tion step, immediately cyclizes under the reaction conditions to the isomeric tetrazole.

5-Nitroaminotetrazole has been prepared previously by diazotizing nitroaminoguanidine in buffered acetic acid solution,[3] or by cyclizing nitroguanylazide in the presence of inorganic or organic bases[3,4] or by nitrating 5-aminotetrazole.[5]

Experimental

Sodium Azide and N-Methyl-N-nitroso-N'-nitroguanidine.—A slurry, consisting of 0.75 g. (0.115 mole) of sodium azide, 1.5 g. (0.0102 mole) of methylnitrosonitroguanidine and 15 ml. of water, was allowed to stand at room temperature; a slow evolution of gas began immediately. Complete solution was obtained after 16 hours. The solution was then heated on the steam-bath for 30 minutes, treated with 1.3 g. of guanidine nitrate, adjusted to *p*H 3.5, diluted with 10 ml. of water and cooled to 5°. The crystalline product was removed by filtration, washed with three 5-ml. portions of cold water and dried. The yield was 1.1 g. (58.2%); m.p. 217–218° dec. One recrystallization from 25 ml. of water raised the melting point to 222–223° dec.; a mixture melting point with an authentic sample of guanidinium 5-nitroaminotetrazole[3] was not depressed. X-Ray powder patterns[6] were also identical. The calculated equivalent weight is 189.15; found, 188.7.

Sodium Azide and N-Nitro-S-methylisothiourea.—When a slurry of sodium azide (1.1 g., 0.0169 mole), N-nitro-S-methylisothiourea (1.95 g., 0.0144 mole) and 20 ml. of water was refluxed on the steam-bath for 12 hours, methyl mercaptan was evolved slowly (very little reaction occurred during 16 hours at room temperature). The product, which was isolated in the same manner as described in the previous experiment, weighed 1.7 g (62.2%) and melted at 220° dec. A mixture melting point with a known sample of guanidinium 5-nitroaminotetrazole was not depressed; X-ray powder patterns were also identical.

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Enzymatic Hydrolysis Studies on Certain Flavonoid Glycosides

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Definite 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) had not been previously recorded, to our knowledge, for isoquercitrin (quercetin-3-glucoside) and quercimeritrin (quercetin-7-glucoside). The present study was, therefore, undertaken to aid in the establishment or confirmation 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,[1] and in hesperetin-7-glucoside and naringenin-7-glucoside.

Using an α-glucosidase prepared by utilizing paper electrophoresis, and also emulsin in buffers of *p*H 5, with controls in every experiment, and following any hydrolysis by means of paper chromatography on the resulting flavonoid aglucone, each of the above-mentioned glucosides was found to be hydrolyzed by the emulsin preparation, but not by the α-glucosidase, as seen in Table I.

TABLE I

Flavonoid glucoside	Hydrolysis catalyzed by Emulsin	Hydrolysis catalyzed by α-Glucosidase
Hesperetin-7-glucoside	Yes	No
Isoquercitrin	Yes	No
Naringenin-7-glucoside	Yes	No
Quercimeritrin	Yes	No
Unknown apricot[1] quercetin glucoside	Yes	No

A relatively simple method for obtaining directly from takadiastase an α-glucosidase free from β-glucosidase activity in a micro amount for further paper chromatographic enzyme studies has been described below. The α-glucosidase was obtained by filter paper electrophoresis over a 40-hr. period, using Whatman 3 MM. paper saturated with citrate buffer of *p*H 3.

Experimental

Preparation of the α-Glucosidase.—Forty milligrams of takadiastase (Parke, Davis and Co.) was dissolved in 0.125 *M* citrate buffer, *p*H 3, and spotted as a band at the center of a Whatman 3 MM. paper strip, 46 × 19 cm. The paper strip, saturated with the citrate buffer of *p*H 3, was placed between two 38 × 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, and then 110 v. direct current was applied for 40 hr. 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) on 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. Each eluant was divided into two portions. To one portion was added an equal volume of 0.5 *M* citrate buffer of *p*H 7, containing maltose (16 mg./ml.). To the second portion was added an equal volume of the *p*H 7 citrate buffer containing cellobiose (16 mg./ml.). The solutions were mixed by shaking and allowed to incubate at room temperature for 6 hr. Aliquots of the solutions were spotted on Whatman No. 1 paper and developed for 12 hr. with a butanol–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.[2]

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 cellobiose. 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 β-glucoside salicin and on methyl α-D-glucoside. The fraction no. 2 hydrolyzed both of these glucosides; whereas fractions no. 3 catalyzed hydrolysis of methyl α-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.

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The Emulsin Preparation.—Tests similar to the above, but using an emulsin preparation (Nutritional Biochemical Corp., Cleveland, Ohio) were performed on cellobiose, salicin, methyl α-D-glucoside and maltose. The emulsin behaved as it was supposed to do, catalyzing the hydrolysis of the two β-glucosides, but not of the α-glucosides.

Enzymatic Hydrolysis of Isoquercitrin.—A preparation of α-glucosidase was obtained as described above and its *p*H was adjusted to approximately 5 by the addition of an equal volume of 0.5 *M* citrate buffer of *p*H 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 hr.

For the emulsin studies, a few crystals of the isoquercitrin were added to 0.5 ml. of a citrate buffer of *p*H 5 and brought nto 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 hr. 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 was 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 α-glucosidase preparation, but practically complete hydrolysis of the isoquercitrin was observed with the emulsin under the experimental conditions used.

A sample of isoquercitrin prepared by the partial hydrolysis of rutin[3] and chromatographically identical with isoquercitrin from grapes likewise was hydrolyzed by emulsin but not by the α-glucosidase.

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. The first two of these were prepared as described by Fox, *et al.*,[3] and the quercimeritrin was obtained through the courtesy of T. R. Seshadri, Delhi, India. All four of these were hydrolyzed with emulsin, but not with the α-glucosidase.

Hesperidin, from which the hesperetin-7-glucoside had been prepared, has been shown by Zemplén and co-workers[4] to be a β-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)[5] and to naringin (naringenin-7-rhamnoglucoside), then from the results reported here these two rhamnoglucosides could possibly be considered to have the glucosyl unit attached beta onto the aglycone. Experimentally, rutin is not hydrolyzed with emulsin or the α-glucosidase.

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Products of the Oxidative Degradation of Thiophene by Nitric Acid[1a]

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The action of nitric acid on thiophene results in the oxidative decomposition of the thiophene molecule with, apparently, a quantitative conversion of the sulfur to sulfuric acid.[2] In the present research, an attempt was made to discover something of the nature of the intermediate products and by-products of this interesting reaction.

It has been observed that 3,4-dibromothiophene and tetrabromothiophene both give dibromomaleic acid when subjected to treatment with cold concentrated nitric acid.[3] In a similar manner, 3-methyl-2,4,5-tribromothiophene gave rise to bromocitraconic acid, $HOOCC(Br)=C(CH_3)COOH$, and the 2-methyl-3,4,5-tribromo isomer was converted to dibromoacetylacrylic acid, $CH_3CO—CBr=CBr—COOH$.[3] The action of ozone on a cold suspension of thiophene in water gave oxalic acid, carbon dioxide and sulfuric acid.[4] With oxygen in the presence of light, formic acid was formed in addition to oxalic acid.[5] On the other hand, succinic acid (dithallous succinate) is said to be formed by bubbling air through a suspension of thiophene in aqueous thallous hydroxide.[6] The action of potassium chlorate on tetraphenylthiophene (thionessal) produced dibenzoylstilbene, $C_6H_5CO—\underset{\displaystyle C_6H_5}{\underset{|}{C}}=\underset{\displaystyle C_6H_5}{\underset{|}{C}}—COC_6H_5$, in good yield.[7] The products of the oxidation of thiophene and its bromo derivatives by hydrogen peroxide,[8] hypochlorous acid[9] or calcium hypochlorite[10] were not identified.

Experimental

For the purpose of isolating and identifying the reaction products, 30 ml. of a 5% solution of thiophene in cyclohexane was refluxed with 300 ml. of 8 *N* nitric acid. The reaction mixture went through the following color changes: colorless, turbid, yellow, orange, clear yellow. The reaction was terminated a few minutes after attaining the clear yellow stage. The solution was allowed to cool, and the cyclohexane layer was removed by means of a separatory funnel. The cyclohexane phase was then extracted with distilled water and the washings added to the yellow aqueous solution.

The Disappearance of Thiophene.—A small portion of the yellow aqueous solution was tested with isatin in concentrated sulfuric acid, but no color was obtained. This indicates that all the thiophene in the aqueous phase had already been consumed.

The Presence of Sulfate.—Another small portion of the solution was diluted with distilled water and tested with barium chloride solution. The resulting copious precipitate of barium sulfate bore evidence to the fact that the reaction had proceeded at least to the point where sulfuric acid was being formed.

Isolation of 2-Nitrothiophene.—A 150-ml. portion of the acidic yellow solution was neutralized with solid sodium hydroxide, whereupon the color changed to dark reddish-orange. A little concentrated hydrochloric acid was added until the solution became bright yellow again and the slightly acidic solution was steam distilled. Yellow-white crystals formed within the condenser. On recrystallizing from ethyl alcohol, long white crystals of 2-nitrothiophene were obtained; m.p. 45–46°. A mixed melting point with 2-nitrothiophene, obtained by nitration of thiophene in acetic acid solution,[11] showed no depression. It was confirmed

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