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# TRANSFORMATION REACTIONS OF PHLORIDZIN IN THE PRESENCE OF APPLE LEAF ENZYMES

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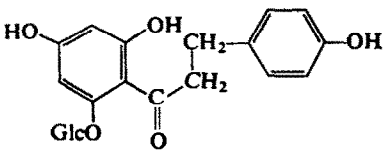
**Abstract**—The transformation reactions of phloridzin in apple leaf homogenates and in the presence of crude apple leaf enzymes have been elucidated. Simultaneously with hydrolysis to phloretin, phloridzin is oxidized via 3-hydroxyphloridzin to the corresponding *o*-quinone. The second oxidation step, *o*-diphenol → *o*-quinone, can be inhibited with cysteine or ascorbic acid. The *o*-quinone is a transient intermediate which rapidly undergoes polymerization reactions. 4-Methylcatechol and phloroacylophenones react under the influence of oxidizing agents to form coupling products. Structures of these compounds have been elucidated. It is suggested that in leaf homogenates the *o*-quinone—from 3-hydroxyphloridzin reacts correspondingly with phloretin and phloretin derivatives.

## INTRODUCTION

PHLORIDZIN (I) is the main phenol present in apple leaves. Studies by Noveroske *et al.*[1,2] on the transformation reactions of phloridzin in the presence of apple leaf enzymes indicated that oxidation products may be involved in the defence mechanism of apple leaves against the scab fungus *Venturia inaequalis*. They found that phloridzin, incubated at pH 5 with a crude enzyme preparation of apple leaves, rapidly yielded the aglycone, phloretin, which was subsequently oxidized. The first oxidation product of phloretin was suggested to be a diphenol. This substance was not isolated.




I

Phloridzin

Manometric measurements indicated approximately a mole-to-mole relationship between loss of substrate and uptake of oxygen. This reaction sequence is inconsistent with their observation that phloretin was not oxidized when added to the enzyme preparation instead of phloridzin. Moreover, the conclusion of Noveroske *et al.* that spore germination of *V. inaequalis* was inhibited by an oxidation product of phloretin is not in accordance with the experimental data which showed that maximum degree of inhibition was obtained before oxidation had occurred.

The present investigation was undertaken to elucidate the transformation reactions of

[1] R. L. NOVEROSKE, J. KUĆ and E. B. WILLIAMS, *Phytopathology* **54**, 92 (1964).
[2] R. L. NOVEROSKE, E. B. WILLIAMS and J. KUĆ, *Phytopathology* **54**, 98 (1964).

phloridzin in the presence of crude enzyme preparations of apple leaves and in leaf homogenates. The role of the transformation products of phloridzin in the defence of apple leaves against attack by *V. inaequalis* will be the subject of future publications.

## RESULTS

### 1. *Enzymatic Transformation Reactions of Phloridzin*

Only the very young leaves of scab-susceptible apple plants are susceptible.[3] Fully unfolded leaves are resistant. The pH value of sap pressed from young apple leaves of susceptible as well as resistant varieties was about 6, whereas sap of the mature leaves had a pH of about 5. Enzymatic transformation reactions of phloridzin in the presence o acetone powders of young apple leaves were studied. The results to be presented were obtained with enzyme preparations of the susceptible variety Cox's Orange Pippin.

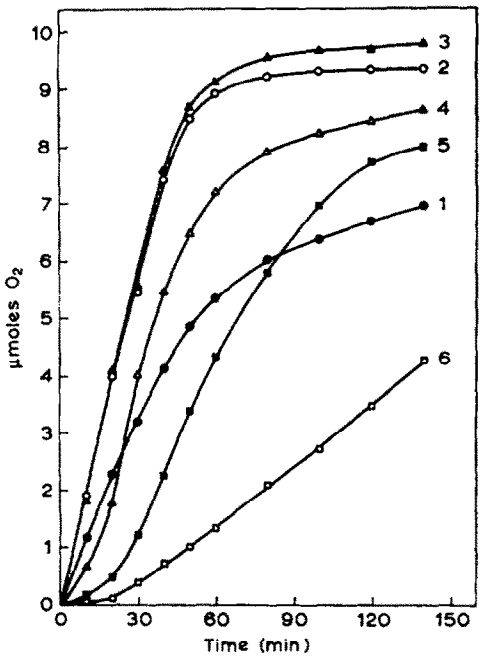



FIG. 1. OXYGEN CONSUMPTION (µmoles $O_2$) AT DIFFERENT pH VALUES IN INCUBATION MIXTURES OF 8 µmoles ($3{\cdot}2 \times 10^{-3}$ M) PHLORIDZIN AND A CRUDE ENZYME PREPARATION (0·3 mg N/ml) OF YOUNG LEAVES OF COX'S ORANGE PIPPIN.
Curve 1: pH 3·4; curve 2: pH 4·0; curve 3: pH 4·8; curve 4: pH 5·2; curve 5: pH 5·8; curve 6: pH 6·5.

Figure 1 shows the enzymatic (polyphenol oxidase = *o*-diphenol: oxygen oxidoreductase) oxidation of 8 µmoles phloridzin at different pH values. The oxidations were of zero order kinetics at all pH values. The oxidation started with a lag-phase which was most pronounced at high pH. Figure 2 shows the highest rates of oxidation of phloridzin (curve 1) (derived from Fig. 1) and phloretin (curve 3) at different pH. Curve 2 shows the rate of formation of phloretin from phloridzin in identical incubation mixtures under anaerobic conditions.

Since the rate of formation of phloretin was higher than the rate of its oxidation, phloretin should accumulate in aerobic incubation mixtures of phloridzin and the apple leaf enzymes.

[3] E. J. BUTLER and S. G. JONES, *Plant Pathology*, p. 735. Macmillan, London (1949).

Curves 1 and 3 in Fig. 3 show the phloridzin concentrations in incubation mixtures at pH 5 and 6 respectively, curves 2 and 4 show the corresponding phloretin concentrations. The initial rates of formation of phloretin were about the same as in the corresponding anaerobic incubation mixtures, but the maximum concentration was reached when only about 3 $\mu$moles of phloridzin had disappeared. Since the rate of formation of phloretin in

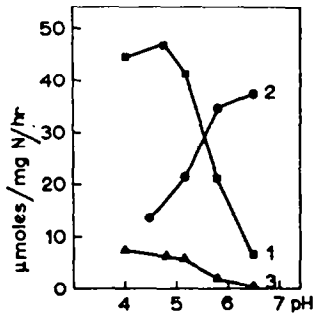



FIG. 2. THE INFLUENCE OF THE pH ON POLYPHENOL OXIDASE ACTIVITY ($O_2$ UPTAKE) WITH PHLORIDZIN (CURVE 1) OR PHLORETIN (CURVE 3) AS SUBSTRATE AND $\beta$-GLUCOSIDASE ACTIVITY (PHLORETIN FORMATION) (CURVE 2) IN A CRUDE ENZYME PREPARATION FROM YOUNG LEAVES OF COX'S ORANGE PIPPIN.

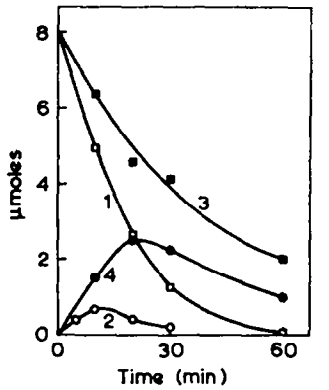



FIG. 3. VARIATIONS IN AMOUNTS OF PHLORIDZIN AND PHLORETIN IN AEROBIC INCUBATION MIXTURES OF 8 $\mu$moles PHLORIDZIN ($3{\cdot}2 \times 10^{-3}$ M) AND A CRUDE ENZYME PREPARATION OF YOUNG LEAVES OF COX'S ORANGE PIPPIN (0·3 mg N/ml).
Curve 1 and curve 3 show the amounts of phloridzin in incubation mixtures with pH 5 and 6, respectively. Curves 2 and 4 show the corresponding phloretin concentrations.

anaerobic incubation mixtures was constant until about 7 $\mu$moles of phloridzin had been transformed, it must be concluded that under aerobic conditions, either the $\beta$-glucosidase has been inactivated during oxidation of phloridzin or the oxidation products of phloridzin have strongly stimulated the oxidation of phloretin. Corresponding results were obtained when enzyme preparations of young leaves of scab-resistant apple varieties were employed.

Figure 4 shows the decrease in phloridzin concentration in homogenates of mature (curve 1) and young (curve 3) leaves of Cox's Orange Pippin. Curves 2 and 4 show the corresponding phloretin concentrations. Phloretin was formed in higher concentrations in young than in mature leaves most probably owing to the higher pH value of the former. Also, in this case, phloretin was formed in considerably lower concentrations than expected

according to the initial rates of phloretin formation. The results obtained with homogenates of leaves from a scab-resistant Antonovka hybrid were essentially the same.

Neither leaf homogenates nor incubation mixtures of phloridzin and apple leaf enzyme contained significant quantities of *o*-diphenols. However, two *o*-diphenols accumulated when cysteine or ascorbic acid was present in incubation mixtures of phloridzin and acetone powders of apple leaves.

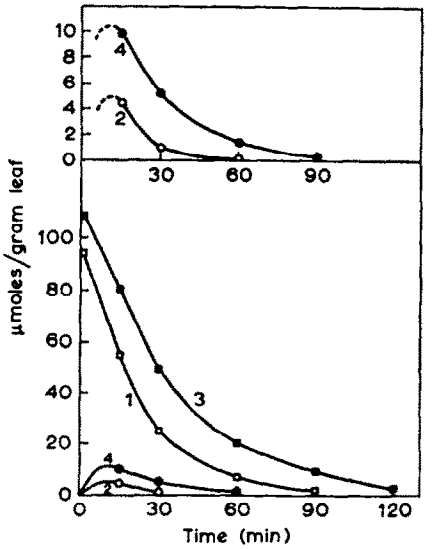



FIG. 4. CONCENTRATIONS OF PHLORIDZIN (CURVE 1) AND PHLORETIN (CURVE 2) IN HOMOGENATES OF MATURE LEAVES OF COX'S ORANGE PIPPIN. CURVE 3 AND CURVE 4 SHOW THE CONCENTRATIONS OF PHLORIDZIN AND PHLORETIN RESPECTIVELY IN HOMOGENATES OF YOUNG LEAVES.

After chromatographing on silica gel thin-layer plates with chloroform–methanol, 5:1, these substances appeared as brown spots at $R_f$ 0·8 and 0·25 after spraying with 0·5 per cent $KIO_3$. They were presumed to be 3-hydroxyphloretin and 3-hydroxyphloridzin respectively. To confirm this hypothesis 100 g of apple leaves of Cox's Orange Pippin were homogenized in 200 ml of 0·1 M $H_3PO_4/KH_2PO_4$ (pH = 4·0) containing 10 g of ascorbic acid. After shaking for 24 hr the pH was raised to 6·5, the optimum for $\beta$-glucosidase activity. After standing for 2 hr the *o*-diphenol at $R_f$ 0·25 had disappeared. The homogenate was extracted with ethyl acetate. The ethyl acetate contained the *o*-diphenol with $R_f$ 0·8 and a small amount of phloretin. Purification was effected by column chromatography on silica gel. Elution was performed with chloroform–methanol, 9:1. The *o*-diphenol was crystallized from dilute ethanol. The substance proved to be identical with synthetic 3-hydroxyphloretin. $H_2O$ (mixed melting point, i.r. and NMR spectrum). Accordingly the *o*-diphenol at $R_f$ 0·25 is considered to be 3-hydroxyphloridzin. At all pH values 3-hydroxyphloretin as well as other *o*-diphenols were enzymatically oxidized at about twice the rate of phloridzin. This fact explains why the intermediate *o*-diphenols do not accumulate during enzymatic oxidation of phloridzin in the absence of reducing substances.

2. *Polymerization Reactions*

The experiments presented above have revealed that the oxidation of phloridzin proceeds via 3-hydroxyphloridzin to its corresponding *o*-quinone. These reactions should consume 1 mole oxygen per mole phloridzin oxidized. Since the experimental values were about

1·25 at the optimum pH interval of the polyphenol oxidase (Fig. 1), it is evident that the *o*-quinone is further oxidized. To gain more knowledge of these oxidation reactions the enzymatic oxidation of 3,4-dihydroxydihydrochalcone and of 2′,4′,6′-trihydroxydihydrochalcone and of their mixture was studied (Fig. 5). 3,4-Dihydroxydihydrochalcone (curve 1) was oxidized at about the same initial rate as 3-hydroxyphloretin (curve 2), but the total amount of oxygen consumed was less with the former compound.

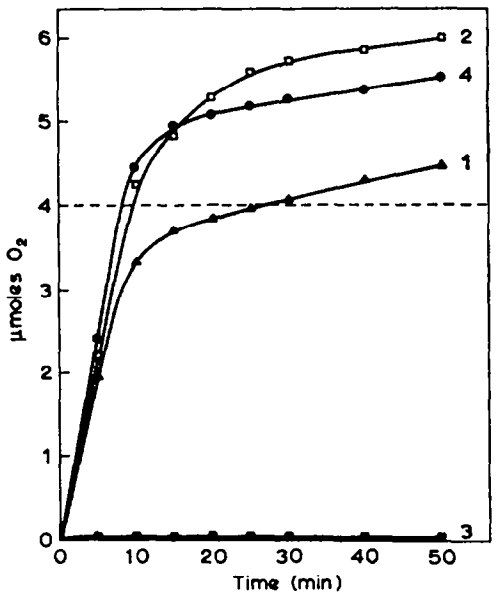



FIG. 5. THE OXYGEN UPTAKE AT pH 4·8 IN INCUBATION MIXTURES OF A CRUDE ENZYME PREPARATION (0·3 mg N/ml) OF YOUNG LEAVES OF COX'S ORANGE PIPPIN AND DIFFERENT PHENOLS.
Curve 1: 8 µmoles ($=3·2 \times 10^{-3}$ M) 3,4-dihydroxydihydrochalcone.
Curve 2: 8 µmoles 3-hydroxyphloretin.
Curve 3: 8 µmoles 2′,4′,6′-trihydroxydihydrochalcone.
Curve 4: a mixture of 8 µmoles 3,4-dihydroxydihydrochalcone and 8 µmoles 2′,4′,6′-trihydroxydihydrochalcone.

2′,4′,6′-Trihydroxydihydrochalcone was not oxidized in the presence of the enzyme preparation (curve 3). However, in a mixture of this substance with 3,4-dihydroxydihydrochalcone (curve 4) the total amount of oxygen consumed was about the same as in the oxidation of 3-hydroxyphloretin. This shows that the *o*-quinone of 3,4-dihydroxydihydrochalcone can react with the phloroglucinol nucleus of 2′,4′,6′-trihydroxydihydrochalcone. Most probably the *o*-quinone from 3-hydroxyphloridzin can react in a similar way. In this case repeated head-to-tail reactions can lead to polymeric substances. It appears from Table 1 that the characteristic brown-reddish colour of the oxidation products of phloridzin is associated with the products of the oxidative coupling reaction.

Both 3-hydroxyphloretin-2′,4′-diglucoside and 3,4-dihydroxydihydrochalcone yielded yellow oxidation products which gradually turned greyish. The oxidation curves with these two compounds were also quite identical. Consequently, glucosidation of the OH-groups in 2′ and 4′ position renders the phloroglucinol nucleus unreactive with the *o*-quinone.

TABLE 1

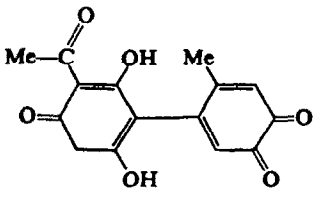
| Phenols enzymatically oxidized | Colour of the oxidation products |
|---|---|
| 1. Phloridzin | Brown-reddish |
| 2. 3-Hydroxyphloretin | Brown-reddish |
| 3. 3-Hydroxyphloretin-2′,4′-diglucoside | Yellow, gradually becoming greyish |
| 4. 3,4-Dihydroxydihydrochalcone | Yellow, gradually becoming greyish |
| 5. 4-Methylcatechol | Yellow brownish |
| 6. 2′,4′,6′-Trihydroxydihydrochalcone | Not enzymatically oxidized (colourless) |
| 7. Mixture of 3 and 6 | Brown–red |
| 8. Mixture of 4 and 6 | Brown–red |
| 9. Mixture of 5 and 6 | Brown–red |

3. *Chemical Model Experiments*

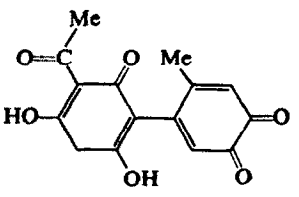
In order to reveal the nature of the supposed coupling reaction of the *o*-quinone from 3-hydroxyphloridzin with phloroacylophenones, some chemical oxidations with model compounds have been carried out. Equimolar amounts of phloroacetophenone and 4-methylcatechol reacted under the influence of oxidizing agents like $KIO_3$ or $K_3Fe(CN)_6$ to form a yellow coupling product, $C_{15}H_{12}O_6$. Since the 5 position of 4-methyl-1,2-benzoquinone is most liable to nucleophilic attack it is likely that phloroacetophenone adds to the 5 position of the quinone ring with the transfer of two hydrogen atoms. The intermediate quinol is then further oxidized to the quinone $C_{15}H_{12}O_6$. The most convenient way to carry out the reaction was oxidation with $KIO_3$ in an unbuffered aqueous solution containing enough acetone to keep the reactants in solution. From the red solution a yellow substance precipitated. The yellow compound is sparingly soluble in the usual organic solvents. It dissolves in alkali with a red colour. From this solution it is precipitated unchanged upon acidification. Reduction of an alkaline solution with bisulphite gives a light orange solution which turns slightly yellow upon acidification. After standing for some time exposed to air the original compound precipitates again.

An alcoholic solution gives a brown colour with $FeCl_3$. The NMR spectrum (taken in dimethyl sulphoxide-$d_6$) is in accordance with one of the structures IIa, IIb, or IIc.

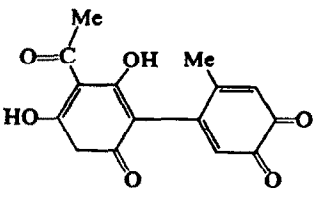
The following resonances were observed*: 1·58δ (Me), 2·53δ (COMe), 3·43δ ($CH_2$), 6·02 and 6·59δ (quinone protons) and a very broad absorption attributable to the OH groups at about 14δ. Structure II was further confirmed by a methylation experiment. This yielded



IIa



IIb



IIc

as the main product a compound $C_{18}H_{18}O_6$ (A) showing the following NMR absorptions (in $CDCl_3$ solution): 1·15δ (doublet $J=7·2$ Hz, CH*Me*), 1·62δ (quinone Me), 2·58δ (COMe), 3·55δ (quartet $J=7·2$ Hz, C*H*Me), 4·01 and 4·08δ (OMe groups), 6·22 and 6·91δ (quinone protons). It is evident that both the OH groups and the $CH_2$ group in the phloroacetophenone ring are methylated. This compound reacted in acetic acid solution with *o*-phenylene

* δ values are given in ppm downfield from tetramethylsilane.

diamine to give the phenazine derivative. This reaction proves that the compound is an *ortho*-quinone.

Oxidative coupling also took place between 2′,6′-dihydroxy-4′-methoxyacetophenone and 4-methylcatechol. The product (B) upon methylation yielded compound A. Since compound B must have a structure similar to IIa or IIb (the 2′-OH group replaced by an OMe group) structure IIc for the reaction product of phloroacetophenone and 4-methylcatechol is excluded.

Phloretin and 2′,4′,6′-trihydroxydihydrochalcone reacted in the same way as did phloroacetophenone. Structures analogous to II were confirmed by NMR spectroscopy. By thin-layer chromatography it was shown that enzymatic oxidation and chemical oxidation by means of $KIO_3$ of a mixture of 4-methylcatechol and 2′,4′,6′-trihydroxydihydrochalcone yielded the same coupling product. Oxidative coupling of 4-methylcatechol with phloridzin and with 4′,6′-dihydroxy-2′-methoxyacetophenone failed. Although the reaction mixtures showed a colour which differed from that of a solution of only 4-methylcatechol oxidized with $KIO_3$, no yellow product could be isolated. After 24 hr phloridzin and 4′,6′-dihydroxy-2′-methoxyacetophenone could be reisolated from ethyl acetate extracts of the reaction mixtures.

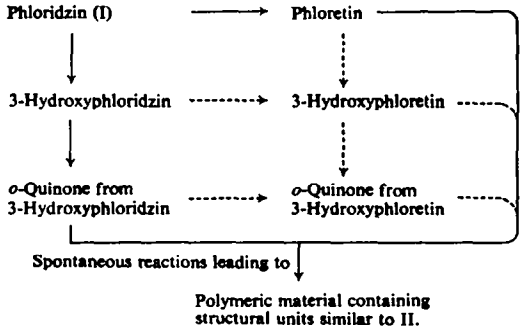
Compounds closely related to phloroacetophenone, e.g. 2′,6′-dihydroxyacetophenone and 2′,6′-dihydroxy-4′-methylacetophenone also did not react to an appreciable extent under the same circumstances. After 24 hr the starting materials were detected chromatographically.

Although it is not relevant to the problem of enzymatic conversions of phloridzin it is noteworthy that oxidative coupling of methylphloroacetophenone with 4-methylcatechol was easily effected. It is known that methylphloroacetophenone can be oxidized to a dimer, which upon dehydration yields usnic acid.[4] Apparently the reaction with 4-methyl-1,2-benzoquinone is more rapid. The NMR spectrum (in DMSO-$d_6$) of the product showed absorptions at 1·59δ (quinone Me), 1·92δ (tertiary Me), 2·54δ (COMe), 3·45δ $CH_2$), 6·87δ (broad absorption, alcoholic OH), 6·97δ (quinone proton) and 14·3δ (phenolic OH).

The spectrum indicates that two carbon atoms in the 4-methyl-1,2-benzoquinone ring are bonded to two carbon atoms in the methylphloroacetophenone nucleus, firstly to the C-atom bearing the methyl group and secondly to a C-atom bearing an hydroxyl group. This leaves several possibilities for the structure of the compound.

## DISCUSSION

The experiments have revealed the following sequence of reactions of phloridzin in the presence of apple leaf enzymes:




[4] D. H. R. Barton, A. M. Deflorin and O. E. Edwards, *J. Chem. Soc.* 530 (1956).

The optimum pH of the polyphenol oxidase lies between 4 and 5, without any distinct maximum. Its specific activity with phloridzin as substrate is seven to ten times higher than with phloretin.

The optimum pH of the β-glucosidase is about 6·5. In accordance herewith, phloretin was formed in higher yields in homogenates of young apple leaves (pH 6) than in those of mature leaves (pH 5). Chemical model experiments have revealed that *o*-quinones can react with phloroacylophenones. Glucosidation of the hydroxyl group in 2′-position, as in phloridzin, prevented this reaction almost completely. The *o*-quinones formed by phloridzin oxidation can therefore couple only with the aglycones of the reaction scheme presented. Thus, the fate of the *o*-quinone formed from phloridzin depends very much on the pH of the medium. At high pH when the activity of the β-glucosidase is near its optimum and phloretin is formed in highest yields, the *o*-quinone/phloretin coupling reaction may be the predominant one, whereas the fate of the *o*-quinone is uncertain at pH < 5. The products of the coupling reaction were found to be brown-reddish. The fact that phloretin did not reach a high concentration either in leaf homogenates or in model incubations of phloridzin with the acetone powder of young apple leaves can in part be explained by the coupling reaction. It seems probable, however, that inactivation of β-glucosidase by unstable oxidation products of phloridzin also adds to this result.

The importance of phloridzin and its transformation products in the resistance mechanism of apple leaves against *Venturia inaequalis* has been discussed briefly.[5]

## EXPERIMENTAL

Melting points were determined with a Reichert melting-point microscope. U.v. spectra were recorded with a Cary spectrophotometer. I.r. spectra were recorded in KBr disks with a Perkin–Elmer model 137 "Infracord". 100 Mc NMR spectra were measured with a Varian HA-100.

*Acetone powders* were prepared from young leaves of the scab-susceptible apple variety Cox's Orange Pippin and of a scab-resistant $F_1$ hybrid of the variety Antonovka. 40 g of apple leaves were homogenized for 1 min in 250 ml ice-cold water containing 2–3 drops Triton X-100.[6] The homogenate was rapidly squeezed through a fine meshed nylon cloth into 2·5 l. cold acetone (−12°). The plant debris was re-extracted by homogenizing in water for 1 min. The acetone precipitate was collected by centrifugation and washed by repeated suspension in cold acetone and centrifugation. A white or white-greyish residue was obtained. The residue was dried in a vacuum desiccator. The dried powder weighing about 2·5 g contained 10·8–11·2% N.

### *Manometric Estimations*

Oxidations of phenols in the presence of acetone powders was measured by conventional Warburg technique. The volumes of the flasks were approximately 15 ml. The main compartments normally contained 1·0 ml 0·1 M phosphate buffer of the desired pH and 1·0 ml of an aqueous solution (or suspension) of the phenol to be oxidized. The sidearm contained 0·5 ml of an aqueous suspension of the acetone powder, containing 1·5 mg N/ml. All experiments were carried out at 25°.

### *Quantitative Determinations of Phloridzin and Phloretin*

Parallel to manometric estimations of phloridzin oxidation, the concentrations of phloridzin and phloretin were determined in larger incubation mixtures of identical composition. After varying incubation times, samples of 4·0 ml were withdrawn and added to 2·0 ml 1 N TCA in 96% ethanol. After neutralizing with NaOH to pH 5–7, the mixture was extracted twice with 10 ml ethyl acetate.

The ethyl acetate fractions were dried ($Na_2SO_4$) and evaporated. The residue was dissolved in 1·0 ml absolute ethanol. Using a micropipette, 50 μl was brought on a silica gel thin-layer plate and chromatographed in acetone–chloroform, 3:2. Phloretin ($R_f = 0·8$) and phloridzin ($R_f = 0·15$) were extracted from the silica

[5] J. RAA, *Neth. J. Plant Path.* In press (1968).
[6] E. HAREL, A. M. MAYER and Y. SHAIN, *Phytochem.* 4, 783 (1965).

gel by 80% ethanol and the concentration of each of the compounds was calculated from the extinction values at 286 nm in the eluates.

*The β-glucosidase activity* was determined by measuring the rate of formation of phloretin from phloridzin under pure, oxygen-free nitrogen. *Leaf homogenates* were prepared by grinding leaves in a chilled mortar containing a small quantity of dry ice ($CO_2$). The fine powder which was obtained was kept at −30° until all $CO_2$ had evaporated. The leaf powder was then poured into an empty flask standing in a water bath at 25°. At different times samples were withdrawn and weighed exactly before extraction with boiling 70% ethanol. After removing the chlorophylls by extraction with light petroleum, the phenols were extracted into ethyl acetate and their concentrations determined as described.

*Reference Phenols*

*Phloridzin* and *phloretin* were commercial preparations. *3-Hydroxyphloretin-2',4'-di-β-D-glucoside* was prepared according to Zemplén, *et al.*[7] (cf. Ref. 8). *3-Hydroxyphloretin* was obtained from the diglucoside by acid hydrolysis,[7,8] larger quantities being synthesized using a method analogous to the classic phloretin synthesis of Fischer and Nouri.[9]

(i) *3,4-Dihydroxyphenylpropionamide.* Dihydrocaffeic acid methyl ester[10] (60 g) was dissolved in methanol (400 ml). In a 2 l. autoclave the solution was saturated with $NH_3$ and heated to 100°. The pressure was 30 atm. After 20 hr at this temperature the solution was evaporated *in vacuo*. The dark-coloured residue was extracted in a Soxhlet apparatus with ethyl acetate. Most of the colour remained undissolved. Evaporation of the ethyl acetate afforded crystals (49 g) with m.p. 140° (lit. m.p. 140°[11]).

(ii) *3,4-Diacetoxyphenylpropionamide.* The dihydroxy compound (44 g) was treated with acetic anhydride (160 ml) and pyridine (175 ml). After standing for 24 hr at room temperature the solution was poured onto ice. Extraction with ethyl acetate and evaporation of the solvent yielded a residue which was recrystallized from benzene–light petroleum. Yield 46 g, m.p. 109°. (Found: C, 58·48; H, 5·68. Calc. for $C_{13}H_{15}O_5N$: C, 58·86; H, 5·70%.)

(iii) *3,4-Diacetoxyphenylpropionitrile.* A mixture of the amide (46 g), dry $CHCl_3$ (180 ml) and phosphor oxychloride (90 g) was refluxed for 1 hr. Subsequently the solution was evaporated *in vacuo*. The residue was decomposed with ice water. The product was then extracted with ethyl acetate. The ethyl acetate solution was washed with 5% $NaHCO_3$ and then with water. After drying ($Na_2SO_4$) the solvent was evaporated and the residue was fractionated *in vacuo*. The fraction with b.p. 185°/0·3 mm (23 g) was used in the next step.

(iv) *3-Hydroxyphloretin.* A mixture of the nitrile (15 g), dry phloroglucinol (10 g), $ZnCl_2$ (3·5 g) and ether (50 ml) was shaken for about 20 min. The mixture was then cooled in an ice–salt mixture and dry HCl was passed in. After about 1 hr the solution was saturated. The mixture was left overnight and subsequently 2 N $H_2SO_4$ was cautiously added. The ether layer was decanted and the residue which consisted of a thick oil and an aqueous solution was slightly warmed. The nearly clear solution was then cooled and the acid layer was decanted from the oily residue. The oil was boiled in water for about 15 min. After cooling, crystals separated which were once recrystallized from dilute ethanol. Slightly yellowish needles were obtained (15 g) with m.p. 230–232°. The compound crystallized with 1 molecule of $H_2O$ and proved to be identical with 3-hydroxyphloretin prepared according to Zemplén *et al.*[7] (mixed m.p., i.r. spectrum and chromatographic behaviour). It is remarkable that in this reaction the acetyl groups were quantitatively removed. (Found: C, 58·54; H, 5·45. Calc. for $C_{15}H_{14}O_6 \cdot H_2O$: C, 58·44; H, 5·23%.) It should be noted that the most convenient method to prepare 3-hydroxyphloretin is the new procedure starting with apple leaves as described in section 1 under Results.

*3,4-Dihydroxydihydrochalcone*

3,4-Dihydroxychalcone[12] (12 g) was dissolved in methanol (200 ml). Hydrogenation was started with a 10% Pd/C (2 g). After 15 min the theoretical quantity of $H_2$ was taken up. Reduction was stopped, and after evaporation of the filtered methanol solution, the residue was recrystallized from benzene. This yielded 10 g of yellow product which contained the desired compound and small amounts of other reduction products, and of the chalcone as was shown by TLC on silica gel ($CHCl_3$–acetone, 5:1, detection with diazonium salt reagent). A chromatographically pure sample was obtained by column chromatography over silica gel. The compound crystallized from benzene in nearly colourless crystals with m.p. 108·5–109°. (Found: C, 74·14; H, 5·88. Calc. for $C_{15}H_{14}O_3$: C, 74·36; H, 5·83%.)

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*4-Methylcatechol* was prepared from vanillin analogous to the synthesis starting with veratric aldehyde as described by Adams *et al.*[13] *4',6'-Dihydroxy-2'-methoxyacetophenone*,[14] *2',6'-dihydroxyacetophenone*[15] and *2',6'-dihydroxy-4'-methylacetophenone*[16] were prepared as described in the literature.

*Coupling Reactions*

*5'-Acetyl-2',6' (or 2',4')-dihydroxy-2-methyl-4,5,3',4' (or 4,5,3',6') tetrahydro-4,5,4' (or 4,5,6')-trioxobiphenyl (II).* To a solution of phloroacetophenone[17] (5·04 g, 0·03 mole) and 4-methylcatechol (3·72 g, 0·03 mole) in acetone (50 ml) was added a solution of $KIO_3$ (4·28 g, 0·02 mole) in water (200 ml). After standing for about 1 hr at room temperature a yellow precipitate started to separate from the red solution.

After 2 hr the precipitate was collected and washed with water and subsequently with acetone. Yield 5·0 g (60%). The compound dissolved after prolonged boiling in ethanol. Cooling of this solution afforded small yellow needles. The compound melted to a red–brown tar at 255–260°. (Found: C, 62·15; H, 4·27; O, 33·05. Calc. for $C_{15}H_{12}O_6$: C, 62·50; H, 4·20; O, 33·30%); $\lambda_{max}$ (EtOH) 275 and 380 nm (log $\epsilon$ 4·26 and 4·22); i.r. bands in CO stretching region at 1739, 1642 and a broad absorption at 1615–1550 $cm^{-1}$.

In a similar way were prepared:

(a) *5'-Dihydrocinnamoyl-2',6' (or 2',4')-dihydroxy-2-methyl-4,5,3',4' (or 4,5,3',6')-tetrahydro-4,5,4' (or 4,5,6')-trioxobiphenyl*, starting with 2',4',6'-trihydroxydihydrochalcone.[18] Yield 55%, m.p. 230–235° (dec.). (Found: C, 69·33; H, 4·76; O, 25·52. Calc. for $C_{22}H_{18}O_6$: C, 69·83; H, 4·80; O, 25·37%); $\lambda_{max}$ (EtOH) 277 and 380 nm (log $\epsilon$ 4·25 and 4·20); i.r. bands at 1736, 1642 and 1615–1550 $cm^{-1}$.

(b) *5'-[3-(4-Hydroxyphenyl)propionyl]-2',6' (or 2',4')-dihydroxy-2-methyl-4,5,3',4' (or 4,5,3',6')-tetrahydro-4,5,4' (or 4,5,6')-trioxobiphenyl* starting with phloretin. Yield 50%, m.p. 225–230° (dec.). (Found: C, 66·20; H, 4·74; O, 28·43. Calc. for $C_{22}H_{18}O_7$: C, 67·00; H, 4·60; O, 28·40%); $\lambda_{max}$ (EtOH) 277 and 385–389 nm (log $\epsilon$ 4·30 and 4·25); i.r. bands at 1733, 1642 and 1615–1550 $cm^{-1}$.

(c) *5'-Acetyl-6' (or 4')-hydroxy-2'-methoxy-2-methyl-4,5,3',4' (or 4,5,3',6')-tetrahydro-4,5,4' (or 4,5,6')-trioxobiphenyl* (B) from 2',6'-dihydroxy-4'-methoxyacetophenone.[19] Yield 88%, m.p. 215–230° (dec.). (Found: C, 63·04; H, 5·00; O, 31·21. Calc. for $C_{16}H_{14}O_6$: C, 63·57; H, 4·67; O, 31·76%); $\lambda_{max}$ (EtOH) 268 and 373 nm (log $\epsilon$ 4·26 and 4·21); i.r. bands at 1724, 1642 and 1625–1550 $cm^{-1}$.

*5'-Acetyl-2',6' (or 2',4')-dimethoxy-2,3'-dimethyl-4,5,3',4' (or 4,5,3',6')-tetrahydro-4,5,4' (or 4,5,6')-trioxobiphenyl (A).* A suspension of compound II (2 g) in dry $CHCl_3$ (30 ml) was shaken with freshly prepared $Ag_2O$ (4 g) and MeI (4 ml). Compound II slowly dissolved. After 20 hr it was shown by TLC (silica gel, solvent, $CHCl_3$–ethyl acetate, 9:1) that the starting material had completely disappeared. The reaction mixture contained a major yellow product ($R_f$ 0·3) next to some brown material and small amounts of two or three faster-moving yellow compounds. The reaction was stopped and the filtered solution was evaporated.

The residue was washed with ether and the ether-insoluble material (1 g), which mainly consisted of the major reaction product, was further purified by column chromatography on silica gel. Elution was performed with $CHCl_3$–ethyl acetate, 9:1. The pure product was once recrystallized from a small amount of ethanol which yielded bright yellow crystals (0·8 g). The compound has a long melting range from about 200° to 230° with decomposition. (Found: C, 65·17; H, 5·46. Calc. for $C_{18}H_{18}O_6$: C, 65·45; H, 5·49%); $\lambda_{max}$ (EtOH) 256 and 393 nm (log $\epsilon$ 4·23 and 4·39),* i.r. bands at 1733, 1692, 1672, and 1620–1575 $cm^{-1}$.

The same compound was prepared by methylation of the oxidative coupling product of 2',6'-dihydroxy-4'-methoxyacetophenone and 4-methylcatechol (identical i.r. and NMR spectra).

The *phenazin derivative* was obtained by reacting a solution of 100 mg of the compound in 10 ml of acetic acid with 100 mg of *o*-phenylenediamine on the water bath. After 30 min the solution was evaporated and the residue was purified by column chromatography on silica gel. The orange–yellow band was eluted with $CHCl_3$–ethyl acetate, 9:1. The product was recrystallized from benzene–light petroleum which yielded 95 mg of yellow needles, m.p. 230° after previous sintering. The compound gives an intense violet colour with conc. $H_2SO_4$ which becomes orange–red upon dilution. (Found: C, 71·67; H, 5·60; N, 6·18. Calc. for $C_{24}H_{22}O_4N_2$: C, 71·63; H, 5·51; N, 6·96%.) The highest wavelength absorption maximum in ethanol was found at 405 nm (log $\epsilon$ 4·57), i.r. bands at 1686, 1650, 1608 and 1560 $cm^{-1}$.

* The highest wavelength absorption band of the hydroxy compound is not symmetrical and very broad. This explains why the hydroxy compound has a stronger colour than this methylated product despite the fact that the absorption maximum of the latter is at higher wavelength and has a higher extinction value.

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*Oxidative Coupling of Methylphloroacetophenone with 4-Methylcatechol*

To a solution of methylphloroacetophenone[20] (3·64 g) and 4-methylcatechol (2·48 g) in acetone (75 ml) was added a solution of $KIO_3$ (2·84 g) in water (200 ml). After standing overnight the yellow precipitate was collected and washed with water and then with ethanol. Yield 3·4 g (56%). Recrystallization from about 750 ml of ethanol afforded a mixture of yellow and orange prisms. The orange crystals contained about 3 moles of ethanol as was shown by the NMR spectrum. They were converted into the yellow form by drying at 100°. On thin-layer chromatograms the compound gave a single spot, m.p. 220–230° (dec.). (Found: C, 63·46; H, 5·10. Calc. for $C_{16}H_{14}O_6$: C, 63·57; H, 4·67%); $\lambda_{max}$ (EtOH) 309 and 380 and a shoulder at 405 nm (log $\epsilon$ 4·44, 4·24 and 4·17), i.r. bands at 1739 and a very broad absorption at 1665–1540 $cm^{-1}$.

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