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ISOLATION AND IDENTIFICATION OF FLAVONOID COMPOUNDS IN COTTONSEED BY PAPER CHROMATOGRAPHY

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ISOLATION AND IDENTIFICATION OF FLAVONOID COMPOUNDS IN COTTONSEED BY PAPER CHROMATOGRAPHY

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CHAPTER I

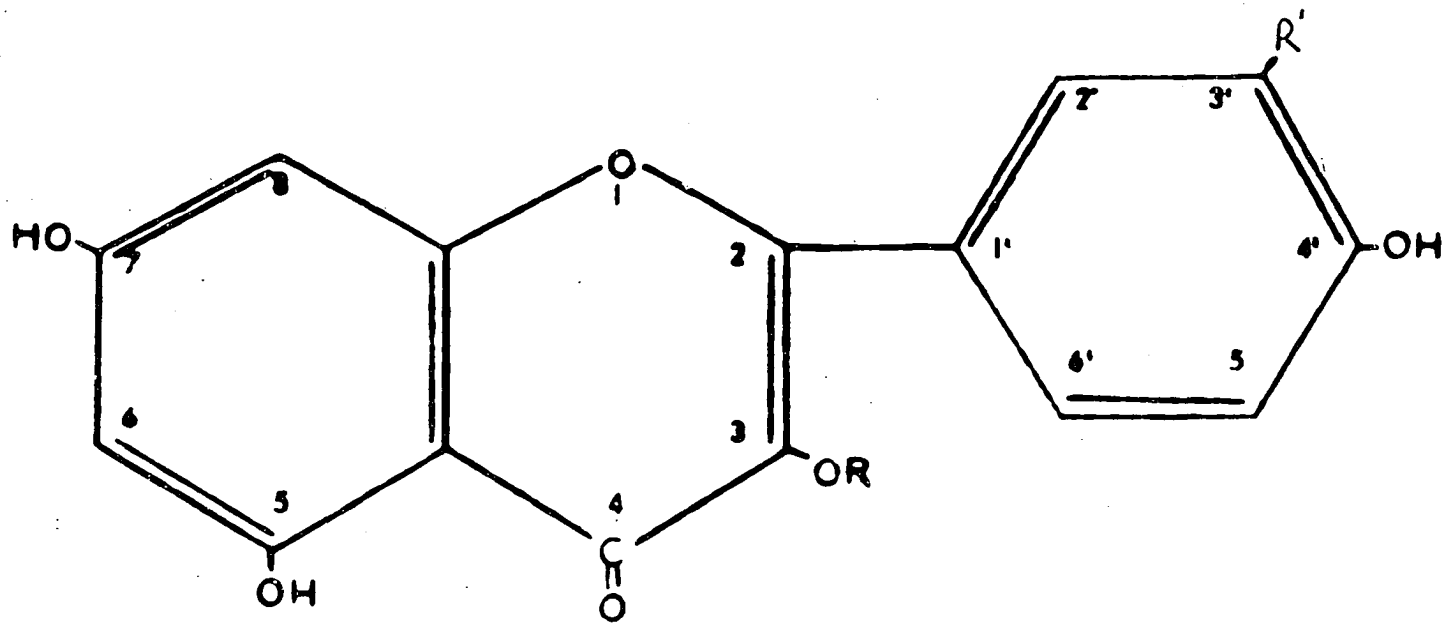
INTRODUCTION

In studies of flavonoid compounds in natural products where the quantity of any one particular compound is very small, paper chromatography has become an extremely useful tool in both isolation and analyses. The work reported here is an example of the important application of paper chromatography to isolation and identification of flavonoid compounds in cottonseed.

In the early 1890's Perkins (1) did extensive work on the flavonoids of cotton flowers. Among the compounds he isolated were gossypetin (3,5,7,8,3',4' - hexahydroxy flavone) and its glucoside, gossypitrin, quercetin (3,5,7,3',4' - pentahydroxy flavone), quercimeritrin (7-glucosyl-quercetin), quercitrin (3-rhamnosyl quercetin), and isoquercitrin (3-glucosyl quercetin). Though there is no rule as yet by which the existence of these compounds in a specific part of a plant can be predicted with any certainty, it was believed that at least some of the compounds occurring in the flowers would be found in the seed, either as the same compounds or as derivatives of them.

FIGURE 1

STRUCTURAL FORMULAE OF QUERCETIN, AND KAEMPFEROL



QUERCETIN R = H and R' = OH

KAEMPFEROL R = H and R' = H

CHAPTER II

EXPERIMENTAL

Isolation of Kaempferol-3-rhamnoglucoside and Quercetin-3-glucosylglucoside

Five kilograms of mechanically delinted cottonseed were crushed with a Waring blendor and extracted with 85% isopropyl alcohol-water solution until the solvent in contact with the seed was almost colorless. The extract was concentrated under reduced pressure to one-tenth the original volume (to about 2 liters). This was washed with an equal volume of water and allowed to stand in the separatory funnel a few hours (2-5 hours). The oil separated as the upper layer. The lower aqueous layer was withdrawn, and the oil was washed a second time with water. This process of washing was repeated until the washings were colorless (usually 4 times). The washings were combined and evaporated under reduced pressure to a volume of 400-500 ml. This combined concentrate is hereafter referred to as the concentrated extract.

The concentrated extract was streaked, with a medicine dropper, on 18 x 22 1/2 in. Whatman No. 3MM rectangular filter paper, 3 inches from the top of the paper and dried with hot air from a hairdryer. The papers were placed in chromatography chambers and developed 12-16 hours in the solvent system n-butyl alcohol-acetic acid-water

(6:1:2 v/v). The papers were removed from the chambers or tanks and allowed to dry at room temperature. Three main zones appeared on the dried chromatograms. They were labeled 1, 2, and 3. See Figure 2. Zone 1 had the smallest R_f value and Zone 3, the largest.

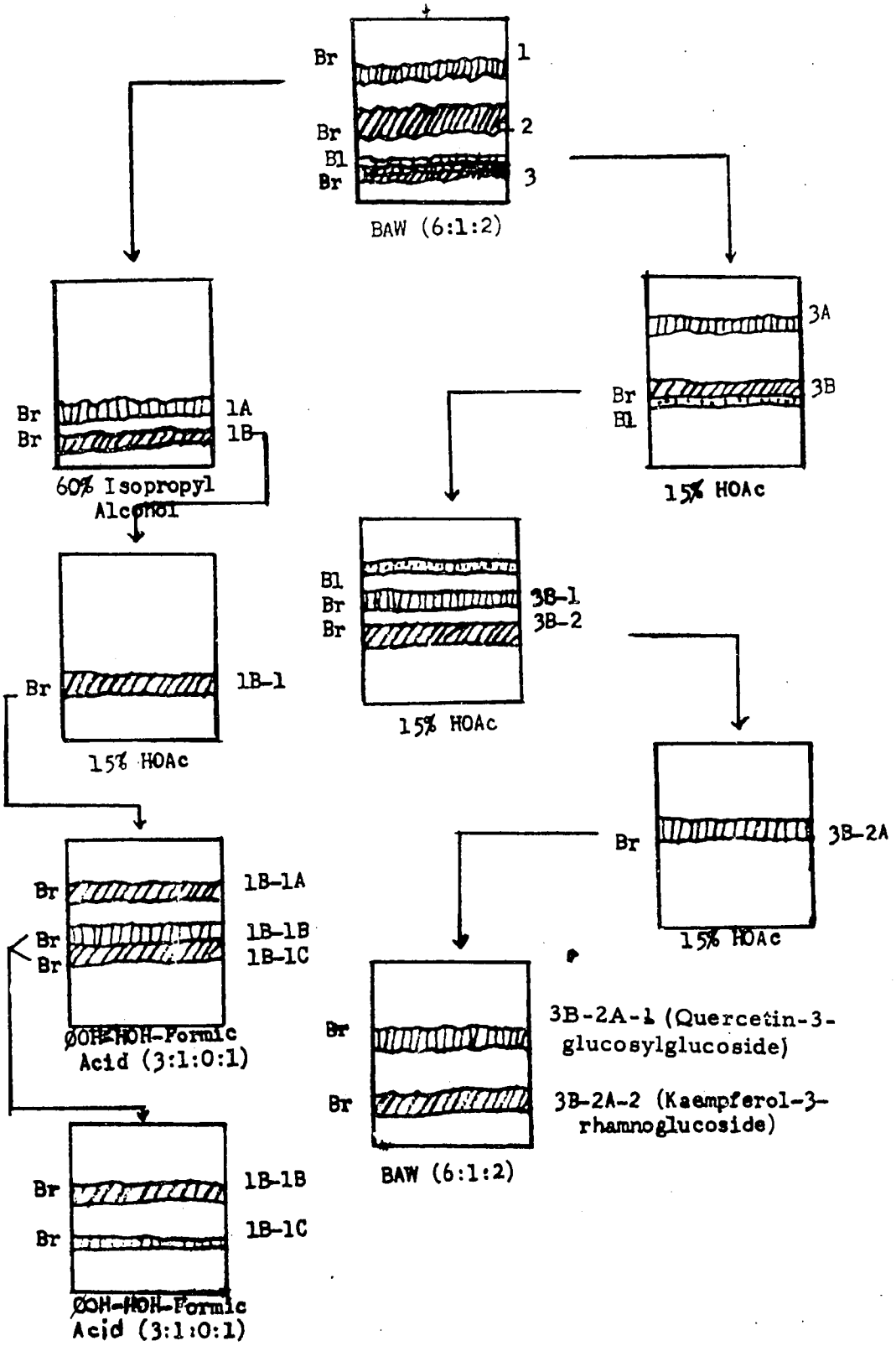
Zone 3 was cut, and then sewed on new sheets of Whatman No. 3MM filter paper, 18 x 22 1/2 in., 3 inches from the top of the sheet. The paper back of the sewed strip was removed. After new sheets containing zone 3 were developed in a 15% acetic acid-water solvent system for 5-6 hours, two zones labeled 3A and 3B were seen. 3A was the zone which had been previously found to contain isoquercitrin (2) and 3B was the zone of higher R_f.

In order to identify the flavonoid compound present in zone 3B, the zone was cut and sewed on new sheets of Whatman 3MM filter paper and developed a second time in the 15% acetic acid-water solvent. Two zones now appeared and were labeled 3B-1 and 3B-2. 3B-1 had the smaller R_f value and appeared to be identical with 3A. It was therefore removed as an impurity, and 3B-2 was investigated.

Zone 3B-2 was cut and sewed on new paper and developed again in the 15% acetic acid-water system. This time only one zone appeared (3B-2A). Zone 3B-2A was cut and sewed on new sheets of paper and developed 14-18 hours in the n-butyl alcohol-acetic acid-water system. Two zones now appeared and were labeled 3B-2A-1 and 3B-2A-2. Each of these zones was cut and sewed on new papers and each was

FIGURE 2

PAPER CHROMATOGRAPHIC SEPARATION OF FLAVONOIDS IN COTTONSEED CONCENTRATED EXTRACT



developed once more in n-butyl alcohol-acetic acid-water. Each zone now appeared to contain only one compound.

Chromatographic Comparison of the Glycosides

The compound "3B-2A-2" was compared by paper chromatography with authentic samples of the following glycosides: rutin, quercitrin, isoquercitrin, and kaempferol-3-rhamnoglucoside. The R_f values of these glycosides in four different solvent systems using Whatman No. 1 filter paper, are given in Table 1.

The R_f values indicate that "3B-2A-2" is not rutin, quercitrin, or isoquercitrin, and that it is chromatographically identical in the solvent systems used with kaempferol-3-rhamnoglucoside. "3B-2A-1" is not any of these compounds.

For further confirmation of the identity of "3B-2A-2", a spot of kaempferol-3-rhamnoglucoside was placed in the upper left-hand corner, about 8 cm. from either edge of a sheet of Whatman No. 1 filter paper. A spot of "3B-2A-2" was superimposed on the kaempferol glycoside spot, and when the mixed spot was dry, the chromatogram was developed in one direction in the n-butyl alcohol-acetic acid-water system. The chromatogram was removed from the tank and air dried. It was then developed in a direction perpendicular to the first direction in 15% acetic acid-water solvent system.

The dried two-dimensional chromatogram was sprayed with aluminum chloride (1% in methanol), and then examined under

TABLE I

R_f Values of Isolated Compounds and Known Standards

Compound	Solvent Systems[a]			
	(1)	(2)	(3)	(4)
Isoquercitrin	0.68	0.48	0.69	0.89
Quercitrin	0.80	0.58	0.75	0.95
Rutin	0.51	0.60	0.71	0.88
"3B-2A-1"	0.59	0.67	0.76	0.91
Kaempferol-3-rhamnoglucoside	0.60	0.79	0.80	0.93
"3B-2A-2"	0.60	0.80	0.80	0.92
Quercetin	0.77	0.07	0.39	--
Aglycone from "3B-2A-1"	0.75	0.07	0.38	--
Kaempferol	0.84	0.14	0.49	--
Aglycone from "3B-2A-2"	0.84	0.15	0.48	--

[a]Solvent systems; (1) n-butyl alcohol--acetic acid--water (6:1:2 v/v); (2) 15% acetic acid--water; (3) 60% acetic acid--water; and (4) 60% isopropyl alcohol--water.

ultraviolet light to see if there had been any separation of the two compounds. Only one spot was seen.

Comparison of spectral curves. In order further to establish the identity of "3B-2A-2", a curve of the ultraviolet absorption spectrum was drawn and compared with that of authentic kaempferol-3-rhamnoglucoside. (See Figure 3).

An ultraviolet absorption curve of the aglycone resulting from the hydrolysis of "3B-2A-2" was compared with authentic kaempferol. (See Figure 4). Both the glycoside curves and the aglycone curves are almost identical with kaempferol-3-rhamnoglucoside and kaempferol respectively.

By comparison of R_f values, two-dimensional mixed chromatograms, and ultraviolet absorption curves of "3B-2A-2" with those of authentic kaempferol-3-rhamnoglucoside, the two compounds appeared to be identical.

Hydrolysis of the Kaempferol Glycoside

Ten mg of "3B-2A-2" were dissolved in 30 ml of 50% ethyl alcohol-water solution containing 2% v/v sulfuric acid and refluxed 2 1/2 hours. The ethyl alcohol was distilled off, and the hydrolysis mixture was extracted twice with ethyl acetate. The ethyl acetate layer was concentrated and co-chromatographed with authentic kaempferol and found to be identical with it in the solvent systems, n-butyl alcohol-acetic acid-water, 60% acetic acid-water, 15% acetic acid-water, and

FIGURE 3

ULTRAVIOLET SPECTRA OF KAEMPFEROL-3-RHAMNOGLUCOSIDE AND "3B-2A-2"

____Kaempferol-3-Rhamnoglucoside

- - - -Glycoside "3B-2A-2"

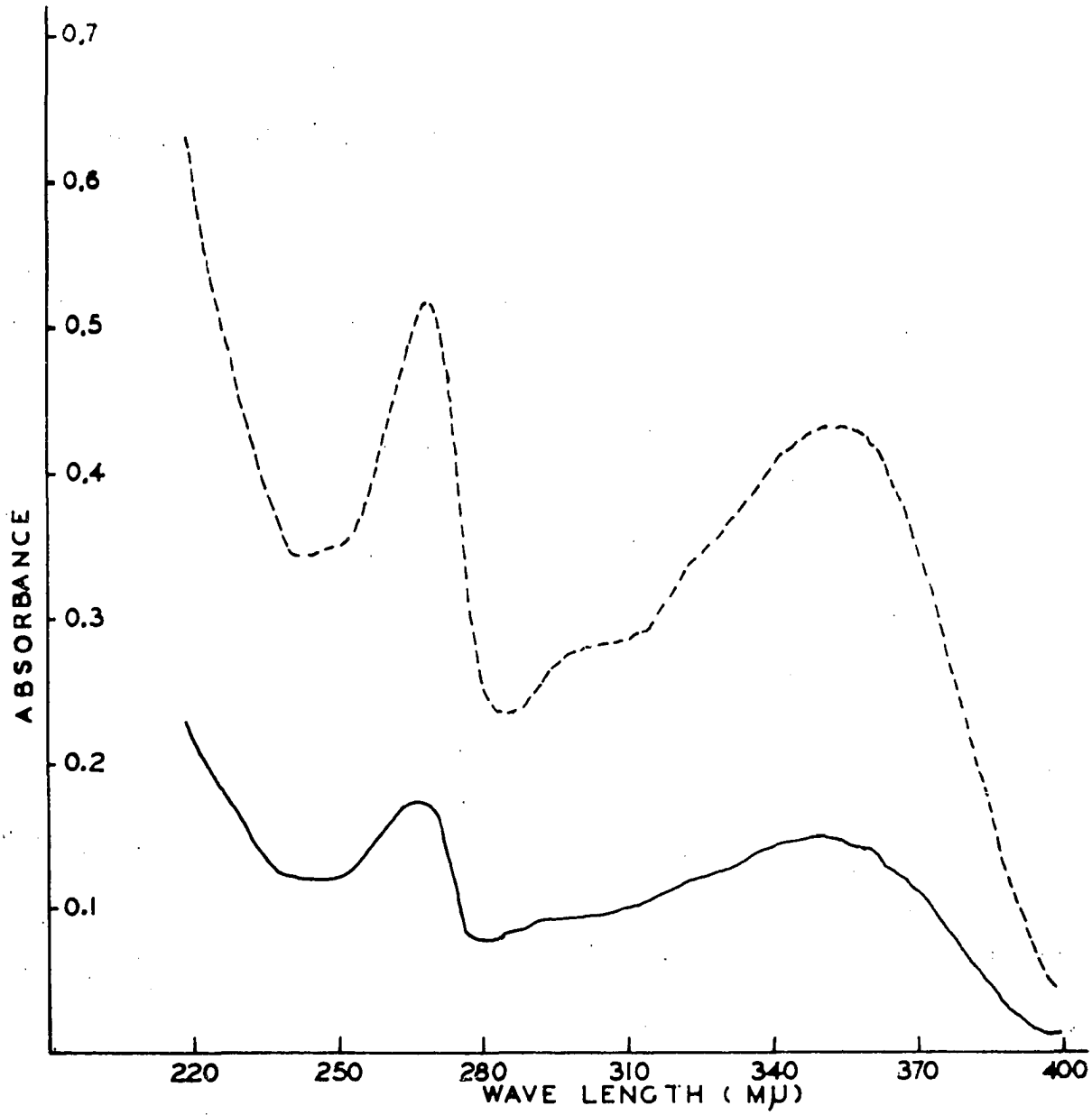
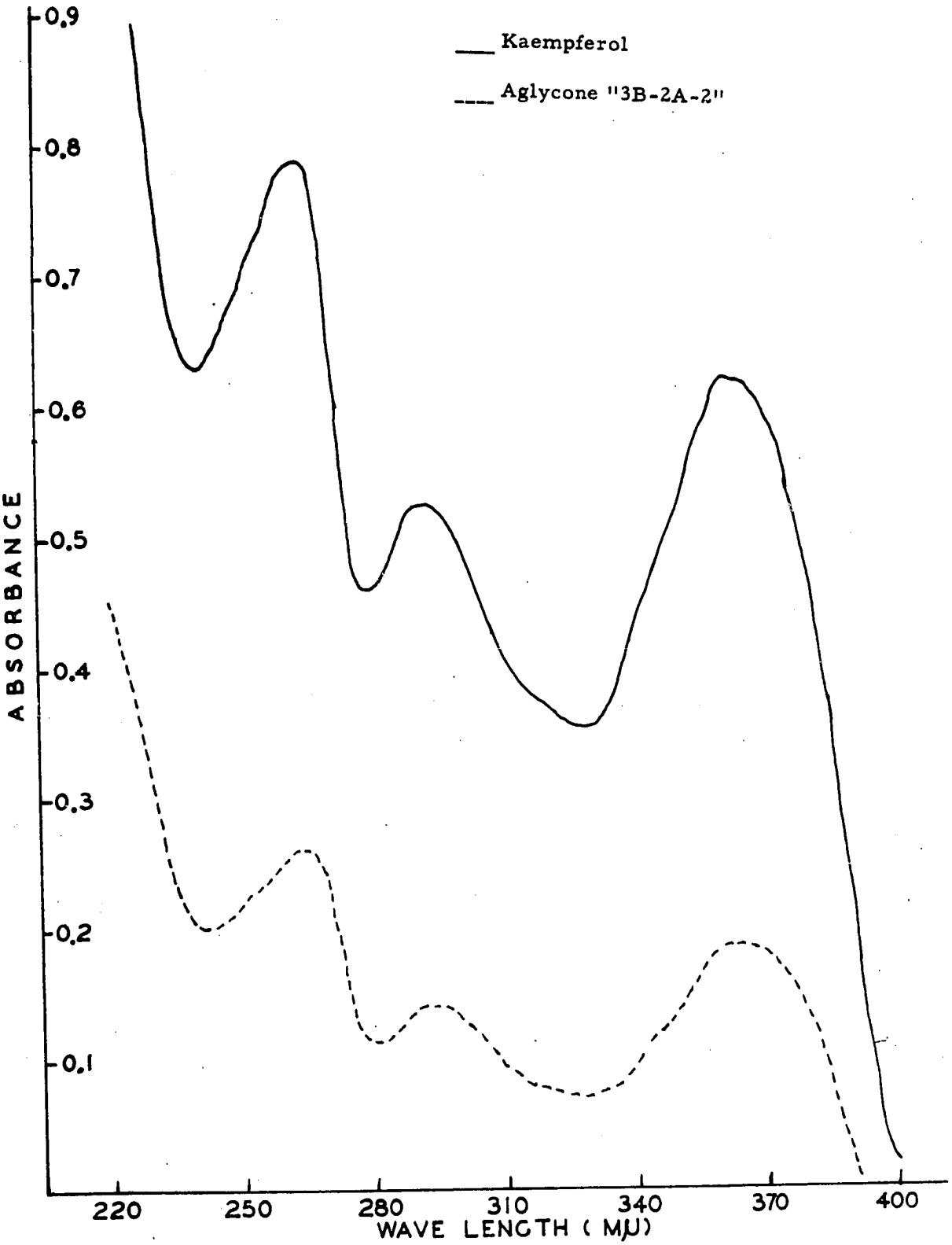


FIGURE 4

ULTRAVIOLET SPECTRA OF KAEMPFEROL AND THE AGLYCONE OF "3B-2A-2"



nitromethane-benzene-water (3:2:5 v/v).

The aqueous layer of the extracted hydrolyzate was passed through a cation-anion mixed bed ion exchange resin (Amberlite MB-1, XE-81) to remove the acid. The neutral sugar solution was concentrated to about 2 ml. under reduced pressure. This concentrated sugar solution was co-chromatographed with several known sugars, in the solvent system, n-butyl alcohol-pyridine-benzene-water (5:3:1:3 v/v), and also in the n-butyl alcohol-acetic acid-water system. The sugars of "3B-2A-2" were found to be glucose and rhamnose. Since the glycoside had appeared to be identical with kaempferol-3-rhamnoglucoside and the hydrolysis products were kaempferol, glucose, and rhamnose, it was concluded that the glycoside is kaempferol-3-rhamnoglucoside.

Analysis of the Glycoside "3B-2A-1"

About 10 mg. of compound "3B-2A-1" were hydrolyzed according to the procedure described previously for the kaempferol glycoside, and the hydrolysis products were analyzed quantitatively by paper chromatography. In order to accomplish this, curves on known quantities of materials had to be prepared. This was done as follows:

Quercetin curve. Quercetin (113.0 mg.) was dissolved in 95% ethyl alcohol, and the solution was diluted to a volume of 100 ml.

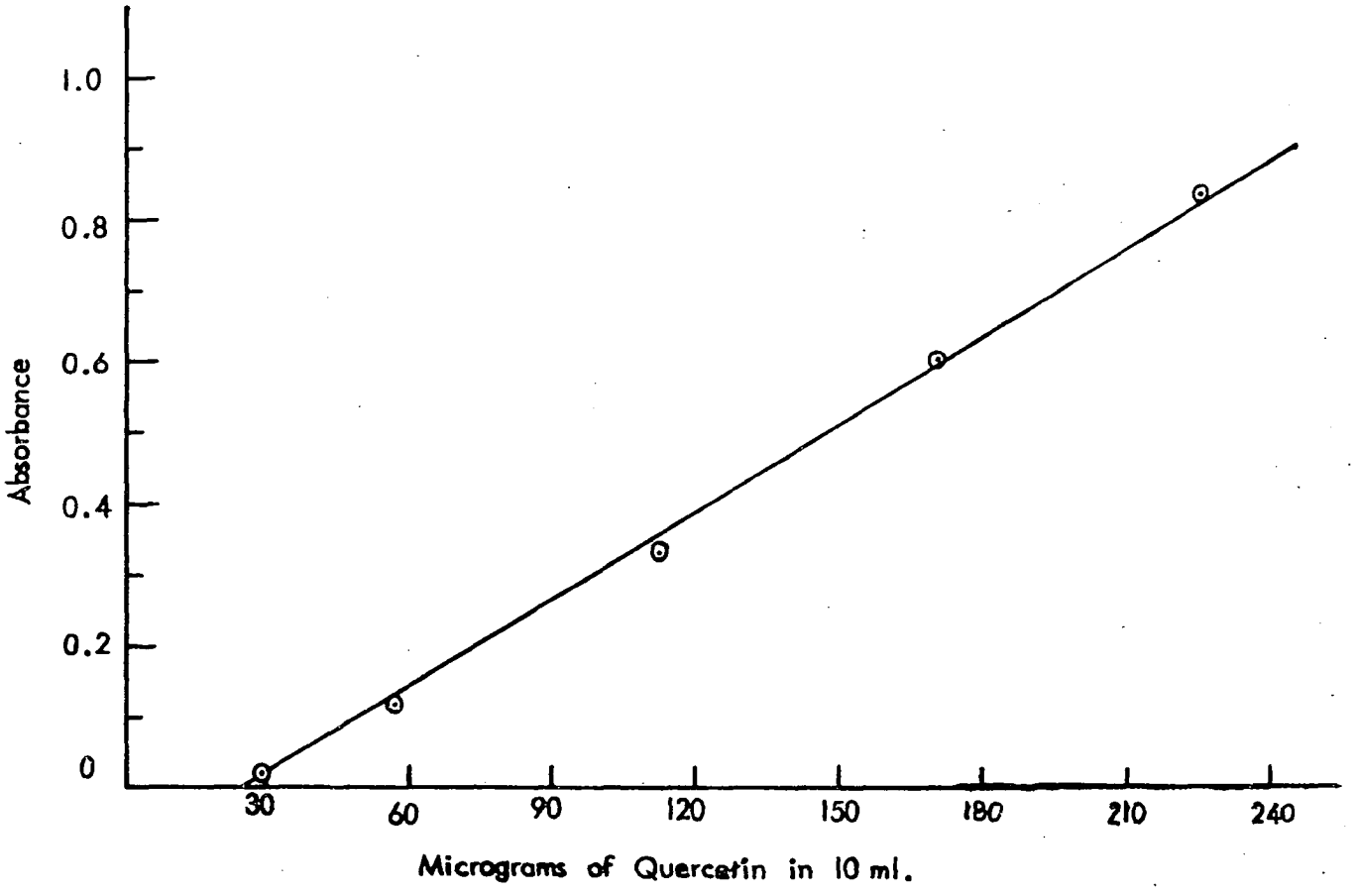
One large sheet of Whatman No. 1 filter paper (18 x 22 1/2 in.) was marked off into five equal spaces. The spaces were streaked with 25, 50, 100, 150, and 200 lambdas, respectively, of the stock solution.

The streaks did not overlap, but rather a space of about one inch was left between them. The whole sheet was developed in n-butyl alcohol-acetic acid-water for 14 hours. After drying, each strip was cut from the sheet and eluted in a solution of ethyl alcohol and water (4:1) for 24 hours. A strip of blank paper was placed in the same elution solvent and its eluant collected to be used as a blank. Each eluant was collected separately and diluted to 10 ml. From the strength of the stock solution, the concentration of samples was calculated to contain, after dilution to 10 ml., 2.82, 5.65, 11.3, and 22.6 gamma/ml., respectively. The quercetin absorption curve has its highest peak at 374 millimicrons (4). At this wave length, absorbance reading were taken of each sample. The straight line thus obtained is shown in Figure 5.

Glucose curve. A stock solution of glucose was made containing 200 mg. of glucose in 100 ml. of aqueous solution. A strip of Whatman No.1 filter paper, 30 cm. wide, was marked with a line three inches from the top, and beginning 1 cm. from the left. This line was extended 13 cm. A space of 2 cm. was left, and the line was continued to within 1 cm. of the right side of the paper. Four such papers were marked, and each was streaked with glucose stock solution in the amounts of 25, 50, 75 and 100 lambdas, respectively. From the concentration of stock solution, this gives for the streaks, 50, 100, 150, and 200 gammas, respectively. The strips were developed for 14 hours in the n-butyl alcohol-acetic acid-water system. After drying, each

FIGURE 5

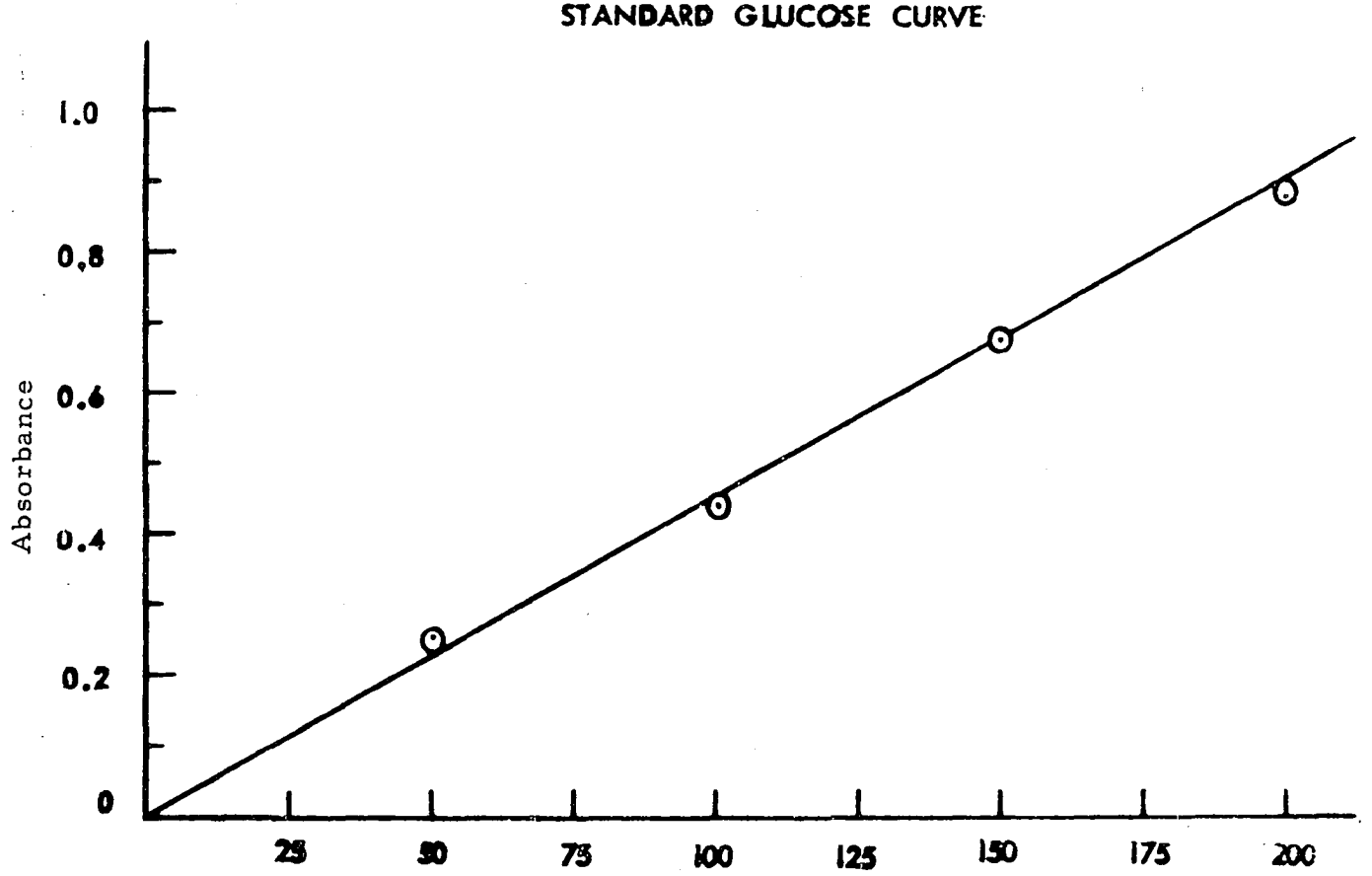
STANDARD QUERCETIN CURVE



30 cm. strip was split down the middle, and one of each of the two resulting strips was sprayed with a reagent spray consisting of 3 grams of 2-aminobiphenyl dissolved in 100 ml. of glacial acetic acid to which had been added 1.3 ml. of phosphoric acid. After spraying, the strip were allowed to dry, then sprayed again, dried and heated in the oven at 100°C. until a brown band appeared (1-3 minutes). Each strip was then placed beside the strip from which it had been cut in order to locate the glucose area on the non-sprayed paper. This area was marked and eluted with water. At the same time a blank strip of Whatman No. 1 paper was eluted to obtain a standard blank. When 4-6 ml. of eluant was collected, it was treated as follows to obtain a standard curve. Each sample was diluted to 10 ml. One ml. of each was transferred by pipette to a glass stoppered test tube, and to it was added 5 ml. of a .4% solution of 2-aminobiphenyl in glacial acetic acid. The blank was treated likewise. The tubes were inserted in a beaker of boiling water and heated for one hour. A green solution was obtained. Absorption readings were taken at a wave length of 380 millimicrons, and a straight line was plotted of absorbancy vs concentration (Figure 6).

By comparison of R_f values in several solvent systems, and by chromogenic sprays, the aglycone of compound "3B-2A-1" was found to be quercetin. The only sugar found to be present was glucose. The glycoside was then co-chromatographed with two known quercetin glucosides, quercimeritrin and isoquercitrin. Since these had different R_f

FIGURE 6


STANDARD GLUCOSE CURVE
Absorbance
1.0
0.8
0.6
0.4
0.2
0
25
50
75
100
125
150
175
200
Micrograms of Glucose in 10 ml.

values than did the unknown, it appeared that the difference in R_f values might be found to be a difference in the number of sugar units per molecule. An analysis of the quantitative relationship of glucose to quercetin was carried out as follows: "3B-2A-1" was hydrolyzed by refluxing with 2% sulfuric acid for 2 1/2 hours. The hydrolyzate was cooled and extracted twice with ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness under reduced pressure. The solid aglycone was taken up in 95% ethyl alcohol, and the volume was adjusted to 5 ml. Of this 5 ml., 0.4 ml. was streaked on Whatman No. 1 filter paper and developed for 14 hours in the n-butyl alcohol-acetic acid-water solvent system. The chromatogram was dried and the aglycone, quercetin, was eluted with 95% ethyl alcohol. This volume of eluant was adjusted to 10 ml. A sample of it was placed in the Beckman DU spectrophotometer and its absorbance taken at 374 millimicrons. The absorbancy at this wave length was 0.085. From the standard quercetin curve, Figure 5, this value is equivalent to 45 micrograms of quercetin per 10 ml. Since this 10 ml. sample represents 0.4 ml. taken from the original 5 ml. of aglycone, it therefore was 8% of the total amount of the aglycone. The 45 micrograms then was 8% of the total weight of quercetin resulting from hydrolysis, namely 562.5 micrograms. The number of moles of quercetin which this represents is 0.563 mg/3.02 x 10^5 mg/mol or 0.186 x 10^{-5} moles.

Quantitative Analysis of the Sugar

The aqueous layer remaining from the extraction of the hydrolyzate from glycoside "3B-2A-1" was passed through a cation-anion mixed bed ion exchange resin (Amberlite MB-1, XE-81) to remove the sulfuric acid. The neutral solution was then concentrated and the volume adjusted to 2 ml. Of this 2 ml., 0.6 ml. were streaked on Whatman No. 1 filter paper. On the same line, and two inches from the end of this streak, a spot of the glucose solution was placed in order to determine the location of glucose after development. The chromatogram was developed 14 hours in the pyridine solvent system, n-butyl alcohol-pyridine-benzene-water (5:3:1:3 v/v). After drying, the sugar was eluted with water. This eluant was concentrated to 5ml. One ml of this sugar solution was transferred to a glass stoppered test tube, and 5 ml. of a 0.4% solution of 2-aminobiphenyl in glacial acetic acid were added. The solution was heated in the water bath for one hour.

A blank was prepared by eluting paper which had been placed in the solvent system used and developed along with the sugar chromatogram. The volume of this blank-eluant was adjusted to 5 ml. To one ml. of the blank were added 5 ml. of the 2-aminobiphenyl solution, and this was heated in the water bath along with the sugar sample. A duplicate was made of both the blank and the sugar sample. The treated samples were allowed to cool overnight and the absorbance was measured at 380 millimicrons. The absorbance of the sugar was 0.134, which, as seen

from the standard glucose curve is equivalent to 35 micrograms. This 35 micrograms represent 1/5 of the 0.6 ml. of streaked sugar. Therefore, the 0.6 ml. which was streaked, contained 5 x 35 or 175 micrograms of glucose and in the original 2 ml. there were (175/0.6) x 2 or 583 micrograms. In terms of moles of glucose, this represents 0.583 mg/1.80 x 10^5 mg/mol or 0.324 x 10^{-5} mol. The ratio of moles of quercetin to moles of glucose, then is 0.186 x 10^{-5}/0.324 x 10^{-5} or 1:1.74. Within experimental error, this amounts to a 1:2 ratio as would be expected from the R_f values of quercetin glycosides.

Spectral Studies

In order to locate the position or positions of attachment of the sugars, a bathochromic shift study was carried out according to the method of Jurd (2, 3). These studies involving the use of such reagents as sodium acetate, boric acid and aluminum chloride, are designed to indicate which hydroxyl groups of the flavonoid are not covered by a sugar, methoxy, etc. Since the aglycone was quercetin, the glycoside linkages could occur at positions 3, 5, 7, 3' or 4'.

To determine whether the No. 7 position was open, sodium acetate was used. The ultraviolet spectrum of "3B-2A-1" in absolute ethyl alcohol was determined on the Beckman DK-1 recording spectrophotometer. Excess anhydrous fused sodium acetate was added to the sample cell and to the blank. The spectrum was determined after 5-10 minutes. There was a shift of the first maximum of 12 millimicrons

FIGURE 7

EFFECTS OF SODIUM ACETATE ON ULTRAVIOLET SPECTRUM OF GLYCOSIDE "3B-2A-1"

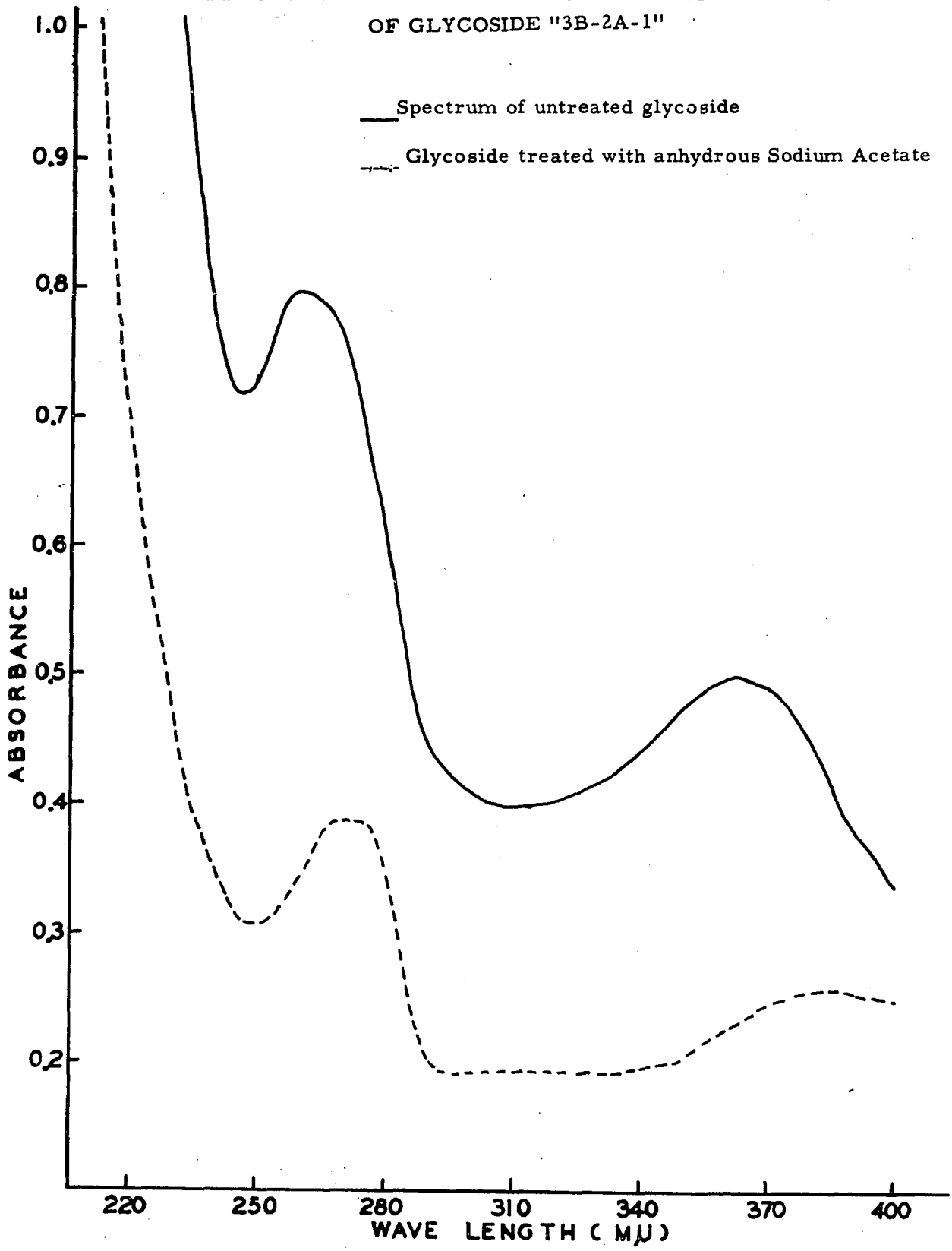
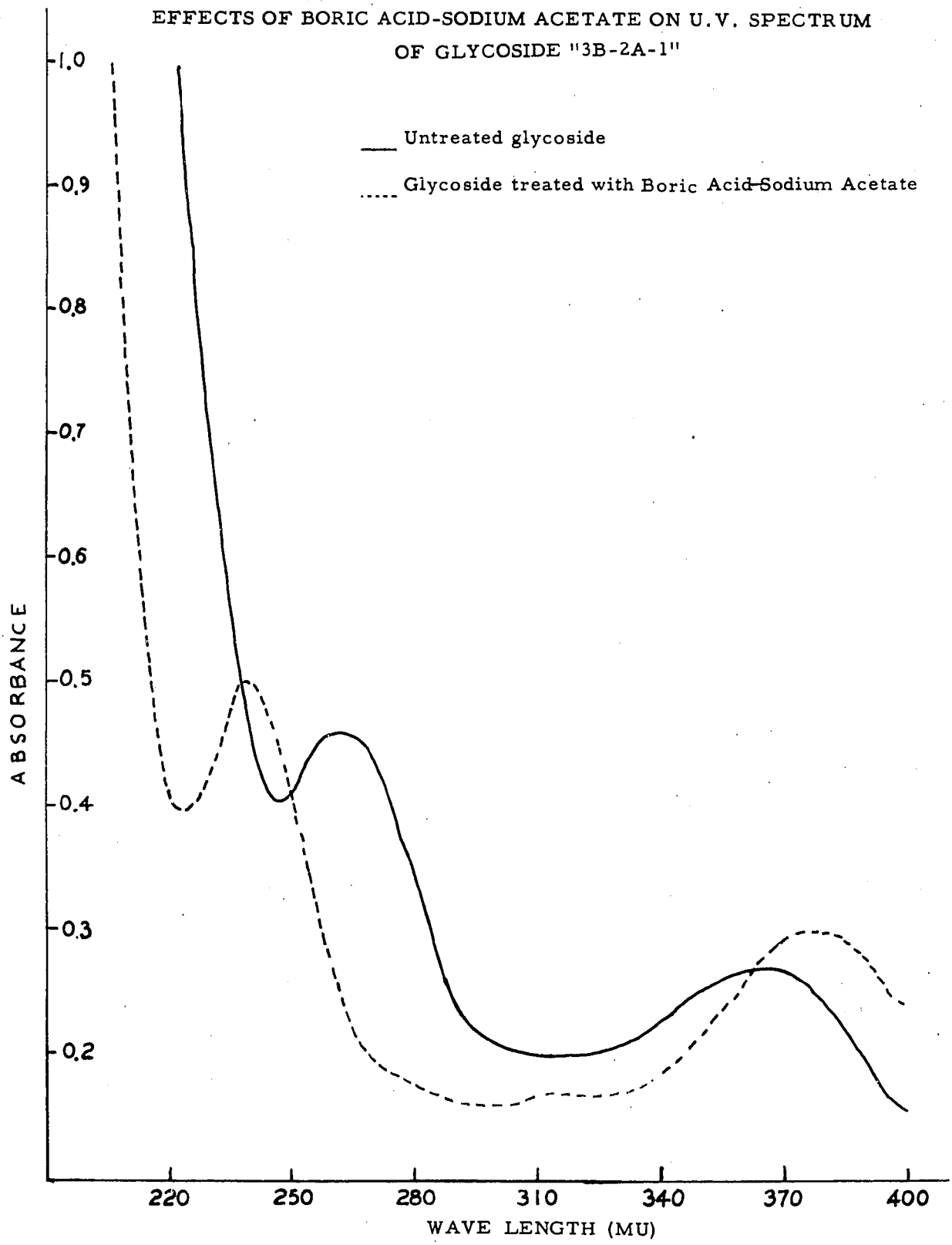


FIGURE 8

EFFECTS OF BORIC ACID-SODIUM ACETATE ON U.V. SPECTRUM OF GLYCOSIDE "3B-2A-1"



toward the longer wave length (See Figure 7) thus indicating that the No. 7 position was open and not covered by a sugar.

To 2 ml. of the "3B-2A-1" stock solution (approximately 0.0001 M) in absolute ethyl alcohol was added 2 ml. of a saturated solution of boric acid in absolute ethyl alcohol. The solution was diluted to 10 ml. with absolute ethyl alcohol, and an excess of sodium acetate was added. After shaking the solution and allowing it to settle for 10-20 minutes, the spectrum of the solution was recorded on the same graph with the untreated sample. The maximum peak which had occurred around 260 millimicrons was shifted about 23 millimicrons toward the shorter wave lengths. The second maximum, which was originally about 370 millimicrons, was shifted about 15 millimicrons toward the longer wave lengths (See Figure 8). According to Jurd, these shifts would indicate the presence of o-dihydroxy groups. With quercetin, this would mean that the 3',4' positions are open and not covered by a sugar.

With the evidence that the No. 7, 3' and 4' positions are open, this places the sugars either on the No. 3 or No. 5 position, or one molecule of glucose on each of these positions. The compound chelates with aluminum chloride, which indicates that the No. 5 position is open. This leaves only the No. 3 position. Further evidence that the No. 3 position is covered is afforded by the fact that the glycoside fluoresces brown rather than yellow.

According to the information obtained at this point, "3B-2A-1"

appeared to be a quercetin-3-glucosylglucoside. The data substantiating this belief are:

(1) R_f values in four different solvent systems are more nearly those of a diglycoside rather than of a mono-or triglycoside.

(2) The hydrolysis products are quercetin and glucose.

(3) The hydrolysis products occur in the ratio of quercetin to glucose in an approximate 1:2 ratio.

(4) Spectral and chelation studies indicate that positions No. 5, 7, 3' and 4' are open.

(5) The brown fluorescence indicates that the No. 3 position is covered.

Isolation of Glycosides from Zone 1

Zone 1 from the n-butyl alcohol-acetic acid-water system (See Figure 2) was cut and sewed on new Whatman #3MM filter papers (18 x 22 1/2 in.) and developed for 16 hours in a 60% isopropyl alcohol-water solvent system. Two zones, 1A and 1B, appeared. Zone 1A had the smaller R_f value and showed only a faint fluorescence with ammonia vapors. It was not further examined. Zone 1B, which was brown and showed a strong fluorescence with ammonia vapors, was cut and sewed on new Whatman #3MM filter papers and developed for 4-6 hours in the 15% acetic acid-water solvent system. Only one zone was seen. It was, therefore, labeled 1B-1. The zone 1B-1 was eluted with water and concentrated, under reduced pressure. The glycoside was precipitated by

by slowly adding, with gently shaking, 5 - 8 volumes of acetone. The precipitated glycoside was filtered and dried on a sintered glass funnel of "medium" porosity. The dried glycoside was thoroughly washed with ethyl acetate, and then with isopropyl alcohol, ether, and finally with absolute ethyl alcohol. It was quite insoluble in these organic solvents, but readily dissolved in water and in ethylene glycol. About 100 mg. of 1B-1 was obtained by successive isolations from paper. The purity of 1B-1 was tested by spotting it and developing in five solvent systems commonly used for flavonol glycosides. In each of these solvents only one spot appeared. However, in the n-butyl alcohol-acetic acid-water system, there was some indication of the presence of more than one compound, but still only one spot appeared. It was therefore, tentatively assumed to be pure and the following hydrolyses were carried out.

Acid Hydrolysis of 1B-1. About 10 mg. of 1B-1 were completely hydrolyzed by boiling for 2 hours with 2% sulfuric acid. The hydrolyzate was extracted twice with ethyl acetate. The ethyl acetate extracts were combined and concentrated and then co-chromatographed with kaempferol and quercetin in the solvent systems, n-butyl alcohol-acetic acid-water, 15% acetic acid-water, 60% acetic acid-water, and nitromethane-benzene-water (3:2:5 v/v). The aglycone appeared to consist of only one compound and the R_f' value was the same as the R_f value of quercetin.

The aqueous portion of the hydrolyzate was neutralized by its pass-

age through a cation-anion mixed bed ion exchange resin (Amberlite MB-1, XE-81) and then concentrated under reduced pressure. The concentrated sugar solution was co-chromatographed with several known sugars, and found to contain galactose, glucose, arabinose, xylose, and rhamnose. From the R_f values of the glycoside, it seemed unlikely that this was a pentaglycoside of quercetin, therefore, partial hydrolysis was attempted by boiling the glycoside with 2% sulfuric acid for short periods of time ranging from 5 minutes to 2 hours. After 5 minutes, no hydrolysis had occurred, and after 2 hours the glycoside was completely hydrolyzed. Intermediate times gave varying degrees of hydrolysis, but the most significant was a 15 minute period, after which the sugar found to be present, by chromatographic comparison, were a trace of glucose, rutinose (6(beta-1-L-rhamnosido)-D-glucose), xylose, and another pentose. When 1B-1 was hydrolyzed 30 - 45 minutes, and the aqueous portion of the hydrolyzate chromatographed after being neutralized as described previously, four zones appeared. One corresponded to rhamnose, one to glucose, and two zones had R_f values smaller than the R_f value of glucose. Isolation and further hydrolysis of one of these zones showed that it contained galactose and glucose. Upon co-chromatographing this galactose-glucose sugar with lactose, the two were found not to be identical. The glycosidic linkage between the galactose and glucose must be different than in the case of lactose.

Enzymic Hydrolysis of 1B-1. The compound 1B-1 was treated with varying concentrations of the enzyme, emulsin, for periods of time, ranging from 1 hour to 10 days, and no evidence of hydrolysis could be observed when the mixture was chromatographed on paper. Therefore, a hydrolysis was carried out using maltase. Maltase, obtained from "Delta Chemicals", was made up to a 10% aqueous solution, and 5 ml. of the enzyme solution were added to 5 ml. of a concentrated aqueous solution of the glycoside. The mixture was allowed to stand for 3 hours at room temperature (35 degrees centigrade). The pH of the solution was approximately 6. The hydrolysis mixture was streaked on Whatman #3MM filter paper (18 x 22 1/2 in.) and developed for 16 hours in the n-butyl alcohol-acetic acid-water solvent system. A strip (3 x 22 1/2 in.) was cut from the dried chromatogram and sprayed with a solution consisting of 3 grams of 2-aminobiphenyl in 100 ml. of glacial acetic acid, to which had been added 1 ml. of phosphoric acid. The sugars detected were glucose, galactose, and a trace of a pentose.

Examination, in ultra-violet light, of the remaining chromatograms revealed that a glycoside was still present which had an R_f value the same as that of the original glycoside. This glycoside zone was cut out and sewed on new Whatman #3MM filter papers and developed in the 15% acetic acid-water solvent system in order to remove the trace of pentose which was found to be in the same general area. The pentose moved

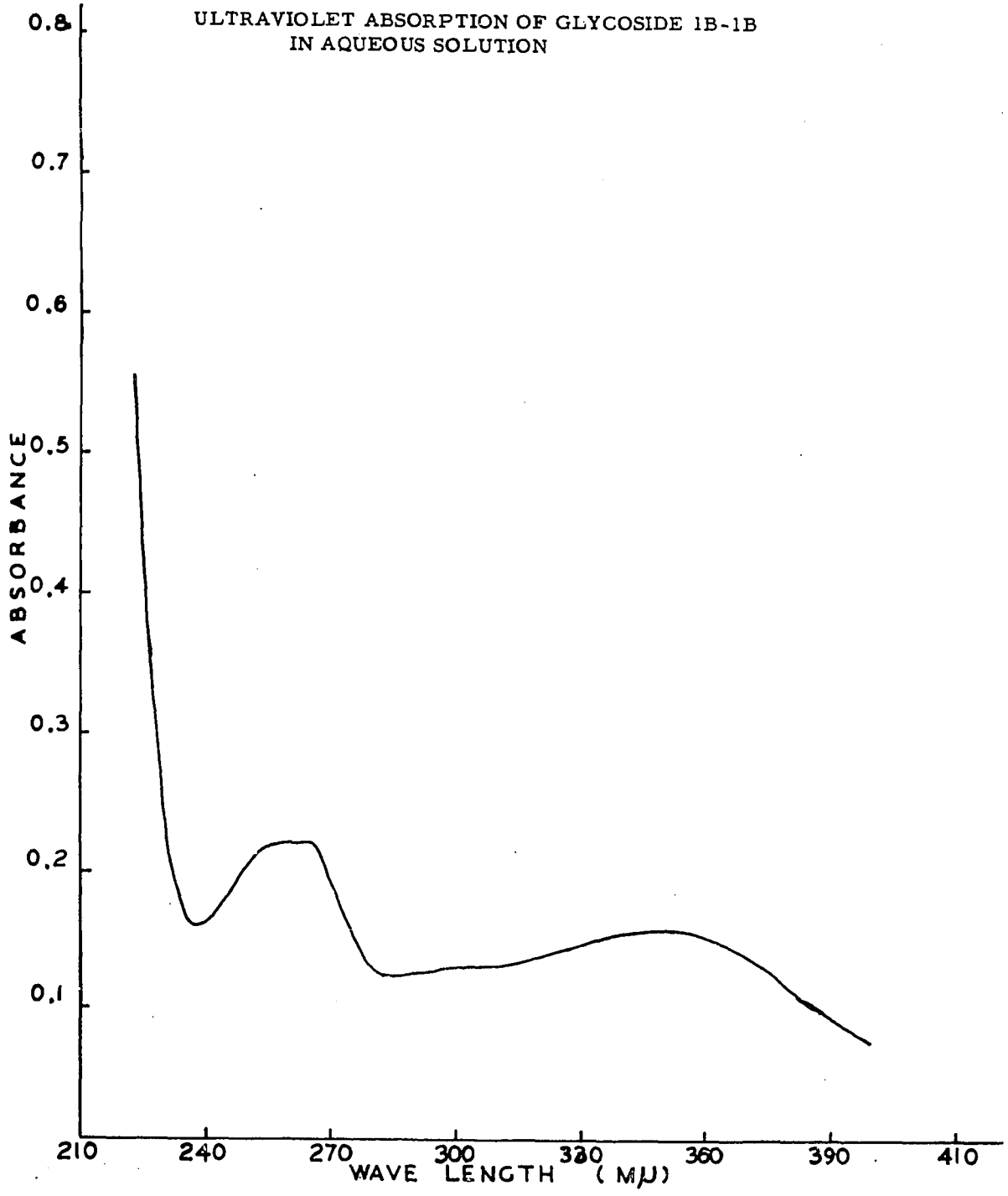
ahead of the glycoside. The glycoside zone was cut from the dried chromatograms and the compound was eluted with water. The eluant was concentrated and hydrolyzed 4 hours with 2% sulfuric acid. The hydrolysis products were analyzed and found to be quercetin, glucose, rhamnose, xylose, and arabinose.

Further Separation of Zone 1B-1. From the results obtained by the partial hydrolysis of 1B-1, both by sulfuric acid and by maltase, it was concluded that this must be a mixture, rather than a pure single compound; therefore, new solvent systems were tried in order to find one which could bring about the desired separation. The system which gave suitable results was one containing phenol-water-formic acid (3:1:0.1 w/w). The solid glycoside, 1B-1, was dissolved in water and streaked on Whatman #3MM filter paper (18 x 22 1/2 in.), and developed for 16 hours in the phenol-water-formic acid system. One major zone, 1B-1A, separated from two other zones which overlapped each other. These two zones were labeled 1B-1B, and 1B-1C. The two zones were cut as one and sewed on new Whatman #3MM filter papers and developed a second time in the phenol-water-formic acid system. After 20 hours of developing, the two zones were completely separated. Since the phenol-water-formic acid solvent should give R_f values opposite to those of the 60% isopropyl alochol-water solvent, 1B-1C appeared to be a portion of 1A which had not separated in the 15% acetic acid-water system. At any rate, its small quantity discouraged its complete

investigation. Nevertheless, the zone was eluted and the eluant was concentrated and hydrolyzed. It was found to contain glucose as the only sugar.

Examination of 1B-1B. The chromatograms containing zone 1B-1B were air dried for 8 hours. At the end of this time they still possessed a strong odor of phenol. Zone 1B-1B was cut from each of the papers and thoroughly washed by immersing and agitating them in chloroform to remove the residual phenol. The strips were dried and the flavonoid was eluted with water. The eluant was concentrated, under reduced pressure. Five ml. of the concentrated solution were hydrolyzed by boiling for 4 hours with 2% sulfuric acid. The hydrolyzate was cooled and extracted twice with ethyl acetate. The extracts were combined and evaporated to dryness. The solid aglycone was taken up in 95% ethyl alcohol. This aglycone was co-chromatographed with several flavonols in the solvent systems listed in table 1. The aglycone was found to have an R_f value identical with that of quercetin. The aqueous portion of the hydrolyzate was neutralized by passing it through a cation-anion mixed bed ion exchange resin (Amberlite MB-1, XE-81). The neutral solution was concentrated to a volume of 1 1/2 ml., and a portion of it was co-chromatographed with galactose, glucose, arabinose, xylose, and rhamnose, in the solvent system, n-butyl alcohol-benzene-pyridine-water (5:1:3:3 v/v). The sugars found to be present were glucose, arabinose, xylose, and rhamnose.

FIGURE 9


ULTRAVIOLET ABSORPTION OF GLYCOSIDE 1B-1B
IN AQUEOUS SOLUTION
ABSORBANCE
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
210
240
270
300
330
360
390
410
WAVE LENGTH (MμJ)

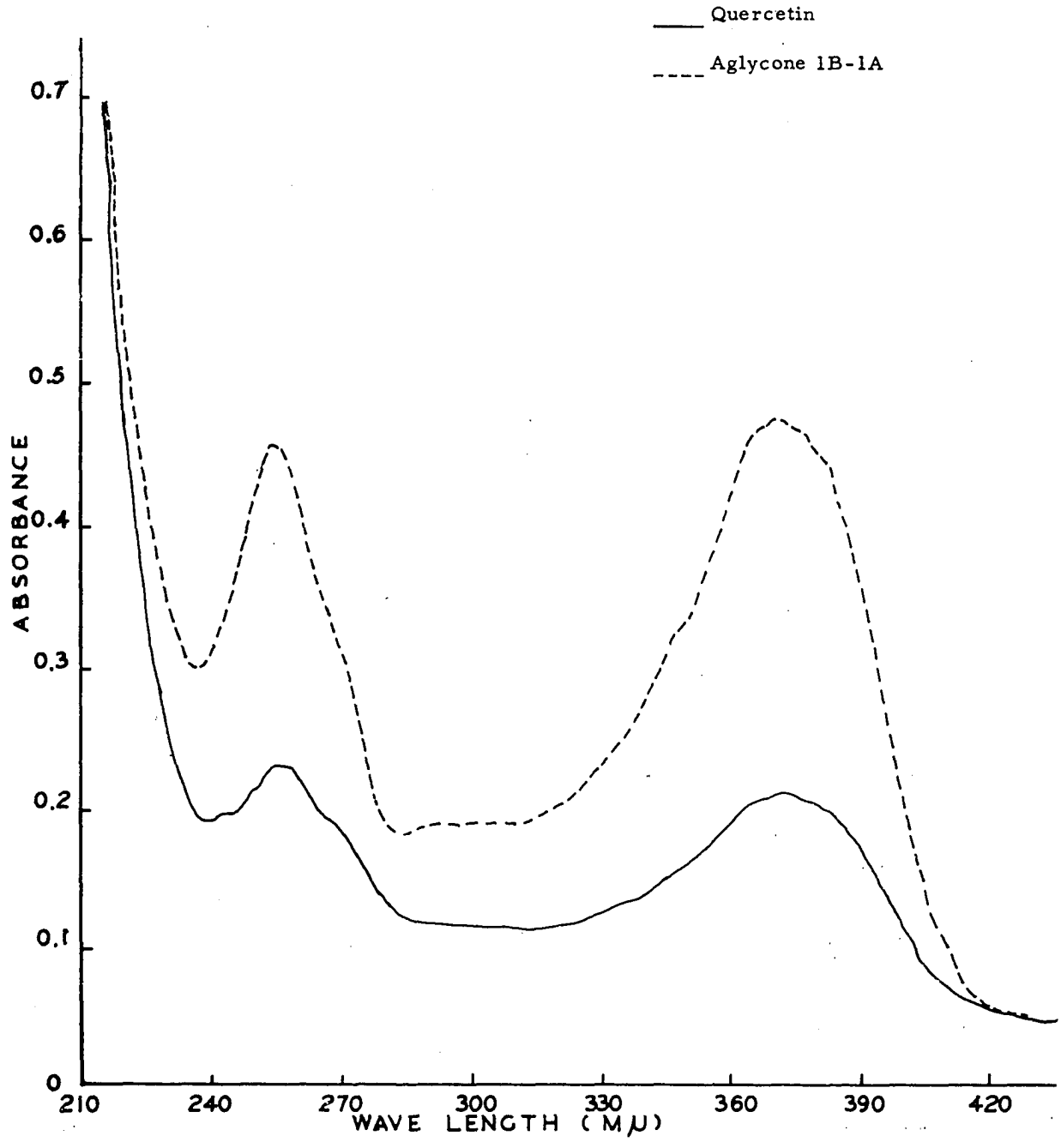
Compound 1B-1B gave an ultraviolet absorption curve in aqueous solution which is typical for flavonol glycosides (See Figure 9).

Examination of 1B-1A. Zone 1B-1A was cut from the dried chromatograms and washed thoroughly with chloroform to remove the residual phenol. The dried strips were eluted with water and the eluant was concentrated, under reduced pressure, to about 10 ml. About 5 ml. of the glycoside solution were hydrolyzed by boiling for 4 hours with 2% sulfuric acid. The hydrolyzate was cooled and extracted twice with ethyl acetate. The extracts were combined and evaporated to dryness, and then taken up in 95% ethyl alcohol. This solution was made to a volume of 5 ml. The R_f values of the aglycone were determined in four different solvent systems. The R_f values in the n-butyl alcohol-acetic acid-water system, and in the 60% acetic acid-water system were very slightly different from the R_f values of quercetin in these two solvent systems. The aglycone was then co-chromatographed with several flavonols of similar structure, including kaempferol, robinetin, rhamnetin, morin, and myricetin. It was found not to be identical with any of these. The aglycone was then suspected of being gossypetin. In order to compare it with gossypetin, the latter had to be synthesized. A modified procedure of Seshadri (7) was used. The aglycone was found not to be identical with the synthetic product.

Even though the exact structure of the aglycone of 1B-1A was not known, it was desirable to estimate the ration of sugar to aglycone in the original glycoside. This was done by streaking one ml. of the 5 ml.

FIGURE 10

ULTRAVIOLET ABSORPTION OF QUERCETIN AND AGLYCONE 1B-1A
IN 95% ETHYL ALCOHOL



volume, mentioned above, on a strip (7 1/2 x 22 1/2 in.) of Whatman #1 filter paper and developing it for 8 hours in the 60% acetic acid-water system. The chromatogram was dried, and the aglycone was eluted with 95% ethyl alcohol, and this made up to a total volume of 5 ml. The absorption of the solution was taken at 374 millimicrons in the Beckman DU spectrophotometer. The 374 millimicron wave length was chosen because this is the area of the broad absorption peak as was obtained by determining the abosrption curve from 220 to 420 millimicrons on the Beckman DK-1 recording spectrophotometer (figure 10).

The aqueous portion of the hydrolyzate of 1B-1A was neutralized by passing it through a cation-anion mixed bed ion exchange resin (Amberlite MB-1, XE-81). The neutral solution was concentrated and the volume adjusted to 1.5 ml. Six hundred lambdas (0.6 ml.) of the solution were streaked on a strip (3 1/2 in x 22 1/2 in.) of Whatman #1 filter paper, and for location purposes, another spot of the solution was placed beside the streak. On the other side of the location spot was placed a spot containing the known sugars, galactose, glucose, arabinose, xylose, and rhamnose. The chromatogram was developed for 30 hours in the n-butyl alcohol-pyridine-benzene-water (5:3:1:3 v/v) system. The chromatogram was dried and a strip containing the location sugar spot and the know sugars was cut and sprayed with a solution consisting of 3 grams of 2-aminobiphenyl dissolved in 100 ml. of glacial

acetic acid to which had been added 1 ml. of phosphoric acid. The sprayed strip was allowed to dry and was then heated in the oven at 100 degrees centigrade for 1 - 3 minutes. From the location spot two brown spots appeared which corresponded to galactose and glucose. (Previous qualitative work in different solvent systems had indicated that 1B-1A did contain galactose and glucose only). The sprayed and heated strip was then placed beside the original chromatogram from which it had been cut, and the galactose and glucose areas were marked off on the unsprayed chromatogram. These areas were cut out and eluted separately with water. Each eluant was concentrated and its volume adjusted to 5 ml. The solutions were prepared for quantitative ultraviolet absorption readings as described for glucose in the quercetin-3-glucosylglucoside. The absorbance for glucose at 380 millimicrons was 0.027 and for galactose it was 0.025. Timell found that galactose, glucose, and mannose give the same standard curve of absorbance vs. concentration. Therefore, figure 6 was used to determine the concentration of galactose as well as the concentration of glucose. From a glance at the reading, 0.027 for glucose, and 0.025 for galactose, it is seen that the two sugars are in a 1:1 ratio. Determining the molecular ratio of aglycone to sugar is a little more difficult since the exact structure of the aglycone is not known. From the close R_f values of this aglycone to those of quercetin, the colors produced with ammonia vapors and with aluminum chloride spray, and

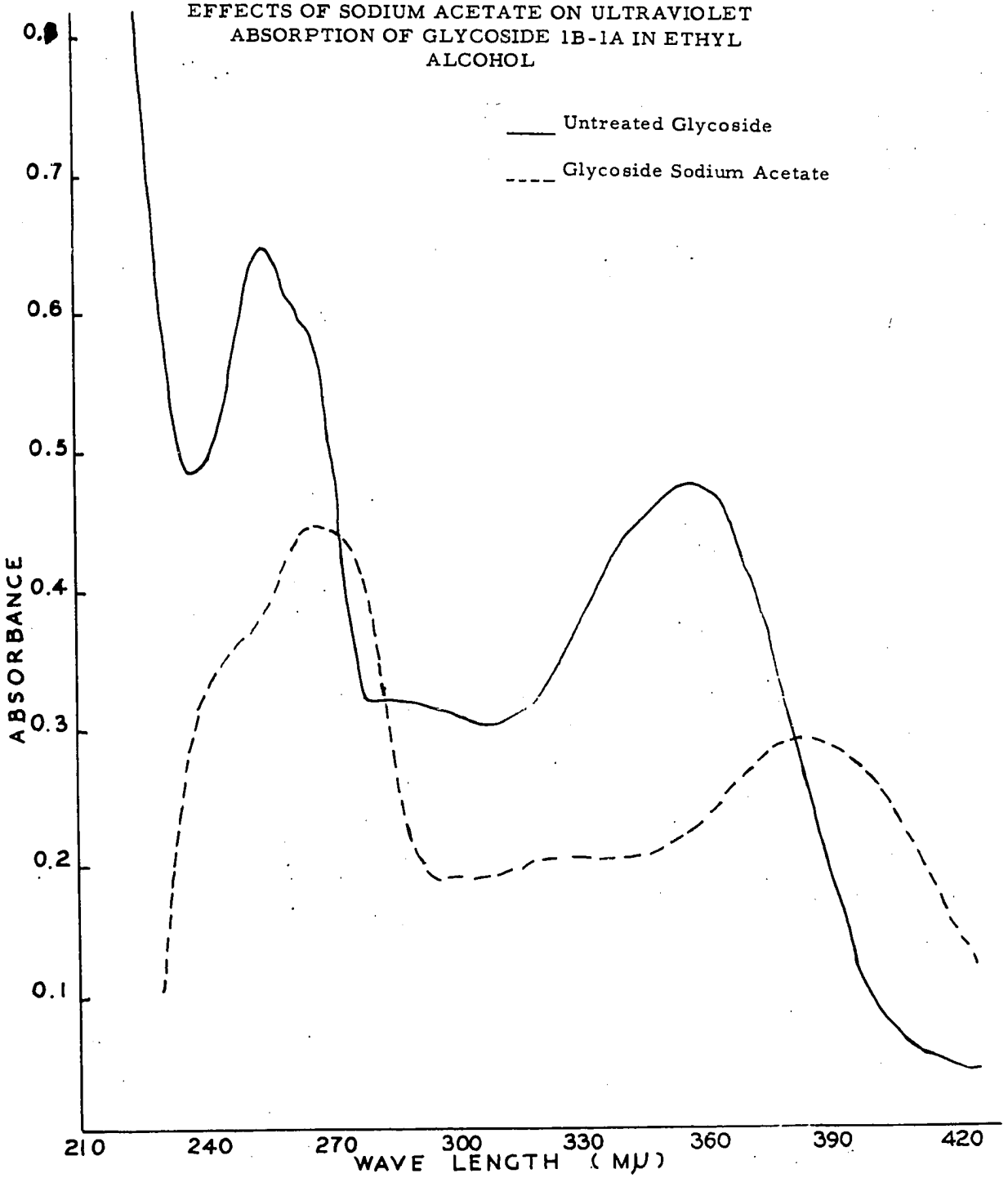
the sharp resemblance of the ultraviolet curves of these two flavonols (figure 10), one is almost forced to conclude that whatever the exact structure of the aglycone of 1B-1A may be, it is quite like that of quercetin. Chromatograms of the aglycone in nitromethane-benzene-water (3:2:5 v/v) system indicate no methoxy groups to be present.

The absorbance of the 5 ml. sample of the aglycone at 374 millimicrons was 0.093. By diluting the sample to 10 ml. a reading of 0.0465 would be obtained. If it can be assumed, on the basis of likeness of their absorption curves and other characteristics, that equal concentrations of the aglycone and quercetin would have equal absorbance at the same wave length, 374 millimicrons, then the standard curve for quercetin could be used to determine the concentration of aglycone 1B-1A. The absorbance reading of 0.047 represents a concentration of 35 micrograms of quercetin. This 35 micrograms represent 1/5 of the total quantity of aglycone obtained from the hydrolysis. Therefore, there must have been 5 x 35 or 175 micrograms of aglycone resulting from the hydrolysis. This can not be converted into moles unless the exact molecular weight is known. But it terms of quercetin, this can be shown to be 0.175 mg./3.02 x 10^{-5} mg./mol. or 5.8 x 10^{-5} moles of quercetin.

In order to compare the moles of quercetin to the moles of sugar, the number of moles of sugar are calculated. The reading of 0.027 for glucose represents 6.4 micrograms of glucose per milliliter or 32

FIGURE 11

EFFECTS OF SODIUM ACETATE ON ULTRAVIOLET ABSORPTION OF GLYCOSIDE 1B-1A IN ETHYL ALCOHOL



micrograms for the 5 ml. sample. Since only 0.6 ml. of the total 1.5 ml. were streaked, this 32 micrograms represent 40% of the total amount of glucose resulting from the hydrolysis. The total quantity was then 80 micrograms. The number of moles of glucose produced on hydrolysis were 0.080 mg./1.8 x 10^5 mg./mol or 4.45 x 10^{-7} moles. The number of moles of galactose were determined by the same method as that described for glucose, and was found to be 4.16 x 10^{-7} moles. The ratio of glucose to galactose is as 1.06:1, and the ratio of these sugars to quercetin would be galactose:quercetin:1:1.39, and glucose: quercetin::1:1.30.

Ultraviolet Spectra of 1B-1A. In order to localize, if not determine exactly, the position of attachment of the sugar to the aglycone, a bathochromic shift study was carried out. Five ml. of aqueous solution of the glycoside 1B-1A were evaporated to dryness, under reduced pressure. The solid remaining was further dried by placing a calcium chloride trap between the flask containing the solid and the suction aspirator. With the suction turned on, the solid was allowed to dry overnight. A small quantity of the glycoside was dissolved by adding absolute ethyl alcohol to the flask and heating it. An absorption curve of the glycoside in absolute ethyl alcohol was taken. The curve had absorption maxima at 254 and 355 millimicrons. The sample cell and the blank were saturated wth anhydrous fused sodium acetate and the curve was determined again. This time the maxima were 265 and 385

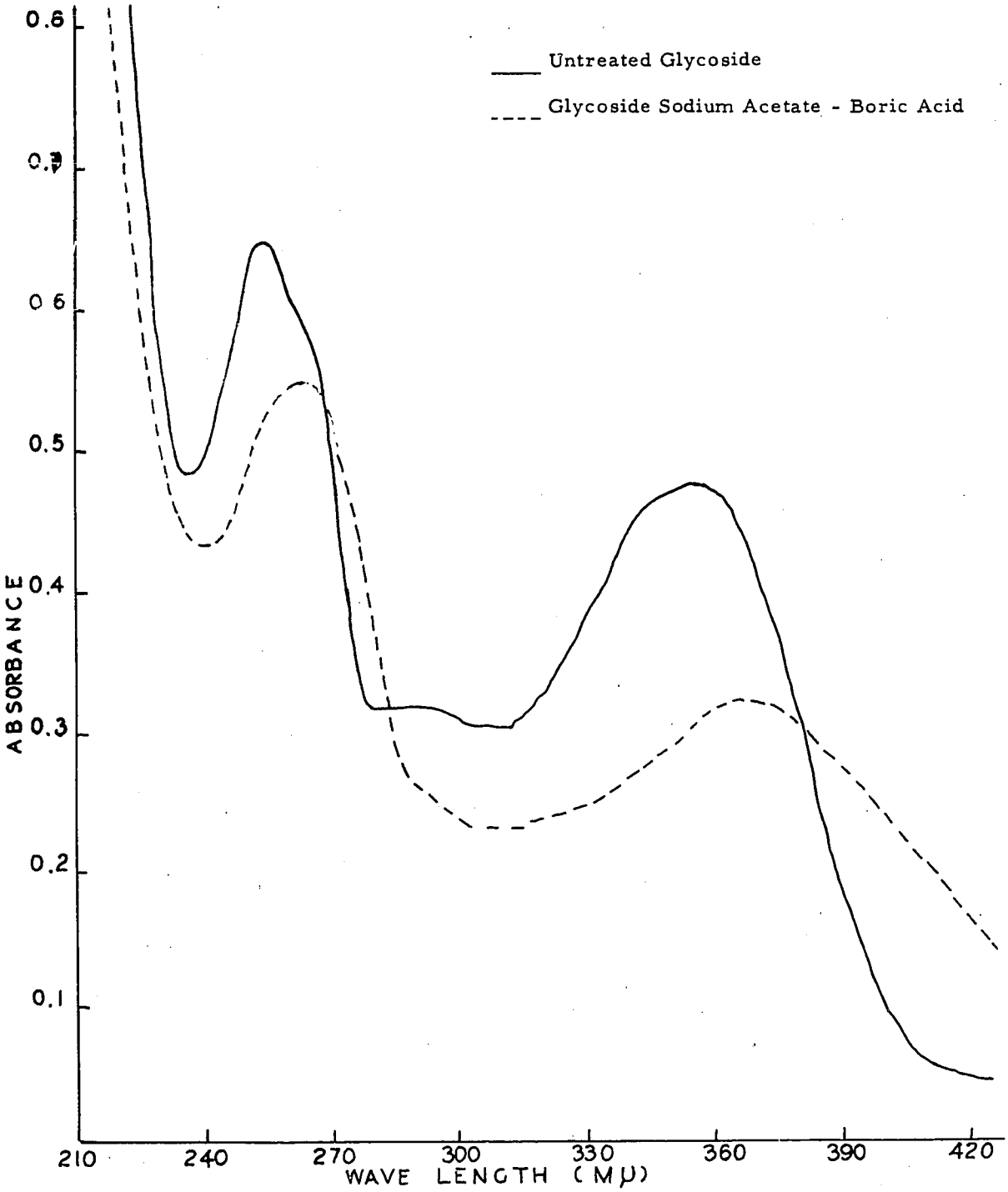
millimicrons (figure 11). According to Jurd (5,6) these shifts are characteristic for flavonols having a hydroxyl group on the #7 position.

In order to determine whether o-dihydroxy were present, 2 ml. of the glycoside solution were added to 2 ml. of saturated boric acid solution in absolute ethyl alcohol, and the mixture was diluted to 10 ml. with absolute ethyl alcohol. A sample of this solution was saturated with anhydrous fused sodium acetate and allowed to settle for 20 minutes. The absorption curve was determined, using as a blank a sodium acetate-boric acid saturated solution in absolute ethyl alcohol. The curve was compared with the original curve (figure 12). In this case the maxima had shifted from 254 to 264, and from 355 to 365 millimicrons. This indicates the presence of 0-dihydroxy groups, but does not show how many or where they are located. The glycoside chelates with aluminum chloride, indicating that the #5 position contains a hydroxyl goup.

From data obtained up to this point, it can be said that the glycoside 1B-1A is probably a quercetin-like diglycoside, consisting of a quercetin-like nucleus and the sugars, glucose and galactose.

FIGURE 12

EFFECTS OF SODIUM ACETATE-BORIC ACID ON ULTRAVIOLET ABSORPTION OF GLYCOSIDE 1B-1A IN ETHYL ALCOHOL



SUMMARY

The existence of flavonoid compounds in cottonseed was not confirmed until Pratt and Wender (2, 3) began their chromatographic investigations of the properties of certain pigments of cottonseed. Cottonseed have now been shown to be a very important source of many flavonoid glycosides. All the flavonoids isolated from this source have been in the form glycosides. There is no evidence that free quercetin, kaempferol, or other aglycone exist in the seed (except as glycosides.)

During this study six different glycosides have been isolated and their properties investigated. All the flavonoids isolated have been flavonol glycosides. They are rutin, isoquercitrin, kaempferol-3-rhamnosylglucoside, quercetin-3-glucosylglucoside, and the two glycosides designated 1B-1A and 1B-1B.

Ultraviolet absorption spectral shifts in absorption maxima, and paper chromatography were used to establish the identity of the isolated compounds.

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