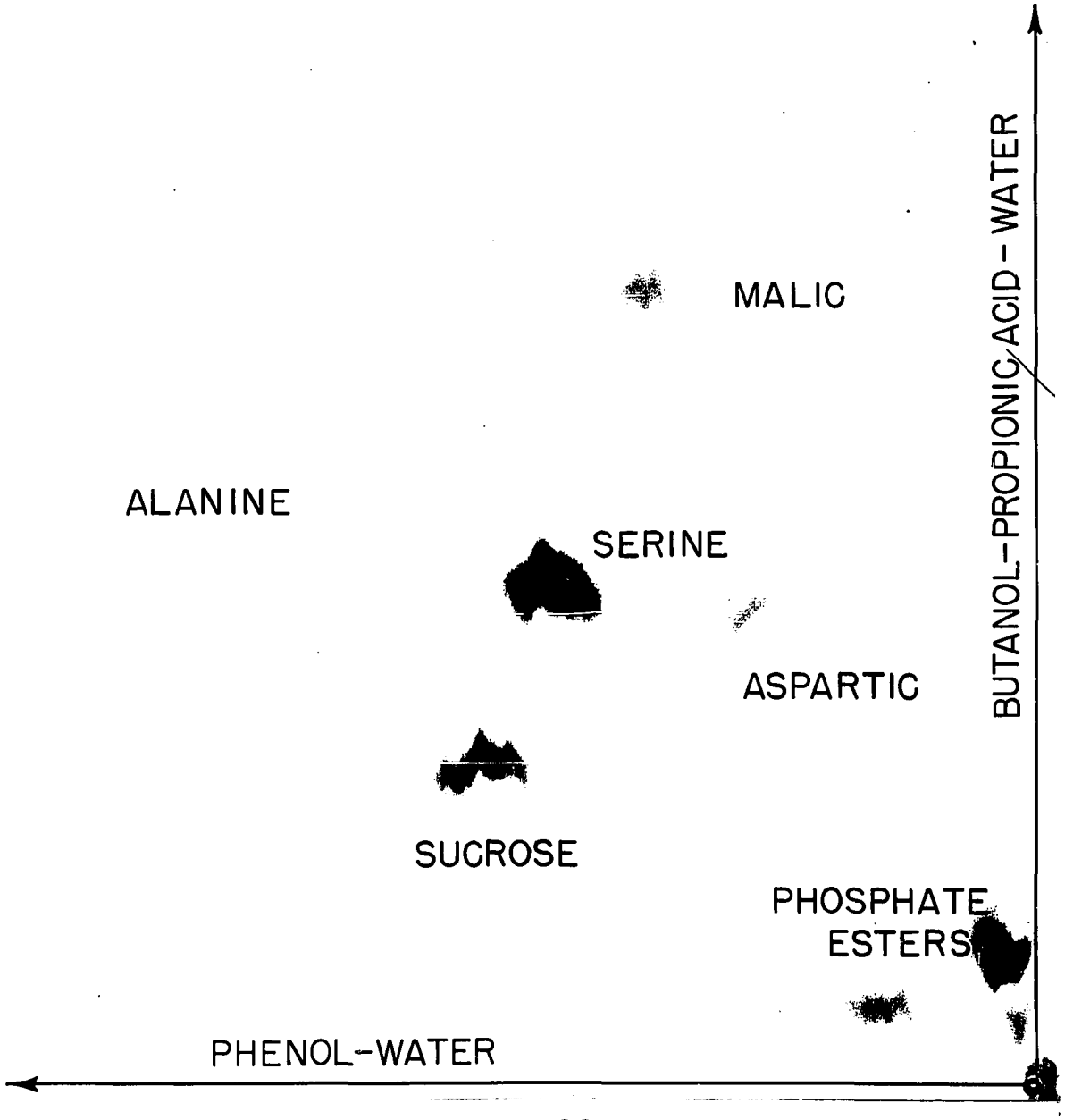


Figure 20

Chromatogram of $C^{14}O_2$ Products in the Coleus Leaf After 10 Minutes Photosynthesis

Examination of the $C^{14}O_2$ fixation pattern as shown in Figures 19 and 20 before and after irradiation may give a possible explanation of the results observed. The important difference between the two chromatograms appears to be the concentration of C^{14} activity in sedoheptulose and sucrose. In the ultraviolet treated Coleus leaf, Figure 17, the sucrose has practically no C^{14} activity; whereas, the sedoheptulose appears to be a major product. In the untreated leaf, Figure 18, the sedoheptulose has practically no C^{14} activity; whereas, the sucrose appears to be a major product on the chromatogram. The high ratio of sedoheptulose to sucrose, observed in the ultraviolet treated Coleus leaf, is typical of $C^{14}O_2$ fixation at very low CO_2 concentrations.[33] If one assumes that for some reason the CO_2 concentration is the limiting factor in the fixation rate, then it would be invalid to assume that the chlorophyll content is the limiting factor as was the case in the untreated leaf. A low internal CO_2 concentration may be the explanation for the lower fixation rate on a chlorophyll basis in the anthocyanin-containing section of the leaf after ultraviolet light irradiation.

It is also possible that the ultraviolet light may have had a differential effect on the stomatal condition of the plant thus depleting the CO_2 concentration to a greater extent in the anthocyanin-containing section of the leaf. Attempts to detect any differences in the stomatal condition of the two sections were, however, unsuccessful.

CHAPTER VI

ISOLATION OF, AND STUDIES ON, A FLAVONOID-PROTEIN COMPLEX

Introduction

The hypothesis that flavonoid and anthocyanin pigments might function in oxidation-reduction processes is based upon the differences found in naturally occuring compounds of this group in the oxidation level of the carbons 2, 3, and 4 of the pyrone ring. If these pigments do function in plants in such processes one might find one of these pigments in close association with an enzyme or protein molecule. An attempt was, therefore, made to investigate the possibility of a flavonoid-protein complex existing in plants.

Extracts of spinach leaves were investigated as a possible source of such a flavonoid-protein complex in that Aronoff[3] had observed a yellow colored protein in the supernatant of his chloroplast preparations from spinach. The yellow color of Aronoff's protein solution could not be removed by dialysis. The color changed to deep yellow in basic solution and to green on the addition of ferric chloride. These are typical reactions of many flavonoid compounds. The fact that the material did not give a positive reduction test with magnesium and hydrochloric acid was considered by Aronoff as sufficient evidence to rule out the presence of flavone like compounds. However, this inter-

pretation does not seem justified since one often has difficulty obtaining a positive reduction test with many flavonoids in the presence of impurities in the usual laboratory test.

Studies were carried out on the yellow colored protein from spinach, and by use of paper chromatography, chromogenic sprays, and colors in ultraviolet light, traces of flavonoid-like materials were shown to be present. Similar studies were carried out on corn extracts and again a flavonoid-like-protein complex was indicated. Examination of the complex from corn by ionophoresis indicated that the flavonoid-like material and the corn protein could be separated.

Another manner in which one might expect the anthocyanins and flavonoids to function in oxidation-reduction reactions is by the oxidation-reduction of the ortho dihydroxy phenyl grouping in some of these pigments. For example, it has been shown that quercetin (3,5,7,3',4'-pentahydroxyflavone) will catalyze the oxidation of ascorbic acid by peroxide in the presence of the enzyme peroxidase.[17] The mechanism of the catalytic effect of the flavonoid is presumed to be by the oxidation of the 3'4'-dihydroxyphenyl group to an ortho quinone. The ortho quinone is then reduced chemically by ascorbic acid, thus, regenerating the dihydroquinone. Thus, the reaction may be written as follows:

$$\text{catechol} + \tfrac{1}{2}\,O_2 \longrightarrow \text{o-quinone} + H_2O$$

$$\text{o-quinone} + \text{ascorbic acid} \longrightarrow \text{dehydro ascorbic acid} + \text{catechol}$$

Ascorbic acid can not be oxidized directly by the peroxide-peroxidase system.

There is also the enzyme tyrosinase or polyphenolase which can oxidize some flavonoids to the ortho quinone form utilizing molecular oxygen.[32] One would, therefore, expect the system containing tyrosinase, quercetin, and molecular oxygen to be able to oxidize ascorbic acid. There is also another enzyme known as ascorbic acid oxidase which can oxidize ascorbic acid directly utilizing molecular oxygen. Both the ascorbic acid oxidase and the tyrosinase have been shown to have copper as their prosthetic group. One might, therefore, suggest that the addition of some flavones to tyrosinase might conceivably make it act as an ascorbic acid oxidase and, therefore, it seemed advisable to check on the possibility that the enzyme ascorbic acid oxidase might contain a flavonoid as a prosthetic group. A preparation of ascorbic acid oxidase was investigated for the presence of flavonoid compounds, before and after acid treatment, using paper chromatographic techniques. No typical flavonoid material could be detected on the paper chromatograms.

Methods

Detection of Flavonoids

The presence of flavonoid pigments was ascertained by the use of paper chromatographic systems and chromogenic sprays for flavonoids.[10]

Location of Protein on Paper Chromatograms

After innophoresis, the paper strips containing the protein were dried and then stained overnight in a solution of bromphenol blue (.01% bromphenol blue, 5% acetic acid, and 5% mercuric chloride in 95% ethyl

alcohol).[27] After staining the strips, they were washed three times by immersion in 2% acetic acid for ten minutes. After blotting, the stained strips were dried on non-absorbent paper in an oven at approximately 60° C. Under these conditions, the proteins appeared as blue bands against a white background.

Experimental

Investigation of Ascorbic Acid Oxidase for Flavonoid Pigments

Part of the method for the preparation of ascorbic acid oxidase described by Powers et al.[31] was used to prepare ascorbic acid oxidase from two kilograms of fresh summer squash. The purification was carried through the first three steps described by these workers. The purification was not carried to any greater length as it was felt that if any flavonoid was present in ascorbic acid oxidase, it most certainly would be present in the impure preparation.

A portion of the ascorbic acid oxidase, equivalent to about one fifth of the whole preparation, was treated with acetone. This resulted in the precipitation of some protein. The supernatant solution was concentrated and applied to paper chromatograms which were developed in the 15% acetic acid-85% water solvent system. The chromatograms were sprayed with Benedict's solution and alcoholic aluminum chloride. No typical flavonoid material could be detected by the method which is sensitive to 40 to 100 micrograms of flavonoid compounds.[10]

A second portion of the preparation was treated with 3% hydrochloric acid for one hour. Part of the protein precipitated and was

separated by centrifugation. The supernatant solution was concentrated and examined chromatographically as above. No evidence for a flavonoid could be detected. There was, however, a blue fluorescent compound present on the chromatograms. Its identity was not ascertained.

Studies on a Flavonoid-Protein Complex in Spinach

Fresh spinach was bought in the local markets and stored in the refrigerator. The stems and large veins were separated from the remaining leaf tissue. Approximately one hundred grams of the leaf tissue were placed in a Waring blendor containing 100 ml. of 0.5 M phosphate buffer (pH = 5) and fifty grams of cracked ice. The material was blended for ten minutes or until thoroughly disintegrated. The mascerate was then filtered through cotton into centrifuge tubes. The filtrate was centrifuged at 3000 g for 30 minutes, the precipitate was discarded, and the supernatant liquid was centrifuged twice again. After the third centrifugation, most of the chlorophyll containing material had been removed, although a faint red fluorescence in the supernatant suggested that there probably still was some chlorophyll in the deep yellow solution.

The supernatant solution was then dialyzed for thirty six hours against distilled water to remove non-bound, dialyzable material, which should include the free flavonoids. At the end of the dialysis, a considerable amount of green colored proteinaceous material, apparently containing chlorophyll, precipitated. This is always observed when the removal of salts denatures some protein. This green material was sepa-

rated from the supernatant liquid by centrifugation, and the absorption spectrum of the supernatant liquid was then determined (Figure 19).

Enough hydrochloric acid was added to a ten ml. portion of the supernatant liquid to make it up to a concentration of 3%, and the mixture was placed in a boiling water bath for one hour. The mixture was concentrated to near dryness and extracted with methyl alcohol. A portion from the methyl alcohol extract was applied to a paper chromatogram, and the chromatogram was developed in the 15% acetic acid-85% water solvent system. A flavonoid-like material could be detected at an R_F of 0.10. The material was colorless in visible light and brown in ultraviolet light. After spraying the chromatograms with Benedict's solution, alcoholic aluminum chloride, or exposure to ammonia, the flavonoid-like material was yellow in visible light. After treatment with the aforementioned spray reagents, the spot appeared orange-brown, orange, and orange-brown respectively in ultraviolet light. Some flavone aglycones behave similarly.

The protein solution was fractionated with ammonium sulfate. Sufficient ammonium sulfate was added to the protein solution to make the concentration 20 g. of ammonium sulfate per 100 ml. of solution, and the resulting protein precipitate was separated by centrifugation. After addition of sufficient ammonium sulfate to raise the concentration to 40 and 60 g. of ammonium sulfate/100 ml. of solution respectively, two additional fractions were collected from the same protein solution. Each of the three protein fractions was analyzed for the presence, and shown to contain some, of the flavonoid-like material. Each fraction of

the precipitated protein was taken up in 0.05 M phosphate buffer (pH = 6.5) and dialyzed against distilled water. Again, a portion of the protein in the solution precipitated during the dialysis. The precipitate contained almost all of the flavonoid material and could not be redissolved in water. Apparently, some irreversible denaturation had occurred.

Studies on a Flavonoid-Protein Complex from Corn

Essentially the same procedure was used for the preparation of a flavonoid-protein complex from corn leaves as was used for the preparation of the complex from spinach leaves. The corn flavonoid-protein complex did not precipitate from solution on dialysis, following the ammonium sulfate fractionation. After acid hydrolysis, the corn flavonoid had an R_F of 0.15 in 15% acetic acid-85% water. In addition to the flavonoid with an R_F of 0.15, there were traces of two other flavonoids, one which had a higher R_F, 0.16, and one with a lower R_F, 0.08. In addition to the flavonoids, there was some blue fluorescent material present.

A large preparation of the corn extract containing the flavonoid-protein after dialysis was lyophilized and stored in a dessicator over Drierite. This material was dissolved in distilled water and used for ionophoresis studies. Aliquots of the material were applied at the center of a paper strip saturated with buffer. A potential of 1000 V. was applied across the paper for four hours. The paper was removed and dried. The position of the flavonoid band was located by exposing

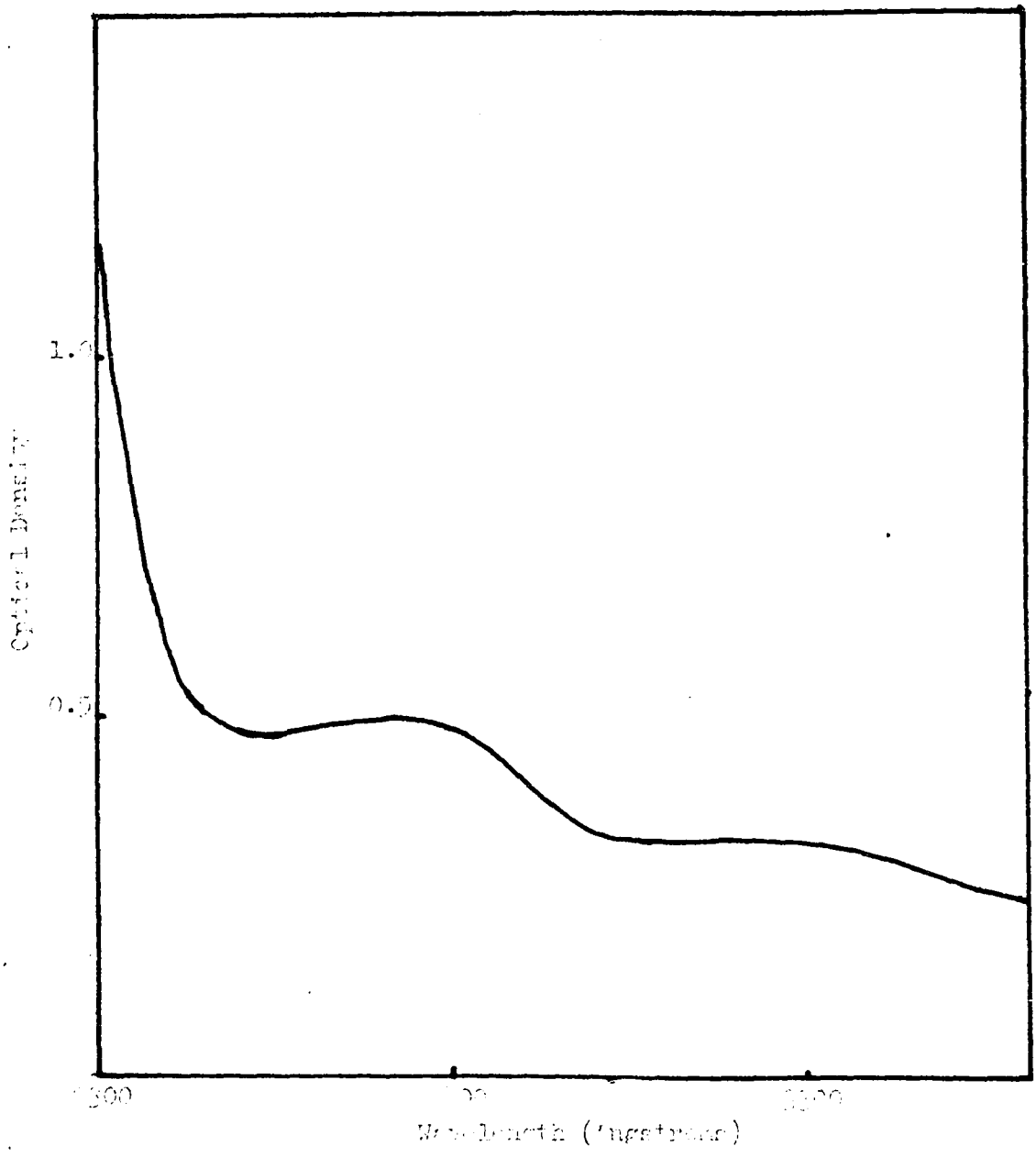


Figure 21

Absorption Spectrum of Starch Extract

After Dialysis Against Distilled Water

the chromatogram to ammonia and observing it under ultraviolet light and looking for the orange colored band. The position of the protein on the chromatogram was located by staining with bromphenol blue. The ionophoresis was carried out in 0.05 M borate buffer (pH = 8); 0.05 M acetate buffer (pH = 5); and 0.05 M phosphate buffer (pH = 6.5). In all cases the flavonoid separated from the protein, as no protein staining material was detected in the position occupied by the flavonoid band.

Discussion

No flavonoid compound could be detected in the experiments conducted on the ascorbic acid oxidase preparations. The studies on the spinach and corn extracts indicate that a flavonoid-like material is associated with the proteins of extracts of these plant materials. The failure of the flavonoid pigments to be dialyzed out indicates that either the flavonoid was bound by a covalent bond or that the dissociation constant of the complex was very high. Although ionophoresis was not used on the spinach flavonoid-protein, the results on the corn flavonoid-protein complex suggest that the bonding was not covalent, as one would not have expected the flavonoid and protein to separate if this were the case. It seems quite possible that the bonding between the flavonoid and protein might be of a metal chelate type which was sufficiently strong to prevent the release of the flavonoid even on prolonged dialysis, although no further experiments were conducted to confirm or refute this possibility.

It may well be that the flavonoid-protein complex is formed in the isolation procedure since the plant is known to contain many metal containing proteins which might complex with the flavonoid during the isolation procedure. On the other hand, the flavonoid-protein complex may be an active component in some enzyme system. The elucidation of the role of the flavonoid-protein complex remains for future investigators.

CHAPTER VII

SUMMARY

The supposedly pure xanthorhamnin obtained commercially was separated by a new paper chromatographic system into its three major component glycosides. Two of these glycosides were characterized as xanthorhamnin and methyl xanthorhamnin. The third glycoside was found to be a derivative of quercetin containing two moles of rhamnose to one mole of galactose. The method of separation provides a satisfactory procedure for obtaining pure xanthorhamnin in experimental quantities.

In studies on the action of the hydrolyzing enzymes, emulsin and alpha glucosidase, it was found that the flavonoid glucosides, isoquercitrin, hesperetin 7-glucoside, naringenin 7-glucoside, and a quercetin glucoside from apricots, were hydrolyzed by emulsin, but not by the alpha glucosidase. The linkage of the glucose to the aglycone must, therefore, be of a beta configuration in all these glucosides. Since the isoquercitrin prepared by partial hydrolysis of rutin is hydrolyzed by emulsin, the attachment of the rhamnoglucoside in rutin to its aglycone, quercetin, is also likely a beta configuration.

Tyrosinase was found to be inactive, under the conditions studied, in introducing an ortho-hydroxyl group into the side phenyl ring of flavonoid compounds with a mono-hydroxy side phenyl ring. It

would seem, therefore, unlikely that the enzyme tyrosinase would be capable of converting flavonoids of the mono-hydroxy side phenyl ring configuration to the corresponding di-hydroxy side phenyl ring compound *in vivo*.

An unidentified compound found in sedoheptulose-C^{14} studies by Tolbert and Zill has been identified in this thesis research as beta-phenylglucoside. It was probably formed in the detoxification of traces of phenol contaminating the biosynthesized sedoheptulose-C^{14} solutions. The fact that the plant detoxified phenol by forming a glucoside is some evidence in support of the theory that the anthocyanin and flavonoid aglycones may be side metabolic products which are detoxified by the formation of glycosides.

The rate of $C^{14}O_2$ fixation found in an anthocyanin-containing section of a *Coleus* leaf as compared to an anthocyanin-free section of the same leaf in red and green light supported the filter effect theory previously reported by Gabrielsen. The rate of $C^{14}O_2$ fixation was directly proportional in red light to the chlorophyll content of the two sections. In green light, the rate was much lower in the anthocyanin section than in the anthocyanin-free section of the leaf.

Studies utilizing the same *Coleus* leaf (which has both anthocyanin-containing and anthocyanin-free leaf tissue) after treatment with ultraviolet light did not give any support to the theory that the anthocyanins protect the plant against the deleterious effects by absorbing out ultraviolet light. In fact, the $C^{14}O_2$ fixation rate after ultraviolet treatment would seem to indicate that the anthocyanin-containing sec-

tions were more sensitive to the deleterious effect of ultraviolet light than the anthocyanin-free sections of the leaf.

A flavonoid-like protein complex was isolated from extracts of spinach and corn leaves. The presence of flavonoid was not established rigorously, but a substance which was bound to the protein gave several typical flavonoid color reactions. The flavonoid-protein complex could not be separated by dialysis and could be precipitated by ammonium sulfate. The complex from corn was, however, separated by paper electrophoresis. The close association of the flavonoid-like compound and the protein suggests that the complex may have some enzymatic function in the plant.

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