THE SYNTHESIS OF PHENOLIC GLUCOSIDES

BY PLANT TISSUES

by

Chitra Roy

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

Department of Botany,
McGill University,
Montreal.

April 1959

ACKNOWLEDGEMENTS

The writer wishes to express her sincere thanks to Dr. G.H.N. Towers for his inspiration and generosity in the direction of the work. She also wishes to thank Dr. P.N. Avadhani for useful criticism and advice. She is grateful too to Dr. A.C. Neish of Prairie Regional Laboratory, National Research Council, Saskatoon, for a gift of a sample of radioactive phloroglucinol. She is indebted to the National Research Council of Canada for the award of a Bursary and for grants to Dr. Towers in aid of apparatus.

TABLE OF CONTENTS

INTRODUCTION

On feeding phloroglucinol to apple tissue in connection with the biosynthesis of the flavonoid, phloridzin, it was found that phlorin, the β-glucoside of phloroglucinol, was formed (Hutchinson, 1958).

Such a ready synthesis of this phenolic glucoside suggested a very good system for the study of the biosynthesis of simple plant glycosides. These compounds occur widely in plants and not very much was known about their biogenesis at the time when this study of the synthesis of phenolic glucosides in plant tissues was undertaken.

REVIEW OF LITERATURE

A. NATURALLY OCCURRING PHENOLIC GLYCOSIDES

Phenolic glycosides are the glycosidic derivatives of the cyclic forms of sugars in which the potential aldehyde group is replaced by condensation with phenol to form a hemi-acetal. The phenolic glycosides of plants show diversity with respect to the sugar component, however, D-glucose is found to be most common. All naturally occurring glycosides, with a few exceptions, have a β -glycosidic linkage which can be split by hot concentrated hydrochloric acid or by the hydrolyzing enzymes, the glycosidases. The enzyme emulsin is specific for β -glucosides (McIlroy,1951).

The naturally occurring phenolic glycosides occur in the plant kingdom almost exclusively. Only a very few glycosides are found in animals, for example, protoaphin, a phenolic glucoside, which occurs in aphids (Brown et al, 1952). According to Trim (1955b), the first discovery of the presence of glycosides in plants was made in 1829 when Leroux characterized the phenolic glucoside, salicin. Since the beginning of the twentieth century several glycosides of phenols have been detected in plants. Stoll and Jucker (1958) list forty-two phenolic glycosides that have been found in plants up to now. Some of the better known among them are arbutin, betuloside, coniferin, monotropitin and salicin. Phlorin, the β -glucoside of phloroglucinol with which this thesis is largely concerned has not been reported to occur naturally, although Bate-Smith (1950) suggested that it is present in the petal extracts of Pelargonium.

An examination of the distribution of phenolic glycosides shows that they are present in several families of plants (McIlroy, 1951). Like the other plant glycosides, the phenolic glycosides occur in roots, leaves, fruits and bark, mainly. Many plants are believed to accumulate their characteristic glycosides in the maximum amounts in the young organs during the cell elongation stage of growth (Trim, 1955b). Freudenberg et al (1952) and Trim (1955a) have found them to be closely associated with the cambium. Heartwood generally contains phenolic derivatives (White, 1958). The glycosides are known to be located in the vacuoles of the cells (Baumann and Pigman, 1957).

The amount of phenolic glycosides varies with different species of plants. It may also vary seasonally and with climatic changes.(McIlroy, 1951). Variations in the content occur at different stages of growth depending upon the equilibrium of synthesis and hydrolysis of the glycosides (Friedrich, 1958). There is believed to be an increase in the amount of intra-cellular phenolic derivatives at the advent of an attack by fungi, bacteria or virus (Dufrenoy, 1936).

Phenolic glycosides are not the only phenolic compounds that occur in plants. Phenolic acids quite commonly occur as esters, for example, chlorogenic acid. Phenols do not generally occur in nature in the free state, although the occurrence of a number of free phenols, such as catechol, resorcinol, hydroquinone, pyrogallol and phloroglucinol has been reported in plant tissues. These phenols are believed to participate in amino acid and carbohydrate metabolism (Clarke and Nord, 1955).

Since most of the experimental work for this thesis has been done with phloroglucinol, it was considered interesting to review the occurrence of this phenol in plants. Waage (1890) said that phloroglucinol is widely spread in the free state in higher plants. According to Karrer (1958), however, phloroglucinol rarely occurs in the free state in plants even though it is often obtained as a degradation product of such substances as tannins, anthocyanins, flavonoids and other compounds. Although phloroglucinol has been implicated in the biosynthesis of flavonoid compounds (Robinson, 1936; Seshadri, 1951), Geissman and Hinreiner (1952) say that phloroglucinol occurs rarely or not at all in the plants containing flavonoid compounds. However, phloroglucinol has been reported to occur in the free state in some plants by a few authors. Keegan (1915) claimedto have isolated phloroglucinol from Lychnis dioica, although it is not known how reliable were his methods of detection. Klein et al (1931) detected and isolated a few free phenols including phloroglucinol, by microsublimation and by extraction under such mild conditions as to prevent degradation, in such plant tissues as bark, wood, seeds and leaves. Green (immature) dates have been reported to contain phloroglucinol along with other phenolic substances, on the basis of qualitative tests (Turrel et al, 1940). In ripening dates phloroglucinol conjugates with glycine (Rindernecht and Jurd, 1958). Phloroglucinol has been reported to be present in the leaves of seven varieties of willow (Kursanov, 1944). Bate-Smith (1950) claims to have detected phloroglucinol and possibly its β -glycoside in extracts of Pelargonium petals. Phloroglucinol occurs free as well as in a combined state along with other phenolic compounds

in the cones of Sequoia sempervirens and S. gigantea (Kritchevsky and Anderson, 1955).

Lower plants and microorganisms are also known to contain phenolic compounds. Phenolic substances have been reported to occur in the red algae. Polysiphonia fastigata is believed to contain the potassium disulphonate of hydroxybenzoic acid (Mastsgli and Augier, 1949). Some fungi are also known to contain phenolic substances. van Sumere et al (1957) have found that the uredospores of Puccinia graminis var. tritici contain derivatives of phenol, catechol and 2-methoxyphenol. The last of these three classes namely 2-methoxyphenol compounds, may be present as glycosides.

B. FATE OF PHENOLS INTRODUCED INTO PLANTS

Phenols introduced into plants may affect metabolism in various ways and they themselves may be variously changed.

1. Toxicity of Phenols

Phenols are generally considered to be toxic substances. There is no agreement as to the definition of the term 'toxic' but by toxic substance one generally means a substance which interferes with the normal functioning of a cell (Currier, 1956). The extent of toxicity of phenols varies with the organism and the nature and the amount of the phenol concerned. Phenols are generally inhibitory in their effect but like other toxic substances, they can be harmless or even stimulating in very low concentration.

Toxicity of a phenol is displayed with respect to respiration

(Boswell and Whiting, 1938; Hackney, 1948) by the formation of active complexes with copper (Currier, 1956). Some of the phenols, such as phloroglucinol, resorcinol or phenol, inhibit the activity of carboxylase (Karrer and Viscontini, 1945). Gavaudan and Brébion (1946) found from manometric studies of oxygen released by Elodea canadensis that phenols inhibit photosynthesis. They affect mitosis also (Gavaudan and Brébion, 1946) and can cause mutations in the cell (Srb and Owen, 1953). It has been suggested that inhibitions in germination of seeds may be caused by phenols, the active hydroxyl group being largely responsible. The inhibitory effect has been found to decrease with increasing number of hydroxyl groups (Evenari, 1949). Eisenmenger (1931) studied the effect of toxicity of phenol on soy bean seedlings. The seedlings were grown in varying concentrations of phenols and toxicity was determined by measuring the relative growth rates. The toxicity was observed to increase with increased concentration of phenol. Chabrolin (1940) designed experiments to determine the effect of various toxic substances on different plants. Among the toxic substances were both free phenols, such as phenol, resorcinol and others and their derivatives either in solution or as emulsions. Using Raphanus raphanistrum as the test plant, one of the most toxic group of substances was found to be the phenols and their derivatives. The lethal dose differed with different species of plants. Papaver rhoeas withstood applications of 10% sulphuric acid but was very sensitive to phenols and their chlorine derivatives. Lathyrus ochrus, on the other hand, was very resistant to chlorophenols.

2. Formation of Glycosides of Phenols

When relatively small concentrations of phenols are introduced into plant tissues, β-glycosides corresponding to those phenols are formed (Ciamician and Ravenna, 1916; Hutchinson et al, 1958; Miller, 1940; Miwa et al, 1957; Nystrom et al, 1959; Pridham, 1958).

It is likely that glycosidation serves as a mechanism of detoxication (Armstrong and Armstrong, 1931; Miller, 1940).

According to Armstrong and Armstrong (1931), Ciamician and Ravenna (1916) first showed that plants form glycosides when they are inoculated with aromatic products of glycoside hydrolysis. They claimed to have isolated salicin from such plants as maize and beans treated with weak solutions of saligenin. However, Pridham (1958) has recently reported that when he fed saligenin to young bean shoots (o-hydroxybenzyl) -β-glucoside, and not salicin ((o-hydroxymethylphenyl)-β-glucoside) was formed.

Miller (1938-1957) has studied the induced synthesis of glycosides of alcohol and phenol quite extensively. He has shown that when certain chemicals, which do not occur naturally in plants, are absorbed by various higher plants, glycoside formation takes place, with these chemicals acting as aglycone groups, even in species and families not known to contain natural glycosides.

He introduced alcohols and phenol either in the vapour form or in nutrient medium. He identified the resultant glycosides by forming acetyl derivatives of the glycosides and then comparing them with the corresponding synthetic glycoside acetates.

Miller found that a great variety was displayed with respect to the sugar molecule of the induced glycosides. The nature of the sugar residue involved in glycosidation varied with different species of plants. Potato tubers (Solanum tuberosum L.) formed β-(2-chloroethyl)-d-glucoside when these tissues were treated with ethylene chlorohydrin (Miller, 1939a), however, the glycosides formed from this compound by Lycopersicon esculentum Mill., which is closely related to Solanum tuberosum, was not a glucoside (Miller, 1939b). When plants were treated with chloral hydrate, this aldehyde was reduced to its corresponding alcohol, which conjugated with sugar to form β-2,2,2,-trichlorethyl-glycoside (Miller, 1941a). The sugar component of this glycoside varied in different species. A glucoside was formed in Zea mays (Miller, 1942), a gentiobioside in Lycopersicon esculentum Mill., (Miller, 1941a), and a mixture of these two glycosides in tobacco (Nicotiana tabacum L. var. Turkish) (Miller, 1943). Yet another kind of glycoside was detected in dandelion (Taraxacum officinale Weber) treated with chloral hydrate. In this case, the sugar component of the resultant glycoside was found to be a disaccharide consisting of glucose and xylose, which is probably primeverose (Miller, 1957).

The sugar components of the glycoside did not vary with different species only, the same plant tissue showed variations with different or even the same aglycone. For example, Gladiolus corms formed β-(2-chloroethyl)-d-glucoside when treated with ethylene chlorohydrin (Miller, 1938) and β-o-chlorophenyl-gentiobioside when o-chlorophenol was introduced (Miller, 1940). It was found that these two glycosides could be synthesized simultaneously when the aglycones concerned were provided

(Miller, 1941b). The leaves of tobacco formed a mixture of the glucoside and the gentiobioside of the same aglycone, trichloroethyl-alcohol (Miller, 1943).

While the work on this thesis was in progress , Miwa et al (1957), Nystrom et al (1959) and Pridham (1958) reported the formation of glucosides in the plants fed with various phenols. The details of their work will be discussed later under "Discussions". Towers et al (1958) found that maleic hydrazide produced a β-glucoside when fed to leaf discs of tobacco.

The lower plants and microorganisms are generally rather sensitive to phenols. However, some of them have been reported to be able to resist them and some are known to use them as a source of carbon. They probably attack them by oxidizing them (Evans, 1958). There is no evidence for the formation of glycosides.

A lot of work has been done on the metabolism of phenols by animals. When a phenol is introduced into an animal, the bulk of it gets detoxified as the glucuronide and the sulphate (Bodansky, 1948; Dubin, 1916; Fishman, 1956; Quick, 1937; Williams, 1951). Glycosides are not known to be formed from introduced phenols in higher animals. Glucoside was suggested as an intermediate stage in the glucuronide synthesis (Fischer and Piloty, 1891) but no support for this hypothesis was found from the experiments of Hemingway et al (1934) and Pryde and Williams (1936). However, phenols have been found to be conjugated with glucose rather than with glucuronic acid in locusts (Myers and Smith, 1954). This has been found to be true of fifteen different insects from five orders

(Smith, 1955).

C. MECHANISM OF SYNTHESIS OF PHENOLIC GLYCOSIDES

1. Chemical Synthesis

According to Armstrong and Armstrong (1931), Michael (1879) was the first one to synthesize phenolic glycosides chemically. He coupled acetochloroglucose with potassium salts of phenols and produced salicin, helicin and methyl arbutin. The β-glucoside of phloroglucinol, phlorin, has been prepared by alkali fission of phloridzin (Cremer and Seuffert, 1912).

2. Enzymic Synthesis

(1) Reversion of hydrolysis

Investigations of the enzymatic hydrolysis of β-glucosides have shown that the equilibrium between glucoside, glucose, aglycone and water in dilute aqueous solution is such that the hydrolysis proceeds virtually to completion, until less than 1% glucoside is left unhydrolyzed. In order to increase the amount of glucoside in the reaction mixture, that is, to bring about an enzymatic synthesis of the glucoside, it is necessary to have the glucose dissolved or suspended in a very concentrated solution of the aglycone. The equilibrium is established after two or three weeks (Veibel, 1950).

$$\text{Aglycone} + \text{Glucose} \underset{\longleftarrow}{\xrightarrow{\text{Glucosidase}}} \text{Glucoside} + \text{water}$$

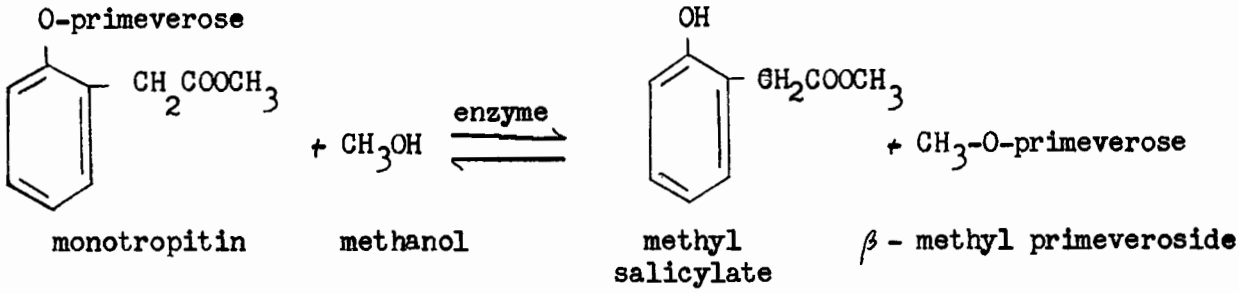
Bourquelot (1913) and his associates synthesized several glycosides

by means of hydrolytic enzymes, that is, enzymes known primarily for their hydrolytic action <u>in vitro</u>. They let emulsin (a β-glucosidase) act for long periods of time on solutions containing glucose and high concentrations of aglycone. This way, they prepared a number of alkyl glucosides, such as the β-glucosides of methanol, ethanol, isopropanol, butanol, and some other alcohols. A few alkyl and benzyl galactosides were also prepared by emulsin. Enzymic synthesis of phenolic-β-glucosides in this manner, however, has not been reported.

(2) Transglycosidation reaction

Glycosides may be formed by transglycosidation reaction which involves the transfer of a sugar molecule from a glycoside to a aglycone (a sugar acceptor) by means of an enzyme. Unlike the reactions involving reversal of hydrolysis which require very high concentration of aglycone, the transglycosidation reactions result in substantial yields of glycoside when the aglycone is used in comparatively low concentrations(Takano, 1956).

Such transfer reactions were first noted by Rabaté (1935). He demonstrated a reaction in which a glycosyl group was transferred from a phenolic glycoside to an alcohol in the following way (Rabaté, 1937):



This reaction, which was mediated by an enzyme preparation from Gaultheria procumbens resulted in a 27% conversion of the phenol-linked primeverose to methyl primeveroside in the presence of as little as 1 to 2% methanol.

The phenomenon of transglycosidation has been illustrated by the work of some of the recent authors. Transfer of a glucose residue from a number of aryl-β-glucosides to certain alcohols has been demonstrated by using β-glucosidase preparations from a number of fungi and higher plants (Jermyn and Thomas, 1953; Takano, 1956; Takano and Miwa, 1950). Pridham (1957) obtained (o-hydroxyphenyl)-β-gentiobioside and glucose on incubating arbutin with a β-glucosidase preparation from Populus grandidentata. In view of such results, it has been suggested that the hydrolytic and transferring activities are shared by the same enzyme molecule. Hydrolysis is regarded by many (Gottschalk, 1958) to be a specific type of a transfer reaction where the water molecule acts as an acceptor.

It is interesting to note that no in vitro reaction has been reported where a phenolic hydroxyl group has been engaged in glycosidation, either by transfer reaction or by Bourquelot's method (reversal of hydrolysis).

(3) Synthesis of glycosides through phosphorylated sugars

While work on this thesis was in progress, Miwa et al (1957) reported their findings on the synthesis of phenolic glucosides. They found that the synthesis of these glucosides in vivo was inhibited in the absence of oxygen. They also found that the activity of the cell-free enzyme preparations was lost by dialysis but regained by the

addition of ribonucleic acid (yeast nucleotide) and adenosine triphosphate. They concluded from this that the synthesis is mediated by the energy obtained from respiration through glycosyl-phosphate compounds.

Cardini and Leloir (1957) and Cardini and Yamaha (1958) also published the results on the synthesis of phenolic glucosides during the period of the present study. They synthesized the β-glucosides of a number of phenols, such as hydroquinone, saligenin, catechol and resorcinol by incubating a mixture of a phenol and uridine diphosphate glucose in the presence of a wheat-germ extract. They also synthesized the gentiobioside of hydroquinone in the same way.

MATERIALS AND METHODS

A. MATERIALS

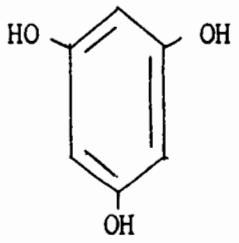
1. Plant Material

Gerbera jamesonii Bolus was used in a few of the earlier experiments but it was not easily available later on and Pelargonium hortorum L.H. Bailey var. Carmin Pink was used instead. Fully grown leaves of plants grown in the McGill greenhouse were used. This plant was chosen because it was easily available throughout the year.

Other plant materials which were used are mentioned in the section "Experiments and Results".

2. Chemical Substances

a) Phloroglucinol



Phloroglucinol was used as the representative phenol. It was chosen because a) it is soluble in water, b) it does not oxidize as readily as most of the other phenols (von Oettingen, 1949), c) phloroglucinol and phlorin can be distinguished easily from many other compounds by a characteristic colour reaction with Erhlich reagent,

d) a sample of phloroglucinol-C^{14} was obtained through the generosity of Dr. A.C. Neish, Prairie Regional Laboratory, N.R.C., Saskatoon.

Other phenols which were used are mentioned in descriptions of the experiments.

b) Radioactive chemicals

Radioactive chemicals were purchased from the Atomic Energy of Canada, Limited. The small amounts of materials, 1 to 5 mg. were made to 10 ml. in distilled water and the solutions were kept frozen when not in use. The specific activities are reported in the section "Experiments and Results".

B. METHODS

1. General Method

The general method used in the experiments can be outlined conveniently by subdividing it into the different major steps involved. They are: feeding, extraction, chromatography and identification by spray reagents.

Leaf discs, 1.2 cm. in diameter, were obtained with a Ganong leaf punch from full grown leaves. The discs were then vacuum infiltrated in tap water by placing them in water in a stoppered suction flask and applying vacuum to the side arm of the flask. On releasing the vacuum, water was drawn into the leaf tissue. This procedure was repeated until the leaf discs, which floated on the water previously, remained suspended in it. (The process of vacuum infiltration is useful because it helps to eliminate the air trapped in intercellular spaces of the tissue.)

The leaf discs were then blotted to remove the excess water from them.

Five discs per sample were then placed in a small (4.2 cm. in diameter) Petri dish containing a solution of the substances that were to be fed. The discs were floated with their lower surface downwards so that the stomata were in contact with the solution. (Pelargonium leaves have more stomata on lower than on upper epidermis; Meyer and Anderson, 1952.) The Petri dish was then covered and placed under constant light of approximately 200 f.c. The discs were allowed to incubate for approximately 20 hours at room temperature (20° to 22°C).

After incubation, the discs were rinsed in tap water and blotted. This was repeated twice. The discs were then killed by placing them in 75 ml. of boiling 80% ethanol and extracted by refluxing. Two or three extractions were made until no more colouring matter (chlorophyll) was extracted into the alcohol. Generally, one extraction was sufficient. The combined extracts were evaporated to dryness at room temperature under an air jet.

Large, descending chromatograms, both one- and two-directional, were used. One-directional chromatograms provided a quick method for the separation of phenolic substances and also enabled the comparison of compounds on the chromatogram with standard or reference compounds.

One-directional chromatograms were prepared by spotting the plant extracts and the standard compounds along a line drawn parallel to and 10 cm. away from the lower edge of the filter paper (Whatman No. 1) measuring 46 x 75 cm^2. The paper was then irrigated by the organic phase (80 ml. per trough) of a mixture of n-butanol-acetic acid-water, 4:1:5 v/v/v (Block et al, 1955) in a chamber equilibrated with the aqueous

phase of the solvent mixture, for a period of 22 hours during which the solvent front ran off the edge of the paper.

In preparing two-directional chromatograms, the plant extract was spotted at the intersection of the lines drawn parallel to and 10 cm. away from the two adjacent edges of a Whatman No. 1 filter paper (46 x 57 cm^2). The maximum amount of the extract was spotted each time, taking care that the spot did not become gummy in texture. The paper was then run in the first solvent, phenol saturated with water (80 ml. per trough) in a direction parallel to the long axis of the paper until the solvent front reached within 5 to 10 cm. of the edge of the paper, which took 24 hours or more depending mainly on the temperature of the room. After drying it for 72 hours in a fume cupboard, it was next run in a second chromatographic chamber containing 80 ml. per trough of n-butanol-acetic acid-water, (4:1:5) as the solvent for about 22 hours.

The combination of these two solvents, namely, phenol-water, and butanol-acetic acid-water, was found to be better for separation of phenolic compounds than any other combination tried (Hutchinson, 1958), although the residual phenol on the paper from the first solvent reacted with phenol detecting sprays. This disadvantage, which may be lessened by drying the papers thoroughly over a longer period of time before using the second solvent, is outweighed by the results obtained. The phenolic compounds were separated very well from other substances as sugars, amino acids and so on. Moreover, they formed discreet and compact, round or elliptical spots.

The phenolic compounds were detected by various spray reagents, such

as vanillin in concentrated hydrochloric acid (which reacts with phloroglucinol), (Lindt, 1887), diazotized sulphanilic acid (Block et al, 1955), diazotized p-nitroaniline (Swain, 1953), and Erhlich reagent (Block et al, 1955). Vanillin in concentrated hydrochloric acid was unsuitable as a spray reagent and was later discarded in favour of other reagents. Diazotized sulphanilic acid reacted with phenols to give colours in the yellow-orange-purple range but this spray was not used except in a few experiments as it produced a very strong yellow background. Diazotized p-nitroaniline was found to be a very sensitive spray. It reacted with phenols and produced colours in the yellow-orange-red spectrum (under acidic conditions). With phloroglucinol and phlorin it gave an orange colour. This spray also gave a light yellow background (before an alkali overspray). Best results were obtained with this reagent when the chromatogram was sprayed with a solution containing 5 ml. of 0.5% p-nitroaniline in 2N hydrochloric acid, 1 ml. sodium nitrite and 20% sodium acetate and then oversprayed with 4% sodium hydroxide. This spray needed to be prepared fresh just before spraying. In this case too, a background colour (bright pink) was obtained after the alkali spray, but it did not interfere with the colours obtained with the phenols and their glycosides. The alkali overspray showed up certain compounds which could not be detected before, for example, arbutin. Erhlich reagent was prepared by dissolving 1 gm. of p-dimethylaminobenzaldehyde in 200 ml. of 95% ethanol and 5 ml. concentrated hydrochloric acid. It is a very stable reagent and was, therefore, found to be convenient for use. It was found to react with those phenolic compounds which contained a phloroglucinol nucleus. It gave a

rose-pink colour with phloroglucinol immediately after spraying and the same colour with phlorin on heating. Practically no background colour was obtained with this reagent.

2. Technique with Radioactive Substances

Radioactive substances (C^{14} compounds) were fed to vacuum infiltrated plant tissue in solution. The extraction of the plant materials was then carried out as described above. The ethanolic extract was made to 1 ml. with 80% ethanol. The radioactivity content of the plant extract was measured by transferring 0.03 ml. of this solution onto a planchette lined with an aluminium foil and then allowing it to dry. The measurement was taken by exposing the sample to a thin-end-window Geiger Muller tube attached to a Berkeley decimal scaler, Model 2001. The sample was counted until the count exceeded 3,000 so that the standard deviation of the count obtained was less than 2%. The radioactivity of the ethanolic extract was determined using three aliquots and the average of these three results was used. This count, after subtraction of the background count, served in expressing the total radioactivity in 80% ethanol-soluble fraction of the plant tissue. The remaining extract was spotted and chromatographed in the way described above. The chromatograms were generally prepared in duplicate when radioactive substances were used. One of each set of chromatograms was sprayed with a chromogenic reagent and the other one was radioautographed to show the distribution of radioactivity in the various compounds in the plant tissue.

A radioautograph was prepared by placing the chromatogram concerned

in contact with a Kodak NO-Screen X-ray film after trimming the chromatograms approximately to the size of the film (35.6 x 43.2 $cm.^2$) by cutting away the edges from the origin. The chromatogram was allowed to remain in contact with the film for 2 to 3 weeks and heavy weights were placed on the holder containing the film. The film was then developed by placing it in Kodak Rapid X-ray developer for seven minutes, dipping it in dilute aqueous solution of acetic acid for one to two minutes, washing it in tap water and finally, fixing it in a solution of Kodak Liquid X-ray Fixer. The film was then washed in running tap water for two hours and dried. The dark patches or "spots" on the radioautograph (developed film) represented the radioactive substances on the chromatogram. The radioautograph was matched with its chromatogram and the dark spots were traced out on the paper. The various spots were identified by their R_f values and also by their behaviour towards different spray reagents. (The intensity of the spot on the radioautograph was found to be proportional to the radioactivity content of the compound corresponding to it, within limits.) The amount of activity in each compound was counted by placing the particular spot of the chromatogram under the end-window of a Geiger Muller tube attached to a Berkeley decimal scaler, Model 2001. Since the spots were often larger than the end-window of this tube, they were divided up into smaller squares which could be covered by the end-window and each of these squares was counted separately, shielding the rest of the area with pieces of cardboard. The counts from each of these small square areas covering the spot representing the particular compound were added up, after subtracting the background count, to get the total radioactivity

content of that compound. This value was then expressed as a fraction of the total count on the paper or in terms of the total of 80% ethanol-soluble fraction of the plant tissue.

In experiments in which a particular compound on the radioautograph was needed for further study, the spot representing that compound was cut out from the corresponding chromatogram and eluted and only the remaining spots were sprayed with chromogenic reagents. The duplicate chromatogram was generally used for spraying to help in the detection and identification of the compounds.

The methods outlined above, both the general method and the technique with radioactive isotopes, were used throughout the course of this study unless otherwise mentioned; for instance, roots might have been used instead of leaf discs or certain factors such as light and temperature might have been varied.

EXPERIMENTS AND RESULTS

A. THE SYNTHESIS OF PHENOLIC GLUCOSIDES IN VIVO

1. Synthesis of Phlorin by Leaf Discs of Pelargonium

Two samples of four leaf discs each were placed in small Petri dishes containing in one case 0.5 ml. of water and 0.2 ml. of a 0.01% glucose-1-C^{14} (2 µc.) and in the other case 0.5 ml. of 2% phloroglucinol and 0.2 ml. of glucose-1-C^{14} (2 µc.). The discs were allowed to incubate under fluorescent lights (200 f.c.) for approximately 20 hours. After rinsing them in tap water and blotting them, they were extracted with boiling 80% ethanol. The extracts were chromatographed both one- and two-directionally as described previously. The radioautographs prepared from these chromatograms showed practically the same pattern, qualitatively, in the two samples. (See Figures 1 and 2.) However, the sample containing phloroglucinol had an additional intensely radioactive spot containing 42% of the radioactivity in the ethanolic extract. The R_f of this new compound was the same as that of phloroglucinol, namely 0.26, in phenol-water solvent, and in butanol-acetic acid-water (4:1:5) solvent, its R_f was 0.20 compared to 0.63 for phloroglucinol. The spot corresponding to this compound on chromatogram reacted with such spray reagents as Lindt's reagent (vanillin in hydrochloric acid), diazotized sulphanilic acid, diazotized p-nitroaniline, and Erhlich reagent, producing the same colour as phloroglucinol in each case.

This compound was found to be radioactive also when leaf discs were fed for 18 to 19 hours with 0.5 ml. of 0.1% solution of phloroglucinol-C^{14}

(0.5 μc. approximately) and inactive glucose. In this case, approximately 96% of the total radioactivity content in the ethanolic extract appeared in this compound. (See Figure 3.)

On eluting this compound with 80% ethanol and then hydrolyzing it with 2N hydrochloric acid or emulsin, phloroglucinol and glucose were obtained. These were identified by chromatography. This showed that this compound consisted of phloroglucinol and glucose and successful hydrolysis with emulsin indicated the presence of a β -linkage between phloroglucinol and glucose.

The identity of this compound as phlorin, the β -glucoside of phloroglucinol, was confirmed by comparing its behaviour on chromatograms with phlorin obtained through alkali fission of phloridzin according to the method of Cremer and Seuffert (1912).

When leaf discs were fed either with phloroglucinol and glucose-C^{14} or with phloroglucinol-C^{14} and glucose, a very weakly radioactive spot was detected on chromatograms (see Figure 3) which moved a little slower than phlorin in both phenol-water and butanol-acetic acid-water solvents. In phenol-water solvent, it travelled a distance of 3.8 cm. from the origin when phlorin travelled 9 cm. In the butanol-acetic acid-water solvent, its distance from the origin was 8 cm. compared to 10.6 cm. for phlorin and 24.6 cm. for phloroglucinol. With p-nitroaniline spray, it gave the same yellow-orange colour as phloroglucinol or phlorin. It was present in extremely small amounts. It is referred to as Unknown # 1 in the later sections of the thesis.

Figure 1. Radioautograph of ethanol-soluble fraction of Pelargonium leaf discs fed glucose-U-C^{14}.

Compounds corresponding to the numbers on the figure are:

1. Glucose
2. Fructose
3. Sucrose

Figure 2. Radioautograph of ethanol-soluble fraction of Pelargonium leaf discs fed glucose-U-C^{14} and phloroglucinol.

Compounds corresponding to the numbers on the figure are:

1. Glucose
2. Fructose
3. Sucrose
4. Phlorin

Figure 3. Radioautograph of ethanol-soluble fraction of Pelargonium leaf discs fed glucose and phloroglucinol-C^{14}.

Compounds corresponding to the numbers on the figure are:

1. Phloroglucinol
2. Phlorin
3. Unknown # 1

2. Variability in Uptake of Glucose-C^{14} and Synthesis of Phlorin by Leaf Discs given Identical Treatment

Four sets of leaf discs were fed with phloroglucinol and glucose-C^{14} in exactly the same manner. In each treatment five leaf discs of Pelargonium were incubated in a solution containing 2 ml. of 10 mM phloroglucinol and 0.2 ml. of 0.015% * glucose-U-C^{14} (2 µc.) for about 16 hours. The discs were then extracted in 80% ethanol and treated in the usual way. The distribution of radioactivity on chromatograms prepared from these extracts is shown in Table 1.

TABLE 1

Distribution of Radioactivity in 80 per cent Ethanol-soluble Fraction of Leaf Discs fed with Phloroglucinol and Glucose-U-C^{14} under Identical Conditions

Set no.	Total activity in ethanolic extract Counts/min.	Per cent radioactivity in 80 per cent ethanol-soluble fraction			
		Phlorin	Glucose	Fructose	Sucrose
1	51500	20	21	21	33
2	53100	27	14	13	30
3	48100	17	22	23	30
4	48300	21	18	17	32

* Glucose-U-C^{14} refers to generally labelled glucose

3. Glycoside Formation Using Various Phenols

Various phenols including catechol, 2,4-dinitrophenol, gallic acid, hydroquinone, p-hydroxybenzaldehyde, orcinol, phenol, phloretin, phloroglucinol, pyrogallol, resorcinol, saligenin, vanillin were fed separately with radioactive or inactive glucose to leaf discs of Gerbera and Pelargonium. In each treatment except in the feeding of catechol, gallic acid, phloretin, and pyrogallol, five leaf discs of Gerbera were fed with 0.5 ml. of a 10 mM solution of a phenol and 0.01 ml. of a 0.015% glucose-1-C^{14} (0.1 μc.). Catechol and gallic acid were fed to leaf discs of Pelargonium in 10 mM solutions of these phenols without using radioactive glucose. In the case of phloretin and pyrogallol, five leaf discs of Pelargonium were given 2 ml. of a 10 mM solution of the phenol and 0.2 ml. of 0.015% glucose-U-C^{14} (2 μc.).

With the exception of 2,4-dinitrophenol and phenol, in which treatments the discs looked badly burnt, each of the other phenols gave rise to compounds which appeared to be the corresponding glucosides. The new compounds had a lower R_f value than their respective phenols in butanol-acetic acid-water and they reacted with diazotized sulphanilic acid and diazotized p-nitroaniline sprays. (See Table 2.) They appeared as intensely radioactive spots on the radioautographs whenever glucose-C^{14} was fed. (See Table 3.)

When phloretin was fed to leaf discs, in addition to naturally occuring compounds, which were present in the control sample, two additional radioactive spots appeared. One of them was identified as phloridzin and the other as phlorin. Phlorin accounted for 2% of the radioactivity in the ethanolic extract whereas phloridzin contained 8%.

TABLE 2

R_f Values and Colours with Diazotized p-Nitroaniline and Diazotized Sulphanilic Acid of Various Phenols and Compounds believed to be their Glucosides

Compounds	R_f	Colour with diazotized p-nitroaniline		Colour with diazotized sulphanilic acid
		Before alkali spray	After alkali spray	
Monohydroxy phenols and their glucosides				
Phenol	-	-	-	-
Phenol glucoside	-	-	-	-
Saligenin	0.82	yellow	magenta	orange-yellow
Saligenin glucoside	0.42	✱	✱	yellow
p-Hydroxybenzaldehyde	-	-	-	-
p-Hydroxybenzaldehyde glucoside	0.36	dark yellow	purple	salmon
Vanillin	-	-	-	-
Vanillin glucoside	0.33	brown-yellow	purple	pink-buff
2,4-Dinitrophenol	0.85	yellow	yellow	yellow
2,4-Dinitrophenol glucoside	-	-	-	-

Table 2 - continued

Compounds	R_f	Colour with diazotized p-nitroaniline		Colour with diazotized sulphanilic acid
		Before alkali spray	After alkali spray	
Dihydroxy phenols and their glucosides				
Catechol	0.80	red-brown	blue	purple
Catechol glucoside	0.55	red-brown	purple	red-brown
Resorcinol	0.80	yellow	violet	yellow
Resorcinol glucoside	0.37	yellow	purple	brown
Hydroquinone	0.80	white	fawn	brown-purple
Arbutin	0.37	-	violet	red-brown
Orcinol	0.84	yellow	orange-yellow	yellow
Orcinol glucoside	0.42	yellow	brown-red	orange
Trihydroxy phenols and their glucosides				
Phloroglucinol	0.64	yellow-orange	brown-orange	brown-yellow
Phlorin	0.20	yellow-orange	brown-orange	brown-yellow
Pyrogallol	0.66	red-brown	blue-gray	purple-brown
Pyrogallol glucoside	0.38	yellow-brown	blue	violet
Gallic acid	0.53	yellow-brown	blue-brown	khaki
Gallic acid glucoside	0.26	mustard yellow	fawn	purple-red
Phloretin	0.87	yellow-orange	orange-brown	red-brown
Phloridzin	0.59	yellow-orange	orange-brown	red-brown

* means not sprayed

\- means no detectable spot on chromatogram

TABLE 3

Distribution of Radioactivity in 80 per cent Ethanol-soluble Fraction of Leaf Discs fed with Labelled Glucose together with a Phenol

Phenol administered	Per cent radioactivity in 80 per cent ethanol-soluble fraction		
	Glucoside	Glucose	Sucrose
Monohydroxy phenols			
Phenol	0	100	0
Saligenin	91	1	5
p-Hydroxybenzaldehyde	39	5	12
Vanillin	59	3	6
2,4-Dinitrophenol	0	100	0
Dihydroxy phenols			
Resorcinol	78	2	5
Hydroquinone	45	21	17
Orcinol	89	1	4
Trihydroxy phenols			
Phloroglucinol	72	2	9
Pyrogallol	44	11	11
Phloretin	8	3	35

4. Competition between Phenols

Experiments were carried out in which two different phenols were fed simultaneously. In one set of samples, phloroglucinol and its isomer pyrogallol were fed for 19 hours in the following way: Sample 1 consisted of five leaf discs incubated in a solution of 2 ml. of 10 mM phloroglucinol and 0.2 ml. of 0.015% glucose-U-C^{14} solution (2 μc), Sample 2 had five leaf discs in 1 ml. of 10 mM phloroglucinol, 1 ml. of 10 mM pyrogallol and glucose-C^{14} (2 μc) and lastly, in Sample 3 five leaf discs were placed in a solution of 2 ml. of 10 mM pyrogallol and glucose-C^{14} (2 μc).

A second series of experiments dealt with the competition between phloroglucinol and resorcinol. It was carried out in the same way as described above, the only difference was that resorcinol was used instead of pyrogallol. The feeding time was 16 hours.

The results obtained from these experiments are indicated in Table 4.

TABLE 4

Relative Distribution of Radioactivity in 80 per cent Ethanol-soluble Fraction of Leaf Discs fed with Glucose-C^{14} and (1) one Phenol or (2) two Phenols

Treatment	Compound administered together with glucose-C^{14}	Total activity in ethanolic extract counts/min.	Distribution of radioactivity of 80 per cent ethanol-soluble fraction in counts/min.			
			Glucoside	Glucose	Fructose	Sucrose
1	Phloroglucinol	42,700	Phlorin: 13,240	5,550	4,700	6,900
2	Phloroglucinol + Pyrogallol	16,200	Phlorin: 1,940 Pyrogallol glucoside: 2,430	2,900	1,600	2,900
3	Pyrogallol	10,100	Pyrogallol glucoside: 4,500	1,100	400	1,100
4	Phloroglucinol	28,300	Phlorin: 8,800	2,550	2,000	5,100
5	Phloroglucinol + Resorcinol	28,800	Phlorin: 2,900 Resorcinol glucoside: 9,500	2,000	2,600	4,600
6	Resorcinol	30,000	Resorcinol glucoside: 12,600	2,700	3,000	5,400

5. Fate of Phloroglucinol fed to Entire Plants

Rooted cuttings of Pelargonium were allowed to take up phloroglucinol by placing them in jars containing a 10 mM solution of phloroglucinol. They were left under fluorescent lights for approximately 24 hours after which roots, stems, petioles and the younger leaves of these plants looked quite healthy (normal) but the laminae of the mature leaves looked grayish and flaccid although their veins appeared normal. The phloroglucinol compounds detected by Erhlich reagent on the chromatograms of the extracts of the various parts of this plant are shown in Table 5.

TABLE 5

Detection of Phloroglucinol Compounds in 80 per cent Ethanol-soluble Fraction of the Various Parts of Pelargonium Plants administered with Phloroglucinol

Parts of the plant	Compounds detected by Erhlich reagent on chromatograms		
	Phlorin	Phloro-glucinol	Other compounds
Roots	xx	x	-
Stems	xxxx	x	-
Petioles (of all leaves)	xxxx	-	-
Mature leaves	xxx	x	-
Young leaves	xxxx	-	Unknown # 1

x indicates presence
- indicates absence

6. Synthesis of Phlorin in Various Plants

An experiment was carried out to examine the ability of various plants in conjugating phloroglucinol with glucose.

Leaf tissues from various plants were fed with phloroglucinol (0.2 ml. of 0.2% solution) and glucose-1-C^{14} (0.1 ml. of a 0.01% solution) (0.1 μc.) for 22 hours. The leaf materials were extracted and treated in the usual way. The results of this experiment are shown in Table 6.

TABLE 6

Distribution of Carbon-14 in 80 per cent Ethanol-soluble Fraction of Leaf Discs or Segments fed Labelled Glucose and Phloroglucinol

Leaf discs or segments	Per cent radioactivity of 80 per cent ethanol-soluble fraction			
	Phlorin	Glucose	Fructose	Sucrose
Adiantum macrophyllum	11	1	1	82
Pinus resinosa	62	1	0	37
Briza media	27	1	0	64
Kalanchoë punctata	3	46	42	9
Pelargonium hortorum	76	3	0	1
Gerbera jamesonii	82	1	0	5
Malus sylvestris	29	1	0	5

7. Feeding of Phloroglucinol to Various Organs and Tissues of a Variety of Plants

Various organs and tissues of plants were tested for their ability to conjugate phloroglucinol with glucose. Tissues from a variety of plants were incubated in a solution containing 10 mM phloroglucinol and 10 mM glucose. The following plant tissues were examined:

(1) Roots of Pelargonium and Salix cut up into 6 to 7 cm. pieces from the tip of the roots.

(2) Potato cylinders (1 cm. in diameter) obtained from a potato by a cork-borer. These cylinders were cut up into thin slices which were washed in running water for approximately two hours.

*(3) Petals from the flowers of Pelargonium hortorum (var. Carmin Pink).

(4) Cylinders (1 cm. in diameter) obtained from the pulp of an apple with a cork-borer. They were cut up into thin slices and washed in running water for one hour.

(5) Twenty-four-hour-old wheat embryos.

(6) A carrot was divided up into epidermis and cortex, phloem and xylem. Thin, 2 to 3 cm. long sections of the cortex and small cubes (3 to 4 mm)^3 of the xylem and phloem were used.

(7) Six leaf discs from Gerbera jamesonii were fed with phloroglucinol and glucose for about twenty hours. After rinsing the discs in tap water and blotting them, they were cut up in such a way as to divide them into rims (edges) and centres of the discs. The ethanolic extracts of these two parts were then examined.

---

* Contrary to the suggestion of Bate-Smith (1950), naturally occurring phloroglucinol and phlorin were not detected in the petals of 16 varieties of Pelargonium examined.

These plant materials were treated in the usual way. The results obtained from the experiment are listed in Table 7.

TABLE 7

Detection of Phloroglucinol Compounds in 80 per cent Ethanol-soluble Fraction of Various Tissues fed with Phloroglucinol

Plant material	Compounds detected on chromatograms		Remarks
	Phlorin	Phloroglucinol	
Pelargonium roots	x	-	-
Salix roots	x	x(trace)	
Potato tuber	x	-	(1)1 mM phloroglucinol was used and no external source of sugar was present. (2)Amount of phlorin formed appeared to increase progressively from 1 to 5 days of incubation. (3)Microscopic examination of the incubated discs showed the cell walls and some of the smaller starch grains to be stained brick red.
Pelargonium petals	x	xxxx	-
Apple pulp	-	x	-
Wheat embryos (24 hr. old)	x	-	The amount of phlorin formed appeared to increase with increase in concentration of phloroglucinol. 1,5 and 10 mM solutions were used

Table 7 - continued

Plant material	Compounds detected on chromatograms		Remarks
	Phlorin	Phloroglucinol	
Carrot tissues			
Epidermis and cortex	x	-	-
Phloem	x	x(trace)	
Xylem	x	-	
Leaf disc			
Rim	x	x	
Centre	x	x	-

x indicates presence
- indicates absence

8. Feeding of Phloroglucinol to Marchantia and Various Algae and Fungi

Marchantia

Small portions of thalli were fed with 0.2% solution of phloroglucinol and 0.1 ml. of 0.01% glucose-1-C^{14}. The plant material was then treated in the usual manner. A very small amount of phlorin-C^{14} was detected in the ethanolic extract. It contained only 5% of the total activity in the 80% ethanol-soluble fraction.

Algae

A number of marine algae were examined to see if they could synthesize phlorin from administered phloroglucinol. Species of

Ulva, Fucus, Laminaria, Agardhiella, and Polysiphonia were incubated in solutions containing 10 mM phloroglucinol and glucose-U-C^{14} (2 μc.) for approximately 20 hours and then treated in the usual manner. The ethanolic extracts of none of these algae contained phlorin although free phloroglucinol was detected in each sample, except in the case of Ulva. In the extract of Fucus, there were two unidentified compounds which produced rose-pink colour with Erhlich reagent. One of these compounds was radioactive.

Fungi

Phloroglucinol-C^{14} was fed to four-day-old shake cultures of Aspergillus giganteum. The culture solution in which it was growing was decanted off and 5 ml. of a 0.12% solution of phloroglucinol-C^{14} containing 0.24 μc. (18,800 counts per minute) were added to 50 ml. of fresh sterile nutrient medium. No activity was found either in the ethanolic extract of the mycelium, or on the chromatogram prepared from the extract. Acetone and methanol extracts were made of the dried up mycelial residue but there was no detectable radioactivity in either of these two extracts. The medium was taken to dryness and an aliquot examined for radioactivity. The activity was too low to permit subsequent examination of the material worthwhile.

9. Effect of Temperature on the Synthesis of Phlorin

Three sets of five-leaf discs of Pelargonium were fed with 2 ml. of 10 mM phloroglucinol and 0.2 ml. of 0.015% glucose-U-C^{14} (2 μc.) for approximately 20 hours in the dark at the following temperatures:

Sample A : 37°C

Sample B : 21 to 22°C

Sample C : 5 to 6°C

After incubation, all the discs in Sample A looked brown and flaccid, in Sample B, two of the discs had small brown patches, the rest being normal and in Sample C, all of them were green and turgid and fresh. The results obtained from these treatments are shown in Table 8.

TABLE 8

Distribution of Radioactivity in 80 per cent Ethanol-soluble Fraction of Leaf Discs incubated in a Solution of Phloroglucinol and Glucose-U-C^{14} at Different Temperatures

Temperature of incubation (°C)	Total activity in ethanolic extract (counts/min.)	Per cent radioactivity of 80 per cent ethanol-soluble fraction			
		Phlorin	Glucose	Fructose	Sucrose
37	107	0	100	0	0
21-22	4707	34	4	14	11
5-6	906	11	5	2	77

10. Relative Synthesis of Phlorin in Light and in Dark

Two sets of four discs each were obtained from the leaves of Gerbera jamesonii. They were vacuum infiltrated and placed in

solutions containing 0.5 ml. of 0.2% phloroglucinol and 0.2 ml. of 0.015% glucose-U-C^{14} (2 μc.). One of the dishes (Sample A) was placed under fluorescent lights and the other (Sample B) in a dark room for approximately 24 hours. After incubation, three of the four discs in the Sample B were found to have dark brown patches whereas the ones in the light were healthy and green. The results are shown in Table 9.

TABLE 9

Relative Distribution of Radioactivity in 80 per cent Ethanol-soluble Fraction of Leaf Discs incubated in a Solution of Phloroglucinol and Glucose-U-C^{14} in Light and in Dark

Condition of incubation	Total activity in ethanolic extract (counts/min.)	Per cent radioactivity of 80 per cent ethanol-soluble fraction			
		Phlorin	Glucose	Fructose	Sucrose
Light	26,700	48	3	2	24
Dark	21,800	55	1	1	14

11. Changes with Time in the Distribution of C^{14} in the Extracts of Leaf Discs Infiltrated with Glucose-C^{14} and Phloroglucinol

Five sets of five-leaf discs each were obtained from Pelargonium and placed in five small suction flasks, each flask containing 2 ml. of 10 mM phloroglucinol and 0.2 ml. of 0.015% glucose-U-C^{14} (2 μc.).

The flasks were connected to a manifold and a current of air was drawn through them. The first four of the samples, which were incubated in light, were taken off and killed in boiling 80% ethanol after 1, 2, 4 and 6 hours from the start of the experiment. The fifth sample was then transferred from light to dark and killed after a further 18 hour period of incubation.

The pattern on the radioautographs was exactly the same in all five samples. The comparative values of radioactivity are illustrated in Figure 4.

12. Effect of Iodoacetate on the Synthesis of Phlorin

An experiment was designed to see if iodoacetate (which is known to inhibit sucrose synthesis) has any effect on the synthesis of phenolic glucosides. Three sets of five-leaf discs of Pelargonium were fed in the following way: Set 1 (control) was given 1 ml. of water and 0.3 ml. of 0.1% phloroglucinol-C^{14} (0.3 µc. approximately), Set 2 had 1 ml. of 1 mM sodium iodoacetate plus 0.3 ml. of 0.1% phloroglucinol-C^{14} and Set 3 was given 0.3 ml. of phloroglucinol-C^{14} after incubating the discs in 1 ml. of 1 mM sodium iodoacetate for 30 minutes. In the case of Set 1, 98% of the radioactivity in the 80% ethanol-soluble fraction of the leaf discs appeared in phlorin, compared to 60% in the last two cases (Sets 2 and 3). In each case, the remaining activity was in free phloroglucinol.

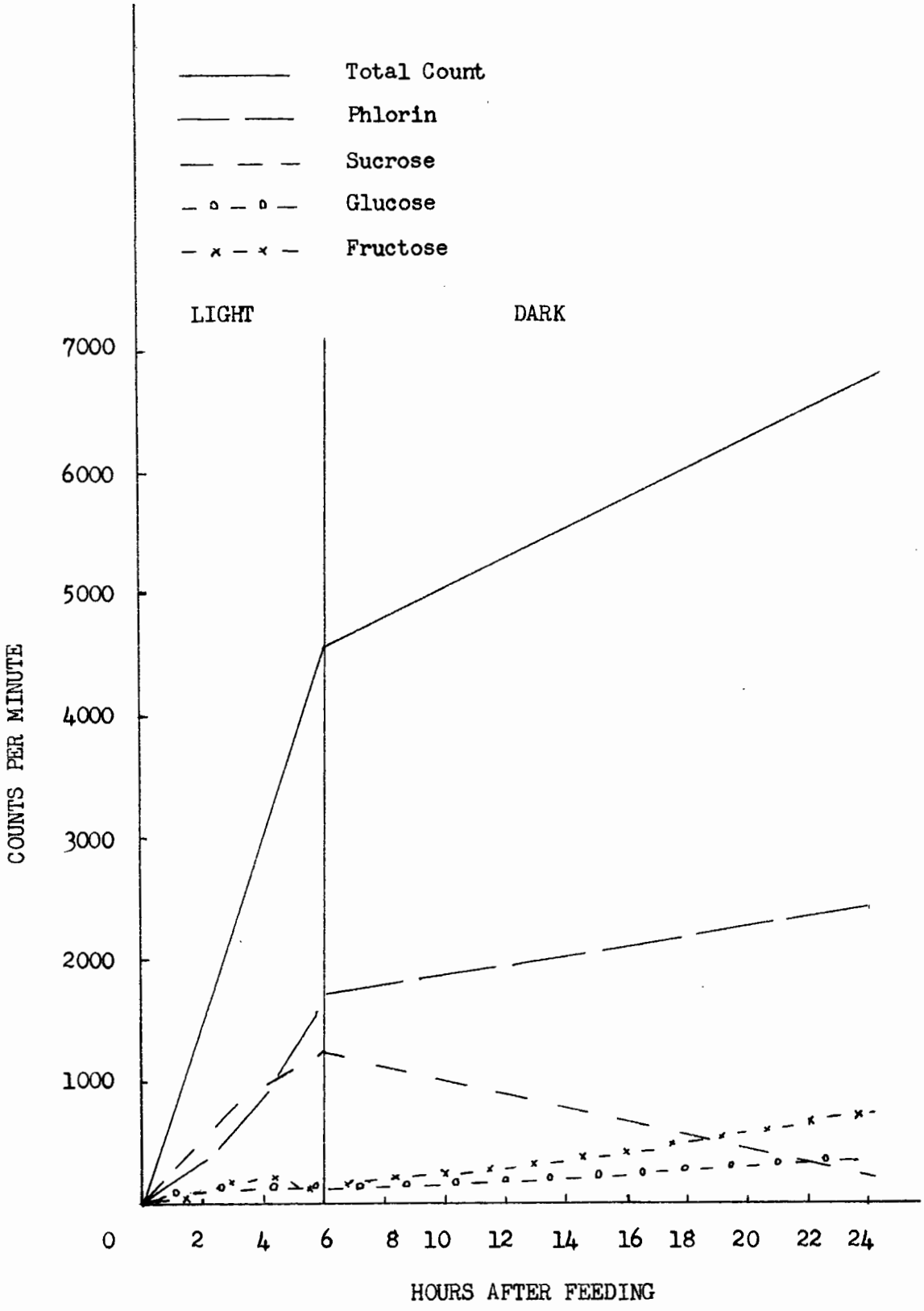


Figure 4. Changes with time in the distribution of C^{14} in the ethanol-soluble fraction of Pelargonium leaf discs fed glucose-C^{14} and phloroglucinol.

13. Feeding of Phloroglucinol without any External Sugar

Phloroglucinol alone was fed to two sets of leaf discs, one taken from a plant grown under normal conditions in the greenhouse and the other from a plant starved for 48 hours by transferring it from the greenhouse to a dark room. The discs were then treated in the usual way. On examining the ethanolic extracts of these samples, it was found that phlorin was formed in both samples and a very small amount of free phloroglucinol was detected in the sample of starved leaves.

14. Feeding of Various Sugars and Sugar Acids together with Phloroglucinol to Leaf Discs

A number of sugars, pentoses and hexoses, and sugar acids were fed together with phloroglucinol to leaf discs of Pelargonium. In each case, five leaf discs were incubated for about 18 hours in 0.5 ml. of 10 mM phloroglucinol and 0.2 ml. of 10 mM solutions of each of the following sugars: L-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, L-rhamnose, D-ribose, and D-xylose and the sugar acids D-galacturonic acid and D-glucuronic acid. The leaf discs were then treated in the usual manner. When the ethanolic extracts were chromatographed, only phlorin and no other glycoside of phloroglucinol was detected.

This experiment was repeated using leaf discs from a plant which had been starved by placing it in a dark room for 48 hours. After incubation, some of the discs in galactose, glucose, rhamnose and ribose turned completely brown or had scattered brown spots. Compared to the

results from the above experiment with normal leaves, the phlorin spots appeared to be less intensely coloured with p-nitroaniline and were smaller in size in the extracts from the discs of the "starved" leaves. Another difference between the two sets was that in extracts of starved leaf discs, free phloroglucinol was present in every case, except in the case of mannose. In other respects, the two sets were similar.

15. Synthesis of Phlorin from Phloroglucinol and Sucrose-C^{14} in Leaf Discs

An experiment using phloroglucinol (0.5 ml. of a 10 mM solution) and generally labelled sucrose-C^{14} (0.2 ml. of a 0.012% solution) (2 μc.) in the incubation of five leaf discs of Pelargonium showed that sucrose served very well as a source of glucose in the formation of the glucoside, phlorin.

No phloroglucinol compound, other than phlorin, was detected on the chromatogram with Erhlich reagent. On counting the radioactivity content of the various spots on the chromatograms it was shown that phlorin contained 42% of the total count on the paper. Sucrose had 32%, glucose contained 5% and fructose accounted for 8%, and several other compounds which appeared on the radioautograph were only weakly radioactive.

16. Relative Formation of Phlorin-C^{14} from Phloroglucinol and Glucose-C^{14} and Glucose-1-phosphate-C^{14}

A comparison was made between glucose-C^{14} and glucose-1-phosphate-C^{14} as a source of sugar in the glucosidation of phloroglucinol. One set of

five leaf discs of Pelargonium was fed with 2 ml. of 10 mM phloroglucinol plus 2.5×10^{-5} M glucose-U-C^{14} (0.4 µc.) and the second set with the same amount of phloroglucinol plus 2.5×10^{-5} M generally labelled glucose-1-phosphate-C^{14} (0.4 µc.). Extraction of these leaf discs showed that phlorin had been formed in both cases and that a larger number of compounds appeared to be radioactive with glucose-C^{14} than with glucose-1-phosphate-C^{14}. The quantitative analysis of the results are shown in Table 10.

TABLE 10

Distribution of Radioactivity in 80 per cent Ethanol-soluble Fraction of Leaf Discs fed with Phloroglucinol and Glucose-U-C^{14} or Glucose-1-phosphate-C^{14} (generally labelled)

Compounds fed with phloroglucinol	Total activity in 80 per cent ethanolic extract (counts/min.)	Per cent radioactivity in 80 per cent ethanol-soluble fraction			
		Phlorin	Glucose	Fructose	Sucrose
Glucose-U-C^{14}	1750	31	20	20	19
^{x}G-1-P-C^{14}	523	58	4	4	16

^{x}G-1-P-C^{14} = glucose-1-phosphate-C^{14} (generally labelled)

17. Feeding of Phloroglucinol together with Pyruvate-C^{14} or Succinate-C^{14}

Leaf discs of Pelargonium were fed with compounds involved in

respiration to see if they had any effect on the synthesis of phlorin. Four sets of five leaf discs were placed in the following solutions:

Sample A : 0.5 ml. of 10 mM phloroglucinol + 0.2 ml. of 0.088% pyruvate-1-C^{14} (2 μc.)

Sample B : 0.5 ml. (tap) water + " " "

Sample C : 0.5 ml. of 10 mM phloroglucinol + 0.2 ml. of 0.036% succinate-C^{14} (2 μc.)

Sample D : 0.5 ml. of (tap) water + " " "

The discs were then left under the fluorescent lights for about an hour. The radioactivity contents of the ethanolic extracts were approximately the same in Samples A and B and in Samples C and D. The radioautographs obtained from this experiment showed that the pattern of C^{14} distribution was qualitatively identical in Samples A and B, except that Sample A had an additional spot for phlorin-C^{14}. The same was found to hold true for Samples C and D. The results are expressed quantitatively in Table 11.

TABLE 11

Distribution of Radioactivity in 80 per cent Ethanol-soluble Fraction of Leaf Discs fed with Pyruvate-1-C^{14} and Succinate-1,4-C^{14} with or without Phloroglucinol

Compounds fed	Total activity in 80 per cent ethanolic extract (counts/min.)	Per cent radioactivity of 80 per cent ethanol-soluble fraction		
		Phlorin	Sucrose	Other Compounds
Pyruvate-1-C^{14}	28,400	-	-	100
Phloroglucinol + pyruvate-1-C^{14}	28,500	1.3	-	98.7
Succinate-1,4-C^{14}	37,400	-	55	45
Phloroglucinol + succinate-1,4-C^{14}	34,000	58	15	27

B. THE SYNTHESIS OF PHLORIN IN VITRO

Several attempts were made to synthesize phlorin with homogenates of plant tissues. The plant tissue from which the homogenate was made, the method of preparation of homogenate, the source of glucose, and the conditions of experiments were changed in various experiments.

The following methods were used in the preparation of homogenates:

(1) Leaves of Gerbera jamesonii were ground in phosphate buffer (pH 7.3) with a mortar and pestle.

(2) Homogenate was obtained by grinding leaves of Pelargonium in acetate buffer (pH 5.96) in the same way as above.

(3) Pelargonium leaves were ground in phosphate buffer (pH 6.6) similarly.

(4) Leaves of Pelargonium were frozen by dipping them in liquid air and then they were quickly ground in a chilled mortar. The powder thus obtained was used as a source of enzyme.

(5) Twenty-four-hour old wheat embryos were frozen and ground up as in (4).

In those experiments where phloroglucinol together with glucose-C^{14} or glucose-1-phosphate-C^{14}, was used as substrates, no synthesis of phlorin occurred. When the source of glucose was sucrose-C^{14} or glucose-1-phosphate-C^{14} plus uridine triphosphate, there was a slight synthesis of phlorin-C^{14}. All the incubations with phloroglucinol-C^{14} gave rise to phlorin-C^{14} to a greater or lesser extent.

The results of some of the more interesting or successful enzyme syntheses are summarized in Tables 12 and 13.

TABLE 12

Formation of Phlorin-C^{14} in the Incubation of Phloroglucinol and Labelled Glucose-derivatives with Homogenate (4) for 20 minutes in Ice-cold Water or Glycine Buffer (pH 7.8)

Substrate				Per cent radioactivity in 80 per cent ethanol-soluble fraction	
Phloroglucinol	Glucose derivative	Cofactor	Buffer or water	Phlorin	Other Compounds
15.8 µM	0.33 µM G-U-C^{14}	4 µM ATP	1 ml. water	0	100 (Glucose Unknown)
15.8 µM	0.33 µM G-U-C^{14}	4 µM ATP	1 ml. buffer	0	100 (Glucose Sucrose Unknown)
15.8 µM	0.33 µM G-U-C^{14}	4 µM ATP $MgSO_4$ (trace)	1 ml. buffer	0	100 (Glucose Sucrose Unknown)
5 µM	0.07 µM sucrose-C^{14}	2 µM ATP $MgSO_4$ (trace)	1 ml. water	trace	100 (approx) (Glucose Fructose Sucrose)
15.8 µM	7.7 µM G-1-P-C^{14} + 4.2 µM UTP	-	2 ml. water	1.6	98.4 (Phosphate compound)

G-U-C^{14} = glucose-U-C^{14}; G-1-P-C^{14} = glucose-1-phosphate-C^{14} (generally labelled)
UTP = uridine triphosphate; ATP = adenosine triphosphate

TABLE 13

Formation of Phlorin-C^{14} in the Incubation of Phloroglucinol-C^{14} and Uridine diphosphate glucose (UDPG) with Homogenates for 20 minutes in Ice-cold Water or Phosphate Buffer (pH 6.6) (a) or Glycine Buffer (pH 7.8) (b)

Substrate					Per cent radioactivity in 80 per cent ethanol-soluble fraction		
Homogenate	Phloroglucinol (μM)	UDPG (μM)	Cofactors	Buffer or water	Phlorin	Phloroglucinol	Others
(4)	15.8	3.5	-	2 ml. water	trace	100	0
(5)	15.8	3.5	ATP (2 μM) $MgSO_4$ (trace)	2 ml. water	3.0	88	9 (Phosphate compound)
* (3)	2.5	0.7	-	1.5 ml. buffer (a)	3.6	96	0.4 (Unknown)
(4)	15.8	3.5	-	1 ml. buffer (b)	14.0	82	4 (Unknown)

* The sample marked with an asterisk was incubated for 90 minutes at room temperature

DISCUSSION

The ready synthesis of phenolic glycosides in plant tissues fed with dilute solutions of phenols has been established by the work of Ciamician and Ravenna (1916), Miller (1938 to 1957), Miwa et al (1957), Nystrom et al (1959), Pridham (1958) and the work reported in this thesis. Paper chromatography and the use of C^{14}-labelled compounds have made the problem of studying the fate of administered compounds to plants comparatively easy. The use of leaf discs makes it possible to work with small amounts of reasonably uniform tissue.

It is seen from Table 1 that there was a difference of less than 6% in the incorporation of C^{14}-labelled glucose in the ethanolic extracts of the different sets of five discs. The radioactivity in sucrose appeared to be constant in all cases. The ratio of activity (approximately 1:1) between the hexoses, glucose and fructose, was also constant. There was, however, a much wider variation in the distribution of radioactivity among phlorin and the hexoses. It is unlikely that it was due to the method of determining radioactivity on chromatograms, since there were very slight variations in the size of spots between the different chromatograms. It is more likely that the variation in percentage difference was due to inherent differences in leaf discs. Five leaf discs per treatment, therefore, did not provide sufficient material for quantitative comparisons unless there were obvious and large differences.

Of the phenols which were administered to the plant tissues, catechol, gallic acid, hydroquinone, orcinol, phloretin, phloroglucinol, pyrogallol

and resorcinol were converted to their corresponding β -glucosides. Pridham (1958) also reported that when young bean shoots were fed with glucose and such phenols as quinol, phloroglucinol, resorcinol, catechol and pyrogallol, the β -glucoside of these phenols were formed.

Saligenin, p-hydroxybenzaldehyde and vanillin produced glucosides which reacted with diazotized p-nitroaniline and diazotized sulphanilic acid indicating that the phenolic hydroxyl group of these phenols was free and not involved in the glycosidic linkage. Miller (1941a) has shown that the aldehyde, chloral hydrate was reduced to the corresponding alcohol which formed β -2,2,2-trichloroethyl-glycoside when fed to plants. It is possible that p-hydroxybenzaldehyde and vanillin are similarly reduced to the corresponding alcohols when fed to leaf discs of Pelargonium and that the alcohol hydroxyl group is preferentially conjugated. This is illustrated in the case of saligenin, where it has been shown that the hydroxyl group of the alcohol and not the phenolic hydroxyl group was linked to glucose when it was fed to young bean shoots (Pridham, 1958). Miwa et al (1957) reported that when glucose and such phenols as vanillin and salicyl aldehyde were infiltrated into leaf discs of various plants, the β -glucosides corresponding to these phenols were formed. However, in view of the present work it seems likely that in these cases the alcoholic hydroxyl group and not the phenolic hydroxyl group was involved in the conjugation with glucose.

When phloretin and glucose-C^{14} were fed to leaf discs of Pelargonium, a radioactive spot corresponding to phloridzin in chromatographic behaviour

was detected. In addition, phlorin-C^{14} was found on the chromatogram indicating that phloretin was hydrolyzed to phloroglucinol and phloretic acid (p-hydroxyphenylpropionic acid) in the tissue and that phloroglucinol was then converted to its glucoside. On the other hand, it is possible that phloridzin which was formed from the administered phloretin, was hydrolyzed into phlorin and phloretic acid. It may be pointed out here that although there is an abundance of phloridzin in species of Malus, phlorin has never been detected in these plants (Hutchinson, 1958).

Phenol and 2,4-dinitrophenol did not form any detectable glycoside. Pridham (1958) also failed to obtain glycoside with phenol. This failure in the formation of a glycoside from phenol or 2,4-dinitrophenol may be explained by the fact that the leaf tissue was killed when subjected to the feeding of these phenols. In both cases, the leaf discs looked badly burnt, and in each case only a weak spot of glucose-C^{14} appeared on the radioautograph. Nystrom et al (1959) have recently reported the formation of a β-phenylglucoside in wheat and barley leaves fed phenol either with glucose-C^{14} or with sedoheptulose-C^{14}. They found that when a solution of 60 μg of phenol in 100 μl of glucose-C^{14} was fed to leaves, 3 to 7% of the total radioactivity fed to the plant appeared in phenylglucoside. They also found that 300 μg of phenol inhibited the metabolism of sugars in the leaf tissue and hence, phenylglucoside synthesis did not occur.

Table 3 shows that there were considerable differences in the amounts of glycoside formed with different phenols. This may be related to the permeability of cell membranes to these phenols or to relative specificity of the enzyme.

An examination of Table 4 shows a few interesting points. The total radioactivity in the ethanol-soluble fraction was four times less when pyrogallol and glucose-C^{14} were fed as compared with phloroglucinol and glucose-C^{14} feeding (Treatments 1 and 3). This could be caused by either an inhibition by pyrogallol of glucose-C^{14} uptake or by a stimulation of respiration at the expense of glucose-C^{14}. When phloroglucinol and pyrogallol were fed separately with glucose-C^{14} (Treatments 1 and 3), there was about three times as much phlorin-C^{14} as pyrogallol glucoside-C^{14} formed. However, when both phenols were administered together with glucose-C^{14} (Treatment 2) phlorin-C^{14} and pyrogallol glucoside-C^{14} were formed equally readily, indicating that phlorin-C^{14} synthesis was inhibited or reduced in this case. It may also be seen in Table 4 that there was a decrease in the incorporation of C^{14} into sucrose from pyrogallol and glucose-C^{14} feeding as compared with phloroglucinol and glucose-C^{14} feeding.

There was no significant difference in total activities of the ethanol-soluble fractions or in activities of the glucosides formed when glucose-C^{14} was administered either with phloroglucinol or with resorcinol (Treatments 4 and 5 in Table 4). When the two phenols and glucose-C^{14} were fed all together, the total radioactivity in the two glucosides, phlorin plus resorcinol glucoside, was the same as in the above treatments. However, the ratio of activities in phlorin to resorcinol glucoside was 1:3. It is possible that resorcinol affected (reduced) the entry of phloroglucinol into leaf discs. It could also be due to competition between these two substrates for the same enzyme system.

When phloroglucinol was fed to an entire plant, phlorin was

identified in the extracts of the various parts of the plant (Table 5). The absence of free phloroglucinol in the petioles of all the leaves and the presence of phlorin in young leaves may be explained by assuming that phlorin is the compound which was translocated and not phloroglucinol. The appearance of free phloroglucinol in stems and mature leaves could be due to hydrolysis of phlorin. Ciamician and Ravenna (1916) found that when salicin was administered to a maize plant, it was partly hydrolyzed. However, according to Miller (1940), once the glycoside has been induced to form by the introduction of chemicals, there is no apparent movement of the compound within the plant. When potato tubers and gladiolus corms containing β -2-chloroethyl-D-glucoside were planted the resulting sprouts did not contain any of the glucoside. Similarly, when gladiolus corms containing o-chlorophenyl gentiobioside were grown until new corms were produced, the glycoside could not be detected either in the resulting shoot or in the daughter corms although the old corm still contained appreciable quantities of the glycoside. The seed produced by tobacco plants which accumulated large amounts of trichloroethyl-β-glycosides in the leaves, stems and roots did not contain these glycosides (Miller, 1943).

It is seen from Table 6 that leaf tissue from all the plants tested could form the β -glucoside, phlorin, from administered phloroglucinol. A comparison could not be made between plants as to the extent to which phloroglucinol was combined with glucose-C^{14} since the same amount of leaf tissue was not used in all cases. However, a study of the distribution of C^{14} in the various compounds of the ethanol-soluble

fraction indicates that the ratio of phlorin-C^{14} to sucrose-C^{14} varied in different plants. (In each case, phlorin-C^{14} and sucrose-C^{14} appear to be inversely proportional.) In Adiantum, it was found that not all of the phenol that entered the tissue was conjugated with glucose.

Leaf discs of Sambucus sieboldiana, Mallotus japonica, Plantanus orientalis and Iris japonica are also able to synthesize β -glucosides when infiltrated with phenols and glucose (Miwa et al, 1957). Synthesis of phenolic glucosides from administered phenols has been observed also in beans (Pridham, 1958), wheat and barley (Nystrom et al, 1959).

Neither algae nor fungi were able to synthesize phlorin from administered phloroglucinol. The absence of phlorin-C^{14} or phloroglucinol-C^{14} in extracts of the fungi fed with phloroglucinol-C^{14} and also the disappearance of phloroglucinol-C^{14} from the fungal medium may be explained by the fact that this compound was metabolized to carbon dioxide. Many micro-organisms are known to be able to oxidize phenols (Evans, 1958).

Table 8 shows that of the three temperatures tried, room temperature (21° - $22^{\circ}C$) resulted in greatest incorporation of radioactivity in the ethanol-soluble fraction. Phlorin synthesis did not occur at $37^{\circ}C$ and it was very much reduced at 5° to $6^{\circ}C$.

Phlorin was found to be formed equally well in light as in the dark (see Table 9). The difference in the amount of sucrose-C^{14} in the two samples is probably a reflection of the difference in the equilibrium between synthesis and hydrolysis of sucrose-C^{14} under the

two conditions. (In the dark, equilibrium is shifted more towards hydrolysis than synthesis.) Nystrom et al also found that phenyl-glucoside could be formed from administered phenol both in the presence or absence of light.

An examination of Figure 4 shows that the rates of phlorin-C^{14} synthesis and the incorporation of C^{14} into the ethanol-soluble fraction bore the same relationship in the light and in the dark - that is, phlorin accounted for 30% of the total activity. Sucrose synthesis paralleled phlorin synthesis in the light. In the dark, sucrose was hydrolyzed which is indicated also in Table 9. The total activity of the sugars (glucose, fructose and sucrose) remained the same during the six to twenty-four hour period, showing that either very little of the C^{14} sugars were respired or that the rate of incorporation of C^{14} into the hexoses, glucose and fructose, was balanced by the rate at which sucrose was used in respiration or in any other process. A contrast between breakdown of sucrose in the dark and the stability of phlorin under these conditions may be seen. The accumulation of phlorin with time indicates that it did not participate in any active metabolism. These results suggest that further experiments along these lines would be very valuable in the understanding of the rate of synthesis and the fate of glycosides. Experiments under constant light or constant darkness over various intervals of time would be useful.

The experiment designed to study the effect of iodoacetate on phlorin synthesis shows that it had an inhibitory effect on the glucosidation of phloroglucinol.

The result of feeding phloroglucinol alone indicates that phlorin synthesis could occur at the expense of endogenous sugars.

Since the administration of phloroglucinol together with various sugars and sugar acids did not give rise to glycosides other than phlorin, even in leaves from plants starved for forty-eight hours, it would appear that the enzyme involved in glycoside formation with phloroglucinol is highly specific for glucose (or its derivatives). Nystrom et al (1959) reported that on feeding phenol and sedoheptulose-C^{14} to leaf tissue the β -glucoside of phenol and not its sedoheptuloside was formed. Miller found that the same alcohol may combine with different sugars in different plants (see page 8). It is possible that the gentiobiosides formed in Miller's experiments resulted from the condensation of a second molecule of glucose with the glucoside of the alcohol, rather than by a condensation of gentiobiose with the alcohol. Cardini and Yamaha (1958) found that the gentiobioside of hydroquinone was formed more efficiently with arbutin as a substrate than with hydroquinone. Thus it seems possible that other sugar molecules may join on to the glucose molecule of phlorin over a period of time.

The fact that Unknown # 1 (see page 23) became radioactive with either phloroglucinol-C^{14} or glucose-C^{14} and that it gave a colour reaction with p-nitroaniline similar to phlorin, suggests that it contains phloroglucinol and two or more glucose molecules. According to Bate-Smith and Westall (1950) the R_f of a phenolic bioside is slightly smaller than that of the corresponding monoglycoside and the difference in R_f between a mono- and a di-glycoside is about as great

as the difference between free phenol and its mono-glycoside. Since the difference in R_f values between phlorin and Unknown # 1 was very slight, this unidentified compound is probably a bioside of phloroglucinol, possibly its gentiobioside.

It is well established that phosphorylated sugars enter cells much less readily than the free sugars. Table 10 shows that the total activity in the ethanol-soluble fraction from the uptake of glucose-1-phosphate-C^{14} was less than one-third of the activity resulting from uptake of glucose-C^{14}. The activity in phlorin-C^{14}, however, was 58% of the total activity in case of glucose-1-phosphate-C^{14} feeding compared to 31% of the total activity in case of glucose-C^{14} feeding. Miwa et al (1957) found that phenolic glucosides were formed more efficiently from glucose-1-phosphate than from glucose. According to Cardini and Leloir (1957), glucose enters into glycosidic conjugation with phenols in the form of uridine diphosphate glucose (UDPG). Glucose-1-phosphate is more readily converted to UDPG than glucose. Glucose-1-phosphate is converted to UDPG in a single step where energy is released (Baldwin, 1957):

$$\text{UTP} + \text{Glucose-1-P} \rightleftharpoons \text{UDPG} + \text{pyrophosphate}$$

(UTP = uridine triphosphate, glucose-1-P = glucose-1-phosphate)
In order for glucose to be converted into UDPG, it has first to be phosphorylated by means of ATP to glucose-6-phosphate, which in turn is changed to glucose-1-phosphate to be converted to UDPG (Krebs, 1954).

Table 11 shows that both pyruvate-C^{14} and succinate-C^{14} could be

converted to a glucose-C^{14} derivative which combined with phloroglucinol to give phlorin. It is somewhat surprising that such a large percentage of the activity of succinate-C^{14} was chanelled into phlorin-C^{14} synthesis. This occured to a certain extent at the expense of sucrose synthesis. The relatively large amount of activity in sucrose from succinate-C^{14} may be explained by the fact that succinate can be converted to glucose more readily than pyruvate. Conversion of pyruvate to glucose involves several intermediary steps. It is first necessary for the pyruvate to be phosphorylated into phosphoenolpyruvate. The energy required for this step is comparatively high (Krebs, 1954):

$$\text{pyruvate}^- + \text{TPNH} + \text{DPN}^+ + \text{ATP}^- \longrightarrow \text{phosphoenolpyruvate} + \text{TPN}^+ + \text{DPNH} + \text{H}^+ + \text{ADP}^- \qquad (\Delta G = +5.9 \text{ K cal.})$$

On the other hand, succinate may be converted to phosphoenolpyruvate via oxaloacetate by means of phosphoenolpyruvic carboxylase (Bandurski and Greiner, 1953). This step does not require ATP. The phosphoenolpyruvate is then probably converted to glucose (on its derivative) by reversal of glycolytic reactions.

Lines 1, 2 and 3 in Table 12 show that on incubating a mixture of Pelargonium leaf homogenate, phloroglucinol, glucose-U-C^{14} and ATP in the presence of glycine buffer, no phlorin synthesis occured. Sucrose-C^{14}, however, was formed. Since UDPG may be a common intermediate in sucrose and in phlorin synthesis (Cardini and Leloir, 1957; Leloir and Cardini, 1953), it is not easy to see why sucrose-C^{14} was synthesized from glucose-C^{14} and phlorin-C^{14} was not. It is possible that endogenous

UDPG combined with traces of fructose-C^{14} which was present as a contamination in the glucose-C^{14} solution.

A trace of phlorin-C^{14} appeared when sucrose-C^{14} was used as a glucose donor together with phloroglucinol (Table 10, line 4). Cardini and Yamaha (1958), however, did not obtain phenolic glucosides from in vitro experiments with wheat-germ extract when they used sucrose as a substrate. It is possible that this synthesis of phlorin-C^{14} from sucrose-C^{14} was brought about by a transglycosidation reaction. Pridham (1957) obtained the gentiobioside of hydroquinone and glucose from arbutin with a β-glucosidase preparation from Gaultheria dentata.

Phlorin-C^{14} synthesis occured with glucose-1-phosphate-C^{14} and phloroglucinol in the presence of UTP (Table 12, line 5). Other experiments, not reported here, in which UTP was omitted failed to yield phlorin. Burma and Mortimer (1956) showed that sugar beet leaf homogenates incubated for 15 minutes at 20° to 25°C, converted radioactive glucose-1-phosphate to glucose and fructose and an unknown, but in the presence of UTP or UDP plus ATP, the product was UDPG.

Phlorin synthesis was demonstrated with phloroglucinol-C^{14} (Table 13). The most successful synthesis was obtained by incubating phloroglucinol-C^{14} and UDPG with Pelargonium leaf homogenate in glycine buffer (pH 7.8).

The preliminary experiments indicate that UDPG is a possible intermediate in the synthesis of phlorin. Whilst this work was in progress, Cardini and Leloir (1957) showed very clearly that UDPG is an intermediate. They found that a phenolic glucoside was produced only when glucose was in the form of UDPG in the substrate mixture. Other

glucose donors such as uridine diphosphate-acetyl glucosamine, guanosine diphosphate mannose, glucose-1-phosphate, sucrose, cellbiose or maltose were not effective in the synthesis (Cardini and Yamaha, 1958). They found this to be true also in the case of the synthesis of the gentiobioside of hydroquinone from arbutin (or hydroquinone) in the presence of the same wheat-germ enzyme preparation which brought about the synthesis of phenolic glucosides. It is interesting to note here that they synthesized sucrose from fructose and UDPG in the presence of the same wheat-germ extract (Cardini et al, 1955).

Cardini and Yamaha (1958) point out that the mechanism involved in their preparation of gentiobioside of hydroquinone is different from mechanism involved in Pridham's (1957) preparation of the same compound by the action of a β-glucosidase preparation on arbutin. Cardini and Yamaha (1958) did not find any hydrolytic or transglycosidic activity in their wheat-germ extract with respect to arbutin.

Cardini and Yamaha (1958) were unable to demonstrate whether the same or two different enzymes are involved in the glycosidation of phenolic hydroxyl groups and in the conjugation of glucosyl group to the glucose residue of arbutin.

It appears that there may be two main ways of formation of phenolic glucosides: 1) transglycosidation involving β-glucosidase and, 2) transfer of glucose from UDPG to a phenolic hydroxyl group by a transferase.

It is interesting to note that two mechanisms have been proposed for the formation of phenol glucuronides in animals (Brodie et al, 1957).

These mechanisms parallel those involved in the synthesis of phenolic glucosides in plant homogenates.

Fishman and Green (1957) have found that a highly purified cell-free enzyme preparation from liver can catalyze the transfer of the glucuronosyl radical from a variety of glucuronides to a number of alcohols. This enzyme, they say, is in many ways indistinguishable from β -glucuronidase.

The donors are relatively non specific with respect to the aglycone. They include saturated cyclic alcohols, a variety of mono- or di-phenols, certain phenol derivatives and naphthol. Nineteen different alcohols and six glycols have been shown to be able to act as acceptors. According to Fishman and Green (1957), the failure of phenols to act as acceptors may in part be due to the difficulty in attaining the desired concentration of those substances in water and to their inhibiting action on the enzyme. It may be pointed out here, as already stated in the review of literature, no successful transglycosidation reaction with plant enzymes has been reported where a phenol served as an acceptor.

The mechanism of the synthesis of glucuronides by Fishman and Green's method may be summarized as:

$$\text{Hydrolysis} \quad RGA + HOH \longrightarrow ROH + GA$$

$$\text{Transfer} \quad RGA + XOH \longrightarrow ROH + XGA$$

RGA = glucuronide (donor), XOH = aglycone (acceptor), GA = glucuronic acid

In the other mechanisms which can bring about the synthesis of glucuronides, glucuronic acid enters into conjugation with a phenol not

directly but in the form of uridine diphosphate glucuronic acid (UDPGA). Glucuronic acid is transferred from UDPGA to the acceptor molecule by means of a transferase enzyme. The reactions involved in this mechanism are (Brodie et al, 1957):

$$\alpha\text{-glucose-1-phosphate} + \text{UTP} \longrightarrow \text{UDPG} + \text{P-P}$$

$$\text{UDPG} + \text{DPN} \longrightarrow \text{UDPGA} + \text{DPNH}$$

$$\text{UDPGA} + \text{ROH} \xrightarrow{\text{transferase}} \text{R-O-glucuronide} + \text{UDP}$$

Brodie et al (1957) point out that since the product is β-glucuronide, there must be an inversion of the α-linkage of glucuronic acid in UDPGA. The transferase involved in this mechanism has no β-glucuronidase activity - unlike the enzyme involved in the mechanism of synthesis of Fishman and Green (1957).

It is not known if the two mechanisms by which plant glucosides are synthesized in vitro are functional in vivo also. If they are so, it is not known which of the two is mainly responsible for the synthesis. It is possible that Cardini and Leloir's method involving UDPG operates in the synthesis of phenolic glucosides in plant tissues. The finding of Miwa et al (1957) that oxygen is necessary for the synthesis of aryl glucosides in plant tissues suggests that phosphorylation may be involved in the process. The fact that iodoacetate inhibited the synthesis of phlorin in leaf discs of Pelargonium (Experiment A-12) also indicates a possibility of the participation of a phosphorylating enzyme. Iodoacetate also inhibits the synthesis of sucrose in vivo (Kriukova, 1940) and Leloir and Cardini (1953) synthesized sucrose in the same way that

they synthesized phenolic glucosides. This supposition that UDPG is involved is further supported by the fact that glucose-1-phosphate participates more efficiently than glucose in the synthesis of phenolic glucosides (Miwa et al, 1957; Experiment A-16).

Although phenolic glucosides have not yet been synthesized in vitro by means of β-glucosidase, there is some evidence that this enzyme may be involved. Trim (1955a) found from his studies on two glycosides of purpurin-3-carboxylic acid, an anthraquinone, that there is a remarkable correlation in the occurence of these glycosides and the enzyme galiosinase, which specifically hydrolyzes these glycosides. The glycosides and the enzyme were found to occur in directly proportional amounts. The glycosides were never found to exist without the enzyme. The enzyme, however, was present where no glycoside could be detected. According to Trim (1955a), in any particular cell the accumulation of purpurin-3-carboxylic acid glycosides takes place after the production of the enzyme by the cell. In drawing conclusions from the results of his experiments, Trim (1955a) says, "These observations provide sufficient ground to speculate upon the participation of galiosinase in the biosynthesis of one or both of the glycosides which it hydrolyzes in vitro". However, he points out that it is possible that the enzyme is produced as an adaptive response to trace amounts of the substrates.

Synthesis of sucrose may be mentioned here as an analogy with synthesis of phenolic glucosides. Hassid (1958) says that it is improbable that sucrose is synthesized in plants by the action of invertase since the equilibrium of the invertase reaction is shifted

very much towards hydrolysis.

Two mechanisms of sucrose synthesis are known to occur *in vitro*, namely, 1) synthesis of sucrose from glucose-1-phosphate and fructose by an enzyme from *Pseudomonas saccharophilia* (which exhibits trans-glycosidation activities) (Hassid and Doudoroff, 1950) and, 2) the synthesis involving UDPG and fructose in a wheat-germ extract (Leloir and Cardini, 1953). It has not been established completely which of these two mechanisms is responsible for the synthesis of sucrose *in vivo*.

SUMMARY

The literature on the natural occurrence of the simpler phenols and their glycosides in plants, and the induced formation of these glycosides by plant tissues, has been reviewed.

Methods were devised for the analysis of plant tissues for phenolic glucosides, particularly phlorin. These methods involved the use of carbon-14 labelled compounds, chromatography and radioautography.

Phenols when introduced into plants were found to be converted to their β -glucosides. Ninety-six per cent of the radioactivity in the ethanolic extract of leaf tissue fed phloroglucinol-C^{14} was converted to phlorin. The alcoholic hydroxyl group of a phenol was found to be preferentially involved in glycosidation. When two phenols were fed together, the respective glucosides were synthesized and the relative quantities formed indicated that phenols may compete with one another for the same enzyme system.

When phloroglucinol was fed to an entire plant through its roots, phlorin and in some cases phloroglucinol, were detected in the various parts of the plant.

A variety of angiosperms (both mono- and di-cotyledons) and species of Pinus, Adiantum and Marchantia synthesized phlorin from administered phloroglucinol. Phlorin synthesis was shown to occur also in roots, stems, leaves, petals, embryos and various tissues such as cortex, xylem and phloem. The only exception was parenchymatous tissue of the apple fruit. Algae and fungi did not form phlorin when incubated in phloroglucinol.

Phlorin synthesis occured both with and without light and was highest at room temperature (21° to 22°C). The synthesis was inhibited by iodoacetate.

Pelargonium leaf discs were fed with glucose-C^{14} and phloroglucinol and analyzed after intervals of 1 hour, 2 hours and 6 hours, in the light and after a further 18-hour period in the dark, for ethanol soluble compounds. The radioactivity in phlorin-C^{14} remained at about 30% of the total activity throughout this period.

Phlorin synthesis was shown to occur with either endogenous or exogenous glucose. No glycoside other than phlorin was formed when leaf discs were infiltrated with phloroglucinol and one of a number of sugars and sugar acids such as ribose and glucuronic acid. Phlorin-C^{14} was formed in leaf discs fed with phloroglucinol and sucrose-C^{14}.

Although glucose-1-phosphate-C^{14} was not taken up as readily as glucose-C^{14} by leaf discs when fed together with phloroglucinol, 58% of the activity of the ethanol extract from the glucose-1-phosphate-C^{14} fed material was found in phlorin compared to 31% from the glucose-C^{14} fed leaf tissue.

Phlorin-C^{14} synthesis was shown to occur when phloroglucinol was fed to leaf discs either with pyruvate-C^{14} or succinate-C^{14}. Fifty-eight per cent of the total activity in the ethanol extract from the succinate-C^{14} feeding was located in phlorin.

Phlorin was synthesized in vitro from phloroglucinol-C^{14} and uridine diphosphate glucose using a homogenate of Pelargonium leaves as a source of the enzyme. UDPG could be replaced by glucose-1-phosphate plus uridine triphosphate. Phlorin-C^{14} was synthesized from

phloroglucinol and sucrose-C^{14} using the same homogenates.

The significance of these results have been discussed.

BIBLIOGRAPHY

Armstrong, E.F. and Armstrong, K.F. 1931. The Glycosides. Longman's Green and Co., London.

Baldwin, E. 1957. Dynamic Aspects of Biochemistry. 3rd edition. Cambridge University Press.

Bandurski, R.S. and Greiner, C.N. 1953. The enzymatic synthesis of oxalacetate from phosphoryl-enol-pyruvate and carbon dioxide. J. Biol. Chem. 204: 781-786.

Bate-Smith, E.C. 1950. Anthocyanins, flavones and other phenolic compounds. Partition Chromatography. Edited by R.T. Williams. Biochemical Society Symposia No. 3, pp. 62-73. Cambridge Univ. Press.

Bate-Smith, E.C. and Westall, R.G. 1950. Chromatographic behaviour and chemical structure. I. Some naturally occurring phenolic substances. Biochem. Biophys. Acta. 4: 427-440.

Baumann, H. and Pigman, W. 1957. Naturally occurring glycosides and glycosidases. The Carbohydrates. Edited by W. Pigman, Chapt. 10, pp. 536-601. Acad. Press Inc., New York.

Block, R.J., Durrum, E.L. and Zweig, G. 1955. A Manual of Paper Chromatography and Paper Electrophoresis. Acad. Press, New York.

Bodansky, O. 1948. The metabolism of Drugs and Toxic Substances. Ann. Rev. Biochem. 17: 306-307.

Boswell, J.G. and Whiting, G.C. 1938. A study of the polyphenol oxidase system in potato tubers. Ann. Bot., N.S. 2: 847-863.

Bourquelot, E. and Bridel, M. 1913. Synthèse des glucosides d'alcools à l'aide de l'emulsine et reversibilité des actions fermentaires. Ann. chim. et phys. (8) XXIX : 145-147.

Brodie, B.B., Gilette, J.R. and La Du, B.N. 1957. Enzymatic metabolism of drugs and other foreign compounds. Ann. Rev. Biochem. 27: 427-454.

Brown, B.R., Ekstrand, T., Johnson, A.W., Macdonald, S.F. and Todd, A.R. 1952. Colouring matters of the Aphididoe. Part VI. The glucosidic nature of protoaphin. J. Chem. Soc. 1952: 4925-4928.

Burma, D.P. and Mortimer, D.C. 1956. The biosynthesis of uridine diphosphate glucose and sucrose in sugar beet leaf. Arch. Biochem. Biophys. 62: 16-28.

Cardini, C.E. and Leloir, L.F. 1957. Biosíntesis de gluéosidos. Ciencia e Investigacion 13: 514.

Cardini, C.E., Leloir, L.F. and Chiriboga, J. 1955. The biosynthesis of sucrose. J. Biol. Chem. 214: 149-155.

Cardini, C.E. and Yamaha, T. 1958. Biosynthesis of plant glycosides from uridine diphosphate glucose. Nature 182: 1446-1447.

Chabrolin, C. 1940. Relations entre la fonction chimique de quelques composés et leur toxicité sélective pour les plantules de Phanérogames. Compt. rend. Acad. Sci. /Paris/ 210: 262-266.

Ciamician, G. and Ravenna, C. 1916. Attireale accad. Lincei 25: 3 (Quoted by Armstrong and Armstrong, 1931).

Clarke, D.D. and Nord, F.F. 1955. Simple benzene derivatives. Modern Methods of Plant Analysis. Edited by K. Paech and M.V. Tracey. Vol. III, pp. 332-350. Springer-Verlag, Berlin.

Cremer, M., and Seuffert, R.W. 1912. Über Phlorin, ein Spaltungsprodukt des Phloridzins. Berichte 45: 2565-2571.

Currier, H.B. 1956. Effects of toxic compounds: stimulation, inhibition, injury and death. Encyclopedia of Plant Physiology. Edited by W. Ruhland. Vol. II, p. 792-825. Springer-Verlag, Berlin.

Dubin, H. 1916. Physiology of Phenols. J. Biol. Chem. 26: 69-91.

Dufrenoy, J. 1936. Cellular immunity. Amer. J. Bot. 23: 70-79.

Eisenmenger, W.S. 1931. Factors modifying the toxicity of phenol. Plant Physiol. 6: 325-332.

Evans, C.W. 1958. Metabolism of aromatic compounds by lower plants. Phenols, quinones, and the problem of the biogenesis of benzene nuclei. Encyclopedia of Plant Physiology. Edited by W. Ruhland. Vol. X. pp. 454-506. Springer-Verlag, Berlin.

Evenari, M. 1949. Germination inhibitors. Bot. Rev. 15: 153-194.

Fischer, E. and Pitoty, O. 1891. Reduction der Zuckersäure. Bor. dtsch. chem. Ges. 24: 521-528.

Fishman, W.H. 1956. Metabolism of drugs and their organic chemicals. Ann. Rev. Biochem. 25: 659-684.

Fishman, W.H. and Green, S. 1957. Enzymic catalysis of glucuronyl transfer. J. Biol. Chem. 225: 435-452.

Freudenberg, K., Reznik, H., Bosenberg, H. and Rasenack, D. 1952. Das an der Verholzung beteiligte Fermentsystem. Chem. Ber. 85: 641-647.

Friedrich, H. 1958. Untersuchungen uber die phenolischen Inhaltstoffe von Pyrus communish. Pharmazie 13: 153-155.

Gavaudan, P. et Brébion, G. 1946. Action sur la photosynthèse de quelques substances inhibitrices de la caryocinese. Trav. Sci. Sta. Essais Bouchet 2: 37-46. (Ref. Biol. Abstracts 22: 2695).

Geissman, T.A. and Hinreiner, E. 1950. Theories of the biogenesis of flavonoid compounds. Parts I and II. Bot. Rev. 18: 77-244.

Gottschalk, A. The enzymes controlling hydrolytic, phosphorolytic and transfer reactions of the oligosaccharides. Encyclopedia of Plant Physiology. Edited by W. Ruhland. Vol. VI, pp. 87-124. Springer-Verlag, Berlin.

Hackney, F.M.V. 1948. Studies in the metabolism of apples. VII. A study of the polyphenolase system in apples. Proc. Linn. Soc. N.S. Wales 73: 439-454.

Hassid, W.Z. 1958. The synthesis and transformation of the oligosaccharides in plants, including their hydrolysis. Encyclopedia of Plant Physiology. Edited by W. Ruhland. Vol. VI, pp. 125-136. Springer-Verlag, Berlin.

Hassid, W.Z. and Doudoroff, M. 1950. Synthesis of disaccharides with bacterial enzymes. Adv. Enzymology 10: 123-143.

Hemingway, A., Pryde, J. and Williams, R.T. 1934. The biochemistry and physiology of glucuronic acid. V. The site and mechanism of the formation of conjugated glucuronic acid. Biochem. J. 28: 136-142.

Hutchinson, A. 1958. Biochemical and physiological studies of Malus rootstocks. Ph.D. Thesis, McGill Univ.

Hutchinson, A., Roy, C. and Towers, G.H.N. 1958. Synthesis of phlorin and other phenolic glucosides by plant tissues. Nature 181: 841-842.

Jermyn, M.A. and Thomas, R. 1953. Transferase activity of the β-glucosidase of Aspergillus oryzae. Austral. J. Biol. Sci. 6: 70-76.

Karrer, W. 1958. Konstitution und Vorkommen der organischen Pflanzenstoffe (exclusive Alkaloide). p. 96, Birkhäuser Verlag Basel und Stuttgart.

Karrer, P. and Viscontini, M. 1947. Einfluss verseheindener Lusätze auf die Wirksamkeit der Cocarboxylase. Helv. chim. Acta 30: 268-271.

Keegan, P.A. 1915. Mitteilungen über Pflanzenchemie. Chem. Zentralbl. II, p. 714-715.

Klein, G., Siersch, E. and Linser, H. 1931. Zum mikrochemischen Nachweis freier Phenole in der Pflanze. Österr. Bot. Z. 80: 223-249.

Krebs, H.A. 1954. Considerations concerning the pathways of synthesis in living matter. Bull. Johns Hopkins Hosp. 95: 19-33.

Kritchevsky, G. and Anderson, A.B. 1955. Chemistry of the genus Sequoia. I. The cone solid of coast redwood (Sequoia sempervirens and giant sequoia (S. gigantea)). J. Org. Chem. 20: 1402-1406.

Kriukova, 1940. Synthesis and hydrolysis of sucrose in the tissue of higher plants under conditions of inhibited phosphorylation. Biochimija 5: 574-583. (Quoted by Hassid, 1958).

Kursanov, A.L. 1944. Changes in tannin substances in the willow during the period of spring growth. Biochimiia 9: 322-336. (From Biol. Abstr. 19: 22803, 1945).

Leloir, L.F. and Cardini, C.E. 1953. The biosynthesis of sucrose. J. Am. Chem. Soc. 75: 6084.

Lindt, O. 1887. Zum mikrochemischen Nachweis des Phloroglucins. Z. anal. Chem. 26: 260-261.

Mastagli, P. and Augier, J. 1949. Etude de la structure du composé phenolique contenu dans Polysiphonia fastigata. Compt. rend. Acad. Sci. [Paris] 229: 775-776.

McIlroy, R.J. 1951. The Plant Glycosides, Edward Arnold and Co., London.

Meyer, B.S. and Anderson, D.B. 1952. Plant Physiology. 2nd edition. D. Van Norstrand Co. Inc., Toronto.

Michael, A. 1879. Synthetical researches in the glucoside group. Amer. Chem. Jour. 1: 305-312.

Miller, L.P. 1938. Formation of β-(2-chloroethyl)-d-glucoside by gladiolus corms from absorbed ethylene chlorohydrin. Contrib. Boyce Thompson Inst. 9: 425-429.

Miller, L.P. 1939a. Synthesis of β-(2-chloroethyl)-d-glucoside by potato tubers treated with ethylene chlorohydrin. ibid. 10: 139-141.

Miller, L.P. 1939b. Glycoside formation in plants from absorbed ethylene chlorohydrin, o-chlorophenol and chloral hydrate. Amer. J. Bot. 26: 143.

Miller, L.P. 1940. Formation of β-o-chlorophenyl-gentiobioside in gladiolus corms from absorbed o-chlorophenol. Contrib. Boyce Thompson Inst. 11: 271-279..

Miller, L.P. 1941a. Formation of a β-2,2,2-trichloroethylgentiobioside into tomato plants frown in media containing chloral hydrate, trichloroethyl alcohol, or chloral cyanohydrin. ibid. 12: 15-24.

Miller, L.P. 1941b. Simultaneous formation of a β-gentiobioside and a β-glucoside in gladiolus corms treated with chemicals. ibid. 12: 163-166.

Miller, L.P. 1942. Synthesis of β-2-trichloroethyl-D-glucoside and its isolation from corn and dandelion plants treated with chloral hydrate. ibid. 12: 465-470.

Miller, L.P. 1943. High yields of β-2-trichloroethyl-d-glucoside and β-2-trichloroethylgentiobioside from tobacco plants treated with chloral hydrate. ibid. 13: 185-200.

Miller, L.P. 1957. Monosaccharide components of an induced glycoside of trichloroethyl alcohol from dandelion tops. ibid. 19: 113-115.

Miwa, T., Nakamura, S., and Shibata, A. 1957. Biosynthesis of arylglycoside in higher plants. Kôso Kagaku Shinpojiumu 12: 48-53. (From Chem. Abstr. 32: 1314, 1958).

Myers, C.M. and Smith, J.N. 1954. Glucoside formation from phenols in Locusts. Biochem. J. 56: 498-503.

Nystrom, C.W., Tolbert, N.E. and Wender, S.H. 1959. Formation of β-phenylglucoside in plant leaves. Plant Physiol. 34: 142-143.

Pridham, J.B. 1957. Determination of phenolic glycosides and aglycones on paper chromatograms. Anal. Chem. 29: 1167-1169.

Pridham, J.B. 1958. Metabolism of phenolic compounds by the broad bean Vicia faba. Nature 182: 795-796.

Pryde, J. and Williams, R.T. 1936. The biochemistry and physiology of glucuronic acid. VI. The excretion of ethereal sulphate by the rabbit following the administration of phenyl glucosides. Biochem. J. 30: 794-798.

Quick, A.J. 1937. Detoxication mechanisms. Ann. Rev. Biochem. 6: 291-302.

Rabaté, J. 1935. Contribution à l'étude biochimique des salicaées. XI. Sur l'hydrolyse du salicoside par la poudre fermentaire de feuilles de Salix purpurea L. et sur quelques phénomènes qui en derivent. Bull. Soc. chim. biol. Paris, 17: 572-601.

Rabaté, J. 1937. Sur le gaulthéroside (éthylprimeveroside). Sa synthèse biochimique. Compt. rend. Acad. Sci. (Paris) 204: 153-155.

Rindernecht, H. and Jurd, L. 1958. A novel non-enzymic browning reaction. Nature 181: 1268-1269.

Robinson, R. 1936. The formation of anthoeyanins in plants. Nature 137: 172.

Seshadri, T.R. 1951. Biochemistry of natural pigments. Ann. Rev. Biochem. 20: 487-509.

Smith, J.N. 1955. Ethereal sulphate and glucoside conjugation in insects. Biochem. J. 60: 436-442.

Srb, A.M. and Owen, R.D. 1953. General Genetics. p. 248. W.H. Freeman and Co., San Francisco.

Stoll, A. and Jucker, E. 1958. Heteroside Aufbau und Vorkommen. Encyclopedia of Plant Physiology. Edited by W. Ruhland: Vol. VI, pp. 534-779. Springer-Verlag, Berlin.

Swain, T. 1953. The identification of coumarins and related compounds by filter paper chromatography. Biochem. J. 53: 200-208.

Takano, K. 1956. Acceptor relations in the enzymatic transfer of β-glucosyl and β-galactosyl groups. J. Biochem. [Tokyo] 43: 205-216.

Takano, K. and Miwa. 1950. Enzymatic transfer of glucose. II. Identity of glucotransferase and β-glucosidase. J. Biochem. [Tokyo] 37: 435-444.

Towers, G.H.N., Hutchinson, A. and Andrea, W.A. 1958. Formation of a glycoside of maleic hydrazide in plants. Nature 181: 1535-1536.

Trim, A.R. 1955a. Histochemical and qualitative studies on the distribution of galiosinase in the stellate shoot. J. Exp. Bot. 6: 100-125.

Trim, A.R. 1955b. Glycosides as a general group. Modern Methods of Plant Analysis. Edited by Paech and Tracey, Vol. II, pp. 295-316. Springer-Verlag, Berlin.

Turrel, F.M., Sinclair, W.B. and Bliss, D.E. 1940. Date Growers Institute, Indigo, California, 17th Ann. Rep. 9. (Quoted by Rindernecht and Jurd, 1958).

van Sumere, C.F., van Sumere-de Peter, C., Vining, L.C. and Ledingham, G.A. 1957. Coumarins and phenolic acids in the uredospores of wheat stem rust. Can. J. Microbiol. 3: 847-862.

Veibel, S. 1950. β -Glucosidase. The Enzymes. Edited by J.B. Sumner and K. Myrback. Vol. I, Part 1, Chapt. 16, pp. 583-620. Acad. Press, New York.

Von Oettingen, W.F. 1949. Phenol and its derivatives: The relation between their chemical constitution and their effect on the organism. Nation. Inst. Health Bull. 190: 1-408.

Waage, T. 1890. Über das Vorkommen und die Rolle des Phloroglucins in der Pflanze. Ber. dtsch. bot. Ges. 8: 250-292.

White, D.E. 1958. Chemistry of plant products. Nature 182: 299-300.

Williams, R.T. 1951. The metabolism of drugs and toxic substances. Ann. Rev. Biochem. 20: 441-464.