BIOSYNTHESIS OF PHENOLIC GLUCOSIDES.

A Thesis submitted by

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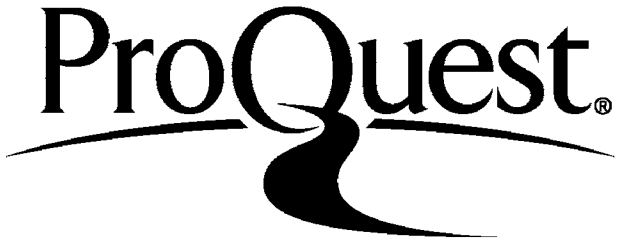

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ABSTRACT.

An investigation of the biosynthesis of phenolic glucosides *in vivo* and *in vitro* has been carried out. Introductory experiments using the broad-bean (Vicia faba) showed that a relatively high yield of mono β-glucosides was obtained when phenols were fed to germinating broad-bean seeds.

The mono β-glucosides of quinol, resorcinol and catechol formed in this way have been characterised. Phenolic and alcoholic glucoside derivatives of both o and p-hydroxybenzyl alcohols were produced, the phenolic derivative predominating in the latter case and o-hydroxybenzyl β-glucoside in the former, and not the naturally occurring phenolic glucoside, salicin. A possible route for the formation of salicin in plants is discussed.

With two trihydric phenols, pyrogallol, and 1,2,4-trihydroxybenzene, all the possible isomeric mono β-glucosides were obtained, with 2,3-dihydroxyphenyl β-glucoside predominating with

pyrogallol and 2,4-dihydroxyphenyl β-glucoside with 1,2,4-trihydroxybenzene. A possible general mechanism for the formation of glucosides *in vivo* is considered in the light of these results.

The acid-soluble nucleotide content of broad-bean seeds was examined in an attempt to find the glucose donor for these syntheses. No definite conclusion could be reached but there were some indications that uridine diphosphate glucose (UDPG) was present.

The formation of mono β-glucosides from phenols and UDPG or precursors of UDPG in the presence of wheat germ or bean enzymes were studied. There appeared to be a close similarity between the glucosides formed by the *in vivo* and *in vitro* syntheses.

o-Hydroxybenzyl β-fructofuranoside was formed from saligenin, and sucrose in the presence of invertase, but β-fructofuranosyl transfer to a phenolic hydroxyl group could not be accomplished in this way.

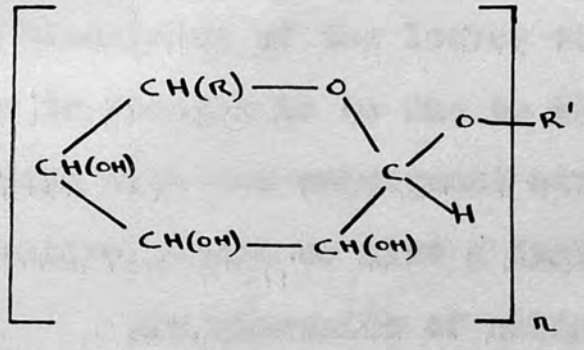
Acidic derivatives of the mono β-glucosides

of the dihydric phenols were detected. An investigation of the structure of the acidic derivative of resorcinol β-glucoside suggested that the 6-position of the glucose was substituted and the substituent was tentatively identified as a sulphate. The reactions of this compound and in particular the action of alkali were difficult to interpret so a model compound ,6'-tosyl arbutin, was synthesised and its reactions studied.

C O N T E N T S.

INTRODUCTION

A large proportion of the naturally occurring low molecular weight phenols in plants exist as glycosides. Bourquelot (1) stated that glycosides were present in 205 out of the 281 species of seed plants which he examined. The general structure of these compounds is :- (2)



R:-CH_2OH,H,CH_3,COOH.

R' :- Aromatic

n:- 1-4

With only one or two exceptions the naturally occurring glycosides have β-links and the sugar is in the pyranose ring form. The glycosides most frequently encountered are D-glucosides but D- and L-galactose, D-mannose, D-fructose, L-rhamnose, D-fucose, D-xylose, D-ribose and L-arabinose have also been isolated.(3) Disaccharide glycosides have been extracted from plant materials, for example gentiobiosides (6-O-β-D-glucopyranosyl-D-glucopyranosides) and rutinosides (6-O-β-L-rhamnopyranosyl-

D-glucopyranosides), while tri- and tetrasaccharides have been isolated from steroid glycosides (4). The cardiac glycosides are known to contain deoxy sugars which are not encountered elsewhere in nature (3).

Among the substituted phenyl glycosides, arbutin, the mono-β-glucoside of quinol, is known to occur in conjunction with methyl arbutin in plants including the Arbutus and Pyrus families. The blackening of the leaves of certain varieties of pear is thought to be due to enzymic hydrolysis of arbutin with the subsequent air oxidation of the resulting quinol to give a dark product (3).

The glucoside of saligenin, which is found in the bark, leaves and flowers of Salix and Populus species, is salicin (o-hydroxymethylphenyl β-D-glucoside). Rabaté, in his biochemical study of the Salicaceae (5), showed that salicin was present in all members of the family as the predominant glycoside. Salicin is frequently encountered together with a 6-benzoyl derivative, populin. The 2-benzoyl derivative, tremuloidin (6) has also been extracted from the bark of Populus tremuloides.

The possible function of phenolic glycosides in plants has long been a subject for speculation. Frey-Wyssling in his review of the subject [7] listed the following five possible functions of glycosides:- reserves of sugars in plants, control of osmotic pressure, stabilisation of labile aglycones, detoxification and action as plant hormones. Pfeffer[8] suggested that they formed sugar reserves and Bridel[9] found that glycosides disappeared during the germination of seeds of annual plants. However, Kerstan[10] stated that during the unfolding of the leaves the glucose of aesculin disappeared only from the wood and buds of Salix fragilis and Aesculus carnea and not from the bark of twigs although it comprised 18% of the store of sugar in the latter. Starvation experiments showed that the glucoside sugar did not form an important reserve. Weevers [11] stated that salicin had the function of a reserve substance and was hydrolysed by salicinase of the emulsin complex. He also showed that salicin accumulated in the leaves during illumination and was depleted during darkness. At the present time it is still not clear whether phenolic glycosides

are important sugar reserves. The glucosides are probably not but other glycosides of rarer sugars may be important storage compounds.

Another theory is that glycosides are only waste products of the plant metabolism [12] but when the variety of glycosidic residues is considered this does not seem feasible. For example, phenolic derivatives of D-glucose , D-galactose, L-arabinose, D-xylose and rutinose are all found in apple skin.[13] The detoxification of phenols in the plant by glucosylation may be of importance [14, 15] . Phenols are usually lipophilic but they also contain hydrophilic groups and are therefore surface active compounds. In view of this they could be deposited in the tonoplast membrane which contains lipoids and consequently upset the function of the cell vacuole. Glucosylation, however, would increase the hydrophilic character of the phenol and the glucoside would pass into the cell vacuole [7] . Support for the detoxification theory would appear to be given by the fact that beans germinate more readily in a solution of arbutin than in an equimolar solution of quinol.[16] In this way

glucoside formation in plants is similar to glucuronate formation in animals. The formation of glucuronates has also been demonstrated in plant material (17), flavone glucuronates having been isolated.

Glycosides might also govern the biological reactivity of the phenolic portion of the molecule by determining which enzyme can attack the phenol. Some support for this idea is given by the strophanthidin glycosides which have widely differing biological activities depending on the nature of the glycosidic group (18). In this connection it is also interesting to note that in grapefruit the 2-O-β-L-rhamnosyl-D-glucoside of diosmetin imparts a bitter flavour to the fruit but the 6-O-β-L-rhamnosyl-D-glucoside is tasteless.(19)

Glycosides are accompanied in the plant by specific hydrolysing enzymes known as glycosidases which cleave the glycosidic bond of mono- -glycosides to give the aglycone and monosaccharide. Disaccharidases have also been extracted from plant tissue (Rhamnus and Viburnum species) which hydrolyse glycosides of primverose (20) (6-O-β-D -xlopyranosyl-D-glucose)

rutinose and p-vinylphenyl 6-O-β-D-apiosyl-D-glucoside[21] to give disaccharide and aglycone, in each case.

The first investigation of the biosynthesis of phenolic glycosides was carried out by Ciamician and Ravenna [22] who fed maize seedlings with a solution of saligenin and claimed that salicin was produced. In further experiments they claimed that concentrated aqueous extracts of maize seedlings [23] and whole germinated maize or french bean seeds all converted saligenin to salicin.[24] They also replaced saligenin by quinol and benzyl alcohol and tentatively suggested that glucosides were formed with these compounds.[24]

Miller obtained 2-chloroethyl-β-D-glucoside from potato tubers, gladioli corms and wheat seedlings by exposing them to ethylene chlorohydrin [25] and 2,2,2-trichloroethyl β-D-glucoside from several different plants treated with chloral hydrate [26-28] With both chloral hydrate and o-chlorophenol he also obtained gentiobiosides [29,26]

Miwa and coworkers [30] infused leaf discs with D-glucose and quinol and found that arbutin was

produced. With vanillin and salicylaldehyde the corresponding aryl glucosides were again formed. Replacement of D-glucose by α-D-glucose-1-phosphate gave increased yields but grinding the leaves caused loss of activity. Dialysis of cell-free preparations gave an inactive preparation but activity was restored on addition of 'yeast nucleotide' and adenosine triphosphate (ATP).

The uptake of aqueous solutions of phenols by broad bean shoots has been studied by Pridham (15) who found that catechol, quinol, resorcinol and phoroglucinol were all converted to the corresponding mono β-glucosides. He found that phenol was highly toxic to the plant but Nystrom et al (31) fed traces of phenol to barley and wheat leaves and obtained phenyl β-D-glucoside. Recently Trevelloni (32) has extended the work of Meyers and Smith (33) and has shown that arbutin is produced when quinol is injected into locusts and that m-aminophenol is also glucosylated under these conditions. Smith and Turbert (34) used 4-methylumbelliferone in the same way and obtained the corresponding glucoside. Dutton and

Duncan [35] have shown that o-aminophenol is glucosylated in both beetroot and cockroach. The glucoside of indole acetic acid was found by Zenk[36] to be formed when epicotyls of Pisum sp. were treated with the acid. Harborne and Corner [37] isolated D-glucose esters of p-coumaric, caffeic and ferulic acids from Antirrhinum majus leaves which had been fed with the free acids.

The first glucoside syntheses in vitro were accomplished by Bourquelot et al [38,40] who synthesised glucosides of alcohols from excess D-glucose with β-glucosidase and alcohols. Salicyl β-glucoside was synthesised in this manner [41], and this compound was also found, together with saligenin and D-glucose, when salicin was hydrolysed by enzymes from powdered willow leaves, [5] almonds, and broad-beans[42]. p-Hydroxyphenyl β-D-gentiobioside has been synthesised by incubating arbutin with dialysed protein preparations from trembling aspen (Populus tremuloides) [43] and sweet almond and broad-bean seeds [42]. Courtois and Leclerc [44] found that the glucose from β-D-glucosides could be transferred to a primary or

secondary alcohol group but not to a tertiary group by almond β-glucosidase. Synthesis of phenolic glucosides *in vitro* could not be accomplished by simple transferase systems. (42,45)

In 1950, Leloir and associates (46,47) isolated uridine diphosphate glucose (U D P G) during an investigation of the interconversion of α-D-galactose-1-phosphate and α-D-glucose-1-phosphate.

(1) U D P G + α-D-galactose-1-phosphate $\underset{\text{transferase}}{\overset{\text{uridyl}}{\rightleftharpoons}}$ U D P Gal. + α-D-glucose-1-phosphate

(2) U D P G $\overset{\text{galactowaldenase}}{\rightleftharpoons}$ U D P Gal.

The second reaction requires the presence of diphosphopyridine nucleotide (D P N) and proceeds via an intermediate 4-keto derivative. U D P G is formed enzymically from α-D-glucose-1-phosphate and uridine triphosphate (U T P). (48-52)

(3) U T P + α-D-glucose-1-phosphate $\overset{\text{pyrophosphorylase}}{\rightleftharpoons}$ U.D.P.G. + pyrophosphate

Many reactions with U D P G have been studied. For example, Leloir and Cardini showed that the

synthesis of sucrose via U D P G can proceed by two paths (53-55):-

(4) U D P G + D-fructose $\rightleftharpoons$ U D P + sucrose

(5) U D P G + D-fructose-6-phosphate $\rightleftharpoons$ U D P + sucrose phosphate

In the latter reaction free sucrose could be formed by the action of sucrose phosphorylase. Trehalose phosphate is synthesised from U D P G and D-glucose-6-phosphate (56). Polysaccharides have also been synthesised from U D P G, for example, a β-1, 3-glucan was obtained from a mung-bean seedling preparation using ^{14}C labelled U D P G (90), and starch has been synthesised by Leloir et al (57).

Sugar nucleotides can also serve as glycosyl donors to non-carbohydrate acceptor molecules. Liver microsomes have been shown to transfer the D-glucopyranuronosyl moiety of U D P G Glucuronic acid to a wide variety of aromatic and aliphatic alcohols, carboxylic acids and amines (58,59).

As the D-glucose residue in U D P G has an α-linkage, the formation of β-linked polymers or

β-glucosides must proceed with inversion of configuration in contrast to sucrose synthesis where the α-linkage is retained.

The acid-soluble nucleotide content of plants has been investigated by several workers. Only nucleotides with a uracil base will be considered here. Cabib and coworkers (60) examined the nucleotides present in yeast and isolated uridine monophosphate (U M P -5'), U D P G and U D P -N-acetylglucosamine. U D P derivatives of D-glucose, D-galactose, D-xylose and L-arabinose were isolated from mung-bean seedlings by Ginsburg et al (61). Using a different extraction technique Bergvist (62,63) studied the acid-soluble nucleotides present in wheat, barley, and oat plants, and found U M P -5', U D P G and U T P. Edelman (64) also examined wheat seedlings with similar results. The presence of U D P G in banana fruit was shown by Rowan (65).

It was decided to investigate the structure of the glycosides formed when solutions of various phenols were fed to broad-beans (Vicia faba) in order to determine the possible specificity of reaction.

These plants were selected because of ease of germination and because the seeds were relatively large and easily handled. It was then planned to investigate the acid-soluble nucleotide content of the broad-bean in an attempt to locate U D P G which was considered to be the likely glucose donor. Finally it was hoped to carry out *in vitro* syntheses of phenolic glucosides and to compare the structures of these compounds with those obtained from the *in vivo* experiments.

MAIN SECTION.

A preliminary investigation was carried out to determine which organ of the plant and which stage of development gave maximum glucoside formation in the presence of aqueous solutions of phenols. Cut shoots, dormant and germinated whole seeds and isolated embryos and cotyledons were tested. It was established that bean seeds which had been allowed to germinate for 24 hr., before feeding with the phenols, gave the highest concentrations of phenolic glucosides. Extracts prepared from this source also contained fewer interfering phenolic compounds thus facilitating the separation and characterisation of the glucosides. However, even the germinated seed extracts contained at least 20 other phenolic compounds in addition to sugars, amino-acids etc.

Experiments using aqueous solutions of quinol at different concentrations showed that 1%, w/v gave a high yield of arbutin with little of the blackening of the tissues which resulted with higher concentrations.

The addition of D-glucose to the phenol

solution appeared to have little effect on the yield of glucoside and consequently it was not included in the large-scale preparations. Aqueous methanol (90%, w/v) was used for the extraction of the tissues, as the glucosides were soluble in this solvent and as fewer contaminating phenolic constituents were extracted than with, for example, water. The alcohol also inhibited the attack of the glucosides by β-glucosidase action. Only the formation of mono β-glucosides and acidic derivatives was studied; the formation of gentiobiosides and other disaccharide derivatives of the phenolic compounds was not investigated nor were the glucosides formed by substitution of more than one phenolic hydroxyl group. This was to a great extent due to the difficulty experienced in separating small amounts of di- or trisaccharide derivatives of phenols from other components present in plant extracts.

It is also difficult to locate accurately glucosides with no free phenolic hydroxyl groups on paper chromatograms. Diazotised p-nitroaniline/NaOH reagent (A) does not normally react with O-substituted phenols and reagents such as silver nitrate (C) and

potassium periodatocuprate (F) are not specific.

The mono β-glucosides formed were identified by paper chromatographic and electrophoretic comparison with standard compounds, where these were available. When none were available, chromatographic and electrophoretic mobilities were used to decide whether a compound was mono β-glucosylated. Mono- and di-glucosides are readily distinguished by these methods, eg arbutin has R_F 0.40 and 0.51 and p-hydroxyphenyl β-gentiobioside R_F 0.07 and 0.17 (solvents A and B respectively). Melting points and mixed melting points were obtained when possible although most of the glucosides examined did not crystallise and were readily oxidised by air to dark coloured products. The presence of a glycosidic link was shown by the fact that glucose and the parent phenol were produced on treatment with acid. Glucose was identified by chromatographic comparison with a standard in solvent B using spray B. Two solvent systems (A and B) and standard compounds were used to identify the aglycones. Hydrolysis with β-glucosidase confirmed the configuration of the glycosidic bond, the glucose and the phenol produced being again

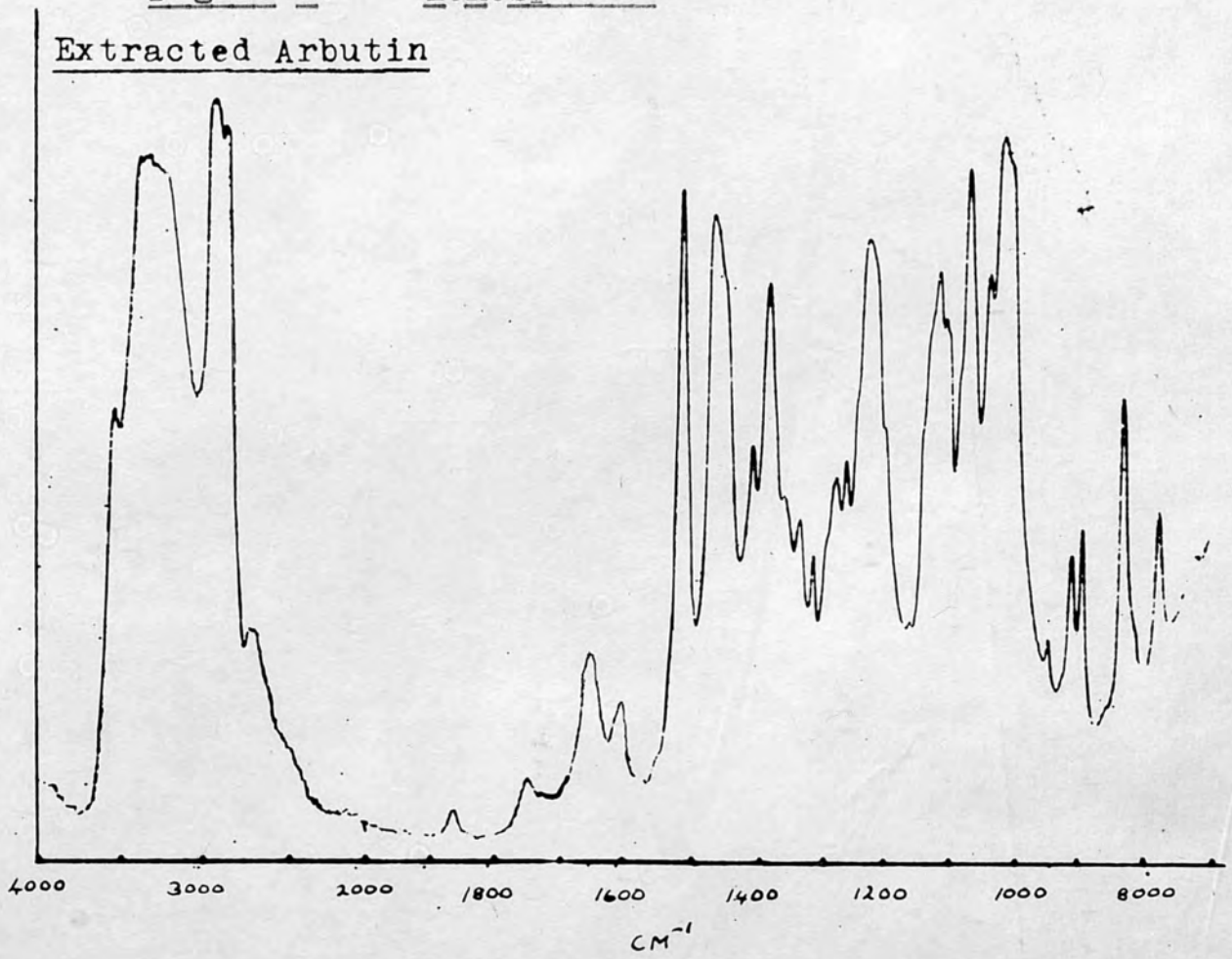
identified by chromatographic methods.

The presence of a benzene ring, free phenolic hydroxyl groups and adjacent phenolic hydroxyl groups were shown by u.v. measurements using recognised methods involving pH change and addition of complexing anions.(66,67)

The mono β-glucoside of quinol, ie arbutin, has well established properties and can be readily crystallised. The formation and structure of compound (I), arbutin, obtained by feeding germinated broad-bean seeds with quinol, were therefore examined in some detail (Experiment 11.) A firm identification of this compound supports the structures proposed for other β-glucosides which were less stable or were obtained in low yield and thus could be examined only by indirect methods. Compound (I) extracted from the bean seeds was crystallised and identified as arbutin by determination of melting and mixed melting points, analysis and u.v. and i.r. spectrophotometry (Fig1). (I) also gave a pentaacetyl derivative with the same melting and mixed melting points and i.r. spectrum as authentic pentaacetyl arbutin (Fig.2.) and a correct analysis. Hydrolysis of (I) with both acid and β-glucosidase

Figure 1. I.R.Spectra

Extracted Arbutin



Standard Arbutin

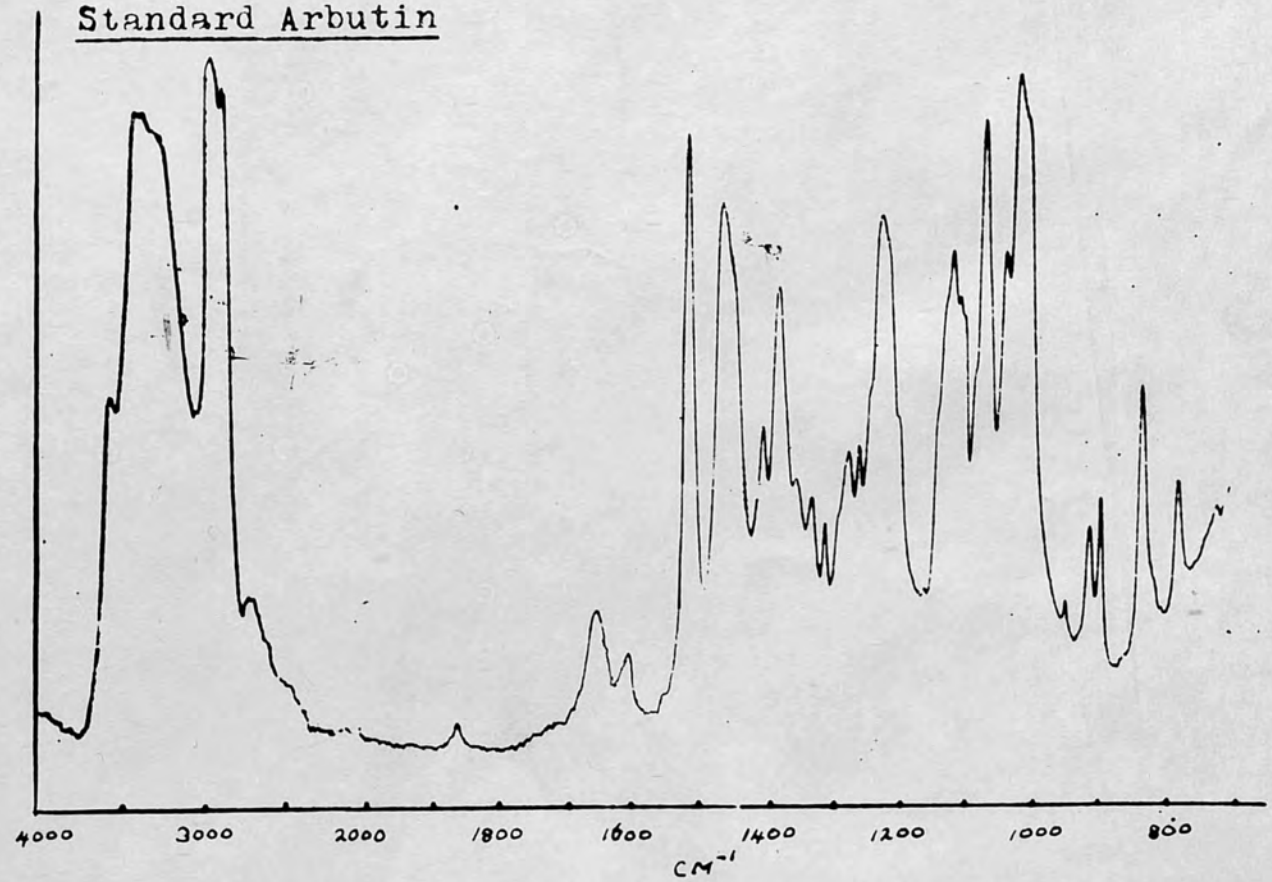
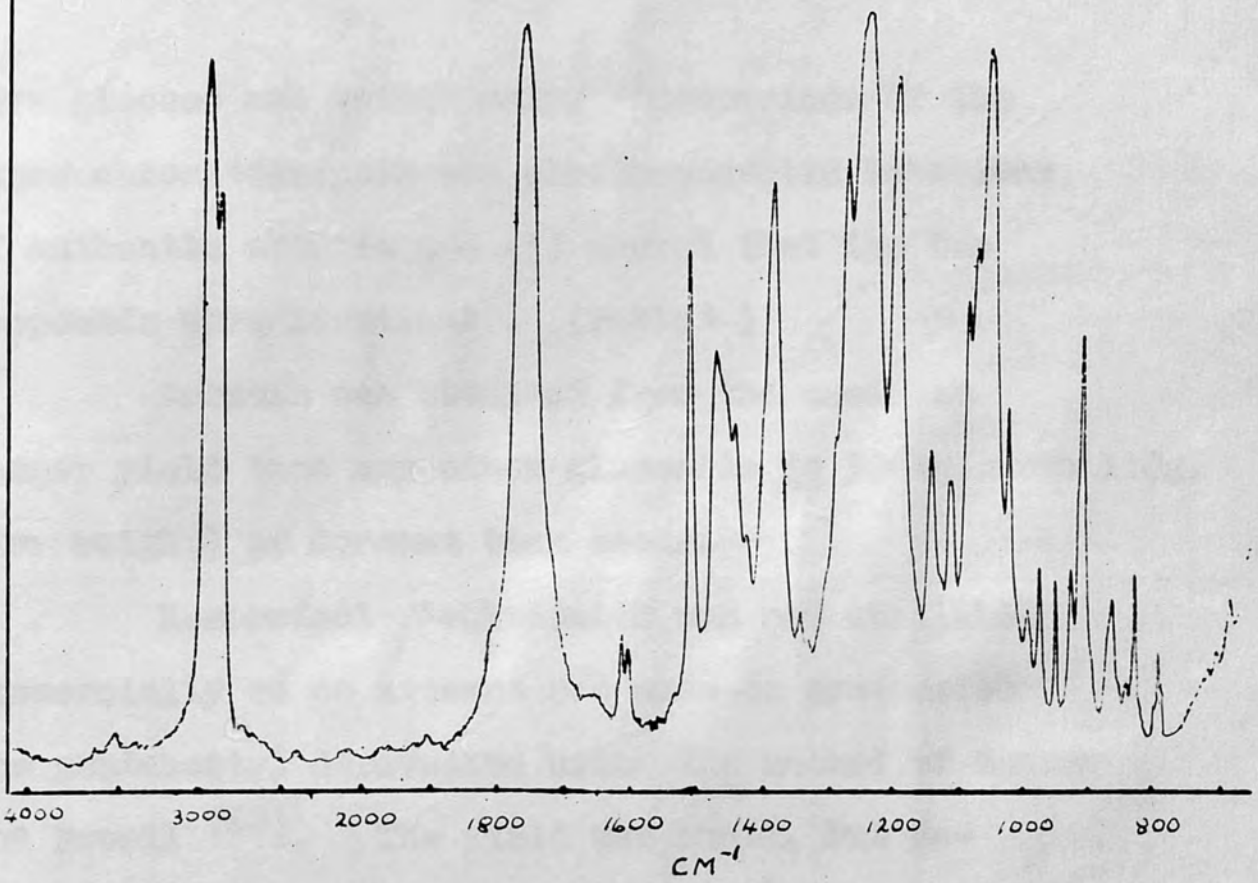
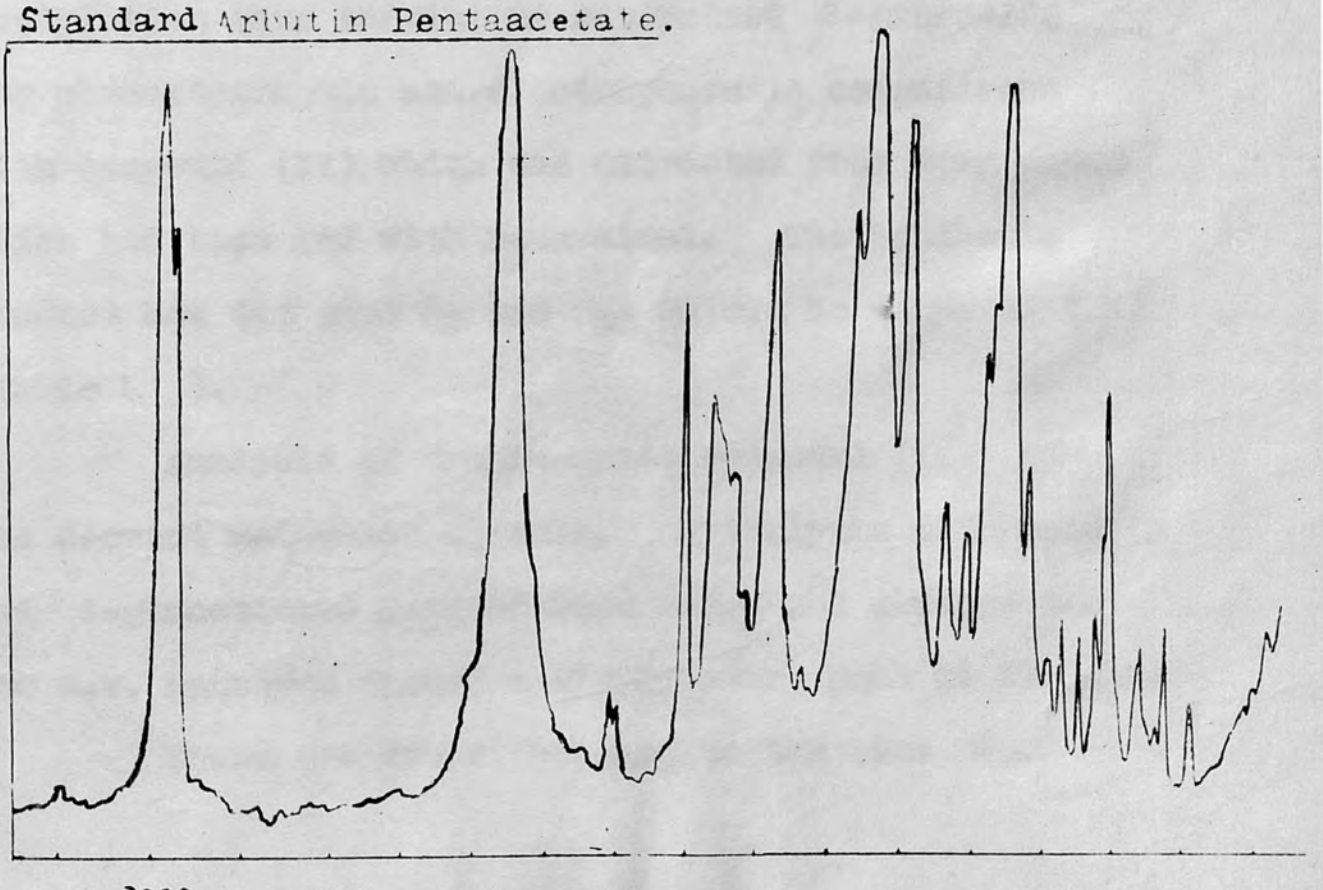


Figure 2. I.R. Spectra.

Extracted Arbutin Pentaacetate.



Standard Arbutin Pentaacetate.



gave glucose and quinol only. Comparison of the paper chromatographic and electrophoretic behaviour of authentic arbutin and (I) showed that the two compounds were identical . (Table 1.)

Arbutin was obtained from the seeds in larger yield than any other glucoside ie 300mg. from 110g. (dry weight) of dormant bean seeds.

Resorcinol β-glucoside was not available commercially so an attempt was made to synthesise the pentaacetyl derivative using the method of Bembry and Powell (68). The yield was small, but de-acetylation gave sufficient resorcinol β-glucoside for chromatographic and electrophoretic comparisons with compound (II), which was extracted from bean seeds which had been fed with resorcinol. The synthetic product had the same R_F and M_{SA} values as compound (II) (Table 1.).

Analysis of freeze-dried compound (II) gave the correct molecular formula. Hydrolysis with acid and β-glucosidase yielded resorcinol and glucose and the u.v. spectrum showed a single sharp peak at 271 mμ.

There are few references to the mono β-

TABLE 1.

Compound	Solvent (R_F values)		Buffer (M_{SA} values)			
	A.	B.	A.	B.	C.	D.
Compound (I)	0.47	0.45	0.22	0.16	0.00	0.00
Arbutin	0.48	0.45	0.22	0.16	0.00	0.00
Compound (II)	0.50	0.47	0.43	0.34	0.00	0.00
Resorcinol β-glucoside	0.51	0.47	0.44	0.35	0.00	0.00
Compound (III)	0.61	0.59	0.40	0.41	0.00	0.00
Catechol β-glucoside	0.62	0.59	0.41	0.43	0.00	0.00

glucoside of catechol in the literature [69]. Some impure catechol 2,3,4,6-tetra-O-acetyl-β-D-glucoside was acetylated and then de-acetylated to give the glucoside. Chromatographic and electrophoretic comparison of this compound with compound (III) obtained from bean seeds treated with catechol showed that they were identical (Table 1.). Compound (III) was hydrolysed with both acid and β-glucosidase yielding catechol and glucose.

Two mono β-glucosides, compounds (IV) and (V) in the approximate proportions of 3:1, were obtained when pyrogallol was fed to broad-bean seeds (Experiment 14). Compound (IV) gave a brown coloured complex with molybdate ions and had a high electrophoretic mobility in buffer C but was immobile in acetate buffer (D) of the same pH. It was also visible as a brown spot on paper chromatograms treated with molybdate and moved very slowly when these papers were developed with solvents A and B. Only compounds possessing an o-dihydroxy grouping will complex under these conditions.[70] Compound (V) was unaffected by the presence of molybdate on paper

TABLE 2.

Solvent	Paper	R_F values	
		Compound IV	Compound V
A.	Normal	0.37	0.52
	molybdate	0.03 *	0.55
B.	Normal	0.37	0.61
	molybdate	0.12 *	0.62
Buffer	**pH**	**M_{SA} values**	
A.	10.0	0.56	0.46
B.	10.0	0.43	0.33
C.	5.0	0.69 *	0.00
D.	5.0	0.08	0.00

* Brown spot visible before spraying.

chromatograms and did not move on electrophoresis in buffer C (Table 2.)

Both compounds yielded pyrogallol and glucose on hydrolysis with acid and β-glucosidase.

Further proof of the structures of the compounds was obtained from their u.v.spectra. Bathochromic shifts of the maximaof both compounds were observed on addition of hydroxyl ions but on addition of borate ions to these solutions a hypsochromic shift was observed only with the spectrum of compound (IV). Borate ions complex with o-dihydroxy groupings and produce a shift towards the u.v. end of the spectrum.(67)

Compound (IV) must therefore be 2,3-dihydroxyphenyl β-D-glucoside and compound (V), 2,6-dihydroxyphenyl β-D-glucoside.

Extracts prepared after feeding 1,2,4-trihydroxybenzene to broad-bean seeds contained three compounds (VI),(VII) and (VIII) resembling mono-β-glucosides on chromatograms , in the approximate proportions of 6:2:1. Hydrolysis of compounds (VI) and (VII) with both acid and β-glucosidase yielded 1,2,4-trihydroxybenzene and glucose. Insufficient

TABLE 3.

Solvent	Compound (R_F values) (VI)	(VII)	(VIII)
A	0.51	0.40	0.33
A + molybdate	0.56	0.06*	0.34
B	0.40	0.30	0.49
B + molybdate	0.45	0.05*	0.50
Buffer		**M_{SA} values**	
A	0.46	0.50	0.78
B	0.42	---	---
C	0.00	0.77*	0.45
D	0.00	0.05	0.44

* brown spot visible before spraying.

(VIII) could be extracted in a pure state for hydrolysis. This material also seemed to be more unstable than (VI) and (VII).

Compound (VII) gave a brown complex on paper chromatograms buffered with molybdate ions and a high M_{SA} value was recorded on electrophoresis in buffer C. Compound (VII) was stationary in buffer D (Table 3.).

A shift of the absorption maxima of both compounds (VI) and (VII) was observed on addition of alkali to their solutions. Subsequent addition of borate ions induced a hypsochromic shift in the spectrum of (VII) thus confirming the presence of an o-dihydroxy grouping. The absorption maximum of (VIII) disappeared on addition of alkali.

Oxidation of all three compounds with a solution of ferric chloride followed by chromatographic examination, showed that only compound (VI) was not oxidised and therefore did not possess a potential quinonoid structure.

Methylation of compound (VI) was carried out using diazo-methane; previous attempts to obtain a

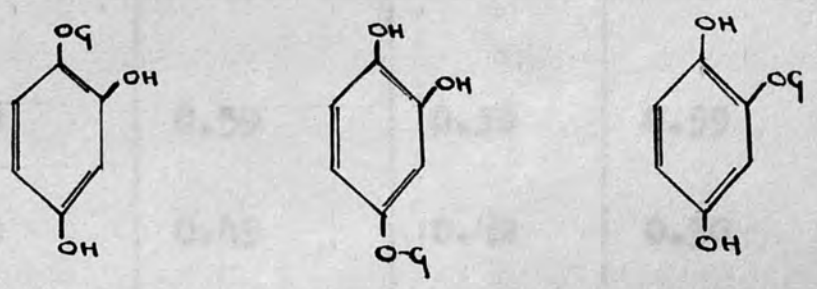
methylated phenol with N,N-dimethylformamide, methyl iodide and silver or barium oxide were unsuccessful. Hydrolysis of the glycosidic bond of the methylated product was followed by chromatographic examination of the products using 2,4-, 2,5- and 2,6-dimethoxyphenols as standards. A compound co-chromatographing with 2,4-dimethoxyphenol and giving the same blue colour with spray A was present.

A control methylation and hydrolysis of authentic arbutin under the same conditions yielded quinol monomethyl ether. Examination for methylated derivatives of D-glucose showed that in both experiments only partial methylation of the sugar moiety had occurred[71].

Authentic 2,5-dimethoxyphenol was synthesised as follows. The sodium sulphonate of quinol dimethylether was obtained by direct sulphonation of the dimethyl ether. The identity of this compound was checked by preparing the p-toluidide derivative. Fusion of the sodium sulphonate with alkali, chloroform extraction and chromatographic examination of the products showed the presence of a compound

which stained blue with the diazo spray A, and had chromatographic properties typical of a dimethoxyphenol.

The evidence strongly suggests that compounds (VI) , (VII), and (VIII) have the structures shown below:-



(VI) (VII) (VIII)

p-Dihydroxy groupings are more readily oxidised to p-benzoquinones than a corresponding o-dihydroxy compound (72). This fact may account for the apparent instability of compound (VIII).

Salicin, the β-glucoside of saligenin, is widespread in plant tissues but in the course of the present studies (Experiments 17-19), it was found that saligenin was converted predominantly to salicyl β-glucoside (IX) (o-hydroxybenzyl β-D-glucoside) by germinating broad-bean seeds. Compound (IX) was identified by hydrolysis to saligenin and glucose with both acid and β-glucosidase and by chromatographic and

Table 4.

Solvent	R_F values				
	Saligenin	Salicyl β-glucoside	Salicin	Compound (IX)	Compound (X)
A	0.89	0.59	0.56	0.59	0.55
B.	0.89	0.49	0.49	0.50	0.48
D.	0.91	0.78	0.73	0.78	0.74
Buffer		**M_{SA} values**			
A.	0.81	0.28	0.00	0.29	0.00
B.	0.30	0.27	0.00	0.27	0.00
C.	0.18	0.00	0.00	0.00	0.00
D.	0.09	0.00	0.00	0.00	0.00

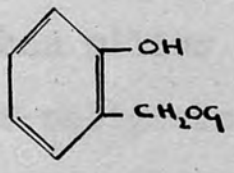
electrophoretic examination using authentic salicyl β-glucoside as a standard. Authentic salicyl β-glucoside and compound (IX) both gave a red colour with the diazo spray A. A small quantity of salicin (X) was detected in the bean extract by viewing the paper chromatograms under a short wave u.v.lamp, when it appeared as an absorbing spot. Salicin does not react with the diazo spray. Separation of compound (X) from the extract was achieved by chromatography in solvent A, elution of the band containing both compounds (IX) and (X) and, finally fractionation of these two compounds by paper electrophoresis in buffer A. Compound (X) has no free phenolic hydroxyl group and is therefore electrophoretically immobile. β-Glucosidase hydrolysis of this compound yielded saligenin and glucose and the R_F and M_{SA} values of (X) were the same as those of authentic salicin.

Addition of alkali to a solution of (IX) caused a bathochromic shift of the absorption maximum. Neutralisation of the alkaline solution of (X) obtained after elution of the compound from

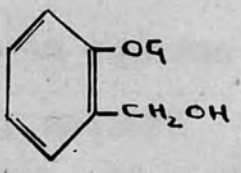
TABLE 5.

Compound	Absorption maximum	Addition of hydroxyl ions.
Saligenin	275mμ	
Salicin	268mμ	268mμ
Salicyl-β-glucoside	274mμ	297mμ
p-Hydroxybenzyl alcohol	277mμ	
p-hydroxymethyl phenyl β-glucoside	264mμ	264mμ
p-Hydroxybenzyl β-glucoside	279mμ	290mμ

electrophoretograms caused no change in the position of the absorption maximum (Table 5). Only compound (IX) can therefore possess a free phenolic hydroxyl group. This is also supported by the reaction of (IX) but not (X) with the diazo reagent (A). Rabaté and Ramart-Lucas [73] showed that substitution of phenolic hydroxyl groups resulted in a hypsochromic shift of the absorption maximum whereas substitution of an alcoholic hydroxyl group in an aromatic ring had little effect. As can be seen (Table 5) the spectra of salicyl β-glucoside (IX) and salicin (X) are consistent with these observations. The structure of compounds (IX) and (X) are therefore as shown below:-



(IX)



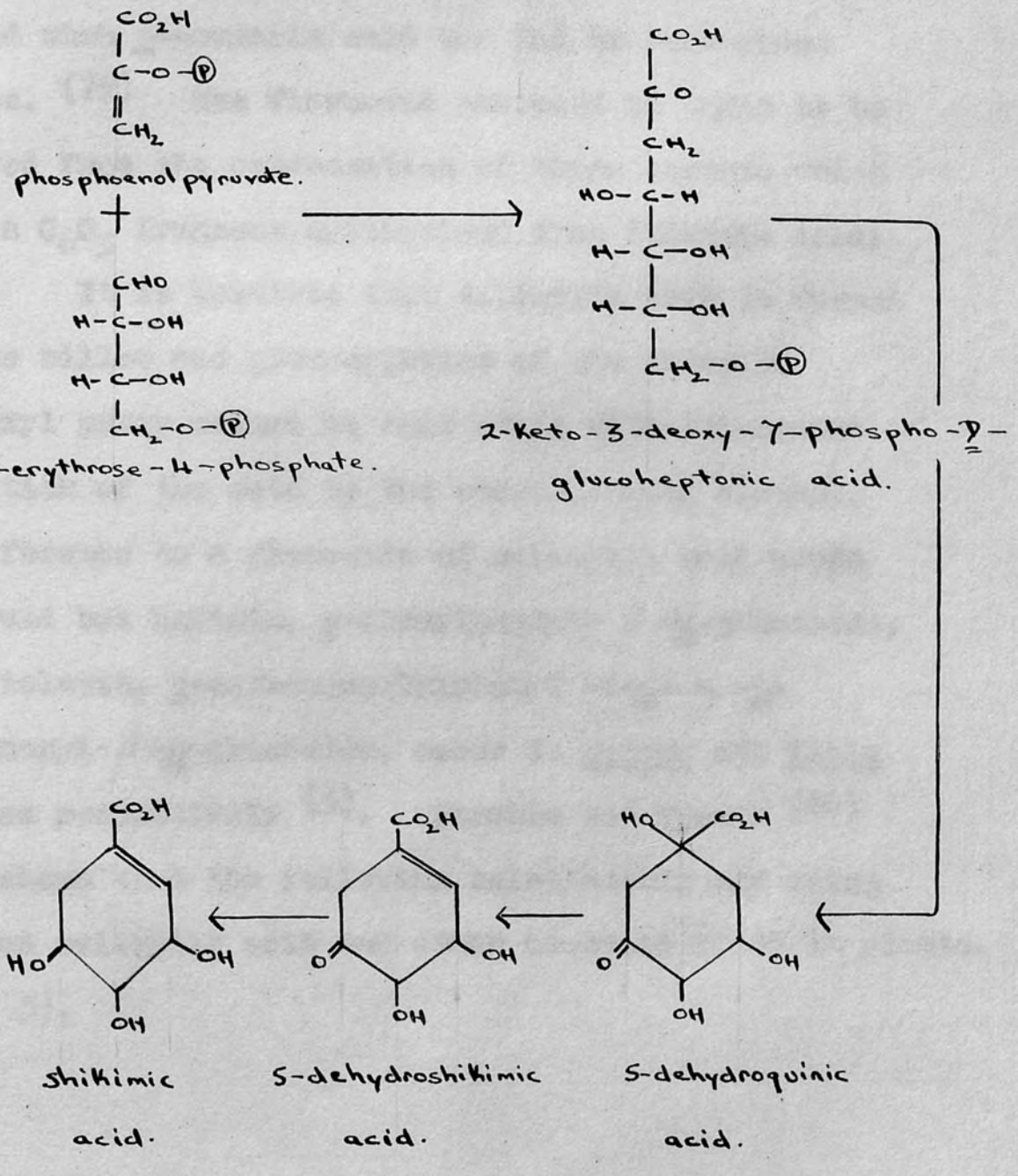
(X)

Ciamician and Ravenna [22] stated that salicin was formed when saligenin was fed to maize seedlings. In view of the results obtained with bean seeds it was decided to repeat this Italian work (Experiment 19)

using maize seeds at various stages of growth. In all cases salicyl β-glucoside (IX) was readily formed but salicin could not be detected. Ciamician and Ravenna identified salicin in their experiments by melting point, carbon and hydrogen analysis, the formation of a red colour with sulphuric acid and enzymic hydrolysis to saligenin and glucose. No crystalline salicyl β-glucoside was available for a melting point determination but this glucoside is isomeric with salicin, it gives a red colour with sulphuric acid and yields the same products on hydrolysis with β-glucosidase. It seems probable, therefore, that the compound extracted by Ciamician and Ravenna was salicyl β-glucoside and not salicin.

When willow shoots were kept in an aqueous solution of saligenin and then extracted, salicyl β-glucoside and salicin were found. The latter was also present in a control extract, however. This result may imply that saligenin is not the precursor of salicin in the willow. It is possible that saligenin fed externally does not reach the correct site of synthesis. Another possibility is that salicin is

formed <u>via</u> shikimic acid. The biosynthetic pathway of the latter is :- (74-76)

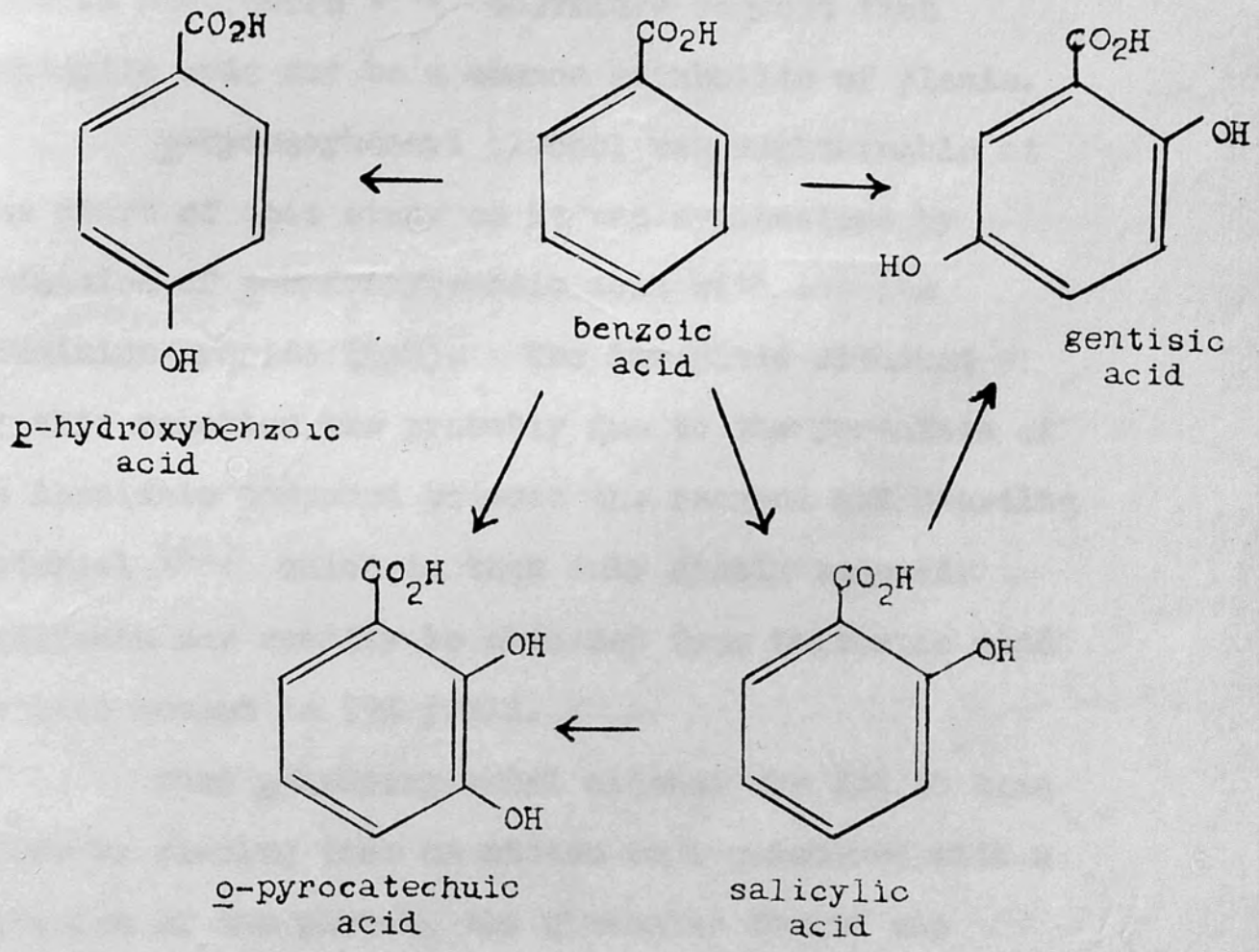


Shikimic acid is known to be a precursor of protocatechuic acid (3,4-dihydroxybenzoic acid)[77] and it has been proposed that cinnamic acids are also formed from this same precursor [78]. Quercetin was formed when p-coumaric acid was fed to buck-wheat plants. [79] The flavonoid skeleton is known to be derived from the condensation of three acetate units with a C_6C_3 fragment synthesised from shikimic acid.

It is possible that salicylic acid is formed in the willow and glucosylation of the phenolic hydroxyl group occurs at that stage with subsequent reduction of the acid to the corresponding alcohol. No reference to a glucoside of salicylic acid could be found but helicin, o-formylphenyl-β-D-glucoside, and violutin, o-carboxymethylphenyl-6-O-α-L-arabinosyl-β-D-glucoside, occur in Spirea and Viola species respectively [3]. Ibrahim and Towers [80] have shown that the following relationship may exist between salicylic acid and other aromatic acids in plants. (Fig. 3).

Figure 3

Interconversions of Aromatic Acids in Plants



These reactions can occur even in those plants which do not contain these acids in either free or bound forms. Ibrahim and Towers (80) therefore suggest that salicylic acid may be a common metabolite of plants.

p-Hydroxybenzyl alcohol was unobtainable at the start of this study so it was synthesised by reduction of p-hydroxybenzoic acid with lithium aluminium hydride (LAH). The low yield obtained by this reaction was probably due to the formation of an insoluble compound between the reagent and starting material (81) which is then only slowly reduced. Saligenin may readily be obtained from salicylic acid by this method in 99% yield.

When p-hydroxybenzyl alcohol was fed to bean seeds by placing them on cotton wool moistened with a solution of the phenol, the glucoside formed was p-hydroxymethylphenyl β-glucoside (XI). If bean seeds were partially covered with an aqueous solution of the phenol, however, p-hydroxybenzyl β-glucoside (XII) was also obtained but in lower yield than (XI).

Compound (XI) was visible on paper chromatograms and electrophoretograms (Table II.) as an absorbing

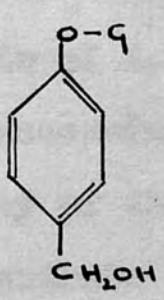
band under a short wave u.v. lamp but could not be detected with spray A. Compound (XII) on the other hand gave a pink-purple colour with spray A. Hydrolysis of compound (XI) with both acid and β-glucosidase yielded p-hydroxybenzyl alcohol and glucose. Compound (XI) had an absorption maximum of 266mμ which was unaffected by alkali, thus confirming that the phenolic hydroxyl group was substituted.

p-Hydroxybenzyl β-glucoside was synthesised by the biochemical method of Bourquelot and Herissey[41] using excess D-glucose, β-glucosidase and the phenolic alcohol. β-Glucosidase will not catalyse the glucosylation of phenolic hydroxyl groups.[82] The product gave the same pink-purple colour as (XII) with the diazo spray (A) and was hydrolysed with both acid and β-glucosidase to p-hydroxybenzyl alcohol and glucose. The spectrum of the compound had an absorption maximum at 279mμ; addition of alkali caused a shift to 290mμ showing that the compound contained a free phenolic hydroxyl group. The Rp and M_{SA} values of this compound and the extracted compound

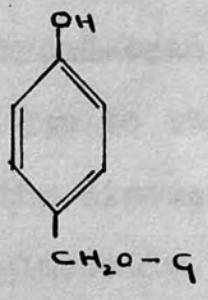
(XII) were identical (Table 11.). Insufficient compound (XII) was obtained from bean seeds for further characterisation.

A consideration of the u.v. spectra of compounds (XI), synthetic (XII) and p-hydroxybenzyl alcohol shows that the absorption maximum of (XI) is close to that of the parent phenol whilst that of compound (XII) is shifted towards the u.v. end of the spectrum. These results are again similar to those obtained with the glucosides of saligenin and agree with the observations of Rabaté and Ramart-Lucas[73] regarding the substitution of phenolic and primary hydroxyl groups (Table 5).

The two glucosides (XI) and (XII) must therefore possess the formulae shown :-



(XI)



(XII)

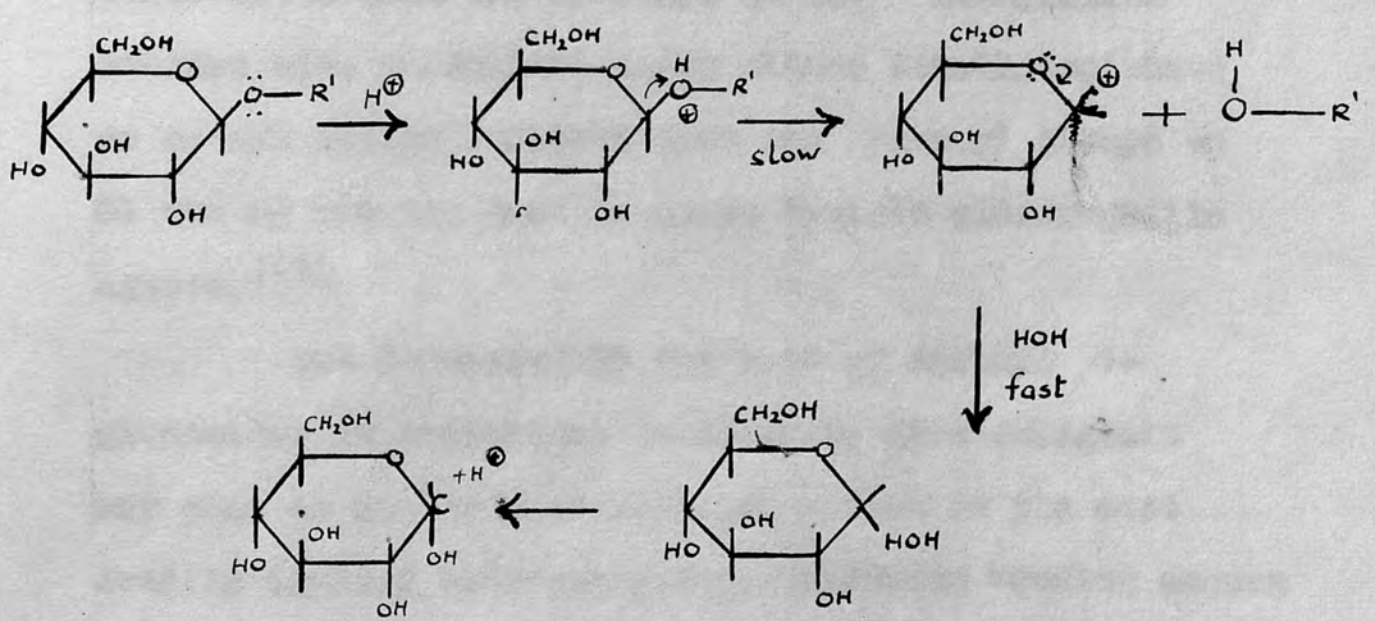
From these results it appears that when only a small quantity of phenol was present in the tissues, glucosylation of the phenolic hydroxyl group occurred predominantly, but as the concentration of the phenol increased the alcoholic group was also glucosylated.

Glucosylation of the phenolic hydroxyl group occurred when p-nitrophenol was fed to broad-bean seeds. The glucoside formed (XIII) could be detected on paper chromatograms by reducing it to the amine with $TiCl_3$ and subsequently spraying with Ehrlich's reagent (p-dimethylaminobenzaldehyde) (83). Hydrolysis with acid and β-glucosidase yielded p-nitrophenol and glucose. The u.v. spectrum of the compound was scanned and a single sharp peak found at 294mμ. Compound (XIII) was electrophoretically immobile in all buffers used. The attempted formation of a glucoside of 2,4-dinitrophenol using broad-bean seeds was unsuccessful. This may have been due to the inability of the phenol to penetrate the tissues or to the extreme instability of such a glucoside.(84)

The experiments have shown that dihydric

phenols are readily converted to mono β-glucosides by broad-bean seeds. With the two trihydric phenols which were examined it was evident that one isomer in each case was formed preferentially although all the theoretically possible isomers were present.

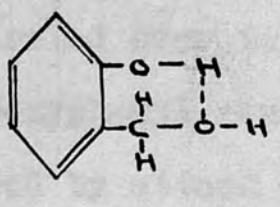
The enzymic hydrolysis of β-glucosides is known to proceed <u>via</u> hexose-oxygen fission, (85) Bunton and co-workers (86) and recently Banks and associates (87) have shown that acid-catalysed hydrolysis of aryl β-glucosides also involves fission of the hexose-oxygen bond with formation of a carbonium ion.



If enzymic synthesis proceeds by a similar mechanism then synthesis of oligosaccharides and glycosides by transglycosylation may also involve attack of an acceptor molecule by an electrophilic agent. Thus centres of high electron density eg phenate ions, will be glucosylated most readily. Using this assumption it is possible in many cases to understand why certain phenolic groups are glucosylated more readily than others by the bean seed. For example, pyrogallol in vivo gave 2,3-dihydroxyphenyl β-glucoside in greater yield than 2,6-dihydroxyphenyl β-glucoside. This is to be expected because the hydroxyl groups on C1 and C3 of the ring are more dissociated than the hydroxyl on C2. Methylation studies with pyrogallol using either dimethylsulphate or methyl iodide indicate that the hydroxyl groups on C1 and C3 are the most reactive towards electrophilic agents.(88)

The formation by the bean of salicyl β-glucoside, in preference to salicin, from saligenin may also be due to electrophilic attack on the most readily ionised hydroxyl group. Hydrogen bonding occurs

in saligenin as shown below:-



thus facilitating proton release from the alcoholic group by stabilisation of the alkoxide ion. The alcohol group in saligenin is known to be highly reactive eg on heating saligenin, 2,2'-dihydroxydibenzyl ether is formed.(89)

The phenolic hydroxyl group was glucosylated almost exclusively in the case of p-hydroxybenzyl alcohol. With this compound it may again be postulated that glucosylation occurs predominantly by substitution at the phenate ion. The tendency of the compound to form this ion is greater than its tendency to give an alkoxide ion. The mesomeric effect of the primary alcohol group is not likely to be strong enough to suppress, significantly, the ionisation of the phenol.

With 1,2,4-trihydroxybenzene the results are more difficult to explain. Three mono β-glucosides

were formed but 2,4-dihydroxyphenyl β-glucoside was present in the highest concentration in the seed extracts. If the preceding argument is carried over to this phenol then 2,5- and 3,4-dihydroxyphenyl glucosides would be expected to be produced by attack of an electrophile on the most readily ionised hydroxyl groups ie those on carbons 2 and 4 of the ring. However, these latter glucosides were present in the lowest concentration in the seed extracts. It is not impossible, however, that all three glucosides were formed but those possessing o- and p-dihydroxy groups ie the 2,5- and 3,4-dihydroxyphenyl β-glucosides were oxidatively decomposed chemically and/or enzymically (cf the action of $FeCl_3$ on these glucosides P 29.).
Oxidases and peroxidases are known to occur widely in plant tissues. It might further be postulated, from the redox potentials of o- and p-benzoquinones ($E_o$aq. 0.792 and 0.699v. respectively), that the 3,4-dihydroxyphenyl β-glucoside would be more stable to oxidising agents than the 2,5-isomer. This explanation could account for the relative concentrations of the three isomers ie 2,4- ≫ 3,4- ≫ 2,5- ≫

dihydroxyphenyl β-glucoside in the extracts.

Glucosylation of the phenolic hydroxyl group also occurred with p-nitrophenol: the presence of the nitro group stabilises the phenate ion in this case.

The results of this study suggest that the glucosylating system of the seed is unspecific but that the reaction may be to some extent controlled by the reactivity of the hydroxyl groups. Steric considerations may also in some cases play a part but these effects were not apparent in this present work. The validity of the hypothesis needs to be tested by feeding a wider variety of phenolic compounds, bearing in mind the complications which may arise due to instability of some glucosides and lack of penetration of the tissues by certain phenolic compounds.

It was shown that glucosylation occurred when phenols were fed to germinating broad-bean seeds and therefore it was decided to investigate the nature of the glucose donor in the seeds. Recent work had shown that many glucosylation reactions involved uridine diphosphate glucose (UDPG) (90).

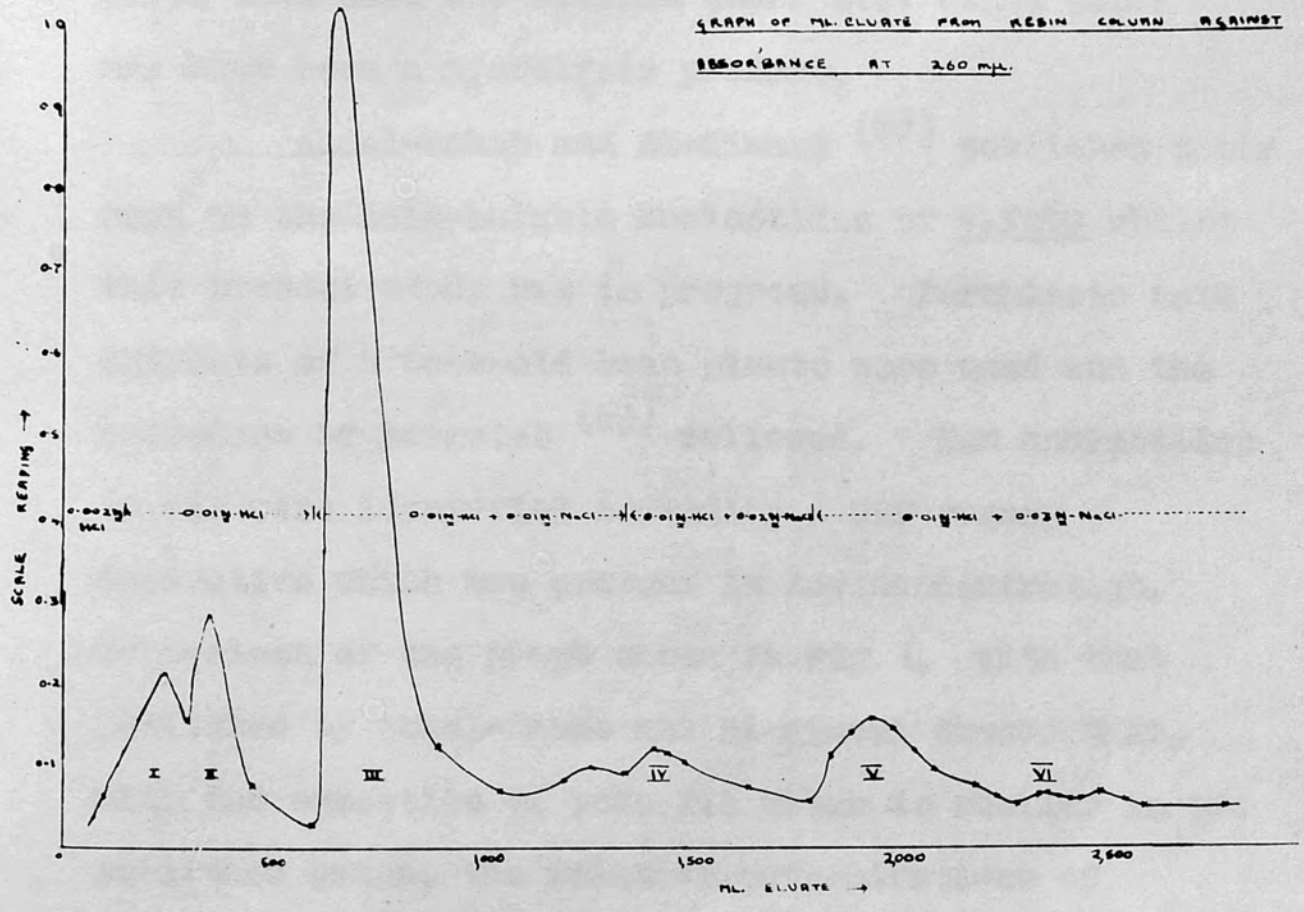
The separation of ribonucleotides using an anion-exchange resin and elution with acid containing increasing concentrations of a salt of the acid was first developed by Cohn (91). Cabib et al (60) extracted nucleotides from yeast with ethanol and precipitated them with mercuric acetate. The precipitate was then decomposed with H_2S, and separation of the nucleotides achieved by the use of a column of Dowex-1 (Cl^- form), which was eluted with increasing concentrations of HCl and NaCl. Ginsburg and co-workers (61) used a similar method for investigating the nucleotides in mung-bean seedlings and Rowan (65) with banana nucleotides. Bergvist (62) extracted wheat, barley and oat plants with ice-cold 10% perchloric acid, adsorbed the nucleotides on norite and then eluted them with aqueous ethanol containing conc. ammonia. The solution was finally adsorbed on Dowex-1 which was eluted with a solution of formic acid and sodium formate. Edelman (64) extracted the nucleotides from wheat seedlings by preparing an ethanolic extract, passing it through a squat carbon column and eluting with aqueous ethanol. This method

was tried without success in this present study.

The method finally adopted was that of Cabib et al[60], and an extract prepared of bean seeds which had been allowed to germinate for 6 days. A simplified elution procedure was employed, however, as the nucleotide of main interest was UDPG. 0.002N-HCl was used initially as the eluent and then 0.01N-HCl with increasing concentrations of NaCl(0.01N-0.03N). Fig. 4 shows the absorbance at 260mμ plotted against the numbers of the fractions collected. The purine and pyrimidine nuclei absorb strongly in this region of the spectrum. Comparison of this graph with those of Cabib and of Ginsburg and associates enabled the nucleotides to be tentatively located:- peak I would be expected to contain adenosine monophosphate (AMP-5') and DPN: II, UMP-5' and guanosine monophosphate (GMP-5'): III, adenosine diphosphate sugar (ADP-sugar) with some adenosine diphosphate (ADP) and AMP-5': IV, mainly ADP: V, UDP-sugars with some UDP and UMP-5': VI, UDP-sugars and UDP.

Concentration of the extracts was attempted by adsorption, on squat carbon columns, of the nucleotides

Figure 4. Separation of nucleotides on a resin column.



but chromatographic examination of the ethanol eluates of these columns did not reveal any UDPG even after concentration. There may have been insufficient UDPG present to be detected under u.v. light. It is known that acid will hydrolyse nucleotides (92) and a compound with a higher Rf value than UDPG was visible under u.v. light which may have been a hydrolysis product.

Abdel-Wahab and El-Kinawi (93) published their work on the acid-soluble nucleotides of **V.faba** whilst this present study was in progress. Perchloric acid extracts of 4 week-old bean plants were used and the procedure of Bergvist (63) followed. Ten nucleotides in all were identified including a UDP sugar derivative which was present in low concentration. Comparison of the graph shown in Fig. 4 with that published by Abdel-Wahab and El-Kinawi showed that, with the exception of peak III which is smaller in the published graph, the relative concentrations of nucleotide fractions were very similar. The authors stated that only a low yield of nucleotides was obtained but in a later note (94) claimed that 2

week-old seedlings gave a maximum yield of nucleotides. The UDP sugar derivative ,which was probably in the main UDPG, was not further identified; the concentration of this compound at different stages of development was found to be as follows:-

	Dormant seeds	1st	2nd	3rd	4th week of germination
UDPX	0.08	0.22	0.50	0.24	0.24 μmoles

The concentration of the UDP sugar derivative reached a maximum after the second week of germination and this may explain the poor yield of UDPG in the experiment described herein. The bean seeds used in the current work were 6 days old and at this stage Abdel-Wahab and El-Kinawi found the lowest concentration of'UDPX' . It seems probable that at this stage of germination the metabolic pool contains only very little UDPG and other UDPsugars. Ginsburg and co-workers used 10 kilos of 6 day-old mung-bean seedlings and extracted only '2.7m.moles nucleotides about one half of which were derivatives of uridine'.

During the course of the work described in this thesis Cardini and Yamaha (45) published a note

recording the formation of phenolic glucosides from UDPG, mono-,di- and tri-hydric phenols and a wheat germ enzyme. This was an extension of an earlier note (95) in which it was stated that arbutin was formed when quinol was used as a substrate. Mono β-glucosides of quinol, resorcinol, carechol, 1,2,4-trihydroxybenzene, phloroglucinol, pyrogallol and methoxyhydroquinone were all tentatively identified by chromatographic methods (96) Only in the case of resorcinol β-glucoside was a standard compound available for comparison. No structures were proposed for the glucosides of pyrogallol and 1,2,4-trihydroxybenzene.

In the present study some of Cardini and Yamaha's work with wheat germ (96) has been repeated and extended. The crude wheat germ enzyme with UDPG and resorcinol gave the corresponding mono β-glucoside which was characterised by chromatography, using two solvent systems with a resorcinol β-glucoside (II) standard. (Table 6) Arbutin (I) was formed when quinol was used as an acceptor and catechol β-glucoside (III) was obtained from catechol. With saligenin, glucosylation

TABLE 6.

Phenol	Enzyme	Buffer (pH 7.4)	Compound Produced
Quinol	I	Tris-HCl	Arbutin(I)
	III	Acetate	-----
Resorcinol	I	Tris-HCl	Resorcinol β-glucoside (II)
	III	Acetate	(II)
Catechol	I	Tris-HCl	Catechol β-glucoside (III)
Pyrogallol	I	Tris-HCl	2,3 dihydroxy phenyl β-glucoside (IV)
1,2,4-Trihydroxy-benzene	I	Tris-HCl	2,4-dihydroxy phenyl β-glucoside (VI)
Saligenin	I	Tris-HCl	Salicyl β-glucoside (IX)
		Acetate	(IX)
	III	Tris-HCl	(IX)
		Acetate	(IX)
p-Hydroxybenzyl alcohol	I	Tris-HCl	----

of the alcoholic group occurred exclusively to give salicyl β-glucoside (IX). Yamaha and Cardini (96) stated that 'a compound migrating on paper like salicin' was produced under these conditions but in the present study no evidence for the presence of salicin could be obtained. The formation of salicyl β-glucoside from UDPG and saligenin was in keeping with the finding that it was the major glucoside produced when saligenin was fed to broad-bean seeds.

Pyrogallol gave one glucoside which was chromatographically and electrophoretically identical with the 2,3-dihydroxyphenyl β-glucoside (IV) obtained from the *in vivo* experiments, whilst 2,4-dihydroxyphenyl β-glucoside (VI) was obtained with 1,2,4-trihydroxybenzene . In both instances the products of the *in vitro* experiments were the same as the major glucosides formed by the germinating bean.

No glucoside formation was observed when UDPG was replaced in the digests by other potential glucose donors such as α-D-glucose-1-phosphate, maltose, cellobiose, or excess D-glucose. (*cf.* Yamaha and Cardini (96).

The following reactions can occur with plant enzyme systems :-

(3) UTP + α-D-glucose-1-phosphate ⇌ UDPG+P-P (49)

(6) UDPG + Ar-OH ⇌ UDP + Ar-O-G (96)

(7) UDP + ATP $\overset{Mg^{++}}{\rightleftharpoons}$ UTP + ADP (97)

and it has now been shown that incubation of phenols with UTP, ATP, α-D-glucose-1-phosphate and wheat germ enzyme produces phenolic glucosides. The mono β-glucoside of resorcinol, catechol, and quinol were all produced by this system and saligenin gave salicyl β-glucoside. The compounds were identified by comparison with standard compounds.

α-D-Glucose-1-phosphate was replaced by α-D-galactose-1-phosphate in an unsuccessful attempt to synthesise quinol galactoside.

The formation of UDPG by reversal of equation (6) was also attempted by incubating arbutin separately with UDP and UTP but no UDPG was produced, although a compound chromatographically identical with UMP-5' was formed. This suggested that a nucleotidase was present in the enzyme. Incubation of UDPG and ATP with the enzyme resulted in complete destruction of these

compounds. In the presence of NaF, a pyrophosphatase inhibitor (98), degradation was not completely inhibited but in this instance UMP-5' and UDP, and AMP-5' and ADP were produced from UDPG and ATP respectively.

An enzyme was also prepared from bean seeds which catalysed the synthesis of phenolic glucosides from UDPG. Several unsuccessful attempts were made to prepare this enzyme using germinated bean seeds, dormant embryos and germinated embryos. The enzyme was finally prepared from whole dormant bean seeds macerated in an 'atomix' to give a concentrated extract.

The mono β-glucosides of quinol, catechol and resorcinol and salicyl β-glucoside were all synthesised by the bean enzyme and identified by paper chromatographic methods. Fractional precipitation of the enzyme with ammonium sulphate did not appear to increase the activity of the enzyme.

Incubation of the bean enzyme with UDPG and ATP both with and without NaF showed that a nucleotidase was present but was less active than in wheat germ enzyme. Even when NaF was absent the original

nucleotides could still be detected, after 20hr. incubation. UMP-5' was the main product from UDPG and AMP-5' from ATP. Therefore the relative inactivity of the bean preparation was probably not due to the presence of a nucleotidase but other inhibitors of the glucosylating enzyme eg phenols could have been present. A more likely explanation of the low activity is that wheat germ is a richer source of protein than bean and therefore possibly a higher concentration of the glucoside-synthesising enzyme is present in the former.

It is therefore suggested that UDPG is responsible for the glucosylation of the phenolic compounds which were fed to bean seeds and is presumably also a substrate for the formation of the naturally occurring glucosides. Other UDPsugars derivatives may give rise to other phenolic glycosides in the plant.

Alcoholic glycosides were synthesised in vitro by Bourquelot and co-workers (38-41) without fission of 'high-energy' bonds, eg glucosides of glycerol, ethylene, and propylene glycols and disaccharides such as cellobiose and gentiobiose. The first

biosynthesis of a phenolic glucoside in the absence of UDPG was carried out by Pridham [82], who obtained resorcinol α-glucoside from resorcinol, maltose and an A. niger enzyme. In the present study the formation of a fructose-containing derivative, (XIV), of saligenin was observed when this phenol was incubated with sucrose and invertase. The positive reaction of this compound with the diazo spray (A) showed that the alcoholic group of saligenin was substituted in (XIV) and not the phenolic hydroxyl group. The R_F values of (XIV) (Experiment 34) were greater than those of salicyl β-glucoside (IX) in both solvents A and B. Compound (XIV) was not hydrolysed by β-glucosidase but was hydrolysed by yeast invertase (a β-fructofuranosidase) yielding fructose and saligenin. Hydrolysis with 0.01N-H_2SO_4 was complete after 5 min; salicyl β-glucoside was not hydrolysed under these conditions. Compound (XIV) could be detected on paper chromatograms with urea hydrochloride, a spray reagent which is specific for ketoses [99]. The evidence strongly suggests that (XIV) is o-hydroxybenzyl-β-fructofuranoside.

Attempts to form fructosides from di- and tri-

hydric phenols by the same method were unsuccessful. Incubation of di-and tri-hydric phenols with β-glucosidase and excess D-glucose also did not yield any glucosides . Replacement of D-glucose by other potential glucose donors such as arbutin, maltose, and cellobiose had no effect. It appears, therefore, that in most cases β-glucosylation of a phenolic hydroxyl group does not occur by a 'simple' transferase reaction and it is possible that the energy requirements are too great unless a 'high-energy' glucose donor, eg UDPG is present. However, in the case of A. niger (82) there appears to be an enzyme capable of synthesising phenolic α-glucosides using maltose or isomaltose as glucose donors.

It was observed in the preliminary experiments that acidic derivatives were formed, in addition to the glucosides, when phenols were fed to broad-bean seeds. These compounds (XVI), (XVII), (XVIII) and (XIX) were found when beans were treated with resorcinol,catechol, quinol and saligenin respectively. All the acidic compounds had lower R_F values in solvent A and slightly higher values in solvent B than the corresponding

TABLE 7.

Compound	Solvent (R_F values)		Buffer (M_{SA} values)	
	A	B	A	C
Resorcinol	0.50	0.47	0.44	0.00
β-glucoside (II)				
(XVI)	0.18	0.54	0.74	0.45
Arbutin (I)	0.47	0.45	0.22	0.00
(XVII)	0.13	0.52	0.48	0.47
Catechol	0.62	0.59	0.41	0.00
β-glucoside (III)				
(XVIII)	0.20	0.66	0.88	0.52
Salicyl	0.50	0.48	0.28	0.00
β-glucoside (IX)				
(XIX)	0.17	0.60	0.39	0.58

glucosides, and migrated on paper electrophoretograms at pH values as low as 3.5. Simple glucosides do not migrate under acidic conditions. The results showed that these derivatives were strongly acidic. The u.v.spectra were similar to those of the apparently related glucosides.

Further chromatographic examination of (XVI), showed that the presence of 'Cetavlon' in a solvent (A) increased the R_F value thus confirming the acidity of the compound (100).

Aqueous and alcoholic solutions of (XVI), even at low temperatures, decomposed to form resorcinol β-glucoside (II).

Initially it was believed that the acidic derivatives of the glucosides were phosphates. Phosphate, containing ^{32}P, and quinol were therefore fed to broad-bean seeds in an attempt to obtain a labelled specimen of compound (XVI). However, no radioactivity could be detected in the acidic compound which was isolated.

Acid hydrolysis of compound (XVI) which had been purified by separation on two cellulose columns using an acidic solvent B followed by a neutral solvent A,

yielded resorcinol and glucose. Hydrolysis was complete after 30 min. under the standard conditions (1.5N-HCl; 100°.) but 2hr. were required for complete hydrolysis with 0.5N-HCl.

Treatment of (XVI) with alkali caused loss of the acidic grouping and the formation of a compound which was chromatographically and electrophoretically identical with resorcinol β-glucoside. The latter yielded resorcinol and glucose on hydrolysis with both acid and β-glucosidase. Hydrolysis of compound (XVI) with 0.5N-NaOH at 70° was complete after 1min. Compound (XVI) was not hydrolysed by α- or β-glucosidase.(101)

Compound (XVI) therefore appeared to be a derivative of resorcinol β-glucoside with an alkali-labile acidic substituent. As (XVI) was not hydrolysed by β-glucosidase it seemed probable that the substituent was on the glucose portion of the molecule. The activity of this enzyme is strongly affected by small changes in the carbohydrate component of glycosides. If carbons 2,3, or 4 , are substituted by methoxy or tosyloxy groups, the resulting glycoside cannot be hydrolysed by β-glucosidase. The effect of

substitution at carbon 6 is normally not so marked, but if a large group such as benzoyl or tosyl is present the rate of hydrolysis is extremely slow. Changes in the structure of the aglycone normally have only slight effects on the rate of enzymic hydrolysis.

The u.v. spectrum and the positive reaction with the diazo reagent (A) confirmed that (XVI) possessed a free phenolic hydroxyl group.

The presence of sulphur in the molecule was demonstrated by sodium fusion followed by a positive nitroprusside test. Alkaline hydrolysis and paper electrophoretic examination of the hydrolysis products in buffer F , showed that an acidic compound which could be detected with BDH Universal Indicator spray (E) was present. It behaved like sulphuric acid on paper chromatograms and electrophoretograms.

A determination of the ash content of compound (XVI) gave 16.7% residue. A flame test on this showed that sodium and probably some potassium ions were present. A strongly positive sulphur test was obtained with the residue using the nitroprusside reagent after sodium

fusion. Chromatographic examination of the residue again showed the presence of a fragment which co-chromatographed with sulphuric acid. Assuming the residue to be composed of sodium sulphate, and compound (XVI) to be the sodium salt of the 6'-sulphonate of resorcinol β-glucoside, M.W. 374, the residue would be 19%. The analysis figures for sulphur and sulphate in (XVI), however, were very much lower than the calculated figures for a phenolic glucoside sulphate.

The position of the substituent on (XVI) was determined by a Kuhn methylation of the compound followed by hydrolysis of the alkali-labile substituent and finally hydrolysis of the glycosidic bond. The tri-O-methyl glucose obtained was identified chromatographically as 2,3,4-tri-O-methyl-D-glucose. A trace of 2,3,4,6-tetra-O-methyl-D-glucose was also detected from the resorcinol β-glucoside impurity. A control methylation of resorcinol β-glucoside yielded only 2,3,4,6-tetra-O-methyl-D-glucose.

A second attempt to obtain a labelled sample of (XVI) was carried out and ^{35}S fed as sulphate, together with resorcinol, to broad-bean seeds, but the

TABLE 8.

Compound	Solvent (R_F values)			Colour with spray B
	A	B	F	
2,3,4-tri-O-methyl-D-glucose	0.70	0.74	0.73	yellow-brown
2,3,6-tri-O-methyl-D-glucose	0.68	0.75	0.71	pink-brown.
2,4,6-tri-O-methyl-D-glucose	0.66	0.70	0.70	red-brown
2,3,4,6-tetra-O-methyl-D-glucose	0.80	0.85	0.86	pink-red
Methylation products	0.70 0.80	0.74 0.85	0.73 0.86	yellow-brown pink-red

(XVI) produced was not radioactive. Autoradiographs of bean cotyledons after 2 days in the ^{35} sulphate solution showed that the radioactivity was localised around the periphery of the cotyledon (Fig.5.). Further autoradiographs of longitudinal sections of bean cotyledons after 8 days in the radioactive solution showed that even after that time very little penetration of the radioactive sulphate had occurred (Fig.5.). This suggests that the sulphate was not absorbed sufficiently into the bean tissues to be metabolised.

To summarise, hydrolysis of (XVI) under alkaline conditions readily yielded resorcinol β-glucoside, and under acidic conditions resorcinol and glucose (and an acid fragment). Clancy and Turvey (102) obtained methanol, glucose and sulphate on acid hydrolysis of a methyl glucoside sulphate. Percival (103) stated that alkali-catalysed hydrolysis of sulphates of α-and β-methyl glucopyranosides and galactopyranosides gave the corresponding methyl 3,6-anhydrohexosides. He also said that unless the removal of a sulphate group led to the formation of an anhydro ring, the hydrolysis of the sulphate group proceeded slowly.

i) after 2 days.

ii)after 8 days.

Figure 5.

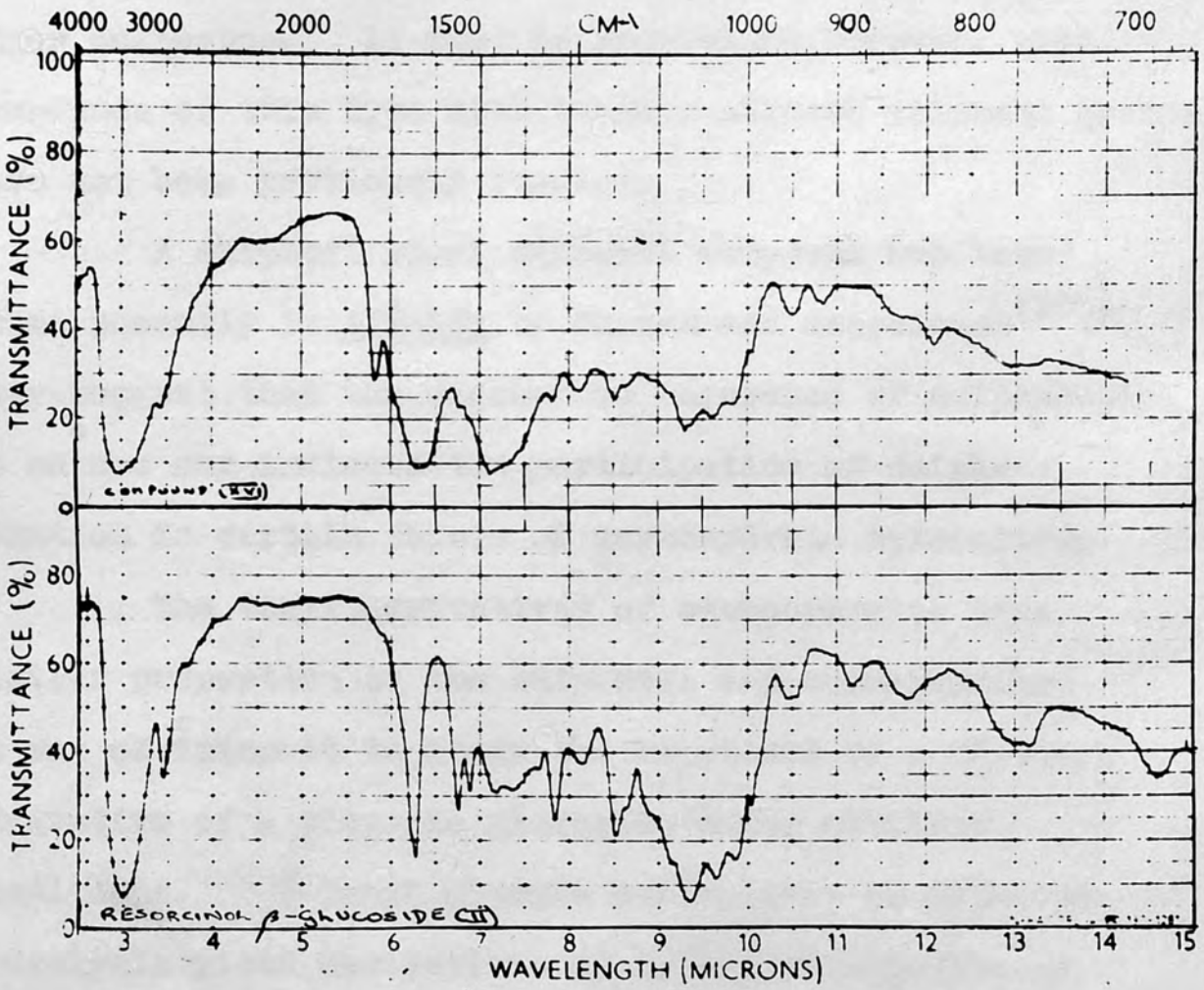
Autoradiographs of broad-bean seeds fed with ^{35}Sulphate.

The alkaline hydrolysis of compound (XVI) does not appear to obey either of these rules, for the substituent, which would appear to be on C-6 of the hexose, is removed yielding a primary alcohol group.

The i.r. spectra of resorcinol β-glucoside (II) and compound (XVI) were scanned (Fig.6.). No strong peaks for sulphate groups were visible but the spectrum of (XVI) possessed additional peaks ie not present in (II), at approx. 830, 930, 1,400 and 1,750 cm^{-1} Lloyd and Dodgson (104) carried out i.r. studies on sulphate esters and found a peak due to the C-O-S bonds in the region 820 - 850 cm^{-1} and a second characteristic peak at 1,240 cm^{-1}.

From these results it can be finally concluded that the derivative of resorcinol β-glucoside formed by bean seeds has an acidic substituent on the 6-position of the sugar moiety. This substituent is removed by alkali and an acidic fragment resembling sulphate results. Sulphur is definitely present in the molecule but quantitative analytical data for this element were unsatisfactory. Some support for the presence of a sulphate group in (XVI) is given by the i.r. spectrum.

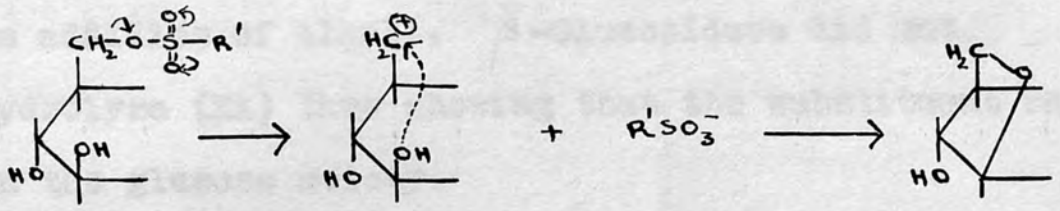
Figure 6. I.R.Spectra.



It is possible therefore that compound (XVI) is the sodium salt of resorcinol 6-O-sulphonyl-β-D-glucoside although at present it is difficult to interpret some of its reactions in the light of the published data on other sugar sulphates. It must be emphasised, however, that compounds of this type with large β-linked aglycone groups have not been previously studied.

A sulphoglycosyl glycerol compound has been found recently in Alfalfa by Benson and associates(105). They suggest that the widespread occurence of sulphosugars in nature may indicate the participation of sulphate fixation in certain phases of carbohydrate metabolism.

The tosyl derivatives of carbohydrates have similar properties to the sulphates and consequently it was of interest to study the reactions of a 6'-tosyl derivative of a phenolic glucoside under alkaline conditions. 6-Tosyl glucose derivatives on alkaline hydrolysis yield derivatives of 3,6-anhydro-D-glucose. The mechanism of this reaction is as shown overleaf(106).



OR $RSO_3R' + NaOH \longrightarrow ROH + NaSO_3R'$

The reaction therefore proceeds by alkyl-oxygen fission with the formation of a carbonium ion unlike the hydrolysis of carboxylic acid esters which generally involves acyl-oxygen fission. The presence of an unsubstituted hydroxyl group at C-3 is essential for hydrofuranol ring formation.(107).

6'-Tosyl arbutin (XX) was therefore synthesised from molar proportions of p-toluenesulphonyl chloride and arbutin in pyridine using Chloroform as diluent. Peat et al (108) have recently shown that direct sulphonation of aldohexoses occurs mainly at C-6. Compound (XX) had the correct molecular formula and the presence of a free phenolic hydroxyl group was indicated by reaction with the diazo spray (A) and a

shift of the absorption maximum from 286mμ to 302mμ on addition of alkali. β-Glucosidase did not hydrolyse (XX) thus showing that the substituent was on the glucose moiety.

Methylation of compound (XX) using the Kuhn procedure followed by hydrolysis gave a compound which was chromatographically indistinguishable from 2,3,4-tri-O-methyl-D-glucose, thus confirming that the tosyl group was substituted on C-6 of the glucose portion.

The treatment of compound (XX) by LAH in an attempt to obtain a 6-deoxy compound as further proof of the structure of (XX)[109] was unsuccessful. Acid hydrolysis of the product yielded 3,6-anhydroglucose and quinol. The examples of this reductive desulphonylation which are quoted by Peat [107] are of methyl glucoside derivatives and it may be that the phenyl group has a steric effect of the reaction.

Alkaline hydrolysis of (XX) ,under the same conditions as those employed for (XVI), and chromatographic examination of the products showed that the glucoside, arbutin, was produced together with small amounts of

3,6-anhydroglucose and quinol. It appears, therefore, that the reaction follows a similar course to the alkaline hydrolysis of (XVI) and involves cleavage of the tosyl group with subsequent formation of a primary hydroxyl group. This mechanism ,giving the effect of acyl-oxygen fission, is known to operate when the tosyl group is substituted on an 'isolated' primary hydroxyl group; however in this case fairly drastic conditions of hydrolysis are apparently required. In the alkaline hydrolysis of (XVI) and (XX) it is therefore postulated that some steric hindrance by the phenolic group occurs thus inhibiting the formation of the anhydro ring or making the anhydrohexoside very unstable. In the case of (XX) some anhydro ring formation apparently occurs together with hydrolysis of the glycosidic bonds. Arbutin,when treated with alkali, yielded glucose and not 1,6-anhydro-D-glucose although the normal product of alkaline hydrolysis of many substituted phenyl β-D-glucosides is 1,6-anhydro-D-glucose (110).

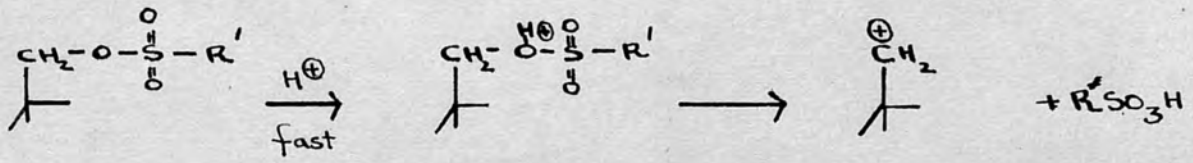
Foster et al (111) hydrolysed phenyl 6-O-toluene p-sulphonyl α-D-glucoside under alkaline

conditions and obtained phenyl 3,6-anhydro-D-glucoside but no reports have been found in the literature concerning the corresponding β-isomer and its reactions or of any 6-tosyl aldohexoside with a bulky β-linked aglycone group.

In view of the result obtained on LAH treatment and subsequent hydrolysis of (XX) the products of acid hydrolysis of (XX) were investigated. It was found that 3,6-anhydroglucose, glucose and quinol were all formed. The presence of 3,6-anhydroglucose was confirmed by chromatographic examination in solvent B and electrophoresis in buffers A and C against a standard. (D-Glucose and its derivatives in ring forms do not normally complex with molybdate ions (113). however 3,6-anhydro-D-glucose has an $M_{sorbitol}$ value of 0.33. The presence of an anhydro ring in the molecule enables the compound to exist in a boat form so that a complex can be formed across the hydroxyl groups on C-1, C-2 and C-4. In the 1C conformation, the two hydroxyl groups on C-2 and C-4 of 3,6-anhydro-D-glucose are in axial positions and consequently the formation of a boat form with three axial hydroxyl groups will

not require as much energy as a normal chair-boat transformation).

Tosyl groups are reported to be stable to dilute aqueous mineral acids. On theoretical grounds when tosyl esters are treated with acid it is possible that bond fission could occur in the same manner as in alkaline hydrolysis ie by alkyl-oxygen bond fission (112) and anhydro ring formation result.



Why this apparently does not occur with other tosylates is not understood, however. Clancy and Turvey (102) have shown recently that the rate of hydrolysis of a sulphate group is greater than that of the glycosidic bond. Quinol and 6-tosyl glucose may be formed by cleavage of the glycosidic bond but no trace of the latter derivative could be detected in the hydrolysate, of (XX).

Hydrolysis of the 6'-tosyl group could also yield arbutin which would be further hydrolysed to give quinol and glucose. In the second case the hydrolysis would follow an analogous course to alkaline hydrolysis.

EXPERIMENTAL.

GENERAL METHODS.

Paper chromatography.

Paper chromatography was carried out on Whatman No.1 or No.3. papers using the descending technique and the following solvent systems; all proportions given are by volume unless otherwise stated.

A — Butan-1-ol, ethanol, water (40:11:19) [114]

B — Ethyl acetate, acetic acid, water. (9:2:2) [115]

C — Ethyl acetate, acetic acid, formic acid, water (9:1.5:0.5:2) [116]

D — Ethyl acetate, pyridine, water (2:1:2, organic phase). [117]

E — Butan-1-ol, acetic acid, water (2:1:1, organic phase) [115]

F — Butan-1-ol, pyridine, water (10:3:3) [118]

G — Amyl alcohol, acetic acid, water (4:1:5) [119]

H — Butan-1-ol, ethanol, water (3:1:1) containing 'Cetavlon' (3%, w/v) [100]

J — Ethanol, ammonia, water (80:4:16) [120]

K — Ethanol, M-ammonium acetate buffer (pH3.7) (75:30) [121].

L Ethanol, M-ammonium acetate buffer (pH7.5) (75:30) [121]

M Propan-2-ol, hydrochloric acid, water (6.5:10) containing 0.2g.mol. HCl in 100ml. [63]

N N,N-dimethylformamide (20% in benzene), stationary phase and petroleum ether (60°-80°) as the mobile phase. [122]

P N,N-dimethylsulphoxide (20% in benzene) and di-iso propyl ether. [122]

Papers buffered with 0.2M-sodium borate were dipped in the solution and the solution then allowed to dry at room temperature.[123] The same procedure was employed to obtain 'molybdate' papers using 8.1×10^{-3}M-ammonium molybdate solution.[70]

The rates of movement of phenolic compounds were calculated as R_F values

$$R_F = \frac{\text{distance travelled by the substance.}}{\text{distance travelled by the solvent front.}}$$

Mobilities of carbohydrates were expressed as $R_{Gluc.}$ values.

$$R_{Gluc.} = \frac{\text{distance travelled by substance.}}{\text{distance travelled by glucose.}}$$

Paper electrophoresis.

Paper electrophoretic examination of compounds was carried out using Whatman No.1 and No.3. papers for 30 min. at 55V/cm. The buffers used were :-

A 0.2M-sodium borate (pH 10.0) [124]

B 0.05M- glycine (pH10.0) [125]

C 8.1×10^{-3}M-ammonium molybdate (pH 5.2) [113]

D 0.2M-sodium acetate (pH5.2) [125]

E 0.2M-sodium acetate (pH3.5) [125]

F 0.2M-sodium phosphate (pH 7.2) [125]

G 0.1N-sodium hydroxide. [126]

The mobilities of compounds were expressed as M_{SA} values ie relative to the movement of salicylic acid. [70]

Spray reagents.

The following spray reagents were used to detect compounds on paper chromatograms and electrophoretograms .

A Diazotised p-nitroaniline solution and N-sodium hydroxide (Phenols) [127]

B p-anisidine hydrochloride followed by heating at 100° for 5 min. (Reducing sugars) [128]

C Silver nitrate in acetone and ethanolic sodium hydroxide (Polyhydroxy compounds) [129]

D — Perchloric acid and ammonium molybdate solution followed by heating to 85° and then spraying with a solution of quinol (Phosphates) (130)

E — BDH Universal indicator adjusted to pH 8.5-9.0 by addition of dil. ammonia solution. (Acids)

F — Potassium periodatocuprate solution and rosaniline in acetic acid (Polyhydroxy compounds) (131)

EXPERIMENT 1. Investigation of the portion of the plant yielding the highest concentration of glucoside.

The cut ends of bean shoots were allowed to stand in (a) an aqueous solution of resorcinol (0.5%,w/v) and (b) an aqueous solution containing resorcinal (0.5%, w/v) and D-glucose (0.5%,w/v). The experiment was repeated using whole bean seeds which had been soaked in tap water for 24 hr. and then transferred to layers of cotton wool soaked in the solutions (a) and (b) described above.

After 1,2,3, and 4 days respectively extracts were prepared by grinding the tissues in cold aqueous methanol, after removing the testas from the whole seeds, and filtering at the pump. The clear yellow solutions obtained were concentrated using a rotary evaporator and examined on paper chromatograms using solvents A and B.

Both whole bean seeds and shoots produced high concentrations of resorcinol β-glucoside, which stained pink with spray A and co-chromatographed with a standard compound. The shoots,however, contained many more contaminating phenolic compounds than the seeds. The effect of the added glucose on the yield of glucoside

was negligible in all cases.

In addition to resorcinol β-glucoside, a second compound (XVI) staining the same colour with the diazo spray (A) as resorcinol β-glucoside was observed. This compound had a lower R_F value in solvent A and slightly higher R_F value in solvent B than the mono glucoside and appeared to be present only in the extracts from the seeds.

EXPT. 2. The portion of the seed yielding the highest concentration of glucoside.

The following were placed between layers of cotton wool soaked in an aqueous solution of resorcinol (1%,w/v).

(i) Dormant bean seeds.

(ii) Germinated seeds (ie kept moist at room temperature for 24 hr.).

(iii). Cotyledons (testas removed; ex germinated seeds.)

(iv). Embryos. (ex seeds kept moist at room temperature for three days)

After 3 days methanol extracts were prepared, as described in Experiment 1, and examined on paper chromatograms using solvents A and B. The intensity of resorcinol β-glucoside was greatest on the chromatograms of the extract from 24 hr. germinated seeds.

EXPT.3. The effect of varying the concentration of the phenolic solution

Bean seeds soaked in water for 24hrs. at room temperature were placed on cotton wool soaked in aqueous solutions of quinol (concentrations ranging from 0.5% ,w/v to 5% ,w/v) with and without D-glucose (1%,w/v).Extracts were prepared after 3 days and examined on paper chromatograms using arbutin (p-hydroxyphenyl β-D-glucopyranoside) as a standard. Darkening of the cotyledons had occurred in the more concentrated solutions. A compound which co-chromatographed with arbutin was detected together with a less intense phenolic spot having a slightly higher R_F value in solvent B and lower value in solvent A than arbutin. The increase in concentration of the phenol did not appear to have affected the rate of formation of the glucoside. The presence of glucose appeared to increase the yield slightly.

The experiment was repeated using seeds that had been germinated for 6 days on damp cotton wool. The results obtained were similar but glucose did not increase the yield appreciably in this case.

EXPT.4. Germination of beans under anaerobic conditions.

Conductivity water was used to make up solutions of quinol (1%,w/v) with and without D-glucose (1%,w/v). Dormant bean seeds were placed in these solutions and the beakers stored in the dark. Some sterilised cotton wool was placed on top of the solutions to prevent the beans from floating. After 3 days methanol extracts were prepared and examined chromatographically. A compound which co-chromatographed with arbutin was present but only a trace of compound (XVI) could be detected.

EXPT.5. Extraction of the bean with different solvents.

Bean seeds were soaked in water for 24hr. and then transferred to cotton wool soaked in an aqueous solution of resorcinol (1%,w/v) for 3 days. The beans were then divided into three groups and water, dimethyl formamide and methanol used as different extracting agents. Concentration and chromatographic examination of the extracts showed that all the extracts contained similar concentrations of resorcinol β-glucoside but many more phenolic compounds were present in the aqueous extract.

EXPT.6. Injection of phenols into the plant.

Well developed bean plants ,approx.12 ins. in height, were used. A fine capillary,connected to a reservoir filled with an aqueous solution of resorcinol (1%,w/v), was injected into the vascular bundle of the stem and the plant left for 5 days.Methanol extracts were then prepared of portions of the stem and leaves and examined on paper chromatograms. No resorcinol could be detected in the extracts but resorcinol β-glucoside was presentin the extracts from the regions of the stem directly above and below the point of injection. A trace of the glucoside was observed in the extract from the leaf directly above the injection site. There was no evidence for the presence of compound (XVI).

INVESTIGATION OF MONO-GLUCOSIDE FORMATION.

General Methods

EXPT.7. Production of glucosides by the broad bean.

1st.Method. Beans were allowed to soak for 24 hr. in tap water at room temperature; they were then transferred to a tray and placed between layers of cotton wool soaked in an aqueous solution (1%,w/v) of the phenol for three days. If necessary ,water was added during this period to replace that lost by evaporation.

2nd.Method. Dormant bean seeds were placed in an aqueous solution of the phenol (1%,w/v) for 24 hrs. They were then removed, washed well and aerated continuously for 3 days in water at room temperature.

Extracts were prepared in both cases by removing the testas and grinding in cold aqueous methanol (90%,v/v) in an 'Atomix'. The thick slurries obtained were centrifuged (3,000 r.p.m.;15 min; 5°) and the clear yellow liquids decanted off. These were concentrated to small volumes,keeping the temperature below 50°, using a rotary evaporator. At this stage the presence of glucosides was confirmed by paper

chromatographic examination and the extracts then resolved using cellulose columns. Solvent A was used to develop the columns and 25 ml. fractions were collected using an automatic fraction collector. The fractions containing the glucosides were combined and concentrated using a rotary evaporator (<50°).

Small scale preparative separations were also carried out in some instances using Whatman No.3. paper and solvents A and B.

EXPT. 8. Hydrolysis of glucosides with β-glucosidase.

Hydrolyses using β-glucosidase were carried out in 0.02M - sodium acetate buffer (pH 5.5; 27°; 2 days) Toluene was added to each digest. Control experiments using boiled β-glucosidase were also prepared in each case.

EXPT. 9. Acid Hydrolysis of glucosides.

The glucosides were heated (100°; 4 hr.) with 1.5N-HCl in an ignition tube connected to an air condenser. The solutions were then evaporated to dryness over sodium hydroxide in a vacuum desiccator and the solids thus obtained dissolved in a little water.

The hydrolysis products from Experiments 8 and 9

were examined on paper chromatograms and compared with standard compounds. The phenolic aglycone was detected by spray A after the solution had been separated on Whatman No.3. paper in solvents A and B. The sugar moiety was detected on Whatman No.1.paper with spray B after a long development with solvent B .

EXPT. 10. Ultra-violet spectra of the glucosides.

The u.v. spectra of the glucosides were measured in aqueous solutions unless otherwise stated. The presence of free phenolic hydroxyl groups were detected by a bathochromic shift of the absorption maximum in the presence of 0.002N- NaOH[66] . O-Dihydroxy groupings were revealed by a hypsochromic shift on addition of 0.002M - boric acid to the alkaline solutions [67].

EXPT.11 Formation of Arbutin by the broad bean.

A methanolic extract was prepared from approximately 60g. of bean seeds fed with an aqueous solution of quinol (1%.w/v) on cotton wool. The pale yellow syrup obtained was shown to contain a compound (I) which co-chromatographed with authentic arbutin and gave the same purple colour with spray A. The syrup was fractionated on a cellulose column and crystals of compound (I) (0.40g.)m.p. 200.5°

were obtained. Recrystallisation from water produced compound (I) m.p. 199° (sinters 167°). Commercial arbutin, on recrystallisation from water has m.p. 198.5° (sinters 167°). Mixed m.p. 199°. [Found : C,49.7; H,6.3. Calc. for $C_{12}H_{16}O_7 . H_2O$: C,49.9; H,6.4% $[\alpha]_D$-62° in water (c,1.8) Lit. value $[\alpha]_D$-60.3°]

Hydrolysis of (I) with β-glucosidase and 1.5N-HCl gave quinol and glucose.

The R_F and M_{SA} values of compound (I) and arbutin are shown in Table 1.

The u.v. absorption spectra of authentic and extracted arbutin showed absorption maxima at 281mμ and 278mμ respectively. Infra-red spectra of the two as Nujol mulls were superimposable , as seen in Fig.1.

The pentaacetyl derivative of compound (I) was prepared using sodium acetate and acetic anhydride as acetylating agents. Recrystallisation of the product from methylated spirits gave the acetate m.p. 146.5°-147°. (Lit.value 145°-146°)[132] Mixed m.p. 146.5° [Found: C,54.3; H,5.6: Calc. for $C_{22}H_{26}O_{12}$: C,54.7; H,5.4%] Infra-red spectra of the pentaacetyl derivatives of both authentic arbutin and compound (I) were superimposable (Fig. 2)

EXPERIMENT 12. The formation of resorcinol glucoside by broad bean seeds.

100 broad bean seeds (approx) were soaked in water for 4 hrs. and then transferred to a tray containing cotton wool soaked in an aqueous solution of resorcinol (1%,w/v). An extract was prepared after 3 days and transferred to the top of a cellulose column. A phenolic compound (II) staining pink with spray A was then eluted. The syrup obtained after concentration was dissolved in water, made acidic by addition of acetic acid (2N) extracted four times with ether to remove traces of resorcinol, and the aqueous solution finally concentrated and stored in the refrigerator. When a few drops of ethanol were added to this aqueous solution a cloudiness was observed, however, no solid separated so the solution was again concentrated to a meringue. This was dissolved in water (2 ml.) and freeze-dried. A brownish-white solid was left. [Found: C,49.8; H,5.8. Calc. for $C_{12}H_{16}O_7.H_2O$: C,49.7: H,6.2%].

Compound (II) and resorcinol β-glucoside had similar R_F and M_{SA} values in 2 solvent and 4 buffer systems (Table 1.).

Hydrolysis of compound (II) with β-glucosidase yielded resorcinol and glucose. Acid hydrolysis of (II)

gave the same products.

The u.v. spectrum showed a single sharp peak $\lambda_{max.}$ 271mμ Resorcinol has $\lambda_{max.}$ 276mμ.

EXPERIMENT 13.

The formation of catechol glucoside.

An extract of beans fed with an aqueous solution of catechol (1%, w/v) was prepared and fractionated on a cellulose column. A compound (III) staining red-pink with spray A was obtained. Attempts to obtain a crystalline specimen failed so compound (III) was dissolved in water, (3 ml.) and freeze-dried.

The R_F and M_{SA} values of compound (III) and authentic catechol β-glucoside were similar (Table 1).

Hydrolysis of compound (III) with β-glucosidase yielded catechol and glucose. Acid hydrolysis gave the same products.

EXPERIMENT 14. The formation of pyrogallol glucosides by the bean.

A methanolic extract of beans fed with an aqueous solution of pyrogallol (1%,w/v) was prepared. Chromatographic examination of the extract showed that two phenolic compounds were present, compound (IV) staining a prussian-blue colour, and compound (V) a green colour with spray A.

The main extract was then resolved by chromatography on large sheets of paper and compounds (IV) and (V) eluted and examined on plain and molybdate-treated papers using solvents A and B. The R_F values of the two compounds are listed in Table 2. Compound (IV) ran as a brown spot on the molybdate-treated papers and had a high mobility on paper electrophoretograms using buffer C. The M_{SA} values of both compounds are shown in Table 2.

Hydrolysis of both compounds (IV) and (V) with β-glucosidase gave pyrogallol and glucose. Acid hydrolysis also yielded pyrogallol and glucose in both cases.

The u.v. spectra of the compounds were scanned. Compound (IV) showed $\lambda_{max.}$ 270mμ; addition of alkali

caused a bathochromic shift to give $\lambda_{max.}$ 283mμ, and on further addition of boric acid a hypsochromic shift to $\lambda_{max.}$ 277mμ was observed. Compound (V) had $\lambda_{max.}$ 276mμ shifting to $\lambda_{max.}$ 281mμ under alkaline conditions but no satisfactory spectrum could be plotted with borate.

EXPERIMENT 15 — The glucosides obtained from 1,2,4-trihydroxybenzene.

Bean seeds were aerated in water for 3 hr. and then transferred to cotton wool soaked in an aqueous solution of 1,2,4-trihydroxybenzene (0.5%,w/v). The tray was kept in the dark for 3 days before an extract was prepared in the usual way.

Chromatography of the extract in solvents A and B showed that three compounds had been formed staining blue-green (VI), pale-blue (VII) and pale-blue (VIII) with apray A. The proportion of concentrations of the three compounds was approx. 6:2:1 as determined by visual estimation of chromatograms. A small scale separation of the three compounds was carried out on thick paper and compounds (VI) and (VII) obtained fairly pure in a freeze-dried condition.

Compound (VIII) was further purified by chromatographic methods and freeze dried.

The R_F and M_{SA} values of the three compounds are listed in Table 3 . Compound (VII) was visible as a brown spot before treatment with spray A, when molybdate-treated paper was used for chromatography, and had a relatively high M_{SA} value in buffer C but was stationary in buffer D.

Hydrolysis of compounds (VI) and (VII) with β-glucosidase yielded 1,2,4-trihydroxybenzene and glucose. Acid hydrolysis gave the same products.

The u.v. spectrum of compound (VI) showed $\lambda_{max.}$ 284mμ shifting to 292mμ in the presence of alkali; compound (VII) had $\lambda_{max.}$ 280mμ shifting to 291mμ on addition of alkali; compound (VIII) had $\lambda_{max.}$ 271mμ .

The three compounds were each spotted onto two sheets of Whatman No.3 paper and the starting line of one sheet sprayed with aqueous M-$FeCl_3$ solution. The two papers were then developed with solvent A and detected with spray A. Compound (VI) had R_F 0.50 on both papers but (VII) and (VIII) could not be

detected on the paper sprayed with $FeCl_3$.

Compound (VI) (10mg.) was shaken with N,N-dimethyl formamide (0.2ml.), methyl iodide (0.04ml.) and barium oxide (0.05g.) for 6 hr.[133] The mixture was centrifuged and the clear solution heated with 2N-methanolic HCl (30min.,100°), concentrated and then heated with aqueous N-HCl (3hr.,100°). Chromatographic examination of the solution showed that no methylated phenol was present. Barium hydroxide was replaced with silver oxide[134] and the experiment repeated but again unsuccessfully.

Diazomethane was prepared by adding slowly with shaking N-nitrosomethylurea (5g.) to KOH (10g.) dissolved in water (15ml.) and ether (50ml.) at 0°.[135] The ether layer became yellow and was decanted off and dried over KOH pellets (2hr.,0°).

Freeze-dried compound (VI) (10mg.) was dissolved in dry methanol (10ml.) and excess diazomethane in ether (10ml.) added. The flask was kept at 0° overnight. Glacial acetic acid was then added dropwise until the yellow colour disappeared. The solution was concentrated to dryness, heated with

2N-MeOH/HCl (1hr.,100°), and evaporated to dryness over NaOH in a vacuum dessicator. The dark residue was dissolved in chloroform and compared chromatographically with 2,4-, 2,6-, 2,5-dimethoxyphenol using the two phase solvent systems N and P. The methylated compound co-chromatographed with 2,4-dimethoxyphenol (see Table 9.).

TABLE 9.

Compound	Solvent R_F values. N	P	Colour with spray A
Methylated compound(VI)	0.36	0.48	pale-blue
2,4-dimethoxyphenol			
2,5-dimethoxyphenol	0.08	0.21	pale-blue
2,6-dimethoxyphenol	0.33	0.38	deep-blue

A portion of the residue was hydrolysed with acid neutralised and the solid dissolved in water and examined chromatographically in solvent A against standard tri- and tetra-O-methyl-D-glucoses. Partially

methylated sugars only were detected in the hydrolysate by spray B.

EXPERIMENT 16 — Methylation of quinol with diazomethane.

Arbutin (0.5g.) was dissolved in dry methanol (10ml.) and diazomethane in ether (15ml.) added. The solution was left overnight (0°). Excess diazomethane was decomposed by addition of glacial acetic acid drop by drop. The solution was then evaporated to dryness, heated with 2N-MeOH/HCl (100°, 1 hr.) and evaporated to dryness over NaOH in a vacuum desiccator.

Chromatography of the product in solvents N and P against standard quinol monomethyl ether showed that the two gave the same colour with spray A and had identical R_F values in both solvent systems.

EXPERIMENT 17. The glucosides obtained from saligenin.

A methanolic extract was prepared from beans which had been fed with saligenin (o-hydroxybenzyl alcohol). Chromatographic examination showed the presence of a compound (IX), staining pink with spray A which co-chromatographed with authentic salicyl β-glucoside (o-hydroxybenzyl β-D-glucopyranoside).[42] The extract was therefore fractionated on a cellulose column and the chromatographic and electrophorectic behaviour of (IX) examined. Salicin, (o-hydroxymethylphenyl β-D-glucopyranoside), saligenin and salicyl β-glucoside were used as standards and the papers viewed under u.v. light before spraying. Salicin cannot be detected by spray A but it is visible under u.v. as an absorbing spot.

Hydrolysis of freeze-dried compound (IX) with β-glucosidase yielded saligenin and glucose. Acid hydrolysis gave the same products.

The u.v. spectrum of compound (IX) showed a single peak $\lambda_{max.}$ 274mμ; addition of alkali caused a shift to $\lambda_{max.}$ 297mμ. Authentic salicyl β-glucoside behaved similarly.[42] Salicin has $\lambda_{max.}$ 268mμ and is unaffected by addition of alkali; the absorption maximum is 275mμ for saligenin.

In order to establish that compound (IX) was the major component produced after feeding beans with saligenin, further chromatographic examination of the extract was carried out using both Whatman No.1 and No.3. papers. The chromatograms were developed in solvents A, B and D. and viewed under u.v. light and sprayed with sprays A, C and G. A trace of an absorbing spot (X) could be detected under u.v. light which co-chromatographed with salicin in all three solvents. When the papers were sprayed with A , however, compound (IX) could be seen to cover almost the same area.

The extract was therefore streaked onto Whatman No.3. papers which were developed in solvent A and the absorbing band , together with salicyl β-glucoside (IX) eluted with water. The solution was concentrated and streaked onto two electrophoretograms which were developed in buffer A. These were also viewed under u.v. light and the absorbing band (X) Rg_A0.0 eluted with water. The u.v. spectrum of this solution, which was alkaline, had $\lambda_{max.}$ 267mμ, addition of acid had no effect on the spectrum.

'Biodeminerolite' (carbonate form) was added to the aqueous solution which was then filtered and freeze-dried.

β-Glucosidase hydrolysis of compound (X) yielded saligenin and glucose.

The R_F and M_{SA} values of compounds (IX) and (X) and those of the standard compounds are listed in Table

EXPERIMENT 18 Willow shoots fed with saligenin.

The cut ends of 4 willow shoots (Salix daphnoides) were placed in an aqueous solution (1%, w/v) for 2 days at room temperature. An aqueous methanolic extract of the whole shoot was then prepared and chromatographed against a control extract prepared from willow shoots placed in water under the same conditions.

Saligenin, salicyl β-glucoside and salicin were all present in the experimental extract but of these only salicin could be detected in the control.

The experiment was repeated using willow shoots placed in an aqueous solution of saligenin (1%,w/v) for 2 days. Chromatographic examination of the extract revealed the presence of saligenin and salicyl β-glucoside which were not present in the control extract.

EXPERIMENT 19 Maize seedlings fed with saligenin.

Maize seedlings were incubated with an aqueous solution of saligenin (1%, w/v) for 3 days at 25°.[22] A methanolic extract was thenprepared and concentrated to a syrup at room temperature. Paper chromatography of this extract against salicin using solvents A and B and paper electrophoresis in buffer A showed no absorbing spot under u.v. which corresponded to salicin. Spray A detected a compound staining pink which behaved identically with compound (IX) and standard salicyl β-glucoside on chromatograms and electrophoretograms.

The experiment was repeated using 6 day old maize seedlings which were left on cotton wool soaked in an aqueous solution of saligenin (1%, w/v) for 24 hr. The extract was prepared as before and chromatograms developed in solvents A, B and D against the same standard compounds. The papers were viewed under u.v. light before spraying ; one half of the paper together with the salicin standard was then sprayed with G and the other half, containing the salicyl β-glucoside standard with spray A.

Maize seedlings were finally left for 3 weeks on cotton wool soaked in the saligenin solution and after this time an extract was prepared and examined on chromatograms. Again no dark band corresponding to salicin was visible under u.v.light but a compound staining pink with spray A and co-chromatographing with a salicyl β-glucoside was present.

TABLE 10

SOLVENT	Rf VALUES.			
	Salicin	Salicyl β-glucoside	Dark band from maize	Pink band from maize
A	0. 48	0. 53	0. 21	0. 53
B	0. 51	0. 54	0. 46	0. 56
D.	0. 81	0. 83	0. 76	0. 84

EXPERIMENT 20 The glucosides obtained from p -hydroxybenzyl alcohol.

A methanolic extract was prepared from bean seeds which had been allowed to germinate between layers of cotton wool soaked in an aqueous solution of p-hydroxybenzyl alcohol (0.5%,w/v). The extract was concentrated and examined on paper chromatograms, each paper was viewed under u.v. light then cut in half and one half was sprayed with A and the other with spray C. No compound could be detected with the diazo spray (A) but a compound (XI) was visible as a strongly absorbing spot under u.v. and could be detected with spray C.

The main extract was then resolved on large sheets of Whatman No.3. paper. Compound (XI) was located under u.v. light and eluted with aqueous methanol (90%,v/v) from the appropriate strips. The u.v.spectrum of this solution was scanned and a single sharp peak $\lambda_{max.}$ 266mμ observed. Addition of alkali did not produce a bathochromic shift.

Hydrolysis of compound (XI) with β-glucosidase yielded p-hydroxybenzyl alcohol and glucose. Acid hydrolysis gave the same products. The R_F and M_{SA} values of (XI) are listed in Table W.

EXPERIMENT 21.

p-Hydroxybenzyl alcohol (0.05g.) was dissolved in 0.02M sodium acetate buffer(pH5.8, 2 ml.) and D-glucose (0.2g) added together with β-glucosidase (0.05g).[41] The flask was incubated for 48 hrs. at 27°. Chromatograms of the digest after that period showed the presence of a compound (XII) staining pinkish-purple with spray A.

The whole digest was then resolved on large sheets of Whatman No.3. paper, and (XII) eluted from the strips with aqueous methanol (90%,w/v). This solution was evaporated to dryness and the solid dissolved in absolute ethanol. The u.v. spectrum of this solution showed λ_{max} 279mμ shifting to 290mμ on addition of alkali.

Hydrolysis of an aqueous solution with β-glucosidase yielded p-hydroxybenzyl alcohol and glucose. Acid hydrolysis gave glucose but the alcohol could not be detected. The R_F and M_{SA} values of this compound are shown in Table 11.

EXPERIMENT 22

The following experiments were set up :-

(i) 6 beans were soaked in tap water for 3 hr., transferred to a petri dish and partially covered with an aqueous solution of p-hydroxybenzyl alcohol (1%,w/v).

(ii) 6 beans, similarly soaked in water, were placed on cotton wool dampened with the same solution of phenol.

After incubation at 25° for 2 days methanolic extracts were prepared and concentrated. Chromatography of these extracts in solvents A and B followed by examination of the papers under u.v. light and spraying with spray A, showed that extract (ii) contained a strongly absorbing band (compound XI) but no compound which reacted with the diazo spray A. Extract (i) however, contained (XI) and also a trace of a compound (XII) staining pink with spray A and co-chromatographing with p-hydroxybenzyl glucoside from Experiment (Table II.).

These experiments were repeated, the incubation period being increased to 4 days. Chromatographic

examination of the extracts prepared showed the presence of compound (XI) in both extracts and of an increased amount of (XII) in extract (i).

TABLE.11

Compound	Solvent (R_F values)		Buffer (M_{SA} values)	
	A	B	A	C
(XI)	0.49	0.52	0.00	0.00
(XII) from β-glucosidase	0.42	0.47	0.43	0.00
(XII) from bean extract	0.43	0.47	0.44	0.00
p-Hydroxy benzyl alcohol	0.92	0.86	0.53	0.00

EXPERIMENT 23. The glucoside obtained from p-nitrophenol

A methanolic extract was prepared from 20 bean seeds which had been fed with an aqueous solution of p-nitrophenol (1%,w/v) for 3 days. Chromatographic examination of the extract in solvent B, showed the presence of a compound (XIII) which could be detected as a yellow spot by spraying the chromatogram with an acid solution of $TiCl_3$, heating (50°,15 min) and then dipping in an alcoholic solution of Ehrlich's reagent (p-dimethylaminobenzaldehyde).[83] The compound did not give a colour reaction with spray A.

Compound (XIII) was separated from the extract by chromatography on thick paper. The R_F and M_{SA} values of (XIII) were :-

	Solvent R_F values		Buffer. M_{SA} values	
	A	B	A	D
Compound (XIII)	0.60	0.71	0.00	0.00
p-Nitrophenol	0.91	0.95	0.79	0.00

Hydrolysis of this compound (XIII) with β-glucosidase yielded p-nitrophenol and glucose.

Acid hydrolysis gave the same result.

The u.v. spectrum of (XIII) had $\lambda_{max.}$ 294mμ and was unaffected by the addition of alkali. p-Nitrophenol has $\lambda_{max.}$ 311mμ.

EXPERIMENT 24 2,4-Dinitrophenol fed to bean seeds.

Bean seeds were fed with a 5×10^{-5} M solution of 2,4-dinitrophenol on cotton wool for 3 days. A methanolic extract was prepared and examined chromatographically in solvent B but no phenol could be detected. The experiment was repeated using solutions of 1×10^{-5} M and 1×10^{-4} M concentration but no 2,4-dinitrophenol could be detected in the extracts.

EXPERIMENT 25. Attempted detection of U.D.P.G. in bean seeds.

The fractionation technique of Cabib [60] et al. was used. All experimental procedure was carried out at 0° unless otherwise stated.

6 day-old peeled bean seeds (1,450g.) were extracted with absolute ethanol (1.5l .) The solution was heated to the boiling point, allowed to cool and then centrifuged (2,000 r.p.m., 15 min.) 5N-Nitric acid was added until the solution was acid to Congo red paper and the solution again centrifuged. Mercuric acetate solution [136] (33 ml.) was added and the solution left overnight. The precipitate was filtered off, suspended in M -ammonium acetate solution (600 ml.) and left at room temperature for 2 hr. Mercuric acetate solution (18 ml.) was again added together with an equal volume of ethanol (600 ml.), the solution was stirred well and left for 2 hr. The precipitate was filtered off, suspended in water (140ml.) and H_2S bubbled through for 2½ hr. After filtration the solution was aerated for 3 hr. filtered again and the pH adjusted to 7.5 with dilute ammonia.

A column of De-acidite 'FF' (SRA 63, 100-200 mesh, 2-3% cross-linked, Cl^- form) diameter 3 cm., height

18 cm., was washed first with N-HCl and then distilled water until the absorbance at 260mμ fell below 0.03. The nucleotide solution was then passed down the column at a flow rate of 6 ml/min. and then the column again washed with water until the absorbance fell below 0.06. The eluent and receiving vessel were colled in ice.

As the nucleotide of main interest was U.D.P.G., the resin column was eluted successively with 0.002N-HCl; 0.01N-HCl; 0.01N-NaCl in 0.01N-HCl; 0.02N-NaCl in 0.01N-HCl; 0.03N-NaCl in 0.01N-HCl. 50 ml. fractions were collected and each eluent passed through the column until the absorbance of the eluate at 260mμ dropped to 0.05; in all 3 litres of eluate were collected. The graph of absorbance at 260mμ against ml. of eluate is shown in Fig. 4.

The fractions from the first peak using 0.03N-NaCl in 0.01N-HCl as eluent were combined and concentrated by adsorption on a charcoal column followed by elution with a solution of ethanol-ammonia-water (40ml. of 95% EtOH, 1 ml. conc. NH_3 made up to 100 ml. with water) Fractions ml.) were collected and the solutions were immediately adjusted to pH 5.6 by the addition of acetic acid. The fractions from the second

peak were similarly concentrated. Chromatographic examination of the eluates from the carbon columns was carried out using acetic-acid washed Whatman No.1. papers in solvents K, L , and M with U.D.P.G., U.D.P. and U.M.P. as standards. No absorbing compounds co-chromatographing with these standards were present. U.v. spectra of the combined eluates showed a shoulder at approx. 255mμ but no definite peak.

EXPERIMENT 26. Preparation of wheat germ enzyme.

The method of Cardini and Yamaha[96] was used for extraction of the wheat germ.

Commercial wheat germ (100g) was suspended in 0.05 M phosphate buffer (pH 7.0; 300ml.; 0°.) with occasional stirring. The extract was centrifuged (16,000g; 20 min,; 0°.) and the supernatent decanted off and dialysed against buffer overnight 5°). A crude protein extract (ENZYME I) was thus obtained.

This extract was fractionated by adding solid ammonium sulphate and the protein fraction precipitating between 0.2 and 0.5 saturation centrifuged off. The precipitate was re-dissolved in 0.05M-phosphate buffer

(100ml.;pH 7.0 containing 0.01M- ethylene diamine tetra-acetic acid (EDTA) and 0.01M-cysteine) and the solution dialysed against a neutral solution of 10^{-4}M-EDTA and 10^{-4}M-cysteine (21.;5°;2hr.). The dialysate was further fractionated by addition of saturated ammonium sulphate solution (pH7.0) and the protein precipitating between 0.2 and 0.33vol. redissolved in the phosphate -EDTA-cysteine buffer (ENZYME II). The pH of this solution was adjusted to 4.7 with N-acetic acid and 0.05 vol. of saturated ammonium sulphate solution (pH4.7) added. The precipitate was discarded and 0.02 vol. of saturated ammonium sulphate solution added to the supernatent. The precipitate obtained after centrifuging was redissolved in the phosphate-EDTA-cysteine buffer (ENZYME III).

These three enzyme solutions were stored at 5° for up to one week ,after that time new extracts were prepared as required.

EXPERIMENT 27. Preparation of mono-β-glucosides.

The enzyme extracts were incubated with phenols and U.D.P.G. in the following proportions:-

10mg. phenol.

6mg . U.D.P.G.

0.1ml enzyme

0.1ml buffer.

The buffer used was Tris (hydroxymethyl)-aminomethane-HCl (Tris-HCl) (125) pH 7.4, 0.05M-sodium acetate buffer was used initially but this was less satisfactory. A control, without U.D.P.G., was included in each case and a few drops of toluene placed in all the tubes which were incubated at 37. The enzyme used in each experiment is shown in Table 6 . Samples were removed after 0.5hr., 2 hr. and 5.hr. and spotted on paper chromatograms which were developed with solvents A and B. The papers were viewed under u.v. light before spraying with spray A .

The phenolic compounds used as acceptors and the glucosides produced are listed in Table 6 . The latter were identified by comparison with standard compounds in both solvent systems.

EXPERIMENT 28. Replacement of U.D.P.G. by alternative glucose donors.

Enzymes II and III were combined and incubated with resorcinol and the following potential glucose donors. (The same substrate concentrations were used as in the previous experiment(No 27).

α- D-glucose-1-phosphate.

Methyl α-D-glucoside.

Maltose

Cellobiose

U.D.P.G.

Samples were removed after 0.5 hr. and 2 hrs. and examined on paper chromatograms using solvents A and B. Resorcinol β-glucoside was formed only when U.D.P.G. was present.

EXPERIMENT 29. Formation of glucosides from U.T.P. and α-D-glucose-1-phosphate.

Adenosine triphosphate (A.T.P.,2.4 mg.), uridine triphosphate (U.T.P., 1.4 mg.) α-D-glucose-1-phosphate (G-1-P.,2.1 mg) and catechol (1.3 mg.) were incubated with enzyme I (0.1 ml.) and tris-HCl buffer (pH 7.4, 0.1 ml.) A control reaction containing only catechol, enzyme and buffer was also prepared. Magnesium chloride (0.5mg.) and sodium fluoride (2mg.)

were added and a few drops of toluene placed on top of both solutions before the tubes were incubated at 37°. Samples were removed after 2, 8 and 24 hr. and examined on paper chromatograms using solvents A and B and a catechol β-glucoside standard. After 8 hr. a trace of compound(III) ,staining pink with spray A and co-chromatographing with the standard glucoside, was present in the incubation, the concentration of (III) had increased after 20 hr.

The experiment was repeated using quinol and saligenin in place of catechol. Small amounts of phenolic compounds were detected after 2 hr. incubation which co-chromatographed with arbutin and salicyl β-glucoside standards, in both A and B. After 24 hr. there was a marked increase in concentrations of these compounds in the digests. There was no evidence for any glucoside formation in the control solutions.

α-D-glucose-1-phosphate was replaced by α-D-galactose-1-phosphate and incubated with quinol, the other substrates and conditions remaining unchanged. Chromatographic examination of this solution did not reveal the presence of a galactoside,however.

EXPERIMENT 30. Reversal of glucosylation

Arbutin (3.0mg.), enzyme I (0.1ml.) and tris-HCl buffer (pH 7.4, 0.1ml.) were incubated with a) U.D.P. (0.5 mg.) and b) U.T.P. (0.5 mg.) as described in the previous experiment. Samples were removed immediately on mixing, and after 1,2,3 and 16 hr., and examinedon paper chromatograms in solvent L with U.D.P., U.T.P. and U.D.P.G. as reference compounds. The papers were viewed under u.v. light but no absorbing spot corresponding to U.D.P.G. other than the standard, was present.

EXPERIMENT 31. Nucleotidases in the wheat germ.

The following reaction mixtures were prepared:-

No:	U.D.P.G.	A.T.P.	Enzyme I	Tris-HCl buffer	NaF
1.	0.5mg.	------	0.1ml.	0.1ml.	---
2.	0.5mg.	------	0.1ml.	0.1ml.	2.0mg.
3.	0.5mg.	-------	------	0.1ml.	--------
4.	0.5mg.	-------	------	0.1ml.	2.0mg.
5.	-----	0.8mg.	0.1ml	0.1ml	------
6.	-----	0.8mg.	0.1ml.	0.1ml.	2.0mg.

Each solution was covered with toluene and the tubes incubated at 37° for 24 hr. Chromatographic

examination of the solutions was carried out using solvent L and the papers examined under u.v.light. Compounds co-chromatographing with U.D.P. and U.M.P.-5' were shown to be present in solution 2. No u.v. absorbing compounds were present in tube 1, and 3 and 4 contained only U.D.P.G. No compounds visible under u.v. were present in 5, but compounds which co-chromatographed with A.D.P. and A.M.P.-5' were present in 6.

EXPERIMENT 32. 'Simple' transferase reactions.

Quinol (3.0mg.), D-glucose (26 mg.) and almond β-glucosidase were dissolved in 0.05M-sodium acetate buffer (pH 5.6, 0.2ml.) and incubated at 27° for 24 hr. Chromatographic examination of the solution did not reveal the presence of any arbutin. The experiment was repeated and samples removed after 8, 24hr. and 3 days but there was no evidence that any glucosylation had occurred.

Resorcinol and 1,2,4-trihydroxybenzene were used in place of quinol and the experiment repeated. No glucoside formation was evident however.

1,2,4-Trihydroxybenzene (1.4mg.) was incubated separately with arbutin (14.0mg.) and cellobiose (29mg.) using β-glucosidase in 0.05M-sodium acetate buffer

(0.2ml.) No evidence of glucosylation was obtained.

EXPERIMENT 33. Preparation of a glucoside-synthesising enzyme from bean seeds.

Dormant bean seeds (35g) were extracted, after removal of the testas, with 0.05M-phosphate buffer (105ml.) in an 'atomix'. The slurry obtained was left to stand (2hr.5°), centrifuged (16,000g;0°;20min.) and the supernatent liquid decanted off and dialysed against 0.05M-phosphate buffer (500ml.;5°).

Resorcinol was then incubated with this enzyme together with U.D.P.G. in tris-HCl buffer using the same concentrations and conditions as in Experiment 27. Samples were removed after 0.5, 3, and 10hr and examined chromatographically using solvents A and B with resorcinol β-glucoside (II) as a standard. A trace of a compound staining pink with spray A and co-chromatographing with resorcinol β-glucoside in both solvent systems was present after 30 min. The concentration increased with prolonged incubation.

This experiment was repeated replacing resorcinol with quinol, catechol, and saligenin. Compounds were produced which co-chromatographed with authentic arbutin (I), catechol β-glucoside (III)

and salicyl β-glucoside (IX) respectively in both solvents A and B. The concentration of glucoside was much greater after 10hr. incubation.

1,2,4-Trihydroxybenzene and prygallol were also used as acceptors but no evidence of glucosylation was obtained with these compounds.

The enzyme was purified by fractionation with solid ammonium sulphate and the fraction precipitating between 0.2 and 0.5 saturation was redissolved in 0.05M-phosphate buffer containing 0.001M-EDTA and 0.001M-cysteine. After dialysis against a neutral solution of 10^{-4}M-EDTA and 10^{-4}M-cysteine, (16hr.;5°) this enzyme was incubated with resorcinol and UDPG (see Experiment 27). A small amount of resorcinol β-glucoside was present after 1hr.

EXPERIMENT 34 Incubation of saligenin with yeast invertase and sucrose

Saligenin (10mg.), sucrose(20mg.)and invertase (BDH concentrate; 0.2ml.) and 0.1M-sodium phosphate buffer (pH 7.5; 0.2ml.) were incubated at 25°. A control solution containing boiled invertase and buffer

was also prepared and incubated at 25°. Samples were removed after 5 min. and examined on paper chromatograms which were developed with solvent A. One chromatogram was sprayed with A and the other with urea hydrochloride.[99] Both spray reagents revealed a compound (XIV) which had R_F 0.68 and did not co-chromatograph with salicyl β-glucoside, R_F 0.59. The compound was purified by fractionation of the digest on thick paper chromatograms (solvent A). The product obtained was dissolved in water (0.2ml.) and incubated with invertase in 0.1M-phosphate buffer (30min.;25°;0.2ml.). Samples were removed and examined chromatographically using D-glucose, D-fructose and saligenin as standards. Compound (XIV) was completely hydrolysed and fructose and saligenin were detected by sprays B and A respectively.

On incubation of compound (XIV) with β-glucosidase no hydrolysis was evident after 2 days.

Hydrolysis with 0.01N-H_2SO_4 (100°) was complete after 5 min. Chromatographic examination of the solution showed the presence of saligenin. Salicyl β-glucoside (IX) was not hydrolysed under these conditions.

EXPERIMENT 35. Formation of an acidic compound by bean seeds.

Bean seeds were fed with an aqueous solution of resorcinol (1%,w/v) and an extract prepared as previously described . Chromatographic examination of this extract revealed the presence of resorcinol β-glucoside (II) and also of a compound (XVI) staining the same colour with spray A. (Experiment 1). The approximate proportion of (II) to (XVI) was 4:1. The R_F and M_{SA} values of compound (XVI) are shown in Table 7. together with those of resorcinol β-glucoside.

The extract was therefore fractionated on large sheets of Whatman No.3 paper using solvent B. A broad band containing both compounds (II) and (XVI) was eluted with aqueous methanol (90%,w/v) and the solution concentrated ($< 50^{\circ}$). This was then resolved on paper using solvent A and compound (XVI) eluted from the appropriate strips. The solution was concentrated ($< 50^{\circ}$) and stored in the refrigerator (5°). Shortly after however, a further chromatographic examination of the purified compound (XVI) showed that small amounts of resorcinol β-glucoside were present. Compound (XVI) was therefore again purified by paper chromatography using solvent

A and the product obtained after elution was freeze-dried and stored in the refrigerator .(5°).

The u.v.spectrum of resorcinol has $\lambda_{max.}$ 271mμ and that of compound (XVI) has $\lambda_{max.}$ 270-271mμ.

The experiment was repeated and extracts of bean seeds fed with aqueous solutions of (i) quinol (1%,w/v) (ii)catechol (1%,w/v) and (iii) saligenin (1%,w/v) for three days were prepared and examined chromatographically in solvents A and B. In all the extracts low concentrations of compounds were present which resembled the corresponding mono β-glucosides, in that they stained the same colour with spray A. These compounds were therefore isolated from the plant extracts by chromatography and elution from thick papers using solvents B and A as described for compound (XVI). The R_F and M_{SA} values of the three mono β-glucosides and compounds (XVII), (XVIII), and(XIX), resulting from quinol, catechol and saligenin respectively are shown in Table 7. .

The u.v. spectrum of (XVII) showed a peak at 265mμ; arbutin has $\lambda_{max.}$ 266mμ. Salicyl β-glucoside and(XIX) have $\lambda_{max.}$ 274mμ.

Examination of the structure of compound (XVI)

EXPERIMENT 36. General Investigations.

The compound (XVI) appeared to be more stable than (XVII), (XVIII), or (XIX) and was therefore chosen for further investigations. Compound (XVI) (100mg.) was therefore isolated from a plant extract prepared from 180g. (dry weight) bean seeds. As the separation was on a large scale two cellulose columns were used, the first was eluted with solvent B and the second with solvent A. The compound was freeze-dried; in the presence of water, resorcinol β-glucoside was rapidly formed.

A more detailed study of the chromatographic and electrophoretic behaviour of compound (XVI) was undertaken. Chromatography in solvent H ie solvent A containing 'Cetavlon', showed that the R_F value was increased in this solvent system.

Compound	Solvent (R_F values)		Buffer (M_{SA} values)
	A	H	D
Resorcinol β-glucoside (II)	0.48	0.48	0.01
Compound (XVI)	0.15	0.41	0.50

Electrophoresis in buffers of low pH values showed that compound (XVI) was a relatively strong acid which had a high M_{SA} value even at pH 2.6.

The i.r. spectra of resorcinol β-glucoside (II) and compound (XVI) as smears deposited on evaporation from methanol were scanned.

EXPERIMENT 37. Introduction of ^{32}P into broad bean seeds.

^{32}Phosphate (1mC.; carrier free in dil.HCl, pH 2-3) was diluted to 150 ml. with distilled water. A portion of this solution (50 ml.) was taken and quinol (0.5g.) dissolved in it. Dormant bean seeds were then allowed to soak in this solution for 3 days, washed well with water and an extract prepared by crushing in methanol. A control extract was prepared from bean seeds left in a further portion (50ml.) of the ^{32}Phosphate solution. The extracts were streaked onto Whatman No.3. papers which were developed with solvents A and B. Arbutin and compound (XVII) were located by spraying strips cut from the sheets. The distribution of radioactivity was measured by counting 1cm.square of a further strip using a Geiger-Muller end window

tube with an EKCO scalar (type N592A). Graphs were plotted of counts per min. against strip number for both extracts and the areas covered by arbutin and compound (XVII) marked. Electrophoretograms in buffer E were examined in the same way.

As no correlation between the activity and the position of compound (XVII) could be found a further experiment was devised. Bean seeds were allowed to soak in a portion (50ml.) of the ^{32}Phosphate solution overnight. Quinol (0.5g.) was then dissolved in the solution and the whole left for 3 days. An extract was prepared and examined on paper chromatograms against the two previous extracts. No increase in the concentration of compound (XVII) was apparent on spraying with A and on counting the strips no relation between the position of compound (XVII) on the chromatograms and the degree of radioactivity was observed.

EXPERIMENT 38. Hydrolyses of compound (XVI).

Incubation of β-glucosidase with a solution of compound (XVI) containing some resorcinol β-glucoside showed that the latter was hydrolysed but

compound (XVI) was unaffected. Incubation with $\alpha\beta$-glucosidase also did not hydrolyse (XVI).

Hydrolysis with acid under standard conditions yielded resorcinol and glucose. Further examination of (XVI) under conditions of acid hydrolysis was carried out using different concentrations of sulphuric acid, different temperatures and removing samples after 5,10,15,30 min., 1, 1½, and 2 hr. The samples were neutralised by $BaCO_3$ and spotted onto a Whatman No.3. paper which was developed with solvent A. The results are summarised in Table 12. below.

Normality	Temperature	Result	Products.
0.5	80°	Hydrolysis complete after 2 hr.	Resorcinol & D-glucose
1.5	100°	Hydrolysis complete after 30 min.	Resorcinol & D-glucose

Alkaline hydrolysis of compound (XVI) (0.5N-NaOH; 70°; 45 min.) followed by neutralisation with IR -120 (H^+ form) and chromatographic examination with solvents A and B showed that only resorcinol β-glucoside was present in the solution. The hydrolysis was therefore repeated and samples removed and neutralised

after 1,5,10,15, 20 and 45 min. It was found that compound (XVI) was completely hydrolysed after 1 min. The initial solution contained a proportion of resorcinol β-glucoside (3:1 approx., XVI:II); after 1 min. the concentration of the latter increased and it was the only detectable phenolic compound present.

Hydrolysis with 0.5N-NaOH (60°; 10 min.), treatment with IR -120 (H^+ form) and further hydrolysis of the solution with 0.5N-H_2SO_4 (80°; 1 hr.) gave resorcinol and glucose.

Compound (XVI) was treated with 0.5N-NaOH (60°; 10 min.) and the solution neutralised as before and filtered. This solution (0.5ml) was then incubated with β-glucosidase and 0.05M-sodium acetate buffer (0.5ml.; pH5.2; 27°; 2 days.). Chromatographic examination of the digest after that time showed that complete hydrolysis had occurred yielding resorcinol and glucose.

EXPERIMENT 39. Investigation of the acidic component of (XVI)

Sodium fusion of compound (XVI) followed by addition of a dilute solution of sodium nitroprusside

gave a transient purple colouration. A control test was carried out as follows. An extract from beans germinated on damp cotton wool was separated on thick papers which were developed with solvent B. The strips having R_F values between 0.5 and 0.6, corresponding to compound (XVI), were eluted and then concentrated to dryness. Sodium fusion, followed by addition of nitroprusside, gave no reaction for sulphur.

Compound (XVI) was treated with 0.5N-NaOH (A.R.; 60°; 10 min.) neutralised with IR-120 (H^+ form, analytical grade) and filtered: Electrophoretic examination of this solution, compound (XVI), 0.01N-H_2SO_4 and salicylic acid in buffer F and spraying with E showed the presence of a fragment from the hydrolysate staining pink and having the same M_{SA} value as sulphuric acid. Further examination of the solution in the same buffer (F) against 0.01N-solutions of phosphoric, oxalic and sulphuric acids showed that all three acids and the compound produced on alkaline hydrolysis of (XVI) had similar M_{SA} values.

Chromatographic examination of the hydrolysate on Whatman No.1. paper with solvent J (40 hr. development) and detection of acidic compounds by spray E also showed the presence of a fragment staining pink and

co-chromatographing with sulphuric acid. Oxalic acid and phosphoric acids move with lower R_F values than sulphuric acid in this solvent.

A determination of the ash content of compound (XVI) was carried out. A crucible was weighed to constant weight and compound (XVI) (4.2mg.) introduced. The heating was continued until the crucible again reached constant weight. The weight of residue was 0.7mg. ie 16.7%. A flame test on this residue showed that it contained sodium and some potassium. Sodium fusion of the residue followed by addition of nitroprusside gave a strong purple colouration. A test for nitrogen with ferrous sulphate ferric chloride and acid was negative.

The residue obtained from ashing (XVI) on a nickel spatula was dissolved in distilled water (0.5ml.) containing IR-120 (H^+ form) and examined chromatographically with solvent J using solutions of sodium sulphate, compound (XVI) and 0.01N-H_2SO_4 as standards. Spray E showed that the sodium salt of the acid gave a reailing spot. Compounds staining pink with the indicator spray (E) and co-chromatographing with sulphuric acid were present in both ashed (XVI) and a

solution of (XVI) but in a lower concentration in the latter.

Analyses of compound (XVI) gave the following percentages of sulphur :- 1.91,0.62 and the percentage of sulphate as determined by Dr.Rees was 2.8.

EXPERIMENT 40. Methylation of compound XVI.

Freeze-dried (XVI) (11mg.) was methylated using the method of Kuhn et al (134) with N,N-dimethylformamide (0.48ml.),methyl iodide (0.08ml.) and silver oxide (90mg.). The dry chloroform solution finally obtained was concentrated and the resulting solution heated with 0.05N-methanolic NaOH (0.3ml.;70°;5 min.). The solution was neutralised with IR-120 (H^+ form) and the liquid filtered and concentrated. An equal volume of methanolic HCl (4%,w/v) was added and the mixture heated (100°; 30 min: 50°; 12 hr.) and then evaporated to dryness. Aqueous N-HCl was then added and the solution again heated (80°; 3 hr.). The acid was neutralised with IRA-400 (HCO_3^- form) and the resulting solution of methylated sugars examined chromatographically with solvents A,B ,F and P and detected with spray B. The following compounds were

used as standards:- 2,3,4-, 2,3,6-, and 2,4,6-tri-O-methyl-D-glucose. A trace of a compound which co-chromatographed with 2,3,4,6-tetra-O-methyl-D-glucose was present but the major product was chromatographically indistinguishable from 2,3,4-tri-O-methyl-D-glucose in all four solvent systems and stained the same yellow-brown colour with spray B. (Table 8).

A compound staining deep red with spray A and co-chromatographing with resorcinol monomethylether in solvents A and B was also shown to be present.

A better chromatographic separation of 2,3,4- and 2,3,6-tri-O-methyl-D-glucose was sought and solvent G tried. However, the R_F values found were 0.61 and 0.63 respectively. In buffer A both compounds have M_{SA} values of 0.0. Buffer G was also used and the paper treated with a chromium trioxide/potassium permangonate spray reagent.[126] 2,3,6-tri-O-methyl-D-glucose showed clearly as a yellow spot on a pink background but the other methylated sugars could not be detected. Both Whatman No.1 and No.3. papers were used but with no improvement in detection.

EXPERIMENT 41. Methylation of resorcinol β-glucoside (II).

Resorcinol β-glucoside (25mg.) was methylated by the method of Kuhn et al.[134]. N,N-dimethylformamide (1.2ml), methyl iodide (0.4ml.) and silver oxide (400mg.) were added and the same procedure followed as in the previous experiment omitting the treatment with methanolic NaOH. Chromatographic examination of the products in solvents A and B showed that only a compound co-chromatographing with 2,3,4,6-tetra-O-methyl-D-glucose was present.

EXPERIMENT 42. Introduction of ^{35}Sulphur in broad bean seeds.

^{35}S as sulphate (1mC; 0.9ml.; pH7.0) was diluted to 100ml. with distilled water. A portion (800ml) was taken and further diluted to 300ml (800μC). Dormant bean seeds (100g.) were allowed to soak in this solution overnight. Resorcinol (3g.) was then dissolved in the solution and the whole aerated for 2 days. The beans were then removed, washed well with water and left on damp cotton wool for a further 24 hr.

Chromatographic examination of an extract with solvent A showed that compounds (II) and (XVI) were both present. The radioactivity present on a strip (1cm.wide) of the chromatogram was therefore counted as described previously (Experiment 37.) and a graph plotted of strip number against counts per min. The radioactivity was concentrated near the origin of the chromatogram.

The experiment was repeated using bean seeds germinated for 24 hr. before applying the solution of resorcinol and ^{35}sulphate. After 2 days 2 bean cotyledons were removed, washed well with water and an autoradiograph taken using a piece of thin polythene, to prevent wetting of the X-ray paper. The autoradiograph was developed after 20 hr. (Fig. 5) Further autoradiographs of bean seeds were taken after 8 days in the aerated radioactive solution. Longitudinal sections of 6 beans were cut and left in contact with the X-ray paper for 30 hr. before development, two sections are shown in Fig 5 . An extract which was prepared from the same batch of beans and examined chromatographically with solvent A contained both compounds (II) and (XVI) An autoradiograph of this chromatogram showed that there was no radioactivity in the region of compound (XVI).

EXPERIMENT 43. Preparation of p-hydroxyphenyl 6-O-toluene-p-sulphonyl- β -D- glucopyranoside.

Arbutin (2.7g.), p-toluenesulphonyl chloride (2.1g.) and pyridine (0.8ml.) were mixed and the solution diluted with dry chloroform (5ml.) The flask was shaken at room temperature for 4 hr. and water (3ml.) then slowly added. The resulting organic layer was separated and shaken successively with dil. H_2SO_4, water, aqueous $NaHCO_3$, and water and finally dried over anhydrous Na_2SO_4. The solution was concentrated under reduced pressure, cooled and a white solid obtained. After recrystallisation from aqueous methanol fine white crystals of compound (XX) were obtained in low yield. m.p. 69-70° [Found: C,52.7; H,5.3; S,7.3. $C_{19}H_{22}SO_9 \cdot CH_3OH$ requires C,52.4; H,5.7; S,7.2%]

Compound (XX) gave the same purple colouration as arbutin with spray A. The R_F and M_{SA} values of arbutin and compound (XX) are shown below:-

Table 13.

Compound	R_F values		M_{SA} values	
	A	B	A	D
(XX)	0.80	0.86	0.00	0.00
Arbutin (I)	0.48	0.45	0.22	0.00

Compound (XX) had $\lambda_{max.}$ 286mμ shifting to 302mμ on addition of alkali.

Incubation with β-glucosidase did not hydrolyse compound (XX). Hydrolysis of an aqueous methanolic solution of compound (XX) with HCl yielded a sugar moiety which was detected with sprays B and C, having the same R_F value (0.45) in solvent B as 3, 6-anhydro-D-glucose.

Alkaline hydrolysis of (XX) with 0.5N-MeOH/NaOH (70°; 15 min.) was followed by neutralisation using IR-120 (H^+ form) and examination on paper chromatograms. A phenolic compound was detected with spray A having the same R_F value as arbutin in the solution. Electrophoretic examination using buffer A showed that this phenolic compound and arbutin had identical M_{SA} values. Sprays B and C detected a low concentration of glucose and a compound which was chromatographically indistinguishable from 3,6-anhydro-D-glucose in solvent B. The hydrolysate was therefore fractionated by chromatography on thick paper and the suspected arbutin component eluted and concentrated. Hydrolysis of this compound with acid yielded glucose and a

trace of 3,6-anhydro-D-glucose but the phenol could not be detected.

Compound (XX) (60mg.) and LAH (6mg.) were dissolved in dry tetrahydrofuran (THF; 2ml.) and heated at 65° for 12hr. More LAH (6mg.) was added after that time and the solution heated for a further 24hr. After decomposition of excess LAH by ethyl acetate, ice-water (1ml.) was added dropwise and the organic layer separated and concentrated to dryness. The solid was triturated twice with cold ethanol, filtered through Whatman No.50 paper and the solution concentrated to dryness. The solid was hydrolysed with acid and the products examined on paper chromatograms using solvent B with D-glucose, 6-deoxy-D-glucose and 3,6-anhydro-D-glucose as standards. A compound was detected with spray B from the hydrolysis which co-chromatographed with 3,6-anhydro-D-glucose. No 6-deoxy-D-glucose was apparently present.

TABLE 14.

Solvent	Compound R_F values			
	D-Glucose	6-deoxy-D-glucose	3,6-anhydro-D-glucose	Hydrolysis product.
B	0.12	0.32	0.43	0.43
Buffer	$M_{Gluc.}$ values.			
A	1.00	0.72	0.84	0.84
C	1.00	0.00	5.00	5.00

Compound (XX) (39mg.) was methylated by the method of Kuhn et al.[134] using N,N-dimethylformamide (0.45ml.), methyl iodide (0.16ml.) and silver oxide (0.18g.). The methylated compound was then heated with N-methanolic NaOH (80°; 2 hr.) the solution was then neutralised with IR-120 (H^+) and concentrated to dryness. 2N-Methanolic HCl was then added, the solution heated (100°; 2 hr.) and evaporated to dryness. 2N-aqueous HCl was added and the solution again heated (100°; 3hr.). The acid was neutralised with IRA-400 (carbonate form) and the solution filtered, concentrated and examined on paper chromatograms using solvent A. 2,3,4- 2,3,6- 2,4,6-Tri-O-methyl-D-glucoses and 2,3,4,6-tetra-O-methyl-D-glucose were used as standards. A compound,

staining yellow-brown with spray B, and co-chromatographing with 2,3,4-tri-O-methyl-D-glucose was present.

SYNTHESES.

EXPERIMENT 44. **Preparation of p-acetoxyphenyl 2,3,4,6-tetra-O-acetyl β-D-glucoside.**

Arbutin (0.5g.) was acetylated by heating with acetic anhydride (2.5ml.) and sodium acetate (0.25g.). A white crystalline solid was obtained, m.p.146.5-147°[132] [Found: C,54.8;H,5.6. Calc. for $C_{22}H_{26}O_{12}$: C,54.7;H,5.4%]

EXPERIMENT 45. **Preparation of 1,2,3,4,6-penta-O-acetyl-D-glucose.**

D-Glucose (5g.) was acetylated in the usual way with anhydrous sodium acetate (4g.) and acetic anhydride (25ml.). A solid (4.2g.) was obtained which was recrystallised from methanol, m.p.131°. (Lit.value 130-131°). [Found: C,48.8; H,5.6. Calc.for $C_{16}H_{22}O_{11}$: C,49.2; H,5.6% . $[\alpha]_D^{+}4.8°$ in $CHCl_3$ (c,7). Lit. value 3.8]

EXPERIMENT 46. **Preparation of resorcinol 2,3,4,6-tetra-O-acetyl β-D-glucoside.**[68]

Resorcinol (2.2g.) , 1,2,3,4,6-penta-O-acetyl-D-glucose (7.5g.) and redistilled phosphorus

oxychloride (0.6ml.; containing water 1%,w/v) were dissolved in dry benzene and heated under reflux for 3 hr. After cooling the reaction mixture was extracted with ice-cold water. The benzene layer was shaken with N-NaOH and water and then dried over $CaCl_2$. The solution was evaporated to dryness under reduced pressure and the resulting white solid recrystallised from ethanol. The crystals (1.35g.) melted over a range and appeared to be a mixture of two compounds, one of which was readily soluble in benzene and afforded yellow crystals from this solvent, and the other only slightly soluble in benzene. De-acetylation of small portions of these solids with sodium in methanol showed that only the former yielded a compound which stained red with spray A. Small samples of this compound were therefore de-acetylated when required as a standard for compound (II).

EXPERIMENT 47. Preparation of o-acetoxyphenyl 2,3,4,6-tetra-O-acetyl β-D-glucoside.

Impure catechol glucoside tetraacetate (1.0g.)

dry pyridine (10ml.) and acetic anhydride (4ml.) were refluxed together on an oil bath for 5 min. After cooling the reaction mixture was poured into ice-water and stirred well. The solid obtained was filtered and recrystallised from methylated spirits. m.p.136.5-137°. [Found : C,55.0; H,5.5.Calc. for $C_{22}H_{26}O_{12}$: C, 54. 7; H,5. 4%]

EXPERIMENT 48. Preparation of p-hydroxybenzyl alcohol.(138, 81)

Lithium aluminium hydride (LAH) (3.7g.) was dissolved in dry ether (50ml.) and the solution transferred to a three-necked flask fitted with a condenser and stirrer. p-Hydroxybenzoic acid (13.5g.) was also dissolved in dry ether and added slowly by means of a dropping funnel. The flask was cooled in ice-water until the addition of acid was complete and the mixture then refluxed for 1hr. Ethyl acetate (5ml.) was added to destroy excess LAH followed by water (approx.200ml.). The ether layer was separated and evaporated to dryness. The resulting white solid was recrystallised twice from water.m.p.113°. (Lit.value

124°) m.p. authentic p-hydroxybenzyl alcohol 114°; mixed m.p. 114°. [Found C,67.7;H,6.5. Calc. for $C_7H_8O_2$: C,67.7;H,6.5%.] The compound had an absorption maximum of 277mμ in water, p-hydroxybenzaldehyde had $\lambda_{max.}$ 282mμ in water. Paper electrophoresis of the alcohol in buffer D showed that it was homogeneous(M_{SA} 0.20) and it gave an orange-pink colour with spray A. p-Hydroxybenzoic acid has M_{SA} 0.80 and saligenin has M_{SA} 0.20. The alcohol was also chromatographically homogeneous R_F 0.86, p-hydroxybenzoic acid has R_F 0.95 and saligenin R_F 0.89 (solvent B).

EXPERIMENT 49. Preparation of 1,2,4-trihydroxybenzene (139).

1,2,4-Triacetoxybenzene (5g.), methanol (10ml.) and conc. HCl (10ml.) were refluxed for 1hr. The mixture was then distilled under reduced pressure ($< 30°$). The colour of the solution changed from dark green to brown and on cooling a brown solid separated. This was recrystallised from ether, m.p.136°. (Lit. value 140 .5°.)

EXPERIMENT 50. Preparation of 2,5-dimethoxybenzene sulphonic acid.

Conc. H_2SO_4 (3ml.) was added to quinol dimethylether (6g.) and the mixture heated on an oil-bath under reflux for 1 hr.[140] The dark green solution obtained was cooled and then poured into ice-water. $NaHCO_3$ was added to pH6-7, the solution heated to boiling, saturated with NaCl and filtered hot. On cooling the solution deposited white crystals. Sodium fusion showed the presence of sulphur in this compound.

The p-toluidide of the sodium sulphonate of 2,5-dimethoxybenzene was prepared and recrystallised from hot water. m.p. 203° (Lit. value 202-203°).[141]

EXPERIMENT 51. Preparation of 2,5-dimethoxyphenol.

2,5-Dimethoxybenzene sulphonic acid, sodium salt, (6g.) was fused with NaOH (10g.) and KOH (4g.) in an iron crucible.[140] The solid was dissolved in water, sulphuric acid added to pH 5-6 and the liquid extracted with ether. Concentration of the ethereal solution yielded an oil. Chromatographic examination of this compound in solvents A and N showed the presence of

a phenolic compound staining blue with spray A together with impurities from fusion. The compound was therefore further purified by chromatography in solvent A and used as a standard for the examination of the methylated phenol obtained from compound (VI).

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The Formation of Phenolic Glycosides by Germinating Broad-bean (*Vicia faba*) Seeds.

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The majority of the naturally occurring low-molecular-weight phenols are present in plant tissues as glycosides (Baruah & Swain, 1957). D-Glucosides are most common although other monosaccharides and short oligosaccharide chains are also found as phenolic derivatives in plants. Previous studies on the biosynthesis of glucosides have been reviewed by Pridham (1960). Hutchinson, Roy & Towers (1958) and Pridham (1958), for example, have infiltrated plant tissues with various phenols and demonstrated the formation of the corresponding glucosides.

It is now shown that glucosides are formed when broad-bean seeds are allowed to germinate between layers of cotton wool which have been soaked in aqueous solutions (1 %, w/v) of various di- and trihydric phenols. Catechol, resorcinol, quinol and phloroglucinol give the corresponding mono-β-glucosides and 2:3- and 2:6-dihydroxyphenyl-β-glucosides are produced on infiltration with pyrogallol. 2-Hydroxybenzyl-β-glucoside is the main product when saligenin (2-hydroxybenzyl alcohol) is used as the substrate.

Cardini & Leloir (1957) found that phenolic glucosides can be produced when uridine diphosphate glucose is incubated with phenols in presence of a wheat-germ extract. This work has been substantiated using enzyme preparations from the bean. Phenolic glucosides apparently cannot be synthesized by transferase reactions involving a phenolic acceptor molecule, a potential glucose donor (e.g. salicin or cellobiose) and β-glucosidase.

The variety and complexity of the naturally occurring plant glycosides suggests that these compounds are biochemically important. The possible function of these compounds *in vivo* is under consideration.

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