THE ENZYMIC HYDROLYSIS OF PHLORIDZIN

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The Structure of Phloridzin

Phloridzin is the name given by de Koninck to a substance which had been found by Geiger to occur in the rootbark of the apple tree. The empirical formula ascribed to it by Roser—C₂₁H₂₄O₁₀·2H₂O—was endorsed by Strecker after a review of the analytical data previously published by Stass, by Liebig and by Mulder. The constitution of phloridzin may be represented as follows:

\[
\begin{align*}
&\text{HO-} \quad \text{O-C₆H₄O₄} \\
&\text{O-CO-CH₃-CH₂-C₆H₄-OH}
\end{align*}
\]

Hydrolysis by dilute mineral acids yields phloretin and glucose. Warm baryta converts phloridzin into phloretic acid and phlorin, which is identical with the phloroglucinol-β-glucoside synthesized by Fischer and Strauss.

The Hydrolysis of Phloridzin by Acid

A polarimetric examination of the rate of hydrolysis of phloridzin under the influence of hydrochloric acid showed the reaction to be kinetically unimolecular, as for glucoside hydrolyses generally. On comparing the velocity coefficient for the hydrolysis of phloridzin with values determined for several other glucosides at the same temperature and under identical catalytic conditions, it is found that phloridzin is the most labile glucoside that has hitherto been examined.

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much stress is not, however, to be laid on the velocity constant as a
guide to relative stability; the critical increment or the energy of
activation for the reaction, calculated by means of the Arrhenius
equation is a much more significant quantity than velocity itself.
Considered from this standpoint, phloridzin still appears to be the
most unstable glucoside yet encountered. The data quoted in
Table I emphasize an analogy between phloridzin and the \( \gamma \)-fructo-
sides, which is more pronounced than that between phloridzin and
the normal glucosides. It should, perhaps, be pointed out that the
data relating to the hydrolysis of the two trisaccharides raffinose and
melezitose refer to the cleavage of the \( \gamma \)-fructosidic linkage in each
case.

\[
\begin{array}{|c|c|c|}
\hline
\text{Compounds hydrolysed} & \text{Rate of hydrolysis (seconds \(^{-1}\) at 25°C. and pH = 6.0)} & \text{Critical increment (calories/gram mole)} \\
\hline
\text{Maltose} & 6.75 & 30,970 \\
\text{Salicin} & 6.43 \times 10^{-8} & 31,630 \\
\text{Arbutin} & 18.10 & 30,750 \\
\text{Phloridzin} & 1.96 \times 10^{-4} & 22,920 \\
\text{Sucrose} & 1.48 & 25,830 \\
\text{Raffinose} & 1.25 \times 10^{-3} & 25,340 \\
\text{Melezitose} & 0.52 & 25,000 \\
\hline
\end{array}
\]

\[
\text{The Diabetic Action of Phloridzin}
\]

The apparently anomalous behaviour of phloridzin during hydroly-
sis by acids is of interest in that this glucoside is exceptional also in
its physiological properties. The glycosuria which van Mering found was induced in animals after injection of phloridzin was shown
by Moritz and Prausnitz to be similar to the most severe forms of
human diabetes. It seems not improbable that this physiological
action of the glucoside is more closely connected with the sugar portion
of the molecule than with the aglykon (non-sugar moiety). Fromm states that the minimal dose of phloridzin required to induce glycosuria
in a dog weighing 7 kg. is 1 mg., whereas the threshold value for
diabetic action in the case of phloretin is 250 mg.
To these two exceptional properties (lability towards acids; diabetic action) attributable to phloridzin may be added the observation of Dann and Quastel\textsuperscript{34} that phloridzin is the only glucoside, of those examined, which exerts a marked retarding effect on the rate of fermentation of glucose by zymin.

**The Purification of Phloridzin**

The general lack of agreement in the values of the simpler physical constants recorded by various workers for phloridzin may, perhaps, be ascribed to the presence of a small quantity of a compound closely similar to phloridzin—a view which is rendered probable by the fact that phloretin is known to occur in nature combined with sugars other than glucose. Thus glycyphyllin is a condensation product of phloretin with rhamnose, a methylaldopentose.\textsuperscript{28} Phloridzin dihydrate is said to melt at 108°C.\textsuperscript{24} or 109°C.\textsuperscript{28}, and to exhibit a specific rotation (D line) of $-49.0^\circ$. The phloridzin used in this work was purified by repeated fractional crystallization from water. The dihydrate thus obtained melted at 113.5 to 114.0°C.; a 1.35 per cent solution in absolute alcohol gave

$$\left[\alpha\right]_{D}^{20^\circ} = -61.48^\circ$$

and

$$\left[\alpha\right]_{D}^{25^\circ} = -52.40^\circ.$$

**Previous Work on the Enzymic Hydrolysis of Phloridzin**

Little is known of the behaviour of phloridzin towards enzymes. Although it is not quite clear as to the precise meaning which is to be attached to the term enzymic specificity in the case of the hexosidases,\textsuperscript{28} experiments show that derivatives of $\beta$-glucose are generally hydrolysed by emulsin; $\alpha$-glucosides are hydrolysed by maltase. The optical rotation of phloridzin and the prevalence of $\beta$-glucosides in nature suggest that phloridzin is a derivative of $\beta$-glucose. Euler,\textsuperscript{27} in fact, goes so far as to classify it with the $\beta$-glucosides, in spite of the statement made by Armstrong\textsuperscript{28} that emulsin is without action upon phloridzin. In this connection it is of interest to note that phloroglucinol-$\beta$-glucoside, of which phloridzin is regarded as a derivative, is attacked by emulsin.\textsuperscript{14} It is fairly certain, however, that phloridzin, although apparently not hydrolysed by emulsin, is attacked by certain other enzymes occurring in the secretions of certain organisms. Charlier,\textsuperscript{29}
by artificially circulating defibrinated blood containing phloridzin through the kidneys of various animals, finds that the kidney of the horse (only) contains an enzyme capable of decomposing phloridzin. The juice secreted by the salivary gland and the hepato-pancreas* of the snail hydrolyses salicin, arbutin, coniferin, convolvulin, quercitrin, salonin, saponin, amygdalin, aesculin, helicin, phloridzin, lactose and maltose. From these results of Bierry and Giaza it is not clear whether phloridzin is hydrolysed by emulsin or maltase, since the secretion examined appears to contain both these enzymes. According to Giaza and GompeP the fresh digestive juice pumped from the stomach of the crab is capable of hydrolysing lactose, raffinose, maltose, several β-glucosides, and phloridzin. The phloridzin in this case may have been hydrolyzed by emulsin, maltase, or saccharase.

**Experimental Procedure**

In attempting to study polarimetrically the action of emulsin, maltase, and saccharase on phloridzin, a difficulty arises out of the low solubility of this glucoside. At 20°C, phloridzin dihydrate is soluble in water only to the extent of 0.189 gm. per 100 cc.; this value varying slightly with the addition of salts to the solution. A saturated solution of phloridzin in water at 20°C. exhibits an optical rotation in a 2 dm. tube of $-0.23^\circ$ ($\lambda = 5461\AA$); quantitative conversion of this into glucose would correspond to a final rotation of $+0.10^\circ$. It is evident from this that it is not possible to study the kinetics of the enzymic hydrolysis of phloridzin polarimetrically, since the total rotational change theoretically possible is less than a third of a degree. It is possible merely to detect chemical change and, in some cases, to measure its extent after relatively long periods. In order to do this, “blank” readings must be taken with enzyme and buffer solutions at various stages of reaction to ensure the absence of bacterial attack. Experiments have been conducted throughout at 30°C. with solutions of phloridzin previously saturated in buffer solutions at 20°C. Rotations have in all cases been taken in 2 dm. tubes at 20°C., using the green line of the mercury spectrum ($\lambda = 5461\AA$). The pH has been controlled by employing the usual Scavenen HCl-sodium-citrate and Na$_2$HPO$_4$-KH$_2$PO$_4$ buffer solutions. For enzyme preparations extracted without glycerol, the solutions have been kept sterile by the presence of toluol or thymol. The saccharase preparation employed was optically inactive; 0.1 gm. of emulsin preparation in 100 cc. of solution gave a rotation of $+0.02^\circ$; 1 cc. of maltase extract in 100 cc. exhibited a rotation of $-0.02^\circ$, which changed to $+0.02^\circ$ after 40 hours, and remained constant for the remaining 160 hours. In some cases, as

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* A duct from the liver unites with a duct from the pancreas before entering the intestine; the secretion referred to is, presumably, obtained from this “joint” duct.
with solutions containing maltase after prolonged maintenance at 30°C., it became necessary, in order to take optical measurements, to remove the enzyme by centrifugal treatment after adsorption on alumina.

The Action of Emulsin on Phloridzin

The emulsin used was extracted with water from the dry product supplied by the British Drug Houses. As the following table (Table II) shows, phloridzin kept in contact with emulsin for 3 days at 30°C. (pH = 4.45) does not undergo chemical change. At the end of this period the enzyme is still active and readily hydrolyses salicin, so that phloridzin cannot be regarded as a catalytic poison towards emulsin. The optimal pH given by Josephson for the β-glucosidase of emulsin is 4.4. This experiment has been repeated at different temperatures (30°C. to 50°C.) and different pH values (4.45 to 6.98) with the same result. There can be no doubt that emulsin does not hydrolyse phloridzin—a conclusion which is difficult to reconcile with the fact that emulsin attacks most of the naturally occurring β-glucosides and the synthetic β-glucosides, including those which are structurally akin to phloridzin, e.g., β-phenyl-glucoside and phloroglucinol-β-glucoside.

### Table II

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial rotation of 0.1 gm. emulsin in 100 cc. solution at pH = 4.45</td>
<td>+0.02°</td>
</tr>
<tr>
<td>Initial rotation of 0.189 gm. phloridzin in 100 cc. solution at pH = 4.45</td>
<td>-0.24°</td>
</tr>
<tr>
<td>Initial rotation of emulsin-phloridzin mixture</td>
<td>-0.22°</td>
</tr>
<tr>
<td>Rotation of mixture after 3 days</td>
<td>-0.24°</td>
</tr>
<tr>
<td>Immediate rotation after addition of 1 gm. salicin per 100 cc.</td>
<td>-2.20°</td>
</tr>
<tr>
<td>Rotation of mixture 2 days after addition of salicin</td>
<td>+0.45°</td>
</tr>
</tbody>
</table>

The Action of Maltase on Phloridzin

The maltase used in this work has been prepared from fresh brewery yeast by a method differing slightly from Willstätter and Steibelt's modification of the procedure originated by Croft Hill and Fischer.

200 gm. of fresh, well-pressed brewery yeast are washed several times with 2,500 cc. of distilled water, filtered in a Buchner funnel, pressed on to dry porous tiles and allowed to crumble into hard granular pieces. After a few days of drying in the air, and then in vacuum over H₂SO₄, the granules are powdered, sieved through a copper gauze (200 mesh) and dried in an oven at 35°C. The light yellow powder (32 gm.) can be kept indefinitely in the dry state. To extract maltase from this...
dried yeast 2 gm. of the powder are treated with 1.2 cc. of N-ammonia, 9.4 cc. of water and 9.4 cc. of glycerol. The mixture is kept at 30°C., with occasional shaking, for 3 hours, after which it is filtered. The ammonia is added to neutralise the acidity produced during the extraction of the enzyme.\textsuperscript{38} The enzyme preparation thus extracted in the presence of glycerol has been found to be much more stable than the water extracts which are usually prepared. 1 cc. of the clear glycerol-water preparation was found to hydrolyse 30 per cent (rotation falls from $+5.98^\circ$ to $+4.90^\circ$) of a 2 per cent solution of maltose monohydrate at 30°C. and pH of 6.98 in 45 minutes. The enzyme extract is thus seen to be somewhat more active than that prepared by Croft Hill\textsuperscript{36} (20 per cent hydrolysis of 2 per cent maltose monohydrate at 30°C. in 40 minutes). The experiments with maltase have been conducted at 30°C. and pH = 6.98. Values published for the optimal pH for this enzyme are: 6.1 to 6.7 (Isaiz\textsuperscript{59}); 6.0 to 6.8 (Rona and Michaelis\textsuperscript{39}); 6.7 to 7.25 (Willstätter and Bamann\textsuperscript{40}).

| TABLE III |
|-----------------|-----------------|
| Initial rotation of 1 cc. of maltase extract in 100 cc. of solution at pH = 6.98 | $=-0.02^\circ$ |
| Initial rotation of phloridzin in buffer solution | $=-0.25^\circ$ |
| Initial rotation of maltase-phloridzin mixture | $=-0.27^\circ$ |
| Rotation of mixture after 19 hours at 30°C. | $=+0.04^\circ$ |

The results given in Table III demonstrate that phloridzin is attacked by yeast maltase. During the period considered, the rotation of the maltase extract in a buffered solution remained sensibly constant, so that the final rotation due to phloridzin and its products of hydrolysis is $+0.06^\circ$. This corresponds to about 90 per cent hydrolysis of the glucoside. By using 3 cc. of enzyme extract it is possible to detect the hydrolysis of phloridzin within less than an hour (negative rotation decreases from $-0.036^\circ$ to $-0.25^\circ$ in 45 minutes). Unfortunately, it cannot be definitely concluded from these results that phloridzin is an $\alpha$-glucoside, because yeast maltase prepared as described above is known to contain, in addition to $\alpha$-glucosidase, traces of saccharase, which can be completely removed only by adsorption processes.\textsuperscript{41} A test with a 2 per cent solution of sucrose buffered at pH = 4.45 showed that the maltase preparation employed contained a small amount of saccharase (rotation falls from an initial value of $+3.12^\circ$ to $2.15^\circ$ after 21 hours at 30°C.). It is not known, therefore, whether maltase or saccharase is responsible for the hydrolysis of phloridzin.
The Action of Saccharase on Phloridzin

The saccharase preparation employed, which was purchased from the Digestive Ferments Company, was capable of hydrolysing 10 times its own weight of sucrose in a 10 per cent neutral solution of the sugar at 25°C. in 2 hours. The data of Table IV refer to the action of this enzyme on maltose (1.667 per cent monohydrate), sucrose (1.667 per cent) and phloridzin (0.189 per cent dihydrate) at 30°C. and pH = 4.45. The optimal pH for saccharase probably lies between 4.2 (Michaelis and Davidsohn) and 4.5 (Waldschmidt-Leitz). It is clear from the data given in the second and third columns that the enzyme preparation employed is rich in saccharase and is free from maltase. Phloridzin is attacked by saccharase under the conditions of experiment, the glucoside being hydrolysed to the extent of over 25 per cent in 20 minutes. Since it has not been found convenient to work with saccharase-free maltase as well as with maltase-free saccharase, it cannot be decided which of these enzymes is responsible for the cleavage of phloridzin. It is reasonable, however, to conclude that phloridzin is attacked by saccharase only, and that the behaviour of the glucoside towards the maltase preparation is due to the presence of saccharase in that product. Wiedenhagen has found that the sucrose-splitting enzyme (saccharase) contains small quantities of α-glucase in addition to β-(γ) fructosidase. α-glucase is said to be inactive at pH = 4.7. If this is true, then it must be concluded that phloridzin is hydrolysed by the γ-hexase, whence phloridzin should be regarded as a derivative of a γ-hexose.

TABLE IV

| Solution Contains 5 Cc. of Saccharase Extract Per 100 Cc. Solution at 30°C. and pH = 4.45 |
| Time (hours) | Rotation of solution (in degrees) |
|             | 1.667% maltose | 1.667% sucrose | 0.189% phloridzin |
| 0.00        | +5.30          | +2.46          | -0.23              |
| 0.33        | +5.24          | -0.10          | -0.14              |
| 4.17        | +5.26          | -0.58          | -0.08              |
| 20.0        | +5.30          | -0.74          | -0.02              |
| 70.0        | +5.30          |                | -0.02              |
ENZYMIC HYDROLYSIS OF PHLORIDZIN

The Sugar of Phloridzin

The behaviour of phloridzin towards emulsin, maltase, and saccharase indicates that this substance can hardly be termed a normal glucoside. The sugar produced during hydrolysis should therefore differ in its properties from those of glucose. Experiment shows this to be the case. A 4.646 per cent solution of phloridzin dihydrate, which was completely hydrolysed in 50 cc. of HCl at 100°C., and from which the precipitated phloretin had been removed, gave a rotation in a 2 dm. tube of +2.00°, which corresponds to a value of +56.42° for \([\alpha]_{546}^\text{D}^\circ\) in the case of the hexose liberated, reckoned as anhydrous sugar. A crystalline monohydrate of phloridzin-sugar was prepared by hydrolysis of the glucoside by 0.2 N H₂SO₄, followed by neutralisation with BaCO₃, filtration, concentration of the syrup under reduced pressure, and crystallization from a mixture of 4 parts of glacial acetic acid to 1 part of water. A 1.204 per cent solution of this sugar in water gave \([\alpha]_{546}^\text{D}^\circ\) = +56.65°, reckoned as anhydrous sugar—a value in agreement with that found by the previous method. It has been found that the ratio of \([\alpha]_{546}^\text{D}^\circ : [\alpha]_{589}^\text{D}^\circ\) for glucose is 1.267, whence \([\alpha]_{546}^\text{D}^\circ\) for phloridzin sugar becomes +44.61°. Hesse, during a careful polarimetric examination of various sugars, found for the sugar of phloridzin \([\alpha]_{546}^\text{D}^\circ = +45.86\), whereas the optical rotatory power of the sugar prepared from honey, grapes, salicin and amygdalin was +51.98°, +52.86°, +52.06° and +54.18°, respectively. The specific rotation for glucose at the concentration considered, i.e., 3 per cent, is +52.58°. The result given in the present investigation is thus seen to be in agreement with the work of Hesse. Unfortunately Fischer, upon whose authority the structure of glucose has been ascribed to phloridzin-sugar, based the identity on the melting point (204°C.) of the osazone, and gave no value for the optical rotation. Roser, who established the sugar as a hexose by measurements of its copper-reducing power, was unable to identify the sugar with glucose. Schiff, by the heat-decomposition of phloridzin, obtained a syrup which he regarded as glucosan. The specific rotation given by Rennie exceeds that of glucose almost as much as Hesse’s value falls short of +52.50°. With the possible exception of the physical constants published by Schunk and Marschewski, it can be concluded that no satisfactory values have yet been found for the melting point of phloridzin-sugar. In
view of the uncertainty which this casts on the true glucosidic nature of phloridzin, it is unfortunate that Johnson and Robertson, after hydrolysing methylated phloridzin, did not examine the methylated hexose thereby produced.

Through the kindness of Professor J. M. Beattie, M.A., M.D., Bacteriologist to the City of Liverpool, it has been possible to compare the effect of various bacilli on sterile solutions of glucose and phloridzin-sugar. In their behaviour towards Bacillus shiga (dysentery), Bacillus paratyphoid B., Bacillus proteus X 19, and Bacillus proteus (Zenker) the two sugars are alike. Bacillus pestis (bubonic plague), however, does not ferment phloridzin-sugar within 24 hours, whereas it does ferment glucose. At a later period, phloridzin-sugar is also fermented.

From the data on the kinetics of the hydrolysis of phloridzin by acids, from experiments on the behaviour of this substance towards the sucrase, and from an examination of the parent sugar, it must be concluded that phloridzin can not be regarded as a normal glucoside. The lability of phloridzin towards acids, and its response to the $\beta$-(\gamma) fructosidase of saccharase suggest that we may here be dealing with a derivative of a $\gamma$-hexose. The matter is in need of further investigation, which, for non-scientific reasons, can not be undertaken by the present writer.

SUMMARY

1. Considering previously published data on the velocity of hydrolysis of glucosides by acids, it is shown that phloridzin, judged from the standpoint of the velocity coefficient and the critical increment for hydrolysis, resembles the $\gamma$-fructosides (sucrose, raffinose and melezitose) more closely than it does the normal glucosides (salicin, arbutin, maltose, etc.).

2. Previous work on the enzymic hydrolysis of phloridzin shows that it is not hydrolysed by emulsin, but that it is hydrolysed by some other enzyme which occurs fairly freely in nature.

3. The difficulty in examining the enzymic hydrolysis of phloridzin lies in its very low solubility. It has been shown, in confirmation of earlier work, that emulsin is definitely without action on phloridzin at various values of pH and of temperature. This result is difficult to reconcile with the $\beta$-glucosidic character commonly ascribed to
phloridzin, and with the fact that emulsin hydrolys (synthetic) phloroglucinol-β-glucoside, of which phlorizin is regarded as a derivative.

4. Phloridzin is hydrolysed by a yeast maltase preparation, known to contain saccharase. Phloridzin is readily attacked by maltase-free saccharase at 30°C. and pH of 4.45. If the α-glucase of the sucrose-splitting enzyme is (as stated) inactive under these conditions, then the enzyme responsible for the hydrolysis of phloridzin is β-(γ) fructosidase.

5. The sugar prepared from phloridzin differs from glucose in its specific rotation and in its action towards Bacillus pestis.

The author is indebted to Professor J. M. Beattie, M.D., Bacteriologist to the City of Liverpool, for his kindness in undertaking the bacteriological examination of several sugars; to Messrs. Daniel Higsons Limited for their courtesy in supplying fresh samples of brewery yeast; to the Department of Scientific and Industrial Research of the British Government for a grant which has enabled him to carry out this work, as Research Assistant to Professor Lewis, F.R.S.; and to Imperial Chemical Industries Limited for a grant made to the Department of Physical Chemistry of this University.

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